EXPLORING THE EPIGENETIC LINK IN CIRCADIAN-DISRUPTION-INDUCED BREAST CANCER

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ABSTRACT

Breast cancer is the leading cause of cancer-related death among women worldwide. Evidence is also mounting that circadian disruption (CD) is probably carcinogenic to humans and is linked to breast cancer development.

The aim of this thesis is to provide new insights into the epigenetic links involved in CD-induced breast cancer. The results presented here show for the first time that CD induces expression changes in breast cancer-relevant and potentially circadian-relevant miRNAs in mammary tissues. Moreover, the results also show that long-term CD can potentially cause gene expression changes on a large enough scale to influence breast cancer-relevant signaling pathways.

These findings align with previous studies that have shown that CD is a warranted concern in breast cancer development, and that the initiation of this process may be linked to aberrant CD-induced miRNA activity in the mammary tissues.

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5-aza – 5-aza-2`deoxycytidine
Ago – argonaute
AKT – protein kinase B
ALU – arthrobacter luteus
BER – base excision repair
BC – breast cancer
BCL – B-cell lymphoma
BID – BH3-interacting domain death agonist
BMAL1 – brain and muscle ARNT-like protein 1
BRCA1 – breast cancer 1, early onset
BTG2 – BTG family member 2
c-Myc – myc avian myelocytomatosis viral oncogene homolog
CaM – calmodulin
cAMP – cyclic adenosine monophosphate
CSC – cancer stem cells
CD – circadian disruption
CDK – cyclin dependent kinase
CHK – check point kinase
CKIδ – casein kinase one delta
CKIε – casein kinase one epsilon
CLOCK – circadian locomotor output cycles kaput
CpG/CG – cytosine guanine dinucleotides
CRY – crytpochrome
DKK3 – dickkopf WNT signaling pathway inhibitor 3
DLX5 – distal-less homeobox 5
DMBA – 7,12-Dimethylbenz[a]anthracene
DDR – DNA damage response
DNA – deoxyribonucleic acid
DNMT – DNA methyltransferase
DSB – double strand breaks
G1 – growth 1/gap 1
G2 – pre-mitotic
E2 – estradiol
ECL – enhanced chemiluminescence
EGF – epidermal growth factor
EMT – epithelial-to-mesenchymal transition
ER/ESR – estrogen receptor
ERBB2 – erb-b2 receptor tyrosine kinase
ERE – estrogen response element

FxR – fragile X mental retardation protein
HER2 – human epidermal growth factor receptor 2
HOXA5 – homeobox A5
HR – homologous recombination
IGF – insulin-like growth factor 1
JAK – Janus kinase
LAN – light at night
LINE-1 – long interspersed nucleotide element-1
MBD – methyl CpG-binding domain
MeCP2 – methyl CpG binding protein 2
miRNA/miR – microRNA
MLT – melatonin
MMP-2 – matrix metalloproteinase-2
MMR – mismatch repair
MRE – meiotic recombination homolog
MRN – MRE11-RAD50-NBN complex
mRNA – messenger RNA
mTOR – mammalian target of rapamycin
NER – nucleotide excision repair
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
nt – nucleotides
NUSAP1 – nucleolar and spindle-associated protein 1
oncomiR – oncogenic miRNA
P-bodies – processing bodies
PACT – protein activator of the interferon-induced protein kinase
PAGE – polyacrylamide gel electrophoresis
PAIP2A – PABP interacting protein 2A
PCR – polymerase chain reaction
$PDCD_4$ – programmed cell death 4
PER – period
PI3K – phosphatidylinositide 3-kinases
PKC – protein kinase C
PLAU/uPA – protease urokinase-type plasminogen activator
PMD – partially methylated domain
PR – progesterone receptor
pre-miRNA – precursor miRNA
pri-miRNA – primary miRNA
PS – photoperiod shifting
PTEN – phosphatase and tensin homolog
PVDF – polyvinylidene difluoride

qPCR – real time PCR
RASSF1A – ras association domain-containing protein 1
RIN – RNA integrity number
RISC – RNA-induced silencing complex
RNA – ribonucleic acid
RT-PCR – reverse transcription PCR
S-phase – synthesis phase
SCN – suprachiasmatic nucleus
SDS – sodium dodecyl sulfate
SEM – standard error of the mean
SNP – single nucleotide polymorphisms
SOX2 – sex determining region Y-box 2
STAT – signal transducer and activator of transcription
TAM – tamoxifen
TP/p – tumor protein
TRPS1 – trichorhinophalangeal syndrome I
TSA – trichostatin
TudorSN – tudor staphylococcal nuclease
ROR – retinoid-related orphan receptors
RORE – retinoid-related orphan receptors element
UTR – untranslated region
WEE1 – WEE1 G2 checkpoint kinase
WGSBS – whole genome shotgun bisulfite sequencing
WIF1 – wnt inhibitory factor 1
Wnt – wingless integrated signaling pathway
ZEB – zinc finger E-box binding homeobox
ZT – zeitgeber time

CHAPTER 1: BREAST CANCER AND CIRCADIAN DISRUPTION: AN EPIGENTIC LINK?

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ABSTRACT

Breast cancer is already the most common malignancy affecting women worldwide, and evidence is mounting that breast cancer induced by circadian disruption is a warranted concern. Numerous studies have investigated various aspects of the circadian clock in relation to breast cancer, and evidence from these studies indicates that melatonin and the core clock genes can play a crucial role in breast cancer development. Even though epigenetics has been increasingly recognized as a key player in the etiology of breast cancer and linked to circadian rhythms, and there is evidence of overlap between epigenetic deregulation and breast cancer induced by circadian disruption (CD), only a handful of studies have directly investigated the role of epigenetics in CD-induced breast cancer. This review explores the circadian clock and breast cancer, and the growing role of epigenetics in breast cancer development and circadian rhythms. We also summarize the current knowledge and next steps for the investigation of the epigenetic link in CD-induced breast cancer.

INTRODUCTION

Breast cancer (BC) is currently the most common malignancy affecting women worldwide. It accounts for 25% of all cancers in women and caused 522,000 deaths worldwide in 2012 (IARC, 2012). Breast cancer is also present in human males, with males having poorer outcomes due to delays in diagnosis (Giordano et al., 2004). Estimates by the American Cancer Society indicate that there will be around 2350 new cases of male invasive breast cancer in the United States in 2015 (AmericanCancerSociety, 2015). The development of breast cancer emerges through a multi-step process, encompassing progressive changes from a normal cell to hyperplasia, carcinoma *in situ*, invasive carcinoma, and metastasis, with the cancer either originating in the milk ducts, milk-producing lobules, or connective tissues (Abeloff et al., 2008; Shackney and Silverman, 2003; Simpson et al., 2005). Breast cancer tumours also vary in gene expression patterns and other characteristics, resulting in different classifications and treatment options.

Currently, breast cancers are separated into two main classes based on specific phenotypes. The two main classes are dependent on the hormone status of the cancer with estrogen receptor (ER) and progesterone receptor (PR) positive cancers being part of the luminal class, and the ER and PR negative cancers being distinguished as non-luminal (Britten et al., 2013). The luminal status of the breast cancer is then combined with the expression status of the erb-b2 receptor tyrosine kinase (*ERBB2*) gene, a proto-oncogene which encodes for the human epidermal growth factor receptor 2 (HER2) protein, in order to determine if an overabundance of HER2 plays a role in tumour progression and to classify the cancer into one of four specific groups: luminal A (group 1), luminal B

(group 2), HER-2 positive (group 3), and basal-like/triple negative (group 4) (Britten et al., 2013). Groups 1 and 2 are the hormone positive breast cancers, with luminal A being HER2 negative and luminal B being HER2 positive. Groups 3 and 4 are the non-luminal cancers, and as the group names suggest, the HER2-positive group only expresses HER2 and the triple negative group is negative for all three of the phenotypes tested.

Based on the four-group classification system discussed, breast cancer patient treatment options have become more specific and efficient, and the prognosis of patients has become better and more accurate. In terms of the luminal breast cancers, these tumours are less aggressive, lead to a better prognosis, and respond to more treatment options. For example, Tamoxifen (TAM), an ER antagonist drug, is the current treatment choice for groups 1 and 2 at any stage in pre- and post-menopausal women (Li et al., 2013; Mourits et al., 2001). However, TAM treatment is not a valid option for groups 3 and 4 because there is no ER expression in these tumours, but non-luminal breast cancers are more aggressive, offer a poorer prognosis, and respond to fewer treatment options (Gadducci et al., 2005; Li et al., 2013). As a result, a potential approach to treating non-luminal breast cancers is to reactivate ER activity through drugs such as 5-aza-2`deoxycytidine (5-aza) or Trichostatin A (TSA), and then apply TAM treatment or chemotherapy (Jang et al., 2004; Li et al., 2013; Yang et al., 2001). Although this approach may not be as efficient and proven as regular hormonal treatment, it represents a step in the right direction that was made possible by investigating the characteristics of different breast cancers.

In 2007, the International Agency for Research on Cancer concluded that "shiftwork that involves circadian disruption is probably carcinogenic to humans"

(Hansen and Stevens, 2012). Recent case studies have supported this claim by providing indirect evidence that shift workers are at higher risk of developing breast cancer. Specifically, studies found that work after midnight significantly increased the risk of breast cancer in women when compared to day work, that the risk increased with the duration and accumulation of overnight shifts, and that rotating shifts between day and night is more disruptive than permanent night work (Hansen and Stevens, 2012; Knutsson et al., 2013). These case study findings, in combination with the fact that one third of the Canadian labour force is not working a regular daytime shift and that shift work may cause a higher prevalence of ER negative breast cancer, warrants further investigation into the mechanisms underlying breast carcinogenesis due to circadian disruption (CD) (Demers et al., 2010; Rabstein et al., 2013). By investigating the characteristics and mechanisms involved in CD-induced breast cancer, valuable insights and knowledge may materialize, thus potentially allowing for different treatment options to emerge and improving the prognosis of patients.

CIRCADIAN RHYTHMS

A circadian rhythm is any biological process that displays an endogenous, entrainable oscillation of approximately 24 hours, with these biological processes being controlled by a circadian system that is organized in a hierarchical manner.

Suprachiasmatic Nucleus

The paired suprachiasmatic nucleus (SCN) of the anterior hypothalamus acts as the master oscillator of circadian rhythms by regulating downstream peripheral oscillators via endocrine and neural signals (Reppert and Weaver, 2002). This master oscillator is composed of two distinct regions, a shell and a core (Antle and Silver, 2005). The shell

region exhibits uniform oscillations of activity, is not immediately responsive to photic stimulation, and can maintain an approximate 24 hour pattern of activity in the absence of environmental stimuli (Abrahamson and Moore, 2001; Welsh et al., 2010). Unlike the shell, the core does not display synchronized, rhythmic patterns of activity in the absence of an external input (Butler and Silver, 2009). Instead, the core is externally synchronized by environmental light through non-visual, photic retinal ganglion cells (Abrahamson and Moore, 2001; Haus and Smolensky, 2013; Silver et al., 1996). Working together, the core interprets and analyzes information regarding the time of day and entrains the oscillator present within the shell (Antle and Silver, 2005). The shell then regulates the output of the SCN, resulting in the organization of bodily processes on an environmentally synchronized schedule for optimal performance (Antle and Silver, 2005; Welsh et al., 2010).

In humans, many biochemical processes, such as hormone secretion, cell cycle, apoptosis, and gene expression, are entrained on a regular 24-hour rhythm by daily periods of light exposure (Stevens, 2009). Repeated unnatural exposure to light during the dark phase of the circadian cycle, such as through shift work, disrupts the activity of the SCN and inhibits synchronizing of the circadian clock to the proper light-dark cycle (Monsees et al., 2012). Although the SCN has the capacity to adjust to new schedules, this adjustment does not occur instantly; instead, it occurs over a certain number of 24-hour cycles (Haus and Smolensky, 2013). Evidence also shows that the majority of shift workers never actually entrain fully to the aberrant light-dark schedule, resulting in continuously desynchronized circadian rhythms (Folkard, 2008; Haus and Smolensky, 2006). Furthermore, peripheral oscillators depend on the SCN for guidance. However, the

SCN adapts faster than peripheral oscillators to environmental stimuli, and as a result, there is a lag that occurs which causes a desynchronizing between the SCN and the peripheral oscillators (Zelinski et al., 2014). During this lag period, a multitude of rhythmic events can become disrupted, resulting in circadian phase shifts that can lead to complications and disease (Zelinski et al., 2014).

Melatonin

Melatonin (MLT), a pineal hormone that plays a role in the control of sleep, is directly linked to circadian rhythm activity through the master oscillator, the suprachiasmatic nucleus. As a result of this integration, not only does the concentration of MLT fluctuate during a circadian cycle, but the ability of melatonin to influence the SCN also changes (Korkmaz et al., 2009). Through a biological timing signal that is internally driven by the central pacemaker in the SCN, MLT is abundant during the night and almost completely absent during the day, with sleep not being necessary to initiate the increase in melatonin levels, but complete darkness being an absolute requirement (Arendt, 2006; Blask, 2009). Therefore, aberrant exposure to light at nighttime can result in lower melatonin levels and potential complications.

Evidence shows that melatonin appears to be involved in cancer development and growth. In experimental rat models of chemical carcinogenesis, the physiological melatonin signal suppresses the initiation phase of tumourigenesis by suppressing the damage and alterations caused by DNA adducts (Blask, 2009; Reiter, 2004). The ability of melatonin to suppress DNA adduct damage is believed to be due to melatonin acting as a potent free-radical scavenger and/or promoting repair of DNA once damage has occurred (Blask, 2009; Reiter, 2004). Studies also show that at nocturnal concentrations,

melatonin may exhibit biochemical and molecular oncostatic actions. In a rat MCF-7 breast cancer cell xenograft study, evidence showed that constant exposure to light resulted in deregulation of the circadian MLT rhythm, and that the tumour xenografts associated with constant light exposure showed significantly increased growth when compared to the light-dark groups (Blask et al., 2003). Another study, utilizing DMBA induced mammary adenocarcinomas in female rats, illustrated that a constant dim light during the dark phase of the circadian cycle caused higher rates of tumour growth and lower levels of survival (Cos et al., 2006).

A possible explanation for the nocturnal, light-induced tumour growth may be that lower levels of MLT cause increased metabolism of fatty acids and aberrations in cancer-dependent pathways. For example, melatonin can cause receptor-mediated inhibition of cyclic adenosine monophosphate (cAMP), resulting in downregulation of the transcriptional expression of ERα in human breast cancer cells and lower fatty acid metabolism by tumour cells through decreased fatty acid transport (Blask, 2009; Blask et al., 2003; Sauer et al., 2001). Calmodulin (CaM) activity, which plays a role in breast cancer tumour development by suppressing apoptosis and increasing survival through EGF-initiated activation of protein kinase B (Akt), is also lowered by melatonin activity through binding of the pineal hormone to CaM and by melatonin stimulating phosphorylation of CaM through protein kinase C α (PKCα) (Benitez-King et al., 1993; Coticchia et al., 2009; Soto-Vega et al., 2004). Further studies have also shown that human breast cancer xenografts exhibit a day-night rhythm of tumour proliferation, fatty acid uptake, metabolism, and signal transduction activity, all believed to be driven by the nocturnal, circadian rhythm melatonin signal (Blask, 2009; Reiter, 2004).

Clock Genes

In mammals, two to ten percent of all gene expression is rhythmic, and these clock genes, located in peripheral oscillators and found in almost all nucleated cells of the human body, regulate practically every biological process and function in a time-specific manner to help maintain circadian rhythms (Reppert and Weaver, 2002; Storch et al., 2002). Aiding in the maintenance of the circadian clock, the core clock genes function through two interlocking regulatory feedback loops driven by the genes *CLOCK/Npas2*, *Bmal1*, *Period* (*Per*) and *Cryptochrome* (*Cry)* (Figure 1.1). In one regulatory loop, two transcriptional activators, BMAL1 (brain and muscle ARNT-like protein 1) and CLOCK (circadian locomotor output cycles kaput), form CLOCK/BMAL1 heterodimers in the nucleus where they activate the expression of the *Per* and *Cry* genes by binding to E-box promoter sequences (Figure 1.1). In the cytoplasm, the PER and CRY proteins form complexes with each other, move into the nucleus, and inhibit the activity of the CLOCK/BMAL1 heterodimers, thus causing transcription of the *Per* and *Cry* genes to stop (Figure 1.1). In the other regulatory loop, the expression of the *Bmal1* gene is controlled through CLOCK/BMAL1 heterodimer formation in the nucleus (Figure 1.1). These heterodimers bind to the E-box promoter sequences of genes that encode the retinoic acid-related orphan nuclear receptors (ROR), Rev-erbα and Roraα; The Rev-erbα and Roraα proteins then compete for the ROR element (RORE) in the *Bmal1* promoter and suppress or activate *Bmal1* expression, respectively (Figure 1.1).

Through utilization of these circadian feedback loops, the precision of the mammalian clock is controlled through post-translational modifications (Ko and Takahashi, 2006). Casein kinase one epsilon (CKIε) and casein kinase one delta (CKIδ),

are the two main kinases responsible for the phosphorylation of the PER and CRY proteins (Reppert and Weaver, 2002). Through the activity of these kinases, by the end of a regular circadian cycle, the PER1 and PER2 proteins have almost entirely undergone degradation, thereby preventing CLOCK/BMAL1 suppression in the nucleus and initiating the start of the next circadian cycle (Eide et al., 2005). Nuclear entry of the PER and CRY proteins also acts as a vital checkpoint for progression of the circadian cycle, with a co-dependency existing between the two proteins (Reppert and Weaver, 2002). The presence of CRY1 and PER2 interaction in the cytoplasm results in PER2 stability by preventing its degradation through CKIε phosphorylation, and thus permits nuclear translocation and suppression of the CLOCK/BMAL1 heterodimer (Figure 1.1). Alterations to the balance between the CRY and PER proteins can therefore lead to changes in the circadian cycle. For example, the *Tau* mutation in the *CKIε* gene leads to altered levels of phosphorylation of PER proteins, resulting in a shortened circadian length (Lowrey et al., 2000). Furthermore, *Per1* and *Per2* respond and adapt faster than *Cry1* to circadian disruption, and as a result, circadian disruption can induce internal phase shifts within the circadian clock (Nakamura et al., 2005).

Clock Genes and Breast Cancer

In addition to helping regulate circadian oscillations, the core clock genes have been shown to influence cancer related mechanisms such as cell proliferation, apoptosis, cell cycle, and tumour-suppressor genes. Studies have shown that overexpression of *Per1* and *Per2* inhibits the growth of various cancer cells. *Period1* plays a role in cell apoptosis in human cancer cells, with its down-regulation stopping apoptosis and its overexpression increasing DNA damage-induced apoptosis (Gery et al., 2006). The *Per2* gene has been

shown to act as a tumour suppressor in luminal breast cancers by linking the circadian oscillator system to the ERα function (Gery et al., 2007). Human *Per2* is estradiol (E2) inducible in mammary cells, causing *Per2* expression to lower ERα activation through the consumption of E2 (Haus and Smolensky, 2013). Since ER activation through E2 results in DNA adduct formation, mammary cell proliferation, and genotoxic waste, the reduction in ERα activation by *Per2* expression results in an oncostatic effect (Parl et al., 2009).

The overexpression of CLOCK has been linked to increased proliferation in luminal breast cancer cells (Xiao et al., 2014). This oncogenic influence of CLOCK is likely associated with estrogen-estrogen receptor α (E2- ERα) signaling, with E2 promoting the binding of ERα to estrogen response elements (EREs) of the *CLOCK* promoter and increasing transcription (Xiao et al., 2014). Numerous single nucleotide polymorphisms (SNPs) in the *CLOCK* gene have been correlated with increased breast cancer risk (Hoffman et al., 2010a). Interestingly, 67% of the SNPs were associated with non-luminal breast cancers, indicating that *CLOCK* expression most likely plays a role in both luminal and non-luminal BCs (Hoffman et al., 2010a). The same study also showed that CLOCK knockdown results in increased expression of various tumour suppressor genes and decreased expression of multiple oncogenes, indicating an oncogenic influence by CLOCK on breast cancer development (Hoffman et al., 2010a).

Unlike CLOCK, BMAL1 has been reported as having oncostatic effects in cancer development. Specifically, BMAL1 has been shown to suppress cancer cell invasion by antagonizing the oncogene B-cell lymphoma-w (*Bcl-w*), thus suppressing matrix metalloproteinase-2 (MMP-2) accumulation and blocking the PI3K-AKT-MMP2

signaling pathway (Jung et al., 2013). Furthermore, loss of *Bmal1* expression has been shown to result in lower transcript levels of *Per1* and *Per2*, and increased protein levels of cyclin D1 (Zeng et al., 2010). The cyclin D1 protein is involved in promoting transition from the G_1 to the S-phase in the cell-cycle by binding to cyclin-dependent kinase 4 (CDK4) or CDK6 proteins, and its overexpression enhances the activity of ERα and counteracts the breast cancer-suppressor gene breast cancer 1, early onset (*BRCA1*) (Wang et al., 2005). In mice and human cancer patients, overexpression of cyclin D1 is associated with mammary tumourigenesis and poor medical prognosis, respectively (Fu et al., 2002; Haus and Smolensky, 2013).

As an extension of the influences of CLOCK and BMAL1 on cancer development, the CLOCK/BMAL1 heterodimer has been shown to regulate cell-cycle genes that are involved in breast cancer progression (Haus and Smolensky, 2013). This regulation occurs in an oncogenic manner through the activation of WEE1 G_2 checkpoint kinase (WEE1), and an oncostatic manner through the suppression of myc avian myelocytomatosis viral oncogene homolog (c-MYC) (Chen-Goodspeed and Lee, 2007; Fu et al., 2002; Haus and Smolensky, 2013; Matsuo et al., 2003). In breast cancer cells, the G_1 cycle checkpoint is often aberrantly regulated, allowing cells with DNA damage to enter the S phase (Vriend et al., 2013). When this occurs, cancer cells rely on the G_2 check point for damage repair before entering mitosis (Vriend et al., 2013). Over expressed in breast cancer cells, the WEE1 protein is a nuclear kinase that helps regulate transition from the G_2 phase to mitosis by inhibiting the activity of the CDK1 protein, thus increasing DNA repair in cancer cells prior to mitosis (Parker and Piwnica-Worms, 1992; Vriend et al., 2013).Overexpressed *c-Myc* causes increased cell proliferation

through cyclin activity and lowered apoptosis through Bcl-2 activity, with *c-Myc* overexpression eventually leading to hyperplasia and tumour development (Fu et al., 2002).

Although both the *Cry* genes may be implicated in breast cancer development, the majority of findings thus far have pinpointed *Cry2* as playing a more prominent role. In a study investigating the potential significance of *Cry2* in breast cancer development, experimenters identified SNPs in the *Cry2* gene as potential biomarkers for increased risk of breast cancer, with the variants having a stronger association with non-luminal breast cancers (Hoffman et al., 2010b). The results also illustrated that the degree of *Cry2* expression can vary between breast cancer types, with the non-luminal breast cancers having higher levels of *Cry2* expression when compared to luminal breast cancers (Hoffman et al., 2010b). Finally, CRY2 knockdown in MCF-7 cancer cells resulted in aberrant regulation of cell differentiation, proliferation, motility, angiogenesis, and apoptosis, thus suggesting a tumour-suppressor role for CRY2 in breast cancer development (Hoffman et al., 2010b).

As discussed thus far, there is clear evidence connecting the circadian clock and its components to breast cancer development through hormone activity and the influence of core clock genes. However, there is another area of study that may provide the crucial link in CD-induced breast cancer.

EPIGENETICS

Epigenetics is the study of heritable changes in gene expression or cellular phenotype by mechanisms that do not involve direct changes to the DNA sequence. Two examples of epigenetic mechanisms are DNA methylation and RNA associated silencing.

DNA Methylation

The biochemical process of DNA methylation involves the addition of a methyl group to the 5` carbon of the cytosine nucleotide (Klose and Bird, 2006). Cytosine DNA methylation is one of the most widely studied and known epigenetic mechanisms (Klose and Bird, 2006). DNA methylation plays a crucial role in normal cell development, cell proliferation, and maintenance of genome stability (Klose and Bird, 2006). In mammals, DNA methylation is mediated by DNA methyltransferase enzymes. DNA methyltransferase 1 (DNMT1) is the major enzyme involved in maintenance DNA methylation and is responsible for copying DNA methylation patterns to daughter strands during DNA replication (Liang et al., 2002). DNA methyltransferase 3a (DNMT3a) and DNMT3b act as de novo methyltransferases that target unmethylated and hemimethylated sites, and are responsible for DNA methylation patterns early in development (Goll and Bestor, 2005). Finally, although DNMT3L has no methyltransferase activity, it interacts with DNMT3a and 3b to help establish maternal methylation imprints and appropriate expression of maternally imprinted genes (Hata et al., 2002).

