AN ABSTRACT OF THE THESIS OF

Kelly L. Hughes for the degree of Master of Science in Veterinary Science presented on March 31, 2014.

Title: Loss of Rhythmic Circadian Clock Gene Expression is Associated with Decreases in Estrogen Receptor Expression in Canine Mammary Cancer

Abstract approved: _____

Patrick E. Chappell

Abstract: The endogenous circadian clock is an intracellular transcriptional feedback loop timing daily patterns of multiple biological rhythms within a 24-hour period. Disturbance in various rhythms leads to alteration of normal biological processes including cellular proliferation and tumor suppression. Endogenous circadian clock rhythms have been found to be disrupted in breast cancer. These findings support the increasing evidence that circadian rhythm interference leads to an increased risk of breast cancer. Estrogens have also been found to play a role in breast cancer by acting on estrogen receptors (ER) to induce gene expression. Both human and canine mammary tumors have been found to express ER. Canine mammary cancer is known to be influenced by estrogen signaling, with early ovariectomy decreasing mammary cancer risk from 0.5 - 24%. There is also evidence of a link between ER and clock genes, which may be important in development of hormone related breast cancer.

A canine mammary cancer cell line with ER expression has been established at the Oregon State University College of Veterinary Medicine and the purpose of this study was to use this *in vitro* model to investigate patterns of clock gene expression and ERα and ERβ. With increased cell passage we observed a loss of rhythmicity of expression of both clock genes

Bmal1 and *Per2* and *esr1/2* (ERα/β). Additionally, preliminary results suggest that manipulation of ER expression may lead to resumption of clock gene rhythmicity. Furthermore, treatment with sirtinol, an inhibitor of the histone deacetylase (HDAC) class III Sirt1, indicated ERβ rhythmicity may be rescued by alteration of clock function via HDAC inhibition. Investigations of the relationship between ER and clock rhythmicity in this cell line are ongoing.

Loss of Rhythmic Circadian Clock Gene Expression is Associated with Decreases in Estrogen Receptor Expression in Canine Mammary Cancer

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Dean of the College of Veterinary Medicine

Dean of the Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Kelly L Hughes, Author

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Chapter 1

Introduction

Comparative aspects of human breast cancer and canine mammary cancer

Breast cancer is the most common cancer in women. Mammary cancer is also one of the most common tumors in female dogs with lifetime risk for malignant tumors varying from 2 to greater than 20% (E.G. MacEwen 1996). Breast cancer is often considered to be a hormone dependent cancer, and there is a clear correlation of hormone dependency in dogs with mammary tumors. The risk of malignant mammary tumors in dogs spayed before the first estrus is 0.05% versus 26% in dogs spayed after the second estrus (E.G. MacEwen 1996). Tumorigenesis in both breast cancer and prostate cancer in humans has been found to be influenced by estrogens and androgens (Gery and Koeffler 2010). There have been found similarities between humans and dogs in association with inherited risk of mammary cancer. *BRCA1* and *BRCA2* have previously been described as breast and ovarian cancer susceptibility genes in human breast cancer. *BRCA1 and BRCA2* normally act as tumor suppressors and mutations led to DNA damage accumulation and increased susceptibility to cancer development (Rivera and von Euler 2011). *BRCA1* and *BRCA2* have also been identified in development of mammary tumors in the English Springer Spaniel (Rivera, Melin et al. 2009; Rivera and von Euler 2011). Canine mammary cancer is a spontaneous disease and dogs are exposed to similar environments as their human counterparts making them an excellent model for spontaneous development of breast cancer and insight into tumorigenesis.

Estrogen acts through receptors, of which estrogen receptorβ may have a protective role in breast cancer

Hormonal affects are mediated through members of the nuclear receptor (NR) superfamily, specifically estrogen and androgen receptors (ER and AR respectively) (Gery and

Koeffler 2010). Estradiol is a steroid hormone which acts by binding to estrogen receptors ERα and ERβ. When ligands bind the ER, activation occurs and there is interaction with estrogen-responsive elements (EREs) found at regulatory regions of estrogen target genes which induce transcription (Cai, Rambaud et al. 2008). Inhibition of ER α is a major tool used to prevent and treat breast cancer (Sommer and Fuqua 2001). ERα and the progesterone receptor (PR), the transcription of/abundance of which are estrogen-regulated, were the first receptors to be studied in human breast cancer (HBC) and canine mammary tumors (CMT). ERα and PR have been identified to have increased expression in malignant and pre-malignant breast tissue compared to normal tissue in humans (Sommer and Fuqua 2001). ERα positivity is found in 50-80% of HBC and ERα and PR are present in more than 50% of CMT (Nieto, Peña et al. 2000; Platet, Cathiard et al. 2004). Estrogen receptor positivity in human breast cancer is considered favorable because it is correlated with a lower grade of cell proliferation, and the greatest benefit of hormone therapy occurs in patients with ER α or PR positive tissue (Platet, Cathiard et al. 2004). Response to estrogen receptor modulators is significantly better in human patients with breast cancer that express ERs and PRs and in dogs with malignant tumors; expression of ERα and PR was associated with a significantly higher survival rate compared with tumors only expressing ERα and not PR (Chang, Tsai et al. 2009). Also similar to humans, in dogs, ERα values were also found to be significantly higher in benign tumors versus malignant tumors in dogs and lower rates of ERα expression were found in mammary tumors that showed distant metastases during the follow up period (Nieto, Peña et al. 2000).

ERβ was discovered after ERα and has been shown to have different functions than ERα, with different tissue distribution. ERα has been identified as having an important role in female reproductive functions while ERβ is a key player in the physiological functions of prostate, colon, cardiovascular, and nervous tissue (Swedenborg, Power et al. 2009). ERβ has been shown to oscillate in a long term manner for the stage of development, i.e., fetal development, and within a

24 hour circadian cycle (Swedenborg, Power et al. 2009). ERβ expression is dynamic over the long term, modulating over stage of development, i.e., fetal development, and oscillates within a 24 hour circadian cycle (Swedenborg, Power et al. 2009).

In normal human mammary glands, ERβ is the predominant receptor; however, in HBC, ERα is the predominant ER (Hartman, Strom et al. 2009). In mice, ERα and ERβ knockouts have shown that ERα is important in ductal development and elongation during puberty, whereas,ERβ is important in the differentiation of epithelium (Helguero, Faulds et al. 2005). ER β has been shown to have an inhibitory effect on the proliferation of estrogen-target tissues (Platet, Cathiard et al. 2004). ERβ is thought to have a protective effect in breast cancer tissue, with an inverse correlation to the marker of cell proliferation, Ki67 (Bardin, Boulle et al. 2004). 17beta-estradiol (E2) has opposite effects on cancer growth depending on which receptor subtype it interacts with. E2 binding to ERα causes cancer promoting responses whereas binding to ERβ causes protection against cancer growth in colon cancer (Mostafaie, Kállay et al. 2009).

Studies have also indicated ER β RNA level was decreased in invasive breast cancer when compared with normal tissue, and decreasing ERβ expression appears to be a common finding in breast and ovarian cancer (Bardin, Boulle et al. 2004; Platet, Cathiard et al. 2004). Studies have also indicated that ERβ RNA was decreased in invasive breast cancer compared to adjacent normal mammary tissue and the ERβ promoter regions were hypermethylated in breast cancer cells compared to normal mammary epithelial cells (Platet, Cathiard et al. 2004; Hartman, Strom et al. 2009), suggesting epigenetic repression. Similarly, in dogs, ERβ expression was found to be high in normal mammary epithelium, and ERβ expression has been found to be higher in benign versus malignant tumors (Martin de las Mulas, Ordas et al. 2004). In summary, ERβ appears to have a protective role in breast cancer, with identification of decreased expression with increasing malignancy in both humans and dogs.

The circadian clock times biological rhythms with external and internal stimuli including hormones within a 24 hour period

Central to regulating endocrine function based on daily, estrual, and seasonal changes is the circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus. The central pacemaker is responsible for synchronization of the environmental cues generating the circadian rhythms and daily peripheral oscillations in various tissues (Fu and Lee 2003). The master circadian clock maintains daily time and rhythms based on external and internal cues (Reppert and Weaver 2001). The endogenous circadian clock is an intracellular transcriptional feedback loop timing the daily patterns of multiple biological rhythms with a 24-hour period. There are at least nine circadian genes: including period 1 (*Per1),* period 2 (*Per2*), period 3 (*Per3*), cryptochrome 1 *(Cry1),* cryptochrome 2 *(Cry2),* circadian locomotor output cycles kaput (*Clock),* brain and muscle ARNT-like protein (*Bmal1),* casein kinase 1ε (CK1ε), and timeless (*TIM*) (Kuo, Chen et al. 2009). The central circadian pacemaker synchronizes peripheral cellular oscillators throughout the endocrine system to the ambient light environment. The SCN also signals to the pineal gland, which directs the synthesis of melatonin (MEL) during the dark phase. With seasonal changes in the day length, alterations in MEL production cause profound changes in the reproductive state of seasonal breeders (McArthur, Hunt et al. 1997). Circadian rhythms regulate numerous functions including sleep, blood pressure, core temperature, production of hormones, and immune activity among many others (Fu and Lee 2003). Disruption in various rhythms leads to alteration of these normal biological processes and a decline in health, including disruption in cellular proliferation and tumor suppression. Cancer in humans has been linked to disruption of these rhythms in peripheral, malignant tissues (Fu and Lee 2003). Both central and peripheral cellular circadian clocks are composed of core circadian transcription factors including CLOCK, BMAL1, Per1, Per2, Cry1, and Cry2. These endogenous clocks regulate the temporal expression of clock-controlled genes (Fu and Lee 2003).

Intracellular clock oscillations occur via interactions among positive and negative feedback loops (Figure 1) involving three *Period* genes and two *Cryptochrome* genes (Reppert and Weaver 2001). The transcription factors, CLOCK and BMAL1 are responsible for stimulating the rhythmic transcription of the *mPer* and *mCry* genes (Reppert and Weaver 2001). CLOCK and BMAL1 bind to the promoters of *Period* (*Per*1, 2 and 3) and *Cryptochrome* genes (*Cry*1 and 2), the proteins of which feed back to inhibit Clock/Bmal1 transcriptional activity in an autoregulatory loop (Chen 2005). During the day, there is accumulation of the PER1-CRY1 heterodimer resulting in a translocation of the complex to the nucleus and interaction with the CLOCK-BMAL1 heterodimers, inhibiting transcription of *per* and *cry* to end the cycle. A secondary feedback loop consists of the nuclear hormone receptors, Rev-erb and Ror. These receptors directly modulate the core feedback loop (Gery and Koeffler 2010) via control of *Bmal1* transcription. There is also regulation at the post-translational level with chromatin remodeling (Gery and Koeffler 2010).

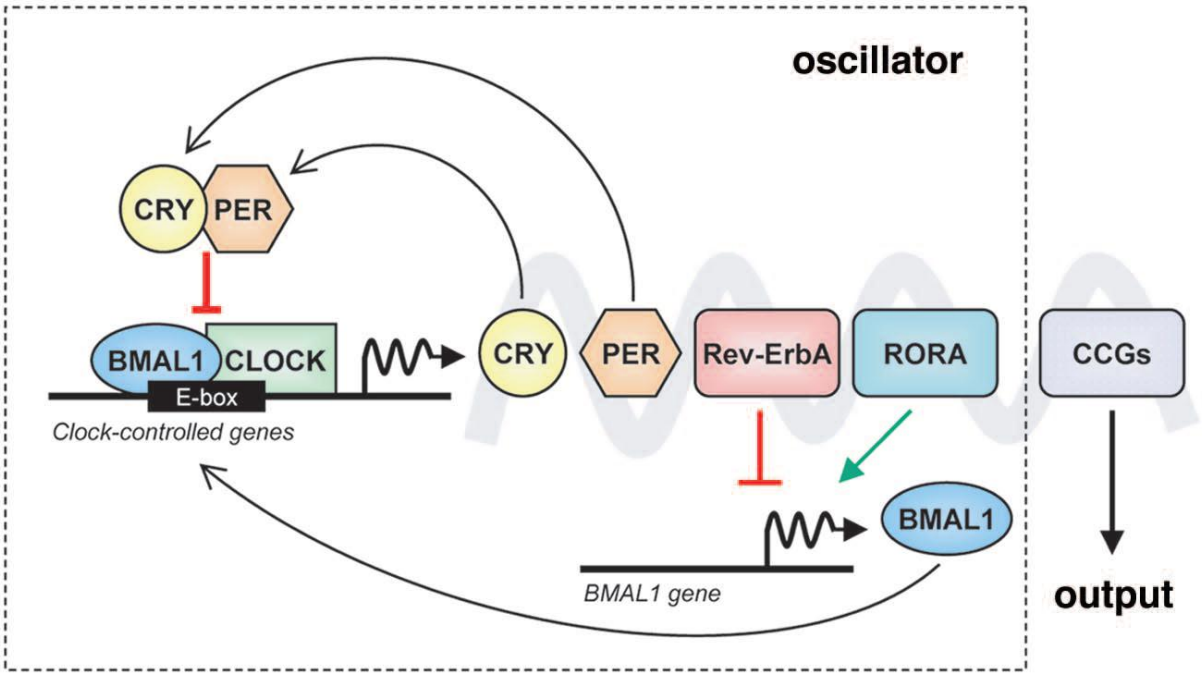

Figure 1: Schematic of the core clock feedback loop. Adapted from:(Rossetti, Esposito et al. 2012).

In SCN neurons, levels of *Clock* within cells are stable throughout a 24 hour period, but *Bmal1* expression levels are high at the beginning of the day and low at the beginning of night (Fu and Lee 2003). High expression of *Bmal1* promotes the formation of BMAL1-CLOCKheterodimers which bind to E-box sequences located in the promoter regions of *Cry, per* and *Rev-Erbα,* and activates transcription (Fu and Lee 2003). It has been shown that CLOCK protein is not actually required for maintaining rhythmic gene expression of *mPer1, mPer2, Bmal1, mCry1* and *Rev-Erbα* (Chen-Goodspeed and Lee 2007). Neuronal PER-ARNT-SIM (PAS) domain protein 2 (*Npas2)* is a paralog of *Clock* and may have a role of redundancy in the circadian clock function (Chen-Goodspeed and Lee 2007). NPAS2 is also able to form a heterodimer with BMAL1 and activate transcription of genes by binding to the E-box elements

(Chen-Goodspeed and Lee 2007). The most well studied positive feedback loop is the transcription of Per2 directly activated by BMAL1/CLOCK heterodimers with nuclear PER2 stimulation of *Bmal1* PAS-mediated transcription (Reppert and Weaver 2001; Fu, Pelicano et al. 2002). Mice that are homozygous for the *mPer2* mutation ($mPer2^{m/m}$) have been shown to have a deficient circadian clock function (Zheng, Larkin et al. 1999).

The three period (*PER)* genes encode PAS domain proteins that do not directly bind to DNA, but function in the nucleus (Chen 2005). The *Clock* and *Bmal1* genes encode PAS helix-loop-helix transcription factors and the mCRY and mPER accumulate and interact with CLOCK and/or BMAL1 to regulate transcription activity (Reppert and Weaver 2001; Fu and Lee 2003). In the nucleus, mCRY proteins work as negative regulators by direct interaction with CLOCK/BMAL1 and inhibit transcriptions, whereas, mPER2 is involved in rhythmic transcription of *Bmal1*(Reppert and Weaver 2001). This results in a rhythmic transcription of *Bmal1,* which is a phase opposite of *mPer/mCry (*Reppert and Weaver 2001).

The SCN neurons are not the only cells containing an intrinsic clock; neurons in peripheral tissue also express functional clocks (Miller, McDearmon et al. 2007). The core circadian clock in the brain coordinates peripheral clocks which coordinate cell relevant functions (Yang, Wood et al. 2009). It has been shown that three *mPer* genes are found in the mouse retina and show circadian expression patterns and these oscillators are independent of the SCN and self-sustaining (Reppert and Weaver 2001). Neurons in other parts of the brain besides the SCN and other tissues including, liver, kidney, and lung all possess functional clocks, and rhythmicity is maintained in the absence of the SCN; however loss of synchrony among organs occurs in SCNx animals (Welsh, Yoo et al. 2004; Miller, McDearmon et al. 2007). *mPer* genes are found in many peripheral tissues, including liver and skeletal muscle, and the RNA oscillations of the *mPer* genes is delayed by 3-9 hours relative to SCN oscillations (Reppert and Weaver 2001). Serum

shock (50% fetal bovine serum plus 50% culture media), and other circulating factors such as glucocorticoid external stimuli have been shown to induce RNA rhythms of several transcription factors normally expressed in peripheral tissue including *Per1* and *Per2* rhythms (Reppert and Weaver 2001). These rhythms are circadian in length and remain for three cycles in tissue culture; it is not clear if the oscillators are unable to sustain prolonged rhythmicity or if there is damping of the clock due to asynchrony of individual cells (Reppert and Weaver 2001). In the mouse fibroblast cell line NIH3T3 it has been shown that the cells contained a self-sustained circadian clock with the same robustness of SCN neurons (Nagoshi, Saini et al. 2004).

Circadian clock genes maintain numerous biological processes including cell cycle progression

The peripheral clocks are synchronized by the central clock and maintain inherent circadian properties that regulate not only temporal expression of clock genes but also hundreds of non-clock genes (Gery and Koeffler 2010). Through expression profiling, it has been demonstrated that up to 15% of transcripts in any tissue are controlled by clock genes (Panda, Antoch et al. 2002; Gery and Koeffler 2010). Cellular processes affected by clock genes include hormone secretion, aging, metabolic pathways, and cell cycle progression (Gery and Koeffler 2010). Disruption in cellular proliferation, loss of tumor suppression, and ultimately cancer have been linked to disruption of these circadian rhythms (Fu and Lee 2003).

The circadian clock regulates the rhythmic transcription of many mammalian genes including cell cycle genes c-*MYC* , *Cyclin D1*, and *WEE1*(Chen-Goodspeed and Lee 2007*)*. Cellular proliferation in normal tissue is gated by the circadian clock (Wood, Du-Quiton et al. 2006) (Yang, Wood et al. 2009). Cell proliferation and tumor suppression genes that normally follow circadian patterns have been found to be deregulated in mPer2$^{m/m}$ mice (Fu, Pelicano et al.

