

Entrainment of breast (cancer) epithelial cells detects distinct circadian oscillation patterns for clock and hormone receptor genes

Stefano Rossetti, Joseph Esposito, Francesca Corlazzoli, Alex Gregorski and Nicoletta Sacchi*

Department of Cancer Genetics; Roswell Park Cancer Institute; Buffalo, NY USA

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Abbreviations: 3D culture, three dimensional culture; CCG(s), clock controlled gene(s); CKIE, casein kinase I ϵ ; CRY1, cryptochrome 1; CRY2, cryptochrome 2; ERA, estrogen receptor alpha; ERE, estrogen response element; PER1, period 1; PER2, period 2; PER3, period 3; qRT-PCR, quantitative RT-PCR; Rev-ErbA, Rev-Erbalpha; RORA, RAR-related orphan receptor alpha

Most physiological and biological processes are regulated by endogenous circadian rhythms under the control of both a master clock, which acts systemically and individual cellular clocks, which act at the single cell level. The cellular clock is based on a network of core clock genes, which drive the circadian expression of non-clock genes involved in many cellular processes. Circadian deregulation of gene expression has emerged to be as important as deregulation of estrogen signaling in breast tumorigenesis. Whether there is a mutual deregulation of circadian and hormone signaling is the question that we address in this study. Here we show that, upon entrainment by serum shock, cultured human mammary epithelial cells maintain an inner circadian oscillator, with key clock genes oscillating in a circadian fashion. In the same cells, the expression of the estrogen receptor α (ERA) gene also oscillates in a circadian fashion. In contrast, ERA-positive and -negative breast cancer epithelial cells show disruption of the inner clock. Further, ERA-positive breast cancer cells do not display circadian oscillation of ERA expression. Our findings suggest that estrogen signaling could be affected not only in ERA-negative breast cancer, but also in ERA-positive breast cancer due to lack of circadian availability of ERA. Entrainment of the inner clock of breast epithelial cells, by taking into consideration the biological time component, provides a novel tool to test mechanistically whether defective circadian mechanisms can affect hormone signaling relevant to breast cancer.

Introduction

As a consequence of the Earth's rotation about its axis, most organisms on this planet have evolved a clock that allows them to tailor their physiology and behavior rhythmically, with a period length of about 24 h. These endogenous, circadian (from the Latin "ca. diem") rhythms can sustain themselves even in the absence of external cues. Disruption of endogenous circadian rhythms seem to have a major impact on overall health and has been correlated with aging and diseases, such as coronary heart disease, various neurodegenerative disorders and cancer.¹⁻⁹

The generation of endogenous circadian rhythms is now known to be an attribute of each individual cell's own molecular time piece, hereafter referred to as circadian oscillator or clock. This clock is based on a genetic network of transcriptional and post-translational negative feedback loops that generate rhythmic 24 h expression of core clock components.^{1,2,10,11} In mammals, the molecular clocks of all cells are centrally controlled by a main coordinator in the brain, the suprachiasmatic nucleus, which,

upon perceiving light from the environment, synchronizes all peripheral clocks to sustain coordinated rhythms for the organism.^{1,2,10,11} Interestingly, even when removed from the organism, isolated cells, such as fibroblasts, maintain their autonomous timing mechanism (ref. 12 and the references within). The core clock components are represented by the genes that encode the proteins necessary for the generation and maintenance of normal circadian rhythms in individual cells^{1,13} (Fig. 1). The primary feedback loop comprises eight core clock genes: *CLOCK*, Casein kinase I ϵ (*CKIE*), Cryptochrome 1 (*CRY1*), Cryptochrome 2 (*CRY2*), Period 1 (*PER1*), Period 2 (*PER2*), Period 3 (*PER3*) and *BMAL1*. Another regulatory loop includes two retinoic acid-related orphan nuclear receptors, *Rev-Erb α* (*RevErbA*) and *ROR α* (*RORA*). *CLOCK* and *BMAL1* genes encode for basic helix-loop-helix transcriptional activators that can heterodimerize and regulate the transcription of the *PER* and *CRY* genes by binding to E-box elements (CACGTG) in their promoters. The *PER* proteins build up in the cytoplasm and are degraded by *CKIE*. The *CRY* proteins, once they accumulate in the

*Correspondence to: Nicoletta Sacchi; Email: nicoletta.sacchi@roswellpark.org
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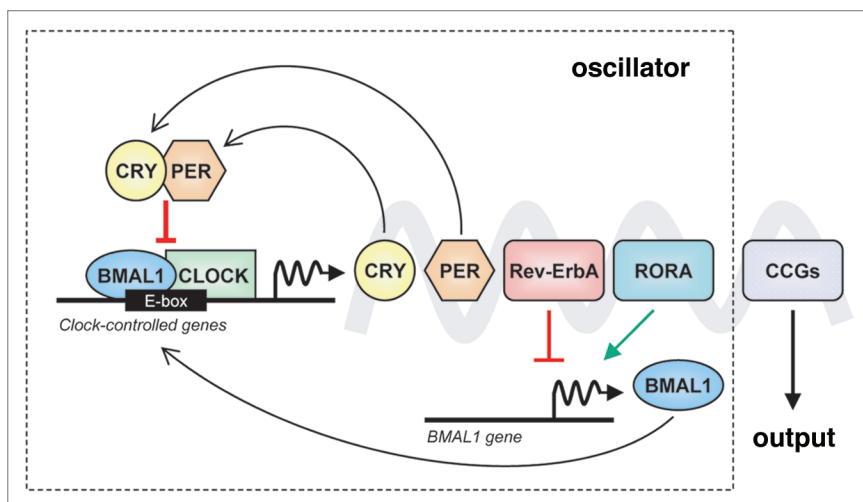


Figure 1. The mammalian circadian clock. Simplified representation of the cell molecular clock, consisting of an oscillator in which clock gene transcripts and proteins accumulate in a circadian fashion due to a complex feedback loop. The output of this inner oscillator is the circadian transcriptional regulation of many clock-controlled genes (CCGs) directly bound by BMAL1-CLOCK.

cytoplasm up to a critical threshold, bind the PER proteins, and form a stable complex that can reenter the nucleus and interfere with the heterodimerization of CLOCK and BMAL1. The CLOCK-BMAL1 heterodimer can also drive the transcription of *Rev-ErbA* and *RORA*, which repress and drive, respectively, the transcription of *BMAL1* itself. The end result of these regulatory pathways is a 24-h rhythmic oscillation of genes of the inner clock. CLOCK and CKIE are the only components of the circadian clock whose expression does not oscillate. Importantly, the CLOCK/BMAL1 complex is able to output to other signaling pathways via clock-controlled genes (CCGs) (Fig. 1), which can influence a wide range of functions outside the clock.^{2,14,15} It is thought that > 10% of all mammalian transcripts undergo circadian oscillations.¹⁶⁻¹⁸

