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Abstract

There is increasing evidence that disruption of circadian rhythms contributes to cancer. Circadian rhythms are regulated by a panel of specific transcription factors, called clock genes, and our current understanding of endogenous cellular rhythmicity is that both positive and negative feedback cycles of clock genes drive the expression of a growing list of other transcription factors and functional genes. It is possible that disruption of circadian control systems, which maintain normal cell function, could lead to malignant transformation into cancer cells. This concept is supported by recent findings that tumors grow faster in mice rendered arrhythmic by destruction of the suprachiasmatic nucleus, the body's circadian "clock". Furthermore, mice carrying a mutation in one of the core clock genes, *per2*, have disrupted circadian rhythmicity and are cancer prone, possibly due to permanent up-regulation of clock-controlled oncogenes.

Rationale

Epidemiological studies have linked shiftwork with an increased risk of breast cancer in humans, suggesting that circadian disruption may influence the development or progression of the disease. Previous studies, including our own have demonstrated rhythmicity of expression of key clock genes in mouse liver, heart, kidney, duodenum, ovary, lung and vascular tissue. As the patterns of expression were highly tissue-specific, it is critical to determine which clock and clock-controlled genes are expressed in mammary tissue and potentially contribute to the increased risk of breast cancer associated with shift work.

Objectives

Hypothesis: Circadian disruption of clock gene rhythmicity in mammary tissue will result in altered gene expression and lead to the loss of growth inhibition and subsequent tumorigenesis.

Experimental: (1) To measure the expression of clock and clock controlled genes in mammary tissue.
(2) To measure the expression, induction and rhythmicity of clock and clock controlled genes in human breast cancer cell lines.

Major findings

- 1) Clock genes (*Clock*, *Bmal1*, *per1*, *per2*, *cry1* and *cry2*) are highly expressed in normal mouse mammary tissue.
- 2) Clock genes are rhythmic in mouse mammary tissue with the timing consistent with rhythmicity driven by the CLOCK/BMAL1 heterodimer complex.
- 3) The expression of genes previously implicated in cell transformation and cell division (*c-myc*, *wee1*, *cyclin D1*, *Gadd45a* and *Tgf β*) is also rhythmic, with up to a 2.5 fold change in expression across 24 hours.
- 4) The timing of the gene expression is consistent with their control by the CLOCK/BMAL1 heterodimer complex.
- 5) Human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-453 and T47D) express *Clock*, *Bmal1*, *per1*, *per2*, *cry1* and *cry2* at confluence.

The demonstration of clock gene driven rhythms in oncogene, cell cycle and growth factor gene expression in the mammary gland suggests a molecular basis for the increased risk of breast cancer in shiftworkers. Shiftworkers not only have disrupted sleep, but also are likely to have disrupted rhythmicity in peripheral tissues due to conflicting hormonal and neural inputs arising from the abnormal timing of food intake, exercise and sleep. The results of the current study will allow us to develop novel hypotheses to investigate the role of rhythm disruption elevating the risk of developing breast cancer.

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Introduction

Clock Genes: Critical Modulators of Breast Cancer Risk?

Background

The increasing prevalence of 24 hour operations in a wide range of industries across the developed and developing countries suggests that the deleterious effects on the health of workers as a result of this work practice will place an increasing burden on the individual workers as well as the community. Emerging epidemiological evidence suggests that prolonged exposure to shiftwork (ie., work outside the accepted normal working hours) is an independent risk factor for the development of breast cancer in women [1-3]. While much attention has been given to the potential role of workplace light during the night [4-6], and the presumed suppression of the hormone melatonin [7; 8] in the development of cancer, the biochemical disruption may occur at a more fundamental level, through the disruption of clock gene function.

It is now known that individual cells exhibit predictable daily rhythms in a wide range of functions, including metabolic homeostasis, hormone secretion and drug metabolism [9; 10]. This cellular rhythmicity is orchestrated by a suite of genes (clock genes) that code for a family of transcription factors. Two genes, *Clock* and *Bmal1* (*MOP3*) play a pivotal role in the initiation and maintenance of rhythms. The protein products of the genes, CLOCK and BMAL1 heterodimerise, enter the nucleus and bind to a regulatory sequence (E-box; CACGTG) in the promoter region of the period and cryptochrome genes to initiate their transcription. The period and cryptochrome proteins (PER1, PER2, CRY1 and CRY2) form a complex with casein kinase 1 ϵ and enter the nucleus to inhibit the actions of CLOCK/BMAL1 and thereby inhibit their own transcription. The CLOCK/BMAL1 heterodimer also induces the transcription of *Rev-erba* and *Rora* genes which interact with Rev-erb/Ror elements (RREs) in the promoter of *Bmal1*. The result of these interactions is cyclicity in the translation of the transcription factors. CLOCK and BMAL1 have an additional central role in cell function by binding to the same regulatory E-box sequence (CACGTG) of other transcription factors and functional genes thus imparting rhythmicity on their transcription and translation.

While the suprachiasmatic nucleus (SCN) of the hypothalamus plays a critical role in the entrainment of rhythmicity to the photoperiodic environment, it is now clear that individual cells outside the brain are also rhythmic [11-14] and that the phasing of rhythms in various tissues and organs can be altered by endogenous factors such as glucocorticoid hormones and glucose [15; 16]. Given this partial autonomy of peripheral tissue rhythm generation and control, it is conceivable that conflicts between environmentally driven rhythmicity and behaviourally driven rhythms could emerge.

This project aims to investigate whether clock transcription factors are rhythmically expressed in normal mammary tissue and in human breast cancer cell lines. In addition we will investigate whether the expression of genes previously associated with breast cancer, including oncogenes, cell cycle modulators and growth factors is also rhythmic. Of particular interest will be the question of rhythmic gene expression in human breast cancer xenografts in mice.

Task 1. To measure expression of clock and clock controlled genes in mammary tissue.

- (a) Obtain mouse mammary tissue at 3 hour intervals, extract mRNA and prepare cDNA.
- (b) Quantitate clock and clock controlled gene expression by Real Time RT-PCR.

Methods

Virgin Balb/c mice (6 weeks old, 6 per cage) were housed from birth on a 12L:12D photoperiod and killed by cervical dislocation at 3 hour intervals over 24 hours. The 3rd and 4th inguinal mammary glands were dissected and placed in RNeasy Lysis Buffer (Qiagen, Crawley, UK) and stored at -20°C. Total mRNA was extracted using a Qiagen RNeasy Lipid Tissue Mini kit. RNA (2 µg in 20 µl) was incubated with 0.4 µg of random hexamer primers (GeneWorks Pty Ltd, Adelaide, Australia) at 70°C for 10 minutes. The tubes were cooled on ice for 5 minutes and 8 µl of 5X RT buffer (Invitrogen, Carlsbad, California), 4 µl of 0.1M DTT (Invitrogen) and 4 µl of 2'-deoxynucleoside 5'-triphosphates (10 mM, Amersham Pharmacia Biotech, Piscataway, NJ) were added. The tubes were incubated at 43°C for 2 minutes, and incubated with 200 units of Superscript III Reverse Transcriptase (Invitrogen) for 90 minutes at 43°C and 5 minutes at 95°C. Ultrapure water (61 µl) was added to a final volume of 100 µl and the cDNA stored at -20°C until further use. Primers were designed with the ABI Prism Primer Express program (Applied Biosystems, Foster City, CA), (see Table 1 for primers used). Amplification of cDNA was performed on a GeneAmp 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). cDNA (5 µl), 2 µl of 0.625 µM forward and reverse primers, 1 µl of water and 10 µl of SYBR green (Applied Biosystems) were added to each well. The samples were amplified in duplicate for 1 cycle of 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. An arbitrary threshold of fluorescence was set within the exponential phase of amplification and the cycle at which the signal exceeded this threshold was designated as the cycle threshold (Ct). The expression of each gene within each sample was normalised against β -actin and expressed relative to a calibrator sample with the use of the formula $2^{-(\Delta\Delta Ct)}$ as described by K. Livak (PE-ABI, Sequence Detector User Bulletin 2). The calibrator sample was designated as the most highly expressed time point for each gene of interest and therefore has a relative expression of 1.

