The circadian rhythm is established by a coordinated network of peripheral clocks interlocked and regulated by a central pacemaker. This network is maintained by the rhythmic expression of core clock genes, which in turn generate oscillatory expression patterns of different sets of target proteins in a tissue-specific manner. Precise regulation of biological processes driven by the body’s circadian network in response to periodic changes in the environment determines healthy life. The delicate balance in the cycling of enzymes, metabolites, cofactors, and immune regulators is essential to achieve cellular homeostasis. Disruption of this circadian homeostasis has been linked with the development and progression of various diseases including cancer. Over the years, circadian regulation of drug metabolism and processing has been employed in the treatment of diabetes, hypertension, peptic ulcers, and allergic rhinitis. Although time dictated drug administration was demonstrated many decades ago, its application in cancer treatment is limited due to insufficient mechanistic data supporting experimental results and inconsistency between clinical trials. However, timed administration of anti-cancer drugs is rapidly gaining attention as studies with animal and human models unveil molecular intricacies involved in the circadian control of biological pathways. In this regard, striking a balance between maximizing tumor responsiveness and minimizing side effects is crucial to achieve positive patient outcomes. This review focuses on regulation of the circadian clock in carcinogenesis outcomes through DNA damage and repair mechanisms and its application in therapy with specific emphasis on skin and breast cancers.
INTRODUCTION

Our daily intrinsic cues for wakefulness, hunger, and sleepiness repeat in about 24-hour cycles driven by our body’s internal rhythms. This circadian rhythmicity evolved as a survival strategy in living organisms and is generated through a genetically-encoded central master clock and unique peripheral clocks in different tissues of the body. Most cellular processes are coupled to this circadian network, which modulates their functions appropriately in response to light-dark and temperature cycles caused by the earth’s rotation in order to sustain life in a best-fit manner [1-5].

However, circadian clocks are subject to disruption, either environmentally from modern living conditions and behaviors such as chronic jetlag, rotating night shift work, and excessive exposure to artificial light at night, or genetically through mutations or altered function in the clock genetic circuit [3,6]. Given that the circadian clock regulates various biological processes, it is not surprising that disruption of the clock may be a plausible cause for the development of various diseases including sleep and metabolic disorders, cardiovascular and Alzheimer’s diseases, and several cancer types [6-9]. Cancer is one of the most studied diseases with respect to circadian disruption. Growing scientific evidence confirms the altered expression patterns of core clock proteins and disruption. Growing scientific evidence confirms the altered expression patterns of core clock proteins and disruption of the circadian clock in DNA damage and repair, carcinogenesis and the up-and-coming treatment strategies, which utilize the body’s clock to achieve better prognosis in cancer patients.

THE CIRCADIAN CLOCK AND ITS FUNCTION IN CELLULAR HOMEOSTASIS

Circadian Clock

In mammals, the suprachiasmatic nucleus (SCN), which houses the master clock, in the anterior hypothalamus perceives signals through visual photoreception and synchronizes peripheral clocks present in other organs of the body including heart, liver, lungs, breast, and skin. This master clock is periodically calibrated through light perception in the morning. One of the vital features of circadian clocks is that they are endogenously driven without any environmental cues due to the self-sustained transcription-translation feedback loop (TTFL) [3,20,21]. As shown in Figure 1, the TTFL is subdivided into the primary feedback loop which is comprised of proteins, namely: brain and muscle ARNT-like protein-1 (BMAL1), circadian locomotor output cycles kaput (CLOCK), cryptochrome (CRY1, CRY2), and period (PER1, PER2, PER3), and the secondary feedback loop made up of retinoic acid orphan receptor alpha (ROA) and reverse-erb receptor alpha (REV-ERB) proteins [1]. CLOCK and BMAL1 dimerize to form a functional transcriptional complex, which binds to specific E/E’-box sequences (CACGTG/CACGTT) in the promoter regions of various target CCGs. The primary transcriptional targets of CLOCK/BMAL1 are PER and CRY genes, which upon transcription and translation, form a heterodimer and shuttle back into the nucleus to bind to BMAL1/CLOCK heterodimeric complex, in order to inhibit its activity [1,22-24]. Simultaneously, the secondary feedback loop components regulate the levels of BMAL1, with ROA increasing and REV-ERB decreasing transcription of ARNTL gene (which encodes BMAL1 protein), thereby maintaining a circadian rhythm of about 24 hours [25]. This 24-hour circadian rhythm influences about 43 percent and 50 percent of protein-coding genes at transcript levels in mice and humans respectively. This influential
rhythmicity of protein-coding genes is collectively from different tissues in the body, in a tissue-specific manner [14,15]. Importantly, different sets of CCGs have been found to exhibit phase variability in different tissues correlating to their tissue-specific functions [26]. Thus, the circadian system regulates various cellular processes such as metabolism, signaling networks, DNA repair, cell cycle, and proliferation. Two of such cellular processes, cell cycle and nucleotide excision repair, will be briefly discussed in this review.

