Light at night activates IGF-1R/PDK1 signaling and accelerates tumor growth in human breast cancer xenografts

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Abstract

Regulation of diurnal and circadian rhythms and cell proliferation are coupled in all mammals including humans. However, the molecular mechanisms by which diurnal and circadian rhythms regulate cell proliferation are relatively poorly understood. In this study, we report that tumor growth in nude rats bearing human steroid receptor-negative MCF-7 breast tumors can be significantly accelerated by exposing the rats to light at night (LAN). Under normal conditions of an alternating light/dark cycle, proliferating cell nuclear antigen (PCNA) levels in tumors were maximal in the early light phase but remained at very low levels throughout the daily 24h cycle period monitored. Surprisingly, PCNA was expressed in tumors continually at a high level throughout the entire 24h period in LAN-exposed nude rats. Daily fluctuations of Akt and MAPK activation in tumors were also disrupted by LAN. These fluctuations did not track with PCNA changes, but we found that activation of the Akt stimulatory kinase PDK1 directly correlated with PCNA levels. Expression of insulin-like growth factor 1 receptor (IGF-1R), an upstream signaling molecule for PDK1, also correlated with fluctuations of PDK1/PCNA in the LAN group. In addition, circulating IGF-1 concentrations were elevated in LAN-exposed tumor-bearing nude rats. Lastly, RNAi-mediated knockdown of PDK1 led to a reduction in PCNA expression and cell proliferation in vitro and tumor growth in vivo, indicating that PDK1 regulates breast cancer growth in a manner correlated with PCNA expression. Taken together, our findings demonstrate that LAN exposure can accelerate tumor growth in vivo, in part through continuous activation of IGF-1R/PDK1 signaling.
Introduction

Circadian rhythm regulation plays an important role in determining the sleep/activity and feeding cycles of all mammals, including humans. Predictable rhythms of core body temperature, metabolism, hormone production, cell proliferation and many other biological activities are linked to circadian regulation (1). Over the long term, disruption of circadian regulation has significant adverse health consequences including the development or exacerbation of cardiovascular diseases, diabetes, depression and cancers (2). Environmental and behavioral conditions such as jet-lag, shift-work, physical activities, stress, and timing of food intake are known to disrupt circadian rhythms; however, the most potent factor in dysregulating normal circadian rhythms is inappropriately timed light, particularly light at night (LAN). LAN-induced circadian disruption can profoundly alter the regulation of circadian hormones such as melatonin and cortisol, and, in turn, alter human physiology and metabolism leading to increased long-term risk for developing or promoting cancer (3-5). The risk of developing breast cancer is up to five times higher in westernized countries than in underdeveloped nations. Industrialized countries have increasingly become 24 hours per day/ 7 days per week societies with a large number of people being exposed to artificial light during the night both in the workplace and at home (6, 7). Night shift work has been designated by the International Agency for Cancer Research (IARC) to be a unique, probable risk factor for developing cancer ostensibly via LAN-induced suppression of melatonin production (8). Indeed, female night shift workers have a increased risk of developing breast (9, 10), colon (11) and endometrial cancer (12), while men working night shifts are also at higher risk of prostate cancer as compared with dayshift workers (13).

Insulin-like growth factor-1 (IGF-1) is a mitogenic factor which regulates normal somatic growth and has been implicated in the regulation of benign and malignant cell growth. The proliferative effect of IGF-1 is mediated mainly by the insulin-like growth factor 1 receptor (IGF-1R) (14, 15). Clinical and epidemiological studies have shown that the IGF-1 system (IGF-1, IGF-1R, and IGF-1R signaling molecules) are involved in the risk, incidence, and tumorigenic responses of a number of human tissues, including the development and growth of breast cancer (16-18). Consequently, the IGF-1 system is used as a biomarker to determine risk and also serves as a marker for intervention in human breast cancer. The circadian rhythm of circulating IGF-1 in human breast cancer patients differs significantly from that of other growth factors and cytokines (19), which has raised the possibility that the IGF-1 system regulates breast cancer in a circadian fashion.