2002). Tumors have shown two growth peaks a day, and two peaks of clock controlled cell cycle genes including *CycD1, c-Myc,* and *vascular endothelial growth factor (Vegf)* while normal host tissue only show one peak of expression of these genes (Yang, Wood et al. 2009). *c-Myc* is involved in cell cycle control including influencing cell proliferation, differentiation, and apoptosis, and overexpression has been found in many human cancers (Chen-Goodspeed and Lee 2007). *c-Myc* has been known to display a temporal difference in gene expression suggesting circadian control (Chen-Goodspeed and Lee 2007). Further evidence of circadian control was demonstrated in *mPer2* mutant mice, where *c*-Myc was found to be deregulated and under direct circadian control through its promoter by a clock-responsive E-box (Gery and Koeffler 2010). In studies done by Fu et al., mutations in *Per1* and *Per2* resulted in uncontrolled c-MYC signaling, overexpression of G1 cyclins, and increased proliferation of osteoblasts (Fu, Patel et al. 2006). Cells derived from double *mPer1/mPer2* mutant mice have shown a significantly shorter cell cycle than cells from wild-type mice (Fu, Patel et al. 2006; Chen-Goodspeed and Lee 2007).

Per2 is thought to play a role in tumor suppression. Mutations in *Per2* can result in a neoplastic growth phase, and in rodents deficient in *Per2* radiation induced DNA-damage has been reported to occur (Fu and Lee 2003). Several genes involved in cell cycle regulation and tumor suppression are deregulated in PER2 deficient mice, including Cyclin D1, Cyclin A, and Mdm2 (Fu, Pelicano et al. 2002; Gery and Koeffler 2010). Other supporting evidence of a role of Per2 in tumor suppression includes findings that induction of *Per2* expression in cancer cells leads to inhibition of growth, arrest of the cell cycle and apoptosis (Gery, Gombart et al. 2005). *Per2* levels were also found to be reduced in lymphoma cell lines (Gery, Gombart et al. 2005). PER2 mutant mice not only show cancer-prone phenotypes but overexpression of *Per2* inhibits cancer cell growth in vivo and in vitro (Yang, Wood et al. 2009). Similar to *Per2,*overexpression of Per1 has been found to inhibit human cancer cell growth in culture, and cancer cells are

sensitized to DNA damage-induced apoptosis suggesting Per1 also has tumor suppressor properties (Yang, Wood et al. 2009).

Clock genes are disrupted in cancer and more specifically hormone responsive cancer

Mutations in *Per1* and *Per2* have also been found in hormone responsive cancer, particularly human breast cancer (Sjöblom, Jones et al. 2006; Yang, Wood et al. 2009).

Disruption of the circadian rhythm has been linked to increased risk of breast cancer in women. Studies have indicated that circadian rhythms' expression through constant light or pinealectomy accelerates breast epithelial stem-cell proliferation, and increases spontaneous mammary cancer development in rodents (Fu and Lee 2003). Studies have found that women with night shift work, sleep deprivation, circadian disruption, and exposure to light at night, have an increased risk of breast cancer (Davis and Mirick 2006; Hansen 2006). The circadian rhythm of melatonin secretion is also regulated in the SCN (Srinivasan, Spence et al. 2008). Melatonin has been shown to inhibit the proliferation of MCF-7 human breast cancer cells which are ERα positive cells, whereas, melatonin did not inhibit proliferation in ERα negative MDA-MB231 breast cancer cells (Yuan, Collins et al. 2002). Melatonin has also been shown to limit estrogen-induced transcription activity of ERα, including anti-apoptotic protein Bcl-2, at the same time as expressing growth-inhibitory and apoptotic pathways of TGF-α and Bax (Hill, Blask et al. 2011).

In a study looking at the expression of the *Period* genes in cases of female breast cancer by Chen et al., *Per* genes were found to have a differential expression pattern in cancerous cells in 96% of the cases, compared with their paired adjacent non-cancerous cells (Chen 2005). They also found differential expression of the PER proteins and in ~50% of the cases this was explained by promoter methylation of the *Per* genes, specifically *PER1* promoter methylation(Chen 2005). Different *PER* expression patterns were found in subpopulations within

the same cancer tissue, signifying that there may be numerous asynchronized circadian clocks within the same neoplasm (Chen 2005). PER1 methylation status also has a strong correlation with the expression of the *c-erB2* oncogene (Chen 2005; Kuo, Chen et al. 2009). Forced overexpression of either Per1 or Per2 has been shown to inhibit cancer cell growth in both breast and prostate cancer cells (Gery and Koeffler 2010). Similarly, silencing of Per1/2 through siRNA has been shown to accelerate growth of breast and prostate cancer in culture (Gery and Koeffler 2010).

What is the relationship between circadian clock disruption, estrogen receptors, and breast cancer?

Intimate relationships between NR and central and peripheral circadian clocks have been observed (Gery and Koeffler 2010). *Per1/2* are estrogen-and androgen-responsive genes eliciting a direct link between the circadian clock signals and ER/AR pathways (Gery and Koeffler 2010). PER2 and ER α have been found to interact, resulting in suppression of estrogen-mediated transcription of ER target genes in breast cancer (Gery and Koeffler 2010). *Per2* has been found to be an estrogen-inducible gene (Gery and Koeffler 2010). *Per1* has been implicated in the development of hormone-dependent prostate cancer. *Per1* was found to be downregulated in human prostate cancers when compared with normal prostates. Furthermore Per1 was shown to physically interact with AR and overexpression of *Per1* in those prostate cancer cells lines which showed both growth inhibition and apoptosis (Cao, Gery et al. 2009). Transcription of *Per1* occurred with recruitment of AR to an androgen response element within the *Per1* promoter (Gery and Koeffler 2010). AR mRNA has been shown to oscillate in the prostate tissue of mice habituated to 12 light/dark cycles indicating the role of AR in coupling of hormonal regulation and peripheral clocks (Cao, Gery et al. 2009).

In sleep studies, disruption of the normal light/dark cycle and sleep deprivation resulted in increased circulating estradiol concentrations (Stevens and Rea 2001). In clock-mutated mouse

embryonic fibroblast (MEF) cells, DNA synthesis was found to be reduced compared to wild type (Miller, McDearmon et al. 2007). In the clock MEF cells it was also found that expression of components involved in the G1/S transition, including estradiol receptor (ER), were also significantly downregulated (Miller, McDearmon et al. 2007).

Epigenetic changes have also been identified in correspondence with circadian genes and breast cancer. DNA methylation in CpG islands of promoter regions is associated with silencing of a gene. Aberrant methylation (hypermethylation) has been identified in tumor suppressor genes in cancer. A strong correlation between *Per1* promoter methylation and estrogen receptor expression has been identified in breast cancer (Kuo, Chen et al. 2009). There is an evolutionarily-conserved E box in the 5' promoter region of ERβ in which circadian-regulatory proteins are recruited causing circadian oscillation of ERβ expression in mouse tissues and synchronized cultured cells (Cai, Rambaud et al. 2008). In BMAL1 knockout (KO) mice, ERβ mRNA is persistently high and loses oscillatory expression found in synchronized cultured cells and mouse tissue (Swedenborg, Power et al. 2009). In mouse HC11 cells (mouse mammary epithelial cells), positive and negative circadian components are recruited to the ERβ promoter region and recruitment of PER1 shows rhythmic expression in line with ERβ mRNA expression (Swedenborg, Power et al. 2009).

Additionally, knocking down either CLOCK or PER1 expression led to elevated expression of ERβ and in BMAL1 KO miceERβ oscillation is lost (Cai, Rambaud et al. 2008). *Per2* was found to be associated with ERα in MCF-7 cells and estradiol enhances the interaction (Gery, Virk et al. 2007). *Per2* has been found to be induced by estradiol in mammary epithelial cells (Gery, Virk et al. 2007).

Epigenetic mechanisms are also likely to be involved in the tumorigenesis of breast cancer and circadian rhythm disruption

Cancer results from an accumulation of mutations and epigenetic alterations are more recently identified as having a major role in cancer pathogenesis. Epigenetics are defined as heritable changes in gene expression which due occur withoutalteration in DNA sequence (Esteller 2008). The major epigenetic changes include cytosine methylation and histone modification. Histone modifications are mediated by histoacetyltransferases (HAT) and histone deacetylases (HDAC). A misbalance of histone acetylation has been linked to cancer, either turning on genes that are normally inactive or turning off genes that are normally active. Hypoacetylation of specific lysines in histone tails normally occurs in inactive genes, and hyperacetylation is typical of transcriptionally active genes (Mai, Massa et al. 2005). There are three classes of HDACs and Silencing Information Regular Two (SIRT) is a family of proteins that are class III histone deacetylases (HDAC). SIRT 1 is an NAD^+ dependent deacetylase which is implicated in transcriptional silencing, genome stability and longevity (Asher, Gatfield et al. 2008). A strong interplay between metabolism and circadian clock has also been suggested and the DNA-binding activity of BMAL1-CLOCK has been shown to be strongly enhanced by the reduced forms of NAD/NADP (Rutter, Reick et al. 2001). CLOCK is not only the transcriptional activator required for circadian expression of numerous genes but is also a histone acetyltransferase (Belden and Dunlap 2008). SIRT1 has been shown to promote cell growth and block senescence, and SIRT1 expression has been found to be up-regulated in cancer (Wang, Kim et al. 2012). Besides the link reported with SIRT1 and cancer, SIRT1 has also been linked to the circadian clock. SIRT1 is an NAD+ dependent histone deacetylase that counteracts activity of CLOCK (Asher, Gatfield et al. 2008; Belden and Dunlap 2008). SIRT1 has also been reported to bind to CLOCK/BMAL1, promoting degradation of PER2 (Asher, Gatfield et al. 2008; Hill, Blask et al. 2011). SIRT1 was found to be expressed at high levels in MCF-7 and MDA-MB-231 human breast cancer cell lines and with transfection of a melatonin receptor and treatment with melatonin, SIRT1 expression was dramatically suppressed (Hill, Frasch et al. 2009). Sirtinol is a

specific inhibitor of SIRT1 and has been shown to cause growth arrest in the MCF-7 cells (Ota, Tokunaga et al. 2006). Sirtinol has been shown to significantly reduce the growth of MCF-7 cells in time-and concentration-dependent manners (Wang, Kim et al. 2012). Sirtinol was also found to decrease expression of SIRT1 in MCF-7 cells to cause cell death through cell cycle arrest through the apoptotic pathway, and to induce autophagic death (Wang, Kim et al. 2012).

Is disruption of the circadian clock involved in mammary cancer development and/or estrogen receptor expression?

Gonadotropin-Releasing Hormone (GnRH) neurons express functional endogenous clocks *in vivo,* and in the immortalized GnRH-secreting GT1-7 cell line (Chappell, White et al. 2003; Tonsfeldt and Chappell 2012). Central to reproductive function, GnRH is released in a pulsatile pattern due to neuronal, humoral and exogenous stimuli and perturbation of circadian clock function disrupts normal patterns of GnRH secretion. Perturbation of clock function disrupts the pustile patterns of GnRH secretion, suggesting circadian control of neuroendocrine cycles, specifically the estrus cycle (Chappell, White et al. 2003).

Rhythmic circadian clock gene expression has also been observed in mammalian testis and accessory reproductive tissue (Bebas, Goodall et al. 2009). Studies suggested that there was a tissue-specific, endogenous, peripheral clock involved in determinants of expression levels of key factors in sperm duct luminal environment (Bebas, Goodall et al. 2009). It was also identified that there were tissue-specific variations in activity, phase, amplitude, and stability in clock gene expression profiles in extratesticular accessory tissues (Bebas, Goodall et al. 2009). Since tissue-specific, circadian clocks appear to play a role in these hormone-responsive tissues we wanted to investigate if there were similar mechanisms that might be involved in the development of breast cancer in the mammary gland, an accessory gland of the female reproductive tract.

Knowing that increased exposure of estrogen in dogs is a risk factor for mammary cancer development, and that ERβ expression oscillates in many tissues, we postulated that circadian influence may be involved in the pathogenesis of mammary cancer development. With this previous work and the notion of circadian clock disruption in oncogenesis, the objectives of this study were to develop a canine mammary cancer cell line and determine if there were clock gene expression, and if so, determine if there were disruption in clock gene expression over time. In order to test our hypotheses we worked with a canine mammary cancer cell line (CMAM) developed at the Oregon State University tissue bank. Our hypothesis was that clock gene expression rhythms in the CMAM cell line would be disrupted. In light of the evidence of cellular oscillators in hormone-responsive target organs, and the suggestion of temporally-gated hormonal secretion and the responsiveness of tissue to environmental stimuli (Tonsfeldt and Chappell 2012), we wanted to look at whether loss of clock oscillations in these cells altered estrogen receptor expression patterns. Knowing that ERβ appears to have a protective role in breast cancer and decreased expression of ERβ is common in hormone dependent cancers, we hypothesized that the CMAM cell line would not express high levels of ERβ. Also knowing that ERβ has been shown to oscillate with a circadian rhythm and that circadian rhythm disruption is found in cancer, we hypothesized that ERβ expression would also be without rhythm.

CHAPTER 2:

Materials and Methods

Establishment of canine mammary cancer cell line (CMAM) and cell culture:

Mammary tissue was collected from an 11 year old spayed female Golden Retriever in RPMI-1640 media[a] and established through the Oregon State University Biobank. Briefly, the tissue was bluntly dissected with a scalpel blade and digested with collagenase[b] for two hours. The digested tissue was then rinsed with phosphate buffed saline (PBS) (0.133M NaCl, 0.0086M K2HPO4, and 0.0015 M KH2PO4), and resuspended with RPMI-1640[a] supplemented with 10% Fetal Bovine Serum (FBS)[c], penicillin (100 U/mL), and streptomycin (0.1 mg/mL) (P/S)[d]. Cells were incubated at 37°C with 5% CO_2/95% O_2. The cells were then passaged to stability. After several passages the cells were stable and maintained in RPMI-1640 + 10 % FBS + P/S + or – gentamicin[d]. For RNA isolation and nuclear extract collection, multiple plates depending on length of collection and treatment groups of similar passage (passage 4 – 10) and confluency (60-90%) were all cultured in the same parallel conditions.

Cryopreservation of cells:

Cells were cultured and maintained in media as described above. The media was aspirated and cells were rinsed with 1 X PBS. The cells were then detached using 1.5 mL of trypsin[e] or TrypLE[d] for a duration of 3 - 5 minutes until cells began to lift off the plate. Warm RPMI-1640 media was added to the plate and the sample was the centrifuged. The media was aspirated and cells were resuspended in 3 mL of freezing media per 10 cm plate. Cell suspensions were divided into 1 mL aliquots in cryogenic vials. Cryovials were slowly frozen in Styrofoam at -80 °C for 24 to 72 hours and then stored in liquid nitrogen (-196 °C) until further use.

Serum shock and cell harvesting protocol - time trial:

Each time point required harvesting from one to four cell culture plates of CMAMs depending on treatments and ultimate analysis. The cells were grown to 60% - 70% confluency and harvested

as follows. After the initial collection the cells were harvested every 6 hours for a 48 hour "time trial." Also, after the initial collection the media was changed from 10% FBS to 50% FBS for serum shock synchronization at 0 hour. The media containing 50% FBS was removed after 2 hour and replaced with 10%FBS + RPMI-1640 + P/S + various treatment and subsequent collection occurred 4 hours later (time point 6 hours) and continued at 6 hour intervals for the duration of the experiment (Figure 2)

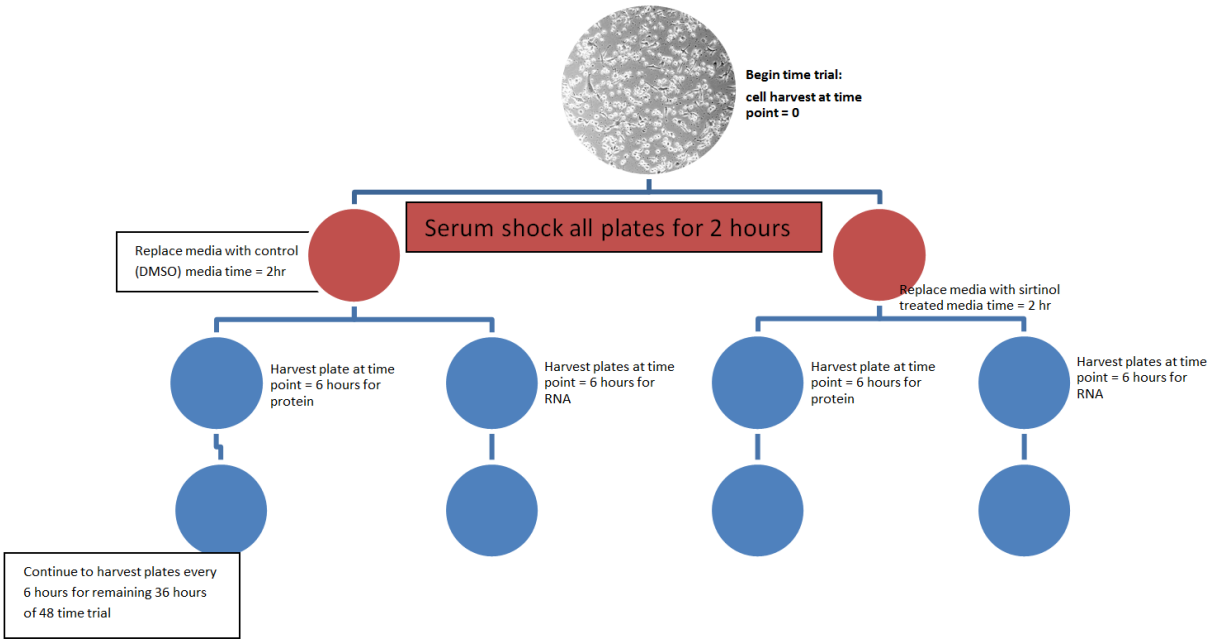

Figure 2: Schematic of time trial. Cell harvesting occurred at time zero and all blue circles.

Cell treatment:

Sirtinol[a] was dissolved in dimethylsulfoxide (DMSO) to a concentration of 100 nM. Valproic Acid[a](an HDAC inhibitor) was dissolved initially in water with treatment concentrations of 1 μM, 0.5 μM, and 0.25 μM. Estrogen[a] was dissolved in DMSO and then ethanol to concentrations of 100 nM and 1 nM. Sirtinol was dissolved in dimethyl sulfoxide (DMSO) and stored at −20°C until use. Reagents were diluted to the appropriate concentrations in culture medium containing

10% FBS. The final concentration of DMSO was <0.1% (vol/vol). Cells used as control were treated with DMSO alone to ensure the vector did not affect results.