Remarkably, nuclear receptors are functionally intertwined with the circadian oscillator.¹⁹ In the mouse, the mRNAs of more than half the nuclear receptors accumulates periodically to enable the rhythmic control of energy, glucose and lipid metabolism.²⁰ Moreover, estrogen receptor α (ERA) function was found to be linked to the circadian oscillator in breast cancer cells.^{21,22} Apparently, the periodic accumulation of PER2 and ERA transcripts/proteins is reciprocally controlled. On one hand, *PER2* is an estrogen-inducible gene that contains an estrogen response element (ERE) in its promoter region that can be directly bound by ERA in the presence of estrogen. On the other hand, the PER2 protein seems capable of interacting directly with the ERA protein, thus affecting its stability. This suggests that PER2 circadian oscillation may influence ERA oscillation as well.

ERA-mediated estrogen signaling plays a key role in mammary gland morphogenesis and development, as shown by the inability of proper mammary gland ductal elongation in ERA-knockout mice.²³ Aberrant estrogen-mediated signaling through ERA is intimately linked to a large majority of breast cancer.²⁴

Several studies over the years have shown a correlation between loss of circadian rhythm and breast cancer in women (reviewed in refs. 25–30).

To understand the reciprocal influence of circadian oscillator and estrogen signaling in breast tumorigenesis, we established a cell system that enables us to couple the analysis of both circadian oscillator and estrogen signaling in human mammary epithelial cells in a circadian fashion. To this end, we took advantage of a serum shock strategy, first described by Balsalobre et al.,³¹ through which mammalian cultured fibroblasts could be induced to synchronously express clock genes in a circadian fashion in response to a short treatment with high concentrations of serum (entrainment). In this study, we slightly modified this method to demonstrate, first, that the circadian oscillator of human mammary epithelial cells can be entrained in culture, and second, that the pattern of circadian mRNA accumulation of both clock genes and *ERA* is different

between human mammary epithelial cells and breast cancer cells. Upon entrainment of the inner clock of the human mammary epithelial cell line HME1, key clock genes are transcribed in a circadian fashion. In contrast, entrainment of ERA-positive and ERA-negative breast cancer epithelial cells shows lack of circadian oscillation of key clock gene expression. Interestingly, we also show that circadian accumulation of *ERA* mRNA occurs in HME1 cells but not in ERA-positive breast cancer cells.

Results

ERA-positive human mammary epithelial HME1 cells are endowed with an inner functional clock. Circadian oscillation of clock genes was first demonstrated in cultured fibroblasts by using a serum shock method.³¹ To assess whether human mammary epithelial cells display circadian oscillation of clock genes, we used a slight modification of that method to entrain HME1, a human mammary cell line derived from a mammaplasty and immortalized with telomerase. HME1 cells have many features of normal mammary epithelial cells: they are ERA-positive, capable of acinar morphogenesis when grown in three-dimensional (3D) culture on reconstituted basement membrane and do not form tumors when xenografted in nude mice (Table 2). Upon HME1 entrainment, we first measured the mRNA accumulation of core circadian genes, *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *Rev-ErbA*, *BMAL1* and *CLOCK*, over a 48 h period and plotted this as a function of time. *PER1*, *PER2* and *PER3* transcription showed oscillations that peaked at 24 and 48 h. The mRNA levels of *Rev-ErbA* peaked at 20 and 44 h. *CRY1* and *CRY2* both had initial peaks at 28 h and began increasing again at approximately 48 h. In contrast, *BMAL1* mRNA oscillated approximately antiphase to the Period genes with peaks at approximately 12 and 36 h. *CLOCK* mRNA did not show any circadian oscillation (Fig. 2).

