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[Circadian Genes in, EWD-8, Triple-Negative Breast Cancer Cells May Demonstrate Rhythmicity in Vitro

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Garza, Kayla; Haughian, James; and Krynski, Luke, "[Circadian Genes in, EWD-8, Triple-Negative Breast Cancer Cells May Demonstrate Rhythmicity in Vitro" (2022). *Capstones & Scholarly Projects*. 108.
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University of Northern Colorado

Greeley, Colorado

Circadian Genes in, EWD-8, Triple-Negative Breast Cancer Cells May Demonstrate Rhythmicity in Vitro

A Thesis Submitted in Partial
Fulfillment for Graduation with Honors Distinction and
the Degree of Bachelor of Science

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December 2022

Circadian Genes in, EWD-8, Triple-Negative Breast Cancer Cells May Demonstrate Rhythmicity in Vitro

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12/1/2022

Abstract

Studies have shown a link between greater rates and risks of breast cancer in women that work night shifts, such that this type of work has been labeled a probable carcinogen. It has been suggested that the disruption of circadian genes- PER and CRY - could be partly responsible for this increased breast cancer risk. The suprachiasmatic nucleus (SCN) in the brain is the central circadian pacemaker; however, how and if Triple-negative breast cancer (TNBC) cells, an aggressive type of breast cancer that is characterized by the absence of estrogen receptors (ER), progesterone receptors (PR), as well as HER-2 growth factor receptors, express particular circadian clock genes when disconnected from the "master clock" is not extensively researched nor understood. This study's goal was to answer the following questions: do triple-negative breast cancer cells (TNBCs) express circadian genes? Do TNBCs exhibit rhythmic circadian gene expression in vitro? Are TNBCs and the circadian genes responsive to external red-light exposure at a wavelength shown to alter mitochondrial function? Cells were cultured in vitro, exposed or not exposed to red light, and then harvested at 6-hour time intervals for 24 hours. The harvested cells endured RNA isolation, followed by cDNA synthesis and PCR to amplify circadian genes PER1, PER2, PER3, CRY1, CRY2. The expression level was compared to constitutively expressed reference genes to determine whether circadian rhythmicity is present in TNBC cells growing in vitro.

Acknowledgements

This project was inspired by my hours of shadowing in the lab with Luke Krynski, my research mentor, who took me under his wing and strengthened my passion for research. I would like to thank both Luke and my faculty thesis advisor, Dr. James Haughian, for their support throughout my undergraduate career.

I also want to express my gratitude to the Office of Undergraduate Research at the University of Northern Colorado and their financial support, as well as their support for cancer research. The completion of such a project was done with the mentorship of the Honors Upper Division Program and the McNair Scholars Program; from networking at conferences to continuously building on my current knowledge in the lab, these organizations helped make me into the scientist I am today.

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Introduction

The circadian rhythm has a known regulatory role in the behavior, metabolism, physiology, and molecular function of the majority of the organisms that inhabit this earth (Sultan et al., 2017). The rhythms are driven by clocks: the master clock and peripheral clocks. Peripheral clocks are said to be coordinated with the master clock, but are specific to the tissue in which they exist. Because tissues are differentiated for diverse tissues such as the heart, lungs, and brain, this results in clock gene expression and circadian activity in these specific tissues.

For every mean solar day of 24 hours, the Earth revolves around the Sun on its axis; these daily intervals of time happen to be extrinsic signals that align with an internal circadian clock in mammals (Masri & Sassone-Corsi, 2018). Within the 24-hour time period, vital processes and activities, such as blood pressure, thermoregulation, hormonal secretions, the internal clock or "sleep-wake cycles," and sporadic environmental cues, change (Li, 2019). These alterations in organisms, known as *circadian rhythms*, regulate a vast array of physiological demands; the respective clock genes are what conspire with elements specialized for particular tissues, hence, why it has been implicated that they are responsible for cancer when they are modified (Ray & Goswami, 2021). As previously mentioned, the circadian clock possesses both clocks or control centers that are central and peripheral (e.g., Li, 2019; Masri & Sassone-Corsi, 2018; Ray & Goswami, 2021). Such a circadian clock conserves oscillations that remain in sync with the light-dark cycle of the day, while jointly coinciding with luminosity, temperature, and sustenance consumption, all of which are deemed "zeitgebers" (Masri & Sassone-Corsi, 2018). The collection of these named "time-

keepers" are what keep time for the organism at the molecular level, which aid in maintaining proper function.

When the biological rhythms of individuals are disrupted, homeostasis is simultaneously thrown off - seemingly small changes have been demonstrated to cause devastating ramifications in the form of disorders, including cancer. Despite the general knowledge of cancer's involvement in the biological clock, there is an absence of research on the magnitude that triple-negative breast cancer (TNBC) may hold in regards to a disrupted circadian rhythm. A 2007 study rendered night shift work to be equivalent to a "human carcinogen," a notion that has inspired the work of others investigating cancer's tie to circadian rhythm disruption (Straif et al., 2007, as cited in Masri & Sassone-Corsi, 2018). With most studies focusing on the broad spectrum of breast cancer in relation to circadian rhythm research, this study aims to fill a small, meaningful part of such a gap.