Harvesting instructions for each time trial:

One plate of cells from each treatment group was removed from the incubator and washed with PBS for RNA isolation. After aspiration, 1 mL Trizol[d] was added to each plate and the Trizol + lysate was pipetted and rinsed back onto the plate multiple times for complete collection. Trizol + lysate were transferred to a 1.5 mL tube for storage at -80˚C. Another plate from each treatment group was also removed for protein isolation. After washing with PBS and aspiration, plates were placed on a cold block and 400 µL of prepared RIPA solution added to each plate and remained on ice block for 5 minutes. Cells were scraped and lysate was transferred to a 1.5 mL tube for storage at -20˚C.

RNA isolation using Trizol:

Cells were harvested by adding 1 mL TRIzol® LS Reagent[d] and scraped from culture plate using a cell scraper. The lysate was transferred into a 1.5 mL tube and stored at -80˚C until extraction. For extraction the lysate was quickly thawed in warm water and 220 µL of chloroform was added and vigorously mixed for ~ 15 seconds. After sitting at room temperate for 2-3 minutes the lysate was centrifuged at 9,500 rpm for 15 minutes at 4 ˚C. The top phase was moved to new tubes and 0.5 mL of isopropanol was added and shaken and left at room temperature for 10 minutes. The supernatant was removed after centrifugation at 9,500 rpm; 4˚C, 10 min and visualization of cell pellet. The pellet was resuspended in 30 µL RNase-free water. The RNA was then purified by adding 10% 3M sodium acetate (pH 5.2), glycogen to a final concentration of 0.05-1.0 µg/µL, and 2.5 volumes of ice cold 100% ethanol and incubated at -80˚C overnight. After incubation the sample was centrifuged for 20 min; 9500 rpm, 4˚C. After visualization of the pellet the supernatant was removed and 250 µL of 70% ethanol was added to wash the pellet and again

centrifuged for 5 min; 9,500 rpm, 4˚C. The supernatant was removed and the pellet was resuspended in 20 µL RNase free water and quantified using UV spectrophotometry[f].

Real-time quantitative-PCR:

Equal RNA concentrations were pooled from various treatment samples obtained at each time point and one microgram of total RNA isolated was converted to cDNA using EcoDry[g] premix PCR master mix. The cDNA program was 1 hour at 42˚ followed by 10 minutes at 70˚. Canine specific primers for estrogen receptor α *(ERα*) (forward primer 5'-GGGATGTGGCTTCTGGCTA-3', reverse primer 5'-GGTGATCTCGCACTCGTTG-3') estrogen receptor β (*ERβ*) (forward primer 5'-CCAAATGTGTTGTGGCCAACT-3', reverse primer 5'-CTGGCACAACTGCTTCCACTA-3'), *Bmal1* (forward primer 5'-GCTGAGGATGGCCGTTCAG-3', reverse primer 5'-GCTGCCCTGAGAATGAGGTG-3'), and Period 2 *(Per2*) (forward primer 5'-CATGCCTCGACCACGCCTTA-3', reverse primer 5'-CCAACACTGACACGGCAGAAG-3'were used to evaluate expression levels of mRNA. Gene-expression profiles were generated for *Bmal1*, *Per1, Per2, ERα, and ERβ* by SYBR green[h] real-time qPCR. The real-time PCR assays were conducted using an Applied Biosystem Step One plus machine. The two-step quantitative real time RT-PCR was performed as described in the manufacturer's protocol. Cycling conditions were as follows: ten minute incubation at 95°C to deactivate then activate with AmpliTaq®Gold DNA polymerase, 40 PCR cycles of fifteen seconds of 95°C followed by 30 seconds of 59°C followed by 35 seconds of 70˚C with a final dissociation curve analysis to confirm the presence of a single amplicon. Relative transcript was calculated by ΔΔCT (ddCT). Tyrosine 3-monooxygenase/tryptophan 5- monooxygenase activation protein, zeta polypeptide (*YWHAZ)* was used as the housekeeping gene for normalizing gene expression. Reactions without cDNA served as negative controls. The relative expression

was determined using triplicate samples for each time point and significant difference between treatment groups was not determined because each group was collected for a single time trial.

Cell viability assay:

Cell viability was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, 5 mg/ml, Sigma). The cultures were initiated in 96-well plates at a density of 2.5×10^3 cells per well. After 48 hours of incubation, the cells were treated with various concentrations of DMSO (control), estrogen, valproic acid, and sirtinol and cultured for 24 or 48 hours. Pure media (no cells) was used as a negative control. At the end of the treatment period, 15 µl of µL of 1X MTT reagent were added to each well and incubated for 1- 4 h. The absorbance per well was measured at 540 nm using the VersaMax Microplate reader[i] at 1 and 2 hours of incubation. Data from three independent experiments were analyzed and then normalized to the absorbance of wells containing medium only (0%) and untreated cells (100%). Cell viability was compared using a one-way ANOVA using Dunnett's multiple comparison test of the control (DMSO) treated group to each drug treated group *($P < 0.05$)*.

Colormetric apoptosis detection:

The cultures were initiated in 96-well plates at a density of 2.0×10^4 to 5.0×10^4 cells per well. After 24 hours of incubation, the cells were treated with either DMSO (control) or sirtinol (100 nM) for 24 hours. Additional controls included pure media (no cells) for background absorbance, TACS Nuclease-treated controls to confirm permeabilization and labeling of the reaction, unlabeled experimental control sample with TdT enzyme omitted from labeling reaction to indicate level of background labeling associated with non-specific binding of the Strep-HRP. At the end of the treatment period, the TiterTACS™ colorimetric apoptosis detection kit[j] assay protocol was followed. Briefly, the cells were centrifuged and media was discarded, cells were

washed with PBS and wells were filled with 3.7% Buffered Formaldehyde solution for 7 minutes. Cells were again centrifuged and washed and post fixed in 100% methanol for 20 minutes and then washed and centrifuged twice. 50 µL of Cytonin™ were added to each well and the plate was incubated for 30 minutes. The plate was centrifuged and buffer discarded and then washed once with dH_2O. The positive control was generated by adding 50 µL of TACS-Nuclease™ solution to appropriate wells and incubated for 10-60 minutes at 37˚C. other samples were covered with PBS. Samples were then washed for 2 minutes in PBS and with plate centrifuge between each wash. The endogenous peroxidase was then quenched by adding 50 µl/well of perioxide soluation and incubated for 5 minutes at room temperature. The cells were then washed with dH_2O and centrifuged. Then 150 µl/well of 1X TdT Labeling Buffer was added and left for 5 minutes. The cells were centrifuged and buffer discarded and 50µl/well of Labeling Reaction mix was added and incubated at 37˚C for 1 hour. To stop the labeling reaction, 150 µl/well of 1X TdT Stop Buffer was added and left for 5 minutes. After centrifugation, discarding of the buffer and washing twice with PBS with centrifuge between washes, 50 µl/well of Strep-HRP soluation was added and incubated at room temperature for 10 minutes. The samples were then washed four times with 200 µl/well of PBS, 0.1 % Tween 20 and centrifuged between each wash. Then 100 µl/well of TACS-Sapphire was added at room temperature. The cells were then incubated in the dark at room temperature for 30 minutes while the kinetics of the reaction were followed at 630 nm to determine the linear range. The reaction was then stopped with 0.2 N HCl per well and the absorbance was measured at 450 nm. The absorbance was compared using multiple T-tests to the control (DMSO) treated cells using multiple T-tests *($P < 0.05$).*

Immunohistochemistry:

Cells from the CMAM B population were grown to ~ 80 % confluency on a 10 cm plate and then harvested by adding 1 mL TRIzol® LS Reagent[d] and scraped from culture plate using a cell scraper. The cell were pelleted by first centrifuging the lysate at 1,000 rpm for 2 minutes with removal of supernatant and then washed twice with PBS. After removal of the supernatant, 10% neutral buffered formalin was added to the tube and submitted to the histology lab for immunohistochemistry processing and staining for cytokeratin AE1/AE3 (CK AE1/AE3) and cytokeratin wide spectrum (CK WSS) for epithelial characteristics and vimentin for mesenchymal characteristics.

Immunohistochemistry for Vimentin (M7020 1:500 dilution)[j] and cytokeratins AE1-AE3 (1:100 dilution)[j] and Wide Spectrum Staining (1:500 dilution)[j] was performed using the following procedure. Cultured cells were collected into a pellet, fixed in 10% neutral buffered formalin, mixed with 1% low melting temperature agarose and treated as tissue. The pellet was processed to paraffin using a Sakura VIP-5 tissue processor. The tissue block was sectioned at 4-5 µm, collected on charged slides, and baked at 60° C for one hour. Slides were rehydrated through two changes of xylene, two changes of 100% ethanol, one change of 80% ethanol and water. For Vimentin, high temperature antigen retrieval (HTAR) was performed in a microwave pressure cooker (Viking Tender cooker) using Dako Target Retrieval solution[j] for 10 minutes after pressure was reached. The pressure cooker was slowly vented and the container containing the slides was allowed to sit for 20 minutes at room temperature. Slides were placed on a Dako Autostainer and washed in Tris Buffered Saline with Tween (TBST)[k] followed by 3% H_2O_2[a] in TBST 10 minutes. The cytokeratins were subjected to enzyme digestion for 5 minutes and then washed with TBST followed by Dako serum -free protein block[j] for 10 minutes and then blown with air. The primary antibodies were diluted in Dako antibody diluent[j] and applied for 30 minutes at room temperature. MaxPoly-One polymer HRP rabbit[l] *was applied for 7 minutes at room temperature and MaxPoly-One polymer HRP mouse[l] * for 10 minutes at room temperature.

Slides were again washed in TBST before the chromagen Nova Red[m] was applied for 5 minutes. Slides were then washed in dH_20 followed by Dako hematoxylin[j] diluted 1:3 in dH_20 for 5 minutes, rinsed in dH_20, rinsed in TBST, run down to xylene and coverslipped. Dako Universal Negative Control-Rabbit[j] was used as the negative control. Positive controls (canine skin) were used as well as a negative control that consisted of Universal Negative Control –Rabbit[j] applied to the cultured cells instead of the primary antibodies.

*Manufacturer name has changed-ImmunoBioScience, Catalog #IH-8064-custom-OrSU or Catalog #IH-8062-custom-OrSU

Chapter 3:

Results

Immunohistochemical identification of CMAMs

We wanted to further characterized the canine mammary cancer cell line that was developed, CMAM. In order to characterize the differentiation of CMAMs, immunohistochemistry of the cells was performed as described in the materials and methods, staining for cytokeratin (epithelial marker) and vimentin (mesenchymal marker). The cells did not stain with either cytokeratin marker (WSS, AE1-AE3) (Figures 3 and 4 respectively) but were positive for vimentin (Figure 5).

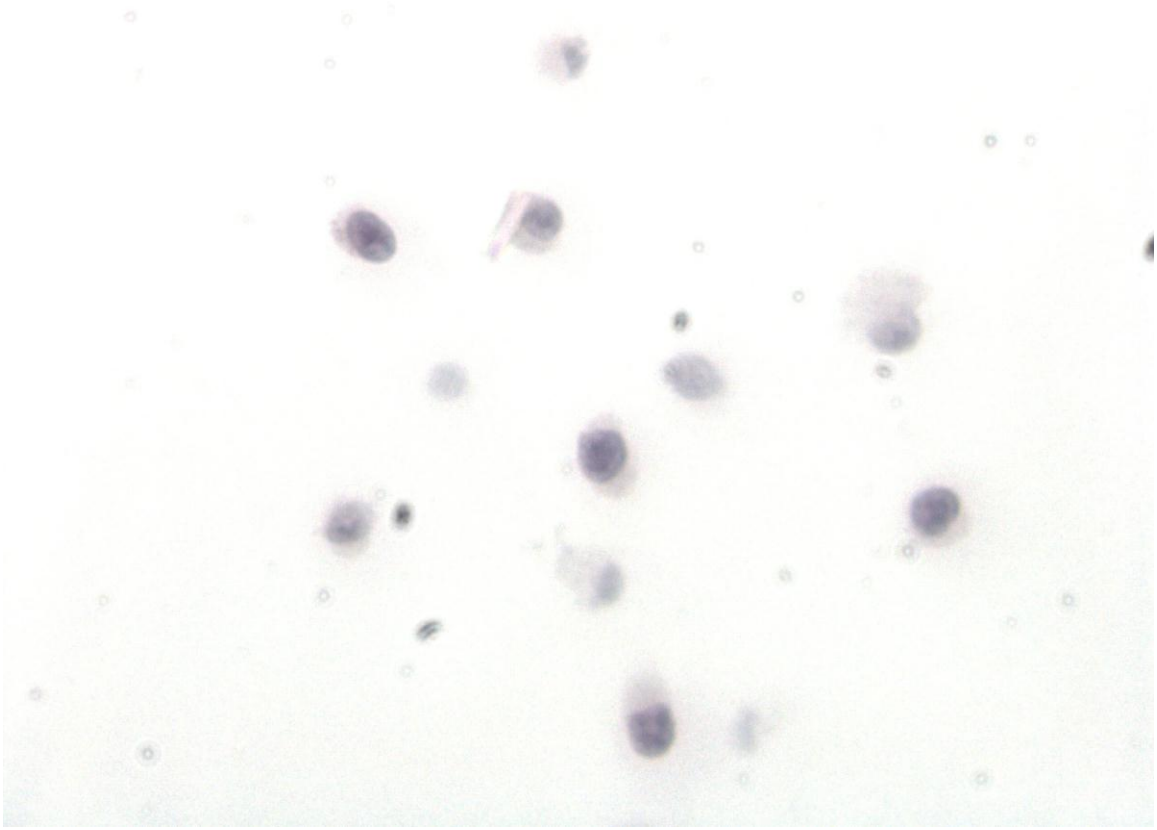

Figure 3: Cytokeratin AE1/AE3 immunohistochemical staining. The cells are negative.

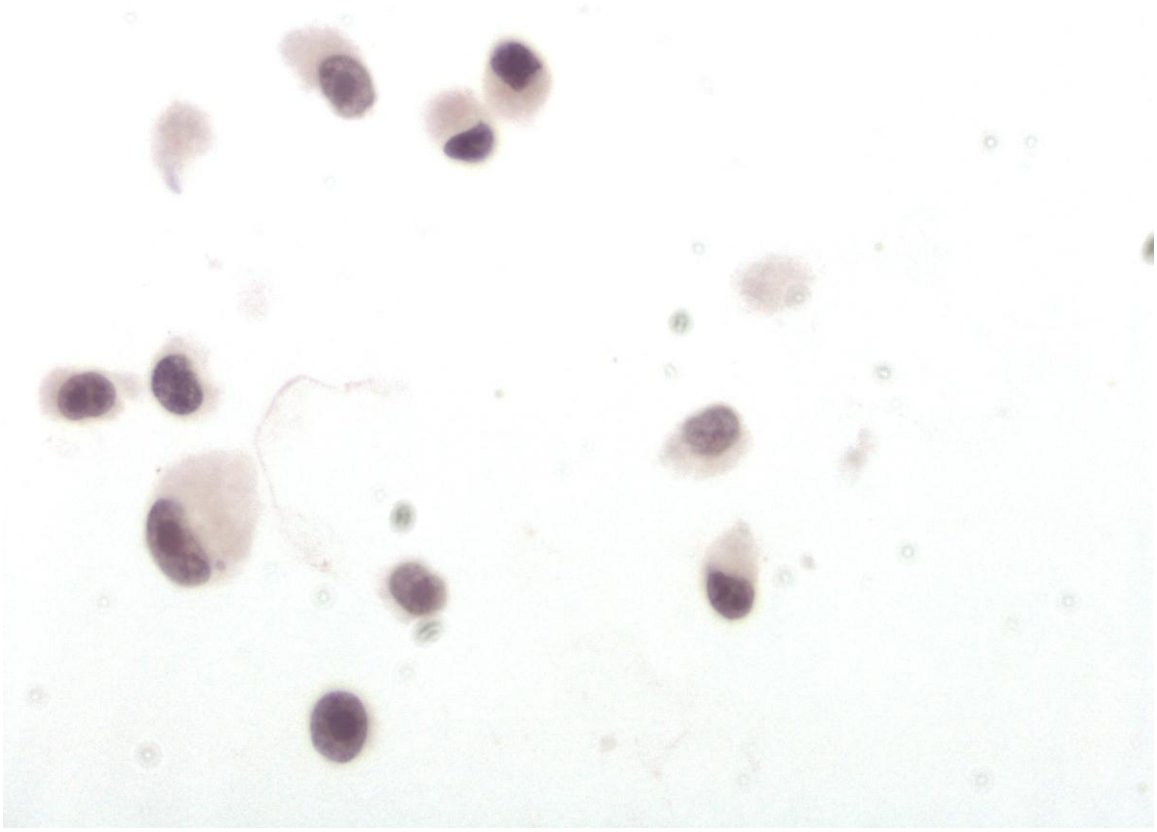

Figure 4: Cytokeratin wide spectrum immunohistochemical staining. The cells are negative.