The expression results were analysed by one way ANOVA. Because the test of homogeneity of variance failed for most genes, data for all genes was log transformed and re-analysed by one way ANOVA. Differences were considered significant if the probability was less than 0.05.

Results

Validation of primers

The primers for mouse *Clock*, *Bmal1*, *per1*, *per2*, *cry1*, *cry2* and *actin* have been validated previously by our group and shown to produced single products of appropriate molecular weight after amplification of cDNA. Moreover the efficiency of the PCR was close to 100% based upon serial dilutions of cDNA.

For the current project the following primers were designed and validated for use; *c-myc*, *Cyclin D1*, *Tgfb*, *tmp*, *veg*, *wee1* and *Gadd45*.

Figure 1 shows the mammary tissue cDNA dilution curves for these primers. The slopes of these curves were all close to 1 and 1:8 dilutions of the cDNA were used in the actual study of the temporal expression patterns of the genes in mammary tissue.

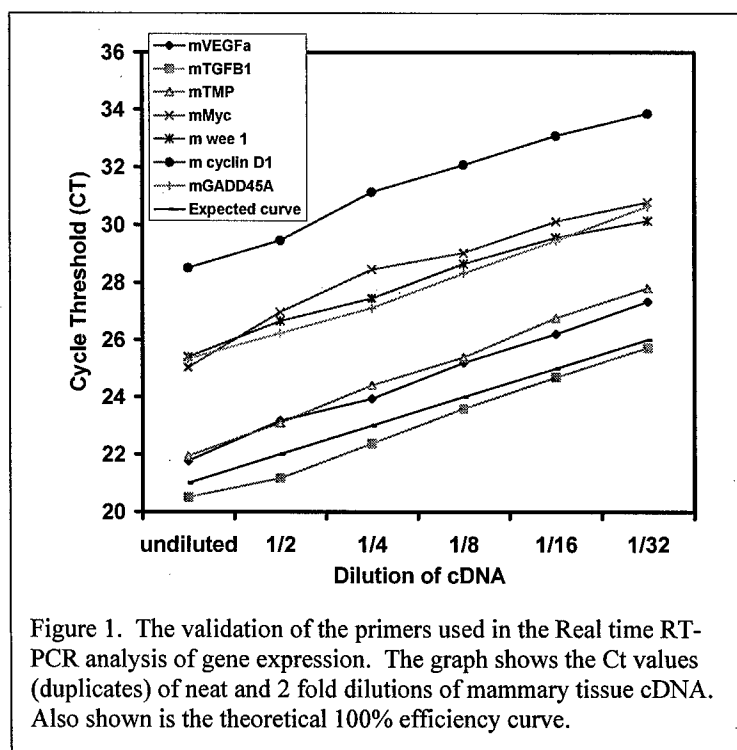


Figure 1. The validation of the primers used in the Real time RT-PCR analysis of gene expression. The graph shows the Ct values (duplicates) of neat and 2 fold dilutions of mammary tissue cDNA. Also shown is the theoretical 100% efficiency curve.

Mammary tissue gene expression across 24 h

All 6 core clock genes (*Bmal1*, *Clock*, *Per1*, *Per2*, *Cry1* and *Cry2*) were easily detectable in mammary tissue, with median Ct values ranging from 22.1 to 24.4. Furthermore expression of all of these genes varied systematically and significantly ($P < 0.05$) across the 24 hours of sampling (Figure 2 and Table 2) with amplitudes ranging from 2.5 fold to 10 fold. Peak expression of *Bmal1* and *Clock* occurred 3 hours before lights on and minimum expression 3 hours before lights off (Figure 2). Peak expression of *per1* and *per2* occurred 3 hours before and at the time of lights off respectively. Of the cryptochromes, highest *cry1* expression occurred 3 hours before lights on, while the *cry2* expression profile was characterised by a broad peak across the day and night followed by a 3 fold decrease at the time of lights on.

The expression of putative clock-controlled genes, *c-myc*, *wee1*, *cyclinD1*, *Gadd45 α* and *Tgfb β* varied significantly and systematically across 24 hours with amplitudes of expression of the order of 2.5 fold (figure 3). Peak expression of all the genes occurred during darkness. Expression of *vegf* and *timp* did not change significantly across 24 hours. Table 3 shows the correlations between the various genes. Of particular interest is the high correlation between *c-myc* expression and *Tgfb β* .

Discussion

In this study we have shown for the first time that the expression of clock and clock controlled genes in mouse mammary tissue changes across 24 hours. All six major (core) clock genes, *Bmal1*, *Clock*, *per1*, *per2*, *cry1* and *cry2* were highly and rhythmically expressed in the mammary

glands of the virgin Balb/c mice. The timing of the peak expression of *Bmal1* and *Clock* (0500h) and *per1* and *per2* between 1700h and 2000h is consistent with the notion that the BMAL1/CLOCK heterodimer complex drives the other clock genes in the mammary tissue as it does in other tissues studied thus far. Moreover, the phase and amplitudes of the rhythms is similar to that observed in other tissues including the liver and heart. Thus the mammary gland possesses the molecular machinery to maintain tissue rhythmicity and rhythmic output. Furthermore the expression of *c-myc* [17], *wee1* [18], *Tgfb β* [19], *Cyclin D1* and *Gadd45 α* [20] was also rhythmic, whereas expression of *vegf* [21] and *timp* [22] did not vary significantly across the day. The amplitude of the expression rhythms was approximately 2.5 fold and expression was generally higher at night.