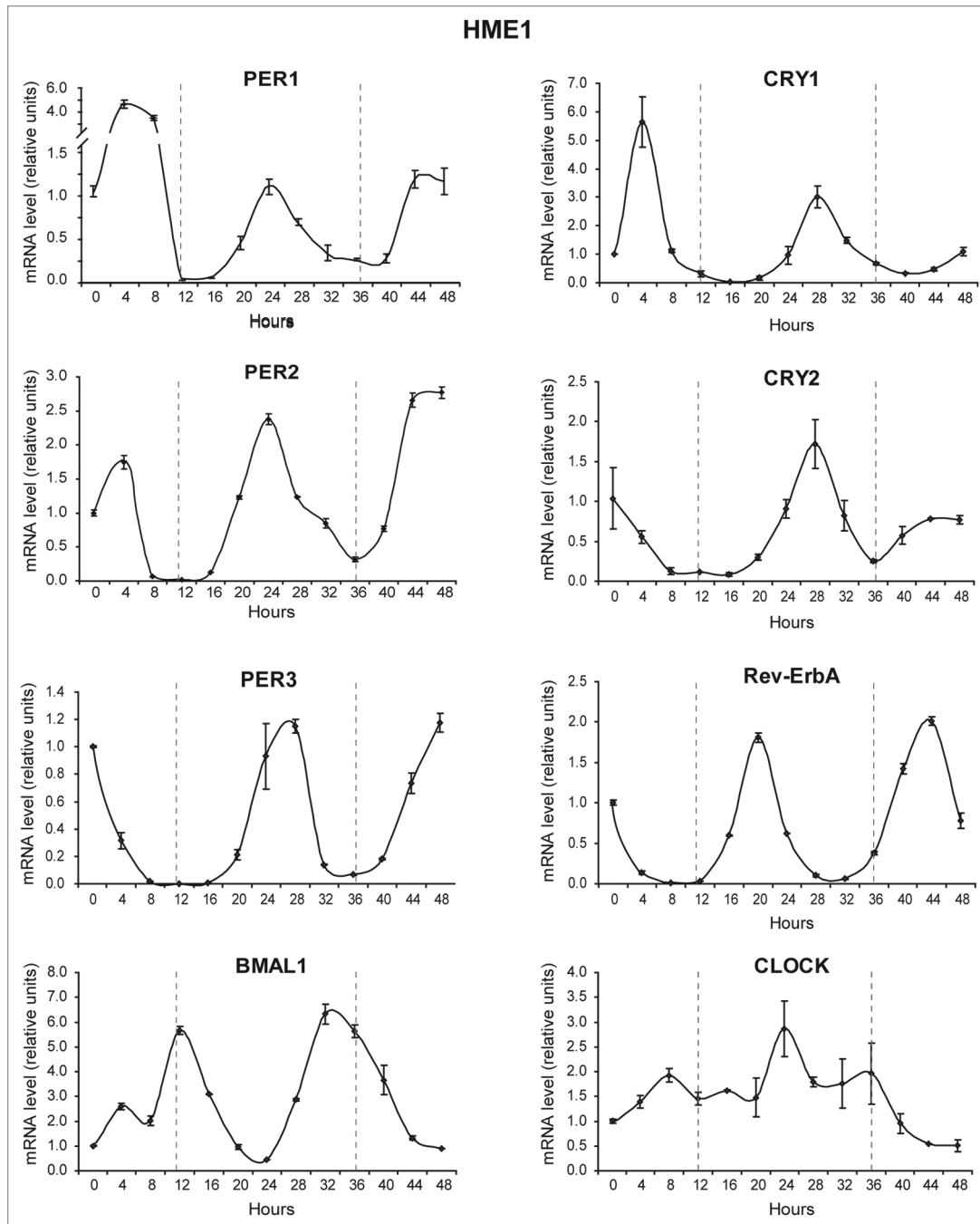


Figure 2. ERA-positive human mammary epithelial HME1 cells are endowed with an inner functional clock. Quantitative RT-PCR analysis on cultured HME1 cells at 4-h intervals after entrainment via serum shock shows that the mRNA of the clock genes *PER1*, *PER2*, *PER3*, *CRY1*, *CRY2*, *RevErbA* and *BMAL1* accumulates in a circadian fashion, while the mRNA of *CLOCK* does not show any circadian oscillation.

Based on these observations, apparently HME1 cells have conserved a functional inner clock that can be entrained *in vitro*.

Entrainment of ERA-negative human mammary epithelial MCF10A cells detects a defective clock. Next, we entrained an immortal, non-tumorigenic human mammary epithelial cell line, MCF10A, which was derived from a fibrocystic breast tissue, and is ERA-negative. MCF10A cells are capable of acinar morphogenesis in 3D culture on reconstituted basement membrane and do not form tumors when xenografted in nude mice

(Table 2). We found that MF10A cells show rhythmic oscillations of clock gene expression, albeit more irregular ones than the ones detected in HME1 cells. Specifically, while the oscillations of *PER1* and *PER3* mRNA were circadian and showed peaks at 24 and 48 h, the oscillation of *PER2* showed a less clear circadian pattern, with an increase in mRNA between the 24 and 48 h peaks. A circadian oscillation was detectable also in *Rev-ErbA* mRNA, with peaks (24 h and 48 h) delayed approximately 4 h relative to HME1 and operating anti-phase to *BMAL1*. *CRY1*

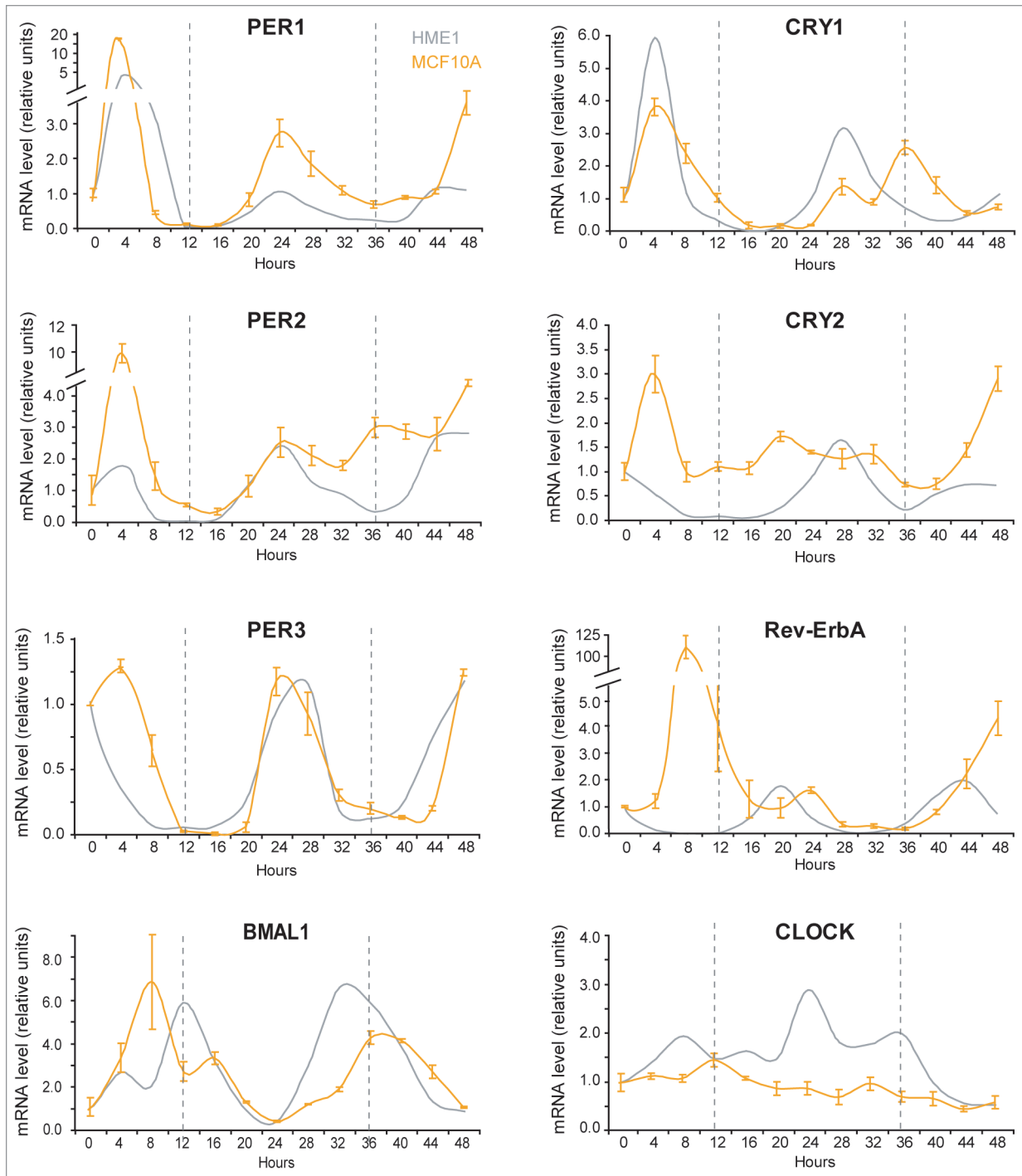


Figure 3. Entrainment of ERA-negative human mammary epithelial MCF10A cells detects a defective clock. Quantitative RT-PCR at 4 h time points after entrainment shows that in cultured MCF10A cells, only *PER1* and *PER3* mRNAs have a circadian oscillation comparable with HME1 cells, while the other clock genes display either an imperfect circadian oscillation (*CRY1*, *PER2*, *RevErbA*, *BMAL1*) or no circadian oscillation (*CRY2*, *CLOCK*).

and *CRY2* mRNAs oscillate irregularly and do not show a circadian pattern. *CLOCK* mRNA did not oscillate (Fig. 3). There is no way for us to conclude whether the MCF10A circadian oscillation pattern is a reflection of loss of ERA expression. However, we cannot discount that the defective clock may be consequent to lack of transactivation of *PER2*, a downstream ERA target gene,²² due to lack of estrogen-ERA signaling in MCF10A cells.