Circadian Control of Cell Cycle and DNA Damage Checkpoint Signaling

The cell cycle is a core cellular mechanism that malfunctions in many cancer types leading to uncontrolled cellular proliferation [27]. Timed progression through various checkpoints in the cell cycle is crucial for maintaining genome stability in normal cells [10,28]. The circadian clock influences the cell cycle either transcriptionally or by protein-protein interactions. For instance, WEE1 kinase, which acts at the G2/M checkpoint, is transcriptionally activated by BMAL1/CLOCK and repressed by PERs or CRYs [29]. Similarly, expression of p21, which inhibits the entry of cells from G1 into S phase, is regulated by BMAL1, and also by PER1 through p53-independent mechanisms, likely through c-MYC stabilization [30,31]. Moreover, PER1 promotes cell cycle arrest during DNA damage by directly interacting with ataxia telangiectasia mutated (ATM) at a protein level, which is responsible for phosphorylation of checkpoint kinase 2 and activation of p53 [32-34]. Also, PER2 helps to stabilize p53 by modulating its ubiquitination through E3 ubiquitin-protein ligase mouse double minute 2 homolog (MDM2) [35]. These interactions are likely mediated by key regulatory factors of the cell cycle and clock, which regulate the coupling or uncoupling of these two systems.

In vivo studies on mouse models have provided physiological relevance to elucidate the mechanistic role of the circadian clock in regulating the cell cycle of the skin and its susceptibility to DNA damage. Our group showed DNA replication to be time-of-day dependent in mouse skin epidermis, with higher BrdU incorporation during DNA synthesis in the morning compared to the evening. To confirm that replication is under the control of the circadian clock, the time-of-day variation of replication was lost in Cry1/2-/- mice [36]. Geyfman et al. further demonstrated that time-of-day dependent cell proliferation in the interfollicular epidermis and upper hair follicles is controlled by BMAL1 intrinsic to keratinocytes. Transcriptome analysis identified distinct circadian genes in anagen (growing) and telogen (resting) skin with the latter exhibiting higher number of oscillating genes. Specifically, the majority of cell cycle- and metabolism-associated genes were elevated in the early morning and early evening respectively. Interestingly, Bmal1-/- mice not only lost circadian control of cellular proliferation but also exhibited a constant elevated cell
proliferation rate indicating a suppressive role of the core clock protein BMAL1 on cell cycle in the interfollicular epidermis [37]. Another intriguing study which utilized abnormal food intake (restricted feeding) as a skin clock disruption strategy observed decreased proportion of cells in S phase, though the shift in clock phase did not alter the phase of DNA synthesis in proliferating interfollicular progenitor cells [38]. This finding suggests that the peripheral clock may not be solely responsible for determining the phase of cell cycle in skin epidermis, even though it is a crucial regulator of cell proliferation [38]. Nevertheless, the importance of the clock control on the cell cycle has also been demonstrated through increased BMAL1-dependent susceptibility of the skin to reactive oxygen species- and UVR-induced DNA damage when the cells are in S-phase [37,38]. These findings collectively demonstrate that the circadian control of skin proliferation is a protective measure against endogenous and UVR-induced genomic instability.