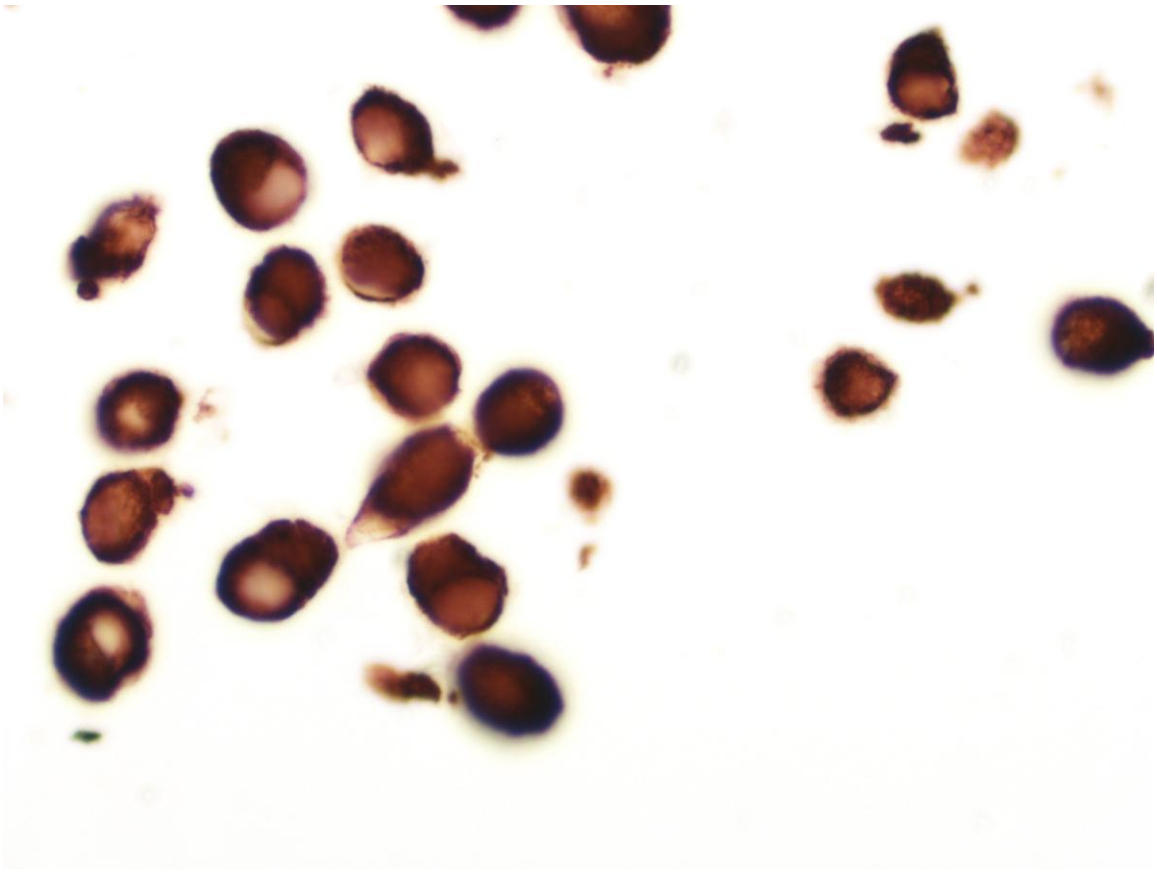

Figure 5: Vimentin immunohistochemical staining. The cells are strongly positive.

Circadian clock genes are expressed and oscillate in early passage cultured CMAM cells and expression and rhythm are lost in late passage cells

To confirm the expression of circadian clock genes in the canine mammary cancer cell line, CMAM, RT-PCR was performed as described. As shown in Figure 1 and 2, the CMAM cell line (early passage – fewer than ten passages from the primary tumor tissue) expresses the characterized mammalian circadian clock genes, including *Bmal1*, and m*Per*2 and expression was lost with increased cell passage (late passage – greater than 10 passages in addition to freeze/thaw cycles). The early passage cells did show circadian rhythm in both *Bmal1* and m*Per2* with *Bmal1* peaking at 6 and twenty-four hours later at 30 hr. m*Per2* was also circadian but out of phase with *Bmal1* peaking at 18 and 42 hours and was overall low.

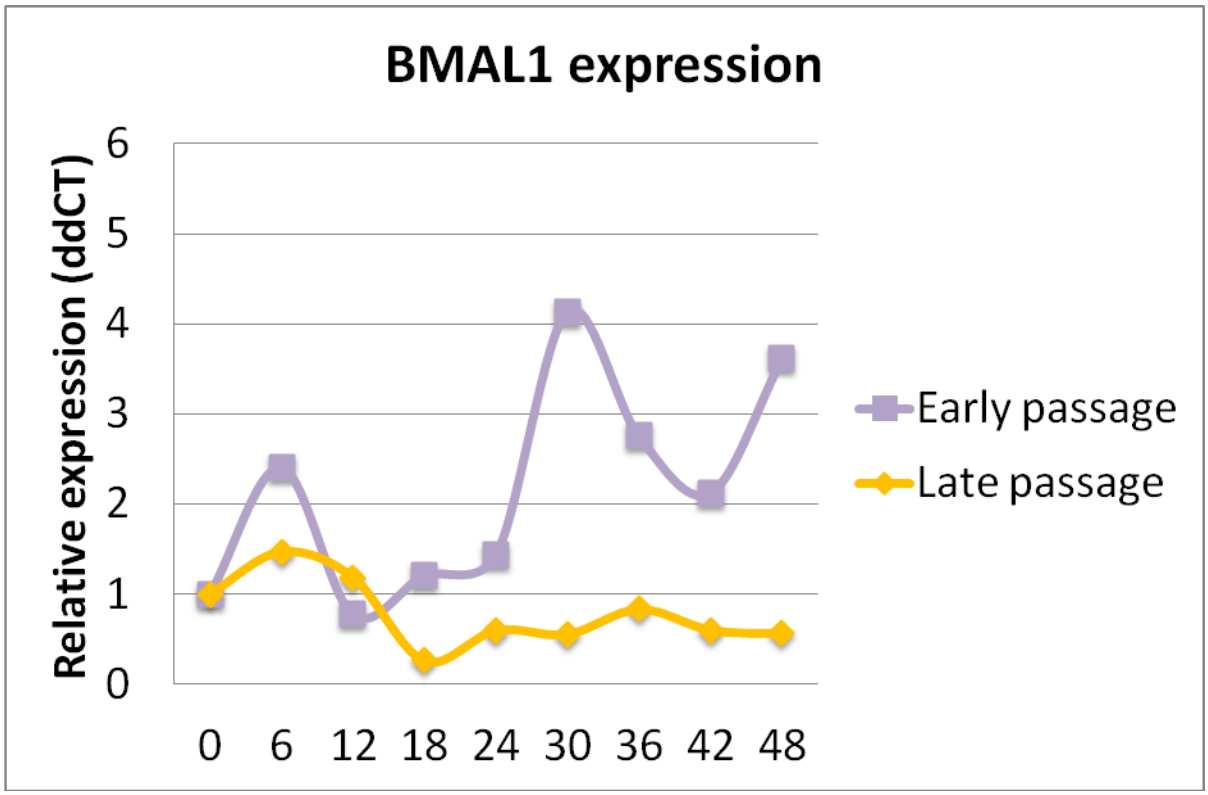

Figure 6: Relative gene expression of *Bmal1* using real-time PCR; x-axis: hours. Early passage cells are indicated in purple and late passage cells are indicated in yellow. The relative expression was determined using the ddCT method standardizing to the YWHAZ gene.

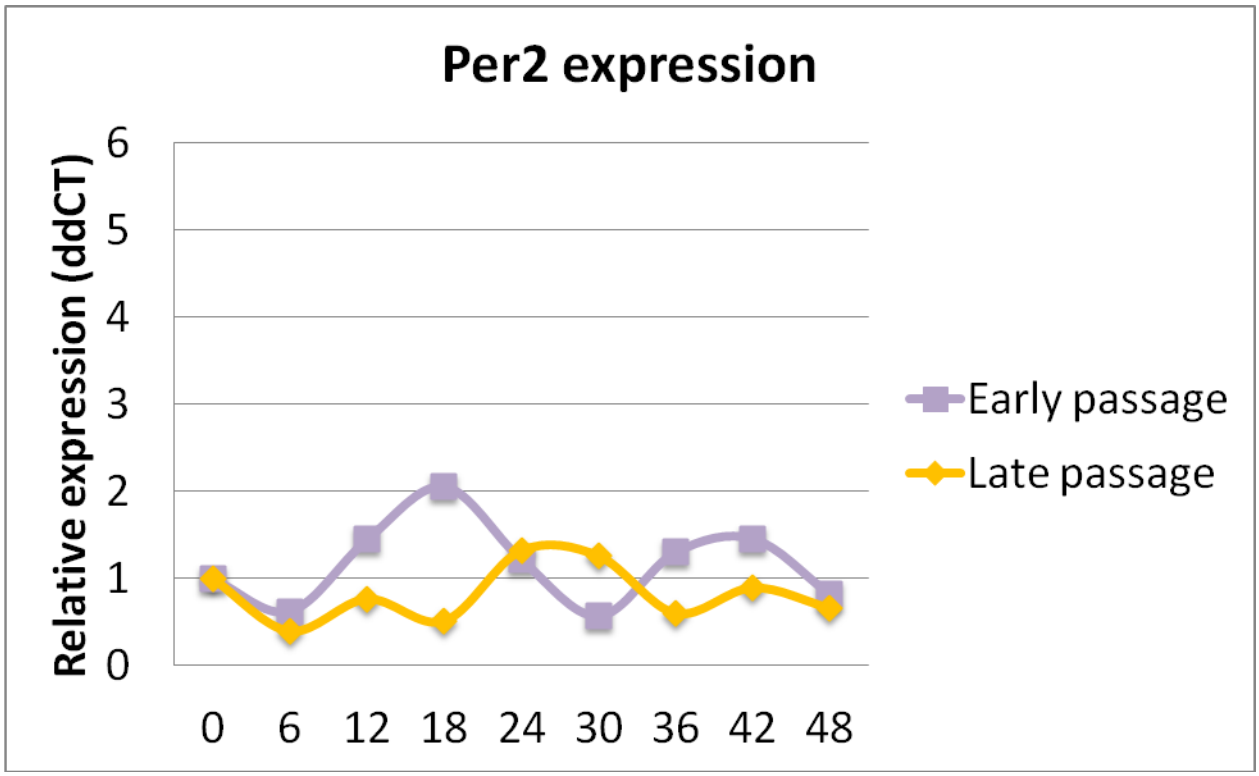

Figure 7: Relative gene expression of *Per2* using real-time PCR. Early passage cells are indicated in purple and late passage cells are indicated in yellow. The relative expression was determined using the ddCT method standardizing to the YWHAZ gene.

Estrogen expression is lost with late passage cells

Similar to *Bmal1* and *Per2*,here was loss of expression of ERβ betweenearly and late passage CMAM cells (Figure 3). ERβ expression did not show circadian rhythm but there were multiple peaks at 18, 30, and 48 hours. ERα expression was overall low and was also decreased with late passage cells and there was no circadian rhythm (Figure 4).

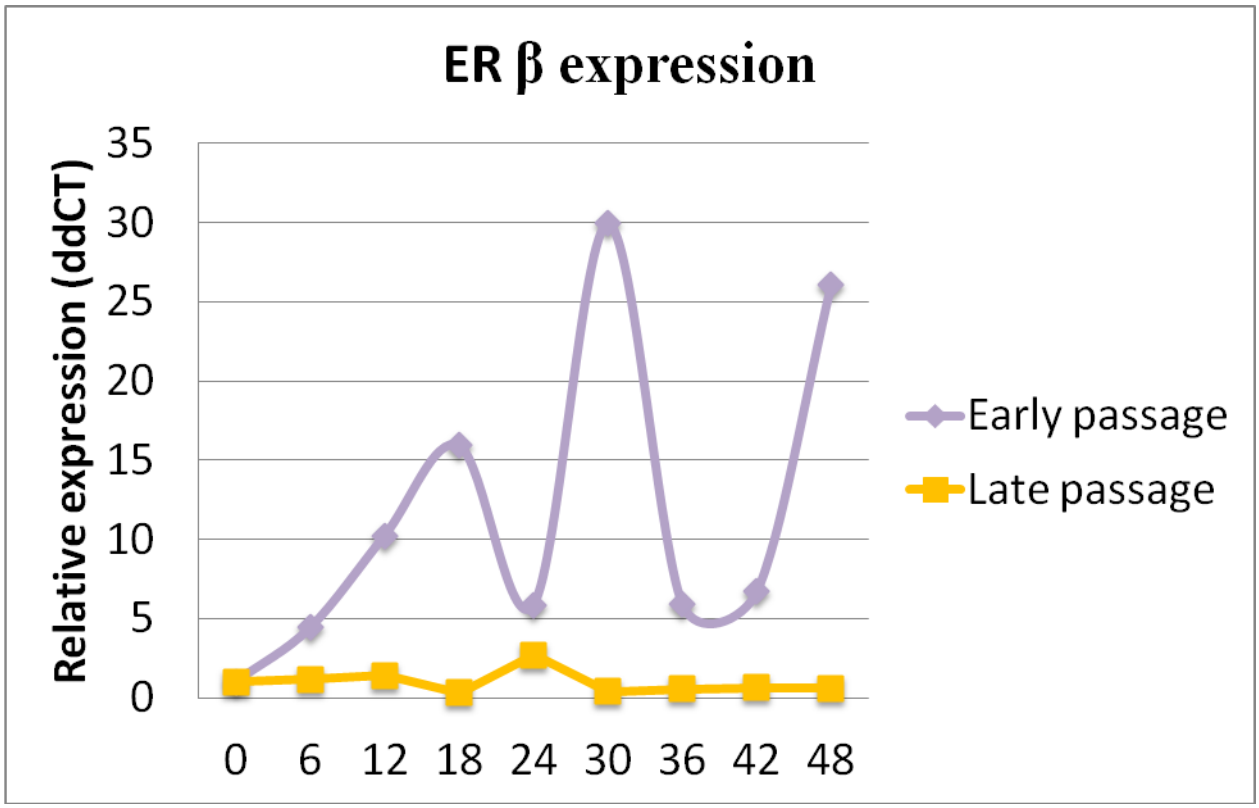

Figure 8: Relative gene expression of ERβ using real-time PCR. Early passage cells are indicated in purple and late passage cells are indicated in yellow. The relative expression was determined using the ddCT method standardizing to the YWHAZ gene.

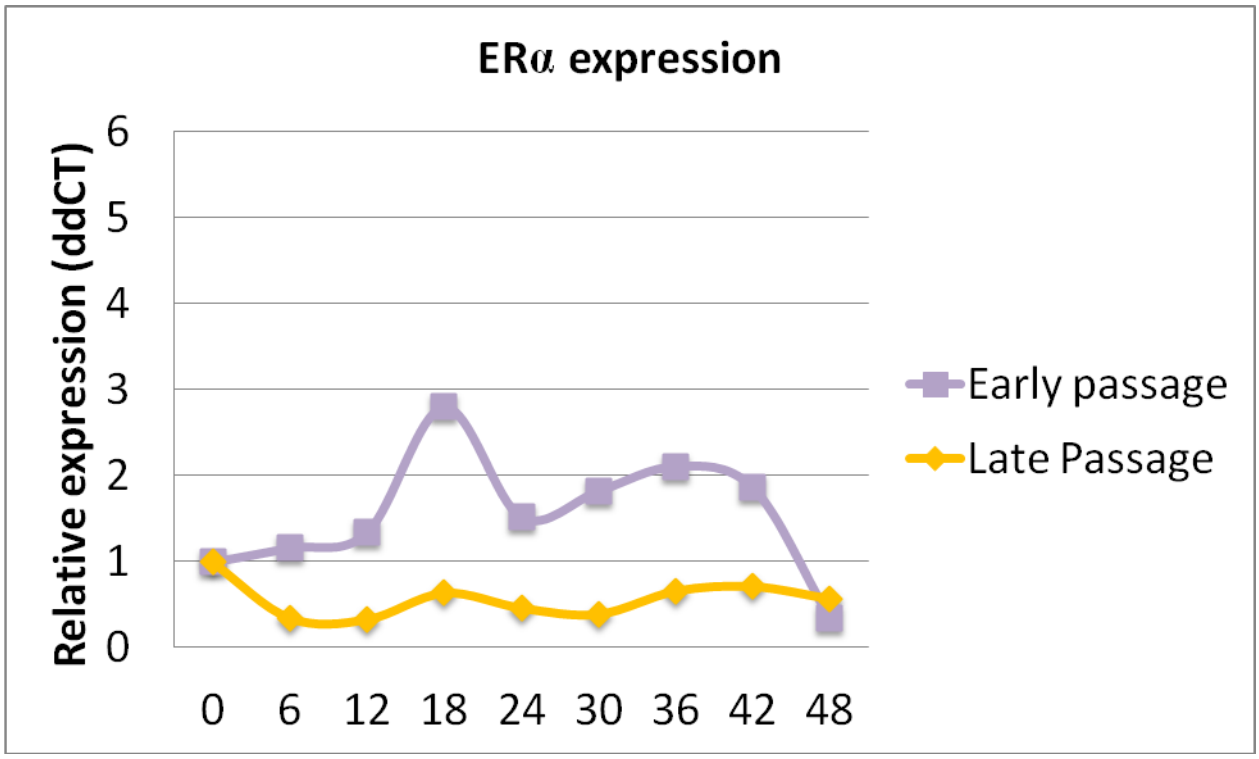

Figure 9 : Relative gene expression of ERα using real-time PCR. Early passage cells are indicated in purple and late passage cells are indicated in yellow. The relative expression was determined using the ddCT method standardizing to the YWHAZ gene.

Loss of expression of circadian genes and estrogen receptors was expected with increased passage of cells. We would expect that as the cells are exposed to the stress of passage that the most aggressive cells would survive. Knowing that circadian rhythm is disrupted in cancer and that estrogen receptor β expression is decreased in more invasive breast cancer and expression of ER α is associated with a more favorable prognosis it would seem that increased passage of CMAMs was consistent with a more aggressive phenotype. We wanted next to determine if the expression truly was lost in CMAMs and if so was there a way to bring back expression and bring back rhythmicity. In order to test these questions we grew up two populations of CMAMs that had been frozen back on different dates by different people. Knowing that SIRT1 is overexpressed in cancer (Wang, Kim et al. 2012) we wanted to use sirtinol, a specific inhibitor of

SIRT1 to see if we could bring back clock gene expression and ERβ expression indicative of a less aggressive cell line. We again harvested cells every 6 hours for 48 hours for the two populations of CMAM A and CMAM B and within each of those populations we had a plate treated with DMSO (control) and a plate treated with 100 nM Sirtinol which was dissolved in DMSO.

Sirtinol appears to jump start expression of *Bmal1* and m*Per2*

In both populations of cells sirtinol treatment induced increases in expression of *Bmal1* at 18 hours particularly evident in the CMAM As and still present but less so in CMAM Bs (Figures 10 and 11). The expression at 18 hours was higher in the sirtinol treated cells (depicted in green) and one of the populations was even higher than the early passage cells (depicted in yellow) shown in Figure 12.