ERA-negative human breast cancer cells show a disrupted inner clock. To gather additional insights as to whether ERA-negative breast cancer cell lines always display a defective clock, we entrained two ERA-negative breast cancer cell lines, HS578T and MDA-MB-231. Analysis of the mRNA oscillation of HS578T clock genes showed that *PER1* and *PER2* mRNAs, after an initial peak following the serum shock, slightly increased at 24 h but

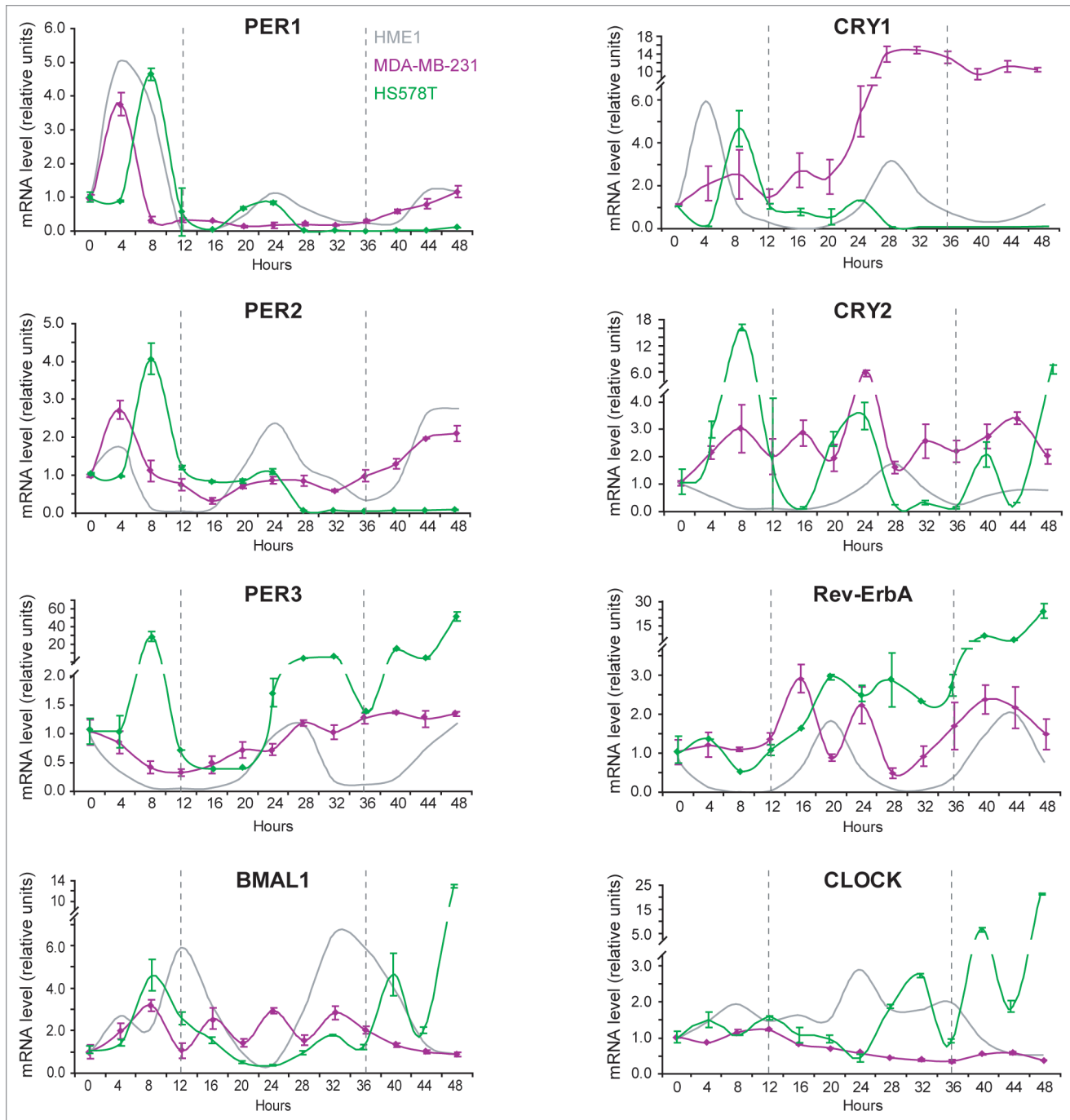


Figure 4. ERA-negative human breast cancer cells show a disrupted inner clock. Quantitative RT-PCR shows that entrainment of the ERA-negative breast cancer cell lines HS578T and MDA-MB-231 cannot induce clock gene mRNA circadian oscillation, as it does in HME1 cells. See the Results section for a more detailed description of the single gene oscillations in each cell line.

sharply declined and remained constant thereafter. *PER3* mRNA showed the lowest levels at approximately 16 h and 36 h, while it accumulated mostly at 28–32 h and at 48 h. *CRY1* mRNA underwent an oscillation similar to *PER1*, with a weak peak at 24 h then declining and remaining constant thereafter. *CRY2* showed non-circadian oscillations with multiple peaks (8 h, 24 h, 40 h, 48 h) within the 48 h. *Rev-ErbA* mRNA increased over time and peaked at 48 h. Similarly, *BMAL1* mRNA did not accumulate in

a circadian fashion and reached the highest level at 48 h. *CLOCK* mRNA did show some oscillations, although not circadian, with peaks at 32, 40 and 48 h (Fig. 4). In entrained MDA-MB-231 cells, *PER1*, *PER2* and *PER3* mRNA remained fairly stable and did not show any oscillation. *CRY1* mRNA remained constant until 24 h then increased and reached a plateau between 28 h and 48 h. Interestingly, *CRY2*, *Rev-ErbA* and *BMAL1* appear to have oscillations with a much greater frequency than in HME1