**Circadian Regulation of Nucleotide Excision Repair**

Nucleotide excision repair (NER) is an exclusive repair mechanism that acts on bulky genomic DNA lesions caused by UV radiation (UVR), cigarette smoke, and platinating agents including cisplatin [13,36]. UVR induces DNA damage through the formation of adjacent bonds between cytosine (C) and thymine (T) residues to create two types of bulky photoproducts, namely: cyclobutane pyrimidine dimers (CPDs) and (6-4) pyrimidine-pyrimidone photoproducts ((6-4) PPs). Consequently, DNA damage response (DDR) signaling gets activated to halt cellular processes such as DNA replication, and initiates DNA repair and/or apoptosis [39-41]. The repair of bulky DNA adducts requires the coordinated activities of numerous repair factors including XPA, XPC, RPA, TFIIH complex, XPG, and XPF-ERCC1. This sequence of events will excise and seal the damaged oligonucleotide containing the lesion. The NER machinery operates through two sub-pathways: transcriptional coupled repair (TC-NER) repairs lesions of actively transcribed genes recognized by RNA polymerase II; global genomic repair (GG-NER) repairs global damage in the genome. These sub-pathways differ in the way the DNA damage is recognized [39,42,43].

The effect of time-of-day is particularly evident in excision repair of UVR and cisplatin-induced DNA damage. Rhythmic NER activity has been observed in brain, liver, and skin tissues—except testis of mice [36,44,45]. Clock control of the NER pathway is executed through direct binding of BMAL1 to E-box sequences in the promoter region of \( \chi_{pa} \) [46]. This results in rhythmic expression of Xpa protein, which is rate limiting in the NER process [44]. In mouse skin, it was shown that DNA replication and excision repair are counter-phased. NER peaks in the evening and slows down in the morning; replication activity peaks in the morning and slows down in the evening [36,37,47]. Consequently, this circadian connection between DNA replication and NER has been exploited in understanding the initiation and progression of UVR-induced skin cancer. UVR-induced DNA damage is the primary causative agent for skin carcinogenesis and since NER activity is directly controlled by the clock, we previously reported up to a five-fold increase in invasive squamous cell carcinomas, when mice were exposed to solar UVR in the morning than in the evening, corresponding to times of decreased and increased NER activity respectively [36]. Since humans are behaviorally anti-phased to mice, humans are expected to have a higher NER rate in the morning when there is higher exposure to UVR compared to the evening [48]. Interestingly, a recent study confirmed this proof-of-concept by showing less sunburn erythema in human subjects exposed UVR in the morning compared to the evening [49,50].

**CIRCADIAN DISRUPTION AS A DRIVING FACTOR IN CARCINOGENESIS**

In the modern competitive world, shift work, particularly at night and across different time zones, is part of the work culture in many industries including law enforcement, healthcare, and technology [6]. These conditions render excessive exposure to artificial light at night and disrupted feeding patterns, which result in circadian misalignment and potential disruption of the CCGs expression pattern [6,51,52]. Several studies conducted with shift workers have shown strong positive associations between shift work and increased risk of various cancers, including skin and breast cancers [53,54]. Based on the available epidemiological evidence, the International Agency for Research on Cancer (IARC) declared that “shiftwork that involves circadian disruption” is a probable carcinogenic factor in humans [55].

Changes in the sleep-wake cycles and feeding routine trigger incongruence in metabolic pathways leading to an imbalance in cellular homeostasis [56,57]. When these changes are chronic, it increases the propensity of metabolic disorders and cancer [56]. Melatonin, a pleiotropic hormone that regulates the sleep-wake cycle and is impacted by light exposure, exerts anti-cancerous effects through multiple pathways in breast cancer by binding to its receptor MT1 [58,59]. It was shown that increased levels of melatonin protect the skin from UVR damage [58]. Furthermore, reduced levels of the nocturnal melatonin in rodents by exposure to artificial light at night enhanced breast tumor development and increased intrinsic resistance to the chemotherapeutic drug tamoxifen compared to the animals under normal 12-hour light/dark cycles [59]. Using genetic mouse models to under-
Clock Dysregulation in Breast Cancer

Breast cancer has been long implicated in circadian disruption outcomes. In the United States, breast cancer is the most common cancer, apart from skin cancers, and is the second leading cause of death in women [66]. The increased incidence of breast cancer is attributed partly to lifestyle changes in the modern world that are often associated with circadian clock dysregulation.