Phosphoinositide-dependent protein kinase 1 (PDK1) plays a pivotal role in mediating IGF-1 signal transduction (20). Phosphorylated PDK1 activates AGC kinases, including Akt and PKC isoforms (21).
Through its effects on these kinases, PDK1 is involved in the regulation of cell metabolism, proliferation and survival of tumor cells (22). Importantly, PDK1 is highly expressed in a large number of invasive human breast cancers (23). Consequently, the IGF-1R/PDK1 system has been identified as a target for molecular therapy with potential benefits for numerous human malignancies (24, 25). The mechanism by which PDK1 induces cellular proliferation involves regulation of the cell cycle machinery. Progression through the cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases (CDK) which associate with cyclins at different phases of the cell cycle. Proliferating cell nuclear antigen (PCNA) binds to the CDK-cyclin complexes as well as to other critical checkpoint proteins, transducing both positive and negative signals (26). Thus, PCNA plays a coordinating role in regulating cell proliferation. Lack of PDK1 in mouse embryonic fibroblasts results in a delayed transition from G0-G1 to the S phase, thus suppressing cell proliferation but not promoting apoptosis (20). Although control of cell cycle progression by PDK1 is dependent on the induction of both cyclin D1 and p27kip1 expression, it is unknown whether PDK1 regulates PCNA. Since the central circadian clock regulates most aspects of physiology and pathology in mammals including humans, it is not surprising that important molecules regulating cell proliferation are expressed and/or activated in a circadian pattern. However, the molecular mechanisms of circadian-mediated cancer growth are not completely understood.

In female nude rats, the nocturnal circadian melatonin signal, controlled by the central circadian pacemaker located in the suprachiasmatic nuclei (SCN) of the hypothalamus, plays a major role in suppressing the growth of tissue-isolated MCF-7 SR- human breast cancer xenografts via melatonin receptor-mediated down-regulation of linoleic acid (LA) uptake/metabolism and related signal transduction mechanisms (29). LAN-induced suppression of nocturnal melatonin production induces accelerated tumor growth, LA metabolism and signal transduction activity (29). Here we use this same unique tumor model system and experimental approach with LAN to test our hypothesis that the central circadian pacemaker may also control diurnal rhythms in peripheral tumor cell proliferation through rhythmic expression of other circulating growth hormones and related signaling cascades.

We not only identified a novel, significant disruption of PCNA in human breast cancer xenografts from rats exposed to dim LAN, but also revealed that PDK1 activation directly correlated with PCNA. In addition, our data show that IGF-1 and the IGF-1R correlates with enhanced breast cancer xenograft growth and that PDK1 may play a central role in circadian-regulated breast cancer cell growth.
Materials and Methods

Animals, light conditions, tumor implantation, and growth.

Weanling (35-50 g), female nude rats (Hsd:RH-\textit{Foxn1nu}) were in laboratory facility light exposure chambers on a 12-hour light (141.5 lux; 345 \( \mu \text{W/cm}^2 \))/12-hour dark (12L:12D) cycle (lights on 0600 – 1800 hrs, i.e. Zeitgeber Time ZT0 – ZT12) and provided standard laboratory rodent chow (Teklad 2018, Harlan, IN) and water in accordance with an Institutional Animal Care and Use Committee approved protocol. No procedures were undertaken that caused more than minimal pain, distress, or discomfort. Following a two-week acclimatization period, animals were separated into two groups of 36 animals per group. Group I (Control) animals were continued to be maintained on 12L:12D (total darkness) lighting regimen. Group II (treatment) animals were subjected to 12L:12LAN (dim light at 0.2 lux = 0.08 \( \mu \text{W/cm}^2 \)). After two weeks, the animals were implanted with MCF-7 steroid receptor negative (SR-) human breast cancer xenografts in tissue-isolated manner, as described previously (27, 28). SR- tumors evolved over several passages from a subset of SR+ xenografts that had become estrogen unresponsive. These tumors were histopathologically determined poorly differentiated grade 3, infiltrating ductal breast adenocarcinoma (29). Latency-to-onset of tumor growth (\textit{i.e.}, time to first palpable, pea-sized mass) and estimated tumor weights were determined for each animal.

When tumors reached sufficient size for measurement, rats were subjected to light CO\textsubscript{2} narcosis, and tumor dimensions were measured through the skin with vernier calipers and were converted to tumor weights. Growth rates (g/day) were generated by linear regression from the estimated tumor weights for the treated and control animals during the course of the study (30). The final tumor weight was determined by weighing at the end of the experiment. Six animals were sacrificed and tumor samples were collected at four-hour interval time points (ZT2, ZT6, ZT10, ZT 14, ZT18, and ZT22).

Tumor extract and immunoblotting.