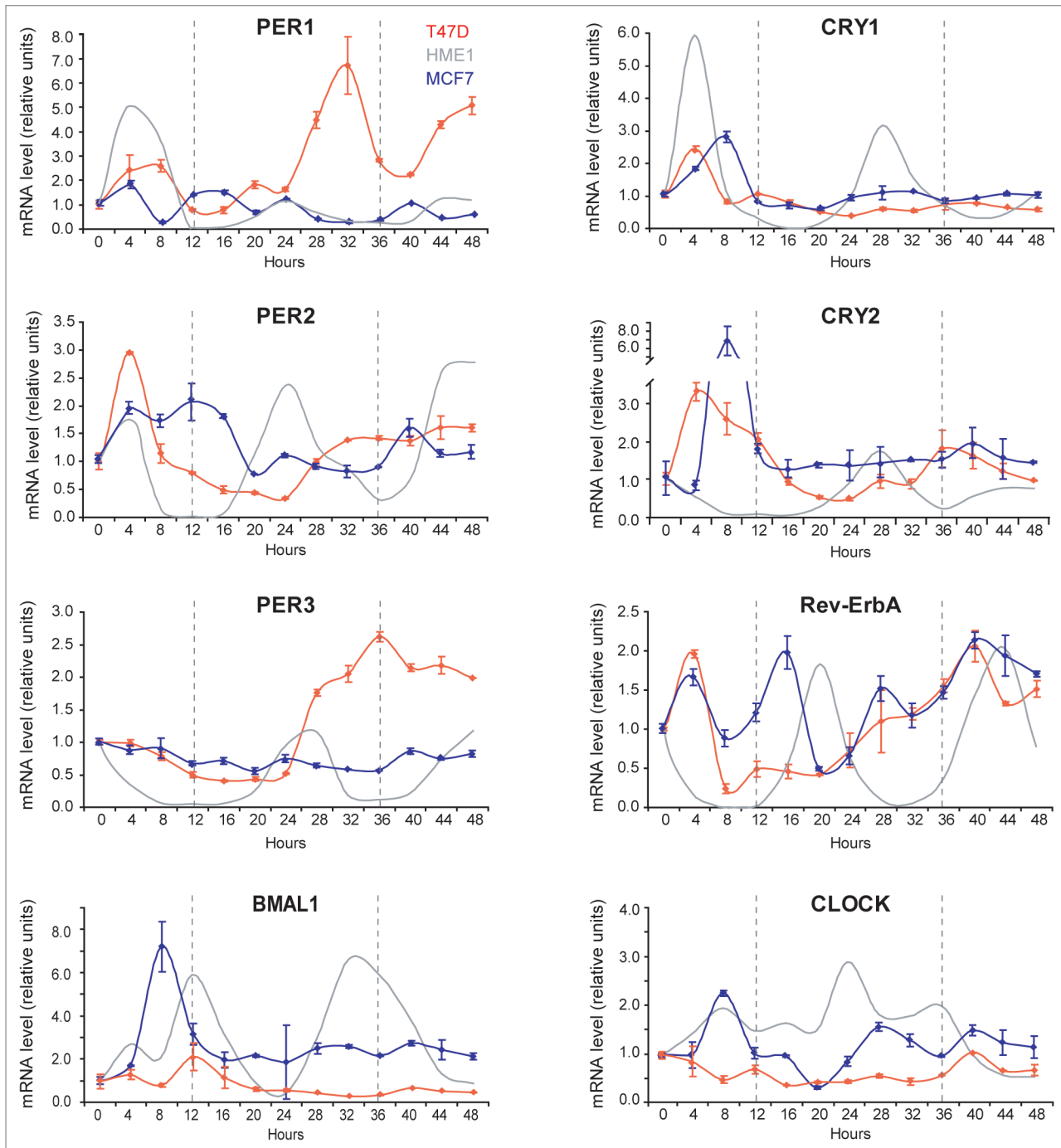


Figure 5. ERA-positive human breast cancer cells also display a disrupted inner clock. Quantitative RT-PCR at different time points after entrainment by serum shock shows that the circadian oscillation of clock genes mRNA is lost in ERA-positive T47D and MCF7 breast cancer cells. See the Results section for a more detailed description of the single gene oscillations in each cell line.

cells, although we could not recognize a defined pattern. *CLOCK* mRNA did not show any oscillation (Fig. 4).

From this qualitative analysis, it is apparent that both ERA-negative breast cancer cell lines have a disrupted inner clock. Again, we cannot conclude whether this disruption is a consequence of loss of ERA expression.

ERA-positive human breast cancer cells also display a disrupted inner clock. Next, we set out to test whether the circadian

oscillation of clock gene expression is present in two ERA-positive breast cancer cell lines, T47D and MCF7, as it is in ERA-positive HME1 cells. As shown in Figure 5, both cell lines do not display circadian oscillations in any of the clock genes analyzed. Specifically, in T47D cells, *PER1* mRNA levels peaked at 32 h and 48 h but showed no circadian rhythm. *PER2* and *PER3* mRNAs show an increase between 24 h and 48 h but do not oscillate. *CRY1* and *CRY2* mRNAs, after an initial peak following

