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Circadian disruption in cancer and regulation of cancer stem cells by circadian clock genes: An updated review

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ABSTRACT

Circadian rhythm, regulated by a time keeping system termed as the circadian clock, is important for many biological processes in eukaryotes. Disordered circadian rhythm is implicated in different human diseases, including cardiovascular disease, neurologic disease, metabolic disorders, and cancer. The stem like-cancer cells (or cancer stem cells, CSCs) are proposed to stand at the top of the heterogeneous hierarchy in different solid tumors, which are responsible for tumor initiation, development, therapy resistance and metastasis. Emerging evidence has shown that circadian clock genes potentially regulate the stemness and features of CSCs in several malignant systems, including leukemia, glioblastoma, breast cancer, colorectal cancer and prostate cancer. The chronotherapies targeting CSCs are therefore of therapeutic potentials in treating malignancies. In this review, we have summarized our current knowledge of circadian clock gene regulation in normal stem/progenitor cells. Moreover, we have provided evidence linking dysregulations of circadian clock genes and cancer development. Importantly, we have listed the potential mechanisms underlying circadian clock gene regulation of CSCs. Finally, we have offered the current attempts of chronotherapy targeting CSCs. Elucidating the molecular regulation of circadian clock gene in CSCs will provide us a novel direction for the development of therapeutics to target CSCs.

1. Introduction

Approximately 50%–80 % protein-coding genes in humans are regulated by the circadian clock, a time keeping system, which coordinates circadian rhythms of physiological, biological, and behavioral functions of eukaryotes [1]. The circadian clock is composed of the central clock in the suprachiasmatic nuclei (SCN) of the hypothalamus, and the peripheral clocks in different tissues, including lung, heart, liver, kidney, and skin, which in turn are regulated by the SCN clock [1]. In response to environmental signals, such as light, the SCN clock synchronizes the peripheral clocks and coordinates circadian outputs on a 24-h cycle [2]. In humans, many biological activities (such as sleep-and-wake cycles, blood pressure, hormonal regulations, metabolisms, body temperature) are circadian rhythmically controlled, and this time keeping system exist in every cell in the body that regulates rhythmic fluctuations by transcriptional, post-transcriptional, post--translational and epigenetic mechanisms [1,2]. Recently, a study demonstrated that exercise during the early active phase leads to bone growth in murine models, suggesting that circadian regulation is also crucial for bone health [3]. Thus, the circadian clock is crucially important in maintaining normal functions and homeostasis in all living organisms.

The core clock machinery consists of a complex but autonomously regulatory transcription-translation feedback loop (TTFL) [1,2]. In the loop, the basic helix-loop-helix heterodimeric transcription factors, BMAL1 (Brain and Muscle Arnt-Like protein) and CLOCK (Circadian Locomotor Output Cycles Kaput) build up the positive regulators and activate transcription of the target genes by binding to the E-box enhancer elements [1,2]. Several key circadian genes are the primary target genes, and they make up the repressor arm, including

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Cryptochrome (CRY1 and CRY2) and Period (PER1, PER2 and PER3), which repress BMAL1/CLOCK activities in the nucleus [1,2]. In addition, another regulatory loop consolidates the TTFL, and the loop is constituted by the nuclear orphan receptors, REV-ERBs (NR1D1/REV-ERBα, NR1D2/REV-ERBβ), and ROR isoforms (RORα and RORβ), which controls gene expression of BMAL1 [1,2] (Table S1). Earlier observations have found that the BMAL1/CLOCK regulates gene expressions of ~10 % of circadian genes that are vital for physiological, biochemical and molecular activities [1,2]. Moreover, other mechanisms also participate and coordinate the clock regulatory network, including kinases, non-coding RNAs and chromatin modifiers [1,2]. Therefore, circadian dysfunctions are linked to jet lag, light/night cycles, sleep disorder, and shift work, and are also likely involved in human pathological conditions such as cardiovascular disease, neurologic and psychological disorders, metabolic and immune diseases, and cancer [1,2,4–6] (Fig. S1).

2. Circadian regulation in normal stem/progenitor cells

Stem cells (SCs) are a small population of cells that can self-renew and have multi-lineage differentiation capacities, which are crucial for tissue regeneration and homeostasis [7]. An egg fertilized by a sperm initiates the cell division and leads to the formation of multi-cellular morula, at which stage cells are totipotent capable of giving rise to three germ layers, i.e., endoderm, mesoderm and ectoderm [7]. The embryonic stem cells (ESCs), derived from the inner cell mass of a blastocyst, are pluripotent whereas multipotent stem cells are able to differentiate into cells in the restricted tissues [7]. There is a large body of studies to investigate if circadian clock exists in either ESCs or adult stem cells, and how circadian clock genes may regulate normal stem/-progenitor cell homeostasis.