While the most common breast cancer tumor subtypes possess positive progesterone receptors (PR) or estrogen receptors (ER), along with negative human epidermal growth factor type 2 receptors, TNBC lacks all three. Though TNBC makes up 12% of all breast cancers, it also has the most poor prognosis. From 2011-2013, a total of 595,789 breast cancer cases were identified in the North American Association of Central Cancer Registries, with triple-negative breast cancer making up 67,903, or 11.4%, of those cases (Moss et al., 2021).

This study was inspired by the following questions: do TNBCs possess circadian clock genes? Are these genes rhythmically expressed throughout a 24-hour day, even in the absence of the master circadian clock? Triple-negative breast cancer (TNBC) is unlike other cancers because it characteristically lacks three markers: estrogen receptors (ER),

progesterone receptors (PR), and Her-2/neu (human epidermal growth factor receptor 2) (Avery, 2018). The hope is that the outcome of this research will help enlighten scientists on the behavior of TNBCs in vitro, and will serve as a first of many stepping stones to curating different treatments for TNBC. If this study can reveal how TNBCs act in vitro, this will help researchers to harness the circadian rhythm as part of the future treatment for patients.

Literature Review

Connections between cancer and circadian rhythm disruption

The prevalence of cancer's link to the disruption of the circadian rhythm has risen as a peak area of interest. Biological interferences in the circadian rhythm may be induced by several factors which can provoke a number of diseases, including cancer (Masri & Sassone-Corsi et al., 2018).

The hypothalamus houses the suprachiasmatic nucleus (SCN), which possesses the central circadian pacemaker which when disrupted, promotes tumorigenesis (Masri & Sassone-Corsi, 2018). Masri & Sassone-Corsi et al. (2018) addressed a gap in the literature: the reason behind the encouragement of tumorigenesis is lacking, but it was proposed that such a disruption exists because of the *asynchrony* of the SCN and circadian rhythm of the periphery. Peripheral clocks can be located in tissues including the brain, heart, lungs, kidneys and liver; however, Li (2019) notes that the peripheral tissues may necessitate a stimulus to preserve the biological rhythms in mammalian organisms. Li (2019) additionally noted that the origin of the stimulus could be the SCN itself, or signals mediated by the SCN.

Circadian genes that have been identified include: PER1, PER2, PER3, CRY1, CRY2, BMAL1, CLOCK, ARNTL, TIM, ROR, NPAS2, NR1D1, NR1D2, and CSNK1E.

Blakeman et al. (2016) speak on behalf of evidence that shows PER1/2 genes to possess apoptotic properties, subduing breast cancer in living model organisms. Thus, when these PER genes are reduced in breast cancer tumors, the ability of PER to suppress tumorigenesis is compromised. A parallel can be drawn in the hematopoietic system involving the formation of cellular blood components, for specific proteins that are clock-affiliated manage the cell cycle, as well as apoptosis (Gery & Koeffler, 2010). Just as circadian regulation is vital for normal cell, organ, and system function, it is essential for hemopoiesis to work properly. Mice with mutations in clock genes demonstrated the magnitude of a normal, functioning circadian order in terms of cell growth and "neoplastic transformation," which is when certain alterations respectively alter the phenotypic or genotypic makeup of the cell, encouraging tumor proliferation (Gery & Koeffler, 2010). Mice with a PER2 mutation deprived of light became "arrhythmic," while mice that lacked PER2 had a predisposition to acquire cancer, though the cancer type was unspecified (Gery & Koeffler, 2010). Moreover, the same mice that endured radiation gained a higher rate of lymphomas, while the wild-type mice lacked the consequence altogether.

As with patient studies, PER2 was shown to be reduced in chronic myeloid leukemia (CML) in contrast to individuals without the condition, a result that was also seen in various lymphomas, such as diffuse large B cell lymphoma (DLBCL) (Gery & Koeffler, 2010). Non-Hodgkin's lymphoma (NHL) risk has been shown by previous researchers to be linked to modified clock genes; going hand in hand with this finding, is additional support noting that an elevated risk of NHL exists in men who work night shifts (Gery & Koeffler, 2010). Gery and Koeffler (2010) accounted for the universal existence of circadian rhythms at the cellular level and its role in the systems of an individual, both of which need to be more thoroughly

understood in order to implement therapeutic advances in cancer treatment. Further, an in vitro experiment induced PER2 expression in K562 cells, human and murine AML and pro-B lymphoid, resulting in apoptosis, hindering of the cell cycle, and slowed progression (Gery & Koeffler, 2010).

The environment, epigenetics, and circadian disruption

The close interconnection of the circadian rhythm and the "oncogenic programs" can result in complex pathophysiological problems as seen with cancer. In cancer, the activated oncogenic routes possess many interrelations with circadian constituents; oncogenic pathways also have the capacity to control the expression of key circadian genes (Astone & Santoro, 2021). A disrupted, or "broken clock," can induce tumor evolution or cause advancement of an already established tumor; genetics and external stimuli (environmental factors) have been found to be associated with a higher risk of cancer development (e.g., Masri & Sassone-Corsi, 2018; Astone & Santoro, 2021). With the disruption of the circadian genes, there is also a change in the expression of the genes themselves (Astone & Santoro, 2021). The mechanism by which circadian gene expression is distorted has long been questioned, but Masri and Sassone-Corsi (2018) acknowledge that the "epigenetic landscape" may be modulated by shift work itself. In Denmark, the DNA of a cohort of female shift workers showed evidence of genome-wide alterations regarding DNA methylation; these alterations were found in both locus specific to cancer and to the circadian clock, "including estrogen receptor α (ESR1) and the circadian genes clock (CLOCK) and cryptochrome 2 (CRY2)" (Masri and Sassone-Corsi, 2018, p. 1796). A total of 5,409 CpG (5'—C—phosphate—G—3') sites were discovered, all of which differed in their methylation in the day versus night shift workers, with 66% of these sites being "hypermethylated."