Clock Dysregulation drives carcinogenesis events. In response to genotoxic stress agents, a healthy circadian clock protects the genome through regulating cell cycle and DNA repair mechanisms to restore the cell to normal function. However, with circadian disruption, both cell cycle and DNA repair pathways are compromised leading to increased DNA replication errors and genomic instability ultimately causing carcinogenesis.
Indeed, several epidemiological studies have demonstrated a strong association between clock disruption and malignancy of breast cancer [54,67]. Two independent research groups reported the absence of rhythmic transcriptional oscillations in canonical circadian genes of breast cancer cells. Interestingly though, these clock genes rhythmicity was reset by the host’s circadian clock or serum shock [68,69]. A detailed study on the expression pattern of the core clock genes revealed a distorted expression profile of ARNTL and PER2 genes in malignant breast cancer cells irrespective of their receptor status [70]. Based on these studies, it is clear that aberrant or broken circadian rhythm is a common feature of many breast cancer cell lines and tissues, specifically the aggressive metastatic carcinomas. Further, the disruption of circadian rhythm is often associated with poor prognosis of breast cancer.

Circadian dysregulation has been associated with breast cancer mainly through the estrogen receptor alpha (ERα, which is encoded by ESR1 gene) signaling network. ERα plays a pivotal role in mammary cell proliferation and mediates hormonal response during puberty [71]. PER2 suppresses transcriptional activation and induces degradation of ERα, but is in turn inducible by estrogen [71]. In breast cancer cells, PER2 functions as a tumor suppressor as the overexpression of PER2 hinders cell proliferation and promotes apoptosis while its knockdown increases ERα-related cell proliferation [72]. Numerous studies also reported low transcript levels of PER2 in breast cancer cells compared to normal breast cells [73-76], suggesting a critical role for PER2 as a tumor suppressor in breast cancers.

**HARNESSING THE CLOCK AS A STRATEGY FOR CANCER TREATMENT**

The concept of "chronotherapy"—the timed administration of treatment—was documented over three decades ago. This treatment strategy harnesses the endogenous circadian cycles to achieve optimal efficacy and minimize side effects for a particular therapy [16]. Given that two of the major limitations with cancer therapy are tumor resistance and toxicity in healthy cells, which can range from mild to life-threatening, chronotherapy provides a promising tool to naturally exploit the specific time of the day, when cellular processes of DNA replication, targeted gene expression, and DNA repair are highly vulnerable in tumor versus normal cells [4,16]. Chemo- and radiation-therapies are widely used in the treatment of most cancers either solely or in combination with other regimens as adjuvant or neoadjuvant options [13]. Importantly, the knowledge of circadian biology has been applied to improve these treatment strategies. Despite the promising prospects of chronotherapy, its application is not yet broadly applied in the clinic. There are a number of successes and failures in studies involving chronotherapy (further discussed below). The failures can be attributed to interindividual variability (including genetics) and hierarchical complexities from molecules to systems in humans. The effectiveness of chronotherapy will depend on a broad range of factors which include uptake/efflux, distribution, metabolism, and elimination [77], which are not yet fully accounted for in chrono-administration of therapy. Though a lot remains unknown, significant progress has been made in the area of DNA repair from the mechanistic to clinical applications of cancer therapies, especially with chronotherapy.

**Chronochemotherapy: Emerging Molecular Connections in Cisplatin-induced Adduct Repair**

Cisplatin or cis-Diaminedichloroplatinum (II) is a very commonly used chemotherapeutic drug. It acts in cells by forming direct crosslinks with purine bases in DNA resulting in bulky adducts, thereby affecting cellular processes of DNA replication, transcription, and DNA repair [78]. Over 30 years ago, a few clinical and in vivo animal studies observed significant advantages of timed dosages of chemotherapeutic agents such as cisplatin and oxaliplatin. One of the premier findings was made in advanced ovarian cancer patients where the administration of cisplatin in the morning resulted in greater delays, reduction, modification, and attenuation of treatments due to toxicity compared to the evening administration [79]. Another similar study in rodents which used oxaliplatin for the treatment of colorectal cancer found encouraging results with evening administration resulting in a threefold increase in tolerability compared to morning administration [80]. This observation was later successfully translated to patients [81]. Some of the well documented clinical studies in renal cell carcinoma, breast and lung cancers that employed circadian timings in treatment administration registered lower toxicity levels at certain times [82-84]. Significantly, more than 40 chemotherapeutic drugs showed dramatic variations in efficacy or tolerance concerning the time of administration in rodent models [85].