The activity of DNA methyltransferases mostly occurs in the context of CG dinucleotides (CpG), with about 60–80% of CpG cytosines being methylated in mammals (Weber and Schubeler, 2007). Hypermethylation of CpG dinucleotides, which is over methylation of DNA, is linked to genomic instability due to silencing of DNA repair genes, silencing of tumour-suppressor genes, and compaction of chromatin (Jovanovic et al., 2010). Hypomethylation of CpG dinucleotides, which is the absence of cytosine methylation, has been linked to gene reactivation, chromosomal instability, and up-regulation of proto-oncogenes (De Smet et al., 2004). The ability of DNA methylation to

cause transcriptional repression is achieved through methyl CpG-binding domain (MBD) proteins, with these proteins selectively interacting with methylated DNA in order to achieve gene silencing (Klose and Bird, 2006). Many genes in mammals also have CpG islands that are found close to promoter regions of genes (Merlo et al., 1995). These short stretches of DNA, located in the 5`-regions of 60% of all genes, contain high concentrations of CpGs, with the methylation status of these islands usually being reversed in tumour cells when compared to normal cells for genes that play a role in cancer development (Jovanovic et al., 2010).

DNA Methylation and Breast Cancer

In recent years, recognition of the role DNA methylation plays in the etiology of breast cancer has increased. In terms of hypermethylation, more than 100 genes have been reported to be hypermethylated in breast tumours or breast cancer cell lines, with many of these genes playing a role in DNA repair, cell-cycle regulation, apoptosis, and metastasis. Among the hypermethylated genes involved in DNA repair are *BRCA1* and RAD9 Homolog A (*RAD9*) (Jovanovic et al., 2010). *BRCA1* is an extensively studied tumour-suppressor gene involved in double-stranded DNA breaks through homologous recombination repair in late S and G_2 phases of the cell-cycle, while RAD9 is involved in base excision repair, mismatch repair, and homologous recombination repair (Jiang et al., 2013; Lieberman et al., 2011). Some of the hypermethylated genes involved in cell-cycle regulation are dickkopf WNT signaling pathway inhibitor 3 (*DKK3*) and wnt inhibitory factor 1 (WIF1), and some of the genes involved in apoptosis are homeobox A5 (*HOXA5*) and target of methylation-induced silencing (*TMS*1) (Jovanovic et al., 2010; Veeck et al., 2009). Both *WIF1* and *DKK3* are Wnt signaling-pathway antagonists, with

increased Wnt pathway activity being associated with lowered cell senescence, resistance to apoptosis, and increased resistance to anti-cancer therapies (Lamb et al., 2013; Veeck et al., 2009). *HOXA5* expression increases apoptotic activity in a p53-dependent manner and through caspase activity (Chen et al., 2004b). Although the exact mechanism by which *TMS1* induces apoptosis has not yet been fully elucidated, *TMS1* is a proapoptotic gene and a sensitive prognostic marker for tumourigenesis (Salminen et al., 2014). Hypermethylation of tumour-suppressor genes can also be an early marker in breast cancer and be compounded by DNMT activity. For example, hypermethylation of the tumour-suppressor gene Ras association domain-containing protein 1 (*RASSF1A*) is an early epigenetic event in breast cancer, and it has been shown to increase linearly in benign breast tissue of women between 23 and 55 years of age (Euhus et al., 2008).

The hypermethylation status of certain genes has also been linked as a predictor of hormone receptor development in breast carcinogenesis. DNA methylation of estrogen receptor1 *(ESR1)* and progesterone receptor *(PR)* promoters has been proposed as a mechanism for the development of ER-negative tumours (Ferguson et al., 1995; Lapidus et al., 1996; Weigel and deConinck, 1993). A recent clinical study has also shown that ERα-promoter hypermethylation has a strong correlation with ER-negative tumours, PR-negative tumours, and non-luminal tumours (Izadi et al., 2012). Furthermore, another study conducted a CpG methylation-pattern analysis on known breast cancer tumour-suppressor genes in order to correlate methylation status with hormone expression. The study found that a combination of reversion-induced LIM (*RIL*) and cadherin 13 (*CDH13*) hypermethylation was strongly correlated with ER- and PR-negative tumours, while a combination of high in normal (*HIN-1*) and *RASSFIA* methylation was associated

with ER- and PR-positive tumours (Feng et al., 2007).

Although DNA hypomethylation was the first epigenetic abnormality discovered in human tumours, it was somewhat cast into the shadows at the expense of tumour-suppressor genes and hypermethylation (Ehrlich, 2009; Feinberg and Vogelstein, 1983). More widely spread across the genome and associated with repetitive DNA elements, DNA hypomethylation was not only ignored, but it was more difficult to pinpoint with limited technology (Brena and Costello, 2007; Ehrlich, 2009). However, as scientific technologies have continued to improve, the role of DNA hypomethylation in breast cancer development has become more illuminated.

Among the types of repetitive DNA elements affected by global hypomethylation are transposable DNA elements (Hormozdiari et al., 2011). Constituting 11% and 17% of the human genome, long interspersed nucleotide element-1 (LINE-1) and Alu are major components of these repetitive transposable elements (Lander et al., 2001). Given their vast distribution in the genome, these regions are often used as markers for genome-wide methylation status (Park et al., 2014). In addition, hypomethylation of these regions has been linked to various cancers, with lower methylation causing transcriptional activation, retrotransposition, genomic instability, and the decrease in methylation correlating with cancer progression (Kitkumthorn and Mutirangura, 2011; Park et al., 2014). In a recent study, researchers investigated the link between Alu and LINE-1 hypomethylation and breast cancer subtypes (Park et al., 2014). The results showed that Alu hypomethylation was correlated with an ER-negative status in invasive breast cancer (IBC), while LINE-1 hypomethylation was correlated with an ER-negative status and HER2 expression (Park et al., 2014). The findings also suggested that LINE-1 hypomethylation is an early event

in breast cancer development, while Alu hypomethylation is a late event in breast cancer progression, and that lower Alu methylation is associated with poorer disease-free patient survival (Park et al., 2014).

Among the advances in technology that have allowed for further insight into DNA hypomethylation and cancer research is whole-genome shotgun bisulfite sequencing (WGSBS). Through the use of this technique, researchers have identified that cystosines methylated in the CpG content are almost completely methylated in pluripotent cells, but are only partially methylated in somatic cells (Lister et al., 2009). Regions of these partially methylated cystosines can be found in nearly 40% of the genome and have been classified as partially methylated domains (PMDs) (Lister et al., 2009). Although hypomethylation has been traditionally associated with increased gene expression, a recent study utilized WGSBS on HER2-positive breast cancer cells and found that global hypomethylation is associated with PMDs and gene silencing (Hon et al., 2012). Specifically, a significant fraction of genes within the hypomethylated regions showed allelic DNA methylation at one allele, and no methylation coupled with repressive chromatin marks H3K9me3 or H3K27me3 at the other allele (Hon et al., 2012). The data from the experiment also suggest that global hypomethylation in breast cancer likely occurs through gradual loss of DNA methylation instead of an active mechanism involving demethylating enzymes, and that this gradual global hypomethylation most likely triggers the repressive chromatin domains (Hon et al., 2012).

Although hypomethylation of transcription regulatory regions in breast cancer is rare when compared to hypermethylation of CpG islands, it still does occur. For example, the gene encoding protease urokinase-type plasminogen activator (*PLAU/uPA*) is

hypomethylated and over expressed in conjugation with tumour progression in breast cancers, with increased *uPA* expression resulting in increased tumour aggression and poor clinical outcome (Annecke et al., 2008; Pakneshan et al., 2004). A high-resolution analysis of DNA methylation in breast cancer identified approximately 1500 regions that are hypomethylated (Jovanovic et al., 2010; Novak et al., 2008; Shann et al., 2008). Taken together, this evidence, in combination with the previous studies discussed, indicates that many of these hypomethylated regions may not only contribute to genomic instability, but probably contain genes and regulatory sequences involved in breast cancer development.

DNA Methylation and Circadian Rhythms

Although not many studies have demonstrated a direct role for DNA methylation in the circadian clock, there is evidence suggesting that this epigenetic process may play a pivotal role in influencing circadian rhythms. Furthermore, through numerous studies, every core clock gene has been identified as being aberrantly methylated in various human malignancies (Joska et al., 2014). Among these malignancies is breast cancer, with many studies illustrating methylation profiles of the core clock genes that are consistent with the aberrant expression patterns previously discussed.

A recent study investigated the role of DNA methylation on circadian behaviour by using a mouse model system and measuring the effect of altered day length on global DNA methylation within the SCN (Azzi et al., 2014). The results showed that mice entrained to a 22-hour day had an altered endogenous free-running period (FRP) and 1, 294 differentially methylated regions when compared to mice entrained to a regular 24-hour day (Azzi et al., 2014). Among the differentially methylated regions were

hypermethylated *Per2* and *Cry1* promoters, and hypomethylated *CLOCK* promoters (Azzi et al., 2014). To verify further the influence of DNA methylation on FRP, the researchers inhibited methylation through the global DNMT inhibitor zebularine, and the results showed a significant suppression of FRP changes in the DNMT-inhibited mice. Finally, the study illustrated that the aberrant changes to FRP through DNA methylation are plastic, with short-day mice entrained back to a regular 24-hour day showing a reversion in FRP and DNA methylation levels (Azzi et al., 2014). These results represent the first evidence of a direct role for dynamic DNA methylation in the circadian clock, and illustrate that the activity of the enzymes catalyzing DNA methylation may be influenced by light-dependent induction or repression.

As discussed previously, the core clock genes can influence breast cancer development through a variety of mechanisms. It is therefore not surprising that promoter methylation profiles in breast cancer correlate with the oncogenic and oncostatic actions of the core clock genes. For example, a pair of studies showed that all the breast cancer types tested had *CLOCK*-promoter hypomethylation and *Cry2*-promoter hypermethylation when compared to controls (Hoffman et al., 2010a; Hoffman et al., 2010b). Another study illustrated that 95% of the breast cancer cells tested in women showed aberrant promoter methylation in the *Period* genes, including *Per1*- and *Per2*-promoter hypermethylation (Chen et al., 2005). The methylation status of the *Per* genes was also linked to increased expression of *ERBB2*, the gene used to help classify breast cancers by type and a prognostic marker that negatively correlates with disease-free survival and overall survival (Chen et al., 2005; Tsutsui et al., 2002).

Even though there is some evidence for the influence of DNA methylation on

circadian rhythms and a consistent promoter-methylation pattern between the core clock genes and breast cancer development, skepticism remains about the actual role of DNA methylation in circadian rhythms. The question remains whether aberrant DNA methylation at clock genes in cancer cells is the cause of circadian disruption, or simply a bystander effect of deregulated circadian rhythms. One theory, based on data from *Neurospora*, suggests that the aberrant methylation does not play a causative role and is instead a readout (Joska et al., 2014). In either case, the epigenetic influence on CD-induced breast cancer likely extends into another epigenetic mechanism.

MicroRNAs

Epigenetic control can also be mediated by means of small regulatory RNAs, specifically microRNAs (miRNAs). Mature miRNAs are abundant, small, single-stranded, noncoding RNAs that are potent regulators of gene expression (Hwang and Mendell, 2006). Functional (mature) miRNAs can originate from either the exons or introns of non-coding genes, or introns of protein-coding genes (Liu et al., 2008a; Liu et al., 2008b; Rodriguez et al., 2004). The miRNAs found within the transcripts of other genes are under direct transcriptional control of those genes, while miRNAs found within intergenic regions are under their own control (Liu et al., 2008a; Liu et al., 2008b). Furthermore, miRNAs can be transcribed as a single unit or part of a polycistron, with polycistron miRNA expression acting as an efficient mechanism to target a single mRNA or to target multiple mRNAs in a signal molecular pathway (Fujita and Iba, 2008; Griffiths-Jones et al., 2008; Koturbash et al., 2011).

The production and development of mature miRNAs in mammals involves multiple steps and numerous proteins and complexes (Ranganna et al., 2013). First,

transcription results in primary miRNA (pri-miRNA) in the nucleus, with the leading candidate for this pri-miRNA transcription being RNA polymerase (pol) II, but evidence showing that RNA pol III may also play a role in some transcripts (Chen et al., 2004a; Lee et al., 2004). The RNase III endonuclease Drosha then forms a microprocessor complex with the protein DGCR8/Pasha, and digests the pri-miRNA to release a 60–70 nucleotide (nt) stem-loop intermediate known as precursor miRNA (pre-miRNA), with the base of the stem loop having a 5` phosphate and a two nucleotide 3` overhang that is characteristic of RNase III cleavage (Krol et al., 2010; Lee et al., 2002). The pre-miRNA is then transported to the cytoplasm by Ran-GTP and the export receptor Exportin5, where it undergoes more processing by the enzyme Dicer (Lee et al., 2002). With the nuclear processing by Drosha defining one end of the pre-miRNA, the other end is processed by Dicer, and as this enzyme is also an RNase III endonuclease, the cleavage results in a 5` phosphate and a two nucleotide 3` overhang in place of the stem loop, as well as the formation of a mature double stranded miRNA duplex (miRNA:miRNA*) (Lee et al., 2003; Lee et al., 2002).

In order to control the translation of their target mRNAs, the mature miRNA duplex associates with the RNA-induced silencing complex (RISC), which is composed of the proteins Argonaute (AGO), protein activator of the interferon-induced protein kinase (PACT), fragile X mental retardation protein (FXR), tudor staphylococcal nuclease (Tudor-SN), as well as other proteins (Hutvagner and Zamore, 2002). However, evidence shows that the miRNA:miRNA* duplex is generally short-lived in comparison to the mature single-stranded miRNA, with fragments from the opposing arm (miRNA*) being found in much smaller quantities in libraries of cloned miRNAs (Lagos-Quintana et

al., 2002; Lim et al., 2003). Although it appears that the AGO protein initiates the dissociation of one of the strands, the exact mechanism of strand selection in mammals has yet to be elucidated (Koturbash et al., 2011). However, one theory suggests that the miRNA* is peeled away and degraded when the miRNA:miRNA* duplex is loaded into RISC because the other arm of the duplex has a less-tightly paired 5` end, and as a result, it is more thermodynamically favourable to separate the duplex in this manner when association with RISC occurs (Khvorova et al., 2003).

Once the miRNA-RISC complex is assembled, it then binds to the 3` UTR (untranslated region) of target mRNAs, resulting in posttranscriptional down regulation of gene expression by one of two mechanisms; mRNA cleavage or translational repression. If there is extensive complementarity between the miRNA and the 3` UTR, then cleavage of the mRNA occurs (Hutvagner and Zamore, 2002). However, if the homology between the miRNA and 3` UTR is not sufficient, productive translation can be repressed through processing bodies (P-bodies) (Liu et al., 2005). In mammals, binding is usually not very high in complementarity, so not only is suppression of gene expression by miRNAs usually achieved through inhibitory machinery, but each miRNA has numerous targets and can influence the expression of many different genes (Thomson et al., 2011).

MicroRNAs and Breast Cancer

The activity of mature miRNAs has an impact on a variety of cancer related processes, such as cellular differentiation, proliferation, apoptosis, and genome stability. One role by which miRNAs can influence these processes is by acting as oncogenic miRNAs (oncomiRs). Among the most well-studied and abundant oncomiRs in various

cancers, including breast cancer, is miR-21 (Koturbash et al., 2011). With its expression being linked to induction by signal transducer and activator of transcription 3 (STAT-3), a protein shown to be overexpressed in breast cancer and linked to various oncogenes, miR-21 is often overexpressed as well, resulting in the negative regulation of various tumour-suppressor genes (Chen et al., 2013b; Koturbash et al., 2011). Specifically, miR-21 has been shown to down regulate the expression of the tumour suppressors phosphatase and tensin homolog (*PTEN*) and programmed cell death 4 (*$PDCD_4$*) (Frankel et al., 2008; Gong et al., 2011). In breast cancer, PTEN coordinates G_1 cell-cycle arrest by down regulating the cyclin D1 protein and increasing p27 expression, causing reduced cell growth and increased apoptosis (Lu et al., 1999; Weng et al., 2001). $PDCD_4$ inhibits CDK1 activity through induction of p21, and loss of $PDCD_4$ is linked to breast cancer development (Frankel et al., 2008; Goke et al., 2004). An experiment also showed that knockdown of miR-21 in MCF-7 and MDA-MB-231 breast cancer cell lines inhibited *in vitro* growth and migration, as well as *in vivo* growth (Yan et al., 2011).

The expression and activity of oncomiRs can also extend into miRNA clusters. For example, the miR-17-92 cluster was among the first miRNA groups discovered to be deregulated in various cancers, including breast cancer (Koturbash et al., 2011). Containing six miRNAs that are transcribed together as a single polycistron, up-regulation of these miRNAs has been linked with increased levels of cell proliferation and lowered apoptosis (Koturbash et al., 2011). Another oncomiR cluster, the miR-221/222 tandem, has been linked to ER-negative and triple negative/basal-like breast cancers (Stinson et al., 2011; Zhao et al., 2008). In terms of ER-negative breast cancer, miR-221/222 was shown to target the 3` UTR of ERα, resulting in lowered ERα protein

levels in MCF-7 breast cancer cell lines and increased resistance to TAM treatment (Zhao et al., 2008). Triple negative breast cancer is notoriously more aggressive than all the other breast cancer groups, and it seems that this aggressiveness may be linked to miR-221/222 expression (Stinson et al., 2011). Epithelial-to-mesenchymal transition (EMT) is the process by which epithelial cells lose the adherent properties and tight junctions that keep them in contact with neighbouring cells and gain mesenchymal properties, causing gene expression changes within the cells, resistance to apoptosis, and the ability to break through the basal membrane and migrate over long distances (Kalluri and Weinberg, 2009). Evidence shows that the miR-221/222 cluster promotes EMT by repressing expression of the trichorhinophalangeal syndrome 1 (TRPS1) protein, which in turn results in an increase of the EMT-promoting protein zinc finger E-box binding homeobox 2 (ZEB2), thus potentially contributing to the more aggressive clinical behaviour of triple negative breast cancer (Stinson et al., 2011).

In addition to acting as oncomiRs, miRNAs can also act as tumour suppressors. Several individual miRNAs have been identified as tumour suppressors in breast cancers. Among these are miR-99a and miR-140. MicroRNA 99a overexpression has been shown to induce G_1 cell-cycle arrest and apoptosis by lowering expression of the mammalian target of rapamycin (*mTOR*) gene, with miR-99a being a potential candidate for use as a therapeutic strategy for effectively controlling breast cancer development (Hu et al., 2014). The transcription factor sex determining region Y-box 2 (SOX2) has been reported to be overexpressed in breast cancers and identified as playing a role in the early steps of tumour initiation through cancer stem cells (CSCs) (Leis et al., 2012). *SOX2* has been identified as a target of miR-140, and in ER-α breast cancer cells, estrogen

stimulation lowers miR-140 expression through ER-α binding to an ERE flanking the mir-140 promoter, thus increasing levels of *SOX2* (Zhang et al., 2012). MicroRNA tumour-suppressor activity can also extend into miRNA clusters and result in more potent regulation than through individual miRNAs. For example, the miR-143/145 cluster has been shown to lower ERBB3 protein levels and cause less proliferation and invasion in breast cancer cells (Yan et al., 2014). Interestingly, although the miRNAs are still capable of causing tumour suppression effects on their own, expression of the miR-143/145 cluster results in more potent tumour suppression through a synergistic effect (Yan et al., 2014).

MicroRNAs and Circadian Rhythms

MicroRNA activity has been shown to be associated with circadian rhythms through rhythmically regulated miRNAs and through direct influence on the circadian cycle. Numerous studies have illustrated that miRNAs oscillate throughout a 24-hour circadian day. For example, in *Arabidopsis*, miRNAs-167, 168, 171, and 398 were shown to oscillate, based on environmental light; daytime levels were also reported as higher than nighttime levels, and the oscillation of these miRNAs was governed through photic control and not an internal clock (Sire et al., 2009). A microarray-based study on mouse livers also revealed that over 13% of the probed miRNAs exhibited circadian expression patterns (Na et al., 2009). Among these miRNAs were miR-181d and 191, with an inversely-correlated expression pattern being observed between these miRNAs and their respective targets, *CLOCK* and *Bmal1* (Na et al., 2009). In another study conducted on HeLa cell lines, results showed that the miR-192/194 cluster directly regulates the entire *Period* gene family, with increased expression of the miR192/194 tandem causing

lowered expression of the *Period* genes and a shortening of the circadian cycle (Nagel et al., 2009).

Of the miRNAs involved in circadian rhythms, miR-132 may potentially play a very prominent role, especially when considering the link between circadian rhythms and breast cancer. A rodent model experiment showed that miR-132 exhibited oscillatory expression in wild-type mice, but not in circadian mutant mice (Cheng et al., 2007). Further investigation has also illustrated that miR-132 is induced by light within the SCN and that it has the capacity to entrain or reset the circadian clock (Alvarez-Saavedra et al., 2011). The proposed model suggests that nocturnal light triggers the chromatin remodeling gene methyl CpG binding protein 2 (*MeCP2*), which triggers the transcription of various light-responsive genes, including the miR-132 promoter, *Per1*, *Per2*, PABP interacting protein 2A (*Paip2A*), and B-cell translocation family member 2 (*Btg2*) (Alvarez-Saavedra et al., 2011). As PERIOD, PAIP2A, and BTG2 protein levels increase, PAIP2A and BTG2 levels eventually reach a certain level and *Per1* and *Per2* transcription is inhibited by these two proteins (Alvarez-Saavedra et al., 2011). Simultaneously, levels of mature miR-132 also increase through miR-132 promoter transcription and processing of pri-miRNA into functional miR-132 (Alvarez-Saavedra et al., 2011). As levels of mature miR-132 increase, translational repression of its targets *Mecp2*, *Paipa2A*, and *BTG2* eventually occurs, thus restoring homeostasis and resetting the induction caused to the circadian clock by nocturnal light (Alvarez-Saavedra et al., 2011).

Interestingly, a recent study has reported that miR-132 levels are lowered in breast cancer cells, with miR-132 being identified as a tumour suppressor by inhibiting

proliferation, migration, invasion, and metastasis (Zhang et al., 2014). Therefore, by extension, the combination of light at night and increased breast cancer risk through shift work could potentially result in an aberrant compounded effect through lower miR-132 expression. Due to increased risk of breast cancer development, miR-132 levels may be lowered, and as a result, further nocturnal exposure to light would not only increase deregulation of the circadian clock due to the inhibition of homeostasis induced by miR-132, but also cause lowered oncostatic activity through increased transcriptional inhibition of the *Period* genes by PAIP2A and BTG2.

Although none of the research discussed thus far has investigated the link between epigenetics, circadian disruption, and breast cancer directly, the fact that the studies discussed have dealt with parts of this equation and illustrate potential overlap, demonstrates that a potentially substantial link may exist between all three factors.

CD-INDUCED BREAST CANCER: AN EPIGENETIC LINK?

As discussed thus far, numerous studies have investigated the link between circadian rhythms and breast cancer, epigenetics and breast cancer, and epigenetics and circadian rhythms. However, to date, only a handful of studies have incorporated all three factors and investigated the link between CD-induced breast cancer and epigenetic modifications. One of these studies incorporated a rodent model to investigate global methylation, while the other studies were conducted on human blood samples and focused on the influence of shift work on DNA methylation in peripheral blood cells.

The rodent model study, conducted to investigate the link between CD-induced breast cancer and epigenetic modifications, involved measuring the effect of circadian disruption on global methylation levels and the effect of melatonin on this relationship

(Schwimmer et al., 2014). Three groups were used in the study: a control group, a light at night (LAN) group, and a LAN plus MLT group, with all three of the groups being injected with the 4T1-murine breast cancer cell line. The results showed that the LAN group had ~30% less global methylation and larger tumour size when compared to the control group, while the LAN+MLT group only showed a ~10% decrease in global methylation and the smallest amount of tumour growth out of all three groups (Schwimmer et al., 2014). These results not only reiterated the important role that MLT plays in breast cancer development, but illustrated the important role that DNA methylation may play in CD-induced breast cancer and that melatonin levels may be linked to the aberrant DNA methylation that is occurring.