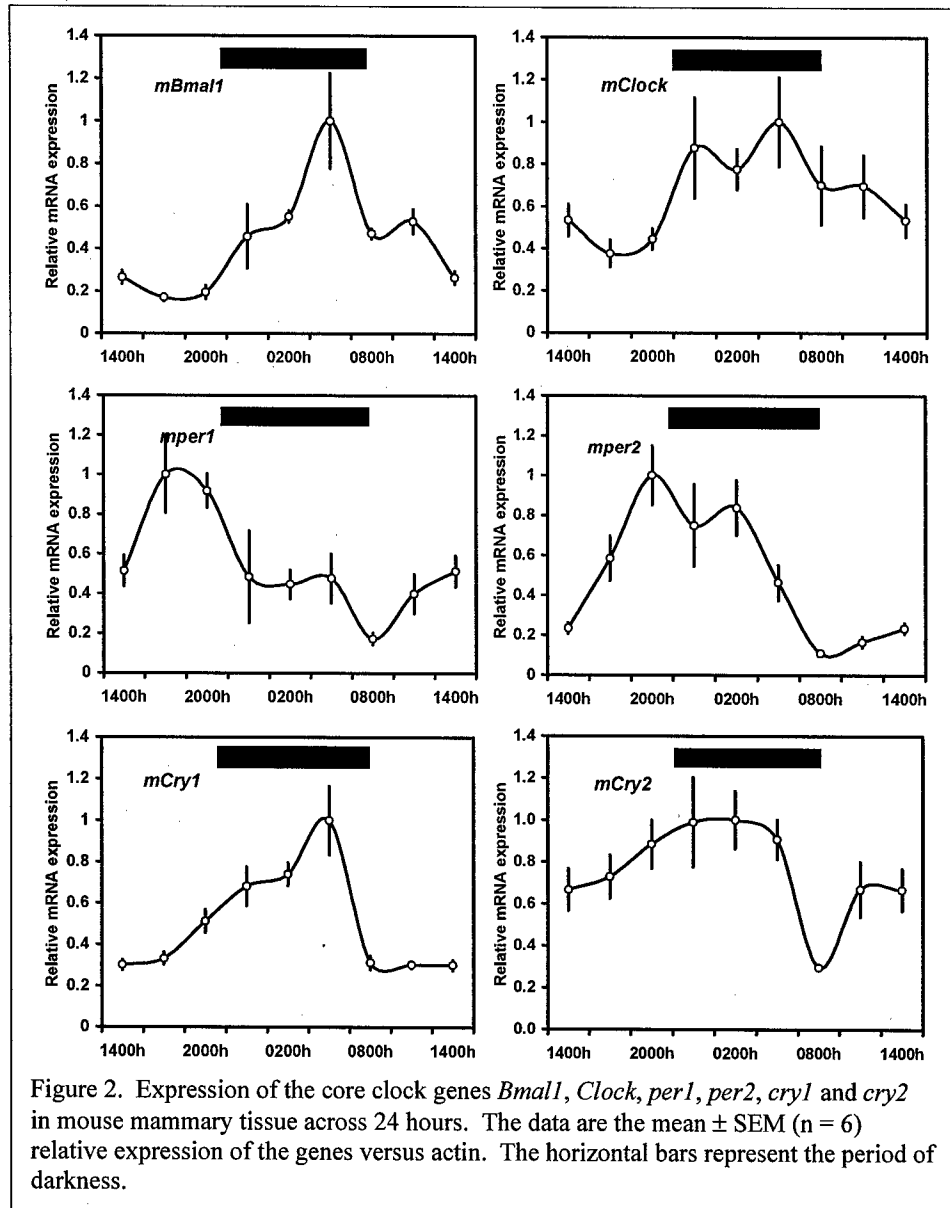
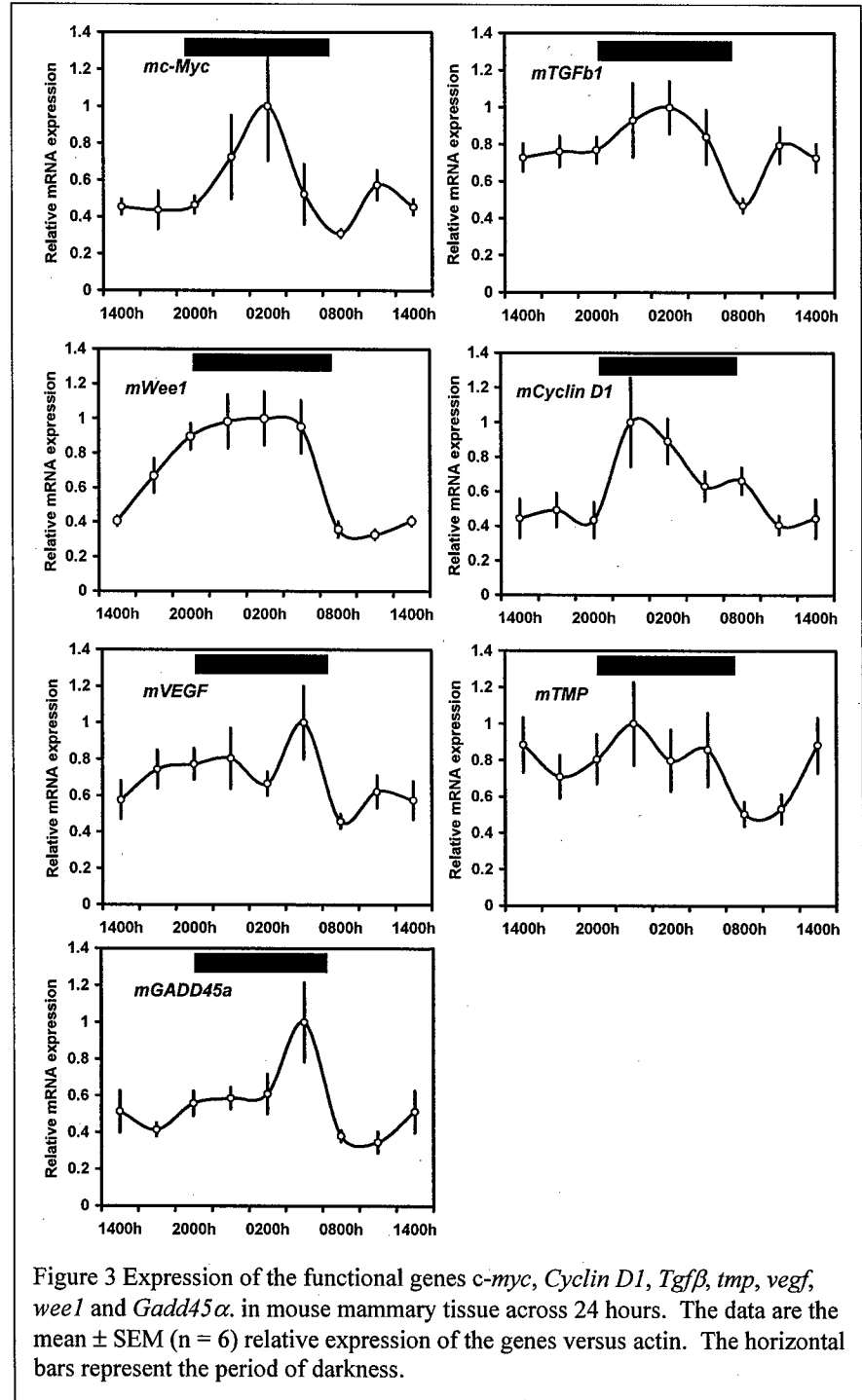


Figure 2. Expression of the core clock genes *Bmal1*, *Clock*, *per1*, *per2*, *cry1* and *cry2* in mouse mammary tissue across 24 hours. The data are the mean $\pm$ SEM (n = 6) relative expression of the genes versus actin. The horizontal bars represent the period of darkness.

These results suggest that cellular function in mammary tissue, like many other tissues is inherently rhythmic. Disruption of cellular clock gene rhythmicity may therefore result in deregulation of oncogene and cell cycle gene expression setting up an environment whereby tumours may evolve.



Task 2. To measure the expression, induction and rhythmicity of clock and clock controlled genes in human breast cancer cell lines.

- Screen a range of estrogen positive and negative breast cancer cell lines and select 2 lines of each showing high expression of *per1* and *per2*.
- Characterize the expression of a wide range of clock and clock controlled genes in the selected cell lines over 48 hours with Real Time RT-PCR.
- Perform feasibility experiments to assess the expression of clock genes in xenografts of the cell lines in mice.

