Cancer clocks in tumourigenesis: The p53 pathway and beyond

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DECLARATION OF INTEREST

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Abstract

Circadian rhythms regulate a vast array of physiological and cellular processes, as well as the hormonal milieu, to keep our cells synchronised to the light-dark cycle. Epidemiologic studies have implicated circadian disruption in the development of breast and other cancers, and numerous clock genes are dysregulated in human tumours. Here we review the evidence that circadian rhythms, when altered at the molecular level, influence cancer growth. We also note some common pitfalls in circadian-cancer research and how they might be avoided to maximise comparable results and minimise misleading data.

Studies of circadian gene mutant mice, and human cancer models *in* vitro and *in vivo,* demonstrate that clock genes can impact tumourigenesis. Clock genes influence important cancer related pathways, ranging from p53-mediated apoptosis to cell cycle progression. Confusingly, clock dysfunction can be both pro- or anti- tumourigenic in a model and cell type specific manner. Due to this duality, there is no canonical mechanism for clock interaction with tumourigenic pathways.

To understand the role of the circadian clock in patients' tumours requires analysis of the molecular clock status compared to healthy tissue. Novel mathematical approaches are under development, but this remains largely aspirational, and is hampered by a lack of temporal information in publicly available datasets.

Current evidence broadly supports the notion that the circadian clock is important for cancer biology. More work is necessary to develop an overarching model of this connection. Future studies would do well to analyse the clock network in addition to alterations in single clock genes.

Alternative Gene Abbreviations: BMAL1 (ARNTL, MOP3), DEC1/2 (BHLHE40/BHLHE41), REV-ERBα/β (NR1D1/NR1D2), CK1δ/ε (CSNK1D, CSNK1E), NFIL3 (E4BP4)

Introduction

In 1729, French astronomer Jean-Jaques d'Ortous de Mairan observed the *Mimosa pudica* plant opening and closing its leaves in accordance with the geophysical day, even when deprived of a light source (de Mairan, 1729). In the centuries since, oscillations that coordinate normal physiology with the 24-hour light/dark cycle have been discovered across the kingdoms of life (Rosbash, 2009). Disrupted circadian rhythms contribute to many human diseases including diabetes, obesity and depression. An epidemiological link between circadian rhythms and cancer was established as early as 1996, and multiple papers on this topic have been subsequently published (Tynes et al., 1996). The WHO classifies circadian disruption as a probable human carcinogen (IARC, 2019), though there is debate as to the validity or strength of this association (Zhang and Papantoniou, 2019, Rivera et al., 2020, Dun et al., 2020, Wegrzyn et al., 2017).

Unsurprisingly, circadian influence over the endocrine system has been implicated in this proposed cancer-circadian link. In 1987, Stevens suggested that melatonin suppression from light at night may play a role in breast cancer biology (Stevens, 1987). This 'melatonin hypothesis' is out-of-vogue currently owing to contradictory data, these discrepancies and potential explanations for them are well reviewed by Hunter and Figueiro (Hunter and Figueiro, 2017). There is established circadian variation in many cancer relevant hormones including melatonin, sex hormones, thyroid hormones and corticosteroids (Prasai et al., 2011). In fact, the link between hormones, circadian rhythms, and cancer is not one way. Pheochromocytomas have been observed disrupting circadian rhythms via aberrant hormone release (Tabebi et al., 2018).

At the cellular level, animal models deficient for one or more core circadian genes are cancer prone (Fu et al., 2002). Cancers are known for aberrant gene expression and therefore, it is possible that the circadian system is dysregulated within tumours, irrespective of the rhythm of the animal as a whole. Indeed, an association between cancer severity and circadian status of the tumour has been documented, and escape from circadian regulation is suggested to be an emerging hallmark of cancer (Papagiannakopoulos et al., 2016, El-Athman and Relógio, 2018). Despite this, our understanding of the role of the circadian system and the influence of specific circadian genes, in tumourigenesis is still developing.

Molecular Clockwork

In most mammals, light is a strong 'Zeitgeber', i.e., an external cue that can entrain circadian rhythms (Duffy and Czeisler, 2009). Light triggers a signal which is transmitted to the suprachiasmatic nuclei (SCN), an area of the ventral hypothalamus referred to as the 'central pacemaker' (Gooley et al., 2001, Hattar et al., 2002). These signals reset the clock in these neurons by activating light inducible elements in core clock gene promoters (Astiz et al., 2019). The SCN then goes on to synchronise peripheral clocks in cells throughout the body. Notably, melatonin and glucocorticoid secretion are under SCN regulation and are thought to have key roles in synchronising peripheral clocks. Glucocorticoids are such strong circadian synchronisers that they are often use to synchronise cells *in vitro* for circadian experiments (Balsalobre et al., 2000, Prasai et al., 2011).

The ultimate units of circadian timekeeping are cell-autonomous transcriptional/translational/post-translational feedback loops (Fig 1). In the core circadian loop, the circadian locomotor output cycles kaput (CLOCK) protein forms a heterodimer with brain and muscle ARNT-like protein 1 (BMAL1). At cycle start, this CLOCK/BMAL1 heterodimer can bind to E-box promoter elements (CANNTG) and promote transcription. Amongst those genes activated in this way are the period (*PER1, PER2* & *PER3*) and cryptochrome (*CRY1 & CRY2*) genes. PER and CRY proteins then accumulate in the cytoplasm and eventually multimerise forming a ~1 megadalton complex along with Casein Kinase 1 δ and ε (CK1δ/CK1ε) and Rab5-activating protein 6 (GAPVD1) (Aryal et al., 2017, Brown et al., 2005).

In the cytoplasm, post-translational modifications fine tune the system. PER2 is post-translationally regulated via a phosphoswitch mechanism (Zhou et al., 2015). CK1 can phosphorylate two distinct regions of PER2. Phosphorylation on the FASP (Familial Advanced Sleep Phase) site leads to PER2 stabilisation, whereas phosphorylation of the degron leads to degradation via β-TrCP. The 'decision making' region appears to be a loop of CK1, the conformation of which can influence site specificity (Philpott et al., 2020). If PER2 is not degraded it can be translocated into the nucleus along with CRY proteins and CK1.

In the nucleus, PERs and CRYs repress activity of the CLOCK/BMAL1 heterodimer by preventing its transcriptional activity or dissociating the complex from DNA (Ye et al.,

2014). Therefore, PERs and CRYs inhibit their own transcription and the transcription of the numerous genes controlled by CLOCK/BMAL1, which closes the loop. It is worth noting that CLOCK/BMAL1 is not the only activating complex that can bind to E-boxes. NPAS2 can substitute for CLOCK at least in some cells (DeBruyne et al., 2007). Moreover, for example, MYC is also capable of binding to E-boxes in direct competition to the CLOCK/BMAL1 complex and, therefore, MYC can dampen or stop the clock (Altman et al., 2015).

A secondary loop adds to the stability and complexity of the system. The *REV-ERBα* and *REV-ERBβ* promoters contain an E-box, and are transcriptionally activated by the CLOCK/BMAL1 heterodimer. REV-ERBα and REV-ERBβ compete with retinoic acid-related orphan receptors (RORα, RORβ & RORγ) for ROR binding sites in the BMAL1 promoter. RORs activate *BMAL1* transcription and REV-ERBs repress it. These primary and secondary feedback loops constitute the canonical circadian system and have analogues across a variety of species (Brown et al., 2012). In mammals, however, there are a number of sub-loops which interlock with either the primary or secondary core loop, proteins involved in these include DBP, DEC1 and DEC2 (Takahashi, 2016, Nakashima et al., 2008).

Manipulation of key components of these interlocking feedback loops has dramatic effects on the clock. This is well studied in circadian gene knockout and mutation models in *Drosophila* and mice. These modifications to the clock elements range from absolute arrhythmicity to changes in circadian period, as measured by activity monitoring in whole animals (Bunger et al., 2000, Lee et al., 2004). Notably, *BMAL1* knockout mice are completely arrhythmic in their behaviour, with nocturnal activity replaced by more random patterns of activity irrespective of time of day (Bunger et al., 2000).

