rijksuniversiteit groningen / faculteit wiskunde en natuurwetenschappen

Circadian Rhythmicity in Cancer & Treatment

Mechanisms and future perspectives

Bachelor Thesis
Geert Dirk Lanting s2254379
Supervised by R.A. Hut

Abstract. The circadian clock is a complex biological program that controls a vast variety of bodily functions. Output signals in the form of genes and proteins that are generated by the cellular molecular clock play a major role in the control of cell division and proliferation. Malfunction of this genetic molecular clock might result in the genesis of cancer. The main components of the molecular clock are Bmal1, Clock, Per and Cry. Bmal1 promotes the transcription of other genes by binding to E-boxes in promoter regions and activates p53 by facilitating p21. Bmal1 also induces the transcription of Myc, an important oncogene. Clock has proven to have important acetyltransferase functions, thereby stimulating transcription and translation. Per has a tumour-suppressor effect by activation of the ataxia telangiectasia, and by regulating cyclin D and β-catenin, both important regulators of the cell cycle. Cry has oncogenic effects, which are enhanced in the absence of Per. The mechanism of this is not well known. A complex and delicate balance exist between the circadian clock genes and their actors in the control of expression of genes required for controlled replication and proliferation. Other elements that control the cell cycle and are temporally controlled include Myc, Wee1, Cyclin D and P21. Circadian driven chances in physiology also have major effects on the genesis of tumours. The immune system is an important safeguard in controlling cancer and it is subject to circadian variation. Natural killer cell count appears to be oscillating and directly influence the avoidance of apoptosis. Melatonin is an important circadian endocrine output signal and has significant tumour-suppressor effects through inhibition of the LA-uptake pathway and lowering of PKA activity, ultimately leading a decrease in transcription factors. This mechanism is only proven in nocturnal animals. There is also circadian variation in chemotherapy resistance. The circadian time-dependant therapeutic index is designed because of this and takes into regards pharmacokinetics and pharmacodynamics. What is most important is that a delicate balance exists between clock genes that prevent uncontrolled cell division, proliferation and ultimately the genesis of cancer.

Keywords: Chronotherapy, Circadian Rhythmicity, Molecular Clock Genes, Bmal1, Clock, Per, Cry, Melatonin, Cancer, Tumourigenesis

TABLE OF CONTENTS

Introduction 3

Circadian Rhythmicity in cells 3

The molecular clock 3
Molecular clock output 4

Defining cancer 5

The Six Classical Hallmarks of Cancer 5
Apoptosis 5
Cell specific vs. tissue specific characteristics 6

Coupling cancer and circadian rhythmicity: De-regulated temporal transcription and output 6

4 ways of affecting the molecular clock and output 6

Mechanisms 7

Molecular clock gene coupled tumourigenesis 7

Bmal1 and Clock 9
Period and Chryptochrome 10
The cell cycle and molecular clock genes 12
Myc 12
Wee1 12
Cyclin D 12
P21 13

Circadian driven changes in physiology and cancer susceptibility 13

The immune system 13
Melatonin 13
The melatonin induced inhibitory pathway 15

Circadian variation in chemotherapy resistance: Chronotherapy 16

Circadian time-dependent therapeutic index 16
Pharmacokinetics 16
Pharmacodynamics 17

Future Perspectives 18

References 20

INTRODUCTION

CIRCADIAN RHYTHMICITY IN CELLS

All cells of the human body contain a complex biological program that is called the circadian clock. The word circadian is derived from the Latin words ''circa'' and ''diem'', which mean respectively ''about'' and ''a day''. It is not hard to imagine that therefore the endogenous circadian clock of the human body is a clock with a period of about 24 hours (Savvidis & Koutsilieris, 2012). The purpose of the circadian timing system is to temporally coordinate the biochemical and physiological functions of the body along the 24 hour day (Levi et al., 2010). The central pacemaker producing endogenous self-sustained oscillations required to temporally coordinate these biochemical and physiological functions is located within the hypothalamus and is called the Suprachiasmatic Nucleus (SCN). .

To entrain the clock of the SCN to the external environment, peripheral input from sensory organs and higher brain areas are integrated in the SCN, and as a result the internal period of the SCN is matched to that of the environment (Lévi et al., 2007). The SCN in turn generates neurological-, physiological- and endocrine signals to which ubiquitous peripheral clocks throughout the entire body are entrained (Innominato et al., 2010). This results in a wonderfully synchronized and harmonic clock resonating throughout the entire body with complex interacting components, which are required to sustain such a system (Gerkema et al., 2013). The resulted synchronous coordination leads to peaks in, for example, the release of hormones like cortisol, catecholamines and melatonin, but also in rhythms in body temperature, autonomous nervous system activity and physical and cognitive performance (Innominato et al., 2010). Next to these functions the biological clock also coordinates the ability to fall asleep and wake, it coordinates the increased motility of the intestines and even immune responses are synchronized to elect an optimized response (Keller et al., 2009). When one takes in consideration all the important functions the biological clock fulfils in the physiology of the human body it is safe to assume that the biological clock is an intriguing and complex biological process which biological and clinical significance should not be underestimated.