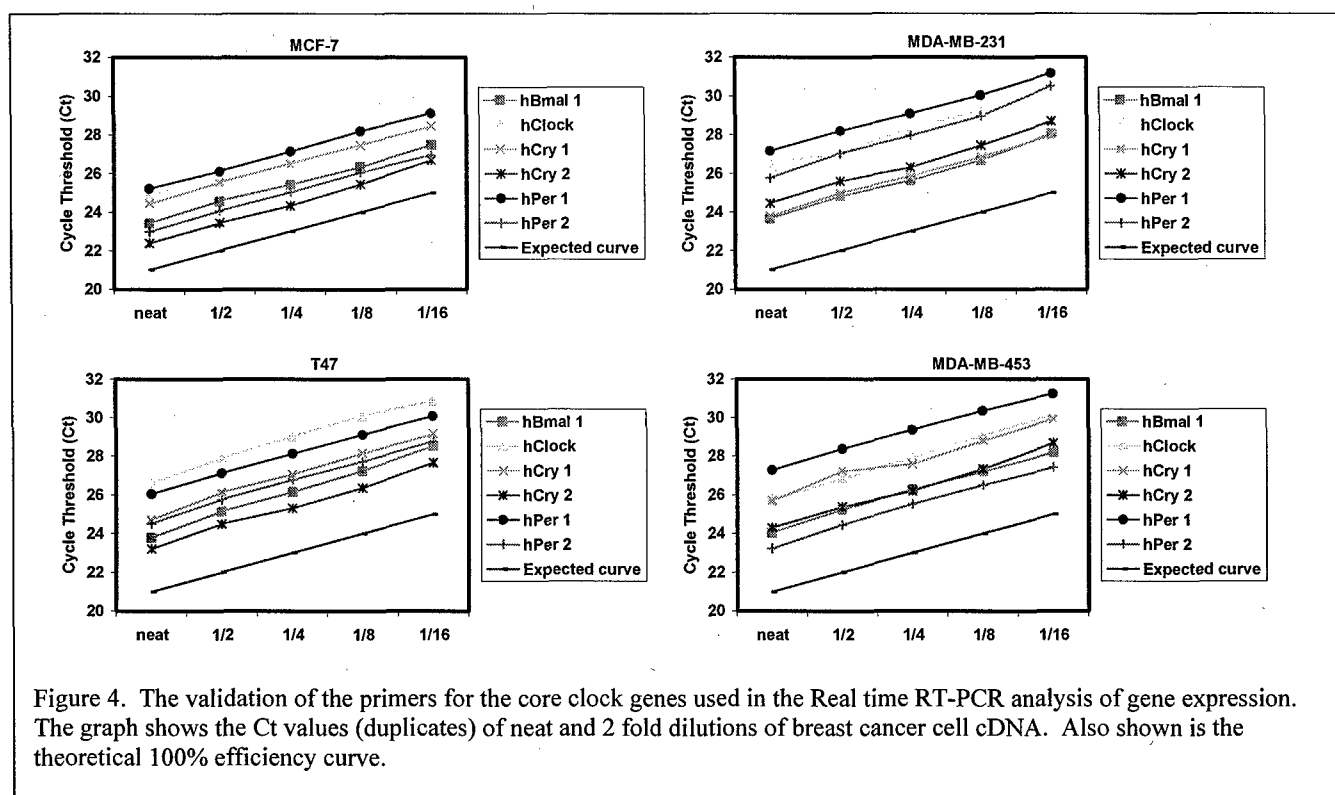
Methods

Validation of human clock gene primers

The following 4 human breast cancer cell lines (MCF-7, T47D, MDA-MB-231 and MDA-MB-453) were grown to confluency, pelleted and frozen prior to RNA extraction. Total numbers of cells in the pellet ranged between 8×10^5 to 10^6 cells. Cells were extracted with 1ml TriReagent using standard procedures. The extracted RNA was DNase treated and 2 μ g reverse transcribed with Superscript III as per the methodology for the mouse mammary tissue.

Figures 4 and 5 show the cycle threshold versus cDNA dilution curves from the 4 cell lines. Note that in all cases the efficiencies were close to 1, indicating that the primers we have designed and the methodology are valid and appropriate for quantitation of changes in clock gene mRNA levels in these cells.

As a further validation of the procedure, the products of the PCR reactions were run on a gel and single products of the appropriate molecular size were identified for actin and the clock genes. Figure 6 shows the results for one cell line (MDA-MB-231) and similar results were obtained for the other lines.



	MDA-MB-231	MDA-MB-453	MCF-7	T47D
hBmal 1	1.0669	1.0203	0.9843	1.1554
hClock	0.9296	1.1011	1.0736	1.0746
hCry 1	1.028	1.0015	0.995	1.087
hCry 2	1.0355	1.0703	1.0581	1.0777
hPer 1	0.9846	0.9911	0.9869	1.0048
hPer 2	1.1387	1.0415	0.9932	1.0461

Figure 5. The slopes of the Ct versus dilution curves for the core clock gene primers in 4 breast cancer cell lines

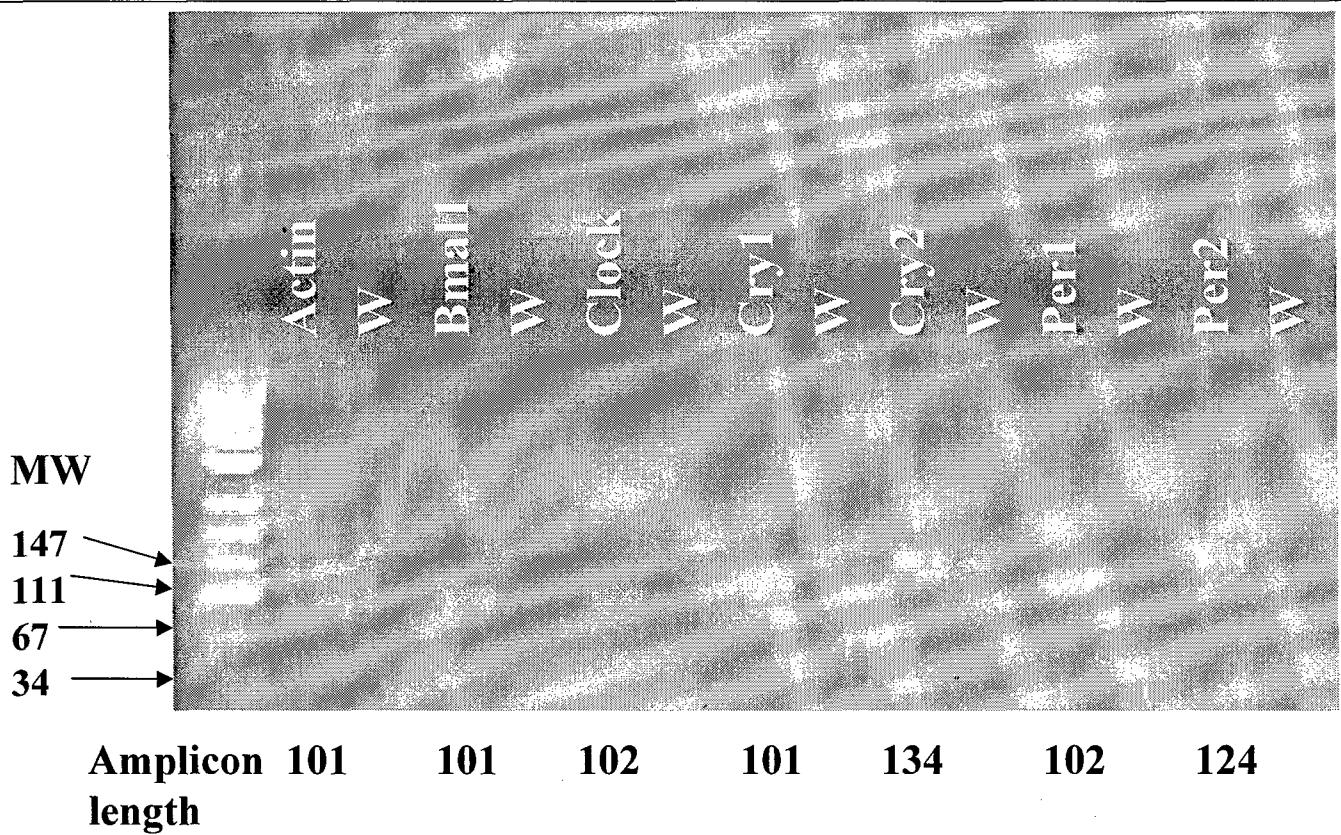


Figure 6. Electrophoresis of PCR products from MDA-MB-231 cells. The left lane shows the molecular weight markers, while the products of actin and the clock genes are alternated with the water control from each reaction. The expected amplicon sizes of each product is indicated under each lane.