2.1. Studies of circadian clock regulation in ESCs

Embryonic development is complicated, and many studies aimed to understand the circadian clock regulation in ESCs have been conducted for a long while. Most observations have found that the circadian TTFL is absent in the early zygotes or gametes. For example, using a bioluminescence imaging system, Yagita et al. did not detect the circadian bioluminescence rhythm in the mouse ESCs *in vitro*, and expression of Sox2, Oct3/4, Klf4 and c-Myc genes led to the reprogramming of differentiated cells without circadian oscillation [8]. In support, other researchers also showed that the circadian clock components are present even before zygotic genome activation, but these clock genes do not constitute a functional feedback loop in the early stages of development [9]. Nevertheless, some reports have suggested that circadian TTFL may be gradually generated during the embryonic development [10]. For instance, Dierickx et al. have found that although the intrinsic functional clock was not seen in undifferentiated human ESCs, the oscillatory expression of core clock genes (BMAL1, CLOCK and PER2) was increased spontaneously during cardiac differentiation [11]. Moreover, this oscillatory network led to a rhythmicity in response to doxorubicin, a widely used anti-tumor drug with side effects in hearts [11]. Recently, Yagita and colleagues reported that premature expression of CLOCK/-BMAL1 interfered with the segmentation clock Hes7 oscillation and the regulatory network in the induced posterior mesoderm and mouse embryonic organoids [12], providing a piece of evidence why CLOCK/-BMAL1 mediated circadian feedback loop is suppressed in the embryonic stages. However, it is still not completely understood when TTFL is initiated during embryonic development, and what possible roles of the TTFL may play in early lineage commitment, all of which waits future in depth investigations.

2.2. Circadian clock regulation in adult stem cells

Hematopoietic stem cells (HSCs) are vital for regenerating blood and immune cells, and the circulation of HSCs as well as the relevant colony generating abilities under circadian clock regulation have been studied [13]. For example, Golan and the colleagues have demonstrated that light and dark onsets have respective peaks, which leads to the release of norepinephrine and TNF in different manners. During the day time, induced norepinephrine regulates HSC egress and differentiation, whereas at night, released TNF is able to promote renewal of HSCs depending on melatonin [13]. In line with this finding, others have recently found that the day and night oscillations of circulating HSCs can also be regulated via the cooperation of central and local cholinergic signals [14]. These studies have provided some evidence on whether and how circadian clock regulates HSCs. Moreover, earlier studies showed that Bmal1 knockout (KO) mice exhibited downregulation of CXCL12 and the release of HSC rhythms, indicating that CXCL12 is a key factor in regulating HSC levels in the blood [15]. In addition, daily clearance of aged neutrophils has been noted to be linked with the regulation of HSC egression. Follow-up studies have also implicated certain signaling mechanisms in regulating the HSC properties, including WNT/β-Catenin, NOTCH1 and TNF secretion [16–18]. A recent study has observed that migration of HSCs is associated with Nlrp3 inflammasome and heme oxygenase-1 (HO-1) activity [19]. Clearly, elucidating both direct and indirect mechanisms for controlling the HSC differentiation and maintenance will provide us more biological insights on the circadian regulation on HSCs.

Epidermal stem cells reside in the interfollicular epidermis, and/or in the basal layer of the skin, which have the proliferative capacities and can differentiate into keratinocytes to populate different layers of the skin [7]. During this process, the circadian clock genes are able to regulate cell proliferation and differentiation of the keratinocytes. For example, Geyfman and the colleagues have found that BMAL1 deletion in mouse keratinocytes led to enhanced cell proliferating phenotypes and elevated level of UVB induced DNA damage in the epidermis [20], suggesting that circadian clock gene (i.e., BMAL1) is able to control the time-of-day dependent cell proliferation. Recently, many studies have devoted to understand the circadian clock gene regulation in epidermal stem cells, and one of the intriguing directions is to determine the role of circadian clock in epidermal stem cell metabolism [7]. A study from Stringari et al. used a noninvasive, label-free, *in vivo* imaging system to examine the relationship between circadian oscillations and energy status in epidermal stem cells of live mice, at the single-cell level, by examining the metabolic levels of the NADH/NAD + ratio [21]. In this study, authors found that the NADH/NAD + ratio was higher during the night, which was related to a higher level of epidermal stem cells in S phase with increased levels of glycolysis [21]. Moreover, BMAL1 deletion in mice disrupted the daily fluctuations in NADH/NAD + ratio in epidermal stem cells, hinting that the circadian clock gene may orchestrate metabolic levels of epidermal stem cells [21]. On the other hand, epidermal stem cells are also located in the bulge of the hair follicle. Earlier studies showed that the circadian clock regulates the timing of the hair cycle, and BMAL1 KO in hair follicles of mice delayed anagen progression, which is initiated due to a block of the cell cycle in G1 phase [22]. In contrast, others reported that BMAL1 KO in keratinocytes of the hair follicles did not delay anagen progression with only a slight increase of S-phase cells in the hair follicles [20]. It is not completely clear the mechanisms underlying these contradictory results, but the latter study implies that changes in other factors may affect the cycling in hair follicles as BMAL1 is knocked out [20]. In line with this conjecture, recent studies indicated that external light promoted proliferation of stem cells in hair follicles through eyes via the hedgehog signaling [23]. Overall, circadian clock disruption in mouse models does not seem to elicit a strong phenotype in the hair follicles or the skin.