The molecular clock

When isolated and cultured each cell has its own endogenous circadian rhythm. However, each cell is synchronized to the phase of neighbouring cells within organs and tissues by the hypothalamic SCN. To sustain such a complex biological program cells are equipped with their own endogenous genetic molecular clock, which consist of a complex interplay of interconnected auto-regulatory transcriptional, post-transcriptional and post-translational negative feedback loops involving ~10-20 clock genes (Innominato et al., 2010). The clock genes work in a complex interplay of transcription and translation and activate or inhibit each other, creating a so called negative feedback loop, as illustrated in Figure 1. The positive limbs of the molecular genetic feedback loop consist of the genes CLOCK and BMAL-1 (Lowrey & Takahashi, 2004). The protein translational products of these genes heterodimerize in the cytoplasm and these heterodimerized proteins promote the transcription of the genes Per1/2 and CRY1/2. The transcription, translation and heterodimerizing of these genes takes some time, and eventually after a few hours delay the dimerized proteins of the Per and Cry genes translocate into the nucleus to inhibit the promotional activity of the proteins of the Clock and Bmal1 genes (Lowrey & Takahashi, 2004). The decreased levels of active translational product of the Clock and Bmal1 genes in turn cause a decreased activation of the Per and Cry genes. Indeed, the activation of the Per and Cry genes indirectly causes

the inhibitions of these genes as well. It is therefore that the Per and Cry genes are called the negative limb of the genetic negative feedback loop which comprises the cellular molecular clock (Lowrey & Takahashi, 2004). Both the positive limb consisting of Clock and Bmal1 as well as the negative limb consisting of Per and Cry are connected by a third gene called Rev-erb-α. Like the transcription of Cry and Per, Rev-erb-α is activated by the translational products of the Clock and Bmal1 genes. Rev-erb-α indirectly inhibits its own transcription however, by inhibiting the transcription of Bmal1 by binding of its translational products to elements in the promoter zone of Bmal1, possibly as well as the promoter zone of Clock and Cry1 genes.

Molecular clock output

It is the delay and the time it takes in which these activations and inhibitions are succeeded by all these molecular components that cause an oscillation in the transcriptional and translational products of the clock genes. These rhythmical oscillations in mRNA and protein levels are the essences that entail the cellular molecular clock, and it is the presence or absence of these products that causes the activation of other specific genes that need to be temporally controlled.

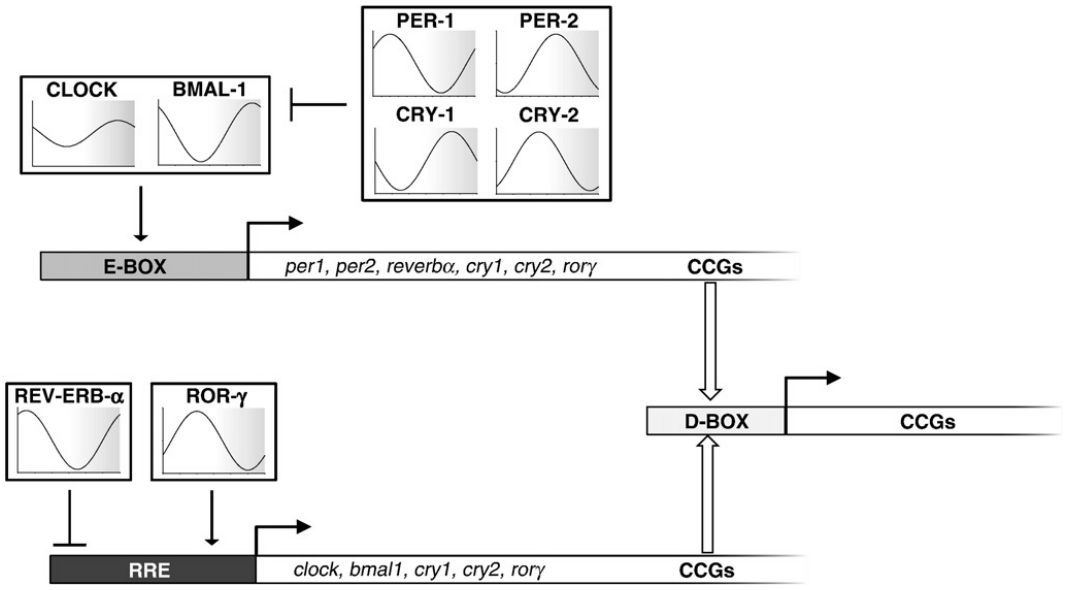

FIGURE 1 Schematic representation of the genetic molecular clock machinery inside the cell by Innominato et al. (2010). What is shown in the bars are specific clock controlled genes (CCGs) with promoter boxes and enhancers lying before them. As previously described, the translational products of the PER and CRY genes inhibit the translational products of CLOCK and BMAL1, thereby directly inhibiting their own transcription. PER and CRY are activated by the heterodimerized products of the BMAL1 and CLOCK genes, and BMAL1 controls its own transcription indirectly by activating Rev-erb-α which inhibits BMAL1 expression. It is these interactions which cause oscillations in transcriptional and translational products which are the fundamental basis of the cellular molecular clock. These oscillations are also shown in the figure.