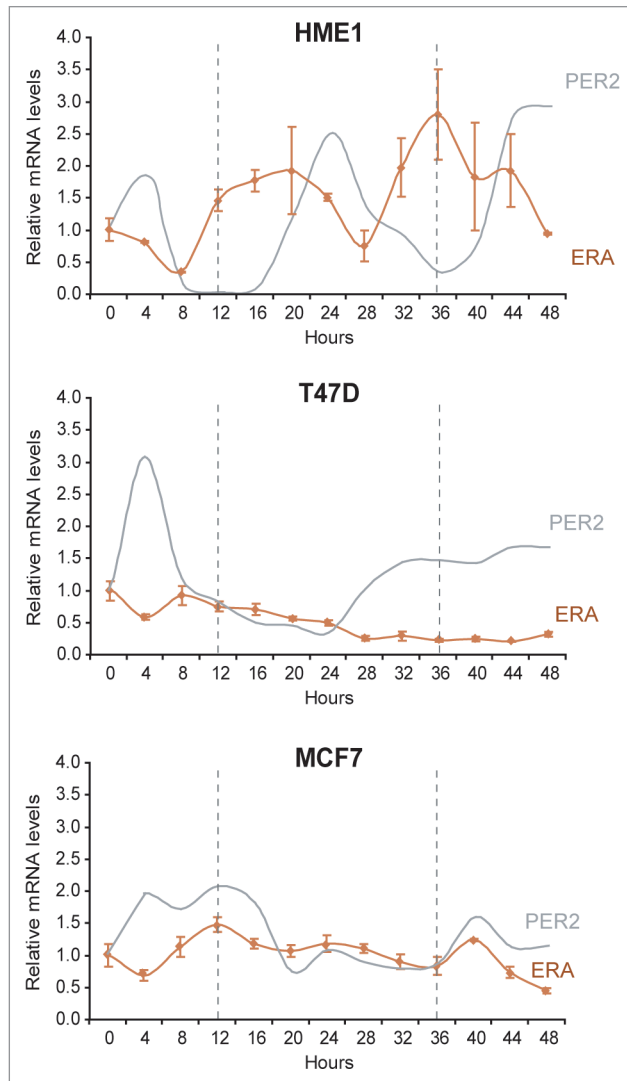


Figure 6. *ERA* mRNA oscillates in a circadian fashion in *ERA*-positive HME1 cells but not in *ERA*-positive breast cancer cells. Quantitative RT-PCR at 4 h time points after entrainment by serum shock shows that *ERA* mRNA oscillates in a circadian fashion, and its accumulation precedes the circadian accumulation of *PER2* mRNA in *ERA*-positive, mammary epithelial HME1 cells. In contrast, *ERA*-positive T47D and MCF7 breast cancer cells, which do not show *PER2* circadian expression, also do not show any *ERA* mRNA circadian rhythmicity.

the serum shock, remained fairly constant throughout the 48 h. *Rev-ErbA* mRNA showed a steady increase from 8 h to 40 h, and did not show any circadian rhythm. *BMAL1* mRNA, after a faint peak at 12 h, decreased and remained constant thereafter. *CLOCK* showed no oscillation (Fig. 5). In MCF7 cells, *PER1* and *PER2* mRNAs underwent irregular oscillations with an increased frequency relative to HME1, while *PER3* mRNA did not show any oscillation. *CRY1* and *CRY2* mRNAs, after an initial increase at 8 h after the serum shock, decreased and remained constant. *Rev-ErbA* mRNA showed oscillations with increased frequency relative to HME1 (period of 12 h rather than 24 h). *BMAL1* and *CLOCK* mRNAs did not oscillate (Fig. 5).

Table 1. List of primers used for real time PCR

Primer name	Orientation	Sequence
BMAL1	sense	5'-AGG ATG GCT GTT CAG CAC ATG A-3'
	antisense	5'-CAA AAA TCC ATC TGC TGC CCT G-3'
CLOCK	sense	5'-AAG TTA GGG CTG AAA GAC GAC GA-3'
	antisense	5'-GAA CTC CGA GAA GAG GCA GAA G-3'
CRY1	sense	5'-GAC AAG ATC ATA GAA CTC AAT GGT-3'
	antisense	5'-AAG CAG AGA ATT CGC ATT CAT TCG-3'
CRY2	sense	5'-TCC CTA GCA TGT CAG CCC GTT-3'
	antisense	5'-AGG ATT TGA GGC ACT GTT CCG A-3'
ERA	sense	5'-AAG AGC TGC CAG GCC TGC C-3'
	antisense	5'-TTG GCA GCT CTC ATG TCT CC-3'
GAPDH	sense	5'-GAA GGT GAA GGT CGG AGT C-3'
	antisense	5'-GAA GAT GGT GAT GGG ATT TC-3'
PER1	sense	5'-GCT GAT TGC AGA GCG CAT CCA-3'
	antisense	5'-GAT GCA GGA ACA GGA GCA CTG-3'
PER2	sense	5'-AAG CAG GTG AAA GCC AAT GAA GA-3'
	antisense	5'-CCA CCG CAA ACA TAT CGG CAT T-3'
PER3	sense	5'-TGC AGG GCA TCC TCC CTT TGA-3'
	antisense	5'-TCC GGC TCC AGG GAT TCA CAA-3'
Rev-ErbA	sense	5'-GAG CAC CAG CAA CAT CAC CAA G-3'
	antisense	5'-TCT TGA AGC GAC ATT GCT GGC A-3'

From this overall analysis, we found of interest that, in contrast to what observed in *ERA*-positive HME1 cells, *PER2* mRNA (like the mRNAs of the other clock genes) does not show a circadian oscillation in the *ERA*-positive breast cancer cell lines T47D and MCF7. Since *PER2* transcription is regulated, at least in part, by *ERA*, we set out to test whether the difference in *PER2* mRNA oscillation between these cell lines was correlated with a difference in *ERA* mRNA oscillation.

ERA mRNA oscillates in a circadian fashion in *ERA*-positive HME1 cells but not in *ERA*-positive breast cancer cells. As shown in Figure 6, we found that in HME1 cells *ERA* mRNA oscillates with a period of 20–24 h, with broad peaks starting at 12 h and 32–36 h and declining at 24–28 h and 44–48 h. Interestingly, *ERA* mRNA accumulation precedes, of approximately 8 h, the *PER2* mRNA accumulation, suggesting that *ERA* and *PER2* circadian oscillations are coordinated (Fig. 6, top). In contrast, T47D and MCF7 breast cancer cells, which did not show *PER2* mRNA circadian oscillation, also did not display any *ERA* mRNA circadian rhythmicity (Fig. 6). In T47D, *ERA* mRNA levels steadily decreased after a faint peak at 8 h (Fig. 6, middle), while in MCF7, *ERA* mRNA, after an initial peak at 12 h, did not significantly oscillate until declining at 48 h.

From these findings, we conclude that *ERA* mRNA displays a circadian expression in *ERA*-positive mammary epithelial cells (HME1) but not in *ERA*-positive breast cancer cells (T47D and MCF7) and correlates with *PER2* mRNA circadian oscillation.

Discussion

The possible link between disruption of circadian rhythms and breast cancer was hypothesized far before the discovery of the molecular clock.³² Among all environmental cues, light is the most powerful circadian synchronizer.⁵ Indeed, epidemiological studies suggested that women experiencing disruption of the circadian clock, such as night-shift workers constantly exposed to artificial light or subjects with sleep deprivation, have an increased risk of developing breast cancer.^{25,27,33-35} Disruption of circadian rhythms has been found associated with breast tumor progression as well. Indeed, patients with metastatic breast cancer, who had impaired circadian rhythms, had earlier mortality than patients with an intact rhythm.³⁶ This observation is further supported by animal studies showing that mammary tumors grow more rapidly, and overall survival decreases, in rodents subjected either to constant light or constant darkness.^{37,38}