Frozen and pulverized tumors (100 mg) from each time point and treatment group were manually homogenized in 50 mM Hepes, pH 7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM Na\textsubscript{3}VO\textsubscript{4}, 100 mM okadaic acid, and 1X protease inhibitor cocktail Set I (Calbiochem/EMD Biosciences, La Jolla, CA). Tumor lysates were collected and microcentrifuged at 15,300 x g for 20 minutes. Cell supernatants were aliquoted and stored at -80\textdegree C. Cell lysates were prepared as previous described (31). The protein concentration was measured by the commercially available bicinchoninic acid kit (Thermo Scientific/Pierce, Rockford, IL). Samples were fractionated by SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Billerica, MA). After blocking, primary antibodies were incubated with the membrane and then secondary HRP-conjugated antibodies, at

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a 1/10,000 dilution. The bands were visualized using Storm Phospholmager (GE Healthcare, Piscataway, NJ). Primary antibodies used were phospho-Akt (Ser473), Akt, phospho-MAPK (Thr202/Tyr204), MAPK, PCNA, phospho-PDK1 (Ser241), PDK1, phospho-GSK3β (Ser9), and PCNA (Cell Signaling Technology, Danvers, MA); Dynamin II (BD Biosciences, San Jose, CA); PDK1, GAPDH, IGF-1R, PKCα, PKCβ, and PKCδ, (Santa Cruz Biotechnology, Santa Cruz, CA); and IRS-1 (Millipore, Billerica, MA).

**Serum IGF-1 measurement.**

Rats were subjected to a series of six low-volume, minimally invasive blood draws via cardiac puncture to collect oxygenated blood, as described previously (29, 32). Briefly, blood collections were designated at 4-hr intervals to include the 24-hr feeding period; each animal was tested only once every 5 days to eliminate the effects on feeding, stress and potential mortality. Animals were lightly anesthetized by CO₂ inhalation (70% CO₂/30% air); 1-ml samples were taken from the left ventricle by cardiac puncture (less than 5% total blood volume). Blood sampling during the dark-phase (i.e., ZT18, ZT22, ZT2) was carried out under a safelight red lamp (Kodak 1A, model B, catalogue #152 1517; 120 V, 15 W, Rochester, NY) in order to preserve the nocturnal melatonin surge. After clotting at room temperature, the sample was centrifuged at 12000 x g for 15 min at 4°C. The serum was removed and stored frozen at -80°C until tested using an ELISA kit (Diagnostic Systems Laboratories, Webster, TX) to measure IGF-1 concentration.

**Generation of stable knockdown MCF-7 cells with decreased PDK1.**

The MCF-7 SR- human breast cancer cells were derived from MCF-7 SR- human breast xenografts (29) and cultured in DMEM or RPMI 1640 medium supplemented with 10% fetal calf serum. RT-PCR using human specific primers was used to verify the steroid receptor status of the cells one month prior to their use in these studies (data not shown). Mission® precloned shRNA plasmids were purchased from Sigma-Aldrich. The following shRNA clones targeting NM_002613 were used: TRCN0000039782, referred to as 1617PDK1 in the text; TRCN0000238781, referred to as 1253PDK1 in the text; and TRCN0000196933, referred to as 676PDK1 in the text. A scrambled luciferase shRNA (referred to as Luc in the text) cloned into pLKO.1 was generated as a control. Eighty percent confluent MCF-7 SR-cells in 6 cm plates were transfected with 5.12 μg of DNA (2.5 μg pLKO.1/1.25 μg pRE/0.63 μg pRev/0.75 μg pMD2G) using Fugene 6 according to the manufacture’s instructions. Twenty-four h post-transfection, fresh medium was added and allowed to incubate at 37°C for 24 h to generate viral supernatant. MCF-7 SR-cells were incubated with viral supernatant for 6 h at 37°C in the presence of 5 μg /ml polybrene. The infection was repeated a second time with viral supernatant collected at 48 h.
Stable cell pools expressing the shRNA constructs were generated by selection for 72 h with 2 μg/ml puromycin and then maintained in 0.2 μg/ml puromycin.

Xenograft growth in nude mice.
Athymic female nude mice (Hsd: Athymic Nude-Foxn1nu) were provided with standard laboratory rodent chow and water ad libitum. Mice (n = 4 - 6) were injected subcutaneously with 1 x 10^7 MCF-7 SR-control cells on the left flank and MCF-7 SR- PDK1 knockdown cells on the right flank. Cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, harvested by gentle trypsinization, centrifuged at 100 x g for 5 min, and resuspended in serum-free RPMI 1640 medium. The xenografts were determined to be poorly differentiated grade 3, infiltrating ductal breast adenocarcinoma by histological examination by a board certified pathologist (data not shown). Tumor measurements were performed every 3 days as described above. All animal studies in nude rats and mice were performed in accordance with IACUC-approved protocols. No procedures were undertaken that caused more than minimal pain, distress, or discomfort.