Previous studies have shown that the circadian clock may influence neurogenesis. For example, circadian rhythms could be observed in neurospheres as they differentiated into glia or neurons [24], and disruption of the circadian clock in Bmal1$^{-/-}$ mice significantly affected migration of neural progenitor cells [25]. These *in vitro* and *in vivo*

studies indicate that the clock genes may regulate neurogenesis by affecting proliferation, migration, and cell fate commitment. Interestingly, neural stem cell activation can also be regulated by N3/β3-adrenoceptor-mediated signaling via blocking BMAL1 [26], potentially implicating the circadian rhythm in controlling mental health.

In other organs/tissues, circadian regulations of adult stem cells have also been proposed. For instance, using a *Drosophila* model, Parasram et al. discovered that circadian clock functions in intestinal stem cells were regulated by WNT and HIPPO pathways [27]. Recently, the same group reported that the clock functions and the intestinal stem cell differentiation were incompatible [28]. In the long run, it will be helpful to uncover the precise mechanisms whereby the circadian rhythm regulates characteristics of intestinal stem cells in defined mammalian models.

3. Circadian disruptions in cancer

3.1. Epidemiological study of circadian regulation and cancer

Approximately 20% of the working population worldwide involves shift-work (including nightwork). Several studies have shown that employees engaged at night shifting work in the long term have higher incidence of solid cancers. In 2007, the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) concluded that shift work is probably carcinogenic to humans based on the epidemiologic and experimental studies [29], which was assessed and supported again in 2019 by IARC. One study examined 78,562 women from the Nurses' Health Study in a 10-year follow-up, who had at least 3 night-shifts in a month, and found a moderate increase in the incidence of breast cancer [30]. Stronger evidence regarding the relationship between breast cancer and night shifting work is supported by population-based and cohort-based case-control studies. For example, Cordina-Duverger et al. conducted a pooled study using data from 5 countries and included 6093 breast cancer patients and 6933 controls, and they observed a positive association of breast cancer risk and night shifting work, especially in pre-menopausal women [31]. Nevertheless, some other studies did not find that night shifting work is inherently carcinogenic. For instance, a generation study cohort from the United Kingdom using questionnaires recruited 102,869 women with a median follow-up of 9.5 years, and demonstrated no support for an increase in the breast cancer risk from women in night shifting work [32]. The seemingly inconsistent conclusions are also noted in prostate cancer (PCa). A population-based cohort study conducted in Germany evaluated if night work affects the incidence of PCa in a 10-year follow-up, and reported that men with night work or shift are more prone to PCa [33]. In contrast, a Canadian population-based case-control study (PROtEus), conducted in 2005–2012, recruited 1904 PCa patients and 1905 controls, revealed no major impact of night shifting work on PCa incidence [34]. In the same cancer type, why did these epidemiological studies reach different conclusions? One possible reason is that patients recruited in different cohorts bear distinct background, including race, nation, and local environment. In addition, investigators in these studies may also utilize different statistical methods for analyzing and interpreting their data. It is also possible that criteria for the definition of shift work and clock disruption in these studies may not reach a consensus [1].

Genome-wide studies and systems analyses have also attempted to establish a relationship between clock disruptions and cancer development. In a meta-analysis, Benna et al. identified several single nucleotide polymorphisms (SNPs) related to breast cancer, including SNPs in the genes encoding CLOCK, NPAS2, and PER3, but the association of clock gene SNPs and cancer susceptibility turned out to be modest [35]. On the other hand, a study across 32 cancer types identified a number of deregulated clock genes that appeared to be associated with tumor stage and patients' survival, and authors suggested that circadian timing may potentially be used in cancer therapy [36]. Overall, a large-scale study using patient samples at distinct time points is needed to ascertain the roles of circadian genes in cancer.

3.2. Studies of circadian rhythm dysregulation and cancer in mouse models

Significant amount of work has been done in genetically engineered mouse models (GEMMs) to interrogate whether circadian clock disruption contributes to tumorigenesis, which has generally revealed tumor suppressive roles of *Per* and *Cry* genes. For example, Fu et al. reported that the *Per2* gene is involved in regulating DNA damage response (DDR) and *Per2*-mutant mice showed a significant increase in tumor development [37]. Subsequent work demonstrated that mice lacking *Per1* and *2*, or *Cry1* and *2* were prone for spontaneous and radiation-induced tumor development [38], although other work found that *Cry1* and *Cry2* mutant mice were not cancer-prone [39]. The seemingly contradictory results may be attributed to different experimental conditions, but the reasons behind distinct phenotypes are not completely understood. Moreover, some earlier studies indicated that CLOCK and BMAL1 may also possess tumor suppressive functions in rodents [2]. However, knocking out *Bmal1* in mice resulted in shortened lifespans and early aging phenotype in mice related to increased levels of reactive oxygen species, but *Bmal1* deficient mice were not predisposed to cancer risk [40]. Similarly, the *Clock* gene knockout in mice also did not lead to spontaneous tumor formation [41]. Lack of tumor-prone phenotypes in *Bmal1* or *Clock* deficient mice may have been related to shortened animal survival. Overall, how loss of circadian clock genes in GEMMs may affect spontaneous tumor development requires further investigations.