Clocks in Tumourigenesis

This intricate system, and disruptions therein, can have profound effects on human health (Roenneberg and Merrow, 2016). Circadian rhythms influence homeostasis and regulate a variety of integral hormones including insulin, glucagon, oestrogen and progesterone, as well as controlling a host of other processes ranging from immunity to metabolism (Petrenko and Dibner, 2017, Rahman et al., 2019). It is, therefore, unsurprising that circadian biology has been implicated in a variety of prevalent conditions, including metabolic syndromes, obesity and cardiovascular disease. This review focuses on the interplay between circadian rhythms and cancer (Bae et al., 2019, Buurma et al., 2019, Noh, 2018).

Expression levels of circadian genes, the amplitude of oscillation, and clock output genes differ between cell types and tissues (Zhang et al., 2014, Mure et al., 2018). Due to this, there may be distinct pro- or anti-tumourigenic roles for each core clock gene in various tumour types. A number of studies have attempted to elucidate the roles of each of the clock genes in various *in vitro* and *in vivo* models (Fig 2).

ISSUES WITH MOLECULAR CLOCK RESEARCH IN TUMOURS

Based on animal as well as human studies, more than 20% of all transcripts are clock regulated and can vary many-fold over the day (Zhang et al., 2014). A lack of timing information for human tissue samples plagues many of the studies to be discussed. If timing information is unavailable for bio-samples it is impossible to know if a difference in expression is due to true differential expression or sampling at different phases of the circadian cycle. Therefore, care must be taken in interpreting claims of up or downregulated oscillating genes. Some studies address this by comparing tumour clock gene expression to that of the adjacent tumour margin (Ye et al., 2018). This approach should still be applied with caution, as tumour margin cells are not equivalent to healthy cells and it is only assumed that the tumour margin and the tumour are in phase with each other (Aran et al., 2017).

Even studies in experimental *in vitro* and *in vivo* models can suffer from a lack of information. Overexpression, knockdown or knockout of circadian genes will result in modified rhythmic gene expression. For example, overexpression of PER2 reduces transcription of *CRY1* & *CRY2* and will, therefore, have profound effects on circadian oscillation (Chen et al., 2009). Therefore, circadian gene manipulations should be proceeded and succeeded by an assessment of the cell's overall circadian function. These knock-on effects may underlie observed phenotypes. Moreover, to compare the consequence of a clock gene modification across cell lines it would be pertinent to have time-of-sampling information even for *in vitro* experiments as this, combined with knowledge of the cells modified rhythmic behaviour, could shed light on the mechanisms behind cell specific tumourigenic effects of clock genes. Additionally, clock genes can have pleiotropic effects independent of their core clock function. For example, CLOCK/BMAL1 can bind to E-box sequences regardless of whether it is regulated in an oscillatory manner. CLOCK/BMAL1 regulated by the clock, CLOCK/BMAL1 unregulated by the clock, and complete loss of CLOCK/BMAL1 proteins each would have different consequences for cell function. These distinctions are likely to apply to most all circadian proteins. To understand clock gene modifications in the context of the

overall circadian clock, i.e., functional analysis via reporter assays, multiple timed samples or the use of one sample combined with the use of a multi-dimensional mathematical model is required.

BMAL1

In humans, *BMAL1* has been associated with a number of cancers including breast, pancreatic and thyroid tumours, though its role in these endocrine cancers is incongruous. BMAL1 is often depicted as the centre of the core circadian system, owing to the BMAL1/CLOCK heterodimer being the canonical positive arm and driver of circadian gene transcription (Fig 1), and *BMAL1* is the only known single gene knockout that is considered to completely stop the clock (Baggs et al., 2009). Unsurprisingly, this has led it to be amongst the best studied circadian regulators in regard to tumourigenesis. In breast cancer, reduced *BMAL1* is consistently associated with increased risk of metastasis (Ramos et al., 2020). Similarly, pancreatic cancer cells are reported to have less *BMAL1* than non-cancerous controls (Jiang et al., 2016). However, thyroid carcinoma and a subset of malignant plural mesothelioma (MPM) had upregulated *BMAL1* (Ye et al., 2018, Elshazley et al., 2012). Despite its links to endocrine related cancers in humans, little evidence thus far has been unearthed linking BMAL1 to endocrine pathways in tumourigenesis.

In xenograft models *BMAL1* appears to be anti-tumourigenic. BMAL1 knockdown (k/d) in a pancreatic cancer cell line with high endogenous BMAL1 expression (BxPC-3) increased subcutaneous tumour size and matrix metalloproteases (MMPs) 9 and 2. BMAL1 overexpression in a pancreatic cell line with low endogenous BMAL1 expression (AsPC-1) showed converse effects (Jiang et al., 2016). This may be a p53 pathway effect as BMAL1 was shown to be bound to the p53 promoter and overexpression of BMAL1 induced an upregulation of phospho-p53 whereas BMAL1 k/d reduced it. It is not clear how transcriptional regulation via BMAL1 could upregulate phospho-p53, but not overall p53 (Jiang et al., 2016). Similarly, in hepatocellular carcinoma (HCC), BMAL1 also appears to inhibit tumour growth. Canonically, hepatocyte nuclear factor 4 alpha (HNF4α) is a tumour suppressing transcriptional repressor. However, a subset of liver tumours expresses an isoform named P2-HNF4α. Both isoforms of HNF4α can repress BMAL1 transcription, however P2-HNF4α has a much stronger effect. Forcing P2-HNF4α expressing HCC cells to re-express BMAL1 caused a reduction in tumour growth subcutaneously in mice and an induction of both p53 and cleaved caspase 3 (Fekry et al., 2018).

BMAL1's role in tumours appears cell type specific in *in vivo* models utilising mouse cancer cells and *in vitro* models. Murine colon adenocarcinoma (C26) cells with BMAL1 k/d formed significantly larger tumours than their wildtype (WT) counterparts (Zeng et al., 2010). In a genetically engineered mouse model of lung adenocarcinoma, tumour specific loss of BMAL1 increased tumour burden, tumour weight, proliferation and MYC production. Notably, this effect was absent if performed in a p53 deficient background, adding credence to the proposed p53-BMAL1 interplay in tumourigenesis (Papagiannakopoulos et al., 2016). Conversely, in an acute myeloid leukaemia (AML) model in mice it was demonstrated that BMAL1 k/d cells are depleted *in vivo* compared to their WT counterparts. This suggests that, in this instance, BMAL1 confers a survival advantage to the cancer cells (Puram et al., 2016). Consistent with these findings, HCT116 (human colorectal carcinoma) cells with BMAL1 k/d formed significantly smaller tumours when grafted onto zebrafish embryos than HCT116 WT. This model system is further removed from mammalian biology and it is unclear if this observation is a result of the cell type or the model. Interestingly, in HCT116 BMAL1 k/d cells, p53 was shown convincingly to oscillate at the mRNA level despite no oscillation at the BMAL1 promoter (Basti et al., 2020). Studies *in vitro* have demonstrated BMAL1 acting as a suppressor of proliferation in BxPC-3, AsPC-1, HCT116, C26 and U87MG (glioblastoma) cell lines and in patient derived glioblastoma stem cells, but acting as a proliferation enhancer in the human MPM cell lines ACC-MESO-1 and NCI-H290 (Jiang et al., 2016, Basti et al., 2020, Zeng et al., 2010, Gwon et al., 2020, Dong et al., 2019, Elshazley et al., 2012).