In vitro assays of cell proliferation.
Cell proliferation of MCF-7 SR- stable cell lines with varying levels of PDK1 protein expression was evaluated by the CyQuant (Molecular Probes, Eugene, OR) and MTS (Promega Corporation, Madison, WI) cell proliferation assays. Briefly, Cells (1 x 10^5 cells/well) from control (parental), luciferase shRNA, 1253PDK1 shRNA, and 1617PDK1 shRNA cell lines were cultured in 96 well plates. Cells were starved for 16 h before the treatment of 5% FBS. Relative fluorescent or optical density units were measured at different time points according to the protocols of CyQuant and MTS Kits, respectively.

Statistical analysis.
All data are presented as the mean ± 1 standard error (S.E.). Statistical significance was determined by a one-way and two-way repeated measures analysis of variance (α = 0.05) using the XLSTAT 2009 program (Addinsoft, New York, NY). Pair-wise comparisons were made using Tukey’s test (α = 0.05)

Results
Light contamination at night accelerates breast cancer growth in vivo
Previously, we assessed the dose-response effects of light exposure during darkness on tumor growth (32, 29). It was observed that dim light was as effective as constant light in promoted rat hepatoma (32) and human breast cancer tumor growth (29) via circadian disruption involving the suppression of melatonin production. However, little is known how disruption of the central circadian rhythm influences
cell proliferative signal transduction in peripheral tissues and in particular in tumors. In order to answer this question, we firstly determined the stimulatory effect of a moderate dose of LAN on the growth of human breast cancer xenografts. Tissue-isolated MCF-7 SR- tumors were implanted in 72 nude rats exposed to control lighting (12L:12D) and experimental condition (12L:12LAN-0.20 lux at night). The differences of latency to onset of tumor appearance and growth rate between LAN and control animals are shown in Table 1. These results, consistent with the previous report (29), confirm that LAN contamination accelerates breast tumor growth in vivo.

**Light at night modulation of signaling in human breast cancer in vivo**

To elucidate the molecular mechanism by which breast cancer tumor growth is accelerated by circadian disruption, tumors were sampled at four-hour intervals over a 24 h period starting at Zeitgeber time (ZT) 2. We first determined whether there is circadian control of Akt and MAPK and their active forms (Fig. 1A and 1B). Phosphorylated Akt levels peaked at mid-light phase (ZT6), were diminished at ZT14 and not present during the dark phase at ZT10 and ZT18, demonstrating that Akt activation in human breast cancer xenografts is under diurnal control. Likewise, phosphorylated MAPK peaked during the early light phase (ZT2), decreased by the mid light phase (ZT6) and was not detected at ZT10. During the dark phase, phosphorylated MAPK remained, for the most part, diminished at ZT14 and ZT18 followed by elevated levels at ZT22. Both total AKT and MAPK varied little over the 24 h period and did not correlate with their active forms, suggesting that diurnal control of Akt and MAPK is primarily mediated by post-translation modification. Maximal protein abundance of PCNA occurred at ZT2 and was undetectable at all other time points during the 24 h period. In contrast, Dynamin II was detected throughout the 24 h period and its abundance did not change. In rats exposed to dim LAN (Fig. 1A and 1B), both phosphorylated Akt and MAPK in the breast cancer xenografts actually peaked at ZT14, rather than being decreased at this time point in animals on 12L:12D, indicating that their expression during the dark phase was disrupted. In contrast, total Akt and MAPK varied little over the 24 h period. Surprisingly, under LAN conditions PCNA was expressed at all time points in the breast cancer xenografts. No variation in Dynamin II was detected throughout the 24 h period under LAN conditions, although Dynamin II relative protein abundance at ZT14 was significantly elevated.