Correspondingly, CpG methylation of the promoters of PER1, PER2, and PER3 paralleled with a 50% rate of change in protein expression within breast tumor tissue, compared to the same patient's normal tissue (Masri & Sassone-Corsi, 2018). Both hypomethylation and hypermethylation of promoters, enhancers, and differentiated sites in malignancies have been reported; DNA methylation typically works to silence or repress the gene that follows the affected promoter (Ehrlich, 2019). These observations collectively give rise to questioning whether the genomic alterations in gene expression are due to the shift-work light exposure; however, Astone and Santoro (2021) question the "loose standardized nature of shift-work" in different studies that draw a connection between cancer and night-shift work. The 2007 classification by the International Agency for Research on Cancer (IARC) had initially deemed shift work as a disruptive agent of the circadian rhythm; yet, in 2019 the terminology was changed to 'night shift work' then, consecutively as "Group 2A" (Takahashi & Koda, 2019). Tacahashi and Koda (2019) urge researchers to consider the limitations given that the carcinogenicity of a night shift cannot be objectively measured in terms of 'toxicity,' in turn creating inconsistencies among studies. For instance, the consideration or lack thereof, of chronotype, rhythmicity of clock genes, sleep pattern and possible polymorphisms are all contributors to shift work research results (Astone & Santoro, 2021).

More extensively, rates of breast cancer have been known to be reduced in women with no perception of light (NPL) as early as 1991, a theory that has been supported by further studies that have revealed a correlation between worsened vision and lower risks of breast cancer (Flynn-Evans, 2009). A study conducted with the intent of mapping out data from women with LP and NPL and their risk level of breast cancer surveyed women in North America, totalling 1,392 individuals in the span of two years. Flynn-Evans et al. (2009)

found that a negative association exists between breast cancer risk and blindness or the lack of light perception; it was expressed that the cause of amplified breast cancer risk in relation to blindness is ambiguous. To add, it was explicitly stated that their results correspond with the higher risks for breast cancer observed in both populations of night shift workers and pilots. Ocular light exposure influences pineal melatonin levels, and the synchrony of the circadian rhythm (Flynn-Evans et al., 2009). Interestingly, the hormone melatonin has its *own* circadian rhythm which is synchronized with the light-dark cycle. The eyes are what mediate the exposure of light at night, which changes the timing of the circadian rhythm. Specifically, it is the ganglion in the retina that juts into the SCN, the location of which the circadian rhythm is produced. Previous research in vitro and in model organisms have added support for the idea that alignment of the circadian rhythm and conserved melatonin could act as protectors against tumorigenesis (Flynn-Evans et al., 2009). Whether blindness impacts the synchrony of the circadian rhythm is unknown. There lies no evident contraindication to the fact that night shift work, rotating shifts, and disruptive light-dark cycle exposure contribute to an elevated risk of breast cancer in women.

TNBC and red light

Because of the absence of three receptors typically used as targets in treatment for other breast cancer types, TNBC is infamous for being aggressive in nature. TNBC is stated to have a higher grade, meaning that the cells fail to take on the normal phenotype for breast cancer cells; in addition, these cells also tend to grow more rapidly and are linked to a poor prognosis. Defining hallmarks of TNBC revolve around significant proliferation, elevated infiltration, phenotypic signatures (mesenchymal and basilar), and the inability to fix breaks

in double stranded DNA (also referred to as homologous recombination deficiency (HRD) (Vankina & Yuan, 2020).

Given that there is a clear association with night shift work that causes circadian rhythm disruption, there are still loose ends. For instance, it is not understood if such a disruption of the clock genes is caused in partial, or fully to tumorigenesis or the evolution of cancer. It is also unknown to what extent this applies to triple-negative breast cancer because there is a lack of primary literature on this particular application. As to how the disruption would work with other hidden factors to promote cancer progression or development is equally as unclear.

At the level of the patient, a range of 40-57% of post-treatment cancer survivors deal with sleep disruption, limitations in completing everyday tasks, and fatigue; one study using different light treatments found that when patients were exposed to dim red light for 14 days straight in 30 minute intervals, their sleep disturbance decreased and sleep efficiency increased (Wu et al., 2021). This begs the question of what red light does that aids in improved sleep quality which is tied to the circadian rhythm that is driven by our external light cues. At the cellular level, it has been argued that the fluorescently-activated mitochondrial enzyme, cytochrome c oxidase, is what triggers ATP production when hit with red light therapy (low level light, LLLT). In conjunction with this idea, it's also been known that with increased ATP in the powerplants of your cells, there's increased efficiency in cell functions ranging from repairing wounds or aiding in cell metabolism. There is evidence that red light has the capacity to excite and stimulate the mitochondria, in turn increasing ATP energy levels, which has been proposed to have "anti-cancer" properties; however, this mechanism is still under much debate. LLLT or low-intensity light therapy has shown to alter

the mitochondria by way of the electron transport chain (ETC) due to its sensitivity to both near-infrared and red light (Tafur & Mills, 2008). The red light at ~670 nm acts by stimulating cytochrome c oxidase which is often blocked in cancer cells by nitric oxide.