Mouse models using other experimental approaches, including SCN destruction, external intervention and sleep deprivation, have also been employed to explore any potential association between circadian disruptions and cancer development [42]. A study from Filipski et al. destroyed SCN in B6D2F1 mice using bilateral electrolytic lesion not only changed the circadian rhythms and serum levels of corticosterone and lymphocyte counts but also significantly accelerated growth of grafted pancreatic adenocarcinoma and Glasgow osteosarcoma in mice with SCN lesions [43]. Moreover, mice subjected to experimental chronic jet lag (CJL) had significantly increased tumorigenicity, faster tumor growth kinetics, and shortened animal survival as compared to the controls, suggesting that the impact of circadian disruptions via CJL is profound on cancer development [42]. Furthermore, previous studies established sleep deprivation models to show that sleep deprivation is capable of promoting tumor progression. For example, Hakim et al. revealed that sleep fragmentation, a sleep deprivation model, enhanced tumor growth and invasiveness, which was closely related to the TLR4 signaling pathway and the recruitment of tumor-associated macrophages [44]. At present, it is still not exactly clear how sleep deprivation misregulates circadian clock and promotes tumor development and progression.

3.3. Disruptions of the core clock genes and their involvement in different cancers

Emerging evidence suggests that disruptions of the core circadian genes are involved in different cancers, and their regulatory functions may be context-specific [1,2]. For example, 'positive' circadian clock regulators BMAL1 and CLOCK are required for hepatocellular carcinoma (HCC) cell proliferation via regulating the cell-cycle regulators p21 and Wee1 [45]. Also, Okazaki et al. showed that BMAL1 and CLOCK rhythmically controlled the transcription of the iron regulatory protein 2 (IRP2) gene and the CLOCK gene mutation delayed colon tumor growth, suggesting that CLOCK regulates colon cancer cell proliferation via an iron-dependent manner [46]. On the other hand, the levels of BMAL1 gene expression were found to be downregulated in pancreatic cancer and highly associated with clinical features of patients [47].

Overexpression of BMAL1 in pancreatic cancer cell lines inhibited proliferation and invasion by inducing cell-cycle arrest, and knocking down BMAL1 enhanced cancer development, suggesting that BMAL1 may function as a tumor suppressor in pancreatic cancer [47]. In line with this, BMAL1 expression was also downregulated in tongue squamous cell carcinoma (TSCC) samples, and functional characterizations revealed that BMAL1 overexpression in TSCC cells induced apoptosis, paclitaxel sensitivity and inhibition of proliferation via recruiting EZH2 to the TERT promoter [48]. Collectively, these studies suggest that BMAL1 and CLCOK may possess either oncogenic or tumor suppressive functions in cancer type and context dependent manners.

Conversely, existing evidence has primarily supported tumor-suppressive functions of PER and CRY [1,2]. For instance, PER1 was downregulated in oral squamous cell carcinoma (OSCC), and PER1 suppressed OSCC progression via regulating glycolysis-mediated cell proliferation and PI3K/AKT dependent mechanisms [49]. The *Per2*-deficient mice had increased tumorigenicity and reduced thymocyte apoptosis induced by gamma irradiation as compared to the wild-type, and the underlying mechanisms involved cell cycle dysregulation and transcription of c-MYC [37]. Furthermore, expression levels of PER1, PER2 and PER3 were all reduced in many other cancers [1]. Interestingly, PER3 induction in PCa cells resulted in increased chemotherapy sensitivity by suppressing the NOTCH signaling [50]. A recent study showed that phosphorylated PER interacted with CK1 and inhibited its kinase activity to regulate circadian rhythms [51]. Similar to *Per* deficiency, *Cry1* and *Cry2* deficient mice are more prone to both spontaneous and radiation-induced tumor development [38]. Mechanistically, CRY proteins have been shown to reduce levels of E2F family members by cooperating with the E3 ligase complex, SKP-CULLIN-FBXL3 (SCF^{FBXL3}) [52], suggesting that CRY members may play their tumor suppressive roles by regulating the cell cycle. Surprisingly, a recent study in a *Drosophila* glioblastoma (GBM) model showed that CRY was functionally required for GBM progression driven by PI3K signaling [53]. More studies are needed to further understand the roles of PER and CRY in tumor development.

4. Cancer stem cell regulation by circadian clock and clock genes

Cancer stem cells (CSCs) are stem-like cancer cells with self-renewal and tumor-regenerating capabilities, and are involved in maintaining tumor homeostasis and driving therapy resistance and metastasis [54,55]. Emerging evidence has implicated the circadian clock and clock genes in regulating the stemness and functional features of CSCs [56,57] (Fig. 1; Table S2).