To directly compare the light associated changes in the signaling cascades between control 12L:12D- and experimental LAN-exposed groups, expression of signaling molecules in breast cancer xenografts are shown side by side in Fig. 1C. Most strikingly, PCNA is elevated at ZT6 through ZT22 in the LAN breast cancer xenografts. Also, elevated Akt phosphorylation was observed in the LAN breast cancer xenografts at ZT6, ZT14, and ZT18 but not at ZT2 and ZT22. We next determined whether activation of signaling molecules upstream and downstream from Akt in the breast cancer xenografts was stimulated by LAN.
Elevated GSK3β phosphorylation followed the same pattern as Akt phosphorylation. In contrast, PDK1 phosphorylation closely correlated with LAN-stimulated PCNA. Elevated MAPK phosphorylation in the LAN breast cancer xenografts was most evident during the dark phase, ZT14, ZT18, and ZT22. LAN had no effect on Dynamin II in breast cancer xenografts but GAPDH, which is often used as a loading control, was elevated at ZT6 through ZT22 as compared with controls.

**Modulation of signaling molecules in breast cancer xenografts but not liver from nude rats exposed to light at night**

Our results showing that PDK1 activation correlates with PCNA expression suggest that PDK1 may play a coordinating role in regulating LAN-stimulated cell proliferation in breast cancer xenografts. To further explore the role of PDK1, we determined the protein abundance of PKC isoforms in breast cancer xenografts. Because PKC isoform protein stability is regulated by PDK1-mediated phosphorylation (33), we reasoned that PKC would correlate with PDK1 activation in breast cancer xenografts. Indeed, PKCβ and PKCδ were elevated in LAN breast cancer xenografts at ZT6 through ZT22 (Fig. 2A), which closely correlates with LAN stimulated PCNA and PDK1 activation. In contrast, PKCα was elevated in control breast cancer xenografts at ZT6 through ZT22, except for ZT18 in the mid dark phase. To determine whether LAN regulates PCNA and PKC isoform in a non-tumor tissue, liver tissue was harvested from nude rats bearing MCF-7 SR- tumors at the same time points that the breast cancer xenografts were collected. As shown in Fig. 2B, LAN has no effect on PKCα, PKCβ, PKCδ, PCNA, or Dynamin II in the liver. In addition, LAN had no effect on Akt, MAPK, and PDK1 activation in the liver (data not shown). Taken together, these data suggest a highly regulated diurnal control of signaling protein abundance in breast cancer xenografts.

**Modulation of IGF-1 receptor signaling in human breast cancer xenografts and liver from nude rats exposed to light at night**

Next we determined whether protein abundance of signaling molecules upstream of PDK1 is regulated in breast cancer xenografts by LAN. As shown in Fig. 3A, IGF-1R β-subunit and IRS-1 were elevated at ZT6 through ZT22 in LAN breast cancer samples as compared with controls, which closely correlates with LAN stimulated PCNA expression and PDK1 activation. Further, IRS-1 Ser312 phosphorylation, which is a physiologically relevant phosphorylation site for insulin signal transduction (34), is elevated at ZT10 through ZT22 in the LAN group. Protein abundance of the IGF-1R β-subunit and IRS-1 was next determined in the liver of tumor-bearing rats exposed to the control lighting condition (Fig. 3B and 3C). IGF-1R β-subunit levels were elevated in the early light phase (ZT2) and the mid to late...
dark phase (ZT18 and ZT22), while maximal IRS-1 levels were observed in the late dark phase (ZT22). The diurnal control of IGF-1R β-subunit and IRS-1 was lost and their abundance was elevated throughout the 24-hour period in nude rats exposed to the LAN (Fig. 3B and 3C), indicating that LAN disrupts the diurnal control of the IGF-1R and IRS-1 protein in both tumor and non-tumor tissues.

**Modulation of circulating IGF-1 in tumor-bearing nude rats exposed to light at night**

Our data show that molecules in the IGF-1R signaling pathway are controlled in a diurnal manner in breast cancer xenografts and that LAN disrupts the control. Thus, it was reasonable to postulate that circulating IGF-1 (the ligand with highest affinity to the IGF-1R) is controlled in a diurnal manner and that LAN disrupts the control and, therefore, may mediate LAN-induced activation of IGF-1R signaling in the breast cancer xenografts. As shown in Fig. 4, under 12L:12D control lighting conditions, the nadir of serum IGF-1 levels was at ZT2 which was immediately followed by the peak in serum IGF-1 levels during the mid-light phase at ZT6, followed by a decrease at ZT10 and a second minor peak during the mid-dark phase at ZT14. In rats exposed to LAN, the diurnal control of serum IGF-1 was disrupted with levels being significantly elevated at ZT2, ZT10, ZT18, and ZT22. The high circulating concentration of IGF-1 in LAN-exposed rats also correlates well with the LAN-induced changes in the IGF-1R system in both liver and tumor groups (Fig. 3). Thus, our data suggest that circulating IGF-1 governs IGF-1R/PDK1 signaling in a diurnal manner.