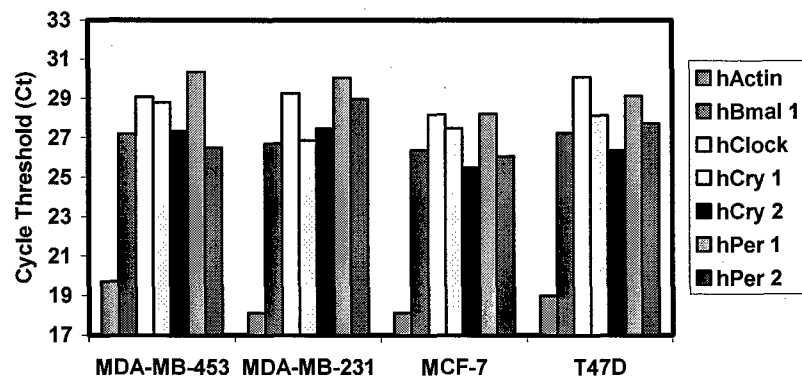


Figure 7. Expression of clock genes in human breast cancer cell lines. The data are the Ct for a 1:8 dilution of cDNA prepared from the cell pellets.

The expression of each of the clock genes was compared between cell lines by plotting the Ct values for a 1:8 dilution of the cDNA. Figure 7 shows that the expression was remarkably similar between lines.

We have decided to use the MCF-7 lines for further experiments as an example of an estrogen dependent line and the MDA-MB-231 as an example of a estrogen independent line.

Induction of clock genes in breast cancer cell lines *in vitro*.

The preceding studies have indicated that the cell lines express clock genes at a significant level at confluency. Previous studies in other systems (eg. Fibroblasts, epithelial cells) have indicated that under these conditions the cultures are arrhythmic. Rhythmicity can be induced in culture by serum shock and a range of other stimuli including forskolin, EGF, etc.

At the time of preparation of this report we have conducted only one induction experiment on MCF-7 cells.

Method

MCF-7 cells were grown in to confluence in 24 well plates and the media was replaced with 50% horse serum or control medium for 2 hours. After 2 hours the media was replaced with normal media in both the treated and control wells. After 0, 30, 60, 120 and 240 minutes of exposure to the serum, the media was removed and 200ul TriReagent was added to the wells. Each time point was assessed in triplicate. The cell lysate was passed through a pipette several times and transferred to tubes and frozen for subsequent analysis. RNA was isolated using the chloroform/isopropanol method as used previously for the cells. There was insufficient RNA to quantify using the optical density method and so the entire RNA preparation was reverse transcribed. Real Time RT-PCR was conducted on *actin*, *c-fos*, *per1* and *per2*.

Results

Actin mRNA expression was detected in the cells before and after the serum shock (Figure 8). The cycle thresholds for the replicate wells for both the control and serum treated cells were extremely variable, ranging from 19 to 32. When the 2 wells with Cts of 32 were excluded the range was still 19-28. This level of variability is of concern since expression of the clock genes is compared against the expression of this gene.

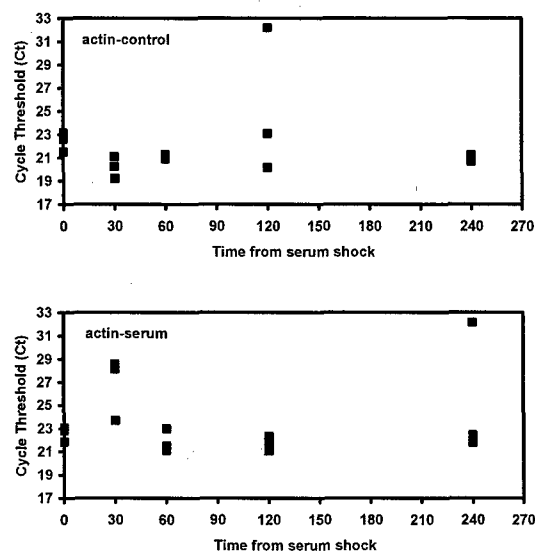


Figure 8. The cycle thresholds for actin expression in MCF-7 cells treated for 2 hours with 50% horse serum or control medium. The data are the Ct values for triplicate wells at various times after treatment.

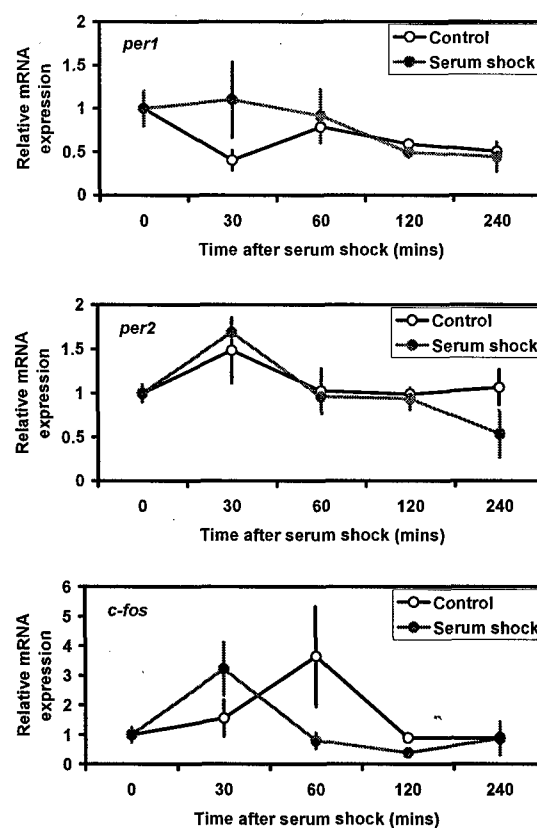


Figure 9. Relative mRNA expression in MCF-7 cells following treatment with 50% horse serum for 2 hours. The data are the mean $\pm$ SEM of 2-3 wells (ie the outliers were excluded).

Figure 9 shows the relative expression of *per1*, *per2* and *c-fos* in relation to actin following serum shock in the MCF-7 cells. While there is a suggestion that there are changes in gene expression with time and in response to serum, the variation within replicates is high and as a consequence any conclusions would be unreliable.

This experiment will be repeated and mRNA extracted using a kit procedure specially designed for precise quantitation of expression in cell lines.

Experiments yet to be performed

- 1) During a no extra cost extension of the project, we will complete the *in vitro* studies of serum induction of the clock genes in MCF-7 and MDA-MB-231 cells using a modified RNA extraction procedure. If we determine that induction of *per1* and *per2* occurs under these conditions, we will proceed to follow clock gene expression for up to 48 hours to establish if the cells maintain rhythmicity *in vitro*.
- 2) MCF-7 and MDA-MB-231 cells will be inoculated into nude mice and subsequent xenografts harvested at 4 hour intervals across 24 hours. The expression of clock genes and clock controlled genes will be determined by Real Time RT-PCR as per the methods used above for mammary tissue.

Discussion

In the course of this project we have shown rhythmic transcription of clock gene transcription factors in normal mouse mammary tissue. Moreover the expression of several other genes, *c-myc*, *wee1*, *cyclin D1*, *Gadd45a* and *Tgfb β* has also been shown to change significantly across 24 hours with highest expression during the dark, active period.

We have also shown that at least 4 different human breast cancer cell lines express the core clock genes at confluence, suggesting that these transcription factors are functional in the tumor cells. It is not yet clear from our studies, however, if the clock system of the cells responds to serum factors by inducing *per1* and *per2* as occurs in other cells.