4.1. Leukemia stem cells (LSCs)

The first direct evidence of CSCs was provided by Dick and colleagues in the early 1990s [58,59], which laid a seminal foundation for the field of CSC research. Recently, in a murine model of acute myeloid leukemia (AML), Puram et al. conducted an *in vivo* RNA interference screen to identify BMAL1 and CLOCK as the critical regulators of LSCs [60]. Both *in vivo* and *in vitro* functional characterizations further revealed that BMAL1 and CLOCK were required for AML cell growth, and the disruption of circadian rhythm impaired cell cycle and induced myeloid differentiation [60]. Interestingly, authors confirmed that myeloid leukemia cells, but not the normal counterparts, were highly

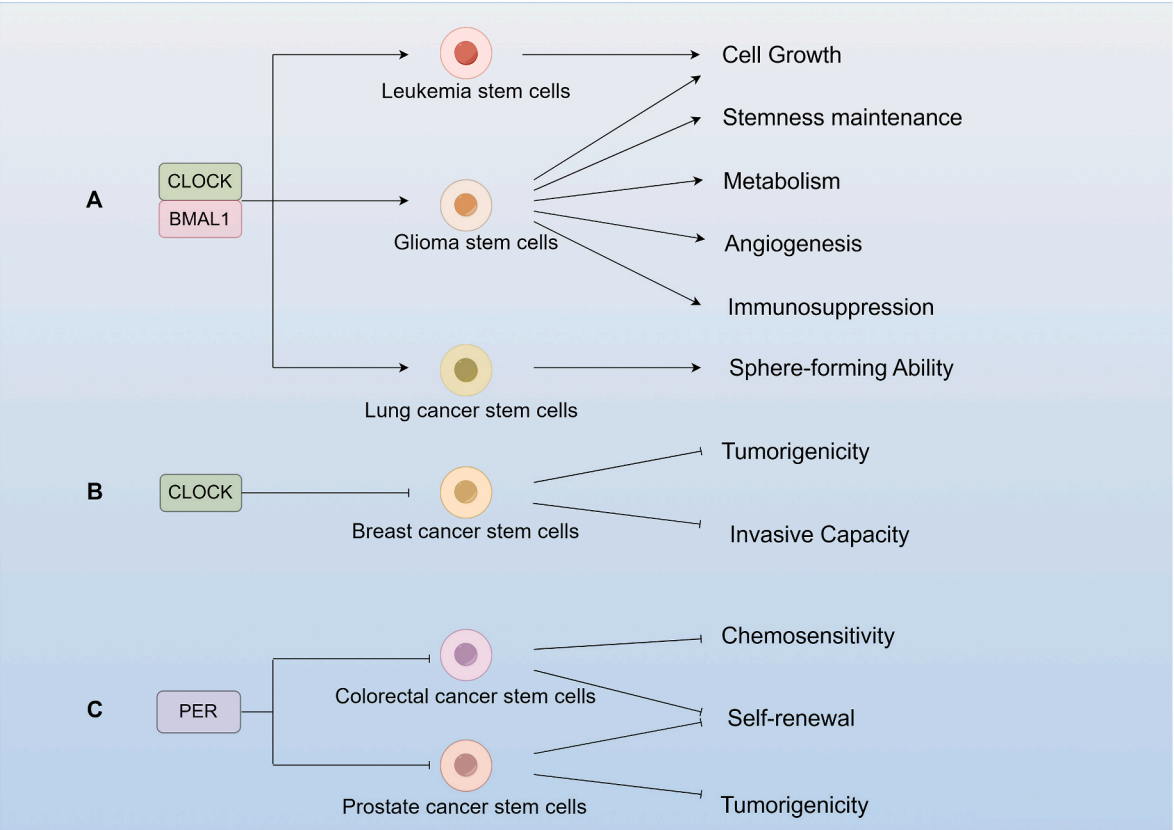

Fig. 1. Studies of circadian clock genes in cancer stem cells (CSCs). (**A**) BMAL1 and CLOCK positively regulate leukemia stem cells for cell growth, glioma stem cells for several features (including stemness maintenance, metabolism, angiogenesis, immunosuppression, and cell growth), and lung cancer stem cells for sphere-forming abilities. (**B**) The CLOCK regulation in CSCs is likely cell-type dependent. For example, CLOCK overexpression in murine breast cancer stem cells in certain models inhibited their tumorigenicity and invasive capacities. (**C**) PER family members negatively regulate colorectal cancer stem cells and prostate cancer stem cells for their CSC features.

dependent on BMAL1 [60]. Work from Numata et al. employed chromatin immunoprecipitation and deep sequencing (ChIP-seq) in mixed lineage leukemia (MLL) AF6 AML cells to report that a circadian clock transcription factor SHARP1 was the most upregulated target gene [61], and that SHARP1 was required for the clonogenic growth, while depletion of SHARP1 markedly attenuated leukemia-initiating capacities, of human MLL-AF6 AML cells. Mechanistically, SHARP1 was able to activate genes for cell survival by binding to transcriptionally active chromatin [61]. These studies implicate multi-faceted roles of circadian clock genes in leukemic development, which could potentially be biomarkers and therapeutic targets for LSCs [62]. At present, it is not clear if circadian rhythm regulations are similar between normal HSCs and LSCs. However, previous findings have hinted that those mechanisms regulating normal HSCs [13] may also likely be involved in regulating the self-renewal and leukemia-initiating abilities of LSCs, but it waits further clarifications.