**PDK1 knockdown in MCF7 cells reduces cell proliferation in vitro**

To confirm the importance of PDK1 in breast cancer cell growth, we generated stable MCF-7 SR-cell lines using lentiviral-mediated shRNAs to reduce PDK1. As shown in Fig. 5A, an approximately 90% reduction in PDK1 but not GAPDH was observed in two independent PDK1 knockdown cell lines, 1253PDK1 and 1617PDK1, compared to cells expressing a control luciferase shRNA. The effect of PDK1 deficiency on cell proliferation was measured using *in vitro* cell proliferation assays. The proliferation rate of the two PDK1 deficient cell lines was significantly slower than that of the parental and control luciferase cells at two separate time points (Fig. 5B and 5C). These results suggest that loss of PDK1 reduces MCF-7 cell proliferation *in vitro*, and further supports a role for PDK1 in breast cancer growth.

**PDK1 knockdown in MCF7 cells reduces breast cancer growth in vivo**

To test whether PDK1 plays a role in breast cancer tumor growth *in vivo*, we implanted MCF-7 SR-cells, stable control luciferase MCF-7 SR- cells or PDK1 knockdown MCF-7 SR- cells into the flanks of athymic nude mice. An approximately 40% and 80% reduction in PDK1 protein was observed in tumors
formed from the 1253PDK1 and 676PDK1 knockdown cell lines, respectively, while an approximately 50% and 90%, respectively, reduction in PDK1 Ser256 phosphorylation was observed (Supplemental Fig. 1). Latency to onset of tumor appearance for the parental and luciferase control cell lines was 15 days (Table 2). In contrast, latency to onset of tumor appearance for the 1253PDK1 and 676PDK1 knockdown cell lines was 17 and 21 days, respectively, suggesting that PDK1 knockdown impaired tumor growth. Consistent with this observation, tumor growth rates for the two PDK1 knockdown xenografts was significantly different from the control xenografts (Table 2). A reduction in final tumor weights for the two PDK1 knockdown xenografts was also observed. Taken together, these results indicate that PDK1 plays a role in regulating breast cancer growth both in vivo and in vitro.

Discussion

A variety of molecular, genomic and epidemiological evidence has linked cell cycle regulation and the circadian clock (35). Most studies of circadian cell-cycle gene regulation have been focused on the components of the transcription/translational-based autoregulatory feedback loops of clock genes that constitute the circadian clock machinery in animals (36, 37) while little is known about how circadian control of extracellular molecules modulate intracellular signaling cascades regulating cancer cell proliferation and tumor growth. Further, a major unanswered question is how disruption of the central circadian clock influences cell proliferation/signal transduction in the periphery and in particular in tumors. Our study establishes that artificial LAN, even at a minimal level, disrupts the circadian rhythm of circulating IGF-1 and IGF-1R signaling, including PDK1 activation which correlated with an upregulation in PCNA and most importantly, accelerated human breast cancer xenograft growth.

Cell proliferation is precisely coordinated and controlled by a number of proteins, including PCNA. In the nucleus, PCNA acts as a cofactor for DNA polymerase, participates in DNA synthesis, and functions in cell cycle progression. Not surprisingly, PCNA mRNA and protein levels are generally low in quiescent cells, but in highly proliferative cells, including cancer cells where PCNA is expressed at elevated levels (38). Consequently, PCNA is widely used as a marker for proliferation in cancer tissues (39). It is well established that the cell cycle is under circadian control in proliferative tissues. However, it has not been reported that PCNA expression has rhythmicity or is diurnally controlled. In the present study, we found that PCNA in the tissue of xenografted human breast cancer reached its peak in the early light phase but remained at very low levels throughout the rest of the 24 h period. In contrast, LAN elevated PCNA at ZT6 through ZT22 in the breast cancer xenografts, thus disrupting the normal diurnal
expression of PCNA. This suggests that continuously elevated PCNA could play an important role in accelerated tumor growth in xenografted human breast cancer.