In a genome methylation analysis of 5,409 CpG sites on nurse blood samples, data showed that 3359 (66.4%) of the sites were hypermethylated and 1,816 (33.6%) were hypomethylated in long-term shift workers, as compared to daytime workers (Zhu et al., 2011). Many of these differentially methylated CpG sites were located near the promoter sequences of circadian-related and cancer-relevant genes. Specifically, aberrant methylation of the circadian genes *CLOCK* and *Cry2* was reported, with shift workers exhibiting *CLOCK* hypomethylation and *Cry2* hypermethylation when compared to day workers, a pattern that has been associated with BC and discussed previously (Hoffman et al., 2010a; Hoffman et al., 2010b). In another study, a total of 50 CpG sites across 26 unique imprinted genes showed significant differences in methylation between shift workers and day workers, with a tendency towards more hypomethylation than hypermethylation (Jacobs et al., 2013). The genes that showed the highest variation in methylation, distal-less homeobox 5 (*DLX5*) and tumour protein 73 (*TP73*), are genes

that are both linked to cancer development (Jacobs et al., 2013). The *DLX5* gene is a transcriptional factor that promotes cell proliferation through up-regulation of *c-MYC* promoter activity and its expression has been linked with breast cancer, while the *TP73* gene is an important component of the p53 family of cell-cycle regulatory proteins and is altered in the majority of cancers (Morini et al., 2010; Sigal and Rotter, 2000). Together, these two studies were among the first to illustrate that shift work can result in aberrant changes to DNA methylation at specific genes that are not only linked to circadian rhythms, but also breast cancer development.

The most recent human blood-analysis study conducted a genome-wide CpG island methylation assay of blood samples at miRNA promoters (Shi et al., 2013). The results illustrated that 50 CpG loci, corresponding to 31 miRNAs, were differentially methylated in shift workers when compared to day workers. Among the differentially methylated genes was hypermethylation of the promoter for miR-219, a miRNA that has been linked to breast cancer development and has been identified as a potential regulator of circadian duration via the modulation of CLOCK- and BMAL1-dependent *Per* transcription (Cheng et al., 2007; Liu and Wang, 2012; Shi et al., 2013). To investigate further, the experimenters performed a genome-wide microarray on an overexpressed miR-219, MCF-7 breast cancer cell line, which identified 319 differentially expressed transcripts (Shi et al., 2013). Among the cancer-relevant pathways affected by these changes were increased apoptosis and immunomediated antitumour activity. In a follow-up study, the experimenters investigated the role of another miRNA that showed significant promoter hypermethylation in the shift worker blood samples, miR-34b (Liu et al., 2015). MCF-7 breast cancer cells were transfected with miR-34b, and 230

differentially expressed transcripts were identified, corresponding again to increased apoptosis and immunomediated anti-tumour activity. Even though these studies analyzed DNA methylation levels at specific locations like the previous experiments discussed, they were the first to illustrate that the epigenetic modifications involved in CD-induced breast cancer may extend into miRNA regulation.

FUTURE DIRECTIONS AND CONCLUSIONS

Although these handful of studies have provided valuable insights into the role of epigenetics in CD-induced breast cancer, there are glaring omissions and new directions that need to be investigated. First, none of the studies investigated the effect of CD on breast cancer development in mammary tissues. Second, although the experiment performed by Schwimmer et al. did incorporate a circadian disruption scheme on a rodent model, the CD scheme was not very in-depth because it did not incorporate varying degrees of CD, and the effect of CD was skewed due to artificial MLT levels. Third, all the studies focused on DNA methylation profiles, and although the Shi et al. study identified aberrant DNA methylation at miRNA promoters, it did not measure direct changes in small RNA expression due to CD. Fourth, none of the studies considered the potential fluctuations in epigenetic modifications due to varying time points within a circadian cycle. Finally, although the studies illustrated epigenetic changes due to CD and their potential role in breast cancer development, none of the studies showed direct downstream consequences due to these epigenetic modifications. Therefore, to shed new light on the epigenetic modifications involved in CD-induced breast cancer, focus needs to be placed on investigating direct CD-induced changes in DNA methylation and miRNA levels in mammary tissues. To accomplish this, environmentally controlled

experiments utilizing model systems and incorporating varying degrees of circadian disruption, mammary tissue extractions at various time points following CD, and mammary tissue extractions at specific time points within the circadian cycle, need to be performed. This approach will allow for a closer investigation into the dynamic and influence of CD on breast cancer development, and new insights and directions may be generated that will lead to a deeper understanding of the potential carcinogenicity of varying degrees of CD, influence of epigenetic circadian fluctuations on tumourigenesis, and the direct effect of CD on epigenetic processes in mammary tissues.

In summary, several conclusions can be drawn from the existing literature:

• Circadian rhythms play a role in breast cancer development. Aberrant exposure to light during the dark phase of the circadian cycle can disrupt the activity of the SCN and cause aberrant changes to downstream processes that influence breast cancer. Among these processes are nocturnal MLT and core clock gene activity, with lower nocturnal MLT levels causing oncogenic effects and the core clock genes influencing a variety of cellular processes related to breast cancer.

• The role of epigenetics has been recognized increasingly in the etiology of breast cancer. Two epigenetic mechanisms, DNA methylation and miRNA activity, influence a variety of cellular processes related to breast cancer development. In addition, these two epigenetic processes have also been linked to circadian rhythms. DNA methylation has been shown to regulate core clock gene expression and may also potentially play a direct role in the circadian clock. While miRNAs have been shown to oscillate with the circadian cycle and directly influence components of the circadian clock.

• Although literature has provided evidence for a link between epigenetic modifications and circadian rhythms, with a clear overlap existing between these two processes and breast cancer development, only a handful of studies have incorporated all three factors and directly studied the epigenetic modifications involved in CD-induced breast cancer. Although these studies represent a good start, there are still many aspects and different directions that need to be addressed when investigating the epigenetic modifications involved in CD-induced breast cancer.

PRESENT STUDY AND HYPOTHESES

In order to address the discussed shortcomings and provide new insights on the epigenetic link in CD-induced breast cancer, the current study employed an environmentally controlled experiment using a rodent model system. To induce circadian disruption, a previously used photoperiod-shifting paradigm that has shown evidence of causing physiological and behavioural changes in rodents was utilized (Craig and McDonald, 2008; Devan et al., 2001). To shed new light on CD-induced BC, the study incorporated varying degrees of circadian disruption, mammary tissue extractions at various time points following CD, mammary tissue extractions at specific time points within the circadian cycle, analysis of gene expression and small RNA levels in the mammary tissues, and investigation into the potential consequences of the aberrant modifications identified.

Given evidence from previous literature, the prediction was that circadian disruption would result in differences between the CD-exposed and control mammary gland tissues in terms of gene expression and miRNA expression. More specifically, there would be differences in the aberrant changes based on the degree of CD, with the

most adamant changes occurring in the groups undergoing the highest degree of CD.

In terms of miRNA expression, the hypothesis was that circadian disruption would result in aberrant changes to miRNAs that have been shown to oscillate with the circadian cycle, miRNAs that target circadian-related genes, and miRNAs that are linked to breast cancer development. There also may be expression patterns linked to these miRNAs that depend on either the degree of CD or the time of tissue extraction relative to the circadian cycle.

In terms of gene expression, the hypothesis was that circadian disruption would result in changes to genes involved in circadian rhythms and breast cancer development, and that the aberrant changes would be at a high enough level to influence breast cancer related pathways.

Finally, we hypothesized that the CD-induced changes in miRNA expression would result in downstream consequences in the mammary tissues through changes in related protein levels.

To test these hypotheses, the study was broken down into two parts: miRNA expression and gene expression. Chapter 2 discusses with the miRNA results, while chapter 3 discusses with the gene expression results.

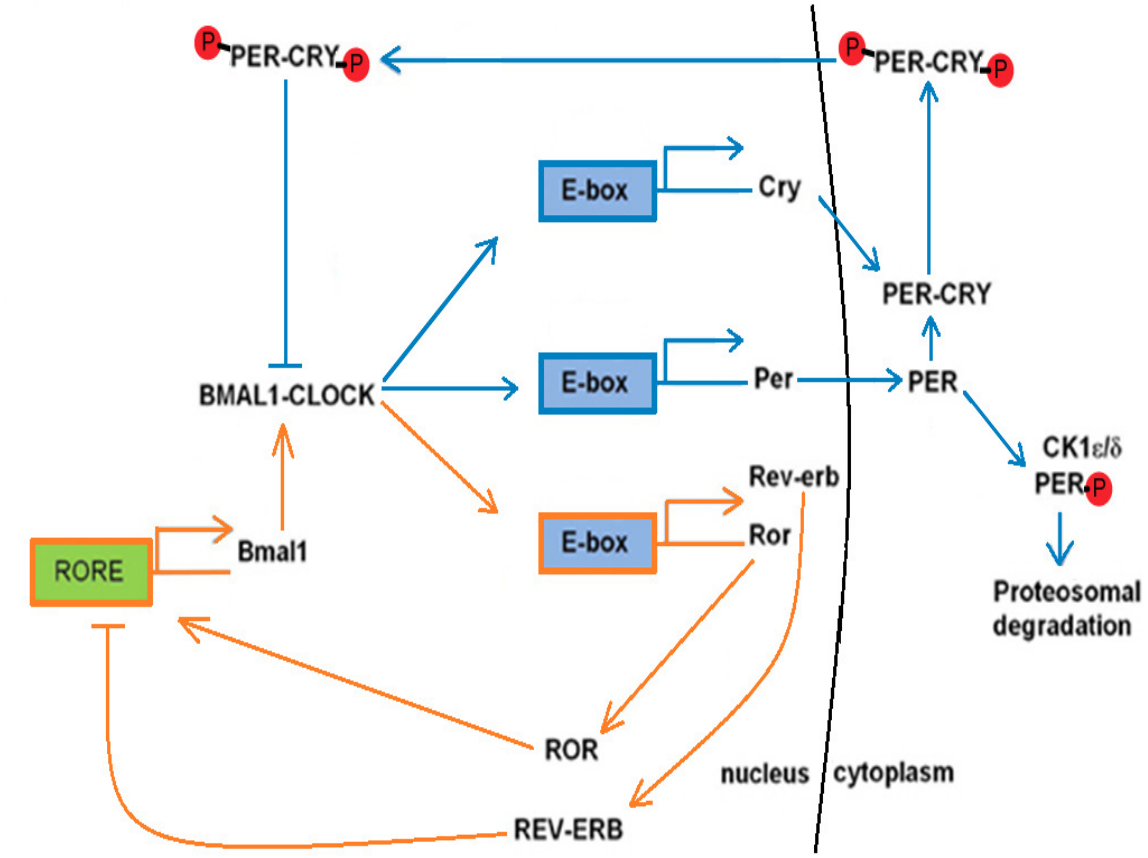

Figure 1.1: Overview of the two interlocking circadian clock regulatory feedback loops. The blue arrows represent the BMAL1/CLOCK regulatory feedback loop which controls the expression of the *Per* and *Cry* genes. The orange arrows represent the regulatory feedback loop that controls the expression of the *Bmal1* gene and formation of the BMAL1/CLOCK heterodimer.

CHAPTER 2: CIRCADIAN DISRUPTION-INDUCED MICRORNAOME DEREGULATION IN RAT MAMMARY TISSUES

Chapter 2 has been submitted in its entirety:

Kochan, D.Z., Ilnytskyy, Y., Golubov, A., Deibel, S.H., McDonald, R.J., and Kovalchuk, O. Circadian disruption-induced microRNAome deregulation in rat mammary tissues. Oncoscience. Accepted April 2015, advance online publication.

ABSTRACT

Breast cancer is already the most common malignancy affecting women worldwide, and evidence is mounting that circadian-disruption-induced breast cancer is a warranted concern. Although studies on the role of epigenetics have provided valuable insights, and although epigenetics has been increasingly recognized in the etiology of breast cancer, relatively few studies have investigated the epigenetic link between circadian disruption (CD) and breast cancer. Using a proven photoperiod-shifting paradigm, differing degrees of CD, various tissue-extraction time points, and Illumina sequencing, we investigated the effect of CD on miRNA expression in the mammary tissues of a rodent model system. To our knowledge, our results are the first to illustrate CD-induced changes in miRNA expressions in mammary tissues. Furthermore, it is likely that these miRNA expression changes exhibit varying time frames of plasticity linked to both the degree of CD and length of reentrainment, and that the expression changes are influenced by the light and dark phases of the 24-hour circadian cycle. Of the differentially expressed miRNAs identified in the present study, all but one have been linked to breast cancer, and many have predicted circadian-relevant targets that play a role in breast cancer development. Based on the analysis of protein levels in the same tissues, we also propose that the initiation and development of CD-induced breast cancer may be linked to an interconnected web of increased NF-κB activity and increased levels of Tudor-SN, STAT3, and BCL6, with aberrant CD-induced downregulation of miR-127 and miR-146b potentially contributing to this dynamic. This study provides direct evidence that CD induces changes in miRNA levels in mammary tissues with potentially malignant consequences, thus indicating that the role of miRNAs in CD-induced breast

cancer should not be dismissed.

INTRODUCTION

In 2007, the International Agency for Research on Cancer concluded "shiftwork that involves circadian disruption is probably carcinogenic to humans" (Hansen and Stevens, 2012). Studies conducted before and since this claim have all contributed evidence that breast cancer induced by circadian disruption (CD) is a warranted concern. Amongst the supporting evidence are aberrant, nocturnal melatonin levels contributing to breast cancer development, a role of circadian-relevant genes in breast cancer, and case study findings showing indirect evidence that night-shift workers are at higher risk of developing breast cancer (Blask et al., 2003; Cos et al., 2006; Hansen and Stevens, 2012; Haus and Smolensky, 2013; Knutsson et al., 2013). Although many studies have been conducted on the potential mechanisms in CD-induced breast cancer, very few of these studies have investigated the epigenetic links that may be involved. One of these potential epigenetic links is the activity of microRNAs (miRNAs), and the role of these small RNAs in both circadian rhythms and the etiology of breast cancer have been increasingly recognized (Jovanovic et al., 2010; Koturbash et al., 2011; Pegoraro and Tauber, 2008).

Mature miRNAs are abundant, small, single-stranded noncoding RNAs that are potent regulators of gene expression (Hwang and Mendell, 2006). Approximately 22 nts long, mature miRNAs associate with the RNA induced silencing complex (RISC) and target the 3` UTR region of target mRNAs, resulting in gene degradation or suppression, depending on the level of complementarity between the miRNA and its target (Koturbash et al., 2011). In mammals, through this mechanism a single miRNA can target multiple genes and influence a broad range of cellular processes related to cancer (Koturbash et

al., 2011). Acting as tumour suppressors, tumour promoters referred to as oncomiRs, or both depending on the degree of malignancy, miRNAs can influence the progression of cancer through various mechanisms such as cellular differentiation, proliferation, and apoptosis (Jovanovic et al., 2010; Koturbash et al., 2011).

In addition to playing a role in breast cancer development, miRNAs have also been shown to oscillate with the circadian cycle and target circadian-relevant genes. In *Arabidopsis*, several miRNAs were shown to oscillate based on environmental light, exhibiting higher levels during the daytime than during the nighttime, and oscillation on the basis of photic control and not an internal clock (Sire et al., 2009). In a study conducted on HeLa cell lines, results showed that the miR-192/194 cluster directly regulates the entire *Period* gene family, with increased expression of the miR192/194 tandem causing lowered expression of the *Period* genes and a shortening of the circadian cycle (Nagel et al., 2009). Further investigation has illustrated that miR-132 expression is induced by light within the suprachiasmatic nucleus (SCN) and that it has the capacity to restore homeostasis and reset the activity caused to the circadian clock by nocturnal light (Alvarez-Saavedra et al., 2011). Interestingly, a recent study has also reported that miR-132 levels are lowered in breast cancer cells, and identified miR-132 as a tumour suppressor that acts by inhibiting proliferation, migration, invasion, and metastasis (Zhang et al., 2014).

Despite the increasing evidence that miRNAs play a role in breast cancer development and in circadian rhythms and that these roles likely overlap, information on the involvement of miRNAs in CD-induced breast cancer remains scarce. In the handful of studies that have investigated epigenetic modifications in CD-induced breast cancer,

all the experiments have focused on changes in DNA methylation (Jacobs et al., 2013; Schwimmer et al., 2014; Zhu et al., 2011). Although the most recent study of epigenetic CD-induced breast cancer conducted a DNA methylation analysis on shift worker blood samples at miRNA promoters and identified increased methylation of breast cancer relevant miRNAs, the study did not measure expression levels of miRNAs or investigate CD-induced changes in mammary tissues (Liu et al., 2015; Shi et al., 2013). Furthermore, although the DNA methylation studies mentioned have provided valuable insights into the epigenetic role in CD-induced breast cancer, none of them have investigated the effect of varying degrees of CD or the influence of fluctuations within a 24-hour circadian cycle. Therefore, to shed new light on the epigenetic mechanisms involved in CD-induced breast cancer, the current study utilized a photoperiod-shifting (PS) paradigm involving various degrees of CD and specific time points within a circadian cycle to investigate the influence of CD on miRNA expression in the mammary tissues of a rodent model system.

MATERIALS AND METHODS

Animal model and circadian-disruption paradigm

Female Sprague Dawley rats from Charles River (Quebec) were housed at the Canadian Center for Behavioural Neuroscience at the University of Lethbridge. The rats were housed in a sterile facility in a temperature-controlled room, two per cage, and given food and water *ad libitum*. Handling and care of the animals was performed in accordance with the recommendations of the Canadian Council on Animal Care, and procedures were approved by the University of Lethbridge Animal Welfare Committee. Before the start of the experiment, all the rats were entrained to a 12-hour light-dark cycle

for 22 days to allow entrainment to a normal light schedule. At 83 days old, the rats were then randomly assigned to different treatment and control groups.

CD was induced by following a photoperiod-shifting paradigm that has been shown to cause physiological and behavioural changes in rodents (Craig and McDonald, 2008; Deibel et al., 2014; Devan et al., 2001; McDonald et al., 2013). In total, 40 female rats underwent this PS paradigm. To stimulate PS, the colony lights were turned on three hours earlier on each successive day of the experiment (Supplementary Tables S1 and S2). To investigate the effect of varying degrees of CD, the 40 rats were separated into acute and chronic CD groups. Twenty rats underwent acute photoperiod shifting, which consisted of lights coming on three hours earlier each day for a total cycle time of six days (Supplementary Table S1). Another 20 rats underwent chronic photoperiod shifting, which consisted of a rotation between a six-day period in which lights came on three hours earlier each day and a 10 day period in which a regular 12-hour light-dark cycle was followed, for a total cycle time of 54 days (Supplementary Table S2). For both the acute (six days) and chronic groups (54 days), following the PS cycle, the rats were exposed to a normal 12-hour light-dark cycle until it was time for tissue extraction.

The acute (20 rats) and chronic (20 rats) CD groups were then separated further based on the time of tissue extraction (Supplementary Figure S1). Mammary tissue extractions occurred 24 hours and two weeks following acute or chronic CD, with 10 rats from each CD group undergoing tissue extraction at each of these times. To account for and investigate the potential influence of specific time points within a 24-hour circadian, two different tissue-extraction time points, each corresponding to a specific zeitgeber time (ZT), were chosen for each tissue-extraction day (24 hours and two weeks following

CD). Half of the rats (five) in each tissue-extraction group were sacrificed at ZT06 (six hours after lights on), and the remaining five rats in each group were sacrificed at ZT19 (19 hours after lights on). These two ZT points were chosen because they represented the light and dark phases of the circadian cycle.

Both the acute (20 rats) and chronic (20) control groups were exposed to a 12-hour light-dark cycle for either six days (acute) or 54 days (chronic). The rats from each CD control group were then exposed to a 12-hour light-dark cycle for either 24 hours (10 rats) or two weeks (10 rats), depending on the time of tissue extraction for the corresponding experimental group. From each tissue-extraction control group (10 rats), five rats were sacrificed at ZT06 and five rats at ZT19 on the corresponding tissue-extraction day.

Euthanasia of the rats was performed through anaesthesia with Isoflurane (4–5 %; oxygen at 2 litres per minute) and decapitation by a guillotine, with euthanasia of the rats alternating between the control and experimental rats. The mammary glands were collected, immediately stored in liquid nitrogen, and stored long-term at -80°C.

Total RNA extraction

Whole mammary tissues were ground in liquid nitrogen using sterile, chilled mortars and pestles. Approximately 0.05 g of ground tissue from each sample was then suspended in Zymo Research tri-reagent (R2053) and lysed using two cycles of the Qiagen Tissue Lyser II for 2 minutes at 25 Hz. Total RNA was then extracted using the direct-zol RNA Miniprep kit from Zymo Research (R2053). The quality of the RNA was checked using NanoDrop 2000c, and quality bioanalysis was conducted using the Agilent 2100 and the Agilent Small RNA Kit and Chip (5067-1548); only samples with an RNA

integrity value (RIN) greater than 8 were used in downstream applications.

Small RNA sequencing and bioinformatics analysis

Three samples from each tissue-extraction and ZT group for both the experimental and control groups were randomly chosen to undergo sequencing analysis. The TruSeq Small RNA Sample Preparation Kit for 25–36 indexes from Illumina (RS-200-0036) was used to prepare small RNA libraries from 1 µg of total RNA. Six-percent SDS-PAGE gels were run to cut out the bands of interest for library validation on the Agilent 2100 using the Agilent High Sensitivity DNA Kit and Chip (5067-4626). Cluster generation for sequencing was performed using cBot and the TruSeq SR Cluster Kit v2-cBot-GA (GD-300-2001) from Illumina. Single-end sequencing was performed using the TruSeq SBS Kit v5-GA (FC-104-5001) from Illumina on the Genome Analyzer GAIIx at 36 cycles.

Bioinformatics analysis was performed on the sequencing data to define expression levels of miRNAs amongst the different experimental groups. Quality of the libraries was evaluated using FastQC v0.10.1 software. Adapter trimming was done using Cutadapt (https://code.google.com/p/cutadapt/). Trimmed reads were converted to fasta using an ad-hoc perl script and collapsed to unique tags with the fastx_collapser program from the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Unique tags in fasta format were aligned to mature miRNA sequences downloaded from miRBase (release 19) using Micro-Razers; only unambiguous alignments were kept (Emde et al., 2010). An ad hoc perl script was used to process the MicroRazers alignment file to produce a tab delimited count file containing miRNA IDs and raw read count columns.

Small RNA qRT-PCR

Validation of the sequencing results was performed by qRT-PCR. Small RNA cDNA was synthesized from 150ng of RNA using the reagents and protocol associated with the GeneCopoeia All-in-One miRNA First-Strand cDNA Synthesis Kit (AMRT-0060). The small RNA qPCRs were performed utilizing SYBR Green on the BioRad C1000 Thermal Cycler and CFX96 Real-Time System, by using the reagents and protocol associated with the GeneCopoeia All-in-One miRNA qRT-PCR detection kit (AOMD-Q060). All reactions were run in triplicate, and the program used was the one recommended by GeneCopoeia (10 min at 95°C; 40 cycles of 10 sec at 95°C, 20 sec at 60°C, and 10 sec at 72°C). Efficiency standard curves for the primers were generated using serial dilutions, and following all the qPCR cycling, melt curve analysis was conducted using the optimal parameters for the BioRad C1000 thermal cycler (65°C to 95°C, increments of 0.5°C).

The reverse primer used for all the reactions was the Universal Adaptor Primer included in the miRNA qRT-PCR detection kit from GeneCopoeia (AOMD-Q060). All the forward primers were miRNA-specific primers ordered from GeneCopoeia based on the target of interest, for example, rno-miR-127-3p (RmiRQP0111). Based on previous publications, recommended endogenous reference genes were ordered from GeneCopoeia and tested for stability (Davoren et al., 2008; Wang et al., 2013). The best combination of two reference genes, rno-miR-16 (RmiRQP0227) and rno-let-7a (RmiRQP0002), was found using the programs NormFinder (http://moma.dk/normfinder-software) and qbase^{plus} (Biogazelle), with the stability values meeting the geNorm stability cutoffs (CV < 0.25, M-Value < 0.5). These two reference genes have been recommended as control

genes for miRNA expression analysis in breast cancer (Davoren et al., 2008).

Western blot analyses

A 0.07 g portion of each mammary tissue sample was lysed and sonicated in 350 µl of 1%SDS+ProteaseInhibitor. Next, the samples were placed in a 95ºC water bath for 5 minutes and then centrifuged at 10,000 g for 10 minutes at 4ºC. The supernatant was collected and centrifuged at 10,000g for 10 minutes at 4ºC. Protein content was determined with the Bradford protein determination assay from BioRad. Equal amounts of lysate protein (10 µg/10 µL) were run on 6–10% SDS-polyacrylamide gels and transferred to PVDF membranes from GE Healthcare.

Western immunoblotting was conducted following well established protocols (Kovalchuk et al., 2008). Unaltered PVDF membranes were stained with Coomassie Blue (BioRad) to ensure even blotting of the proteins. Membranes were incubated with various primary antibodies at different dilutions based on the antibody company, antibodies against rabbit anti-BCL6 (1:1000, Abcam), mouse anti-Tudor-SN (1:500, Santa Cruz), rabbit anti-STAT3 (1:500, Santa Cruz), rabbit anti-phospho-NFκB p65 (Ser311) (1:500, Santa Cruz), rabbit anti-DNMT1 (1:500, Santa Cruz), and mouse anti-βactin (1:1000, Abcam). Antibody binding was revealed by incubation with horseradish peroxidase-conjugated secondary antibodies based on the primary antibody source animal (Santa Cruz) and the ECL Plus immunoblotting detection system (GE Healthcare). Chemiluminescence was detected using the FluorChem HD2 from Cell Biosciences. Signals were quantified using NIH ImageJ software and normalized to the actin protein band.