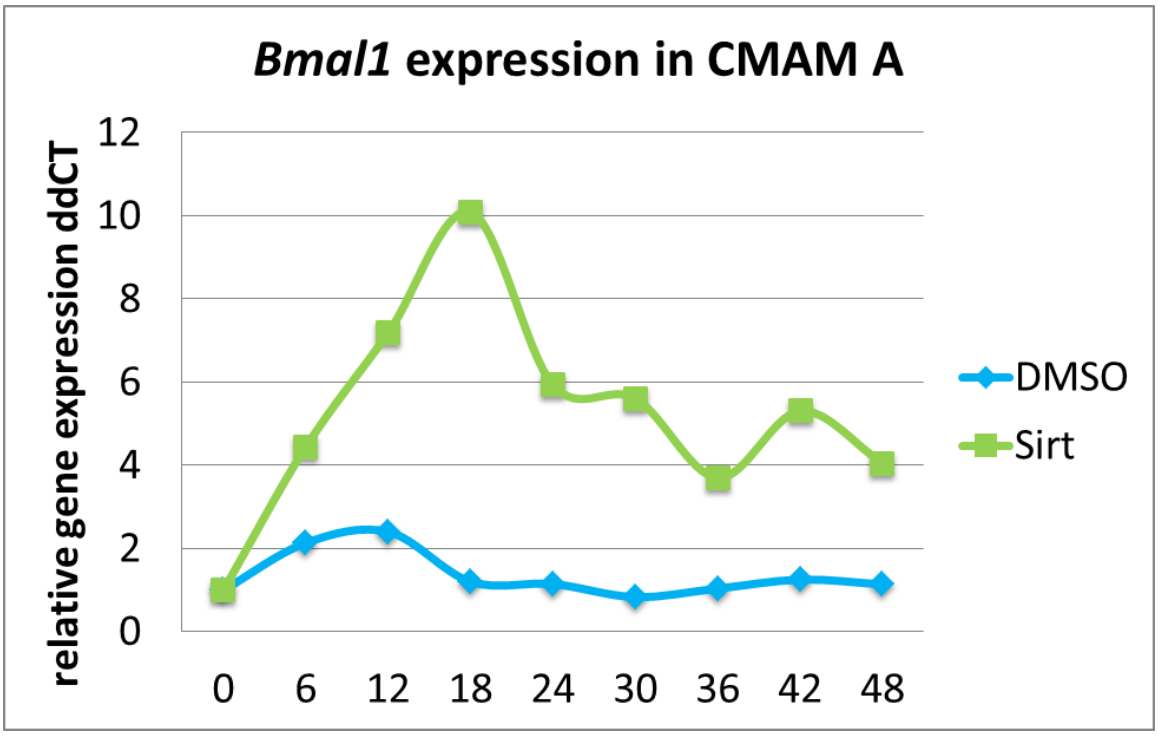

Figure 10: Relative expression of *Bmal1* in CMAM A pass 4. Control cells were treated with DMSO, labeled in blue; treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

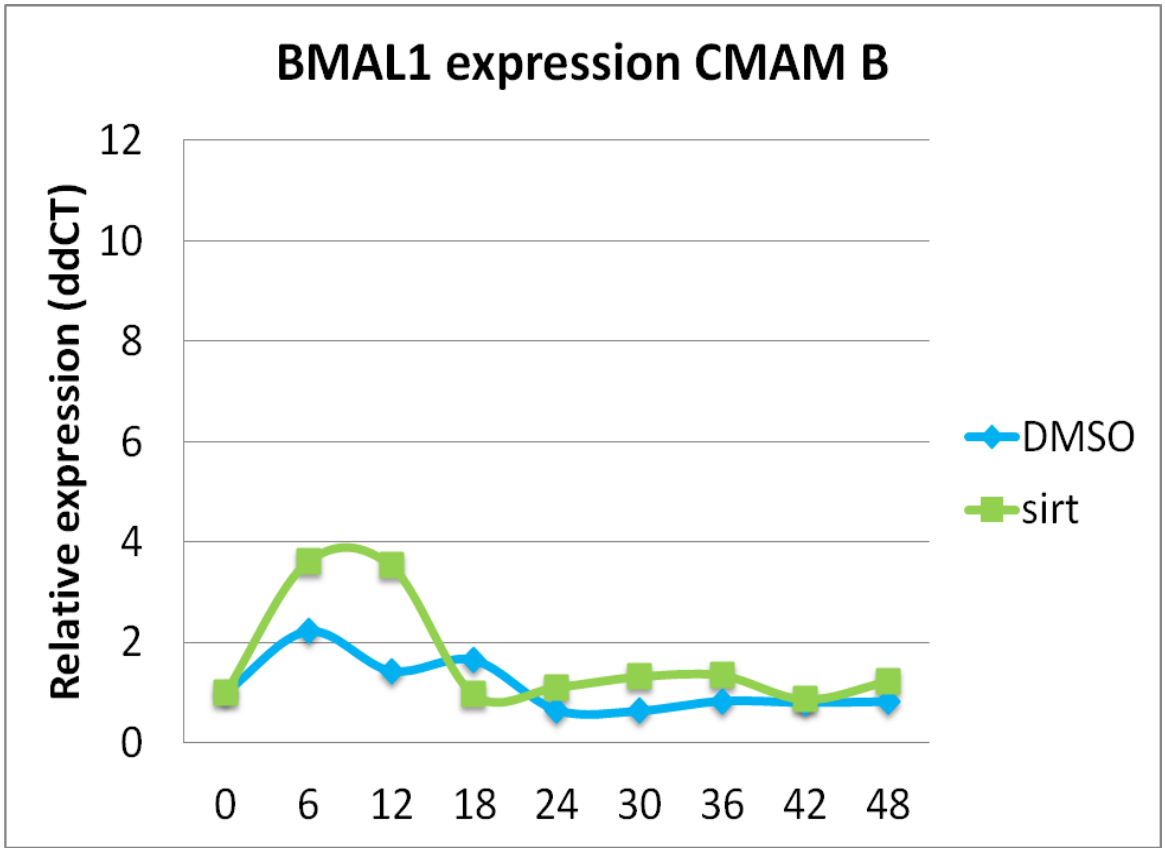

Figure 11: Relative expression of *Bmal1* in CMAM B pass 4. Control cells were treated with DMSO, labeled in blue; treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

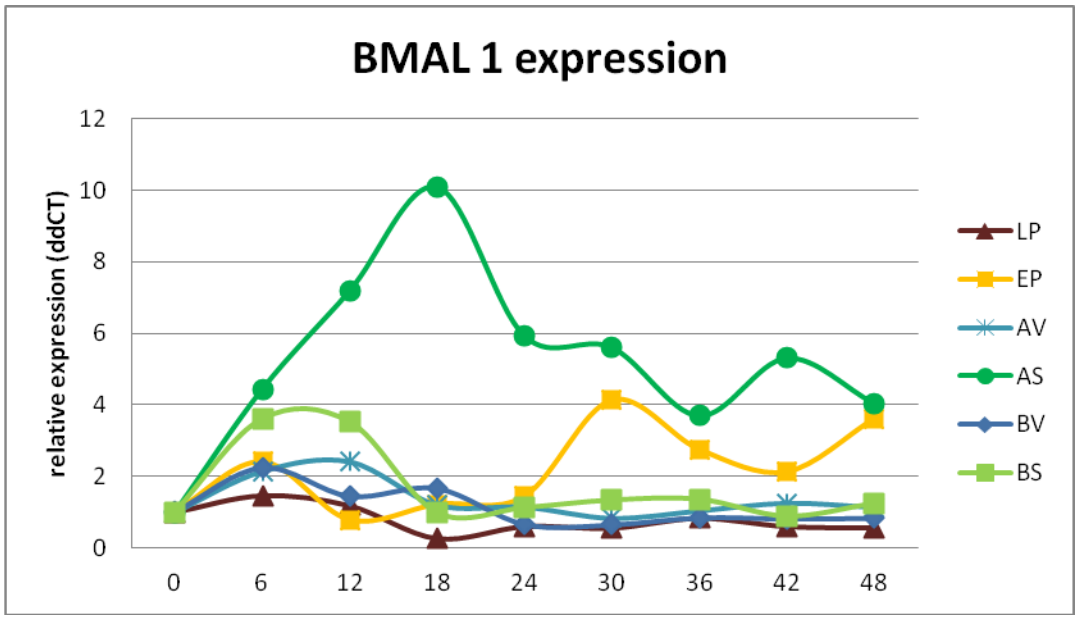

Figure 12: Relative expression *Bmal1* across all the experiments. LP- late passage; EP - early passage; pass 4 CMAM A = AV - CMAM A DMSO treatment (vector) ; AS - CMAM A sirtinol treatment; BV - CMAM B DMSO treatment; BS - CMAM B sirtinol treatment

Similar to *Bmal1*, m*Per2* expression was also jump started with sirtinol treatment with a peak at 18 hours (Figures 13 and 14). There was also a smaller peak at 42 hours. In both populations of cells the peak at 18 hours in the sirtinol treated cells was higher than expression in the early passage cells (Figure 15).

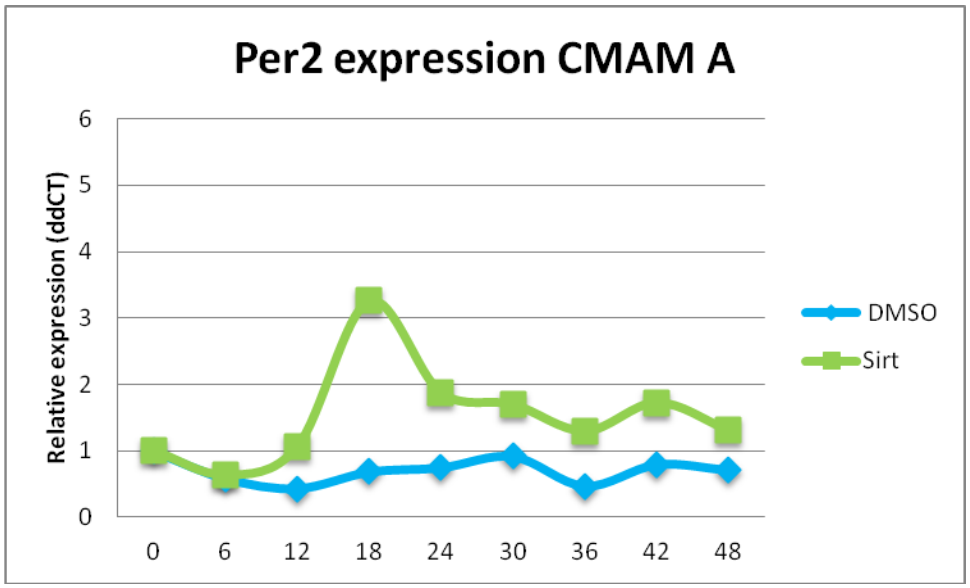

Figure 13: Relative expression of *Per2* in CMAM A pass 4. Control cells were treated with DMSO, labeled in blue, Treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.
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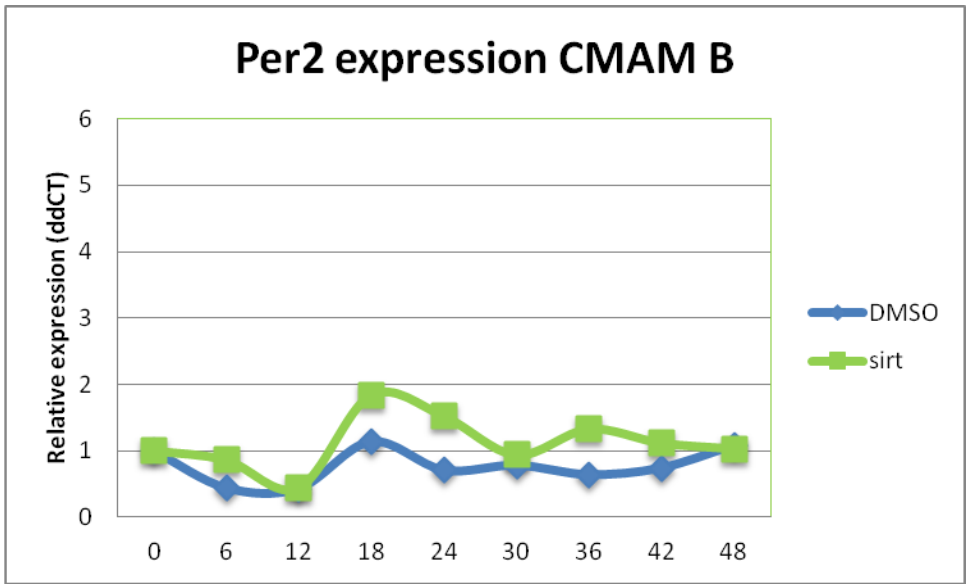

Figure 14: Relative expression of *Per2* in CMAM B pass 4. Control cells were treated with DMSO, labeled in blue, Treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

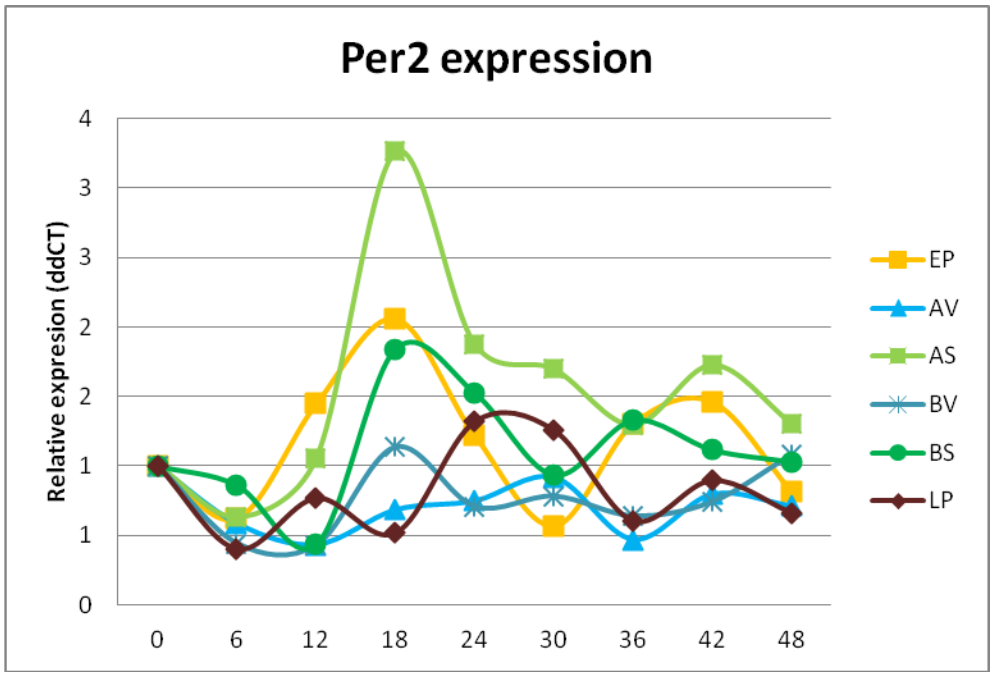

Figure 15: Relative expression of *Per2* across all experiments. LP- late passage; EP - early passage; CMAM A pass 4 : AV - CMAM A DMSO treatment (vector) ; AS - CMAM A sirtinol treatment; BV - CMAM B DMSO treatment; BS - CMAM B sirtinol treatment

ERβ expression showed circadian rhythm with sirtinol treatment

Most striking was ERβ expression in the sirtinol treated cells of the CMAM A population, which showed circadian rhythm with a peak at 18 and twenty-four hours later at 42 hours (Figure 16). In the CMAM B population there was no appreciable difference between the DMSO and sirtinol treated cells (Figure 17). Overall the expression of ERβ was relatively small regardless of treatment or population compared to the early passage cells (Figure 17).

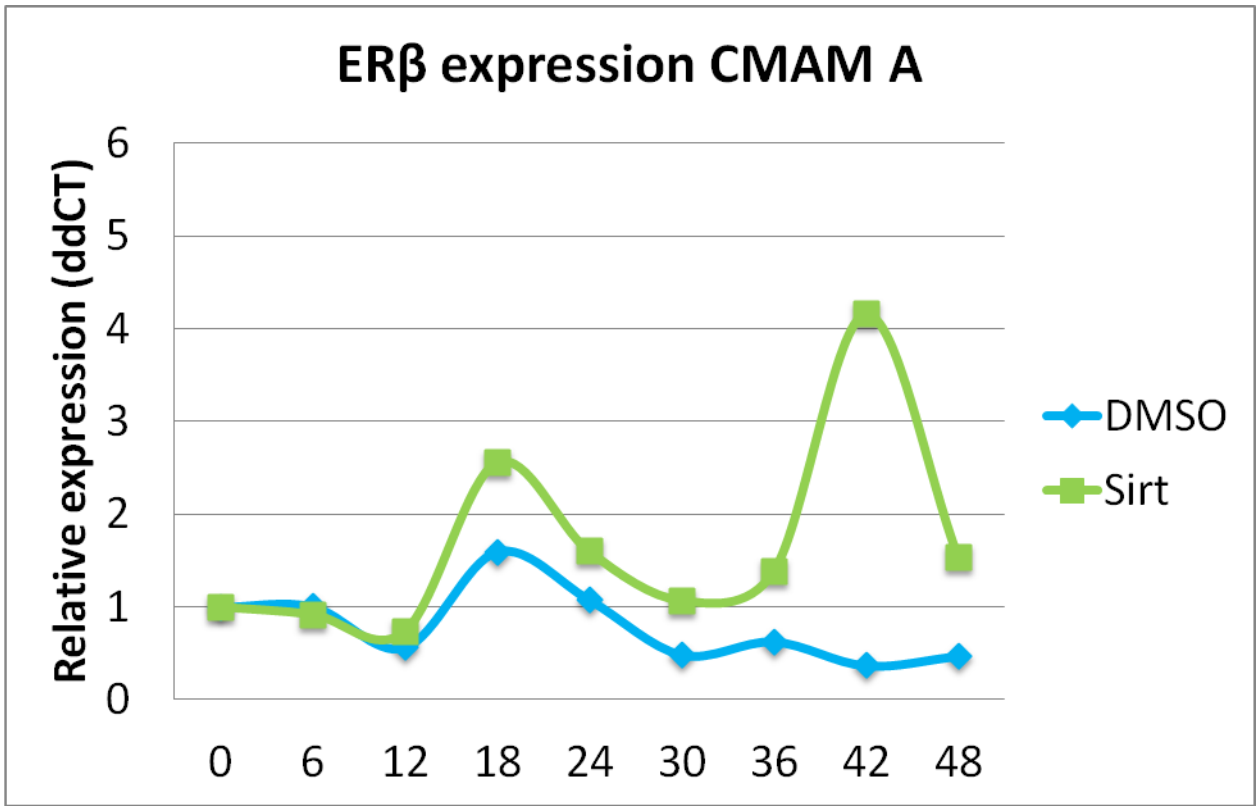

Figure 16: Relative expression of ERβ in CMAM A pass 4. Control cells were treated with DMSO, labeled in blue, Treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