Our finding that PDK1 activation correlated with PCNA in LAN breast cancer xenografts and that PCNA was unaffected in the liver of LAN-exposed rats, suggested that a pro-growth program was activated in part at the level of protein abundance in the breast cancer xenografts. Therefore, given the critical role that PDK1 plays in propagating the IGF-1 signal, we questioned whether: 1) circulating IGF-1 was modulated by exposing rats to LAN and 2) protein abundance of signaling molecules in the IGF-1 signaling pathway both upstream and downstream of PDK1 were regulated in breast cancer xenografts by LAN. First, we found that LAN disrupted the normal circadian rhythm of IGF-1 by elevating the circulating levels of IGF-1 throughout the 24 h period. Second, we found that IGF-1R, IRS-1, PKCβ, and PKCδ expression correlated with PDK1 activation and PCNA expression in LAN breast cancer xenografts. Surprisingly, PKCα inversely correlated with PDK1 activation and PCNA expression in LAN breast cancer xenografts. However, PKCα has been shown to promote apoptosis in MCF-7 cells (40), inhibit proliferation in MF-10A cells (41), and inhibit cell growth in epithelium (42), suggesting that PKCα functions to block growth in highly proliferative tissues. Taken together, these data suggest that a highly regulated pro-growth program involving the IGF-1 signaling pathway is activated in breast cancer xenografts in rats exposed to LAN. Consistent with this conclusion, overexpression of signaling molecules in the IGF-1 pathway has been well documented in cancer tissues (22, 43-46).

PDK1 is essential for mouse embryonic development and appears to regulate cell size, but not cell proliferation in normal mouse tissues (47). However, PDK1 appears to function differently in cancerous cells. In cancer cells with constitutive MAPK activation, PDK1 regulates cell proliferation (48), while in human breast cancer, cell proliferation and tumor growth are potentiated by PDK1 (48). Further, PDK1 activation and overexpression are commonly found in human breast cancers with PIK3CA mutation (45). Our results show that LAN-induced PDK1 activation occurred in the context of disrupted Akt and MAPK activation in the MCF-7 SR- cells, which indicates that multiple signaling pathways are modulated by LAN-mediated circadian disruption. Whether PDK1 plays a critical role in mediating the LAN-induced pro-growth program in the breast cancer xenografts requires further and more formal testing. Although, our results showing that PDK1 regulates MCF-7 SR- cell proliferation in vitro and tumor growth in vivo is consistent with the hypothesis that PDK1 plays a pivotal role in regulating breast cancer cell growth (6, 49, 50). Whether pineal melatonin or some other output of the central circadian clock and its disruption by LAN is involved in regulating IGF-1 and the IGF-1R/PDK1 signaling cascade cannot be determined from the current investigation and requires additional study.
In summary, we have identified a novel, significant disruption of PCNA in an animal model of human breast cancer. Our results suggest that LAN-induced disruption of diurnal control in the IGF-1 and the IGF-1R signaling cascade accelerates tumor growth in vivo through continuous up-regulation of PCNA, providing a new avenue for potentially effective cancer therapy based upon circadian biology and a new mechanism by which breast cancer growth in vivo is regulated.

References


Figures Legends

Figure 1. Light at night modulation of signaling molecules in tissue-isolated MCF-7 SR- human breast cancer xenografts in vivo. Diurnal regulation of signaling molecules in breast cancer xenografts from nude rats exposed to a control 12D:12L light/dark cycle (A, left panel), to light at night (LAN) 12D:12L (0.2 lux at night) (A, right panel) and to both (C). At each different four-hour interval time points (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22), tumor were removed from six animals. Total lysates (four representative samples per each time point, 40 μg of protein per sample) from tumor tissues were analyzed by immunoblotting for Akt, phospho-Akt Ser473 (pAkt), MAPK, phospho-MAPK Thr202/Tyr204 (pMAPK), and PCNA expressions. Dynamin II (Dyn II) was used as a control for the equal loading. B. Quantitation of the immunoreactive bands minus background from the immunoblots presented in (A) are shown as the means +/- SE. Control (■) and LAN (○). Statistical differences between Control and LAN were identified by two-way ANOVA, followed by Tukey’s test for pair-wise comparisons (*, p < 0.05; **, p < 0.01; ***, p < 0.001). C. Total lysates (two representative samples per each time point, 40 μg of protein per sample) from tumor tissues were analyzed by immunoblotting for Akt, pAkt, phospho-PDK1 Ser256 (pPDK1), phospho-GSK3β Ser9 (pGSK3), MAPK, pMAPK, PCNA, and GAPDH expressions. Dynamin II (Dyn II) was used as a control for the equal loading.