BMAL1 is suggested to play a role in multiple endocrine and non-endocrine cancers, though its role does not seem consistent across various models, even within the same cancer type. Interestingly, the tumour types where BMAL1 is suggested to be pro-tumourigenic, thyroid carcinoma, mesothelioma and AML are cancer types with low incidence of p53 mutations in humans with ~1%, 15% and 5%, respectively (Donehower et al., 2019). As p53 is prevalent in downstream analysis of BMAL1 k/d effects it is plausible that some p53 mutations in the tumour may influence tumourigenic effects of BMAL1. Discoveries such as p53 oscillation upon BMAL1 k/d in HCT116 hint at a link to rhythmicity. MMP9 has also been reported to be suppressed by BMAL1 in glioblastoma, pancreatic and breast cancer suggesting that BMAL1 may be tied to metastatic and angiogenic potential (Gwon et al., 2020, Jiang et al., 2016, Wang et al., 2019). It would also be pertinent to have better models in these studies to more effectively demonstrate clinical relevance. Orthotopic xenografts are largely absent in BMAL1 manipulation studies and should be considered for their greater clinical relevance in

BMAL1 and other clock gene studies. Alternatively, as the clock is tied to inflammation and immunity, the use of humanised mouse models or simply immunocompetent mouse models may unearth new pathways by which abrogation of the molecular clock impacts tumourigenesis (Comas et al., 2017).

CLOCK

In humans, CLOCK has been linked to breast cancer and oestrogen signalling (Rossetti et al., 2012, Xiao et al., 2014). Though it is as yet unclear if there are links between CLOCK and other tumour relevant hormones. CLOCK, together with its binding partner BMAL1, is a transcriptional activator. CLOCK is not completely indispensable for circadian function, though CLOCK has other unique functions such as acting as an acetyltransferase (Doi et al., 2006). Cadenas et al. (2014) found an association between clock genes and tumours in humans, combining 766 microarray transcriptomes from several cohorts of breast tumour patients. Prolonged metastasis free survival was associated with higher expression of a number of circadian genes, including *CLOCK* (Cadenas et al., 2014). Fittingly, the six most frequent single nucleotide polymorphisms (SNPs) that occur in *CLOCK* are associated with significant changes in breast cancer risk (Hoffman et al., 2010). Furthermore, in higher grade gliomas and ERα-positive breast tumours *CLOCK* have been reported to be upregulated (Chen et al., 2013, Xiao et al., 2014).

In vitro and *in vivo* models show CLOCK as a tumour enhancer. Though it is significantly less well studied than BMAL1. In a murine AML model cells lacking CLOCK were depleted compared to WT, much like BMAL1 k/d, and both knockdowns prevented cell cycle progression (Puram et al., 2016). Similarly, in a subcutaneous flank model of human colon cancer SW480 cells endogenously expressing low levels of CLOCK, CLOCK overexpression led to larger tumours after two weeks. Mechanistic interrogation found that overexpressing CLOCK caused a decrease in apoptosis related proteins BAX and BID and an increase in p-AKT (Wang et al., 2015b). CLOCK k/d cells also had reduced metastatic potential in mice and reduced expression of HIF1α, ARNT and VEGF, all known to be key players in angiogenesis (Wang et al., 2017). *In vitro,* SW620 cells endogenously expressing high levels of CLOCK showed decreased proliferation and decreased cell migration after CLOCK k/d, while CLOCK overexpression in an endogenously low expressing cell line, SW480, resulted in the opposite effect (Wang et al., 2015b, Wang et al., 2017). CLOCK k/d in U87MG cells (human glioblastoma) increased apoptosis, along with decreased MYC and CCNB1 (Cyclin

B1) (Wang et al., 2016). It is at present unclear if the apoptosis mechanism here is the same as seen in Wang et al. (2015b). Fittingly, CLOCK k/d has also been demonstrated to reduce viability in glioblastoma stem cells and induce cleaved caspase 3 (Dong et al., 2019). In addition to apoptosis and angiogenic related pathways, CLOCK may be integrated closely with oestrogen (E2) and oestrogen receptor α (ERα) signalling in cancer. In ERα positive breast cancer cell lines, E2 has been demonstrated to upregulate CLOCK protein and mRNA by increasing ERα binding to the CLOCK promoter. A CLOCK k/d in these cells reduced proliferation (Xiao et al., 2014).

The two pathways suggested to give rise to a CLOCK mediated effect on tumourigenesis are apoptosis and oestrogen signalling. Oestrogen signalling is an attractive avenue as it has been previously demonstrated that BMAL1 k/d can modify ER signalling and lead to aberrant breast acinar morphogenesis (Rossetti et al., 2012). It may be pertinent to investigate if other circadian genes have similar links to oestrogen. Apoptosis-related proteins have been implicated in this system in multiple studies, therefore it would be useful for future studies to probe for those proteins that have been implicated before. This would demonstrate if apoptosis instigated by CLOCK k/d is via the same mechanism in all cell types. Additionally, some caution must be taken in assuming that CLOCK acts as a tumorigenic enhancer in all cancers. It is conceivable that its effects are just as cell-type specific as those of its heterodimeric partner and the research is simply sparser. Notably, in human patients, an anti-tumourigenic role for CLOCK was suggested that is not seen in xenograft models. NPAS2 is a paralogue of CLOCK that can compensate somewhat for CLOCK loss in the brain, moreover some evidence suggests that it may also be able to do this in the periphery (DeBruyne et al., 2007, Landgraf et al., 2016). In future it would be interesting for studies to take this into account and perform double knockdowns.

PERIOD GENES

Of all clock genes, the PER proteins have perhaps the most variable effects on tumourigenesis, with evidence for a tumour suppressive role and a tumour enhancing role in both human data and in experimental models. PER1 and PER2 are core components of the negative arm of the circadian clock as they suppress the function of the CLOCK/BMAL1 heterodimer. PER2 specifically is a very well researched clock protein with a complex circadian function (Konopka and Benzer, 1971, Philpott et al., 2020). As its expression oscillates distinctly over the circadian cycle, PER2 is a good rhythm marker. Overexpression

of PER2 can stop the circadian clock similar to BMAL1 k/o (Chen et al., 2009). Unlike PER1 and PER2, which have similar and well-defined roles in the clock, PER3 remains elusive. Its role, if any, in cell autonomous clocks appears tissue specific (Pendergast et al., 2012). However, a number of PER3 polymorphisms have been described with a variety of sleep phenotypes (Hida et al., 2014).

Patient sample data thus far suggests a tumour suppressive function for *PER1&3,* with *PER2's* role being less clear. A large study of datasets in The Cancer Genome Atlas suggested an overall tumour suppressive role for the *PERs*, however, *PER1,2&3* expression were associated with pro-tumourigenic inhibition of apoptosis and activation of RAS/MAPK or receptor tyrosine kinase (RTK) signalling in a sizeable minority of the cancer types analysed (Ye et al., 2018). Expression of all three *PERs* differed in 95% of breast tumours relative to margins, though no statistical analysis was presented for this data. Notably, the *PER* expression differed between individuals and between cell populations within tumour slices (Chen et al., 2005). Other groups have found similar results with *PER1&2* being under-expressed in breast tumours and gliomas, and low expression of *PER1,2&3* correlating with poorer overall survival in cases of pancreatic ductal adenocarcinoma (PDA) (Winter et al., 2007, Xia et al., 2010, Relles et al., 2013). In head and neck squamous cell carcinoma, low expression of both *PER1* and *PER3* was predictive of poorer 2-year survival. Low *PER3* expression was associated with larger tumour size and increased invasiveness, which are potentially better metrics than survival as this specific study had a low sample size and high overall 2-year survival (Hsu et al., 2012). *PER1* is reported to be down-regulated in breast tumours relative to healthy controls and in colorectal cancer low expression was associated with increased metastasis (Gery et al., 2006, Oshima et al., 2011). In gastric cancer *PER2* was actually found to be upregulated compared to paired margin biopsies, complicating its potential role (Hu et al., 2014). Finally, hetero- or homozygosity for a particular variant of *PER3* resulted in 1.7-fold elevated risk of breast cancer (Zhu et al., 2005).