Recently, our group has found a mechanistic explanation for the observed clinical outcomes from the previous studies. Using melanoma mouse models, we demonstrated that the time-of-day of administration of cisplatin influenced renal and blood toxicities. There was an enhanced rate of removal of cisplatin-DNA adducts through NER in wild-type mice treated in the evening, and subsequently less toxicity compared to mice treated in the morning, a variation that was lost in genetically clock disrupted (Per1/2 mutated) mice. Interestingly, observations from blood cells, treated ex vivo with cisplatin, from human subjects simulated to day and night sched-
Circadian rhythm was an important factor in dictating hematopoietic stem cell response to ionizing radiation in rats [91,92]. A few decades later, a handful of studies have investigated the effect of circadian timing on radiation therapy (RT) in humans. Patients with non-small cell lung carcinoma (NSCLC), head and neck, cervical, prostate, and breast cancers have been subjected to RT by time-of-day (morning versus evening) and unfortunately, most of the results to date are conflicting [93-100]. Nevertheless, findings have been made using mouse models on the role of the clock in radiation-induced DNA damage response. One of the common side effects of radiation treatment is hair loss and subsequent dermatitis. Plikus et al. identified that mice hair possessed a robust autonomous circadian clock machinery, which translated to periods of maximum hair follicle growth (mornings) and maximum hair follicle repair or restoration (evenings) [101]. Notably, after intense RT there may be no hair regrowth due to permanent damage to hair cells or regrowth may take as long as 6 months. Studies in mice demonstrated increased hair loss upon exposure to γ radiation in the morning when the percentage of mitotic cells were high and Xpa levels were low compared to the evening [101]. This is because radiation severely affects actively proliferating cells in their M phase and low repair rate causing accumulation of DNA damage that is not effectively repaired leading to excess hair loss. The circadian influence on the time-of-day sensitivity of growing hairs to radiation was further demonstrated in wild-type mice, and Cry1/2−/− mice – which did not exhibit rhythmic radioprotective effect [101]. Furthermore, Fu et al. have shown that the inactivation of Per2 gene by mutation sensitized mice to radiation by delaying p53 accumulation.
The implications of the hair circadian system can be translated into future chronotherapy treatment modules aiming at minimizing RT-associated side effects.

Two clinical studies have investigated the circadian regulation of RT in breast cancer patients. The first study published by Noh et al. treated 395 patients in the early morning and early evening times and observed that the early evening treated group experienced significantly higher acute skin reactions compared to the morning treated group [100]. However, a second study by Ishaq et al. involving 140 breast cancer patients who received radiotherapy in the morning, afternoon, and evening time periods showed no statistically significant differences in the incidence of radiation-associated fatigue or dermatitis [102]. The inconsistencies in these studies mean that further clinical and mechanistic investigation is warranted.

**Chronoimmunotherapy: Enhancing Immune Responses via Circadian Clock Regulation**

Immunotherapy is a very promising approach in the future of cancer treatment because it seeks to use the body’s immune system to fight cancer cells. Hence, the possibility of bolstering the immune system to fight off tumor cells will be a critical area of interest moving forward. Remarkably, different studies have demonstrated circadian control of the immune system, which will be discussed.

Time-of-day plays a role in lymphocyte trafficking in and out of the lymph nodes, which in turn regulates the adaptive immune responses. Rhythmic oscillations in pro-migratory factors CCR7 which peaks in the evening and S1P1 which troughs during the day causes lymphocytes (CD4+ T cells, CD8+ T cells, and B cells) to be retained in the lymph nodes during night time and migrate out into the bloodstream during daytime [103].