Statistical analyses

For statistical analyses of the bioinformatics data, raw count files were loaded into R version 3.1.2. All the statistical comparisons were performed using the DESeq2 (version 1.4.5) bioconductor package as described in the package manual (Anders and Huber, 2010). Multiple comparisons adjustment was performed using Benjamini-Hochberg procedure, and microRNAs with an adjusted p-value below 0.1 were considered differentially expressed (Benjamini and Hochberg, 1995). Sample and miRNA clustering based on Euclidian distances was performed using the gplots R package, and the clustering results were displayed graphically as MA plots and heatmap dendrograms. For the qRT-PCR and western blot data, Student's t-test was used for independent variance to determine significance ($p < 0.05$). Statistical analysis and plotting of the data was performed using MS Excel software for Windows, and the results were presented as mean relative expression values ± standard error of the mean (SEM).

RESULTS

Circadian disruption causes aberrant expression of a broad range of miRNAs, and the changes are linked to an early time point in the circadian cycle

In this study, we investigated the effect of varying degrees of CD on miRNA expression in the mammary tissues of Sprague Dawley rats. The influence of light-dependent zeitgeber times was also incorporated to investigate possible fluctuations within a 24-hour circadian cycle. The sequencing results identified a broad range of miRNAs that were differentially expressed between the circadian-disrupted samples and the control samples (Table 2.1). Amongst the differentially expressed miRNAs, all but one play a role in breast cancer development (Table 2.1). Numerous miRNAs that

oscillate with the circadian cycle and/or have predicted circadian-relevant targets were also differentially expressed due to circadian disruption (Table 2.1).

The changes in miRNA expression were observed in both the acute and chronic circadian-disrupted groups, as well as in both the 24-hour and two-week tissue-extraction groups (Figures 2.1–2.3). Only the two-week acute group did not show any changes in miRNA expression (Figures 2.3 and 2.4). Interestingly, the time of tissue extraction based on ZT influenced the results. None of the ZT19 groups showed any changes in miRNA expression (Figure 2.4), whereas with the exception of the two-week acute group, all the ZT06 groups showed differences in miRNA expression compared to the respective control groups (Figures 2.2 and 2.3). The ZT19 and ZT06 extraction time points represented the dark and light phases of the circadian cycle, respectively, indicating that the miRNA expressions correlated to light-dependent time points within the circadian cycle.

Between the ZT06 groups that showed differences in miRNAs, there were no significant correlations or patterns in specific miRNA expression, with only two miRNAs being differentially expressed in more than one group, miR-150-5p and miR-142-5p (Table 2.2). Furthermore, with the exception of no differential expression in the two-week acute ZT06 group, there was no significant difference in the number of differentially expressed miRNAs based on the degree of CD (Table 2.2).

In terms of expression patterns within each individual ZT06 group, the 24-hour acute group had a tandem cluster that was differentially expressed, with downregulation of the tumour suppressor miRNAs, miR-1 and miR-133a (Tables 2.1 and 2.2). Within the 24-hour chronic ZT06 group, two miRNAs from the same gene family that are linked to

breast cancer and circadian rhythms, let-7b and let-7c, were differentially expressed (Tables 2.1 and 2.2). Finally, in the two-week chronic ZT06 group, both miR-146a and miR-146b, which belong to the same gene family, were downregulated (Tables 2.1 and 2.2).

Validation of sequencing results through qRT-PCR verifies miR-127 expression

Of the three ZT06 groups that illustrated differential expression of miRNAs due to CD, emphasis was placed on the two-week chronic ZT06 group due to the differential expression of miRs 146a and 146b, and miR-127 (Figures 2.5A-B and 2.6A). Confirmation of the two-week chronic ZT06 miRNA sequencing results was attempted through small RNA qRT-PCRs. However, the biggest log fold change in miRNA expression based on Illumina sequencing in this group was approximately 1 (Figure 2.2). This made verification of the sequencing results difficult, both because of the sensitivity of qRT-PCR and the fact that qPCR is not an infallible validation method, especially when the technology being validated incorporates PCR-based amplification, as does Illumina (Git et al., 2010). Amongst the qRT-PCRs performed on the differentially expressed miRNAs in the two-week chronic ZT06 group, the miR-127 qPCR results validated the sequencing results. The qPCR data illustrated a similar and consistent change in relative miR-127 expression when compared to the Illumina sequencing (Figures 2.6A and 2.6B).

Circadian-disruption-induced changes in miRNA expression in the two-week chronic ZT06 group correlated to aberrant levels of breast-cancer-relevant proteins

To investigate the potential downstream consequences of changes in miRNA expression due to CD in the two-week chronic ZT06 group, western blot analysis was

performed on relevant proteins linked to the differentially expressed miRNAs and breast cancer development. With respect to miR-146a and 146b activity, the results illustrated higher quantities of the transcriptionally active form of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Figure 2.5). Phospho-NFκB (p65) protein levels showed a nearly 2.5-fold increase in the circadian-disrupted samples compared to the control samples, and a p-value of less than 0.05 (Figure 2.5D). The results also illustrated higher quantities of a verified target of miR-127 and a protein linked to cellular senescence, B-cell lymphoma 6 (BCL6) (Figure 2.6). BCL6 protein levels showed a twofold increase in the circadian-disrupted samples, and a p-value of less than 0.05 (Figure 2.6D). Western blot analysis also showed higher quantities of tudor staphylococcal nuclease (Tudor-SN), a protein liked to NF-kappaB activity and lower miR-127 expression (Figure 2.6). Tudor-SN protein levels showed an almost threefold increase in the circadian-disrupted samples compared to the control samples and a p-value of less than 0.01 (Figure 2.6D). Western blot analysis was also conducted on signal transducer and activator of transcription 3 (STAT3) (Figure 2.7). The data showed that STAT3 protein levels had a 0.5-fold increase in the circadian disrupted samples compared to the control samples, and a p-value of less than 0.05 (Figure 2.7B). Increased STAT3 levels are linked to lower expression of miR-146b and increased NF-κB activity, both of which were present in the two-week chronic ZT06 group (Figure 2.5). To investigate whether DNA methylation differences may have contributed to the aberrant expression of the differentially expressed miRNAs, western blot analysis was conducted on the maintenance DNA methyltransferase, DNMT1 (Figure 2.8). The results showed that DNMT1 protein levels increased more than twofold in the circadian-disrupted

samples compared to the control samples, with a p-value of less than 0.05 (Figure 2.8B).

DISCUSSION

Knowledge on the influence of varying degrees of CD and miRNA activity in CD-induced breast cancer remains scarce. Our study, which induced CD using a photoperiod-shifting paradigm shown to cause behavioural and physiological changes in rodents, is to our knowledge the first to illustrate that CD induces changes in miRNA expression in rodent mammary tissues (Craig and McDonald, 2008; Deibel et al., 2014; Devan et al., 2001; McDonald et al., 2013). Both the acute and chronic CD manipulations resulted in the differential expression of a variety of breast-cancer-relevant and potentially circadian-relevant miRNAs (Table 2.1 and Figure 2.1). The 54 day chronic CD scheme caused changes in miRNA expression in both the 24-hour and two-week chronic extraction groups (Figure 2.2), indicating that long-term CD can have aberrant consequences on breast cancer development through miRNA activity. Because the 6 day acute CD scheme also caused aberrant changes in miRNA expression in the 24-hour group (Figure 2.3), this suggests that even short-term CD may induce potentially harmful changes that can increase the risk of circadian-related diseases.

Amongst the experimental groups in the study, only the two-week acute group did not show any significant changes in miRNA expression (Figures 2.3 and 2.4). The two-week acute group underwent the least rigorous CD-scheme and had the longest reentrainment period before tissue extraction. This information, coupled with the fact that the 24-hour acute and two-week chronic groups did exhibit changes in miRNA expression (Figure 2.1), indicates that the CD-induced changes in miRNA levels may be linked to the degree of CD and a correlated length of reentrainment to restore normal

miRNA expression. These results are supported by evidence from previous studies indicating that CD effects on various processes can be plastic. By utilizing the same acute photoperiod-shifting paradigm used in the present study, a study used behavioural measurements to show that rats required 17 days of a 12-12 light-dark cycle to attain reentrainment (Devan et al., 2001). In terms of epigenetic modifications, a study illustrated that CD-induced changes in DNA methylation can be reversed, showing that circadian disrupted mice that are entrained back to a regular 24-hour day exhibit a reversion in DNA methylation levels (Azzi et al., 2014). Based on our results, it would seem that 14 days or fewer on a 12-12 light-dark cycle is enough time to cause a reversion in miRNA levels due to the acute CD scheme used, but not enough time to cause reversion due to the chronic CD scheme. Along with evidence from previous literature, this indicates that CD-induced miRNA changes are likely plastic, with varying degrees of CD requiring varying lengths of reentrainment to revert the changes in miRNA expression.

Through the incorporation of light-dependent ZT extractions, our results also illustrate that specific time points within a circadian cycle may play an important role in miRNA activity and CD-induced breast cancer. Specifically, the results showed that none of the groups representing the dark phase of the circadian cycle, the ZT19 extraction groups, exhibited any changes in miRNA expression (Figure 2.4). In terms of the ZT06 groups, which represent the light phase of the circadian cycle, all but the two-week acute group showed differences in miRNA levels (Figures 2.2 and 2.3). Previous literature has demonstrated that miRNAs can oscillate throughout a 24-hour circadian cycle. In particular, a microarray-based experiment on mouse liver revealed that over 13% of the

probed miRNAs exhibited circadian expression patterns (Na et al., 2009). Interestingly, some of the miRNAs identified in that study are miRNAs that exhibited differential expression in our study (Table 2.1). Therefore, our findings further stress that specific time points within a 24-hour circadian cycle are important for accurately measuring miRNA levels and activity, and that these details need to be considered when investigating circadian-relevant diseases. For example, melatonin, a pineal hormone that is linked to circadian rhythms and exhibits fluctuations in its daytime and nocturnal concentrations, has been shown to influence human breast cancer xenografts through a day-night rhythm of tumour proliferation, fatty acid uptake, metabolism, and signal transduction activity (Blask, 2009; Reiter, 2004). Research has also reported that DNA methylation activity may be influenced by light-dependent induction or repression of DNA methylation enzymes (Azzi et al., 2014). Therefore, given these previous findings, coupled with the fact that miRNAs can influence cancer-relevant processes and also fluctuate with the circadian cycle, it is not inconceivable that circadian-dependent fluctuations in miRNAs can influence breast cancer development. Our results illustrate a clear pattern of CD-induced changes in miRNA expression based on the light and dark phases of the circadian cycle (Figures 2.2 and 2.4), and all but one of the differentially expressed miRNAs are linked to breast cancer development (Table 2.1). It is therefore likely that circadian fluctuations in miRNA activity are an important component in the development of CD-induced breast cancer and should not be ignored.

In terms of specific miRNAs that were differentially expressed due to CD, all but one have been linked to breast cancer development, with many of the CD-induced expressions correlating to potentially aberrant consequences (Tables 2.1 and 2.2).

Although only two miRNAs were identified in more than one of the ZT06 groups, miR-142-5p and miR150-5p (Table 2.2), there are significant correlations and patterns related to breast cancer development in each ZT06 group. In the 24-hour acute ZT06 group, two miRNAs that are part of the same cluster, miR-133a and miR-1, were both underexpressed (Table 2.2). The miR-1/133a cluster has been shown to be downregulated in a variety of cancers, whereas miRNA-133a has also been shown to act as a tumour suppressor in breast cancer cells by causing S/G_2 phase cell-cycle arrest through activity on phosphorylated protein kinase B (Akt) (Cui et al., 2013; Nohata et al., 2012). Furthermore, two predicted circadian-relevant targets of the miR 1/133a cluster are circadian locomotor output cycles kaput (CLOCK) and TIMELESS (Table 2.1). Previous literature has shown that CLOCK knockdown in breast cancer cells results in increased expression of various tumour suppressor genes and decreased expression of multiple oncogenes, indicating an oncogenic influence by CLOCK that is potentially an early event in breast cancer development (Hoffman et al., 2010a). TIMELESS, a circadian and cell-cycle regulator that may act as a molecular bridge between these two regulatory systems, has been reported to be overexpressed in many breast cancer cells, with the increased expression being linked to increased proliferation and poorer prognostic outcome (Fu et al., 2012; Mao et al., 2013). This indicates that CD-induced down regulation of the miR-1/133a cluster may have oncogenic effects through previously identified mechanisms, as well as through potential circadian relevant targets.

In the two-week chronic ZT06 group, two miRNAs from the same gene family, miR-146a and 146b, were downregulated (Table 2.2). The expression of miR-146a and 146b has been shown to suppress NF-κB activity (Bhaumik et al., 2008; Walker et al.,

2014). A key component of inflammation and innate immunity, NF-κB is a protein complex that controls the transcription of numerous genes and has been implicated as a key component in many steps of cancer initiation and progression (Helbig et al., 2003; Hoesel and Schmid, 2013; Shostak and Chariot, 2011). Activation of NF-κB can result in the upregulation of anti-apoptotic genes and contribution to cell survival and proliferation (Hoesel and Schmid, 2013). In breast cancer, NF-kappaB has been shown to increase cell migration and promote tumour-initiating cells (Helbig et al., 2003; Kendellen et al., 2014). Our western blot results show that activated NF-κB was significantly increased in the circadian-disrupted samples of the two-week chronic ZT06 group (Figure 2.5C-D). This finding is consistent with a previous study that indirectly correlated increased induction of NF-κB pathways to CD-induced promoter methylation of miR-219 (Shi et al., 2013). The present study shows that CD results in a direct increase of NF-κB activity in mammary tissues and that this activity may be linked to CD-induced downregulation of miR-146a and 146b (Figure 2.5).

Cellular senescence is a response to extracellular and intracellular stresses, such as oncogenic stimuli, that causes permanent cell-cycle arrest and acts as a potent tumour suppression mechanism that prevents the oncogenic transformation of primary cells (Campisi, 2013). miRNA-127-3p has been shown to be upregulated in senescent cells, and its downregulation has been shown to promote cell proliferation (Pan et al., 2012; Wang et al., 2011). Small RNA sequencing and validation through qRT-PCR showed that miR-127 is underexpressed in the two-week chronic ZT06 group (Figure 2.6A-B). Because cell senescence plays an important role in tumour initiation, this dynamic warranted further investigation (Rodier and Campisi, 2011). Amongst the proteins linked

to miR-127 activity and breast cancer development, are BCL6 and Tudor-SN. A target of miR-127, the *BCL6* gene has been identified as a potent inhibitor of cell senescence and a contributor to oncogenesis, and the western blot data showed that BCL6 levels were significantly increased in the circadian-disrupted samples (Figure 2.6) (Chen et al., 2013a; Shvarts et al., 2002). Tudor-SN is a member of the RISC complex and also known as SND1, and it has been implicated in various cancers and has been shown to regulate miRNA processing and expression by degrading primary-miRNA transcripts that undergo adenosine deaminase acting on RNA (ADAR) editing (Blanco et al., 2011; Kuruma et al., 2009; Scadden, 2005; Yang et al., 2006). In terms of its link to cancer progression, Tudor-SN has been shown to promote resistance to apoptosis and to be induced by NF-κB, which as mentioned above, was increased in the CD-induced tissues through potentially decreased miR-146a and 146b expression (Figure 2.5) (Armengol et al., 2013; Blanco et al., 2011). Amongst the miRNAs influenced by Tudor-SN, is miR-127, and studies have shown that Tudor-SN triggers miR-127 downregulation in breast cancer cells (Zhao et al., 2013). Our western blot results illustrate that Tudor-SN was significantly increased in the CD-induced tissues (Figure 2.6). This indicates that CD causes aberrant expression of Tudor-SN, which may be linked to increased NF-κβ activity through CD-induced repression of miR-146a and 146b. Consequently, this dynamic potentially results in the decreased expression of miR-127 through increased Tudor-SN activity, which in turn results in increased expression of the proto-oncogene *BCL6*.

Numerous publications have provided evidence that the *STAT3* gene produces oncogenic effects in a variety of cancers, including being active in 50–60% of primary

breast tumours and being linked to the promotion of breast cancer stem cell traits (Chung et al., 2014; Siveen et al., 2014). Our western blot data illustrate that STAT3 levels showed a significant increase in the CD-induced samples in the two-week chronic ZT06 group (Figure 2.7). Interestingly, STAT3 has also been identified as part of a negative feedback loop with miR-146b. In healthy cells, the gene encoding miR-146b is a direct target of STAT3, and as STAT3 levels increase, it results in the activation of miR-146b (Walker et al., 2014; Xiang et al., 2014). The activation of miR-146b results in the attenuation of NF-κB activity, which in turn, results in the subsequent inhibition of STAT3 expression (Walker et al., 2014; Xiang et al., 2014). In breast cancer cells, increased promoter methylation of miR-146b results in inhibition of the negative feedback loop and increased expression of STAT3 (Xiang et al., 2014). As mentioned, miRNA146b levels were downregulated in the circadian-disrupted samples and NF-κB activity was also increased (Figure 2.5). Although no promoter-specific DNA methylation analysis was performed in this study, western blot data indicate that there were increased levels of DNMT1 in the circadian-disrupted samples (Figure 2.8). Together with previous literature that has shown that CD results in increased miRNA promoter methylation, this indicates that the aberrant NF-κB activity and STAT3 levels could potentially be a consequence of miR-146b promoter methylation due to increased levels of DNMT1 (Etoh et al., 2004; Shi et al., 2013). Furthermore, the STAT3 protein has also been shown to induce BCL6 expression by binding to one of two regulatory regions within the *BCL6* gene (Walker et al., 2013). As a result, the CD-induced increases in STAT3 expression through NF-κB activity may not only cause oncogenic effects on their own, but also contribute to the increase in BCL6 levels. In view of the

previously discussed results, it seems that CD results in a cascade of interrelated activity that revolves around increased NF-κB activity and expression of BCL6, with aberrant CD-induced downregulation of miR-127 and miR-146b potentially contributing to this cascade.

Our findings illustrate for the first time that CD induces changes in miRNA levels in mammary tissues. In addition, these CD-induced changes are likely plastic and potentially linked to the light and dark phases of the circadian cycle. Among the differentially expressed miRNAs reported, are breast-cancer-relevant miRNAs that also have predicted circadian-relevant targets linked to breast cancer development. The basis of the link between miRNA activity and CD-induced breast cancer seems to be an interconnected web of increased NF-κB activity and BCL6 expression, likely linked to and promoted by the CD-induced decrease in miR-127 and miR-146b. Because inflammation, innate immunity, and cellular senescence are crucial in tumour initiation and breast cancer progression, and because these two miRNAs have been linked to these mechanisms, it is likely that all of them are key, interrelated components in the potential initiation and development of CD-induced breast cancer. Although our findings are bolstered by previous literature, given that they represent the first evidence of potentially direct consequences of aberrant CD-induced miRNA activity in mammary tissues, further investigation is required. A stricter CD scheme in terms of ZT extractions and extractions following CD is warranted for developing a more accurate range of CD-induced miRNA plasticity and for providing more details on the potential consequences of light-dependent miRNA fluctuations in CD-induced breast cancer. Verification of potential circadian-relevant targets through luciferase reporter experiments, incorporation of the CD schemes

into xenograft models, and further investigation of the dynamic of miR-127 and miR-146b activity in cancer cells are all logical extensions of the present study. In either case, whatever the next avenue may be, this study provides evidence that CD induces changes in miRNA levels in mammary tissues with potentially malignant consequences, thus indicating that the role of miRNAs in CD-induced breast cancer should not be dismissed.

Table 2.1: Circadian disruption induces changes in a broad range of breast cancer relevant miRNAs. Differentially expressed miRNAs based on Illumina sequencing in all the circadian-disrupted samples and their links to breast cancer development and circadian rhythms.

miRNA	Links to Breast Cancer Development	Links to Circadian Rhythms
let-7b-5p	Tumour suppressor linked to cell motility	Cry2; Circadian oscillation
let-7c-5p	Tumour suppressor	Cry2
miR-1-3p	Tumour suppressor in various cancers, possibly linked to BC	CLOCK
miR-10a-5p	Amplified in and linked to ER+ breast cancer	
miR-15b-5p	Tumour suppressor linked to BCL2 expression	
miR-24-3p	Enhances tumour invasion and metastasis	Per2
miR-30b-5p	Linked to tumourigenesis and metastasis	
miR-99a-5p	Tumour suppressor linked to mTOR expression	Circadian oscillation
miR-126a-5p	Linked to metastasis	NPAS2
miR-127-3p	Tumour suppressor linked to BCL6 expression	
miR-130a-3p	OncomiR	
miR-133a-3p	Tumour suppressor linked to tumour growth and invasion	TIMELESS
miR-142-5p	Linked to ER/PR status	Circadian oscillation
miR-146a-5p	Tumour suppressor linked to NFκB	Circadian oscillation
miR-146b-5p	Tumour suppressor linked to STAT3 and NFκB	TIMELESS; Circadian oscillation
miR-150-5p	OncomiR linked to apoptosis	Circadian oscillation
miR-193-3p	Tumour suppressor	Cry2
miR-335	Linked to breast cancer development and BRCA1	CLOCK
miR-672-5p	----------	

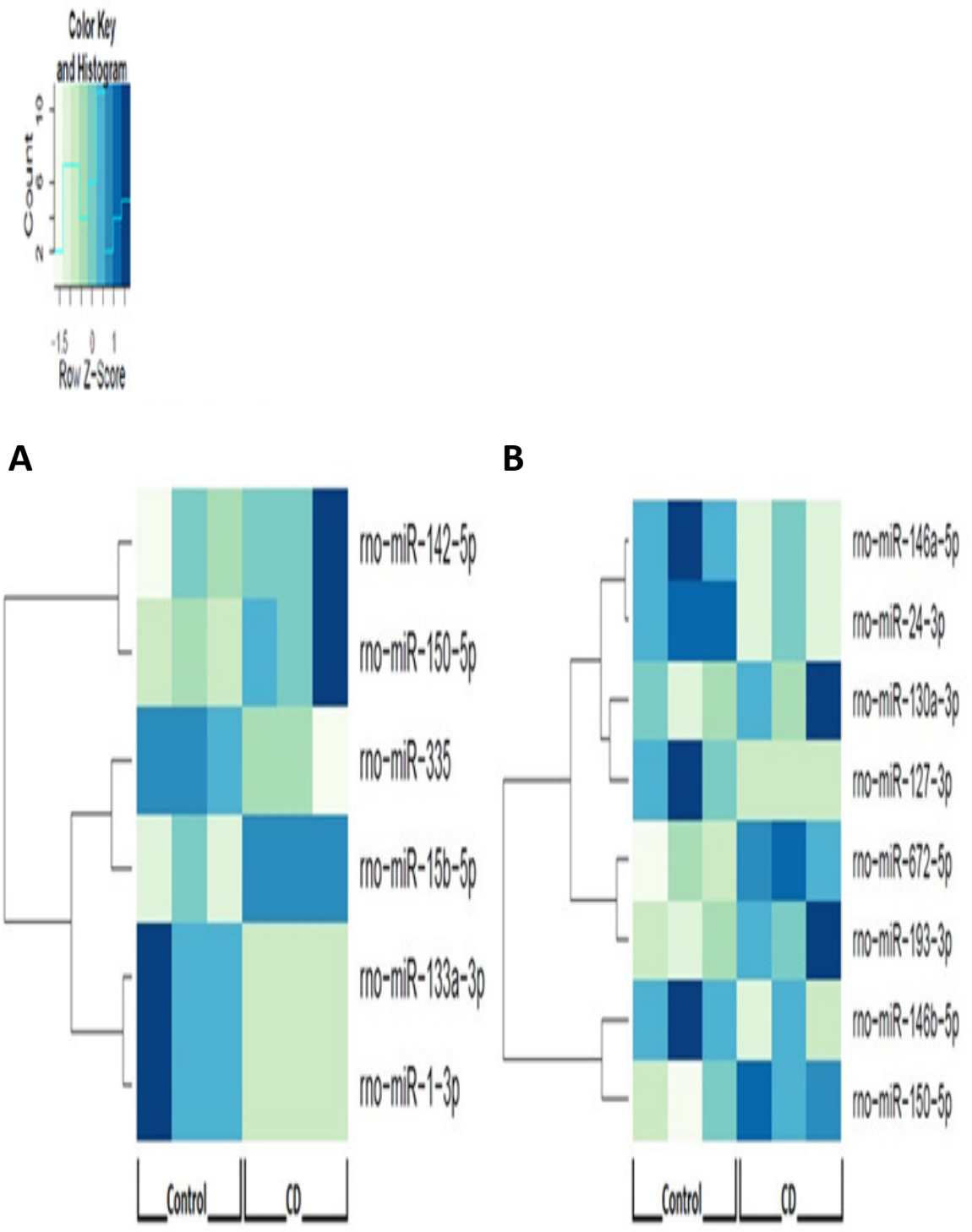

Figure 2.1: Circadian disruption causes changes in miRNA expression in both the acute and chronic groups. Heat map dendrograms based on the Illumina sequencing results for the differentially expressed miRNAs in the **A**. 24-hour acute ZT06 group and **B**. two-week chronic ZT06 group. All the differentially expressed miRNAs represent a significant expression difference, p-adjusted < 0.1, N=6.