Experimentally, PER2 appears anti-tumourigenic *in vivo* and *in vitro*. K562, human chronic myelogenous leukaemia (CML), cells have demonstrated reduced growth capacity in bone marrow upon PER2 overexpression. Induction of p53 as well as reduced CCNB1 and MYC were suggested as potential mechanisms (Sun et al., 2010). Similarly, mutation of PER2 increased proliferation of lung cancer cells in mice, this was conserved in a p53 deficient background (Papagiannakopoulos et al., 2016). In contrast, HCT116 xenografts in zebrafish embryos showed no change upon PER2 k/d (Basti et al., 2020). Overexpression of *PER2* in

murine Lewis Lung Carcinoma or EMT6 (mouse mammary carcinoma) cells *in vitro* results in reduced proliferation and increased apoptosis (Hua et al., 2006). Delivery of PER2 ectopically to murine lung tumours can reduce their size and growth (Hua et al., 2007). In human pancreatic cancer cell lines *PER2* increases BAX and reduces BCL-X in a dose dependant manner, as well as in human osteosarcoma where a reduction in *PER2* increased phospho-AKT and BCL-2 and reduced p27 and p21, implicating both cell cycle and apoptosis pathways (Oda et al., 2009, Qin et al., 2018). Conversely, Basti et al. found, despite the lack of effect *in vivo,* their PER2 k/d in HCT116 conferred the highest rate of proliferation *in vitro* until day 5, similar to PER2's tumour suppressive effects in CML and lung cancer cells *in vitro* (Basti et al., 2020, Sun et al., 2010, Papagiannakopoulos et al., 2016).

Experimental evidence for the role of PER1 and PER3 in tumourigenesis is scarce and that which is available is purely *in vitro*. PER1 overexpression is reported to increase DNA damage induced apoptosis in HCT116 cells, PER1 k/d having the opposite effect (Gery et al., 2006). In PaCa2 (human pancreatic cancer cells) PER1 k/d caused a decrease in proliferation, PER3 also caused a dip in proliferation but it was found to be statistically insignificant. These findings in PaCa2 are interesting as TNFα treatment of PaCa2 increases proliferation and downregulates PER1 and PER3 expression, suggesting PERs may be downstream of this TNFα effect (Suzuki et al., 2008). PaCa-2 and PANC-1 cell lines both demonstrate increased apoptosis upon PER1 k/d and display an increase in BAX and cleaved PARP as well as a decrease in BCL-2 (Sato et al., 2009).

Apoptosis and the cell cycle have both been implicated in the role of the PERs in tumours. PER2 is fairly well studied however, PER1 lacks *in vivo* data and PER3 is generally understudied. The finding that p53 is dispensable for PER2's effect on tumourigenesis in murine lung cancer, contrary to BMAL1, clearly demonstrates that the mechanism of action of each clock gene in tumourigenesis can be distinct, despite phenotypic similarities (Papagiannakopoulos et al., 2016). From the data discussed here it seems likely that the anti-tumourigenic effect of PER2 is via a combination of apoptotic and cell cycle effects, though it remains unclear if the exact same parts of these diverse pathways are involved across multiple tumour types. PER1 is thought to have a similar role in the circadian system as PER2, however the minimal mechanistic information in cancer seems to show an opposite effect. PER1 increases BCL-2 and decreases BAX and vice versa for PER2. A study interrogating the tumourigenic role of both PER1 and PER2 in the same cell line would be ideal. Sato et al. suggest PER1's effect on tumourigenesis may be due to PER1 dysregulation having a wider

impact on the circadian system as a whole, highlighting the aforementioned importance of assessing the whole molecular clock in models (Sato et al., 2009).

CRY1 & CRY2

In human cancer the role of the CRYs is very unclear, with studies suggesting they are tumour suppressive and others suggesting them to be tumour enhancing. The CRY proteins are important for circadian rhythms across the animal kingdom. In mammals they seem to have forgone their ancient light sensing ability, but still remain transcriptional repressors which, along with the PERs form the primary negative arm of the circadian clock (Michael et al., 2017). In humans, *CRY2* is associated with prolonged metastasis free survival in breast cancer and lower *CRY2* expression is associated with worsened overall survival in PDA, suggesting a link between oestrogen related cancer and *CRY* expression which sadly has not been addressed in models (Cadenas et al., 2014, Relles et al., 2013). In colorectal cancer, however, CRY1 expression correlates with worse overall survival, and in gastric cancer high *CRY1* expression relative to paired tumour margin is associated with higher cancer stage (Yu et al., 2013, Hu et al., 2014).

Facilitated by the availability of knock-out mouse models, studies on CRY1- and CRY2-deficient mice are abundant (van der Horst et al., 1999). Tumour autonomous CRY1 and CRY2 data is much scarcer. CRY1 k/d in HOS and U2OS cell lines (human osteosarcomas) led to larger tumours in mice (Zhou et al., 2018). This effect was also seen *in vitro* (Zhou et al., 2018). HCC cells with CRY double knockout in a p53 knockout (k/o) background have been shown to grow at the same rate as the same cells with just p53 k/o, however the CRY loss sensitised them to TNFα treatment, suggesting a link to apoptosis and the NF-κB pathway (Lee and Sancar, 2011). It should be noted that this HCC study was performed in immunodeficient mice; with a functional immune system these tumours would potentially die due to TNFα produced by lymphocytes in the tumour microenvironment without the need for endogenous TNFα.

In vitro, CRY2 appears to be anti-tumourigenic in osteosarcoma but has negligible effects in breast cancer. CRY2 k/d in HOS cells enhanced cell cycle progression, proliferation and migration, reducing p53 expression but increasing MYC, CCND1, MMP-2, β-Catenin and ERK1/2 phosphorylation. The knockdown also modified the circadian landscape in the cells, increasing CRY1, PER1, PER2, BMAL1, and CLOCK expression (Yu et al., 2018). CRY2 k/d in MCF7 cells showed no change in cell cycle distribution or apoptosis with or without methyl

methanesulfonate insult. The only difference observed was a difference in DNA damage accumulation and an upregulation of p21 and Cyclin D1 (Hoffman et al., 2010).

CRY2's inhibition of MYC seems important to its role in cancer. An increase in MYC upon CRY2 k/d is likely due to CRY2's known role in degrading MYC via cooperative action with the E3 ubiquitin ligase FBXL3 (Huber et al., 2016). Indeed, in humans, data concerning PDA, a cancer type inextricably linked to MYC, suggests an increase in CRY2 as beneficial for patient survival. However, the import of this in tumours is unclear as CRY2 k/o has proved insufficient for primary cell transformation; expression of MYC combined with CRY2 k/o was not sufficient to cause colony formation on soft agar (Huber et al., 2016). Therefore, CRY2 may act as an inhibitor of tumour progression but be insufficient to contribute to tumour initiation. In mouse models CRY's role seems reversed when p53 is mutated, with the CRYs acting as tumour enhancers, as loss of CRY acts as a sensitisation factor to TNFα mediated apoptosis (Ozturk et al., 2009). This suggests a link between CRY2 and p53, which is also potentially on display in vitro with p21 accumulation upon CRY2 k/d demonstrated by Hoffman et al. (2010). In human studies it would be interesting to contextualise the role of CRY in terms of the MYC and p53 status of the tumour. More data is required to fully understand the role of CRYs in cancers. There is a wealth of data concerning whole animal knockouts, but they are limited for modelling the clinical situation in human cancers in a meaningful way, there are no known human conditions which completely remove circadian proteins in all body cells.