The first indication that alterations in clock gene function might lead to tumour development came from studies of mice that lacked normal *per2* gene expression due to gene targeting [23; 24]. Not only did these mice have a higher incidence of spontaneous salivary gland hyperplasia, gonadal teratomas (male) and lymphomas, but they were also more susceptible to radiation induced tumour development. Of particular interest was the approximately 8-fold increase in the oncogene *c-myc* in the liver of the *per2* null mutants and also the significant changes in the *c-myc* controlled expression of the *Cyclin D1* and *Gadd45a* genes. Subsequent studies have shown that *c-myc* and *cyclin D1*, have canonical CACGTG E-boxes in their promoter regions. As a consequence these genes are rhythmically expressed in both normal and tumour tissue.

There have been no published studies of the rhythmic expression of clock genes in normal mammary tissue and there has been only one published report addressing the expression of clock genes in human breast cancer. Chen *et al* [25] reported that the expression of *per1*, *per2* and *per3* was "disturbed" in most breast cancerous cells compared to adjacent non-cancerous cells. Methylation of the *per1* and *per2* promoters was altered in the cancer cells and associated with expression of the *c-erbB2* oncogene. Of course in such a study involving single biopsies, no conclusions could be drawn about the degree of actual rhythm disturbance that may have been present in the tissue, but the results are provocative. Yeh *et al* [26] have also shown abnormal expression of *per1* in endometrial carcinoma.

There has been little experimental work reported on possible mechanisms linking rhythm disruption and breast cancer. Those who have proposed the links between shiftwork and breast cancer generally invoke altered melatonin secretion at night through the effects of extraneous light in the workplace. While there are exciting findings emerging that appear to implicate melatonin in breast cancer initiation and growth [7; 8], the evidence that there are major differences in melatonin levels between shiftworkers and day workers is not strong [27].

Workers engaged in night work provide the setting whereby peripheral circadian rhythm disruption could occur. It is well recognised that shiftworkers have reduced sleep duration due to poor sleep during the day [28]. The likely major contributor to the poor sleep is the failure of the central brain clock (SCN) that drives the timing of sleep to actually re-entrain to the new schedule. Thus the drive to produce melatonin and initiate sleep at night persists [27], together with the centrally driven body temperature and cortisol rhythms. Sleep is attempted during the daylight when the core body temperature is high and in the absence of melatonin. Further conflict appears when the shiftworkers eat meals at abnormal times of the night resulting in altered timing of glucose, cortisol and growth hormone levels.

Simulated shiftwork schedules imposed on laboratory animals have been shown to disrupt rhythmic gene expression in peripheral tissue. Filipski *et al* [29] subjected mice to 8 hour phase shifts of the light/dark cycle every 2 days for 3 weeks and reported that the expression of *per2* and *Rev-erba* mRNA in the liver was arrhythmic. When mice that were experiencing this rhythm disruption were implanted with the Glasgow osteosarcoma, the growth of the tumour was accelerated and the expression of *c-myc* and *p53* was altered [30]. The mechanisms underlying the altered tumour growth are not known and the authors did not investigate protein or steroid hormones or any other blood markers.

The conditions of the Filipski *et al* experiments [29; 30] are rather extreme and do not replicate those normally experienced by humans. Penev *et al* [31] subjected cardiomyopathy prone hamsters to a 12 hour phase shift at weekly intervals and reported that the life expectancy of the hamsters was significantly decreased. Thus it is likely that more realistic schedules could be of value in studies of the impact of phase shifting. We have recently shown that subjecting mice to three 12 hour "night shifts" followed by a return to four 12 hour "day shifts" for 4 cycles resulted in the development of glucose intolerance (Kennaway, unpublished results). Thus there is emerging evidence that even small changes in the rhythmic environment can precipitate major physiological changes. We hypothesise that disrupted cellular rhythmicity in the mammary gland may result in a local cellular environment that allows damaged cells to escape normal controls and to transform into a cancer.

Key research accomplishments

- 1) Clock genes (*Clock*, *Bmal1*, *per1*, *per2*, *cry1* and *cry2*) are highly expressed in normal mouse mammary tissue.
- 2) Clock genes are rhythmic in mouse mammary tissue with the timing consistent with rhythmicity driven by the CLOCK/BMAL1 heterodimer complex.
- 3) The expression of genes previously implicated in cell transformation and cell division (*c-myc*, *wee1*, *cyclin D1*, *Gadd45a* and *Tgfβ*) is also rhythmic, with up to a 2.5 fold change in expression across 24 hours.
- 4) The timing of the gene expression is consistent with their control by the CLOCK/BMAL1 heterodimer complex.
- 5) Human breast cancer cell lines (MCF-7, MDA-MB-231, MDA-MB-453 and T47D) express *Clock*, *Bmal1*, *per1*, *per2*, *cry1* and *cry2* at confluence.

Reportable Outcomes

- 1) An abstract was submitted to the 2005 "ERA of Hope Conference" in Philadelphia and was presented as a poster (see appendix 1). The title was "RHYTHMIC CLOCK GENE EXPRESSION IN MOUSE MAMMARY TISSUE".
- 2) A manuscript is in preparation covering the first section of the project and is tentatively titled "Clock gene expression in mouse mammary tissue" by David J Kennaway, Athena Voultsios, Lisa M Butler and Wayne D Tilley.
- 3) Based upon the results of the current project the following two research grant applications have been submitted:

- (a) "Disruption of the circadian rhythms of gene expression and the development of breast cancer"
Department of Defense, Congressionally Directed Medical Research Program, Idea Award.
- (b) "Disruption of the circadian rhythms of gene expression and the development of breast cancer"
Susan G Komen Breast Cancer Foundation, Basic, Clinical and Translational Research Program.

Conclusions

We have shown for the first time that mammary tissue is inherently rhythmic in that it has a rhythm of clock gene transcription factors as well as rhythmicity of functional genes implicated in the development and/or progression of breast cancer. We have also shown that cell lines that are commonly used to investigate cancer cell function and prospective treatments also express the clock genes, although we are yet to show that they are under normal control. We expect to show this in the next few months as we complete our studies.

The results of our project thus far have profound implications for our understanding of both normal and abnormal mammary gland function.

First we highlight the fact that expression of the oncogene *c-myc* is not constant across 24 hours, with expression changing almost 3 fold across the day. This suggests that the timing of treatments aimed at reducing *c-myc* expression may also affect their efficacy depending upon the time of day they are instituted. Given the wide range of genes that have been shown to be rhythmically expressed in other tissues, one can expect that an equally wide range of genes will be rhythmically expressed in mammary tissue aside from those we have investigated here. Any treatment regime must therefore in the future be tested at different times of the day instead of simply when it is convenient for the researcher.

Second the demonstration of clock gene driven rhythms in oncogene, cell cycle and growth factor gene expression in the mammary gland suggests a molecular basis for the increased risk of breast cancer in shiftworkers. Shiftworkers not only have disrupted sleep, but also are likely to have disrupted rhythmicity in peripheral tissues due to conflicting hormonal and neural inputs arising from the abnormal timing of food intake, exercise, light exposure and sleep. Disrupted cellular rhythmicity in the mammary gland may establish a local cellular environment that allows damaged cells to escape normal controls and to transform into a cancer.