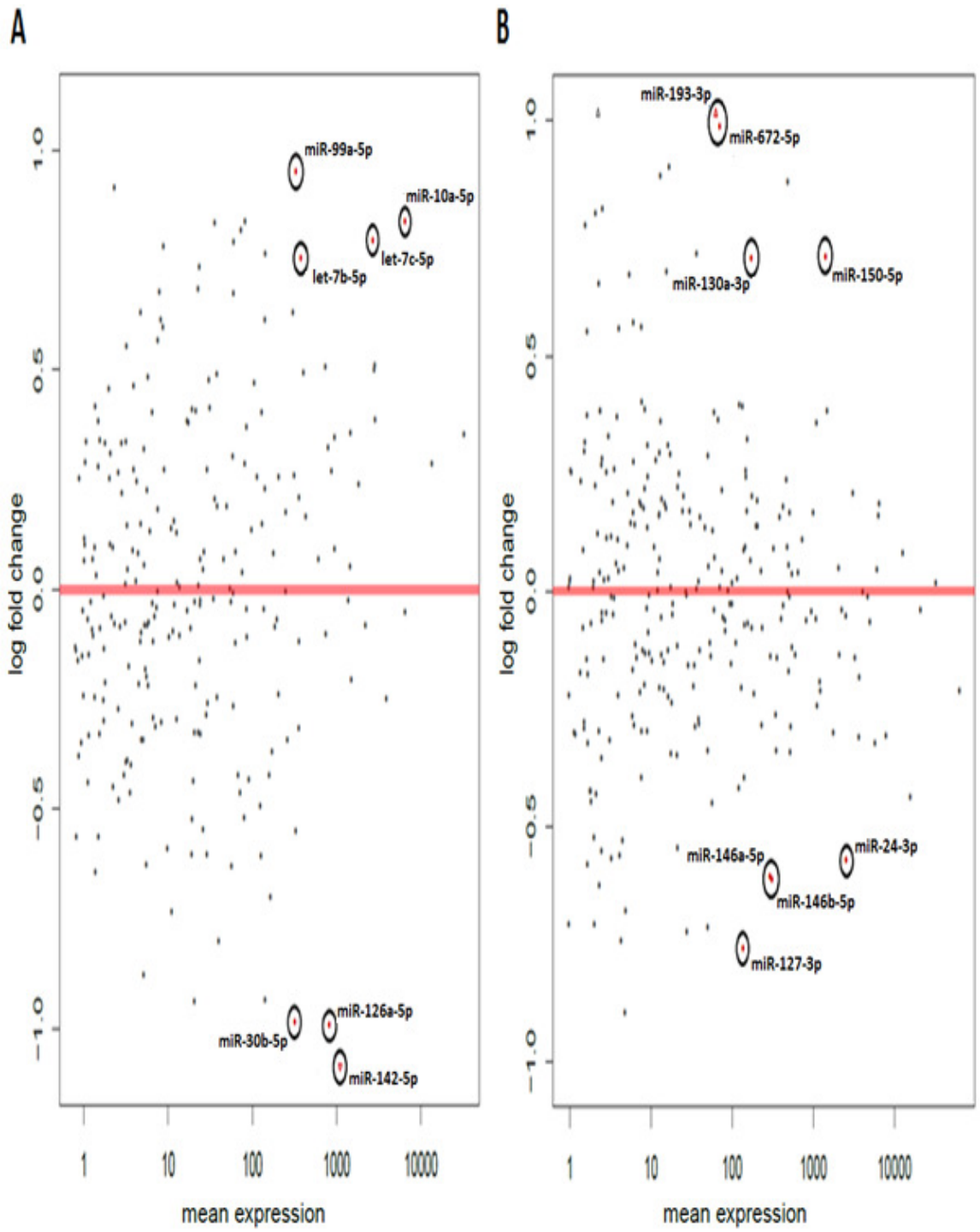

Figure 2.2: Chronic circadian disruption causes significant changes in miRNA expression in the ZT06 groups. MA plot based on the small RNA Illumina sequencing results for the **A**. 24-hour chronic ZT06 group and **B**. two-week chronic ZT06 group. The y-axis represents log fold change, while the x-axis represents mean expression. The plots represent all the miRNA expressions that were identified, and the red plots represent miRNAs that showed a significant expression difference; p-adjusted < 0.1, N=6. Each red plot is identified with the corresponding miRNA.

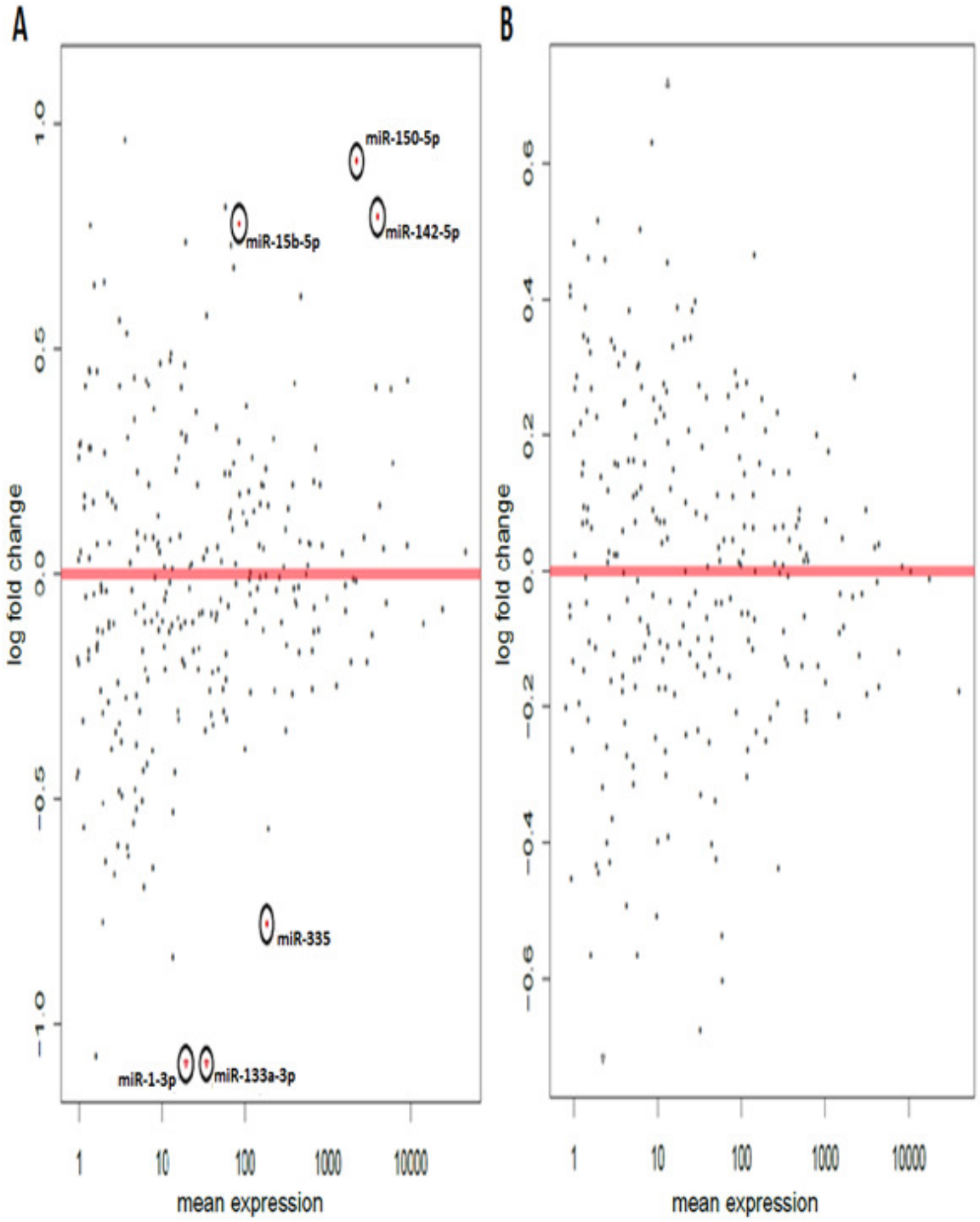

Figure 2.3: Acute circadian disruption causes significant changes in miRNA expression in the 24-hour acute ZT06 group. MA plot based on the small RNA Illumina sequencing results for the **A**. 24-hour acute ZT06 group and **B**. two-week acute ZT06 group. The y-axis represents log fold change, while the x-axis represents mean expression. The plots represent all the miRNA expressions that were identified, and the red plots represent miRNAs that showed a significant expression difference; p-adjusted < 0.1, N=6. Each red plot is identified with the corresponding miRNA.

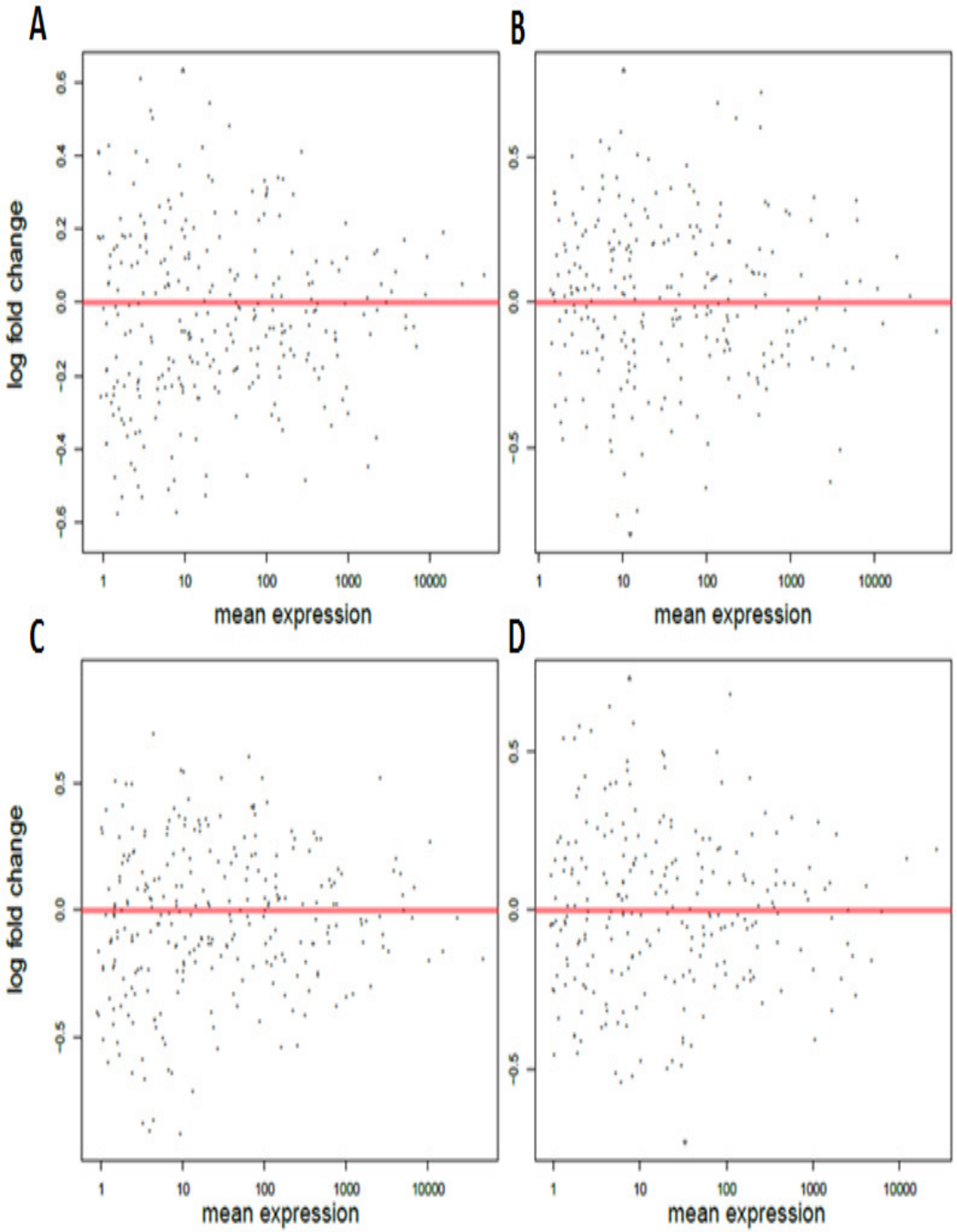

Figure 2.4: Chronic and acute circadian disruption causes no significant changes in miRNA expression in all the ZT19 groups. MA plots based on the small RNA Illumina sequencing results for the **A**. 24-hour chronic ZT19 group, **B**. two-week chronic ZT19 group, C. 24-hour acute ZT19 group, and D. two-week acute ZT19 group. The y-axis represents log fold change, while the x-axis represents mean expression. The plots represent all the miRNA expressions that were identified. None of the miRNAs in the ZT19 groups illustrated significant expression differences.

Table 2.2: Circadian disruption induces potentially aberrant miRNA expression patterns. Expression patterns of all the differentially expressed miRNAs based on Illumina sequencing in the circadian-disrupted groups. Only the ZT specific groups that showed changes in miRNA expression are depicted. Colour coordination represents miRNAs that are part of the same gene family, cluster, or the same miRNA.

24-Hour Acute ZT06	Expression
rno−miR−142−5p	Over
rno−miR−150−5p	Over
rno−miR−335	Under
rno−miR−15b−5p	Over
rno−miR−133a−3p	Under
rno−miR−1−3p	Under
24-Hour Chronic ZT06	**Expression**
rno-miR-126a-5p	Under
rno−miR−30b−5p	Under
rno−let−7b−5p	Over
rno−miR−99a−5p	Over
rno−miR−10a−5p	Over
rno−let−7c−5p	Over
rno−miR−142−5p	Under
2-Week chronic ZT06	**Expression**
rno−miR−146a−5p	Under
rno−miR−24−3p	Under
rno−miR−130a−3p	Over
rno−miR−127−3p	Under
rno−miR−672−5p	Over
rno−miR−193−3p	Over
rno−miR−146b−5p	Under
rno−miR−150−5p	Over

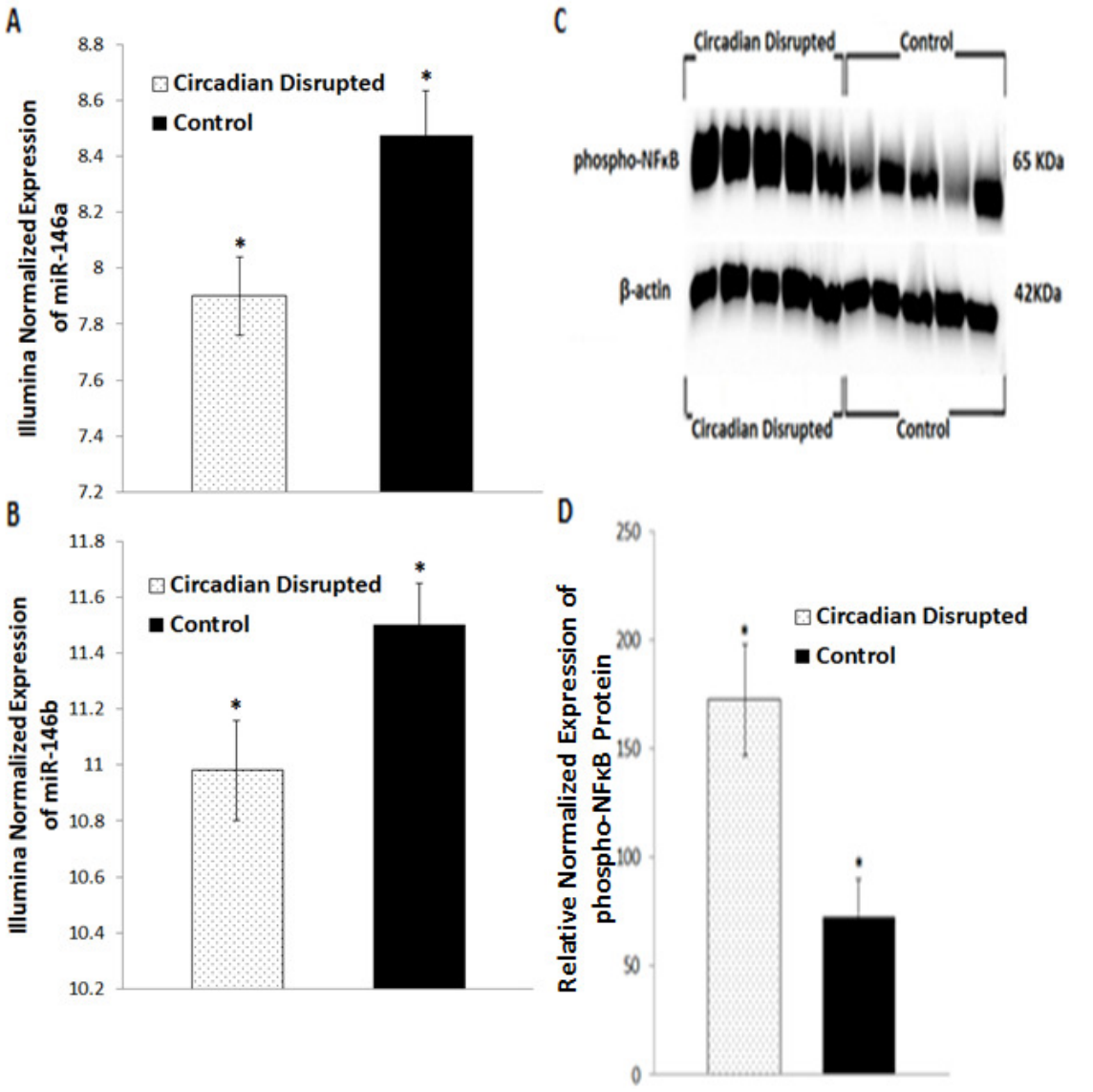

Figure 2.5: Circadian disruption causes lowered expression of miRNAs 146a and 146b, and increased expression of activated NF-κB. A. Mean relative expression of miR-146a based on Illumina sequencing, *p-adjusted < 0.1, N=6. **B**. Mean relative expression of miR-146b based on Illumina sequencing, *p-adjusted < 0.1, N=6. **C**. Western immunoblotting images of phospho-NFκB (p65) and β-actin from a 6% SDS-PAGE gel for the two-week chronic ZT06 group. Images were taken with the FluorChem HD2. The five samples on the left represent the control samples, and the five samples on the right represent the circadian disrupted samples. **D**. Mean relative expression of the phospho-NFκB (p65) protein based on the actin band in C, *p < 0.05, N=10. Error bars represent SEM.

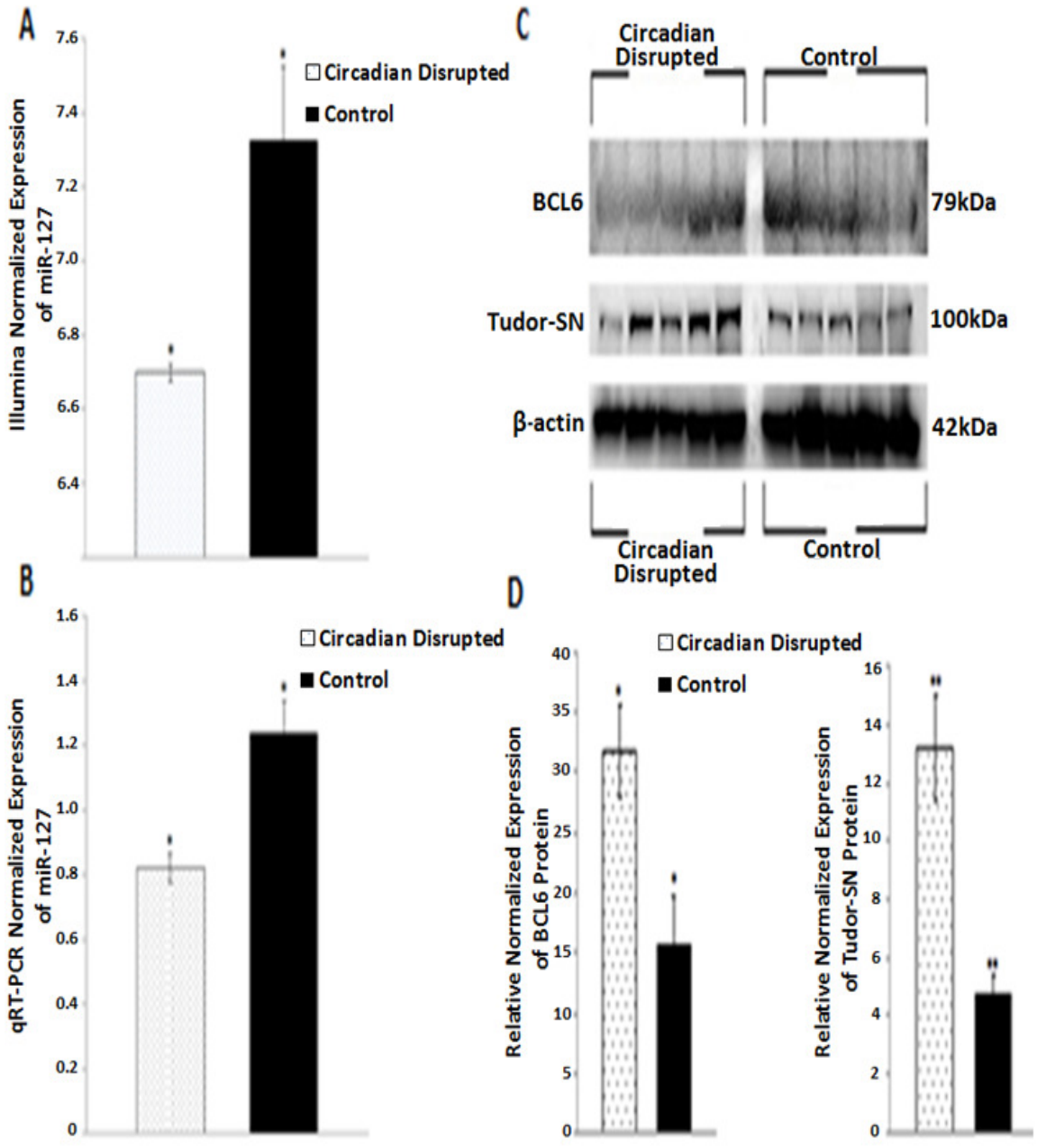

Figure 2.6: Circadian disruption causes decreased expression of miR-127, and increased expression of Tudor-SN and BCL6. **A**. Mean relative expression of miR-127 based on Illumina sequencing, *p-adjusted < 0.1, N=6. **B**. Mean relative expression of miR-127 based on qRT-PCR data, *p < 0.05, N=10. **C**. Western immunoblotting images of BCL6, Tudor-SN, and β-actin from a 10% SDS-PAGE gel for the 2-week chronic ZT06 group. Images were taken with the FluorChem HD2. The five samples on the left represent the circadian disrupted samples, and the five samples on the right represent the control samples. **D**. Mean relative expression of the BCL6 and Tudor-SN proteins based on the actin band in C,*P < 0.05, N=10.