REV-ERBα & REV-ERBβ

There is evidence to suggest that the REV-ERBs play a role in oestrogen related tumours in humans, and in *in vivo* and *in vitro* cell based studies, although the suggested effect on tumourigenesis is occasionally inconsistent. The REV-ERBs are part of the secondary circadian loop as they can bind to elements in the *BMAL1* promoter and suppress its transcription, they have ties to inflammatory pathways and are therefore, attractive targets for anti-cancer compounds. A number of agonists and antagonists of REV-ERBs have been developed (Wang et al., 2020). High expression of REV-ERBα is associated with better prognosis in triple negative breast cancer when the patients were treated with chemotherapy, leading to better overall and disease-free survival (Na et al., 2019).

REV-ERBs seem to be anti-tumourigenic in most, but not all, cell lines tested thus far. There are two available REV-ERB agonists which are used in many of the studies discussed

here, named SR9009 and SR9011. These have proved effective in killing astrocytoma (Becker), leukaemia (Jurkat), breast (MCF7), colon (HCT116) and melanoma (A375) cell lines *in vitro* (Sulli et al., 2018). SR9009 reduced autophagy and increased apoptosis in skin naevi, glioblastoma and subcutaneous small cell lung cancer in mice (Sulli et al., 2018, Shen et al., 2020). In HCT116, REV-ERBα k/d is reported to reduce proliferation and micrometastasis formation *in vivo* in a zebrafish embryo model. This is in contrast to the *in vitro* information from the same study which found enhanced proliferation on REV-ERBα k/d (Basti et al., 2020). In T98G glioblastoma and HepG2 cells introduction of SR9009 is cytotoxic, but in other hepatocellular carcinoma cells (Huh7 and HCCLM3) REV-ERBβ appears to increase cell viability and increase epithelial-mesenchymal transition (Wagner et al., 2019, Tong et al., 2020). Undifferentiated and partially differentiated gastric cancer (BGC-823 & SGC-7901), demonstrated increased proliferation upon REV-ERBα k/d and the glycolysis and pentose phosphate pathway were suggested as the potential mechanism (Tao et al., 2019). It must be noted, however, that these cell lines are known to have misattributed identity, so this data is potentially more related to HeLa cells than gastric cancer (Ye et al., 2015).

REV-ERBα is tied to breast cancer as it and HER-2 lie on the same amplicon. REV-ERBα k/d reduces viability of HER-2 positive breast cancer cells (BT474), potentially due to a link between REV-ERBα and fatty acid synthesis (Kourtidis et al., 2010). Conversely, in other breast cancer cell lines (MDA-MB-231, MCF7, BT474 and MDA-MB-361) SR9011 reduced cell viability. A REV-ERBβ k/d stopped the SR9011 effect therefore, even though SR9011 acts via both REV-ERBs, in these breast cancer cells SR9011 acted via REV-ERBβ (Wang et al., 2015a). Both SR9009 and SR9011 were tested on glioblastoma stem cells and were found to reduce proliferation compared to non-cancerous controls, and they reduced expression of glioblastoma stem cell markers. SR9011 combined with a CRY stabilisation agent reduced glioblastoma stem cell viability synergistically, both agents targeting different molecular feedback loops of the clock (Dong et al., 2019).

REV-ERB research is in an odd position, as there is contradictory data within the same cancer type in both breast cancer and hepatocellular carcinoma. In a number of breast cancer cell lines REV-ERBβ appears to be the important REV-ERB, though this may not be true in all types of breast cancer. From the human data it would be expected that REV-ERB agonists would be anti-tumourigenic which was true in many, but not all, cell lines studied. The comparison is not perfect as the human data was specifically in triple negative breast cancer and involved chemotherapy and the model data was *in vitro*. An experiment involving REV-

ERB knockdowns and agonists in a triple negative breast cancer line (e.g., MDA-MB-231) in a clinically relevant *in vivo* model (e.g., orthotopic) would give greater insight into the role these proteins play in human tumour biology. As there are already small molecules which show good efficacy and high specificity at targeting the clock via REV-ERBs, these genes are a promising route towards circadian based cancer therapies, though it should be noted that the agonists currently available have been suggested to have anti-proliferative effects independent of their REV-ERB agonism (Dierickx et al., 2019, Dong et al., 2019).

TIMELESS

TIMELESS was one of the first circadian proteins to be cloned in flies where it is an integral part to the circadian system (Sehgal et al., 1994). In mammals, a TIMELESS homolog exists, but its function in mammalian circadian biology is poorly understood, presumably in part due to the embryonic lethality of TIMELESS k/o (Gotter et al., 2000, Kurien et al., 2019). In humans, high expression of *TIMELESS* is associated with shorter metastasis free survival in breast cancer, meaning its only link to cancer in humans is in oestrogen related cancer (Cadenas et al., 2014).

This oestrogen related cancer link is also reiterated *in vivo*. In an orthotopic breast cancer model TIMELESS k/d was reported to reduce the viability of MCF7 cells (Chi et al., 2017). Similarly, *in vitro* TIMELESS k/d in MCF7 cells reduces proliferation, and in the hepatocellular carcinoma cell lines HepG2 and Hep3B TIMELESS k/d causes cell cycle arrest and slightly increased apoptosis as well as a drop in migration in Hep3B cells (Mao et al., 2013, Elgohary et al., 2015). Data on TIMELESS is understandably minimal given its unclear role in the circadian system. The orthotopic *in vivo* model used by Chi et al. is an improvement over subcutaneous flank models and could be emulated for breast cancer studies on circadian genes in future.

DEC1 & DEC2

DEC1 and DEC2 are part of a smaller clock sub-loop which interlocks the primary circadian feedback loop. *DEC1* & *DEC2* are E-box containing genes whose corresponding proteins are known to inhibit CLOCK/BMAL1 heterodimer mediated transcription (Nakashima et al., 2008). In AML with MLL-AF6 chromosomal rearrangement, *DEC2* is overexpressed. Downregulation of *DEC2* increases apoptosis of these cells and reduces their viability *in vivo*, though the knockdown does not change the expression of other clock genes

(Numata et al., 2018). DEC1, however, has been reported to prevent cell cycle progression by directly binding to, and hence stabilising, Cyclin E (Bi et al., 2015). Overexpression of *DEC1* in MCF7 cells reduced tumour size in mouse xenografts significantly (Bi et al., 2015). In MCF7 cells, *in vitro*, knockdown of DEC2 increased apoptosis, whereas DEC1 did not. Apoptosis related factors, namely FAS and BAX, were upregulated and MYC expression was decreased (Wu et al., 2015).

Genes outside of the primary and secondary canonical circadian loops, but which nonetheless have a role in circadian oscillations, are interesting and much of their function remains elusive. DEC1 and DEC2 are transcriptional repressors but, as evidenced by the role of DEC1 in stabilisation of Cyclin E, have roles outside of their transcriptional activity. The effect that these proteins have on tumourigenesis, as well as their specific role in tumour rhythms should be studied more closely.

MECHANISM OVERVIEW - P53

The p53 axis is a recurring mechanism linking the clock with cancer biology. This could prove pivotal as p53 is the single most frequently mutated gene in human cancers. Its ability to orchestrate cell cycle arrest via p21 and apoptosis via BCL-2 in response to DNA damage makes removal of p53 function essential for much of malignant growth (Kastenhuber and Lowe, 2017). As explored in this review, p53 has been linked to *BMAL1, PER1, PER2* and *CRY2.* The role p53 plays in the cancer biology of each gene is convoluted, for example *PER2* status modified p53 abundance in K562 cells, though its tumour suppressive role in lung cancer was demonstrated to be p53 independent. Additionally, p53 appears to begin cycling when *BMAL1* expression is lost, antithetical to the canonical idea that no BMAL1 means no circadian oscillation. The ubiquitous role of p53 in cancer makes the cell specific nature of its involvement confusing, though there are some possible explanations. Firstly, some studies do not probe for p53 and therefore it may be playing an unseen role in these cells. Secondly, *BMAL1* exerts pro-tumourigenic effects in cell lines with relatively low p53 mutation rates in patients. Perhaps the natural dependency of the cancer on p53 pathways may give rise to these cell specific effects.