In this report, we discuss for the first time that entrainment of human mammary epithelial cells in culture represents an additional strategy that takes into account the biological time component to advance breast cancer research. Based on the observation that rhythmic expression of core circadian genes is observed in most peripheral tissues³⁹ and can be induced in cultured fibroblasts by serum shock,³¹ we set out to test whether serum-shocked cultured human mammary epithelial cells display an autonomous functional inner clock. We found that HME1 cells, which are immortal, non-tumorigenic and capable, like normal mammary epithelial cells, of proper 3D acinar morphogenesis, display circadian gene expression of key clock genes upon entrainment by serum shock. HME1 cells are ERA-positive and, upon estrogen treatment, express canonical downstream ERA targets, including progesterone receptor (our unpublished observations). Apparently, *ERA* mRNA oscillates in a circadian fashion in HME1 cells entrained in culture. This observation is consistent with previous *in vivo* studies showing that ERA is a nuclear receptor under circadian control.²⁰ ERA is a transcriptional regulator of the *PER2* gene,²² and estradiol induces *PER2* mRNA in HME1 cells (our unpublished observation). The accumulation of *ERA* mRNA in entrained HME1 cells precedes the peaks of *PER2* mRNA accumulation by approximately 8 h, suggesting that *ERA* and *PER2* circadian oscillations are coordinated. In contrast, in the human mammary epithelial cell line MCF10A, which is ERA-negative, we found that the circadian oscillation of many clock genes, including *PER2*, is partially or completely lost. We do not know yet whether lack of ERA expression, by abrogating ERA-mediated estrogen transactivation of *PER2*, had contributed to the development of a defective clock in MCF10A cells. Regardless, neither the lack of ERA nor the defective clock seem to have had an effect on the process of acinar morphogenesis of MCF10A cells, which maintain the ability to form normal, hollow acini lined with polarized cells when grown in 3D culture on basement membrane.^{40,41} Apparently, the microenvironment-dependent morphological architecture is “dominant” over loss of ERA and the defective clock in MCF10A.⁴²

Based on the differences of the circadian oscillator between HME1 and MCF10A cells, we further tested whether there is

an association between loss of ERA expression and defective circadian oscillation of clock genes in breast cancer cells as well. Entrainment of two ERA-negative breast cancer cell lines, HS578T and MDA-MB-231, and two ERA-positive breast cancer cell lines, T47D and MCF7, showed an erratic mRNA oscillation of core clock genes in both ERA-negative and ERA-positive cells. In ERA-positive cancer cell lines, with a disrupted clock, we found that *ERA* mRNA does not undergo circadian oscillation, as it does in HME1 cells with a functional clock. Based on the ERA expression level alone, we always assumed that ERA-mediated estrogen signaling is not impaired in ERA-positive breast cancer cells. However, it is possible that, when *ERA* mRNA does not accumulate in a circadian fashion, the estrogen signaling is somewhat impaired. This observation might be relevant for hormone therapy of ERA-positive cancer. Current hormone therapies targeting ERA-positive breast cancer have been based on the assumption that ERA is constantly expressed. Our data suggest that drugs targeting ERA may be less efficient in breast cancer cells that are ERA-positive yet incapable of accumulating ERA in a circadian fashion.

The circadian clock integrity is deemed to be important for tumor suppression *in vivo*.⁵ However, the intricacy of multiple regulatory mechanisms makes it cumbersome to use *in vivo* models to discriminate whether breast cancer initiation is triggered, either by genetic/epigenetic mutations/variants of clock components, which are capable of influencing ERA oscillation, or by genetic/epigenetic loss of ERA, which regulates inner clock components. To answer these questions requires testing numerous parameters in a mechanistic and circadian fashion. Entrainment of human breast (cancer) epithelial cells provides a convenient tool to perform this type of studies. One of our ongoing studies, using entrained HME1 cells, stems both from published data, showing that women with variants in the *CLOCK* gene have a higher risk of breast cancer and that *CLOCK* expression is higher in ERA-negative tumors vs. ERA-positive tumors,⁴³ and from our observation that *ERA* contains in the first intron an E-box. Thus, ERA could be potentially affected by a dysfunctional *CLOCK*/*BMAL1* heterodimer. Another ongoing study aims at deciphering, mechanistically, the influence of an impaired *PER2*-ERA “regulome” on breast cancer initiation. The rationale is provided by a wealth of observations, including loss of *PER2* expression in breast cancer,^{44,45} evidence of ERA-*PER2* mutual regulation²² and *PER2*'s ability to coordinate circadian outputs via protein-protein interaction with ERA²² and other nuclear receptors⁴⁶ (Fig. 7). The expected outcome of these investigations is to understand whether loss of ERA expression can lead to a disrupted clock due to lack of proper estrogen-mediated *PER2* transactivation, or whether the development of a disrupted clock is a consequence of an impaired estrogen-ERA signaling. Unraveling these mechanisms might improve preventative and therapeutic strategies for breast cancer.

Future studies using entrained normal and breast cancer cells will enable us to focus on understanding whether components of the circadian oscillators play a role in breast cancer by influencing hormone regulation or the other way around. It will be possible to learn from these studies whether breast cancer patients receiving