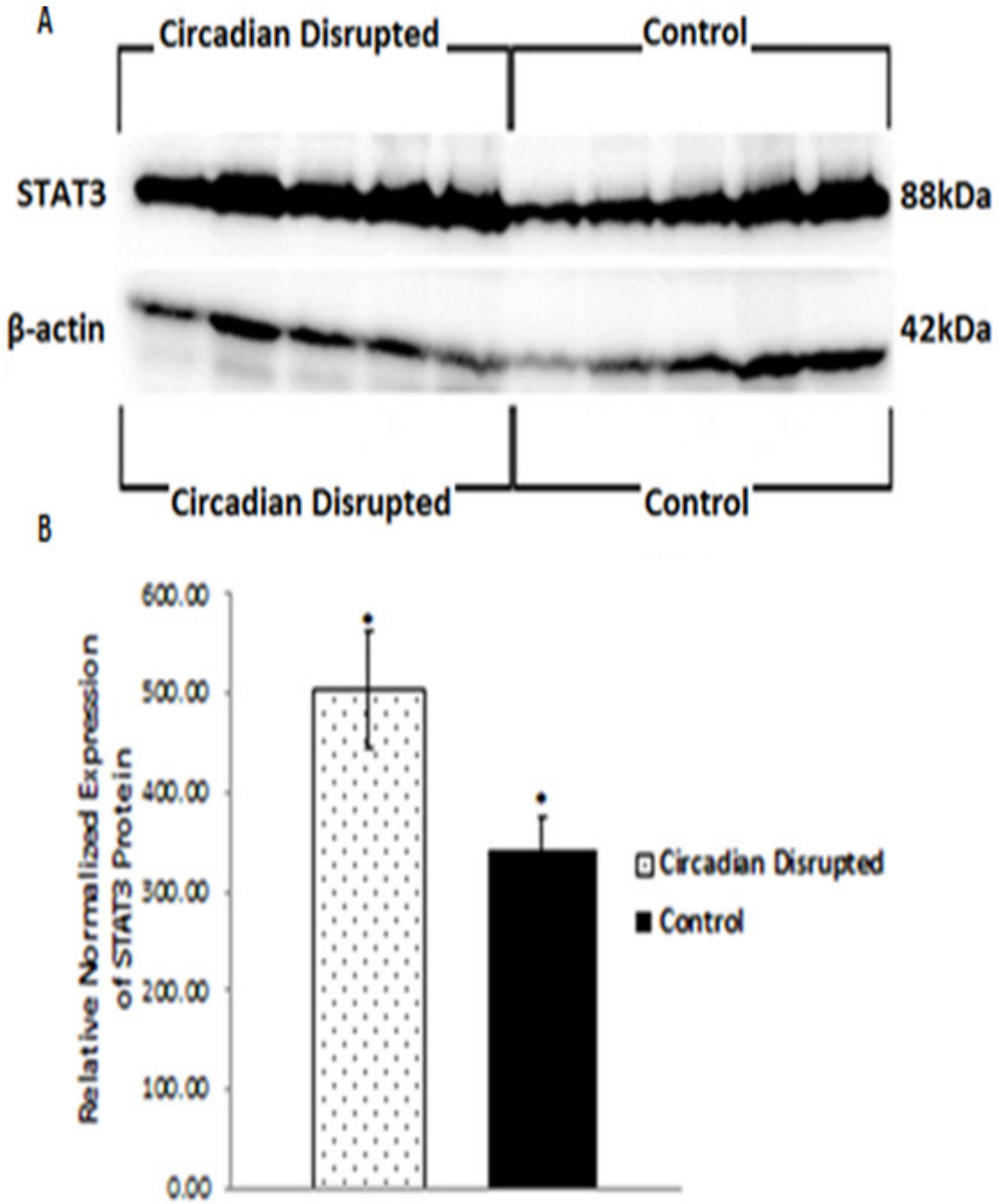

Figure 2.7: Circadian disruption causes increased expression of STAT3. A. Western immunoblotting images of STAT3 and β-actin from a 10% SDS-PAGE gel for the two-week chronic ZT06 group. Images were taken with the FluorChem HD2. The five samples on the left represent the circadian disrupted samples, and the five samples on the right represent the control samples. **B**. Mean relative expression of the STAT3 protein based on western immunoblotting data for the two-week chronic ZT06 group. Expressions were normalized to the actin band in A, *$p < 0.05$, N=10. Error bars represent SEM.

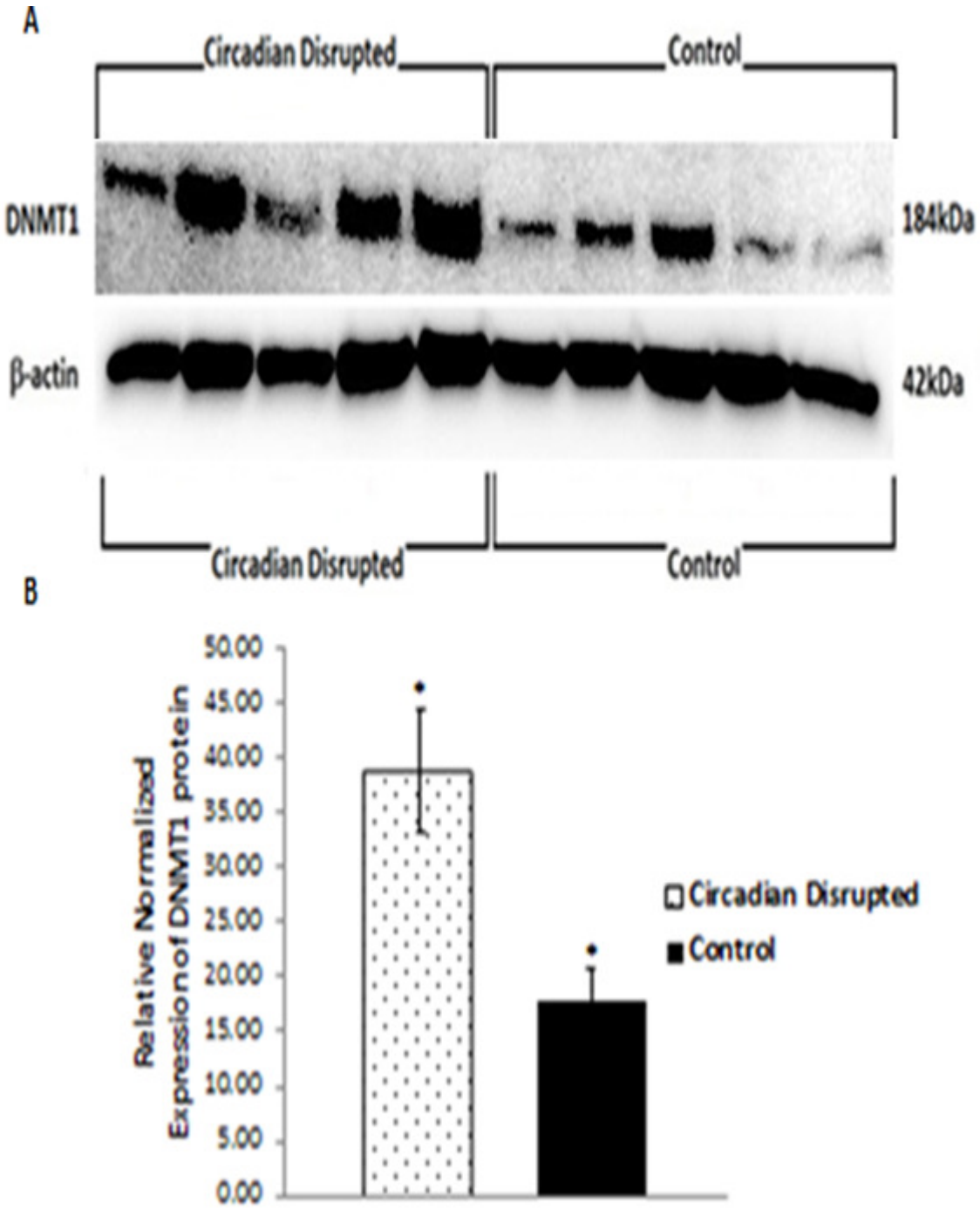

Figure 2.8: Circadian disruption causes increased expression of DNMT1. A. Western immunoblotting images of DNMT1 and β-actin from a 6% SDS-PAGE gel for the two-week chronic ZT06 group. Images were taken with the FluorChem HD2. The five samples on the left represent the control samples, and the five samples on the right represent the circadian disrupted samples. **B**. Mean relative expression of the DNMT1 protein based on western immunoblotting data for the two-week chronic ZT06 group. Expressions were normalized to the actin band in A, *$p < 0.05$, N=10. Error bars represent SEM.

CHAPTER 3: CIRCADIAN DISRUPTION-INDUCED SIGNALLING PATHWAY DEREGULATION IN RAT MAMMARY TISSUES

ABSTRACT

Evidence is mounting that circadian disruption (CD) is a potential carcinogen in breast cancer development. However, very few studies have investigated the potential epigenetic link in CD-induced breast cancer, and no studies have investigated the direct influence of CD on a broad range of transcripts in mammary tissues. Although DNA methylation profiles on peripheral blood samples from shift workers have identified changes in relevant genes, DNA methylation changes in blood are only potentially reflective of what is occurring in the mammary tissues. Since changes in DNA methylation profiles can correlate with changes in transcript levels, the current study utilized a proven photoperiod-shifting paradigm, various degrees of CD, and Illumina sequencing to investigate the direct influence of circadian disruption on transcript levels in the mammary tissues of a rodent model system. Even though our analysis did not identify any significant patterns in mRNA levels based on the degree of CD and the majority of groups did not show changes in gene expression on a large scale, one group (two-week chronic deregulation) displayed 196 differentially expressed genes, 51 of which have been linked to breast cancer. Through gene-specific pathway analysis, the data illustrate that CD may promote breast cancer development through downregulation of DNA repair and p53 signaling pathways, thus promoting genomic instability and cancer development. Although these results have to be interpreted with caution because only a single group illustrated drastic changes in transcript levels, they indicate that CD may directly induce changes in gene expression on a large scale with potentially malignant consequences.

INTRODUCTION

Proper regulation of gene expression is critical for normal cellular function. The study of epigenetics has identified several avenues that can influence the regulation of gene expression. A key component of the epigenome, DNA methylation can directly influence changes in gene expression at the transcriptional level, and changes in DNA methylation profiles can correlate with changes in transcript levels (Jin et al., 2011). One of the most abundant malignancies in the world, breast cancer is already a serious public concern, and evidence has been mounting that circadian disruption is a carcinogen that can trigger and promote the development of breast cancer (Stevens et al., 2014). Although numerous studies have been conducted on CD-induced breast cancer, the majority of these studies have focused on gene-specific analysis, and very few studies have investigated the effect of CD-induced changes across a broad range of genes. As a consequence, the direct effect of circadian disruption in mammary tissues on important cellular mechanisms and pathways, such as DNA damage response (DDR) and p53 mediated signaling, remains largely unknown.

The DNA in every human cell is exposed to tens of thousands of aberrations per day, with stresses originating both endogenously and exogenously (Jackson and Bartek, 2009; Lindahl and Barnes, 2000). Given this constant pressure, cells have evolved mechanisms to detect DNA lesions and to initiate repair of these lesions through signal pathways, with these processes being collectively called the DNA damage response (Jackson and Bartek, 2009). This DNA damage response is a key mechanism to ensure proper cellular function, and pathways related to this mechanism are frequently deregulated in cancers (Jackson and Bartek, 2009). Changes to DNA repair pathways in

tumorigenesis can vary greatly depending on different factors, such as the type of breast cancer and the stage of development (Davis and Lin, 2011; Li et al., 2010). However, in terms of tumour initiation, downregulation of DNA repair pathways has been concretely linked to increased genomic instability and progression of cancer (Negrini et al., 2010).

Cell cycle regulation and apoptosis, which are linked to the DDR system, are necessary for proper cellular function; when they malfunction, they can lead to cancer development (Reinhardt and Schumacher, 2012). Acting as a sort of failsafe system by initiating programmed cell death, apoptosis helps to maintain proper cell function and proliferation (Wong, 2011). Extensive research has shown that decreased levels of apoptosis is a hallmark of cancer progression, with aberrant activity of pro-apoptotic and anti-apoptotic proteins playing a crucial role in this process (Wong, 2011). Amongst the most important pro-apoptotic proteins is p53, a corner stone of tumour suppression activity that has been described as being the guardian of the genome because it responds to various stress signals and helps maintain proper cellular function (Bieging et al., 2014; Lane, 1992). Influencing numerous cancer-relevant pathways besides apoptosis, the p53 signaling pathway is often aberrantly regulated in many malignancies, making it a crucial player in cancer development.

Although numerous studies have investigated links between circadian disruption and breast cancer, surprisingly, not many studies have investigated CD-induced changes through gene expression profiles in mammary tissues (Stevens et al., 2014). Amongst the studies that have investigated gene expression in mammary tissues in relation to circadian rhythms, the focus was not placed on broad gene expression profiles or circadian disruption, but on either the influence of mammary development or chemical induction of

changes to specific circadian genes within the mammary gland (Fang et al., 2010; Metz et al., 2006). In fact, the studies that have come closest to investigating the broad CD-induced gene expression profile in mammary tissues have been the epigenetic experiments on shift worker blood samples that have provided evidence of changes in DNA methylation at important breast cancer relevant genes (Zhu et al., 2011). Since epigenetic profiles of peripheral blood samples have shown a strong correlation with reflective changes in mammary tissues, these DNA methylation profiles may correlate to transcript levels in mammary tissues (Liu et al., 2015; Widschwendter et al., 2008). However, these studies still do not represent a direct attempt to investigate CD-induced gene expression profiles in mammary tissues, and provide no direct information on the potential pathways and cellular mechanisms that may be affected. Therefore, given that transcript levels are an extension of the epigenome, this study will attempt to investigate the effect of varying degrees of circadian disruption on gene expression in mammary tissues by incorporating a proven photoperiod-shifting paradigm and a rodent model system.

MATERIAL AND METHODS

Animal model and circadian disruption paradigm

Female Sprague Dawley rats from Charles River (Quebec) were housed at the Canadian Center for Behavioural Neuroscience at the University of Lethbridge. The rats were housed in a sterile facility in a temperature controlled room, two per cage, and given food and water *ad libitum*. Handling and care of the animals was performed in accordance with the recommendations of the Canadian Council on Animal Care, and the procedures were approved by the University of Lethbridge Animal Welfare Committee.

Before the start of the experiment, all the rats were entrained to a 12-hour light-dark cycle for 22 days to allow entrainment to a normal light schedule. At 83 days old, the rats were then randomly assigned to different treatment and control groups.

Circadian disruption was induced by following a photoperiod-shifting paradigm that has been shown to cause physiological and behavioural changes in rodents (Craig and McDonald, 2008; Deibel et al., 2014; Devan et al., 2001; McDonald et al., 2013). In total, 40 female rats underwent this photoperiod-shifting (PS) paradigm. To stimulate PS, the colony lights were turned on three hours earlier each day. To investigate the effect of varying degrees of CD, the 40 rats were separated into acute and chronic circadian disruption groups. Twenty rats underwent acute photo-period shifting, which consisted of lights coming on three hours earlier each day for a total cycle time of six days. Another 20 rats underwent chronic photoperiod-shifting, which consisted of a rotation between lights coming on three hours earlier each day for six days, and then ten days of a regular 12-hour light-dark cycle, for a total cycle time of 54 days. For both the acute (6 days) and chronic groups (54 days), following the PS cycle, the rats were exposed to a normal 12-hour light-dark cycle until it was time for tissue extractions.

The acute (20 rats) and chronic (20 rats) CD groups were then separated further based on the time of tissue extraction. Mammary tissue extractions occurred 24 hours and two weeks following acute or chronic circadian disruption, with ten rats from each CD group undergoing tissue extractions at each of these times. To account for and investigate the potential influence of specific time points within a 24-hour circadian cycle on CD-induced breast carcinogenesis, two different tissue extraction time points, each corresponding to a specific zeitgeber time, were performed on each tissue extraction day

(24 hours and two weeks following CD). Half of the rats (five) in each tissue extraction group were sacrificed at ZT06 (6 hours after lights on) and the other remaining rats (five) from each group were sacrificed at ZT19 (19 hours after lights on). These two different ZT points were chosen because they represented the light and dark phases of the circadian cycle.

Both the acute (20 rats) and chronic (20) control groups were exposed to a 12-hour light-dark cycle for either six days (acute) or 54 days (chronic). The rats from each CD control group were then exposed to a 12 hour light-dark cycle for either 24 hours (10 rats) or two weeks (10 rats) depending on the time of tissue extraction for the corresponding experimental group. From each tissue extraction control group (10 rats), five rats were sacrificed at ZT06 and five rats were sacrificed at ZT19 on the corresponding tissue extraction day.

Euthanasia of the rats was performed through anesthesia with Isoflurane (4–5 %; oxygen at 2 liters per minute) and decapitation by a guillotine, with euthanasia of the rats alternating between the control and experimental rats. The mammary glands were collected, immediately stored in liquid nitrogen, and stored long-term at a temperature of -80°C.

Total RNA extraction

Whole mammary tissues were ground in liquid nitrogen using sterile, chilled mortars and pestles. Approximately 0.05 g of ground tissue from each sample was then suspended in Zymo Research tri-reagent, and lysed using two cycles of the Qiagen Tissue Lyser II for 2 minutes at 25 Hz. Total RNA was then extracted using the direct-zol RNA Miniprep kit from Zymo research (R2053). The quality of the RNA was then checked

using Nanodrop 2000c, and quality bio-analysis was conducted using the Agilent 2100 and the Agilent Small RNA Kit and Chip (5067-1548), with only samples having a RIN value greater than eight being used in downstream applications.

Gene expression sequencing and bioinformatics analysis

Three samples from each tissue extraction and ZT group for both the experimental and control groups were randomly chosen to undergo sequencing analysis. The TruSeq RNA Sample Preparation Kit V-2/SetA from Illumina (RS-122-2001) was used to prepare small RNA libraries from 1 µg of total RNA. qPCRs were then run to validate the libraries following the PCR program recommended by the Illumina RNA Sample Prep Kit (3 mins at 95°C; 40 cycles of 3 sec. at 95°C, 30 sec. at 60°C). Cluster generation for sequencing was performed using cBot and the TruSeq SR Cluster Kit v2-cBot-GA (GD-300-2001) from Illumina. Single-read sequencing was performed using the TruSeq SBS Kit v5-GA (FC-104-5001) from Illumina on the Genome Analyzer GAIIx at 36 cycles.

Bioinformatics approaches on the sequencing data were used to define gene expression levels amongst the different experimental groups. Basecalling and demultiplexing were performed using CASAVA 1.8.1 pipeline (Illumina) with default settings. The quality of the libraries was evaluated using FastQC v0.10.1 software. Contaminating sequences (adapters, phiX, polyA, polyC, ribosomal RNA) were filtered out, and filtered sequence reads were aligned to the rat genome assembly Rnor 5.0 (Ensembl) from Illumina's iGENOME database. Files in sam format were converted to bam, sorted by chromosomal position, and indexed. Further quality control was performed using the RSeQC_2.3.7 software package, with no libraries being removed due to quality problems.

qRT-PCRs

Validation of the sequencing results was performed by qRT-PCR. cDNA was synthesized using 500 ng of RNA and the iScript Select cDNA Synthesis Kit (#170-8897) from BioRad. The qPCRs were performed utilizing SYBR Green on the BioRad C1000 Thermal Cycler and CFX96 Real-Time System, by using SosoFast EvaGreen Supermix (#172-5201) from BioRad. Gradient PCRs were run for each primer to determine appropriate and optimal annealing temperatures. All qPCRs were run in triplicate, using 2 min at 95°C; 40 cycles of 5 sec at 95°C, 5 sec at primer specific temperature. Efficiency standard curves for the primers were generated using serial dilutions, and after all the qPCR cycling, melt curve analysis was conducted using the optimal parameters for the BioRad C1000 thermal cycler (65°C to 95°C, increments of 0.5°C).

Forward and reverse primers for the genes of interest were designed using the PrimerQuest program from Integrated DNA Technologies, and the custom oligos were ordered from Eurofins Genomics. Based on Hvid et al. 2011, recommended reference genes were ordered from Eurofins Genomics with the same forward and reverse primer sequences. The best combination of two reference genes, ATP5b and Sdha, was found using the programs NormFinder (http://moma.dk/normfinder-software) and qbase^{plus} (Biogazelle), with the stability values meeting the geNorm stability cut offs (CV < 0.25, M-Value < 0.5).

Statistical analyses

Exploratory statistical analyses on the sequencing data and outlier detection were performed using Array Quality Metrics, and gene set enrichment analysis was conducted

using GOStats. Statistical significance between the experimental and control groups was evaluated by one-way ANOVA. Multiple comparisons adjustment was performed using Benjamini-Hochberg procedure, and genes with an adjusted p-value below 0.1 were considered differentially expressed (Benjamini and Hochberg, 1995). The results are expressed as heat maps and MA plots. For the qRT-PCR data, Student's t-test was used for independent variance to determine significance ($p<0.05$). Statistical analysis and plotting of the data was performed using MS Excel software for Windows. The results are presented as mean relative expression values ± standard error of the mean (SEM).

RESULTS

Circadian disruption results in the differential expression of a broad range of genes linked to breast cancer

In this study, we investigated the effect of varying degrees of circadian disruption on gene expression in the mammary tissues of Sprague Dawley rats. The influence of light dependent zeitgeber times was also incorporated to investigate possible fluctuations within a 24-hour circadian cycle. There were no significant patterns in gene expression between the various groups with regards to the degree of CD, tissue extraction time after CD, or ZT (Figure 3.1, Table 3.1). Although the sequencing results identified a broad range of differentially expressed genes, many of which are linked to breast cancer, only two groups illustrated more than ten changes in gene expression, the 24-hour acute ZT06 and two-week chronic ZT19 groups (Table 3.1). The majority of these differences were mainly found in the two-week chronic ZT19 group, in which 196 differentially expressed genes were identified, 51 of which have been linked to breast cancer (Table 3.1; Figure 3.2).

The CD-induced gene expression changes in the two-week chronic ZT19 group correlate to disturbances in breast cancer related pathways

To analyze the significance of the gene expression changes in the two-week chronic ZT19 group in terms of potential complications and breast cancer, pathway analysis was performed. The results illustrated that a substantial number of pathways and processes linked to diseases and complications were altered in these rats (Figure 3.3). Based on this data, gene-specific pathway analysis for some of the breast cancer-relevant pathways was performed to gain more details and insight into the effects of CD. The analysis revealed that numerous DNA repair pathways were altered: base excision repair (BER), homologous recombination (HR), mismatch repair (MMR), and nucleotide excision repair (NER) (Figures 3.4–3.6). The overall gene expression patterns amongst these pathways correlated to decreased DNA repair, a hallmark in the initiation of tumorigenesis. Gene-specific pathway analysis was also performed on the p53 signaling pathway, with the gene expression patterns correlating to decreased p53 signaling, decreased DNA repair, and decreased apoptosis (Figure 3.7). Aberrant regulation of the p53 signaling pathway and decreased apoptosis is a corner stone in the development of most cancers, including breast cancer.

qRT-PCR validation produces the same expression trends, but not significant differences

Validation of the sequencing results was performed on several of the differentially expressed genes through qRT-PCR analysis, including the *Cdk1*, *PDK4*, and *Nusap1* genes (Figure 3.8 and 3.9). Although the qRT-PCR data produced the same expression trends, the data did not produce significant differences (Figure 3.8 and 3.9). In general,

the same pattern was observed in the majority of the genes that were analyzed through qRT-PCR: similar expression trends, but no significant difference. These results may be due to the fact that the two samples that did not undergo Illumina sequencing usually produced qPCR expression trends that were not consistent with the sequencing data, while the three sequenced samples were consistent. Given that multiple reference genes were tested and the stability values for these genes based on recommended programs were well within the recommended ranges (Table 3.2), it is unlikely that the qRT-PCR data is unstable. This indicates that the gene expression differences based on Illumina sequencing in the two-week chronic ZT19 group may have been random, which would explain such drastic expression differences in this experimental group (Table 3.1). However, the qPCR expression trends are consistent when all ten samples are analyzed, indicating that with a larger sample size significant gene expression changes may have been produced through qRT-PCR.

DISCUSSION

Numerous publications have provided consistent evidence that circadian disruption acts as a carcinogen in breast cancer development, but no studies have conducted large-scale gene expression profiles on CD-induced changes in mammary tissues. Therefore, in this study we investigated the effect of varying degrees of CD on gene expression in rat mammary tissues. Although our findings did not produce any significant patterns based on the degree of CD or tissue extraction time, one group, the two-week chronic ZT19 group, did experience drastic changes in gene expression, indicating that long-term circadian disruption may be potentially linked to a cascade of differential gene expression in mammary tissues (Figure 3.1 and 3.2; Table 3.1).

DNA repair mechanisms are key components of the DNA damage response, and the sequencing data illustrated that several DNA repair pathways were aberrantly expressed due to CD in the two-week chronic ZT19 group (Figure 3.3). Specifically, gene-specific pathway analysis showed that key genes in the BER, HR, MMR, and NER pathways were down regulated (Figures 3.4–3.6). Decreased DNA repair has been associated with increased genomic instability and progression of cancer, and these results show that CD may decrease DNA repair on multiple fronts (Negrini et al., 2010). Furthermore, amongst these repair mechanisms, homologous recombination has been shown to play a crucial role in breast cancer development (Helleday, 2010). HR is involved in double strand break (DSB) repairs, and mutations to genes involved in this process are linked to tumours and gene rearrangements; HR can also be viewed as a last resort for DNA repair if DNA lesions are not identified and mended by other repair mechanisms (Helleday, 2010). Gene pathway analysis illustrated that CD-induced down regulation of HR was associated with decreased expression of *BRCA2*, a tumour suppressor gene that has been extensively linked to breast cancer development, as well as lowered expression of *Rad50* and *Mre11*, which are both key components of the MRN complex and are linked to increased breast cancer susceptibility (Figure 3.6) (Bartkova et al., 2008; Shahid et al., 2014). In addition, sequencing results illustrated significant decreased expression of the *Nusap1* gene (Figure 3.9), which has been shown to regulate levels of BRCA1, an extensively studied tumour suppressor gene involved in double strand breaks and breast cancer (Kotian et al., 2014). These results indicate that CD-induced breast cancer development may be linked to aberrant DNA repair through multiple mechanisms, with silencing of important genes involved in homologous

recombination possibly playing a crucial role.