This mechanism is further complicated by circadian-p53 crosstalk. It has been shown previously that a reduction of p53 upon *PER2* k/d can occur due to PER2 preventing MDM2 mediated ubiquitination of p53 (Gotoh et al., 2014). In turn p53 can block CLOCK/BMAL1 binding to the E-box elements in the *PER2* promoter (Miki et al., 2013). If p53 has such strong

links to PER2 then other clock genes that have an effect on p53 may in fact just be modifying *PER2* expression. Nevertheless. p53 remains an interesting mechanism by which the clock and cancer may interact.

MECHANISM OVERVIEW - APOPTOSIS

Another potential mechanism by which these genes influence tumourigenesis is via apoptosis pathways independent of p53. A link between the circadian clock and p53 independent cell death has been previously established, demonstrating a link between NF-κB signalling and CRY in p53 null cells (Lee and Sancar, 2011). Whilst this is a good indicator of a circadian-apoptosis link, the data presented here have shown that aberrations in different clock proteins can lead to differing effects. Across a number of the aforementioned studies, changes in apoptosis related proteins such as p21 and BAX are fairly common. However, it is difficult to know if these effects can be traced back to p53. Apoptosis pathways converge significantly, and the effects of p53 are widespread. Sadly, a number of studies that identify increased apoptosis as a potential mechanism of tumourigenic modifications by circadian genes do not probe for p53 specifically, so it is difficult to ascertain which effects are p53 independent and which are p53 dependant.

MECHANISM OVERVIEW – CELL CYCLE

Cross talk between circadian oscillators and the cell cycle is well established (Farshadi et al., 2020). Dysregulation of the cell cycle is a key driver of increased proliferative capacity. In the majority of studies mentioned here there is a change in proliferation upon clock gene modification. Cell cycle arrest is repeatedly implicated in circadian rhythms and cancer. Primarily cells were found arrested in G2 (Sun et al., 2010, Elgohary et al., 2015, Wang et al., 2015a). It would be presumptuous to assume that this G2 arrest across various genes and cell types is caused by the same mechanism, though the consistency is interesting. There are a number of genes that are important for circadian gene effects on tumourigenesis that also have some function in the cell cycle (*WEE1, MYC, AKT, CYCLINB1, P21, P27, K167*) although many of these are also involved in apoptosis. Splitting up circadian genes' role in apoptosis and proliferation may prove folly as the scope of circadian control is so vast and these pathways overlap to such a degree that it is likely that both are involved on some level. It is also important to note that a number of studies do see an increase in proliferation via MTT assay, colony formation assays or BrdU assays, but do not interrogate the exact mechanism for this increase, so we do not yet have an exhaustive list of what proteins and pathways are involved.

Clocks in the clinic - How to tell if Tumours Tick?

Knowledge of the mechanisms underlying clock influence on human tumours is important, but equally important is the ability to translate this into a clinical setting. As previously mentioned, it is crucial to know the degree of circadian function of a tumour. Understanding the expression of oscillating genes is not as simple as looking for genes that are up or down regulated. Instead, we must ascertain the presence or absence of oscillation, the amplitude and phase of the oscillation, and the abundance of clock genes relative to the point of the circadian cycle. In addition, it is clear that clock gene expression differs based on tumour type, tissue type and the individual. In an age of personalised medicine, bespoke knowledge of the rhythmic function within individual tumours will be paramount. However, taking a biopsy on the hour every hour for 24 hours to assess rhythmicity is unlikely to be a popular option. To state simply, we require an innovative way to know if tumours tick in individuals and what that ticking looks like.

Algorithms have been developed which aim to ascertain the biological time of single samples based on their transcriptome. Several supervised models have been trained using human circadian transcriptome datasets (Agostinelli et al., 2016, Hughey, 2017, Laing et al., 2017, Braun et al., 2018, Wittenbrink et al., 2018). Circadian transcriptomics experiments in mice have demonstrated that expression of each clock gene is usually synchronised in phase across different tissues, but the magnitude of expression and amplitude of oscillation can differ (Zhang et al., 2014). For example, the algorithms ZeitZeiger, PSLR, BIO_CLOCK and BodyTime require that training and test datasets are tissue-matched, or even cell-matched (Wittenbrink et al., 2018). This means these time prediction algorithms are constrained to blood samples, for which training datasets around the clock are available.

Alternatively, other mathematical methods have been suggested that do not rely on serial samples of individuals. CYCLOPS is a largely unsupervised method which requires reasonably large transcriptomic datasets (ideally >250 samples) composed of single samples from different individuals over the course of the day (Anafi et al., 2017). CYCLOPS-ordered hepatocellular carcinoma (HCC) tumour biopsies demonstrated population-wide oscillations in 8 of 9 core clock genes, but showed a reduction in amplitude of oscillation and magnitude of expression relative to CYCLOPS-ordered HCC tumour margins (Lamb et al., 2011, Anafi et al., 2017). Unfortunately, since rhythmic gene expression is estimated from the population,

CYCLOPS cannot estimate the variation in rhythmic gene expression between individuals (Anafi et al., 2017), which limits its clinical use.

Another method that does not require tissue-matched, time-stamped training datasets, known as ΔCCD, is based on calculating the pairwise Spearman correlation of the expressions of all 12 core clock genes previously used to build ZeitZeiger (Hughey et al., 2016, Shilts et al., 2018). For each sample the resulting correlation matrix is then compared to a mouse healthy tissue reference matrix. Using this method, 20 tumour biopsy datasets differed from non-tumour samples, indicating that core clock gene expression of the tumour samples was more different than clock gene expression of non-tumour samples, relative to the mouse reference. This suggests that core circadian gene expression is consistently disrupted in tumours over a population. Interestingly an unrelated algorithm, TimeTeller, trained using a circadian transcriptome data of healthy human oral mucosa biopsies, was used to predict the extent of molecular clock dysfunction in the biopsies of 226 breast cancer patients (Vlachou et al., 2019). Most importantly, clock dysfunction and overall survival correlated, suggesting a functional role for the tumour clock, which warrants further investigation.

Mathematical models have been used to predict population level dysregulation of rhythmic gene expression from human tumour biopsies and have provided some insight into individual clock variation (Vlachou et al., 2019, Anafi et al., 2017, Shilts et al., 2018). Yet, there is poor correlation in the degree of clock gene dysregulation between different tumour types or even between cohorts of the same tumour type (Shilts et al., 2018, Ye et al., 2018). It is unclear whether this is a result of individual molecular clock difference, intertumoral difference, sampling-time difference, or perhaps another factor such as tumour grade. This demonstrates the importance of well-annotated publicly available datasets, ideally samples would be time-annotated.

Conclusion and Future Directions

In conclusion, there is a wealth of evidence at many levels which links the expression of circadian clock proteins to tumour biology and tumourigenesis. Though some of this evidence is limited, such as a lack of timing information for human biospecimen, it still seems overwhelmingly likely that there is a link between circadian clock gene expression and tumour development or progression. There is, however, not a very detailed mechanistic understanding if any given circadian clock gene is pro- or anti-tumourigenic, as the effects of gene's products

appear cell-type specific. It is difficult to understand the cause of this cell specificity without greater knowledge of the underlying mechanisms of the role of circadian clock genes in tumourigenesis in these instances. Additionally, it is not known if the effect of circadian proteins on tumourigenesis is linked to their role in the clock or is a separate function regardless of oscillation.