The initial aim of this study was to answer the following questions: Do triple-negative breast cancer cells (TNBCs) express circadian genes? Do TNBCs exhibit rhythmic circadian gene expression in vitro? In conjunction with this inquiry, lies the following question: Are TNBCs and the circadian genes responsive to external red-light exposure at a wavelength shown to alter mitochondrial function? This research is founded on the fact that there are evident gaps in the knowledge regarding clock genes in triple-negative breast cancer. Triple-negative breast cancer cells may possess potential rhythmicity in vitro due to two known implications that different studies have suggested. The first logical conjecture is the notion that cancer cells manipulate and take advantage of their microenvironment, thus, there could be self-sustainment in regulating their own rhythm to aid in cancer progression. The second reason might include the disruption of the circadian genes to be necessary for the very reason that they have demonstrated the capacity to promote tumorigenesis in other types of breast cancer when altered. Multiple studies have demonstrated there to be a clear connection between profound alterations in clock genes that directly influence apoptosis, proliferation, and cancer propagation (Li, 2019). Mammalian clocks have been widely claimed to sustain their own rhythm, in turn adding to the curiosity that these genes may self-regulate. The cyclic modification of the rhythmicity of the circadian clock and homeostasis within cancer suggests a universal propensity of both cancer cells themselves, and cancer microenvironments to exploit the circadian clock (Astone & Santoro, 2021).

Methods

Triple-negative breast cancer cells (TNBCs, line EWD-8) provided by the University of Northern Colorado were utilized and cultured in Dr. Haughian's lab. Three groups of cells were exposed to different light exposures respectively: ambient light (~400 nm), red light (660 - 680nm wavelength), and no light, with the no light group serving as the control. A total of 5, 6-hour time intervals which were used to compare rhythmicity. The independent variable was light while the dependent variable was rhythmicity that may have, or may not have been expressed in the following genes: PER1, PER2, PER3, CRY1, CRY2, BMAL1, and CLOCK. GAPDH was used as a housekeeping reference gene, as it is constitutively expressed within TNBCs.

Cell Culturing TNBCs

To preserve the viability of the cells throughout the research process, the cells were "passaged" every 3-5 days to ensure that they remained animate and continued to grow. Cells were passaged once 70% confluency was met; this is a procedure that entails re-plating the cells into a new plate with new media to prevent cell death. An electronic pipette with a 5-mL serological pipette tip, media (MEM), and trypsin were needed for culturing. Each time the cells were passaged, 2.5-mL of trypsin was used to fill the initial plate once the media was taken out via a serological pipette. The trypsin allows for *trypsinization*, which is the interference of cell connection to the floor of the plate, in turn causing them to become suspended. The cells incubated with trypsin for 15 minutes, at 36.5 degrees Celsius. Following the completion of trypsinization, the cells were examined under a microscope to ensure the cells had been disrupted (if the cells were still adhered to the floor of the plate, trypsinization was incomplete and incubation was continued). Next, the cell culture with the

TNBCs in the trypsin was centrifuged and the supernatant was discarded. The pellet of cells was re-suspended and re-plated in 3.5-mL of MEM and returned to the incubator.

24-Hour Light Exposure

The two experimental groups and control were kept in tinfoil in the absence of light for 24 hours as a means of "resetting" their rhythm if present. This was done in an attempt to establish a baseline of light exposure. At t = 0 , the initial time interval determined for the EWD-8 cells, the respective well of cells was harvested to obtain a baseline. Once the first interval was collected, the red light group was exposed to 60 minutes of red light (660 - 680 nm) which was placed 7 cm above the plates to expose all 5 well-plates for all time intervals including t = 6, t = 12, t = 18, and t = 24 hours. After a full 60-minutes of red light was shown onto the cells, the cultures were covered in tinfoil as a means of avoiding any unwanted ambient light exposure. The consecutive time intervals were then harvested for all time points for all three groups.

Cell Harvesting

The TNBCs were harvested at 6-hour intervals over the span of 24 hours to detect the circadian rhythm. The "initial interval" served as hour zero, and was completed for the three experimental groups of EWD-8 cells simultaneously. Every 6 hours, a sample of cells from each plate was extracted and preserved in trizol; the samples were then frozen and stored in a freezer to be analyzed once all samples were collected. More specifically, once the cells were harvested for t = 0, four consecutive TNBC extractions were conducted following the same protocol. Thus, a total of 3 samples for 5 different time points (t = 0, t = 6, t = 12, t = 18, t=24), with a total of 15 samples for the experiment.

RNA Isolation, cDNA Synthesis & qPCR Analysis

RNA was isolated from the TNBCs using the PureLink RNA Mini Kit (ThermoFisher). While handling RNA samples, an RNaseZap Reagent was used to eliminate RNases that may contaminate the work space and any equipment being used in the protocol. RNase-free microcentrifuge tubes and pipette tips were used in addition to proper aseptic techniques. RNA samples in sets of 3 were kept on ice while performing isolation. The samples were lysed and homogenized in a Lysis Buffer without 2-mercaptoethanol, followed by the addition of ethanol. A Spin Cartridge was used to allow the RNA to bind to its membrane, leaving any non-RNA debris or impurities in the collecting tube. Purified RNA was followed by elution via RNase-Free Water. A Nanodrop 2000 determined the RNA concentration of the samples; a ratio of 260/280 was obtained, indicating that high quality RNA was isolated.