In understanding the role of the clock in immune response to tumors, our group demonstrated that circadian clock disruption, by the loss of *Per1/2* genes function, enhanced the immune response in melanoma tumors. There were significantly higher levels of CD4+ and CD8+ T cells in circulation and infiltrating the tumors, which rendered cisplatin treatment to be more effective in these mice compared with circadian efficient wild-type group [46]. This finding paves the way for potentially modulating the clock to enhance the adaptive immune response to tumors for immunotherapeutic applications, as well as in combination with other therapies.

In addition to the adaptive immune system, the innate immune system has also been shown to be under circadian control. Pro-inflammatory cytokines and chemokines such as IL-6 and CCL2 respectively secreted by the immune cells were shown to have a rhythmic secretory pattern and recruitment to tissues [104,105]. This circadian control of cytokine and chemokine expression was abolished in myeloid cells of *Bmal1* knockout mice after lipopolysaccharide (LPS) induction. Furthermore, *Clock*Δ19 mice and *Cry1/2* mutant and *Cry1/2−/−* mice showed reduced and increased expressions of cytokines respectively in response to LPS [106,107]. On the secondary arm of the circadian clock feedback loop mechanism, pro-inflammatory cytokines such as IL-6 are strongly regulated by Rev-erba at a molecular level, and in a *Nrt1d1* knockout mice, the circadian rhythmicity in the release of cytokines by macrophages was lost [104]. A mechanistic link between the clock and time-of-day expressions of macrophages was shown in myeloid cells, where *Bmal1* directly controls *Nrf2* transcriptional activity and antioxidant capacity by regulating IL-1β levels [108]. Further studies are still required to elucidate how the circadian system selectively regulates various immune components.

**Potential for Circadian Influence in Breast Cancer Treatment**

Everolimus (EV), a selector inhibitor of the mammalian target of rapamycin mTOR1 complex, is currently used along with anti-estrogens in the treatment of ER-positive breast cancer. Its mode of action is through cell cycle arrest by inhibiting mTORC1 downstream signaling which plays a crucial role in cell cycle progression and transcription [109]. Zhang et al. reported that the efficacy of G0/G1 cell cycle arrest varied up to 4-fold with different times of EV administration in MCF-7 breast cancer cells [110]. The authors showed that although serum shock did not synchronize core clock genes, it induced rhythmicity in mTOR activity, which in turn promoted circadian regulation of the key G1 phase progression proteins (Cyclin D1 and retinoblastoma protein). This study provides mechanistic proof that modulating the circadian clock and clock-controlled mechanisms may be harnessed in targeted therapy for optimal breast cancer treatments. This proof-of-concept can be applied to other forms of cancer, such as melanoma, where the mTOR pathway is also dysregulated [111].

**CONCLUSION: FROM BENCH TO CLINICAL PRACTICE**

The field of chronopharmacology holds significant promise. It deals with applying an endogenous remedy to reducing cancer therapy-associated toxicity while at the same time improving efficacy. However, the pipeline from bench findings to clinical translation or from clinical findings to mechanistic understanding remains quite hollow. Additional knowledge is essential to overcome hurdles in the applicability of chronomodulation in combination therapies, which is often mandatory for the treatment of most cancers. Currently, circadian timing of cancer treatment is not widely used in clinical practice mainly due
to inconsistencies between trials and lack of molecular mechanisms behind the clinical observations in chronotherapy trials. Nevertheless, the little we know so far is very significant and holds promise for the future. DNA damage response mechanisms, particularly XPA regulation by the circadian clock, can be a useful biomarker for personalizing chemotherapy in the clinical setting. Also, the modulation of the clock through pharmacological agents, behavior, and bright light therapy can be powerful tools to improve patient outcomes (Figure 3B). Additionally, the fact that the molecular basis for any cancer therapy rests upon the resistance or sensitivity of cells, which hinge on cellular proliferation and double-strand break repair, means that profiling the circadian clocks and CCGs in normal versus tumor cells is essential. Furthermore, recent advances in targeted gene delivery and integrated “omics” techniques will deepen our understanding of the circadian network in normal and cancerous tissues. Most of the understanding of clock-related genes is at the transcript level, but with newer advances in the field of quantitative proteomics and targeted gene delivery, our understanding of circadian-regulated biological processes can be expanded.

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