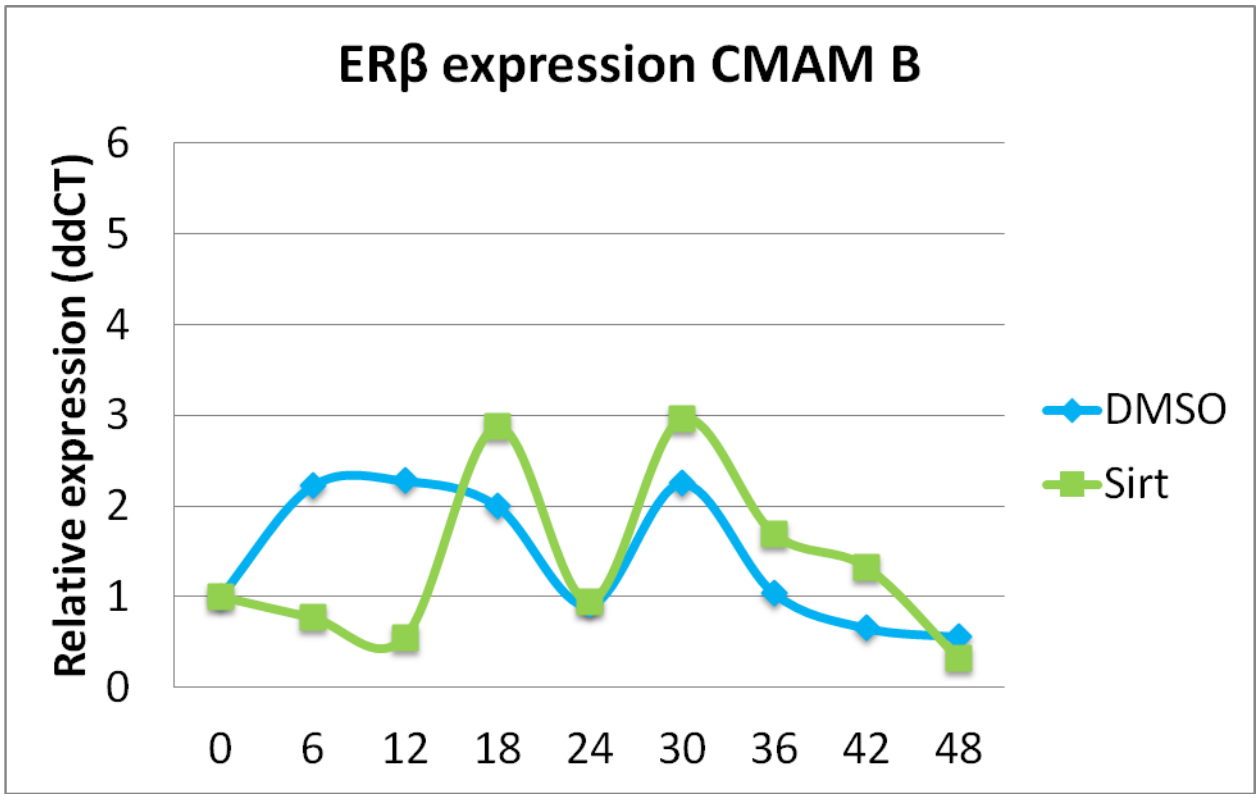

Figure 17: Relative expression of ERβ in CMAM B pass 4. Control cells were treated with DMSO, labeled in blue, Treated cells were treated with 100 nM sirtinol (Sirt), labeled in green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

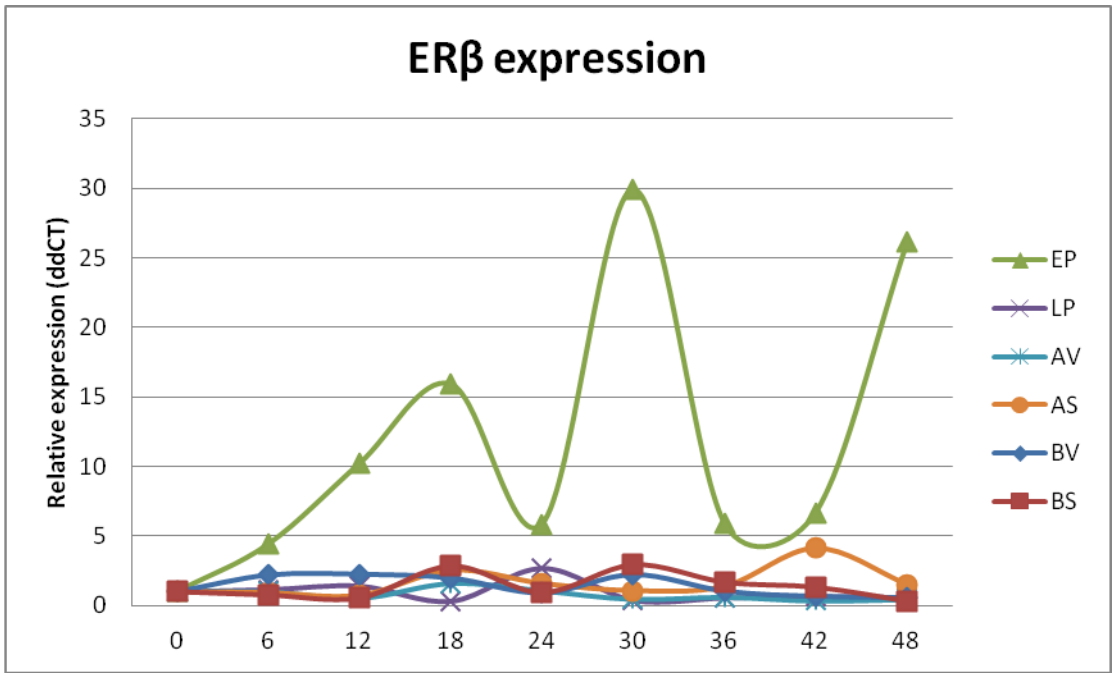

Figure 18: Relative expression of ERβ across all the experiments. LP- late passage; EP - early passage; CMAM A pass 4 AV - CMAM A DMSO treatment (vector) ; AS - CMAM A sirtinol treatment; BV - CMAM B DMSO treatment; BS - CMAM B sirtinol treatment

The expression of ERα was not significantly different between sirtinol and DMSO treated cells and all populations examined. There was never a circadian rhythm and expression was relatively low across the board with respect to ERα expression (Figure 19).

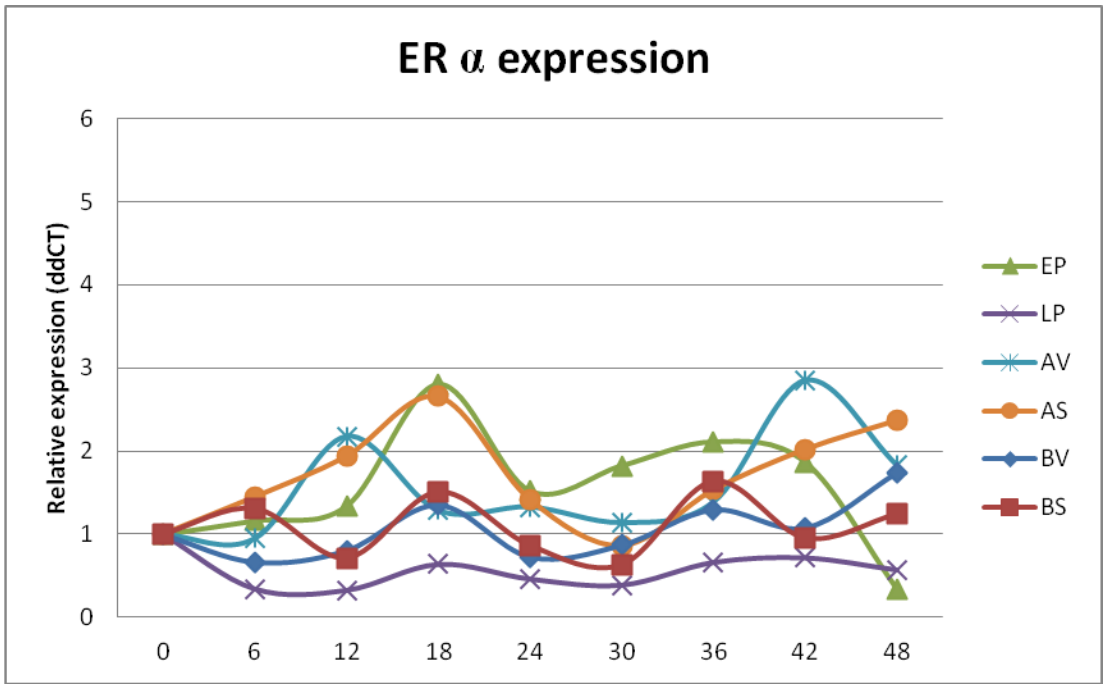

Figure 19: Relative expression of ERα across all the experiments. LP- late passage; EP - early passage; CMAM A pass 4 - AV - CMAM A DMSO treatment (vector) ; AS - CMAM A sirtinol treatment; BV - CMAM B DMSO treatment; BS - CMAM B sirtinol treatment

The above described experiments were performed on the fourth passage of CMAM A and B that were previously frozen. We maintained a plate of each after harvesting for collection and analysis and passed the cells until the tenth passage and then performed the same experiments with harvesting every 6 hours for 48 hours and also performed MMT on the cells at both the forth and tenth passage. In the pass 10 cells we saw a minimal difference between sirtinol treated and DMSO treated cells. We saw very similar results in the expression of *Bmal1* in the pass 10 cells as the pass 4 cells with respect to each population (Figures 20 and 21).

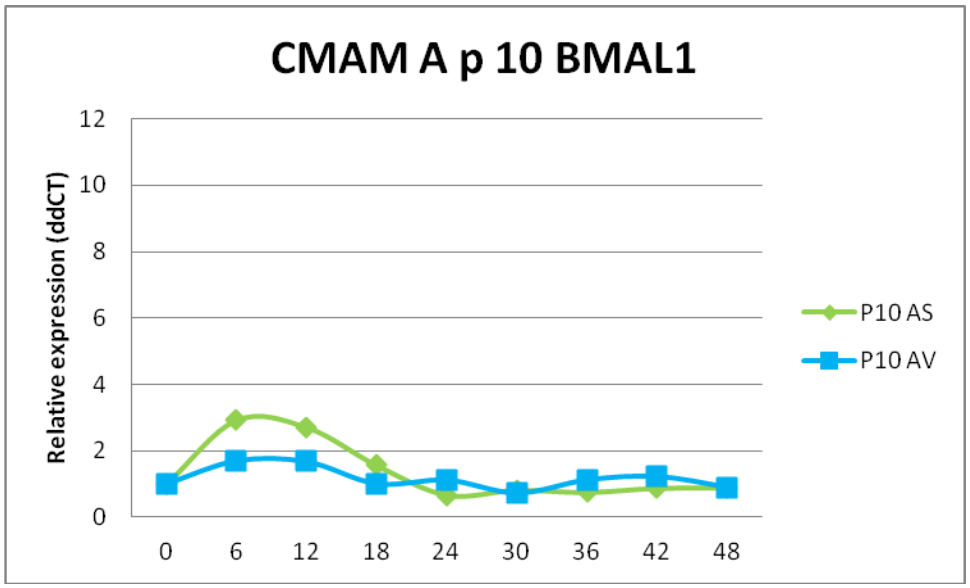

Figure 20: Relative expression of *Bmal1* in CMAM A pass 10. Control (P10 AV (DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 AS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

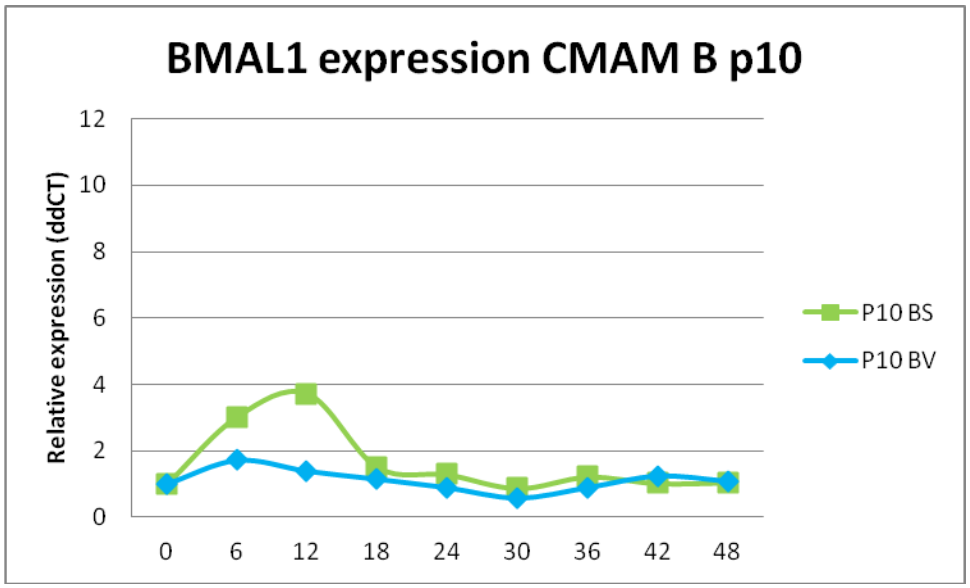

Figure 21: Relative expression of *Bmal1* in CMAM B pass 10. Control (P10 BV (DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 BS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

There were similar findings to *Bmal1* expression with the relative expression of m*Per2, ER β,* and ER α, the effect of sirtinol treatment was markedly dampened or null in cells that had been passed ten times. m*Per2* expression was low in both populations of CMAMs when the cells were analyzed at the tenth passage and sirtinol treatment did not affect expression (Figures 22 and 23).

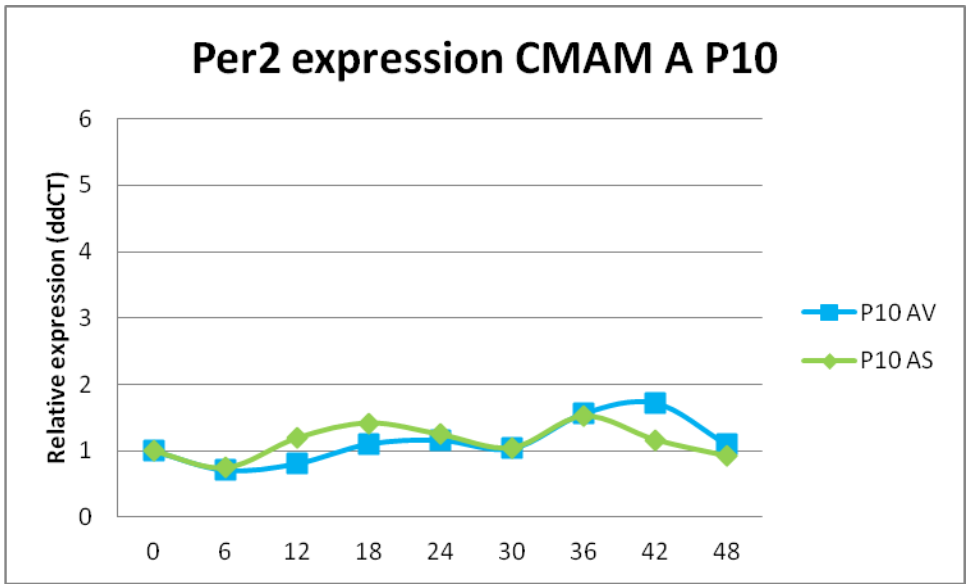

Figure 22: Relative expression of *Per2* in CMAM A pass 10. Control (P10 AV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 AS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

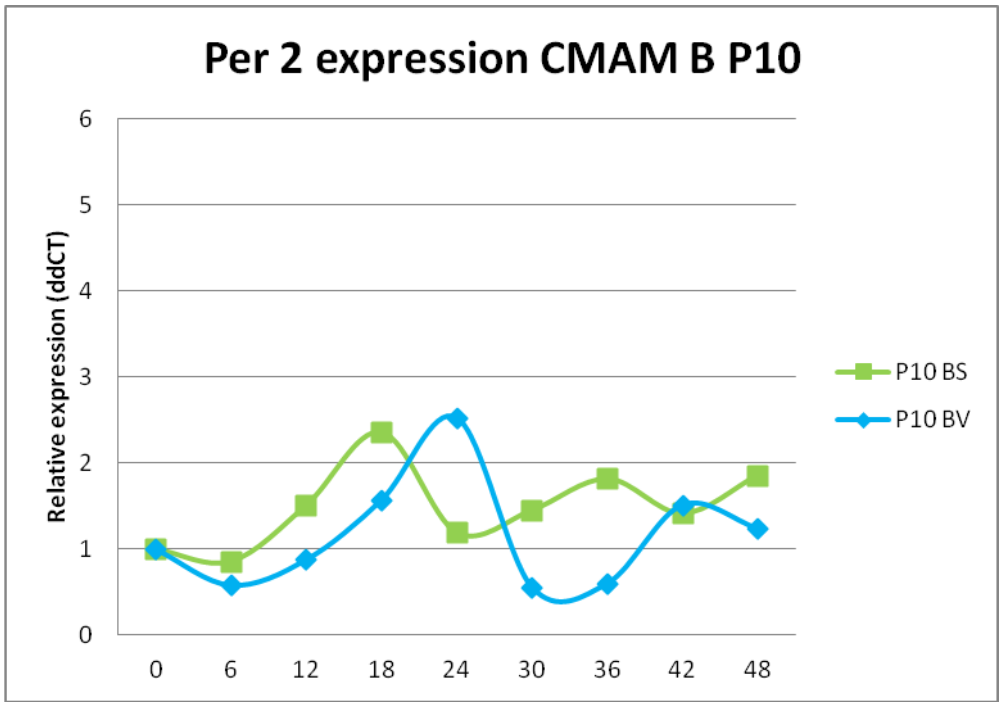

Figure 23: Relative expression of *Per2* in CMAM B pass 10. Control (P10 BV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 BS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

ERβ expression in CMAM A did appear to have circadian rhythm in the sirtinol treated cells, again with peaks at 18 and 42 hours very similar to the cells that had been passed 4 times but there was also a peak in the untreated cells at 12 hours that exceeded either of the sirtinol treated cell peaks (Figure 24). Similar to the pass 4 CMAM B cells the pass 10 cells did not show circadian rhythm and sirtinol did not appear to have an affect (Figure 25).