The isolated RNA was then converted to cDNA using Sensiscript RT Kit (Qiagen). From the concentration of RNA isolated, the amount of Master mix, dNTP Mix, Oligo dT primer, RNase inhibitor, Reverse Transcriptase (RT), RNase-free water, and Template RNA were calculated respectively.

Once the Master Mix (SYBR Green SuperMix, forward primers, reverse primers, and nuclease-free water) was created, the solutions were vortexed and centrifuged. Master Mix and the Template RNA for PER1, PER2, PER3, CRY1, CRY2, BMAL1, CLOCK, and GAPDH were added to separate eppendorf tubes which jointly endured vortexing and centrifugation. Quantitative real-time polymerase chain reaction (RT.qPCR) was then performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX384 thermocycler.

PCR (polymerase chain reaction) is a procedure that allows a particular part of DNA to be analyzed by creating copies of it through specific primers designed for that piece of DNA.

A thermocycler was used to conduct PCR analysis of the cDNA synthesis from each time interval at 95 °C for a half hour; this allowed for the reactions of the activation of polymerase and denaturation of DNA to occur. PCR (polymerase chain reaction) is a procedure that allows a particular part of DNA to be analyzed by creating copies of it through specific primers designed for that piece of DNA. The thermocycler was run 35 times to determine the quantity of each gene expressed. For PER1, PER2, PER3, CRY1, CRY2, BMAL1, and CLOCK, distinct primers were designed by using the NCBI Primer-Blast database. For instance, in this study, specific primers were designed for the PER1/2/3 and CRY1/2 genes in order to target their presence for analysis. The primers are fragments of ssDNA (single-stranded DNA) which were used in every PCR reaction. GAPDH was utilized to compare the expression of the clock genes.

Data Analysis

The primary means of data analysis will include comparing fluctuations or changes in relative gene expression. After all samples were run (CFX384 Real Time PCR System), qPCR excel sheets were pulled from the thermocycler for the samples from every time interval to determine the significance of relative gene expression levels.

Results

The hypothesis stating that rhythmicity would be present in the TNBC cultures exposed to the red light, ambient light, and no light was supported.

Figure 1 shows calculated fold changes which are ratio values for gene expression; positive numbers are an indication of gene upregulation, whereas negative values are indicative of downregulation of that particular gene. For instance, in the group exposed to ambient light, genes PER3, CRY1, BMAL1, and CLOCK are all downregulated at hour 18 compared to the group exposed to ambient light where these respective genes are upregulated. Outliers were eliminated through performing a quartile test, hence why all values are not present for the one trial that was accomplished.

Relative gene expression for the circadian genes in the intervention groups (ambient and red light) were normalized to the gene expression in the control group (no light) using the delta-delta Ct method. Gene oscillations in the ambient light group were less pronounced than that of the red light group. In the ambient light group, PER3 and CRY 1 had an apparent jump from hour 18 to hour 24 (Figure 3). However, PER1, PER2, and CLOCK seemed to stay within a state of stagnancy, but still showed variation within the 0 to 24 hour time point (Figure 3). This set of data had demonstrated rhythmicity in the presence of ambient light due to the relative changes in gene expression of the clock genes. None of the genes flatlined or stayed at the exact gene expression throughout the 24 hour experiment, in turn ruling out the possibility of there being a lack of rhythmicity. Though it should be noted that these relative gene expression changes are not indicative of a proper circadian rhythm. While there appears to be some remnant of a clock that is not completely broken, there are no trends in which BMAL1 and CLOCK are operating in synchronicity, nor is there evidence that PER and CRY are working closely in terms of gene expression.

Figure 6 shows CLOCK to go from a 0.77 to 6.28 level of expression, 5.5-fold higher in the red light group compared to the group exposed to ambient light at hour 12. CLOCK

fell at hour 18 to -0.49 where it was downregulated in the presence of ambient light, but slightly upregulated at 0.02 when exposed to red light. At hour 24, there was a 1.7-fold increase in relative gene expression of CLOCK in the ambient light group compared to the red light group.

Figure 6 shows that BMAL1 was 4.1-fold higher in the red light group even before light exposure, however the trend continues through to hour 12 where BMAL1 is 3.5-fold higher than in ambient light. Together, it can be noted from figure 6 that CLOCK and BMAL1 both peak at hour 12 more so than at any other time interval. Due to exclusion of outliers, PER3 data is limited, but at hour 24 this gene is 15.3-fold higher in ambient light compared to red light.

PER2 shows a consistent average of being higher in relative expression as indicated by a 8.7-fold increase at hour 12 and a 7.8-fold increase at hour 18 in the red light group compared to the ambient light group. In PER1, there was a 0.7-fold increase at hour 12 and a 1.8-fold increase at hour 18 in the ambient light group in terms of gene expression compared to the red light group. While CRY1 data is limited in configuring a reasonable observation, hour 18 exhibits a 6.2-fold downregulation in the ambient light group whereas the red light group remains upregulated at a 0.2 relative gene expression. CRY2 data only demonstrates a trend in upregulation from hour 0 to hour 24; there was a 8-fold increase in relative gene expression in the ambient light group compared to a 1.3-fold increase in the red light group.