Figure 2. Modulation of signaling molecules in tissue-isolated MCF-7 SR- human breast cancer xenografts but not liver from female nude rats exposed to light at night (LAN). Circadian regulation of signaling molecules in breast cancer xenografts from nude rats exposed to a control 12L:12D light/dark cycle or LAN 12D:12L (0.2 lux at night). Total lysates from tumor tissues (A) and liver (B) (two representative samples per each time point, 40 μg of protein per sample) were analyzed by immunoblotting for PKCα, PKCβ, PKCδ and PCNA expressions. Dynamin II was used as a control for equal loading.

Figure 3. Modulation of IGF-1 signaling in tissue-isolated MCF-7 SR- human breast cancer xenografts and liver from nude female rats exposed to LAN. Total lysates from tumor tissues (two representative samples per each time point, 40 μg of protein per sample) (A) and liver (four representative samples per each time point, 40 μg of protein per sample) (B) were analyzed by immunoblotting for IGF-1R, IRS-1 and pIRS-1 (Ser312) expression. Dynamin II was used as a control for equal loading. C. Quantitation of the immunoreactive bands minus background from liver tissue immunoblots (B) are shown as the means
+- SE. Control (■) and LAN (○). Statistical differences between Control and LAN were identified by two-way ANOVA, followed by Tukey’s test for pair-wise comparisons (**, p < 0.01; ***, p < 0.001).

**Figure 4.** Modulation of IGF-1 in tumor-bearing nude rats exposed to LAN. Circulating concentrations of IGF-1 in tumor bearing nude rats exposed to control 12D:12L (●) and LAN 12D:12L (0.2 lux at night) (◇). Statistical differences between Control and LAN were identified by two-way ANOVA, followed by Tukey’s test for pair-wise comparisons (**, p < 0.01; ***, p < 0.001).

**Figure 5.** PDK1 knockdown in MCF7 SR- cells inhibits cell proliferation in vitro. Stable PDK1 knockdown MCF-7 SR- cell lines were generated as described in the Materials and Methods. A. Immunoblotting analysis of PDK1 and PCNA expression. B and C. MCF-7 SR- parental (■), Luc (□), 1253PDK1 (△), and 1617PDK1 cells (☑) were starved for 16 hours before the treatment of 5% FBS. In vitro cell proliferation was evaluated by MTT (B) and CyQuant (C) assays. Fold stimulation is shown as the means +/− SE.
Table 1. Latency to onset and tumor growth in tissue-isolated MCF-7 SR- xenografts

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Latency to Onset (days)</th>
<th>Growth Rate (g/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Light at Night</td>
<td>11</td>
<td>0.56 ± 0.03*</td>
</tr>
</tbody>
</table>

Data was analyzed by ANOVA (*, p < 0.05)
Table 2. Latency to onset and tumor growth in PDK1 knockdown MCF-7 SR- xenografts

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Latency to Onset (days)</th>
<th>Growth Rate (g/day)</th>
<th>Final Tumor Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>15</td>
<td>0.148 ± 0.038</td>
<td>2.78 ± 1.59&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Luc</td>
<td>15</td>
<td>0.166 ± 0.019</td>
<td>3.40 ± 1.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>676PDK1</td>
<td>21</td>
<td>0.104 ± 0.046*</td>
<td>1.78 ± 0.66&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1253PDK1</td>
<td>17</td>
<td>0.090 ± 0.018*</td>
<td>1.76 ± 0.58&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

MCF-7 SR(-) (Parental), MCF-7 SR(-) luciferase control (Luc), and MCF-7 SR(-) PDK1 knockdown (676PDK1 and 1253PDK1) cells. Data was analyzed by ANOVA (*, p < 0.05).

<sup>a</sup>Final tumor weight was determined 39 days after latency to onset

<sup>b</sup>Final tumor weight was determined 34 days after latency to onset
Figure 1
Figure 4

![Graph showing changes in IGF-1 levels over Zeitgeber Time (ZT) with and without LAN exposure.](image)

- **Con**
- **LAN**

**ZT2** **ZT6** **ZT10** **ZT14** **ZT18** **ZT22**

**darkness**

**Zeitgeber Time (ZT)**

- **IGF-1 (µg/ml)**
  - **Con**
  - **LAN**

- **Significance:**
  - `*` for statistical significance
  - `**` for highly significant

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Figure 5
Light at night activates IGF-1R/PDK1 signaling and accelerates tumor growth in human breast cancer xenografts

Jinghai Wu, Robert T. Dauchy, Paul C Tirrell, et al.

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