Table 1 Primers used for the Real Time RT-PCR analysis of mRNA expression in mouse tissue

Gene	Accession Number			Amplicon Length
<i>rActin</i>	NM031144	Forward Primer Reverse Primer	CCTCTGAACCCTAAGGCCAA AGCCTGGATGGCTACGTACA	90bp
<i>Bmal1</i>	AB015203	Forward Primer Reverse Primer	GTCGAATGATTGCCGAGGAA GGGAGGCGTACTTGTGATGTTC	101bp
<i>Clock</i>	NM_007715	Forward Primer Reverse Primer	ATCCCAGAGGGGAGAACATTTCAGA GGGTCTATTGTTCTCTCGAAGCA	140bp
<i>cry1</i>	NM_007771	Forward Primer Reverse Primer	GACACGCGGTTGCCTGTT GCTCCAATCTGCATCAAGCA	101bp
<i>cry2</i>	AF156987	Forward Primer Reverse Primer	ACGGGATGCAGCCATTATGAA GGGTGGTTTCTGCCCATT	118bp
<i>per1</i>	NM_011065	Forward Primer Reverse Primer	TTTGAGAGCTGCAACATTCC TGCTGACGACGGATCTTTCTTG	142bp
<i>per2</i>	NM_011066	Forward Primer Reverse Primer	AGGCACCTCCAACATGCAA GGATGCCCCGCTTCTAGAC	140bp
<i>c-myc</i>	NM_010849	Forward Primer Reverse Primer	ATGTTGCGGTCGCTACGTC CAAGTAACTCGGTCATCATCTCCA	102bp
<i>Tgfβ</i>	NM_011577	Forward Primer Reverse Primer	CTGAACCAAGGAGACGGAATACA GGGCTGATCCCGTTGATTT	101bp
<i>Cyclin D1</i>	NM_007631	Forward Primer Reverse Primer	ATGGAACTGCTTCTGGTGAACA CTCATCCGCCTCTGGCATT	102bp
<i>wee1</i>	NM_009516	Forward Primer Reverse Primer	AGAGCGCAGAGCAGTTACGAA TGCAACTTTGGCTGCCATT	101bp
<i>Vegf</i>	NM_009505	Forward Primer Reverse Primer	TGGATGTCTACCAGCGAAGCTA ACAGGACGGCTTGAAGATGTACTC	101bp
<i>Gadd45α</i>	NM_007836	Forward Primer Reverse Primer	TGGTGACGAACCCACATTCA CACGGGCACCCACTGATC	101bp
<i>tmp</i>	U25633	Forward Primer Reverse Primer	TCCACCATGCTGGTGTGCT CCTTGGTGGCTGCTGGAG	104bp

Table 2 The outcome of ANOVA of the expression of individual genes in mouse mammary tissue

ANOVA			
	Homogeneity of variance of raw data (significance)	F (7, 38)	Significance after log transformation.
<i>Bmal1</i>	0.01*	17.61	0.000*
<i>per1</i>	0.00*	22.23	0.000*
<i>per2</i>	0.16	7.64	0.000*
<i>cry1</i>	0.00*	19.45	0.000*
<i>cry2</i>	0.07	7.74	0.000*
<i>Clock</i>	0.02*	2.81	0.019*
<i>c-myc</i>	0.02*	2.62	0.026*
<i>Tgfβ</i>	0.36	2.92	0.015*
<i>Vegf</i>	0.03*	2.17	0.059 NS
<i>tmp</i>	0.09	1.09	0.391 NS
<i>wee1</i>	0.00*	13.02	0.000*
<i>Cyclin D1</i>	0.36	2.63	0.026*
<i>GADD45α</i>	0.00*	3.79	0.003*

The data in the left panels are the results of the test for homogeneity of variance in a one way ANOVA of the relative expression of each gene. Because the test failed in 8 cases, all data was log transformed and a one way ANOVA again performed (results in right panels). Note that only *veg*f and *tmp* expression was arrhythmic.

Table 3 Correlation matrix for the gene expression data in mouse mammary tissue

	<i>per1</i>	<i>per2</i>	<i>cry1</i>	<i>cry2</i>	<i>Clock</i>	<i>c-myc</i>	<i>TGFβ</i>	<i>Vegf</i>	<i>tmp</i>	<i>wee1</i>	<i>Cyclin D1</i>	<i>GAD D45α</i>
<i>Bmal1</i>	0.06	-0.03	*0.76	0.35	*0.70	0.20	0.27	*0.48	0.13	*0.40	0.33	*0.59
<i>per1</i>		*0.71	*0.44	*0.76	0.24	*0.45	*0.51	*0.45	0.23	*0.83	0.36	0.19
<i>per2</i>			0.12	*0.64	0.14	*0.40	*0.55	*0.41	0.06	*0.49	0.10	0.06
<i>cry1</i>				*0.58	*0.55	0.20	0.31	*0.70	*0.46	*0.77	*0.41	*0.79
<i>cry2</i>					*0.57	*0.66	*0.80	*0.62	0.19	*0.77	0.33	0.36
<i>Clock</i>						*0.41	*0.48	*0.51	0.08	*0.45	*0.46	0.33
<i>c-myc</i>							*0.87	-0.03	-0.32	*0.43	0.30	-0.17
<i>Tgfβ</i>								0.19	-0.12	*0.47	0.32	-0.02
<i>Vegf</i>									*0.59	*0.68	0.17	*0.75
<i>tmp</i>										*0.42	0.15	*0.65
<i>wee1</i>											*0.50	*0.53
<i>Cyclin D1</i>												0.22

The relative expression data for each gene was subjected to multiple correlation analysis. The data are the correlation coefficients and the asterisk indicates where a significant correlation ($P < 0.05$) was found.

Table 4 Primers used for the Real Time RT-PCR analysis of mRNA expression in human tissue

Gene	Accession Number			Amplicon Length
<i>hActin</i>	BC013835	Forward Primer Reverse Primer	GCATGGGTCAGAAGGATTCCT TCGTCCCAGTTGGTGACGAT	106bp
<i>hBmal1</i>	NM_001178	Forward Primer Reverse Primer	GCCGAATGATTGCTGAGGAA GGGAGGCGTACTCGTGATGT	101bp
<i>hClock</i>	NM_004898	Forward Primer Reverse Primer	TCTTCTGGAAATTCATCTAACATCCA GTGTCCAGTATTCATTCCACTTTGA	102bp
<i>hper1</i>	NM_002616	Forward Primer Reverse Primer	TCCCATTTGGCTGCTCATG CGCTCCCGATCCTGCTT	102bp
<i>hper2</i>	NM_022817	Forward Primer Reverse Primer	CAGGTGAAAGCCAATGAAGAGTATT TCACAATGTGCTCAGAGGTAACG	124bp
<i>hcry1</i>	NM_004075	Forward Primer Reverse Primer	ACACCATCCGCTGCGTCTAC AAGATCCTCAAGACACTGAAGCAA	101bp
<i>hcry2</i>	NM_021117	Forward Primer Reverse Primer	CAGAAGCCACCCCTTACATACAA TCGTCTGGTTCTCCTGGAT	134bp
<i>h c-fos</i>	K00650	Forward Primer Reverse Primer	GAACCTGTCAAGAGCATCAGCA CACGGAGCGGGCTGTCT	108bp
<i>h weel</i>	NM_003390	Forward Primer Reverse Primer	GCCATTAAGCGATCAAAAAAGC TACATGAGAATGCTGTCCAAGCA	102bp
<i>hCyclin D1</i>	NM_053056	Forward Primer Reverse Primer	TGGAGGTCTGCGAGGAACA TGCAGGCGGCTCTTTTTC	106bp
<i>hGadd45a</i>	NM_001924	Forward Primer Reverse Primer	TCAACGTCGACCCCGATAAC CGCCTGGATCAGGGTGAA	101bp