In Figure 4, the gene oscillations in the red light group displayed exaggerated relative gene expression levels compared to those of the ambient light group in Figure 3. CLOCK and BMAL1 seemed to follow a similar trajectory increasing in expression from hour 0 up until

hour 12, then falling down around hour 18, but increasing slowly at hour 24 (Figure 4). PER2 also appears to have the highest concentration gradually increasing from 0 up until roughly hour 18, but falls down to below the starting expression at hour 24 when exposed to red light.

Fold Change							
Ambient	Per1	Per2	Per3	Cry1	Cry2	BMAL1	CLOCK
0	4.389684	0.512813	1.360001	0.891422	2.908185	1.101602	1
12	3.092906	0.121611		1.161823	4.954767	1.568636	0.773915
18	3.302233	1.747778	-1.6083	-6.34812	6.816432	-1.66088	-0.48866
24	1.926871		16.67984	2.725222	8.032762		3.038293
Red Light	Per1	Per2	Per3	Cry1	Cry2	BMAL1	CLOCK
0		2.006209	11.56177	3.345435	1.976341	5.15435	1
12	2.434362	8.868717				8.45206	6.284029
18	1.509212	9.557881	0.428331	0.174247		1.981307	0.015249
24	0.991672	1.194058	1.40097	1.362893	1.286156	2.43156	1.374699

Figure 1. **Fold change calculation.** Fold change was calculated for all genes in their respective 24-hour light exposures at each 6 hour time interval.

Delta Ct							
Dark	Per1	Per2	Per3	Cry1	Cry2	BMAL1	CLOCK
0 Hour	14.92329	8.508909	10.31679	12.74669	10.4718	8.499975	
12 Hour	12.7134	10.91666	16.57578	16.2136	12.38659	9.876792	12.87524
18 Hour	9.727129	8.933347	10.32136	15.51622	7.216415	9.563401	7.55278
24 Hour	3.272584	8.118245	6.684232	5.979549	9.643864	7.691586	7.290019
Red	Per1	Per2	Per3	Cry1	Cry2	BMAL1	CLOCK
0 Hour	8.831827	7.504437	6.785501	11.00449	9.488966	6.134185	0
12 Hour	11.42986	7.767936	8.799093	9.371063	4.886479	6.797489	10.22355
18 Hour	12.43557	7.424434	9.936266	11.6889	8.119454	6.916071	13.09921
24 Hour	3.284649	7.862372	6.197806	5.532877	9.280798	6.409704	6.830903
Ambient	Per1	Per2	Per3	Cry1	Cry2	BMAL1	CLOCK
0 Hour	12.78917	9.472405	9.871064	12.9125	8.931678	8.360372	0
12 Hour	11.08444	13.95631	10.0927	15.99721	10.07777	9.227281	13.24499
18 Hour	13.02936	10.68113	8.713065	9.168104	14.03285	7.902524	7.064116
24 Hour	2.326324	0	2.624198	4.533175	6.637967	4.822202	5.686758

Figure 2. **Delta Ct calculations.** Delta Ct values were calculated for all genes in the respective 6 hour time intervals.

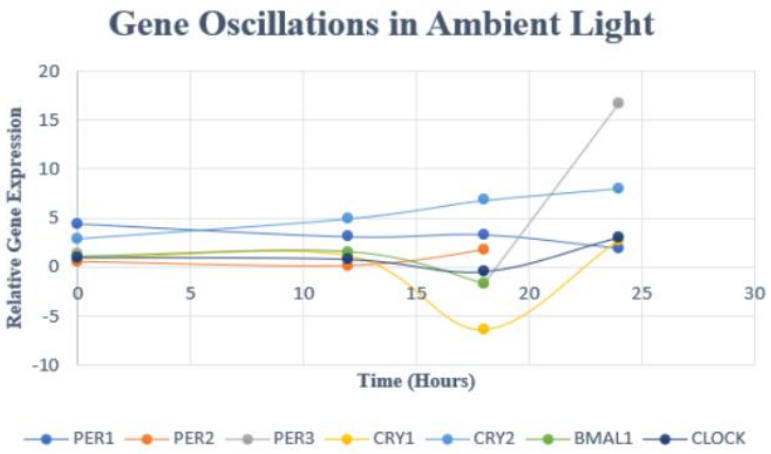

Figure 3: **24-hour, relative circadian gene expressions in the experimental group exposed to ambient light.** Fold change in gene expression for PER1, PER2, PER3, CRY1, CRY2, CLOCK, and BMAL are plotted at 0, 6, 12, 18, and 24 hour time points.