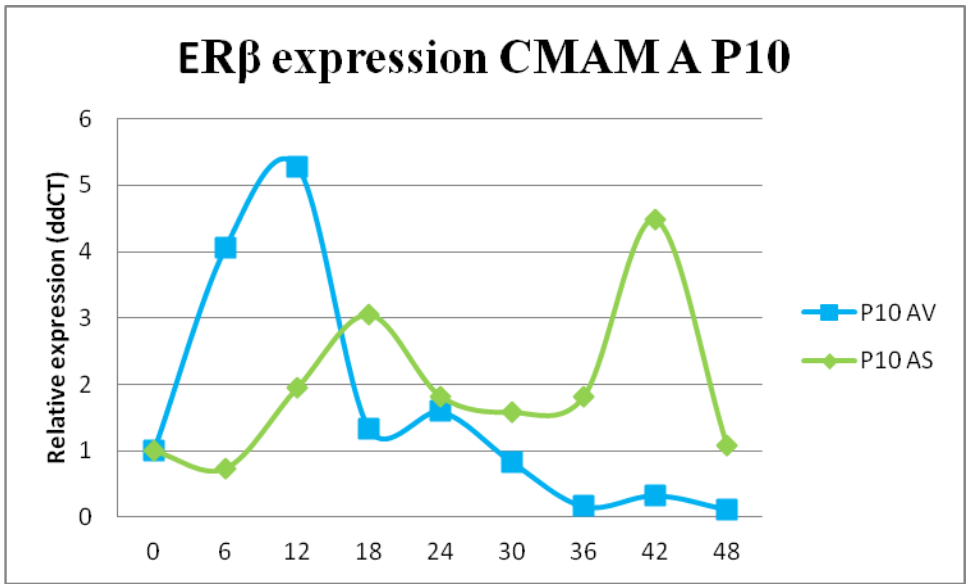

Figure 24: Relative expression of ERβ in CMAM A pass 10. Control (P10 AV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 AS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

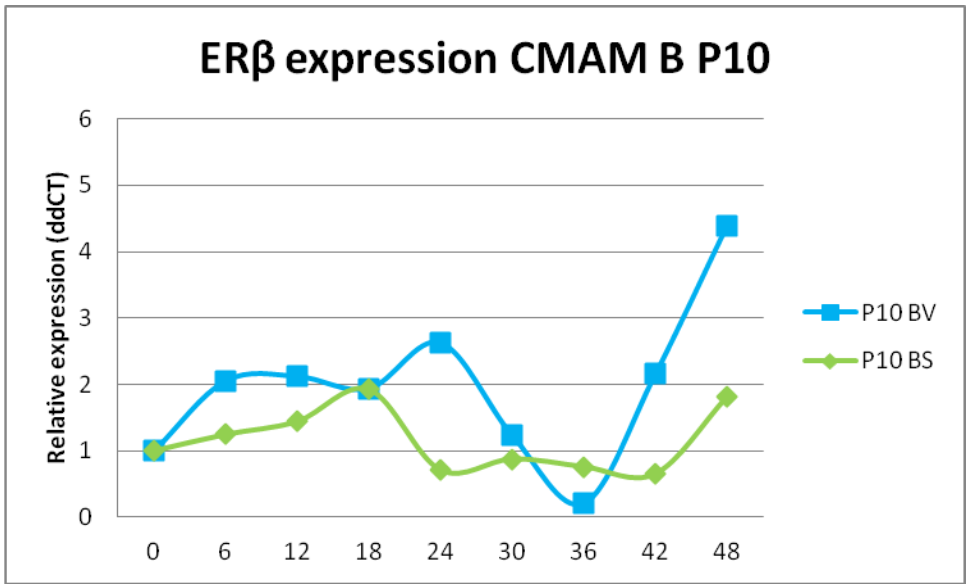

Figure 25: Relative expression of ERβ in CMAM B pass 10. Control (P10 BV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 BS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

Similar to pass 4 cells of both populations, ERα expression was low and not affected by sirtinol in the pass 10 cells (Figure 26 and 27).

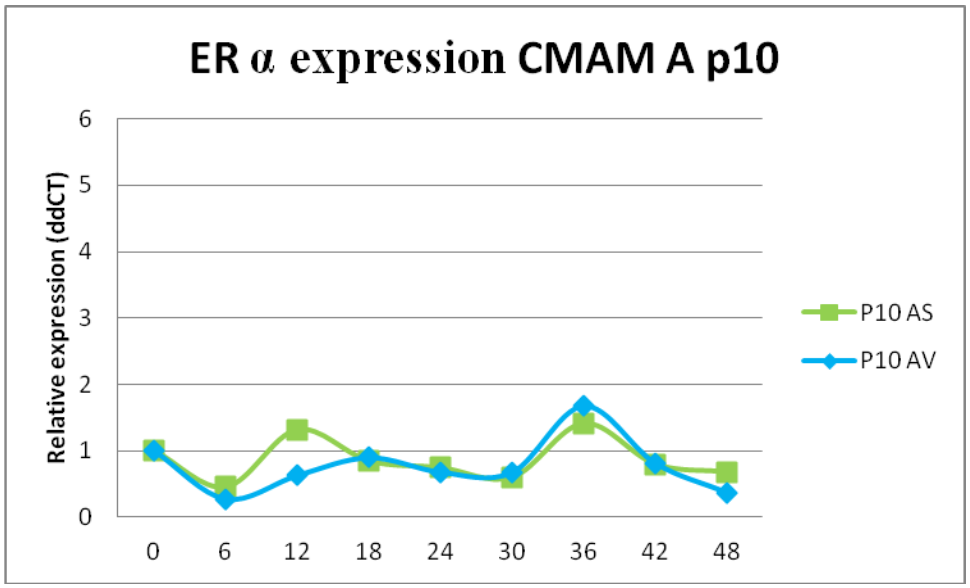

Figure 26: Relative expression of ERα in CMAM A pass 10. Control (P10 AV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 AS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

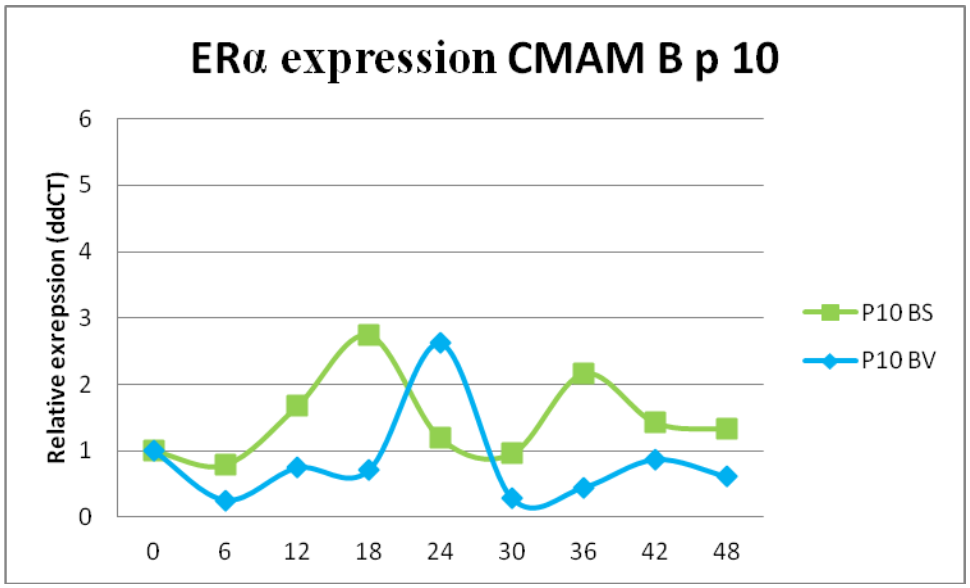

Figure 27: Relative expression of ERα in CMAM B pass 10. Control (P10 BV)(DMSO), labeled blue; cells treated with 100 nM sirtinol (P10 BS) labeled green. The relative expression was determined using ddCT method standardizing to the YWHAZ gene.

We not only wanted to know if sirtinol treatment affected rhythmicity and expression but we also wanted to determine if sirtinol treatment had any affect on the cell viability. In order to

determine cell viability we used the MTT assay as described in the material and methods. In conjunction with cell harvesting experiments we also grew cells on a 96 well plate over a two day period and treated them with DMSO (control), 100 µM Estrogen (E2), 1 µM E2, 0.25 µM, 0.5 µM, and 1.0 µM valproic acid (VPA), and 100 nM sirtinol and the absorbance was determined. MMT assays were not performed on early and late passage cells. In CMAM A and CMAM B we did see a decrease in cell viability in cells treated with Sirtinol (Figure 28). The p-value was significant ($p < 0.05$) in CMAM A pass 10 and CMAM B pass 4 and pass 10. There was no significant difference in CMAM A pass 4 treated with 100 nM sirtinol but there was a wide range for the control (DMSO) treated group. In the CMAM A pass 10 group there was also a significant ($p < 0.05$) for the cells treated with 1 µM E2, 0.5 µM VPA, and 100 nM sirtinol. It is not clear why there was significant difference in this cell population and no others when treated with E2, and VPA. It is also not clear why the low dose of E2 and moderate dose of VPA would show a difference without a difference in higher concentrations. These results are suspicious and repeat or reduplication of the data would need to be performed to validate the results.

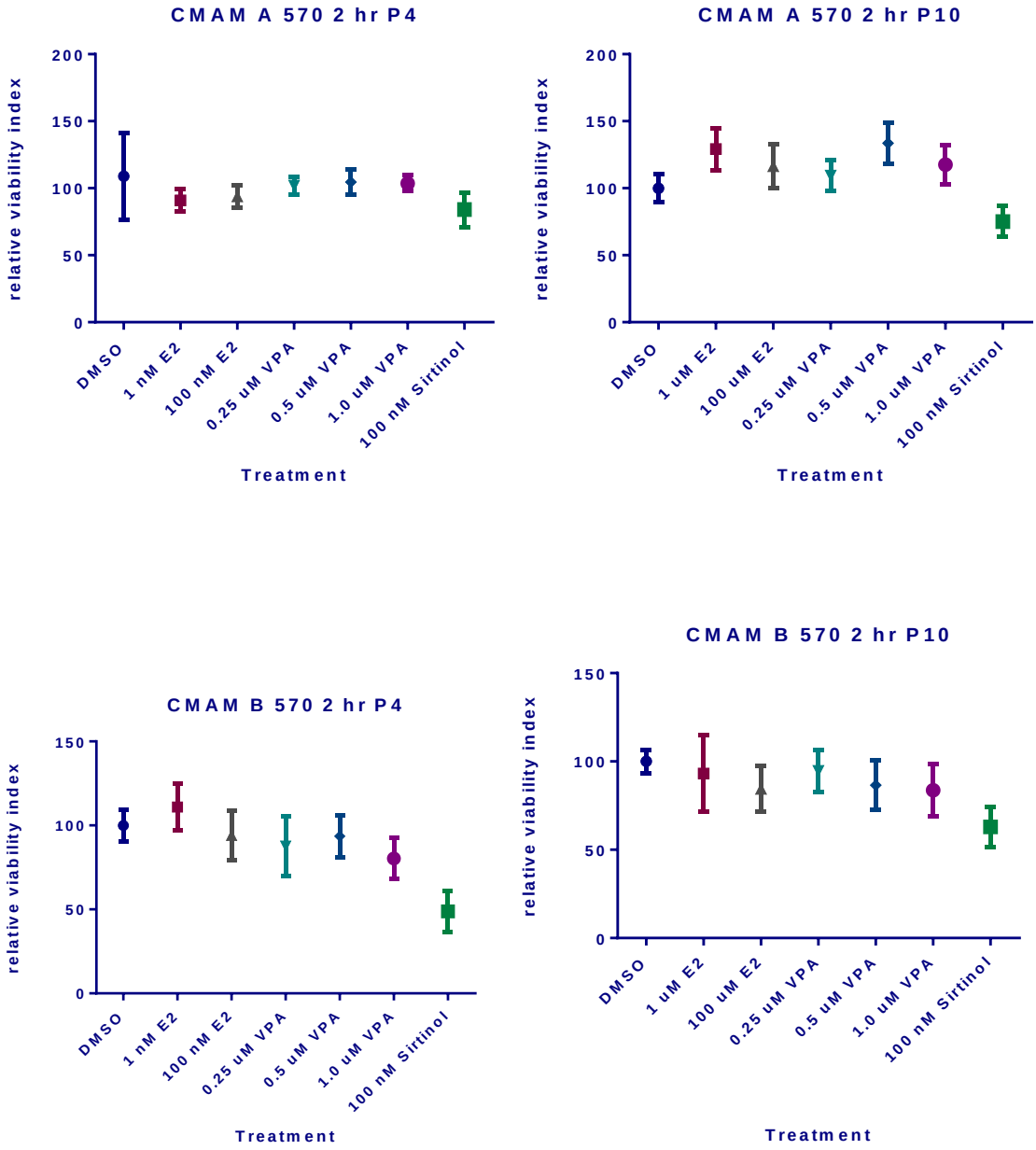

Figure 28: MTT assay. MTT assay for CMAM A and B pass 4 (P4) and pass 10 (P10). Treatments: DMSO (control), E2 – Estrogen; VPA – Valproic Acid; Sirtinol

After identifying that cells viability further investigation was performed to determine if decreased viability was due to apoptosis verses cellular arrest. Using the TiterTACS™ colorimetric apoptosis detection kit, described in the materials and methods, to identify DNA fragmentation of cells undergoing apoptosis we compared several populations of control (DMSO

treated) and sirtinol treated CMAMs (Figure 29).

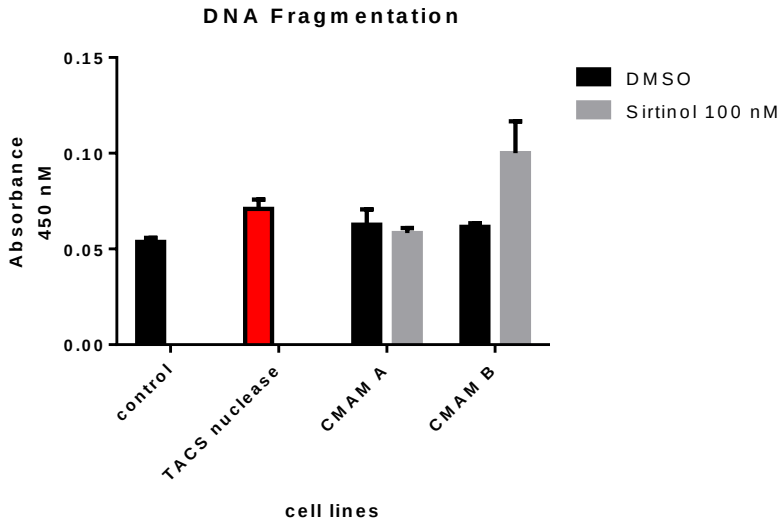

Figure 29: DNA fragmentation, the absorbance at 450 nM; control – negative control, background absorbance; TACS nuclease –positive control. CMAM A and CMAM B were passed 3 times after cryopreservation.

There was a significant increase absorbance indicative of DNA fragmentation and apoptosis in the CMAM B between the sirtinol (100 nM) treated and DMSO treated groups ($p < 0.05$). There was no significant difference in the CMAM A population of cells.

Chapter 4:

Discussion

Breast cancer and mammary cancer are the most common cancers in women and female dogs and therole of estrogen in tumorigenesis and alteration of estrogen receptor expression inbreast cancer and mammary cancer has been demonstrated previously(Anderson 2002; Martin de las Mulas, Ordas et al. 2004; Platet, Cathiard et al. 2004; Hartman, Strom et al. 2009). Disruption in the circadian clock has also been linked to cancer and increased risk of breast cancer and due to circadian clock responsiveness to endocrine cues, breast cancer is particularly susceptible to circadian disruption (Davis and Mirick 2006; Hansen 2006; Rossetti, Esposito et al. 2012).

To the author's knowledge this is the first study to look at the relative gene expression of clock genes in a canine mammary cancer cell line. Using real-time quantitative PCR we identified expression of *Bmal1, Per2, ERα,* and *ERβ* in a canine mammary cancer cell line, finding that expression of these genes was lost or dramatically decreased with later passage cells. This was not unexpected, since increased cell passage often allows only the most aggressive cells to survive. More aggressive cancer cells would be expected to have loss of circadian rhythm as the circadian genes are often disrupted in cancer. The initial relatively high oscillating expression of *ERβ* was somewhat unexpected. The oscillation was not circadian, which again would not be unexpected because in cancer rhythmic disruption has been identified previously. In this study, CMAM cells from early passage showed *Bmal1* and *Per2* rhythms were both circadian and these genes were expressed in antiphase as they are found normally. Expression and normal circadian rhythm of *Bmal1* and *mPer2* were lost with late passage cells. *ERα* was relatively low and not circadian in early passage cells, and later passage cells showed even decreased expression.

Knowing that disruption in circadian rhythm and disrupted expression of estrogen receptors have both been identified in breast cancer, we wanted to see if we could bring back expression of ERβ and the clock genes. SIRT1 functions to counteract CLOCK activity by functioning as a histone deacetylase (Belden and Dunlap 2008). CLOCK/BMAL1 and SIRT1 colocalize forming a chromatin-associated regulatory complex acting on promoters of clock-controlled genes (Nakahata, Kaluzova et al. 2008) By treating the cells with sirtinol, a SIRT1 specific inhibitor, we were hoping to bring back the circadian clock expression and rhythmicity. When the cells were treated with sirtinol there was a difference from the control cells treated with DMSO only. Sirtinol treatment caused increased expression of *Bmal1* and m*Per2,* with a peak at 18 hours. The increased expression was interesting but the expression was not a circadian rhythm and caused both clock genes to peak at 18 hours when normally these genes would be antiphasic to each other.

The most interesting finding was the effect of sirtinol treatment on expression of ERβ. ERβ expression showed circadian rhythm with a peak at 18 hours, and a larger peak at 42 hours. The exact mechanism of this is not known. It is possible that the jumpstart of the circadian genes led to rhythmic expression of ERβ, but since the rhythmicity was still not normal or circadian in *Bmal1* and m*Per2*, this does not seem to be the only contribution. Work is being continued to tease out the relationship between circadian clock gene expression and ER β rhythmicity in mammary cancer in the Chappell lab.

Sirtinol did appear to have an effect on CLOCK activity based on *Bmal1* expression due to the blockage of SIRT1. The effect appeared to be short-lived and did not return clock expression back to normal rhythmicity. The mechanism of loss of circadian expression of *Bmal1* and m*Per2* in the late passage cells is unknown and is likely multifactorial. SIRT1 counteractivates CLOCK activity and may play a role in loss of circadian expression in mammary cancer, but based on evidence from our studies, there appear to be other unknown mechanisms

involved. Unfortunately it has proved difficult to design primers for the canine *Clock* gene, but it would have been interesting to measure *Clock* to see the effect of sirtinol treatment on expression.