Another pathway linked to the DDR system is the extensively studied p53 signaling pathway, with our data showing that CD caused decreased activity in this pathway in the two-week chronic ZT19 group (Figure 3.3). The p53 pathway has long been a corner-stone of cancer research because of its important role in the cell cycle and apoptosis (Bieging et al., 2014). Gene expression pathway analysis on the p53 signalling pathway illustrated that key genes involved in this pathway were mostly down regulated, while the *IGF* gene was up regulated (Figure 3.7). Amongst the most consistently down regulated genes were check point kinase 1 (*CHK1*), *CHK2*, *CASP8*, *Bid*, and sestrins (Figure 3.7). Both CHK1 and CHK2 play a crucial role in cell cycle arrest and apoptosis because they are part of the DDR mechanism and can activate p53 (Jackson and Bartek, 2009). The *CASP8* and *Bid* genes play a role in the signaling cascade that can induce apoptosis, with *CASP8* being able to modify Bid activity (Kooijman, 2006). The *sestrin* genes are induced by p53 upon DNA damage, help regulate stress responses to environmental stimuli, and have been shown to decrease tumour growth in some cancer cells (Lee et al., 2010). Finally, expression of the insulin-like growth factor (*IGF*) was up regulated in all three of the sequenced samples (Figure 3.7). IGF has been identified as an inhibitor of apoptosis in many different cell types through various mechanisms, including inactivation of *CASP8* through FLIP activity (Kooijman, 2006). Taken together, these CD-induced gene expression patterns correlate with decreased apoptosis and increased genomic instability, indicating further contributions to cancer initiation and echoing the theme illustrated by the CD-induced changes in the DNA repair mechanisms previously discussed.

Although the results discussed thus far have provided compounding evidence on the potential influence of CD on breast cancer development through aberrant gene expression patterns, only limited conclusions can be drawn because the changes are isolated to one group (Table 3.1). As mentioned previously, no patterns in gene expression changes were observed based on the degree of CD or tissue extraction times, with only the two-week chronic ZT19 group undergoing changes in gene expression on a high enough scale to significantly influence breast-cancer-relevant pathways (Table 3.1). Furthermore, although the qRT-PCRs generated the same expression trends as the sequencing results, the trends were not significant based on the qRT-PCR data (Figure 3.8 and 3.9). Given that the qPCR reference genes were stable (Table 3.2), these results, coupled with the fact that the non-sequenced samples typically generated opposite expression patterns in the qRT-PCR data, indicate that the drastic expression trend identified in the two-week chronic ZT19 group may have been random, or a reflection of an inter-individual variability in CD responses. However, the offsetting contribution of the non-sequenced samples to the qRT-PCR results was not enough to alter the same expression trend (Figure 3.8 and 3.9). This indicates that with a larger sample size in every group, the same results illustrated in the two-week chronic ZT19 group may have been generated in the other groups as well.

To our knowledge, this study represented the first attempt at directly investigating wide range CD-induced changes to gene expression in rodent mammary tissues. Although the results did not produce significant patterns or findings on the influence of circadian disruption based on the degree of CD or tissue extraction time, the study did provide evidence that CD may directly induce gene expression changes on a significant

scale in mammary tissues. Specifically, the results indicate that circadian disruption may alter DNA damage response mechanisms and p53 signaling in a manner that initiates and promotes breast cancer development. However, based on the results, it seems that the small sample size may either be contributing to the generated results, or masking the extent of the CD-induced changes in gene expression. Therefore, repeating the experimental design with some slight variations and increased group size is a warranted future step. Although these results have to be interpreted with caution, they signify that aberrant CD-induced gene expression changes in mammary tissues might play a role in breast cancer initiation through potentially malignant changes to DNA damage response mechanisms and p53 signaling.

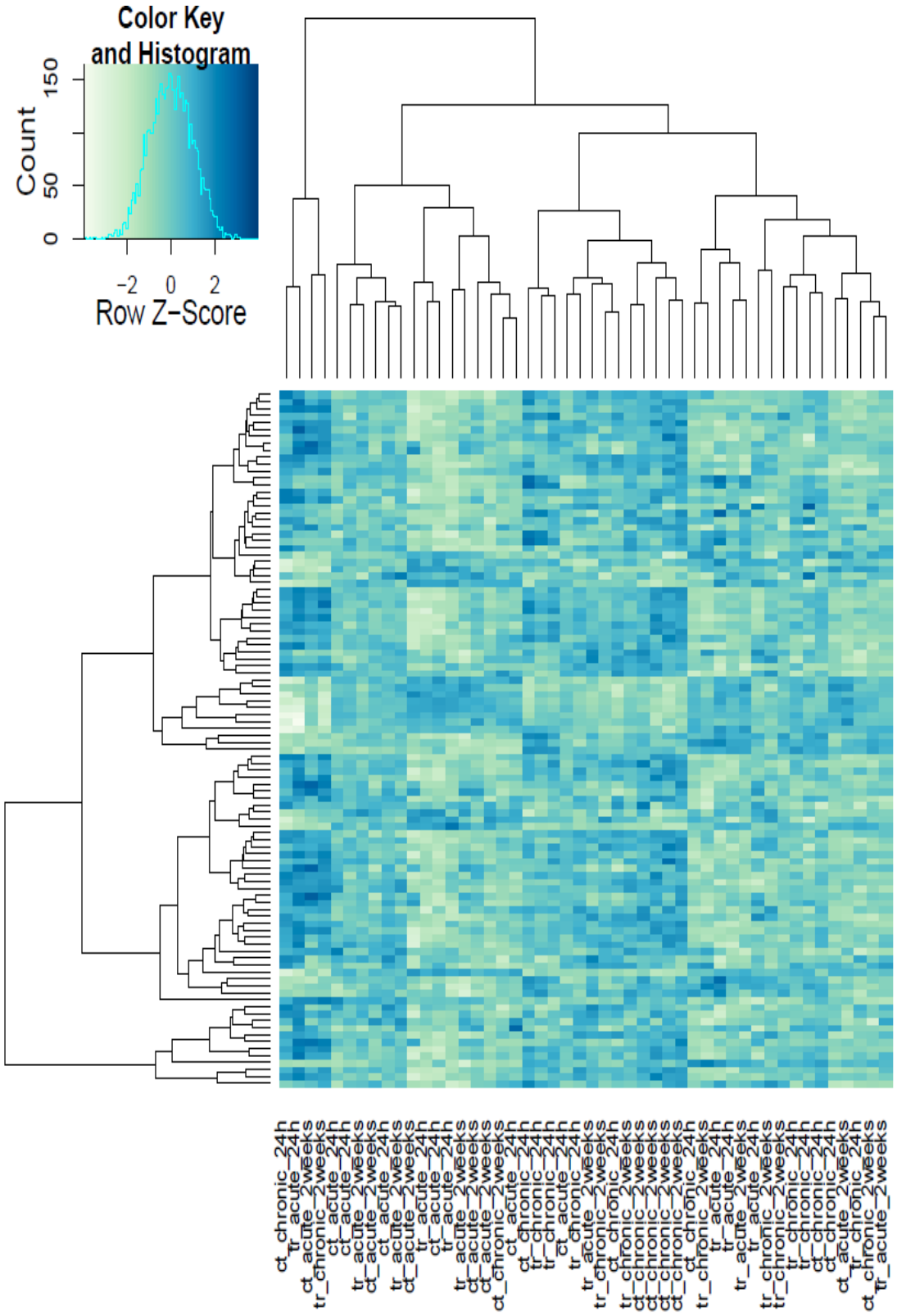

Figure 3.1: Heat map cluster of the Illumina gene sequencing results. All of the sequenced samples based on the degree of circadian disruption and tissue extraction times.

Table 3.1: Circadian disruption induces changes in gene expression. Number of genes differentially expressed in all the experimental groups based on the Illumina sequencing data.

Group	Number of Genes Differentially Expressed	Number of Genes linked to Breast Cancer
24-hr Acute ZT06	15	12
24-hr Acute ZT19	0	0
2-Week Acute ZT06	0	0
2-Week Acute ZT19	2	2
24-hr Chronic ZT06	2	0
24-hr Chronic ZT19	5	4
2-Week Chronic ZT06	4	3
2-Week Chronic ZT19	**196**	**51**

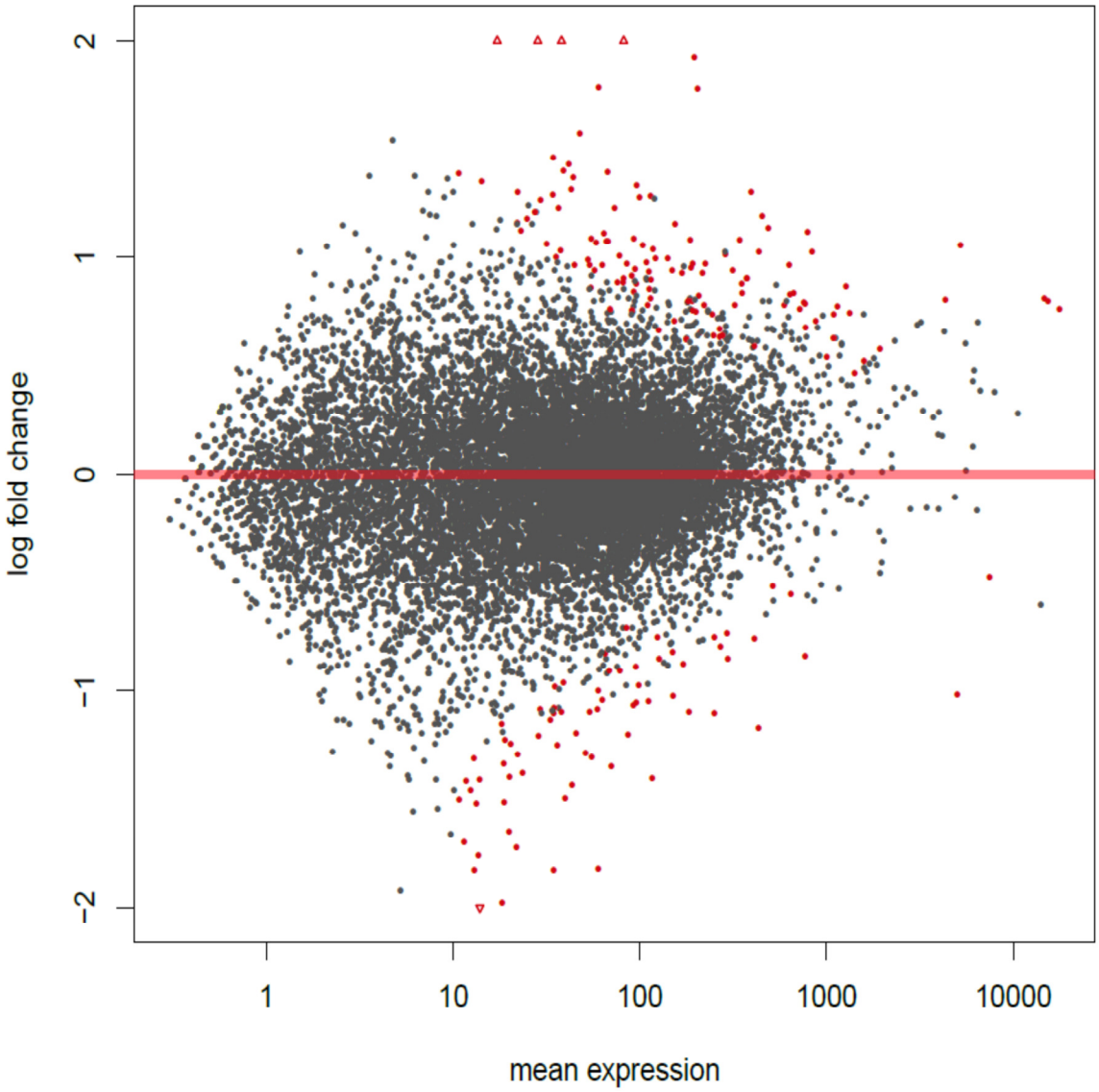

Figure 3.2: Circadian disruption causes large scale gene expression changes in the 2-week chronic ZT19 group. MA plot based on the gene expression Illumina sequencing results for the 2-week chronic ZT19 group. The y-axis represents log fold change, while the x-axis represents mean expression. The plots represent all the gene expressions that were identified, and the red plots represent genes that showed a significant expression difference; P-adjusted < 0.1, N=6.

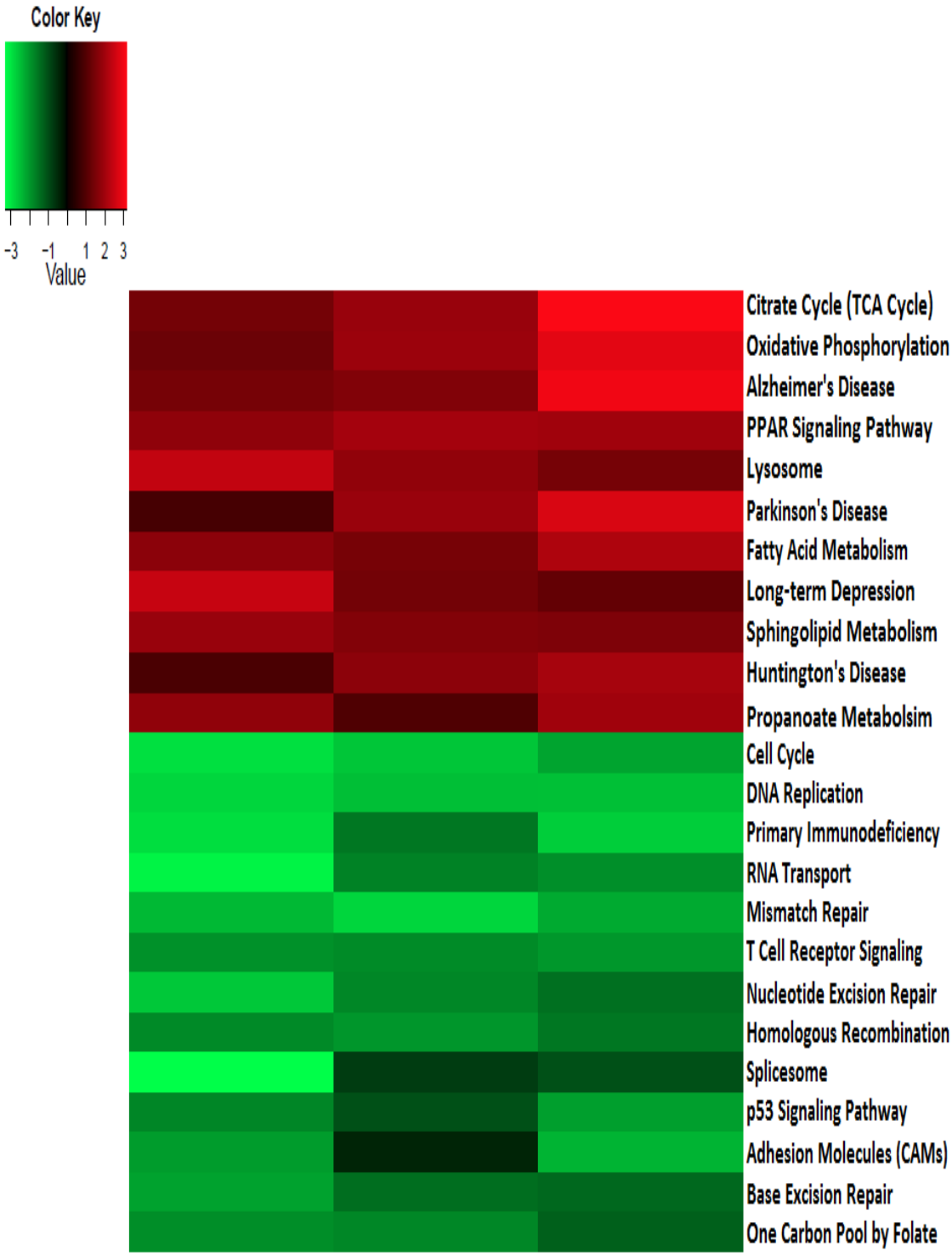

Figure 3.3: Circadian disruption causes changes to disease related and breast cancer relevant pathways in the 2-week chronic ZT19 group. Heat map cluster based on the gene expression Illumina sequencing results for disease related and breast cancer relevant pathways in the 2-week chronic ZT19 group. The red bands represent over expressed pathways and the green bands represent under expressed pathways.

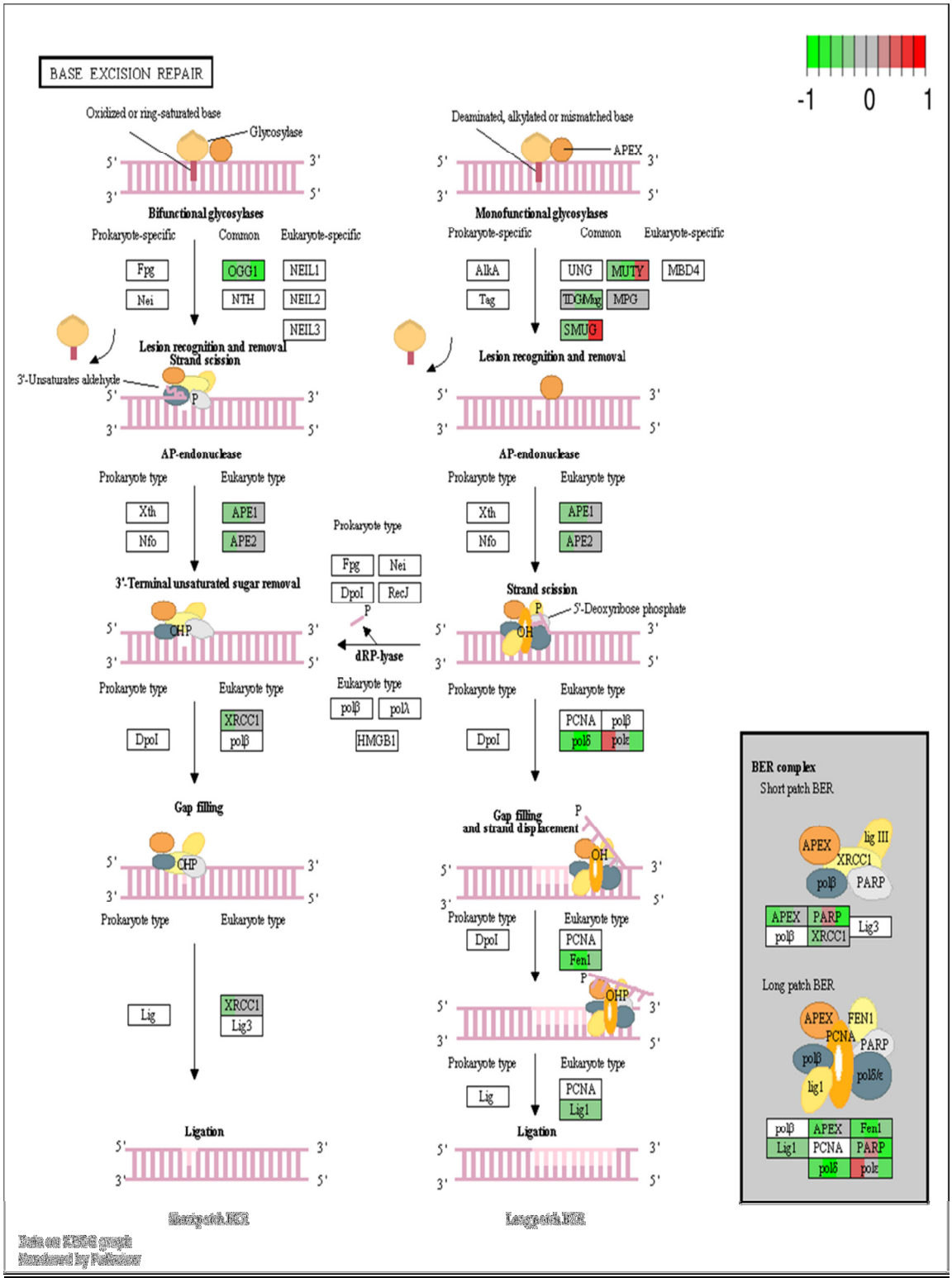

Figure 3.4: Circadian disruption induced changes to the base excision repair pathway. Each gene is broken down into three portions and represents the three sequenced samples, with red correlating to overexpressed and green correlating to underexpressed genes. Pathways were generated using KEGG graphs and rendered by Pathview.

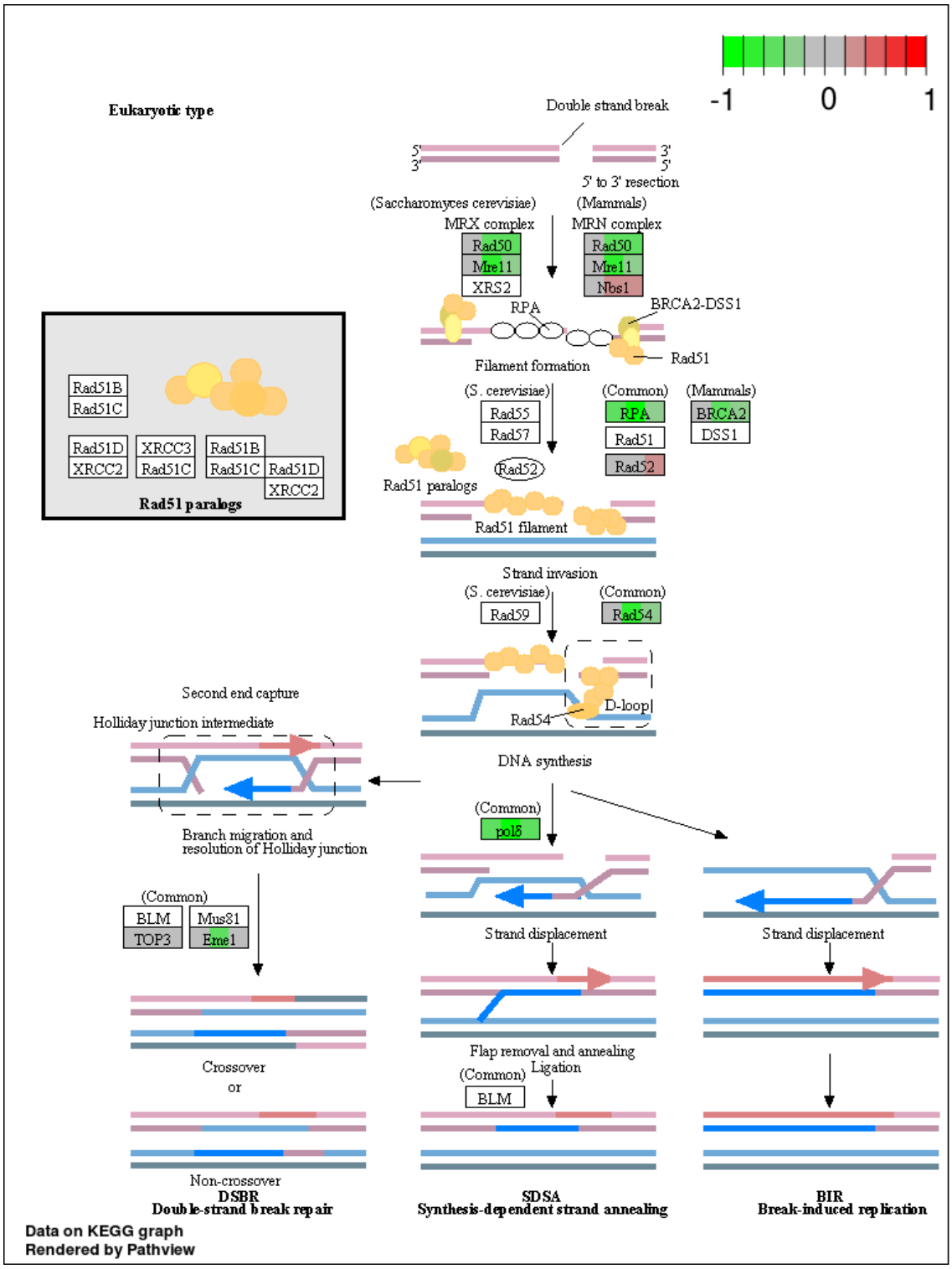

Figure 3.5: Circadian disruption induced changes to the homologous recombination pathway. Each gene is broken down into three portions and represents the three sequenced samples, with red corresponding to overexpressed and green corresponding to underexpressed genes. Pathways were generated using KEGG graphs and rendered by Pathview.