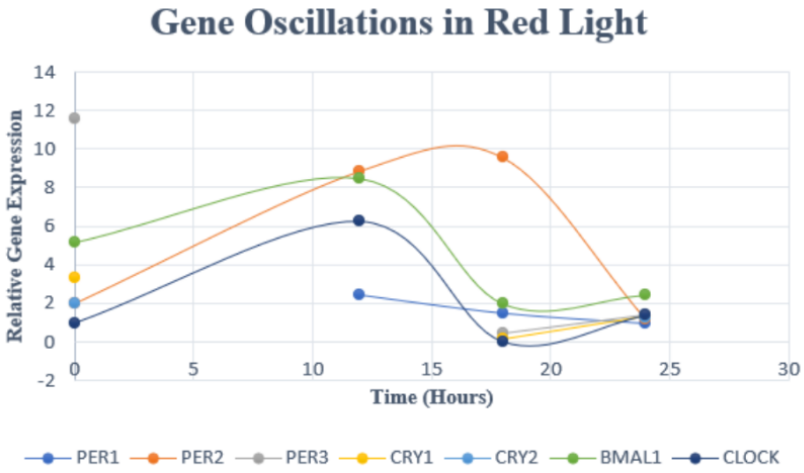

Figure 4: **24-hour, relative circadian gene expressions in the experimental group exposed to red light.** Fold change in gene expression for PER1, PER2, PER3, CRY1, CRY2, CLOCK, and BMAL are plotted at 0, 6, 12, 18, and 24 hour time points.

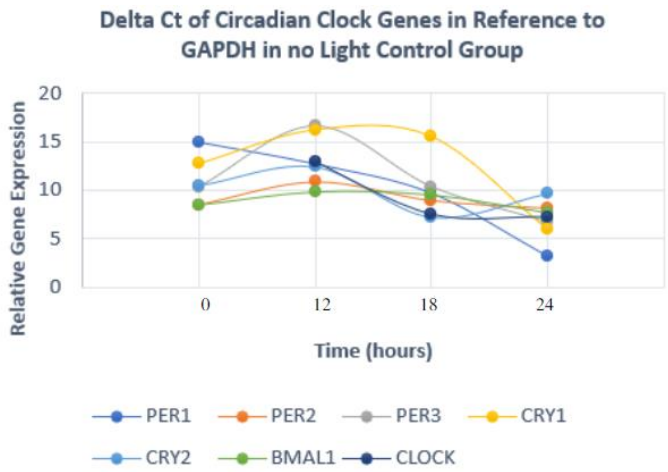

Figure 5: **24-hour, relative circadian gene expressions in the no light group**. Delta Ct values for PER1, PER2, PER3, CRY1, CRY2, CLOCK, and BMAL are plotted at 0, 6, 12, 18, and 24 hour time points.