4.2. Breast cancer stem cells (BCSCs)

Back in 2003, Al-Hajj et al. published a seminal work that breast cancer cells bearing the phenotype of $CD44^{+}CD24^{-/lo}$ had enriched CSC properties [63], which set the foundation for studying CSCs in solid tumors. To explore if CSCs are regulated by circadian rhythms, a Japanese group found that the $ALDH^{+}$ breast cancer stem cells (BCSCs) exhibited circadian changes in a mouse 4T1 breast cancer model, which was reflected in a ALDH3A1-depdent manner and controlled via WNT [64]. Administration of an ALDH inhibitor at ZT14, when the number of $ALDH^{+}$ cells were abundant, elicited dramatic anti-tumor and anti-metastatic effects on triple negative breast cancer (TNBC) cells [64], suggesting that choosing the optimal drug dosing time is of potential importance in treating TNBC by targeting BCSCs. Subsequently, the same group identified that CLOCK level was downregulated in $ALDH^{+}$ cells in the murine 4T1 breast cancer cells [65], and overexpression of CLOCK markedly inhibited tumorigenicity and invasive capacities of 4T1 cells [65]. Post-transcriptionally, miR-182 directly regulated CLOCK and knocking out miR-182 attenuated 4T1 tumor growth [65], providing a piece of evidence on how CLOCK is implicated in breast cancer malignancy. Intriguingly, a recent publication reported that the spontaneous intravasation of circulating tumor cells (CTCs) occurs preferentially during sleep in both human and mouse breast cancer models [66]. Using single-cell RNA sequencing, authors found that mitotic genes were highly upregulated during the rest phase, and circadian rhythm associated hormones (i.e., melatonin, testosterone, and glucocorticoids) were involved in CTC generation and proliferation [66]. Furthermore, treating tumor-bearing mice with insulin significantly affected breast cancer cell proliferation *in vivo* in a time-dependent way [66], hinting that dosing time is also important for treating metastasis prone cancers. However, it remains unclear if CTCs are CSCs, although some CTCs capable of seeding metastasis are enriched in CSC state in some experimental models [67]. Perhaps chronotherapies to target CSCs at a certain time during the night is an option. In the future work, we may need to apply improved methods to detect CTCs with better sensitivity and characterize their CSC properties to better understand the biological functions of these cells.

4.3. Glioma stem cells (GSCs)

Identification, characterizations and clinical implications of GSCs have been widely studied in the past 20 years [68]. However, only recently the circadian characteristics of GSCs have attracted our attention [69,70]. Rich and his colleagues discovered that GSCs display circadian rhythms and BMAL1 and CLOCK are indispensable for maintaining stemness of GSCs in GBM [69]. Mechanistic studies revealed that BMAL1 and CLOCK are highly related to metabolic and chromatin regulations, and administration of small molecule agonists of BMAL1-CLOCK negative regulators specifically and significantly impaired GSC survival and the combinatorial use of cryptochrome and REV-ERB agonists showed profound anti-tumor effects [69]. In line with this finding, DePinho and colleagues have validated that BMAL1 and CLOCK are required for maintaining GSC self-renewal and tumorigenicity by upregulation of OLFML3, which led to the recruitment of the immune-suppressive microglia into tumor microenvironment (TME) [70]. Importantly, targeting CLOCK impaired GSC activities and microglia infiltration, and improved animal survival [70]. To further understand the mechanisms, Pang et al. have shown that CLOCK-mediated OLFML3 stimulated HIF1α-regulated expression of POSTN, a pro-angiogenetic factor, to promote tumor angiogenesis in GBM by activating TBK1 signaling in endothelial cells [71]. In addition, disruption of CLOCK-regulated POSTN-TBK1 signaling dramatically impaired angiogenesis and tumor growth in mouse and patient-derived GBM xenograft models [71]. Together, these studies highlight BMAL1 and CLOCK as potential targets for treating GSCs [69–71], and also hint that we may have different ways to target GSCs, either by directly targeting their circadian machinery, or disrupting their clock regulated signaling pathways.