References

1. Davis S, Mirick DK, Stevens RG: Night shift work, light at night, and risk of breast cancer. *J Natl Cancer Inst* 2001, 93:1557-1562.
2. Schernhammer ES, Laden F, Speizer FE, Willett WC, Hunter DJ, Kawachi I, Colditz GA: Rotating night shifts and risk of breast cancer in women participating in the nurses' health study. *J Natl Cancer Inst* 2001, 93:1563-1568.
3. Hansen J: Increased breast cancer risk among women who work predominantly at night. *Epidemiology* 2001, 12:74-77.
4. Pauley SM: Lighting for the human circadian clock: recent research indicates that lighting has become a public health issue. *Med Hypotheses* 2004, 63:588-596.
5. Stevens RG: Circadian disruption and breast cancer: from melatonin to clock genes. *Epidemiology* 2005, 16:254-258.
6. Stevens RG, Rea MS: Light in the built environment: potential role of circadian disruption in endocrine disruption and breast cancer. *Cancer Causes Control* 2001, 12:279-287.
7. Collins A, Yuan L, Kiefer TL, Cheng Q, Lai L, Hill SM: Overexpression of the MT1 melatonin receptor in MCF-7 human breast cancer cells inhibits mammary tumor formation in nude mice. *Cancer Lett* 2003, 189:49-57.
8. Blask DE, Dauchy RT, Sauer LA, Krause JA, Brainard GC: Growth and fatty acid metabolism of human breast cancer (MCF-7) xenografts in nude rats: impact of constant light-induced nocturnal melatonin suppression. *Breast Cancer Res Treat* 2003, 79:313-320.
9. Reppert SM, Weaver DR: Coordination of circadian timing in mammals. *Nature* 2002, 418:935-941.
10. Hastings MH, Reddy AB, Maywood ES: A clockwork web: Circadian timing in brain and periphery, in health and disease. *Nat Rev Neurosci* 2003, 4:649-661.
11. Panda S, Antoch MP, Miller BH, Su AI, Schook AB, Straume M, Schultz PG, Kay SA, Takahashi JS, Hogenesch JB: Coordinated transcription of key pathways in the mouse by the circadian clock. *Cell* 2002, 109:307-320.
12. Duffield GE: DNA microarray analyses of circadian timing: the genomic basis of biological time. *J Neuroendocrinol* 2003, 15:991-1002.
13. Oishi K, Miyazaki K, Kadota K, Kikuno R, Nagase T, Atsumi GI, Ohkura N, Azama T, Mesaki M, Yukimasa S, Kobayashi H, Iitaka C, Umehara T, Horikoshi M, Kudo T, Shimizu Y, Yano M, Monden M, Machida K, Matsuda J, Shuichi H, Todo T, Ishida N: Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. *J Biol Chem* 2003, 278:41519-41527.
14. Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, Gant TW, Hastings MH, Kyriacou CP: Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. *Curr Biol* 2002, 12:540-550.
15. Balsalobre A, Marcacci L, Schibler U: Multiple signaling pathways elicit circadian gene expression in cultured Rat-1 fibroblasts. *Curr Biol* 2000, 10:1291-1294.

16. Hirota T, Okano T, Kokame K, Shirokuni-Ikejima H, Miyata T, Fukada Y: Glucose down-regulates per1 and per2 mRNA levels and induces circadian gene expression in cultured Rat-1 fibroblasts. *J Biol Chem* 2002, 277:44244-44251.
17. Wanzel M, Herold S, Eilers M: Transcriptional repression by Myc. *Trends Cell Biol* 2003, 13:146-150.
18. Matsuo T, Yamaguchi S, Mitsui S, Emi A, Shimoda F, Okamura H: Control mechanism of the circadian clock for timing of cell division in vivo. *Science* 2003, 302:255-259.
19. Chen CR, Kang Y, Massague J: Defective repression of c-myc in breast cancer cells: A loss at the core of the transforming growth factor beta growth arrest program. *Proc Natl Acad Sci U S A* 2001, 98:992-999.
20. Canale L, Kakizawa T, Laudet V: The days and nights of cancer cells. *Cancer Res* 2003, 63:7545-7552.
21. Sledge GWJ: Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol* 2002, 29:104-110.
22. Ben-Porath I, Yanuka O, Benvenisty N: The tmp gene, encoding a membrane protein, is a c-Myc target with a tumorigenic activity. *Mol Cell Biol* 1999, 19:3529-3539.
23. Fu L, Pelicano H, Liu J, Huang P, Lee C: The circadian gene period2 plays an important role in tumor suppression and DNA damage response in vivo. *Cell* 2002, 111:41.
24. Fu L, Lee CC: The circadian clock: pacemaker and tumour suppressor. *Nat Rev Cancer* 2003, 3:350-361.
25. Chen ST, Choo KB, Hou MF, Yeh KT, Kuo SJ, Chang JG: Deregulated expression of the PER1, PER2 and PER3 genes in breast cancers. *Carcinogenesis* 2005, Epub ahead of print.
26. Yeh KT, Yang MY, Liu TC, Chen JC, Chan WL, Lin SF, Chang JG: Abnormal expression of period 1 (PER1) in endometrial carcinoma. *J Pathol* 2005, 206:111-120.
27. Dumont M, Benhabrou-Brun D, Paquet J: Profile of 24-h light exposure and circadian phase of melatonin secretion in night workers. *J Biol Rhythms* 2001, 16:502-511.
28. Rajaratnam SM, Arendt J: Health in a 24-h society. *Lancet* 2001, 358:999-1005.
29. Filipinski E, Delaunay F, King VM, Wu MW, Claustrat B, Grechez-Cassiau A, Guettier C, Hastings MH, Francis L: Effects of chronic jet lag on tumor progression in mice. *Cancer Res* 2004, 64:7879-7885.
30. Filipinski E, Innominato PF, Wu M, Li XM, Iacobelli S, Xian LJ, Levi F: Effects of light and food schedules on liver and tumor molecular clocks in mice. *J Natl Cancer Inst* 2005, 97:507-517.
31. Penev PD, Kolker DE, Zee PC, Turek FW: Chronic circadian desynchronization decreases the survival of animals with cardiomyopathic heart disease. *Am J Physiol* 1998, 275:H2334-H2337.

Appendix 1

Abstract for Era of Hope Conference 2005

RHYTHMIC CLOCK GENE EXPRESSION IN MOUSE MAMMARY TISSUE

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Epidemiological studies have linked shiftwork with an increased risk of breast cancer, suggesting that circadian disruption may influence the development or progression of the disease. Circadian rhythms are regulated by a panel of specific transcription factors, called clock genes, and our current understanding of endogenous cellular rhythmicity is that both positive and negative feedback cycles of clock genes drive the expression of a growing list of other transcription factors and functional genes. Disruption of circadian control systems, which maintain normal cell function, may lead to malignant transformation into cancer cells. This concept is supported by recent findings that tumors grow faster in arrhythmic mice. Previous studies have demonstrated rhythmicity of expression of clock genes in mouse liver, heart, kidney, and vascular tissue. As the patterns of expression of genes induced by these cyclic transcription factors were highly tissue-specific, it is critical to determine which clock and clock-controlled genes are expressed in mammary tissue and potentially contribute to the increased risk of breast cancer associated with shift work.

General hypothesis: Circadian disruption of clock gene rhythmicity in mammary tissue will result in altered gene expression and lead to the loss of growth inhibition and subsequent tumorigenesis.

Experimental aim: To measure the expression of clock and clock controlled genes in mammary tissue.

Real-Time RT-PCR was used to investigate the mRNA expression of β -actin, *Bmal1* and *per2* in mammary tissue of 6 week old virgin Balb/c female mice every 3h for 24h.

We have shown for the first time that mouse mammary tissue rhythmically expresses the key clock genes, *mper2* and *Bmal1* and that the timing and amplitude of the rhythms is indicative of a fully functional clock system.

Identification of key clock genes and their induction of rhythmicity of functional genes in breast cancer may provide a mechanistic basis for the epidemiological observations linking shift work with an increased risk of breast cancer, and potentially lead to novel targets for the prevention and/or treatment of breast cancer.

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