Deciphering the role of circadian clocks in human tumours requires not only an understanding of the degree of clock function in the tumour, but also an understanding of the degree of clock function in the healthy tissue of the host, ideally in the context of the individual's overall behavioural rhythm. A number of ingenious mathematical models have been developed which have the potential to elucidate clock function from large transcriptomic datasets. However, the availability of timing information in the data, is important both for the training of these models and for their use in matching molecular clock status to clinical information. Important initiatives such as The Cancer Genome Atlas and the International Cancer Genome Consortium do not record time of sampling information, despite recording other important patient-specific metadata such as alcohol and smoking history. Time of sampling information would greatly improve the possible use of such datasets. An additional level of understanding about the patients' individual clocks could be gleaned from biopsies of healthy tissue similar to that of the tumour, this would be the gold standard for comparing the molecular clock of the healthy cells to those of the tumour. However, a more feasible and non-invasive way of gaining this information may be to use a short chronotype questionnaire to determine the patient's behavioural clock function (Ghotbi et al., 2020). This review in part serves as an appeal to patient-facing clinicians involved in research. If circadian-cancer studies in humans are to substantially improve, then more annotation of samples with as much time information as possible is paramount.

As for *in vitro* and *in vivo* modelling of the effects of clock genes on cancers, it will be important to strive for clinically relevant models. Effects of clock k/d *in vitro* do not always hold true *in vivo.* This is somewhat expected as, *in vivo,* tumour cells will be receiving synchronisation information from the SCN much like healthy cells. It is clear that the effects of circadian genes are highly cell type specific, and it is not yet clear what the reasons for this may be. The status of pathways such as p53 in the cells before the circadian aberration may play a key role. Testing circadian genes by a single knockdown in a single cell line gives interesting information but it is slow and cumbersome. A push towards screening more than one clock gene at a time and potentially testing the effects of the entire molecular clock at once

would allow a faster development of knowledge in this area. To ascertain if these effects are actually down to changes in the clock, or due to properties of clock genes which may have come uncoupled from their role in rhythms, it is useful to know the circadian health of a cell line before the modification and after. Some studies already test this but many do not. Better time matched human data and clinically relevant *in vivo* and *in vitro* analysis which takes into account the circadian system as a whole should go some way to allow us to pick apart the complex and varied roles that circadian genes appear to play in tumour biology.

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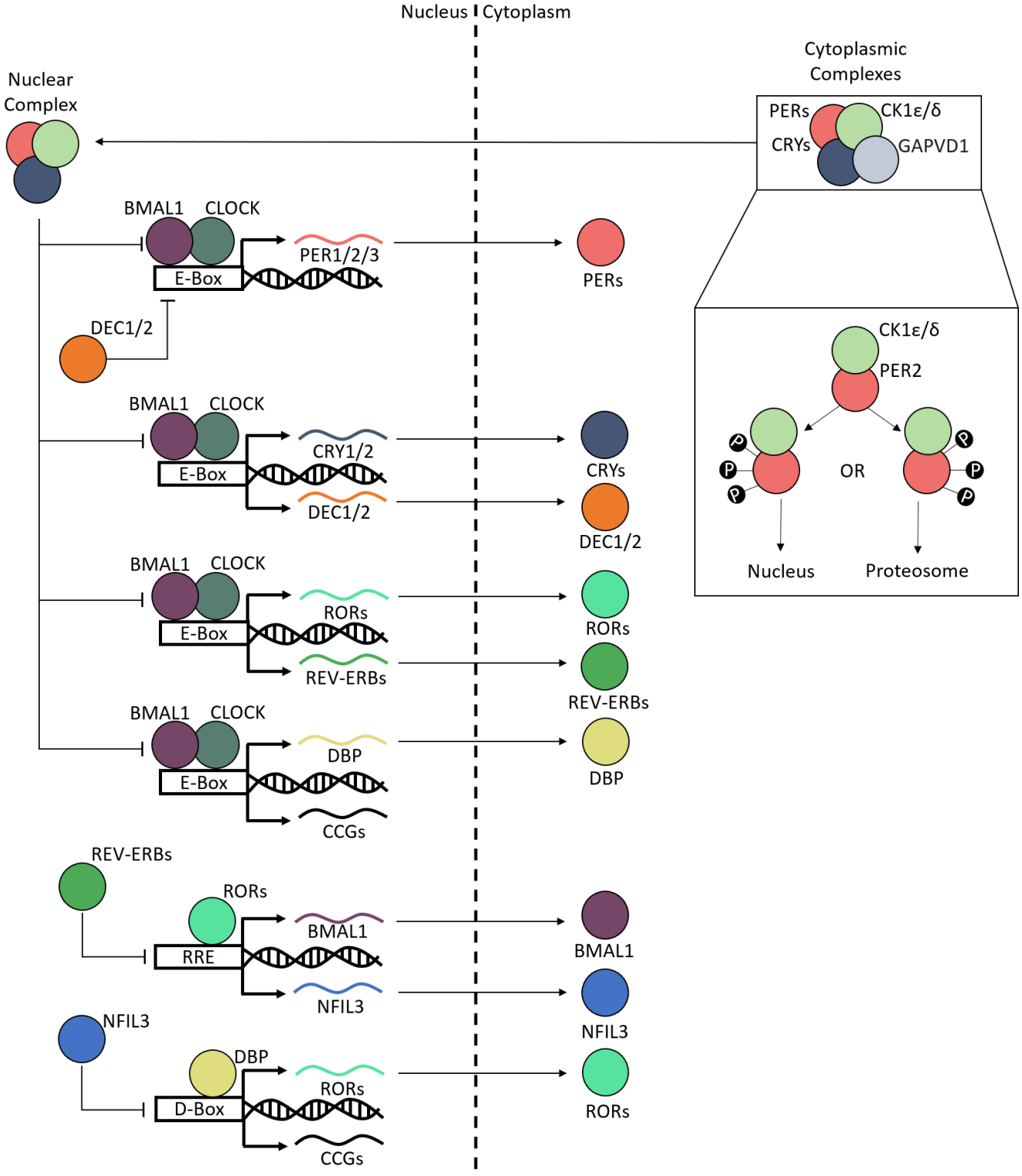
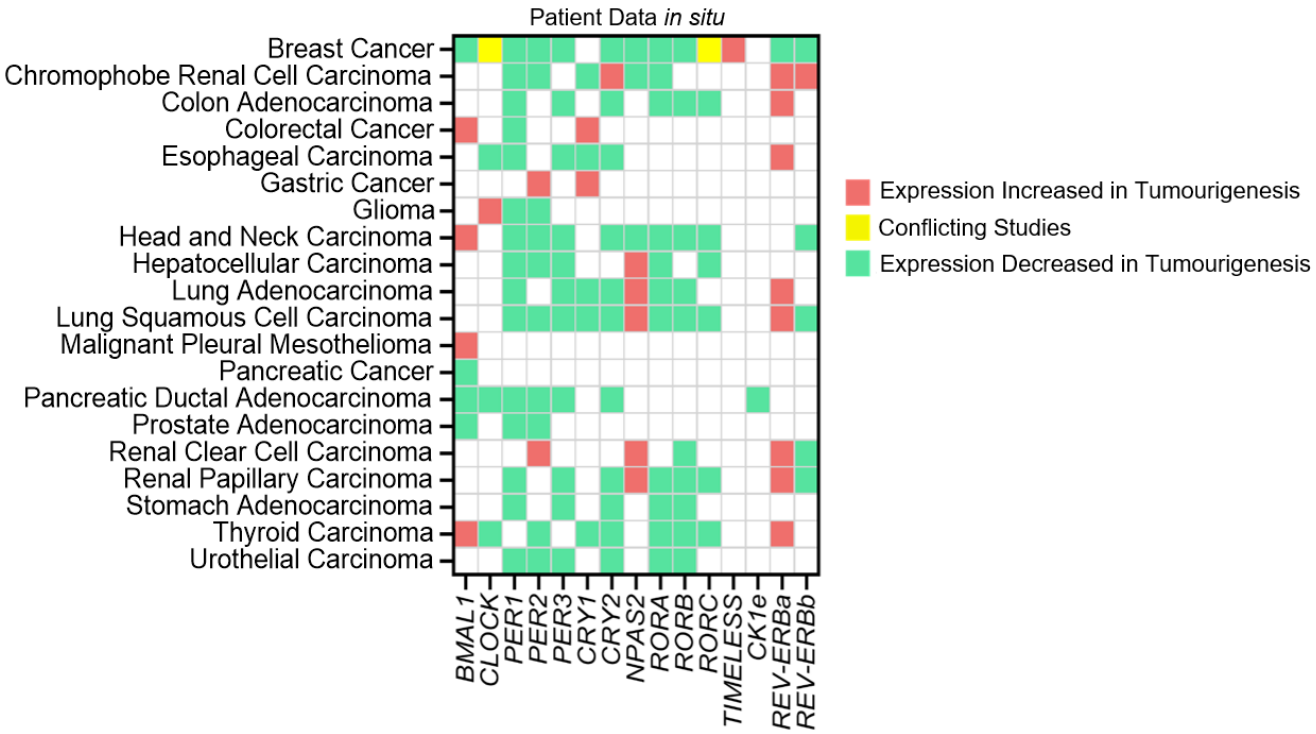
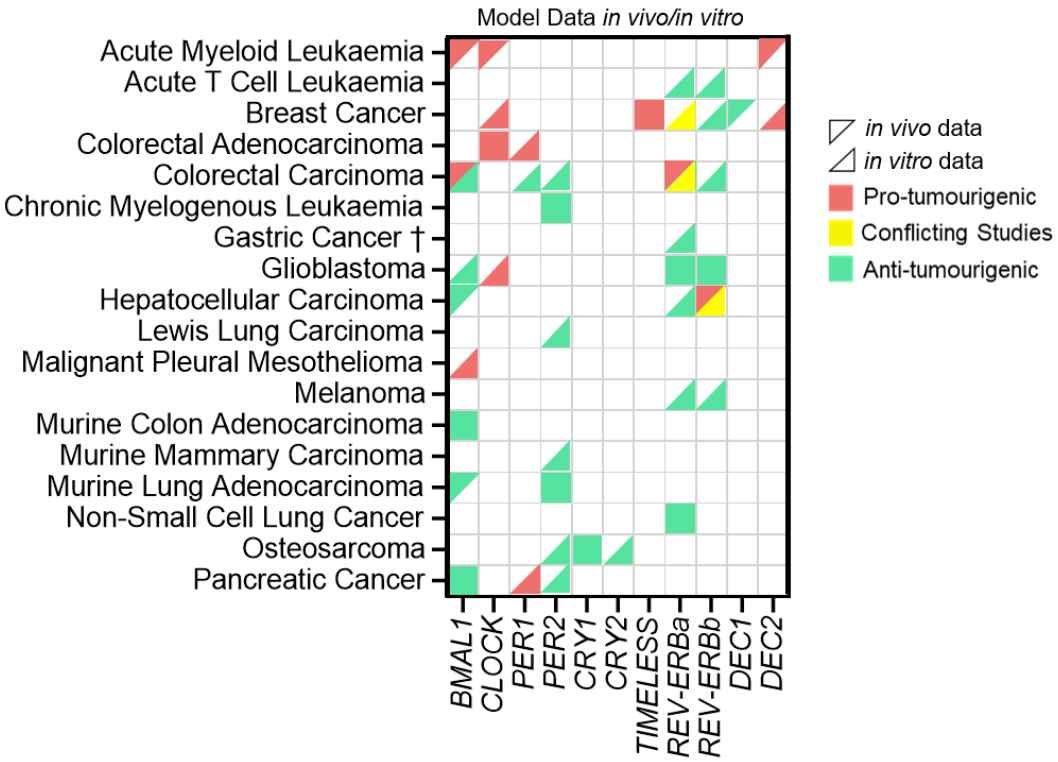
Figure 1. A general overview of the molecular circadian clock. CLOCK/BMAL1 binds to E-box elements in clock-controlled genes (CCGs). CCGs are varied, producing important proteins such as p53, as well as circadian proteins which enter into feedback loops with CLOCK/BMAL1. In the primary circadian loop PER and CRY proteins are translated and accumulate in the cytoplasm. Here they form macromolecular cytoplasmic complexes with other proteins including GAPVD1, who's role in circadian biology remains largely elusive, and CK1ε/δ. During this cytoplasmic phase a number of post-translational modifications occur which modify the activity of the PERs and CRYs. PER2 phosphorylation is pictured as an example, CK1ε/δ phosphorylates PER2 on either the FASP site or the degron site leading to PER2 stabilisation or β-TrCP mediated degradation respectively. Stabilised PER2, along with other members of the cytoplasmic circadian complex can be translocated to the nucleus. Here a nuclear complex of PERs and CRYs can repress CLOCK/BMAL1 mediated transcriptional activation and therefore, repress their own transcription. In the secondary loop RORs and REV-ERBs, who's transcription is regulated by E-boxes, compete for access to ROR response elements (RREs) with RORs promoting *BMAL1* transcription and REV-ERBs repressing it. Pictured here are two of the proposed sub loops. DEC1 and DEC2 repress *PER1* transcription. DBP mediates oscillatory transcription via D-box elements and is repressed by NFIL3, which itself is transcribed via ROR activity at RREs.