4.4. Colorectal cancer stem cells

Regulations of circadian clock in colorectal cancer (CRC) have been studied for the past 30 years [54,55]. For example, it has been reported that BMAL1 and CLOCK contributed to CRC cell proliferation by regulating iron metabolism in a colon-26 model [46]. Moreover, Chakrabarti et al. revealed that circadian clock controlled cellular fate using a single-cell lineage tracking system in human colon cancer cells [72], suggesting the importance of circadian clock in CRC development. Nevertheless, how circadian clock regulates stemness of colorectal cancer stem cells is not completely understood. Recently, Zhang et al. found that BMAL1 is causally involved in EMT, migration and invasion, as well as chemoresistance of colon cancer cells, suggesting that BMAL1 is highly associated with colon cancer metastasis and chemoresistance, and possibly stemness [73]. Interestingly, functional characterizations have revealed that PER3 plays tumor suppressive roles in controlling self-renewal ability and chemoresistance of colorectal CSCs (sphere-forming cells) by inhibiting NOTCH and β-catenin signaling pathways [74]. In the future work, more defined *in vivo* experiments (e.g. lineage tracing) are required to determine if the core clock components are required in CSC maintenance, and if directly targeting some core clock genes in colorectal CSCs affects their stemness and tumorigenicity.

4.5. Prostate cancer stem cells (PCSCs)

Prostate is a hormone-regulated organ, and both androgen and androgen receptor (AR) are important for PCa development [75]. A recent study collected high-risk PCa tissues before and after enzalutamide (an AR antagonist) treatment from a neoadjuvant clinical trial and applied integrative multiomics analyses to understand mechanisms on how PCa cells avoid AR inhibition [76]. Authors in this study [76] identified that treatment-induced FOXA1 was imperative for the survival signals, and post-enzalutamide PCa tissues were highly enriched in the circadian rhythm regulator ARNTL (i.e., BMAL1). Further functional validations confirmed that ARNTL was required for enzalutamide-resistant PCa cell growth [76], nominating ARNTL as a therapeutic target for androgen-independent PCa. Several PCSC populations have been reported in the past ~20 years, and PCSCs play vital roles in castration-resistant prostate cancer (CRPC) [75]. In our study, we have identified that PCa cells bearing $ALDH^{hi}CD44^{+}$ phenotype (termed as double positive/or DP cells) are enriched in PCSCs in CRPC [77]. Using RNA-sequencing and bioinformatic analysis, we found that PER3 was markedly downregulated in PCSCs, which inversely correlated with the prognosis of PCa patients [77]. Mechanistic explorations and functional validations further verified that PER3 negatively regulates stem cell properties of DP cells by targeting WNT/β-catenin

signaling [77], providing a potential strategy to treat CRPC cells via circadian rhythm perturbations. It is still early to study circadian gene regulation in PCSCs, and more reliable systems are needed to be developed to dynamically track and characterize circadian rhythm in PCSCs.

4.6. Cancer stem cells in other solid tumors

At present, there are only scattered reports on circadian rhythm regulation of stem cells or CSCs in other tissue and tumor systems. For instance, Benitah and the colleagues have shown that circadian clock regulates epidermal stem cell heterogeneity and homeostasis [78]. In mouse models, deletion of *Bmal1* led to epidermal aging and reduced skin tumorigenesis [78], possibly by reducing the percentage of tumor-initiating or CSCs. Recently, Mortimer et al. have further shown that the epidermal clock is able to exert a gate-keeping function by integrating and subverting brain signals for skin homeostasis [79]. Moreover, Jiang et al. observed that CLOCK was upregulated in lung CSCs defined by sphere-forming assays, and, interestingly, Epigallocatechin-3-gallate (EGCG) was found to regulate stem cell features of lung CSCs via targeting CLOCK [80]. In addition, Jia et al. established a long-term and quiescent cisplatin-resistant bladder cancer cell model, and authors noted that CRY1 was accumulated in these cells, resulting in attenuation of paclitaxel-induced cellular senescence and degradation of p53 [81]. Overall, potential functions of circadian clock in regulating CSCs in most solid tumors await more thorough investigations.