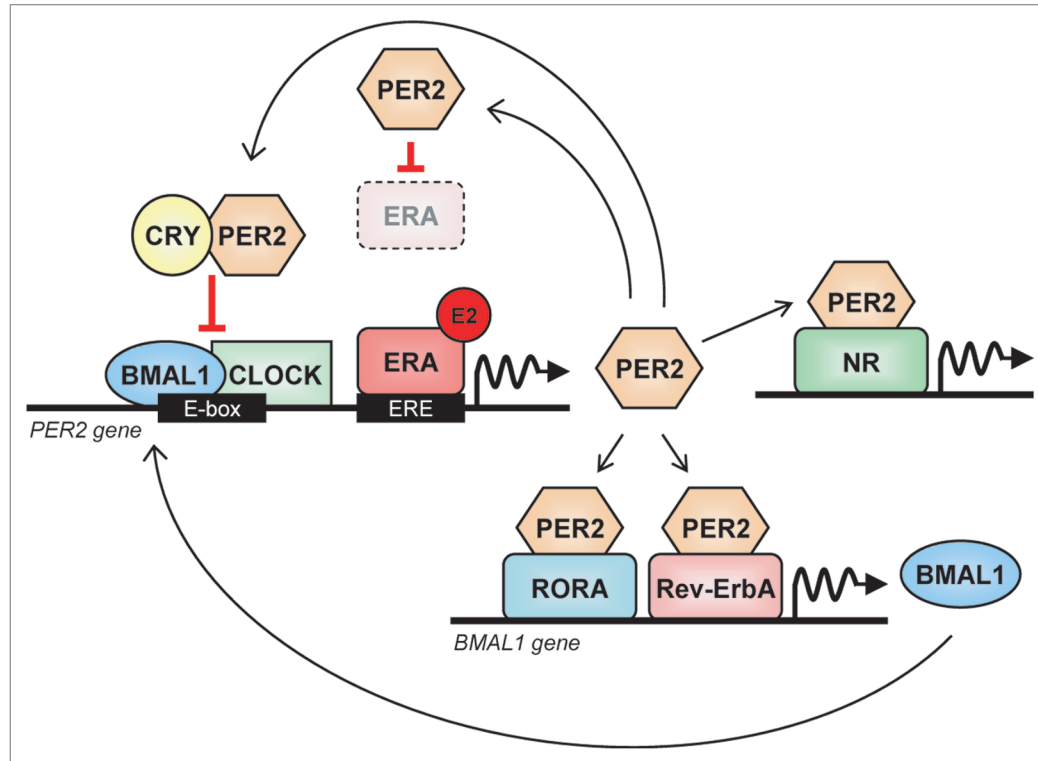


Figure 7. The core clock gene PER2 links the circadian oscillator with ERA signaling. PER2 transcription can be induced by estrogen (E2)-ERA signaling; second, PER2 physically interacts with ERA and leads to its degradation; finally, PER2 interacts with RevErbA, RORA and other nuclear receptors (NR) to modulate the transcription of NR-target genes, including BMAL1. Based on references 22 and 46.

hormone treatment would benefit the most by receiving treatment when ERA expression is least abundant in normal cells and most abundant in cancer cells. Ultimately, we expect that model cell systems that facilitate circadian analysis will accelerate the progress of breast cancer “chronotherapy.” Chronotherapy is a strategy that takes advantage of the asynchronies between normal and malignant tissues by administering therapy at a specific time of the day.

Methods

Cells and cell culture. The human, telomerase-immortalized breast epithelial cell strain h-TERT-HME1 (hereafter called HME1) (Clontech) was grown in mammary epithelial growth medium (MEGM) supplemented with bovine pituitary extract (Lonza). The human, non-tumorigenic epithelial cell line MCF-10A (ATCC, CRL-10317) derived from fibrocystic breast tissue was grown as described in reference 47, in DMEM/F12 supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 μ g/ml Hydrocortisone, 100 ng/ml cholera toxin and 10 μ g/ml insulin and sterile filtered. The human breast cancer cell lines MDA-MB-231 (ATCC, HTB-26), T47D (ATCC, HTB-133), HS578T (ATCC, HTB-126) and MCF7 (ATCC, HTB-22) were grown in DMEM supplemented with 5% fetal bovine serum (FBS) (Invitrogen). All cell lines were grown in a humidified incubator at 37°C and 5% CO₂.

Entrainment of cells by serum shock. Cells were entrained by using a slightly modified serum shock procedure based on

the method originally proposed by Balsalobre et al.³¹ Briefly, cells were seeded into 6-well plates at a density of 1.5×10^5 cells per well and allowed to grow to confluence. After being confluent for 2–3 d, cells were washed twice in PBS (Invitrogen) to remove residual growth factors and starved overnight. For HME1, the starvation medium was mammary epithelial basal medium (MEBM) (Lonza). For MCF10A, the starvation medium was DMEM/F12 (Invitrogen). All other cells were starved in DMEM without FBS. Thereafter, cells were serum shocked using their respective growth media supplemented with 50% horse serum for 2 h. Subsequently, the cells were washed in PBS and returned to starvation conditions. The first time point was taken prior to the serum shock ($t = 0$) and every 4 h thereafter until 48 h.

RNA extraction and cDNA synthesis. Total RNA was extracted by using Trizol Reagent (Invitrogen) according to the manufacturer’s instructions. Briefly, cells grown in 6-well plates were lysed with 1 ml Trizol/well and incubated for 5 min to allow for complete dissociation of the nucleoprotein complexes. After addition of 200 μ l chloroform per 1 ml Trizol, samples were vortexed and centrifuged at 12,000x g for 15 min at 4°C to separate the upper aqueous phase, containing the RNA, from the lower phase. RNA was precipitated from the aqueous phase by addition of 500 μ l of isopropyl alcohol and centrifugation at 12,000x g for 10 min. The RNA pellet was then washed with 1 ml 75% ethanol, briefly dried and resuspended in water. The RNA concentration was measured spectrophotometrically by

Table 2. Molecular and biological features of the cell lines used in this study

	HME1	T47D	MCF7	MCF10A	HS578T	MB-MDA-231
ERA-positive	Yes	Yes	Yes	No	No	No
Immortal	Yes	Yes	Yes	Yes	Yes	Yes
Capable of proper acinar morphogenesis in 3D culture on reconstituted basement membrane	Yes	No	No	Yes	No	No
Capable of anchorage-independent growth in semisolid medium	No	Yes	Yes	No	Yes	Yes
Tumorigenic	No	Yes	Yes*	No	No	Yes
Invasive	No	No	Yes*	No	No	Yes

*In the presence of estrogen.

using the Nanodrop (Thermo Scientific). One μg total RNA was retro-transcribed into cDNA by using the QuantiTect Reverse Transcription Kit (Qiagen), according to the manufacturer's instructions. Briefly, RNA was first incubated with a DNase-containing buffer (gDNA wipeout buffer, Qiagen) to remove possible genomic DNA contamination, then retro-transcribed with random primers in an iCycler thermal cycler (BioRad).

Real-time PCR. Real-time PCR analyses were performed in 96-well plates. The reaction mix (20 μl /well) consisted of 1 μl cDNA (derived from 25 ng RNA), 10 μl iQ SYBR Green Supermix (BioRad), 0.2 μM of each forward and reverse primer specific for each gene of interest (see Table 1), and molecular grade water to reach a final volume of 20 μl . After brief centrifugation, the plates were placed in an iCycler thermal cycler equipped with a MyiQ Real-Time PCR detection system (Bio-Rad) programmed with an initial denaturation step at 95.0°C for 3 min, followed by 45 cycles of 95.0°C for 20 sec, 60.0°C for 30 sec and 72.0°C for 30 sec, with data collection during the extension step at 72.0°C. After the reaction, a melting curve analysis was performed to ascertain that a single-band PCR product

was produced. The transcript levels of the genes of interest were quantified by the Delta delta Ct method, using the housekeeping gene GAPDH for normalization. The amplification efficiency, evaluated from the sample slopes, was similar for all the samples analyzed in the same experiment. The data, corresponding to the mean of at least three independent determinations \pm standard deviation, were reported on the charts by setting the transcript level of the first time point ($t = 0$) as 1 for each gene and each cell line.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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