Figure 2. An overview of the known data linking circadian genes to cancer which will be discussed in this review. Green fill denotes an anti-tumourigenic effect of the gene, red fill denotes a pro-tumourigenic effect of the gene, yellow fill denotes conflicting evidence with some studies suggesting the gene to be anti- and others pro-tumourigenic. ┼ denotes a cell type whos identity was potentially misattributed.


Nucleus
Cytoplasm
Nuclear Complex
Cytoplasmic Complexes
PERs
CK1ε/δ
CRYs
GAPVD1
BMAL1
CLOCK
E-Box
PER1/2/3
PERs
DEC1/2
CRY1/2
CRYs
DEC1/2
RORs
REV-ERBs
DBP
CCGs
RRE
D-Box
NFIL3
PER2
OR
Nucleus
Proteosome


Patient Data *in situ*
Breast Cancer
Chromophobe Renal Cell Carcinoma
Colon Adenocarcinoma
Colorectal Cancer
Esophageal Carcinoma
Gastric Cancer
Glioma
Head and Neck Carcinoma
Hepatocellular Carcinoma
Lung Adenocarcinoma
Lung Squamous Cell Carcinoma
Malignant Pleural Mesothelioma
Pancreatic Cancer
Pancreatic Ductal Adenocarcinoma
Prostate Adenocarcinoma
Renal Clear Cell Carcinoma
Renal Papillary Carcinoma
Stomach Adenocarcinoma
Thyroid Carcinoma
Urothelial Carcinoma
BMAL1
CLOCK
PER1
PER2
PER3
CRY1
CRY2
NPAS2
RORA
RORB
RORC
TIMELESS
CK1e
REV-ERBa
REV-ERBb
Expression Increased in Tumourigenesis
Conflicting Studies
Expression Decreased in Tumourigenesis


Model Data *in vivo/in vitro*
Acute Myeloid Leukaemia
Acute T Cell Leukaemia
Breast Cancer
Colorectal Adenocarcinoma
Colorectal Carcinoma
Chronic Myelogenous Leukaemia
Gastric Cancer †
Glioblastoma
Hepatocellular Carcinoma
Lewis Lung Carcinoma
Malignant Pleural Mesothelioma
Melanoma
Murine Colon Adenocarcinoma
Murine Mammary Carcinoma
Murine Lung Adenocarcinoma
Non-Small Cell Lung Cancer
Osteosarcoma
Pancreatic Cancer
BMAL1
CLOCK
PER1
PER2
CRY1
CRY2
TIMELESS
REV-ERBa
REV-ERBb
DEC1
DEC2
in vivo data
in vitro data
Pro-tumourigenic
Conflicting Studies
Anti-tumourigenic