5. Chronotherapy targeting CSCs

Our current understanding of the molecular mechanisms regulating circadian rhythm suggests that chronotherapies may be developed and applied to target CSCs and treat cancers [82]. For example, pharmacological REV-ERB agonists, including SR9009 and SR9011 [83], have been developed and used in targeting GSCs, which disrupted their self-renewal and tumorigenicity [60,69]. Hirato et al. used a cell-based circadian screening platform to uncover a small molecule, KL001, which activated CRY [84]. KL001 and its oral derivative, SHP656, also inhibited GSC and glioma growth *in vivo* [69,85] (Fig. S2). Interestingly, Hirota and colleagues have also found that a selective inhibitor for CK2, GO289, regulated the circadian clock and inhibited cancer cell growth cell-type dependent manner [86]. Obviously, the drug administration timing during cancer chronotherapy is important. As aforementioned, N, N-diethylaminobenzaldehyde, an ALDH inhibitor, exhibits tumor inhibitory activity at the optimal time ZT14 when the ALDH activity is high in BCSCs [64]. Several anticancer drugs, including the CK4/6 inhibitor Palbociclib [87] and the HSP90 inhibitor 17-AAG [88] have been reported to manifest their anticancer efficacies in a time-of-day manner. Additionally, combining chronotherapy with drugs targeting the TME may represent another therapeutic option. For instance, Wang et al. have demonstrated that dendritic cells (DCs) and $CD8^+$ cells show strong antitumor effects in melanoma cells depending on circadian expression of CD80 [89]. Importantly, cancer immunotherapy is more effective during the day as synchronized with DCs [89]. Recently, many studies have attempted to investigate how circadian regulations are involved in the immunosurveillance and the efficacy of immunotherapy [90–96]. For example, Wang et al. have reported that tumor-infiltrating T cells and endothelial cell play important roles in TME by circadian regulations, which can affect the therapeutic effects of chimeric antigen receptor T (CAR-T) cell therapy and immune checkpoint blockade in cancer treatment via adjusting the time of treatment during the day [97], suggesting the promise of optimizing the combination strategies of chronotherapy and immunotherapy for cancer treatment. Finally, combining chronotherapy and metabolic therapy represents yet another potential approach for cancer treatment. A recent study showed that fatty acid oxidation (FAO) sensed the circadian disruptions caused by sleep deprivation, which dysregulates CLOCK/ASCL1/palmitoyl-coenzyme A (PA-CoA) loop [98]. Authors further showed that combinatorial treatment of chronotherapy and β-endorphin reset the circadian clock and inhibited lung cancer growth [98]. Taken together, it will be important to conduct prospective clinical trials of chronotherapy monotherapy or chromotherapy combination with therapeutic modalities.

6. Conclusions

Circadian clock is crucial in maintaining and regulating normal physiological conditions and its dysregulation is causally implicated in human diseases, including cancer (Fig. S1). CSCs have been considered as the cells driving cancer progression and therapy resistance. Here, we presented some representative and pioneering work that has implicated circadian clock genes and signaling in regulating CSC self-renewal and tumorigenicity. Certainly, there are still many open questions in the field. For example, what are the exact mechanisms whereby the circadian clock genes regulate the stemness of CSCs? Will normal SCs and CSCs share same circadian regulatory mechanisms? How shall we combine chronotherapy and other therapeutic agents (such as chemotherapy, immune checkpoint inhibitor, targeted therapy, and etc.) in cancer treatment? Future work should interrogate interactions among CSCs, components of the TME and circadian clock genes in each cancer type and in the context of intra- and inter-tumor heterogeneity. Recent advances have highlighted the utility of 3-dimesional organoid models to characterize features of CSCs and investigate circadian related diseases [99]. Future studies should also be directed to unveil optimal drug delivery system, time and dosing using genetic mouse models and clinical samples. Finally, many clinical trials have been initiated to investigate circadian rhythms and the efficacy of chronotherapies in different solid tumors (Table S3), and we expect the clinical trials to validate and advance the application of chronotherapy linking to CSC biology in a personalized manner.

CRediT authorship contribution statement

Yiling Zhang: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Qiang Zhang:** Writing – review & editing, Software, Methodology. **Rundong Liu:** Visualization, Methodology, Formal analysis. **Dingxiao Zhang:** Writing – review & editing. **Guangyuan Hu:** Writing – review & editing, Supervision, Project administration. **Xin Chen:** Writing – review & editing, Supervision, Funding acquisition, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

AML	acute myeloid leukemia
AR	androgen receptor
BCSCs	breast cancer stem cells
BM	bone marrow
BMAL1	Brain and Muscle Ant-Like Protein
CAR-T	chimeric antigen receptor T cell therapy
ChIP-Seq	chromatin immunoprecipitation and deep sequencing
CJL	chronic jet lag
CLOCK	Circadian Locomotor Output Cycles Kaput
CRC	colorectal cancer
CRPC	castration-resistant prostate cancer
CRY	cryptochrome
CSCs	cancer stem cells
CTCs	circulating tumor cells
DCs	dendritic cells
DDR	DNA damage response
EGCG	Epigallocatechin-3-gallate
ESCs	embryonic stem cells
FAO	fatty acid oxidation
GBM	glioblastoma
GEMMs	genetically engineered mouse models
GSCs	glioma stem cells
HCC	hepatocellular carcinoma
HO-1	heme oxygenase-1
HSCs	hematopoietic stem cells
HSPCs	hematopoietic stem and progenitor cells
IARC	International Agency for Research on Cancer
IRP2	iron regulatory protein 2
KO	knockout
LSCs	leukemia stem cells
MLL	mixed lineage leukemia
NPAS2	Neuronal PAS Domain Protein 2
OSCC	oral squamous cell carcinoma
PCa	prostate cancer
PCSCs	prostate cancer stem cells
PER	Period
SC	stem cells
SCN	suprachiasmatic nuclei
SNPs	single nucleotide polymorphisms
TNBC	triple negative breast cancer
TME	tumor microenvironment
TSCC	tongue squamous cell carcinoma
TTFL	transcription-translation feedback loop
WHO	World Health Organization

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2024.217391.

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