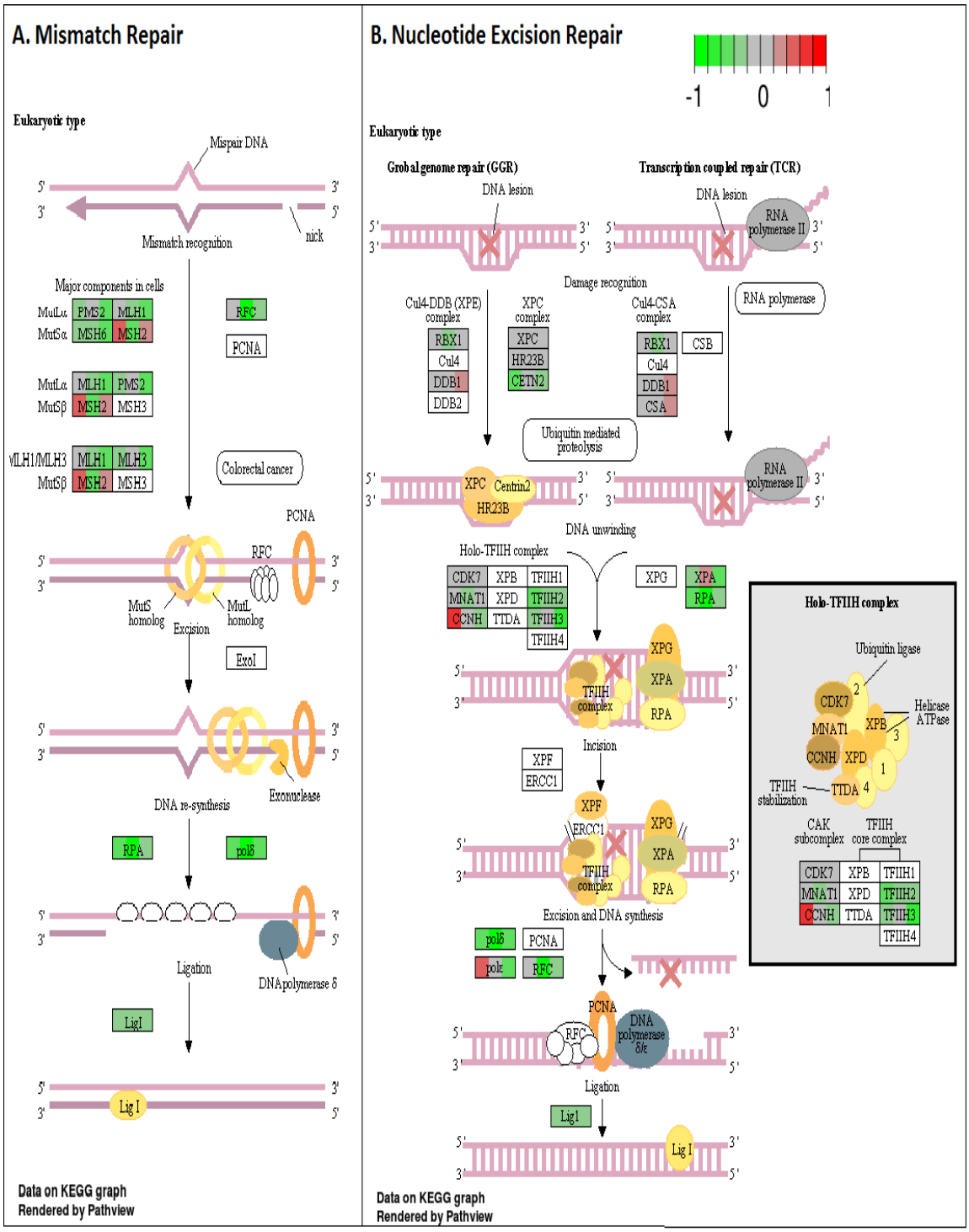

Figure 3.6: Circadian disruption induced changes to the mismatch repair and nucleotide excision repair pathways. A. Mismatch Repair pathway and **B**. Nucleotide Excision Repair pathway based on the Illumina gene sequencing results. Each gene is broken down into three portions and represents the three sequenced samples, with red correlating to overexpressed and green correlating to underexpressed genes. Pathways were generated using KEGG graphs and rendered by Pathview.

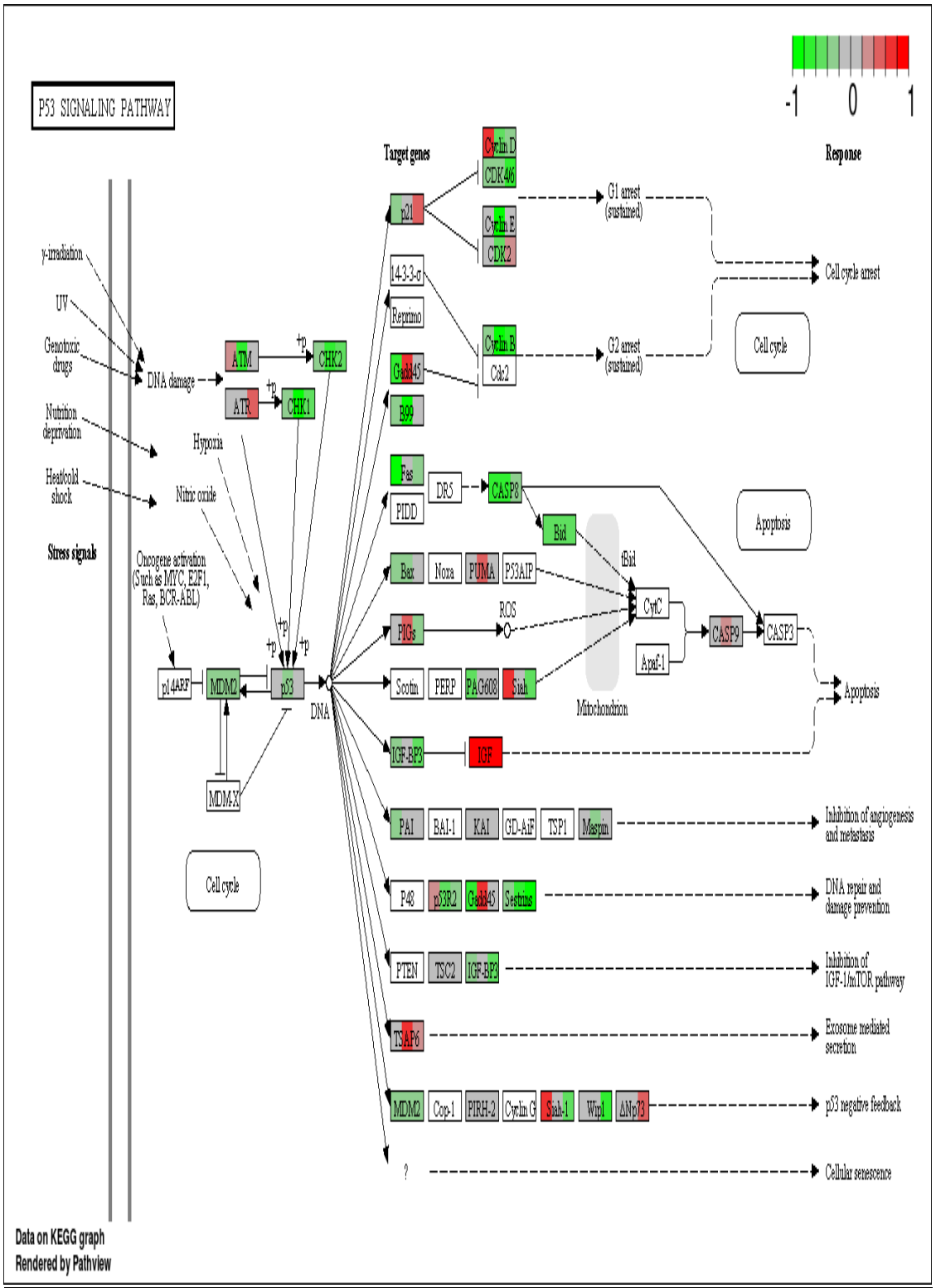

Figure 3.7: Circadian disruption induced changes to the p53 signaling pathway. Each gene is broken down into three portions and represents the three sequenced samples, with red correlating to overexpressed and green correlating to underexpressed genes. Pathways were generated using KEGG graphs and rendered by Pathview.

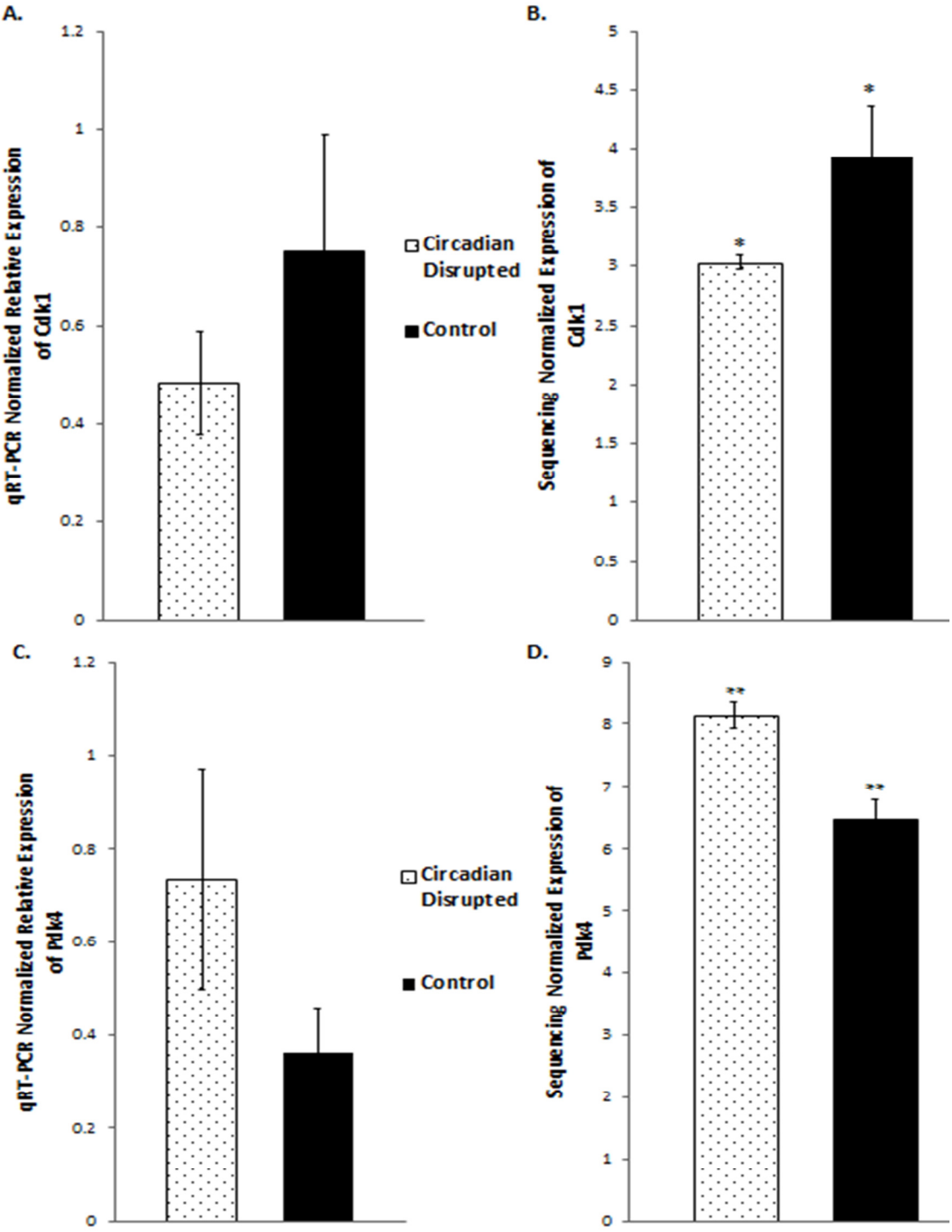

Figure 3.8: qRT-PCR and Illumina sequencing data for the *Cdk1* and *Pdk4* genes. A. Mean relative expression of the *Cdk1* gene based on qRT-PCR, N=10. **B**. Mean Relative expression of the *Cdk1* gene based on Illumina sequencing, N=6, *p-adjusted < 0.05 **C**. Mean Relative expression of the *PDK4* gene based on qRT-PCR, N=10.
D. Mean relative expression of the *PDK4* gene based on Illumina sequencing, N=6, **p-adjusted < 0.01. Error bars represent SEM.

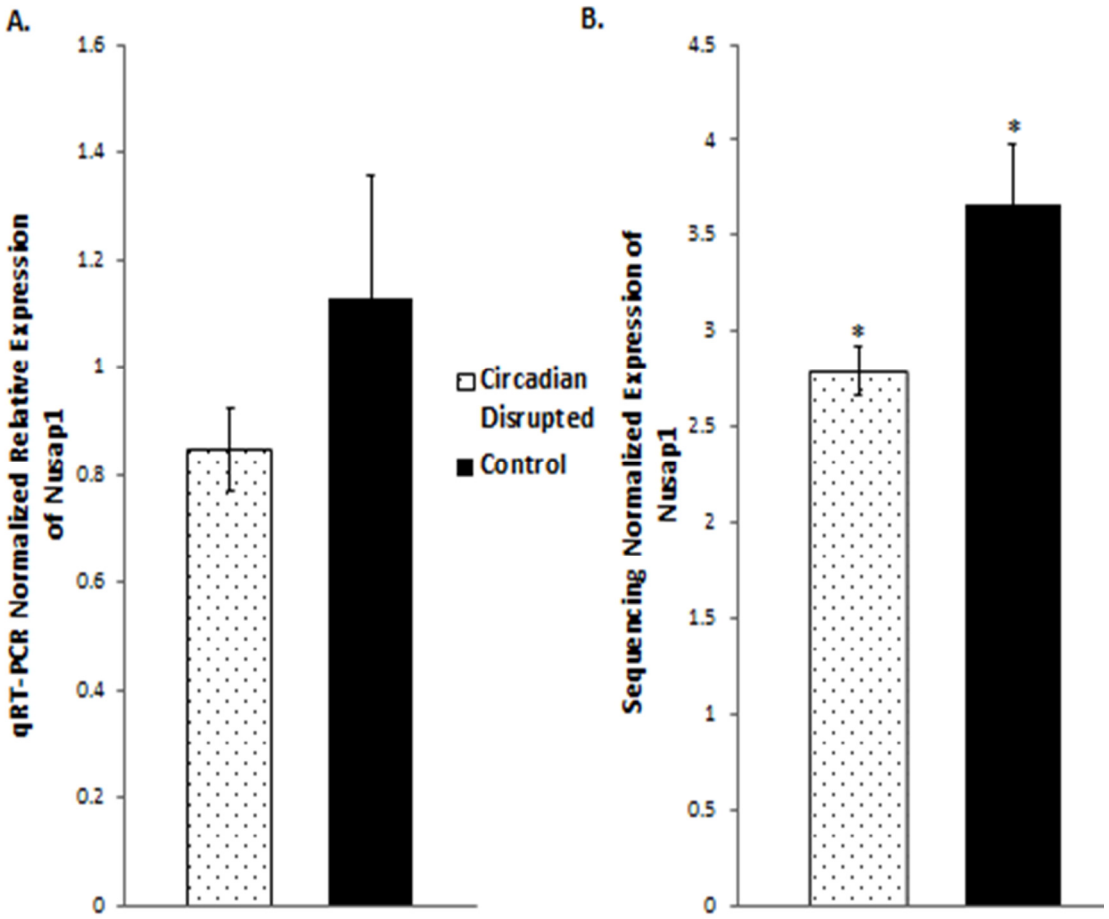

Figure 3.9: qRT-PCR and Illumina sequencing data for the *Nusap1* gene. A. Mean relative expression of the *Nusap1* gene based on qRT-PCR, N=10. **B**. Mean Relative expression of the *Nusap1* gene based on Illumina sequencing, N=6, *p-adjusted < 0.05. Error bars represent SEM.

Table 3.2: Stability data for the qRT-PCR results. Normfinder stability values, and GeNorm coefficient and M-values for the reference genes used in the qRT-PCR analysis.

NormFinder			
Gene Name	Stability Value	Best Combination of Two Genes	Combined Stability Value
ATP5b	0.027	ATP5b and Sdha	0.032
Sdha	0.058		
Tbp	0.115		
GenNorm			
Gene Name	Coefficient Variance	M-Value	
ATP5b	0.0671	0.1929	
Sdha	0.0666	0.1929	

*Cut off for acceptable stability: CV < 0.25; M-Value < 0.5.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSIONS

There is mounting evidence that circadian disruption (CD) is a carcinogen in the development of breast cancer. Given that breast cancer is already the most common malignancy affecting women worldwide and that one-third of the Canadian labour force does not work a regular daytime shift, investigating the mechanisms involved in CD-induced breast cancer is of utmost importance.

Numerous studies have provided evidence of potential mechanisms linking circadian disruption to breast cancer development. These include case studies that provide indirect evidence that shift workers are at a higher risk of developing breast cancer, the consequences of lower nocturnal MLT levels due to CD, the involvement of circadian clock genes in breast cancer development, and model systems showing increased xenograft tumour growth due to CD (Barh et al., 2010; Blask et al., 2003; Cos et al., 2006; Hansen and Stevens, 2012; Haus and Smolensky, 2013; Knutsson et al., 2013). However, despite the fact that these studies have highlighted CD-induced breast cancer as a warranted concern, surprisingly, very few studies have taken different approaches to investigating CD-induced breast cancer.

One of the potential new approaches to investigating CD-induced breast cancer is epigenetics. Two specific epigenetic mechanisms, DNA methylation and miRNAs, have been increasingly recognized in regard to both breast cancer development and circadian rhythms. Studies have shown that both DNA methylation and miRNAs are crucial components in breast cancer development through various mechanisms that can exert both oncogenic and oncostatic influences (Jovanovic et al., 2010; Koturbash et al., 2011). In terms of circadian rhythms, evidence shows that all the core clock genes can undergo aberrant promoter methylation in breast cancer and that DNA methylation may play a

direct role in the circadian clock (Azzi et al., 2014; Joska et al., 2014). miRNAs have been shown to oscillate with the circadian cycle, directly influence the expression of clock genes, and even reset circadian clock signalling pathways (Alvarez-Saavedra et al., 2011; Na et al., 2009; Nagel et al., 2009).

Despite the evidence that epigenetics plays a role in breast cancer development and circadian rhythms, only a handful of studies have directly investigated the role of epigenetics in CD-induced breast cancer. The majority of these studies show that long-term shift work can cause aberrant promoter DNA methylation in breast cancer relevant genes and miRNAs in peripheral blood cells, and one study utilized a rodent model system to exhibit that CD-induced xenograft tumour growth may be linked to increased global DNA methylation (Jacobs et al., 2013; Liu et al., 2015; Schwimmer et al., 2014; Shi et al., 2013; Zhu et al., 2011). However, despite the importance of these findings, all the studies focused on DNA methylation, varying degrees of CD and epigenetic fluctuations within a 24-hour circadian cycle were not investigated, and no analysis was conducted on mammary tissues.

The goal of the current study is to address these shortcomings and provide new insights into CD-induced breast cancer by conducting an environmentally controlled experiment using a rodent model system.

To our knowledge, our results are the first to illustrate that CD directly causes changes in miRNA expressions in mammary tissues and that these changes are likely plastic and linked to the light and dark phases of the circadian cycle. Furthermore, to our knowledge, this study represents the first exploratory attempt at directly investigating wide-range CD-induced changes to gene expression in rodent mammary tissues.

Our major findings are as follows:

1. CD directly induces expression changes in a wide range of breast cancer relevant miRNAs and miRNAs linked to circadian rhythms in rodent mammary tissues.

2. The CD-induced changes in miRNA expression are likely plastic, and this plasticity is linked to the degree of CD and timeframe of reentrainment.

3. The light and dark phase of the circadian cycle potentially influences the CD-induced changes in miRNA expression, indicating that circadian dependent miRNA fluctuations should be considered when investigating CD-induced breast cancer.

4. CD directly induces aberrant expression changes in breast cancer relevant proteins, with these changes potentially being linked to the aberrant CD-induced miRNA expressions.

5. The initiation and development of CD-induced breast cancer may be linked to an interconnected web of increased NF-κB activity and increased levels of Tudor-SN, STAT3, and BCL6, with aberrant CD-induced downregulation of miR-127 and miR-146b potentially contributing to this dynamic.

6. Long-term CD can potentially induce a wide range of gene expression changes on a scale large enough to influence breast cancer relevant pathways.

7. Among the gene signalling pathways that may play a key role in the initiation of CD-induced breast cancer are DNA damage response pathways and p53 mediated signalling.

Interestingly, although the same CD schemes and the same tissues were used for analysis in both the miRNA and gene expression experiments, the results produced differing data in terms of the influence of CD on these processes. The miRNA results

produced a clear pattern of CD-induced changes among the different experimental groups, while the gene expression results only showed drastic gene expression changes in one group and no clear patterns among the experimental groups. It is important to note that gene transcript levels do not correlate to miRNA activity and expression in mammals, because translational suppression is mainly achieved through inhibitory machinery and not degradation. Therefore, the CD-induced changes in miRNA expression are not a representation of the CD-induced gene expression changes reported, and vice versa.

Given that the same tissues were analyzed in both sets of results, this opens the door for numerous possibilities and potential insights when interpreting the miRNA and gene expression data together. For example, transcript levels do correlate with changes in DNA methylation. Previous studies have reported changes in DNA methylation at breast cancer relevant promoters in shift workers, but the analysis was conducted on peripheral blood cells and not on mammary tissues. Although DNA methylation profiles in blood have been correlated to changes in mammary tissues, this still does not provide a direct representation of the CD-induced changes occurring in the mammary glands. Therefore, given our miRNA and gene expression results, it is possible that CD may not induce changes in DNA methylation to the same extent in mammary tissues as in peripheral blood cells. Of course, our gene expression results are also only a correlation in terms of DNA methylation levels, so direct investigation of CD-induced DNA methylation levels in mammary tissues is required. Furthermore, because ours was an environmentally controlled experiment that incorporated a rodent model system, and because there was no bias on the CD-induced changes through artificial MLT levels or xenografts, our results

represent an accurate assessment of the potential carcinogenicity of CD in breast cancer initiation. Based on both sets of results, it would seem that miRNA activity may play a prominent role in the initiation of CD-induced breast cancer, with this activity potentially triggering malignant downstream consequences.

FUTURE DIRECTIONS

Since our results represent the first evidence of potentially direct consequences of aberrant CD-induced miRNA activity and the first attempt to investigate wide-scale CD-induced changes in gene expression in mammary tissues, there are many possibilities for future directions.

1. Investigating the plasticity of CD-induced changes in miRNA expression through stricter CD schemes, reentrainment periods, and ZT extractions.

2. Investigating the specifics and details of light-dependent miRNA fluctuations and their potential role in CD-induced breast cancer.

3. Verifying potential circadian-relevant targets of the differentially expressed miRNAs through luciferase reporter experiments.

4. Incorporating the CD schemes and ZT extractions into xenograft models to investigate the effect of varying degrees of CD on tumour development, and investigating if miRNA and gene expression patterns can fluctuate in tumours based on circadian time points.

5. Investigating the effect and dynamic of miR-127 and miR-146b activity in varying breast cancer cell lines through knockdown or mimic experiments.

6. Repeating the experimental design for both the miRNA and gene expression in only the chronic CD groups, with larger group sizes and DNA methylation analysis.

Overall, this thesis provides the first evidence that CD directly causes changes in miRNA expression in mammary tissues with potentially malignant consequences. Additionally, it represents the first attempt at investigating the direct effect of CD on large scale gene expression changes in mammary tissues. The results align with previous studies that have shown CD as a warranted concern in breast cancer development and that the initiation of this process may be linked to aberrant CD-induced miRNA activity in the mammary tissues.

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APPENDIX A
Supplementary Figures

Supplementary Table S1: Acute photoperiod-shifting schedule.

DAY	Light Off
1	13:30
2	10:30
3	07:30
4	04:30
5	01:30
6	22:30

Supplementary Table S2: Chronic photoperiod-shifting schedule.

DAY	Lights Off	DAY	Lights Off
1	13:30	33	19:30
2	10:30	34	16:30
3	07:30	35	13:30
4	04:30	36	10:30
5	01:30	37	07:30
6	22:30	38	04:30
7-16	Re-entrainment-lights off at 19:30	39-48	Re-entrainment-lights off at 01:30
17	16:30	49	22:30
18	13:30	50	19:30
19	10:30	51	16:30
20	07:30	52	13:30
21	04:30	53	10:30
22	01:30	54	07:30
23-32	Re-entrainment-lights off at 22:30		

Supplementary Figure S1: Breakdown of the circadian disruption tissue extraction groups.