We also wanted to determine if sirtinol treatment not only affected rhythmicity but there was an affect in cell survival. Using the MTT assay we assessed relative cell viability with various treatment groups. Most consistent and significant was treatment with 100 nM of sirtinol, which caused significant decrease in cell viability in CMAM B in both the pass 4 and pass 10 cells, and in CMAM A in the pass 10 cells. There was a wide range for the control (DMSO) treated cells in the CMAM A pass 4 cells which is probably why there was not a significant P value($p = 0.08$). Decrease in cell viability with Sirtinol treatment lends to involvement of SIRT1 in tumorigenesis. By counteracting CLOCK and keeping clock-regulated promoter regions hyperacetylated, SIRT1 may be involved in the resulting circadian rhythm disruption, although other factors are also likely involved. The decrease in cell viability in sirtinol treated cells may have involved mechanisms other that just blocking counteraction of the clock.

SIRT1 has anti-apoptotic properties and is considered to be involved in cell survival in situations of oxidative stress and with DNA damage (Kojima, Ohhashi et al. 2008). The results from the apoptosis assay, identifying DNA fragmentation, appeared to be consistent with the MTT assay and indicated that the decrease in cell viability in cells treated with sirtinol was due to apoptosis. There was no significant difference in the CMAM A population treated with sirtinol but there was also no difference in cell viability in the MTT assay in the CMAM A pass 4 cell population when treated with sirtinol. There was a significant difference ($p < 0.05$) in the CMAM B population when treated with sirtinol compared to control (DMSO) accounting for the decrease in cell viability and consistent with the anti-apoptotic properties identified with SIRT1.

Sirtinol treatment did not really affect ERα expression and expression remained relatively low. In ERα positive human mammary epithelial cells, HME1, ERα expression was found to be circadian and there was accumulation of ERα mRNA preceding peaks of *PER2* mRNA by about

8 hours, suggesting coordination between ERα and m*Per2* (Rossetti, Esposito et al. 2012). We did not see much in the way of ERα expression or rhythmicity. CMAMs likely have different properties than HME1 cells because they are canine in origin and cancer cells; perhaps if there was initially increased expression of ERα in CMAMs we might have seen more differences with sirtinol treatment. Also with the immunohistochemical properties it would suggest that CMAMs are either mesenchymal origin or poorly differentiated myoepithelial cells. If they are mesenchymal in origin their behavior may be very different than epithelial lines. In the human mammary epithelial cell line, MCR10A which is ERα negative, a partial or complete loss of circadian oscillation of *Per2* was identified (Rossetti, Esposito et al. 2012). It is possible that the disruption in *Per2* may have been linked to the lack or low expression of ERα in the CMAMs. Again the histogensis may be responsible for the discrepancy.

We also treated CMAMs with E2 in the MTT assay. There was no significant difference in the high and low (100 μM and 1μM) E2 treatment except in CMAM A p10 cells. This MTT assay showed a significant difference in only the low dose E2 treatment and also showed a significant difference in the moderate dose, 0.5 μM, valproic acid, which was not seen in any of the other experiments. It is possible that there was an error in this plate or that this population of CMAMs developed a phenotype which doesn't respond to sirtinol and with further passages a population developed which was susceptible to sirtinol treatment. Results would need to be confirmed with further experiments and replication. Limitations of the study included that we were not able to confirm gene expression at the protein level. Although gene mRNA was demonstrated in this study that does not directly correlate to the protein. In studies done in the Chappell lab previously looking at clock genes in mouse testis, clock gene mRNA oscillated in the testis but this was not correlated with the protein (Bebas, Goodall et al. 2009). The peak of protein levels does not necessarily occur at the same time as mRNA peaks. For example, rhythmic expression of mPER1 was observed in sections of the epididymis with a protein peak

occurring 6 hours after the peak of mRNA (Bebas, Goodall et al. 2009). Also posttranscriptional modifications to, and degradation of, clock proteins could cause rhythmicity in the absence of mRNA oscillations (Bebas, Goodall et al. 2009).

There are several types of canine mammary cancer which stem from both mesenchymal and epithelial origins. The original mass was diagnosed as a carcinoma but there were some areas with mesenchymal proliferation. The immunohistochemical results suggest that CMAMs are of mesenchymal origin by positive staining of vimentin and negative staining of cytokeratins. It is possible that CMAMs were of mesenchymal origin rather than epithelial origin, or both, as in the case from mixed tumors which is common in the dog. Transformed epithelia stem cells may also fail to differentiate normally and may show characteristics of mesenchymal cells rather than actually differentiate into mesenchymal cells (Klymkowsky and Savagner 2009). It is also possible that CMAMs were epithelial originally and went through epithelial-mesenchymal transformation (EMT). During invasion and metastasis, EMT is a process in which epithelial cells lose cell-cell adhesion structures and cytoskeletal systems change from expression of keratin to vimentin-type intermediate filaments gaining the ability to become motile (Klymkowsky and Savagner 2009). Malignant canine cutaneous epithelial tumors have previously been reported to show EMT characterized by loss of cytokeratin and variable vimentin expression(Bongiovanni, D'Andrea et al. 2013). Although, in this study, generally the loss or reduction of cytokeratin immunostaining appeared to occur more often than development of vimentin expression(Bongiovanni, D'Andrea et al. 2013).

Most human breast tumors show features of luminal epithelial cells (Anderson 2002). Regardless CMAMs were identified to express the clock genes and ERβ for which we were trying to investigate their relationship and tumorigenesis. ERα has been shown to be expressed in about 15-30% of luminal epithelial cells and no other types of cells within human breast tissue, whereas ERβ is expressed in most luminal epithelial cells as well as myoepithlial cells, fibroblasts and

other stromal cells in breast tissue (Anderson 2002). It also would have been ideal to have a normal canine mammary cell line to compare expression levels to. We did try to isolate and culture normal canine mammary cells but there was no way to validate this cell line and the growth rate and sustainability was different than CMAMs.

There are several aspects of circadian clock genes and estrogen receptor that we did not examine and which may play a role or help to decipher the relationship in rhythm disruption and tumorigenesis. These include the effect of the surrounding tissue environment on breast cancer development, other hormones and nuclear receptor involved, epigenetic modifications, cell cycle genes, other clock genes, and melatonin. In clock gene expression studies in mice, Leydig cells specifically demonstrated a rhythm of BMAL1 protein (Bebas, Goodall et al. 2009). Similar to the testes, mammary tissue has both interstitial cells and tubular epithelial cells which may provide different and specific signals. In our studies we had ideally isolated only one cell type and the effect of the entire tissue environment and signals from various cells types were not evaluated. It is likely that other cell types and their relationships are also involved in tumorigenesis and cancer progression and may also regulate circadian rhythm.

There is clearly an effect of estrogen on mammary tissue, but other reproductive hormones play a role in mammary gland development and creating fertile microenvironment for uncontrolled growth. In hormone-deprived animals, treatment with 17-β-estradiol, progesterone, and prolactin in conjunction with cortisol and growth hormone, allows for mammary gland development (Brisken and O'Malley 2010). Any one of these hormones may play a role in developmental dysplasia and subsequent carcinogenesis or progression of malignancy. Progesterone receptor signaling is required for side branching and alveologenesis (Brisken and O'Malley 2010) . Prolactin signaling is necessary for alveologenesis and differentiation of milk producing cells during pregnancy (Brisken and O'Malley 2010). Aberrant expression of either of these hormones may be involved in tumorigenesis. What is more likely is that there is interplay

between multiple hormones and the circadian clock which may lead to disruption and uncontrolled growth and progression to cancer. In normal mammary gland development, prolactin is limiting in alveologenesis but synergism with estrogens and progesterone may be involved in ductal outgrowth and side branching or prolactin and progesterone induction of ER may enhance ductal growth(Brisken and O'Malley 2010). Similarly aberrant expression of prolactin may act with progesterone leading to a decrease in ERβ expression with loss of tumor suppression. Identifying and manipulation of the hormones involved in mammary development may be necessary to understanding perturbations ER expression and clock regulation, as well as tumorigenesis and prevention.

There are numerous and clear relationships between circadian rhythms and reproductive hormones. *Clock* mutant mice that express dominant negative *Clock* gene are subfertile and show lengthened estrous cycles (Chappell, White et al. 2003). Also overexpression of the mutant CLOCK-Δ19 protein in GT1-7 cells (mouse cell line model for GnRH secretion) disrupts the secretion pattern causing a significantly decreased mean pulse frequency (Chappell, White et al. 2003). *Bmal1* knockouts are infertile, linked partially to steroid hormone production (Boden and Kennaway 2006; Tonsfeldt and Chappell 2012). It was demonstrated in the Chappell lab that GT1-7 cells exhibited rhythms of *Kiss1R* (kisspeptin, expressed in neurons involved in signal for GnRH secretion) that were potentiated by E2. Similarly, GT1-7 subclones overexpressing CLOCK-Δ19 showed attenuation E2-induced oscillations in *Kiss1R* expression (Tonsfeldt and Chappell 2012). Treatment of CMAMs with E2 may affect the oscillations of ER α/β as well as clock gene expression. Other hormones, including LH and FSH, might also play a role since they stimulate E2 secretion and looking at other hormones upstream may also enlighten pathways in circadian disruption.

In humans, multiple ERβ isoforms exist, resulting from alternative splicing of exon 8, the last coding exon (Leung, Mak et al. 2006). The qPCR designed in our study corresponded to an early site of the sequence and would not account for isoform differences found at the end of the sequence. There have been differences identified in the ERβ isoform expression in normal mammary tissue compared to human breast cancer cell lines. In one study in humans, looking at two different promoters of the ERβ gene, differences in methylation were identified at the two promoters in normal mammary epithelium and breast cancer cell lines (Zhao, Lam et al. 2003). The methylation status of the 0N promoter region correlated significantly with the level of ERβ mRNA in normal breast epithelial cells culture and ERβ mRNA levels were significantly lower in breast cancer cell lines (Zhao, Lam et al. 2003). They also found that treatment of four breast cancer cell lines with a demethylating agent reactivated the ERβ mRNA of one isoform and was unable to reactivate the other (0N-1 and 0K-1 respectively) (Zhao, Lam et al. 2003). It would be interesting to determine if there was differential expression of ERβ isoform and if treatment with a demethylation agent, such as deoxycytidine (DNA methytransferase inhibitor), restored rhythmicity or had an effect on cell viability.

Progesterone is another hormone important in mammary development and has been implicated in tumorigenesis. In a study of canine mammary tumors, dogs with malignant mammary tumors with expression of both ERα and progesterone receptor (PR) had significantly higher survival rate when compared to dogs with malignant mammary cancer that had expression of only ERα similar to reports in human breast cancer (Chang, Tsai et al. 2009). Overall the presence of PR is considered a good prognostic factor in canine mammary tumors (Subeide 2013). Human patients with tumors that express both ERα and PR have a better prognosis because of the greater probability of response to endocrine therapy compared to tumors that do not contain steroid receptors(Anderson 2002). It is possible that the progesterone receptor may also be oscillating or had an effect on the circadian clock genes or ERα/β expression.

We did not look at any of the cell cycle genes to monitor proliferation in CMAMs. E2 regulates the expression of several cell cycle genes including c-Myc and cyclin D1, and the cyclin D1 promoter is the site at which ERβ opposes ERα-mediated activation (Liu, Albanese et al. 2002; Strom, Hartman et al. 2004). There have been inconsistent results with regards to ERβ antiproliferative effects and induction of cell cycle components, but expression of ERβ in the breast cancer cell line T47D resulted in inhibition of proliferation in response to E2 treatment (Strom, Hartman et al. 2004). Strom et al also identified that expression of cyclin E and cyclin A mRNA (components of active Cdk2 complex from G1 into S phase) was decreased by ERβ (Strom, Hartman et al. 2004). In these studies the cells were stably and transiently transfected with ERβ and the Chappell lab is currently working on transfected CMAMs. The relationship of ERβ and clock genes could be more thoroughly characterized by transfecting CMAMs with either ERβ, Bmal1, or Per2 to determine if the rhythms could be restored. It would also be interesting to determine cell cycle gene expression in CMAMs as well as transfected CMAMs. Determining first if there is cell cycle oscillation and how overexpression of ERβ and clock genes affect cell cycle expression might help to pinpoint where ERβ acts and the steps involved in tumorigenesis.

Clock genes have also been implicated in cell cycle regulation. Cyclin D1, Cyclin A, Mdm-2 are cell cycle genes that show circadian patterns in vivo and are found to be deregulated in mPer2$^{m/m}$mice (Fu, Pelicano et al. 2002). More specifically, circadian regulators control c-myc gene transcription via direct E box-mediated interaction and mutations in *mPer2* resulted in deregulation of *c-myc* in mouse tissue with subsequent decreased apoptosis and genomic instability (Fu, Pelicano et al. 2002). Again it would be interesting to look at the expression of the cell cycle genes since they show circadian patterns and may be affected by ERβ expression.

Another major avenue that we did not explore in this study is the negative limb of the clock. We focused on *Bmal1* and *mPer2,* but knowing what the rhythms of *Clock* and *mPer1* and other genes from the negative limb of clock, *mCry*, mROR, mREV-ERBs may also provide

insight into rhythm disruption. For example, RORα deficient mice show aberrant circadian behavior, providing evidence of RORα involvement in regulation of circadian rhythms (Du and Xu 2012). RORα has been shown to have different effects in ER positive and ER negative breast cancer cells; in ERα positive MCF-7 cells, RORα was shown to activate aromatase expression and accelerate estrogen production leading to proliferation of breast cancer cells (Odawara, Iwasaki et al. 2009; Du and Xu 2012). On the other hand, RORα had some cancer-suppressive effects including inhibiting cell migration and proliferation in ER-negative breast cell lines (Du and Xu 2012). It is possible that RORα plays a role in ER deregulation and circadian rhythm disruption, which may depend on the inherent properties of estrogen receptor expression in different types of mammary cancer.

One of the most potent regulators of the circadian clock is light cues and melatonin (MEL) secretion which is increased during the dark phase and is dependent on light on the retina (Menaker 2003). In general, MEL has been shown to be inhibitory to mammary cancer development. Melatonin reduces the incidence of spontaneous mammary tumors in transgenic mice and mice strains with high tumoral incidence (Sanchez-Barcelo, Cos et al. 2003). There is also a relationship between melatonin and estrogen, with melatonin causing down regulation of estrogen secretion in the ovary (Sanchez-Barcelo, Cos et al. 2003). From various studies it appears melatonin acts as an antiestrogen by binding to its specific receptors allowing interaction with ER-signaling pathways (Sanchez-Barcelo, Cos et al. 2005). Cell viability might be affected with melatonin secretion or perhaps the expression of ERβ and rhythmicity could be rescued with melatonin treatment. Melatonin was also shown to reduce aromatase activity necessary for estrogen synthesis in MCF-7 cells which express aromatase and MT1 melatonin receptors (Zhou, Wang et al. 1993; Cos, Martinez-Campa et al. 2005). Again it would be interesting to measure aromatase concentration in CMAMs to determine if melatonin treatment affected ERα/β expression and rhythmicity and if there was any difference in circadian clock gene expression.

Chapter 5:

Conclusion

Breast cancer in women and mammary tumors in female dogs are the most common causes of cancer showing a need for identification of tumorigenesis to identify possible prevention and/or treatment options. The central role of ovarian hormones in pathogenesis has been previously identified in both dogs and humans (E.G. MacEwen 1996). Estrogen receptor β has been shown to be a protector against breast cancer and has been shown to counteract ERα with inhibition of E2 stimulated proliferation in breast cancer cells lines (Strom, Hartman et al. 2004). Circadian rhythm disruption has become another key player identified in breast cancer development, with industrializing societies showing the highest risk of breast cancer development and identification of exposure to light at night and circadian disruption resulting in increased risk of breast cancer among women (Stevens and Rea 2001; Stevens 2005). The circadian clock controls numerous physiologic rhythms and disruption is linked to disease and cancer (Fu and Lee 2003). More specifically, mutations in the period circadian clock genes have been identified in breast cancer (Chen 2005; Sjöblom, Jones et al. 2006; Yang, Wood et al. 2009). Circadian clock control of ERβ expression has been identified to act though a conserved E-box element on the ERβ promoter (Cai, Rambaud et al. 2008).

We hypothesized that there would be expression of circadian clock genes and estrogen receptors in the canine mammary cancer line - CMAM. We also hypothesized that the circadian rhythm and ERβ rhythm would be lost or that expression of ERβ would be lost in the cancer line. We were surprised that ERβ expression was present in CMAMs and that there was circadian rhythm of *Bmal1* and m*Per2* in the initial early passage cells. The later passage cells lost rhythmicity and expression was lower, in line with our initial suspicions. We believe that circadian rhythm disruption and ERβ expression are linked, and using sirtinol to block SIRT1 which counteracts the CLOCK, we wanted to determine if rhythmicity could be restored. Sirtinol

treatment did have an effect on *Bmal1,* m*Per2*, and ERβ expression, although normal rhythmicity was not restored. Sirtinol treatment also caused a significant decrease in relative cell viability and increased apoptosis.

In conclusion, circadian rhythm was disrupted in late passage CMAMs and ERβ expression was decreased consistent with dedifferentiation and increased malignancy. Sirtinol, by blockage of SIRT1, affected gene expression and decreased relatively cell viability although normal rhythmicity was not obtained. The exact relationship between circadian clock genes and ERβ involvement in canine mammary cancer has not been completely elucidated. Future research will focus on attempting to restore rhythmicity of clock genes and ERβ. It is also still undetermined if circadian rhythm disruption is driving ER β disruption or if ERβ disruption leads to circadian disruption. Attempts at inducing ERβ expression in CMAMs and rescuing clock rhythmicity or inducing clock gene expression and rescuing ERβ expression are currently underway. Future studies are required to determine the extent and mode of regulation between the circadian clock and ERβ and breast cancer tumorigenesis.

Footnotes:

a. Sigma-Aldrich, St. Louis, MO

b. Oregon Health Sciences University, Corvallis, OR

c. Biowest, USA Scientific, Ocala, FL

d. Invitrogen, Carlsbad, CA

e. Cell Applications, Inc. San Diego, CA

f. NanoDrop ND 1000, Thermo Fischer Scientific, Wilmington, DE

g. Clontech Laboratories, Inc. Mountain View, CA

h. Bio-Rad, Hercules, CA

i. Molecular Devices Corp., CA

j. Dako Corporation, Carpenteria, CA

k. Biocare Medical, Concord, CA

l. MaxVision Biosciences, Mukilteo, WA

m. Vector Laboratories, Burlingame, CA

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