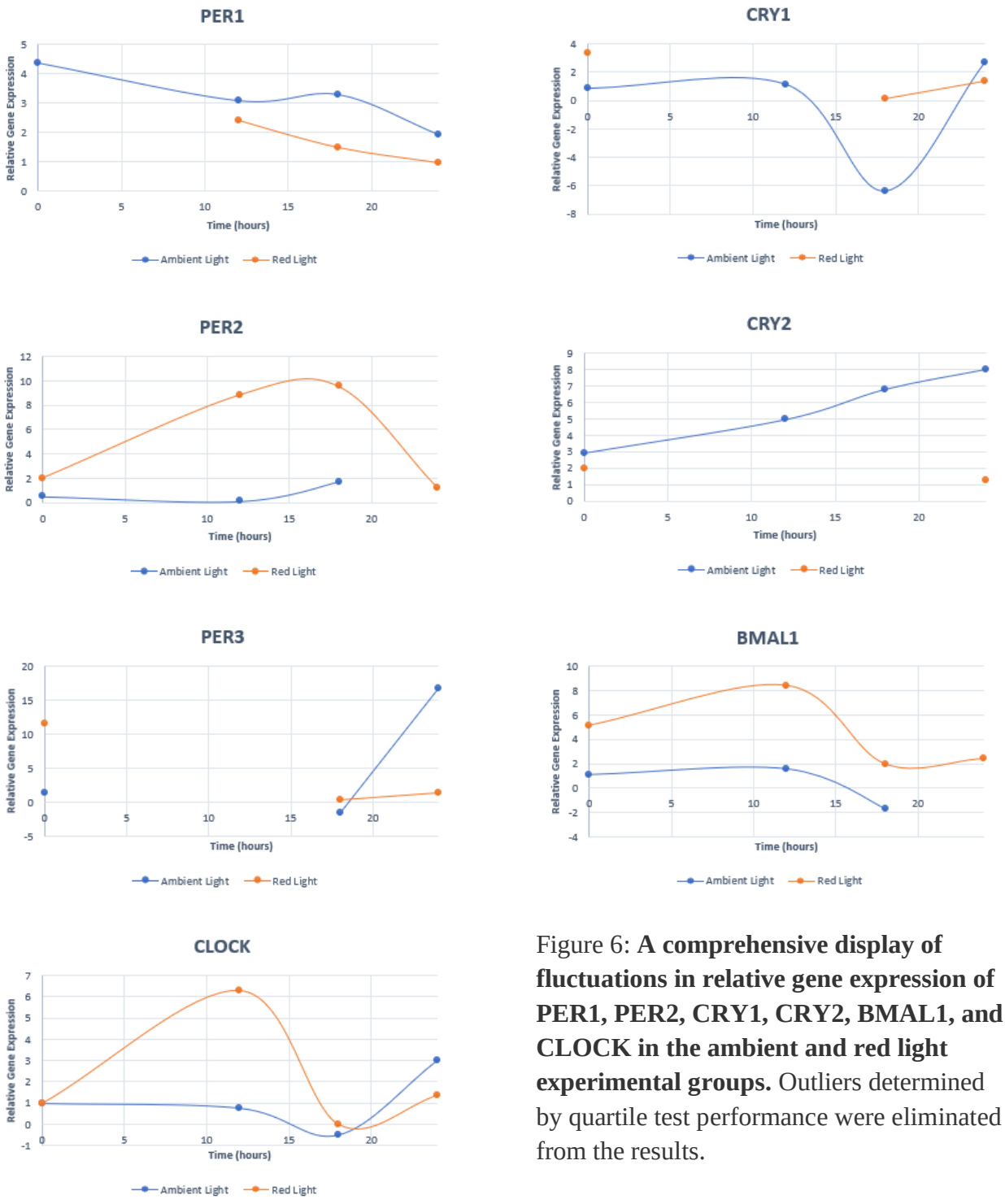

Figure 6: **A comprehensive display of fluctuations in relative gene expression of PER1, PER2, CRY1, CRY2, BMAL1, and CLOCK in the ambient and red light experimental groups.** Outliers determined by quartile test performance were eliminated from the results.

Discussion

The goal of this study was to determine whether circadian genes were expressed in vitro in the absence of an external stimuli, as this has yet to be studied in triple-negative breast cancer cells. In conjunction, another aim was to determine the extent of potential rhythmicity in the cells when exposed to ambient and red light. The hypotheses that these cells would possess clock genes, that EWD-8 cells would demonstrate rhythmicity in the absence and presence of light, and that red light would induce a circadian rhythm were all supported.

As shown in Figure 5, only the Delta Ct value of the circadian genes in reference to GAPDH in the no light group were compared. From this means of analysis, it was found that even without an external stimulus, TNBCs possess rhythmicity; this may point to the potential capacity of these cells to sustain their own rhythm as a means of promoting cancer evolution, tumor growth, or cell proliferation itself. The oscillations observed appear to follow an unsynchronized, “broken” rhythm that fails to take on the nature of a proper circadian rhythm as seen in a living organism. As observed in Figure 4, there are prominent tracks of BMAL1 and CLOCK following a smooth path that aligns with the mechanism by which these genes follow. CLOCK and BMAL1 are bound together and termed heterodimerize, in that they work together in part of a negative feedback loop (Huang et al., 2012). As CLOCK and BMAL1 are expressed, PER and CRY are inhibited. This general biological network in mammals is demonstrated in this experiment as shown by the improved circadian rhythm in the red light group. Interestingly, this result was induced by 60 minutes of red light, a quantity typical for photodynamic light therapy. Future studies could explore effective intervals of time to expose cells or model organisms to red light. These results are promising in the sense that red light could induce a proper circadian rhythm that is often disrupted in cancer. The next steps for this study will include re-running the

samples to obtain values of which were not collected during qPCR due to a potential pipetting error.

The outliers from the data collected were eradicated after performing a quartile test in excel; this was a means of only interpreting the reliable data from qPCR. Outliers and errors in the experiment were likely due to improper pipetting which could have influenced the very specific and sensitive concentrations in the solutions required for a successful PCR run. Additional trials are necessary to pull more precise results and conclusions. The time interval for hour 6 was not collected due to qPCR failing to run the correct cycles. cDNA synthesis can be performed to fill in the gap to obtain a full set of data for interpretation. In collecting data, the morphology of these cultures at the 6-hour intervals were additionally unremarkable, hence why evidence of potential apoptotic properties could not be supported by a single trial of this experiment.

Further research is necessary to determine the biochemistry or why some cultures started out with a higher relative gene depression at their baseline, but it could be argued that not all cells will exhibit a single circadian rhythm at an identical level regarding fold change. Knowing that red light can induce a certain level of rhythmicity in TNBCs leads to a number of questions: if mouse models were exposed to the same 24-hour light exposures, would they exhibit the same results? Would 60 minutes of red light induce an improved oscillatory rhythm? If tumor growth was monitored, could red light show visible signs of decreased growth? With this study being in vitro, there are limitations, but there are implications worth exploring.

Conclusion

The human body functions on a 24-hour period which houses changes in cardiac output, plasma glucose levels, hormone secretions from the neuroendocrine system, thermoregulation, and complicated intrinsic processes, all of which oscillate at different time intervals. Just as clock genes are expressed throughout the body as an evolutionary imprint of time, they are found in vitro even without the ability to mediate light signals through photoreceptors.

Through analyzing pacemaking and oscillatory genes PER1, PER2, PER3, CRY1, CRY2, BMAL1, and CLOCK at the level of the triple-negative breast cancer cell, it was found that these are expressed in the cell line of EWD-8's. Whether they would demonstrate a rhythm in the absence of thermoregulation, the cardiovascular system, brain, or hormones was the ultimate question. Knowing that an out of sync "rhythm" was found in the ambient light group exposed to a much shorter wavelength with a higher frequency could indicate an oncogenic program. The cancer pathways within the TNBCs could be disrupting the clock regardless of the light exposure, but the light exposure could also be driving further disruption of the circadian rhythm itself. The most intriguing finding with this study was the instance that red light does induce an improved rhythm in vitro; though it couldn't be deduced that the cells were entrained by red light, the data still points to red light having a positive effect. This effect has the potential to restore rhythmicity, but several next steps are needed to make accurate interpretations.

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