It is seems likely that defects and dysregulation in the complex circadian genetic interactions can cause biological dysfunction (Innominato et al., 2010); cancer being perhaps the most important dysfunction. For understanding how deregulated cellular rhythms can lead to cancer it is important to describe the basic principles underlying cancer and the hallmarks define it.

The six classical hallmarks

According to the six classical hallmarks of cancer as written by Hanahan and Weinberg (2000) there are six different capabilities and characteristics of cells that can lead to and contribute to the development of uncontrolled cell division and tissue growth, and therefore cancer. These characteristics or hallmarks are almost always present in tumorous cells, though not all of them have to be present for a cell to be defined as cancerous. Under most conditions cancerous cells usually express more than five of the hallmarks. Perhaps the two most important hallmarks are the abilities of the cell to continue to proliferate even in the absence of growth signals as well as being insensitive to anti-growth signals (Fedi et al., 1997). Normal cells require mitogenic growth signals before they can replicate their genetic information and shift into a phase of mitosis and divide. These growth signals can have both extracellular and intracellular sources. Extracellular signals bind receptors on the cell membrane and transduce their signals by means of second messengers to the genetic machinery of the cell and are translated into dividing and proliferating properties. Sometimes these mitotic growth signals are transported across the cell membrane however and bind directly to the nucleus membrane. Intracellular growth signals are often generated by cell division regulatory mechanisms located within the cell, and are often subject to circadian influence. Because tumorous cells do not require these mitotic growth signals and are resistant to anti-growth signals, which work by the same mechanisms as the mitotic growth signals, the body loses its capability of controlling the cell's division and therefore homeostasis in cell numbers is lost (Zuo et al., 1996). Growth signals are still needed for cell division and growth in tumour cells however, it is suggested that tumour cells generate their own mitogenic growth signals.

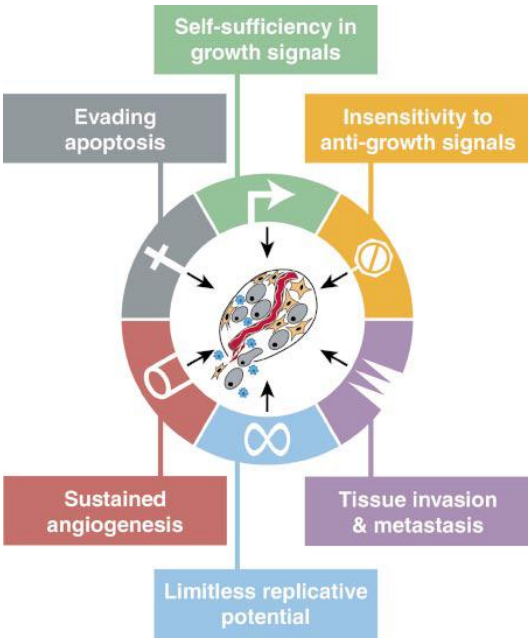

FIGURE 2 The classical hallmarks of cancer. All cancers have usually developed at least five of the six classical hallmarks of cancer. (Hanahan & Weinberg, 2000)

Apoptosis

Besides being self-sufficient in mitogenic growth signals and having the ability to deflect anti-growth signals, cancers possess the ability to evade apoptosis (Hanahan & Weinberg, 2000). Apoptosis is a means of controlled cell death which is induced by the body when a cell is sick or has become redundant. Apoptosis is therefore a means of the body to remain in homeostasis. Usually when cells are damaged and cannot be repaired the body's immune system sends the cell into a path of controlled cell death, thereby destroying the cancerous cell and its ability to spread (Wyllie et al., 1980). This way of stopping affected cells is usually very effective and efficient in stopping cancer growth. However, some cells have gained the ability to avoid apoptosis, and thereby avoiding controlled cell death and gaining the ability proliferate and divide uncontrollably.

Cell specific vs. tissue specific characteristics

The three previously described hallmarks all represent characteristics and abilities of a single mutated cancerous cell. However, tumours do not consist of only one cell. The three remaining classical hallmarks of cancer are attributed to the entire tumour and consist of the ability of a tumour to generate sustained angiogenesis, invade tissue and develop metastasis and lastly gain the ability of limitless replicative potential (Hanahan & Weinberg, 2000). These three hallmarks of cancer are not contributing to the genesis of a tumour but rather to the sustainability and spread of it (Hanahan & Weinberg, 2000).

The abilities of being self-sufficient in cell growth signals, being insensitive to anti-growth signals and the ability to avoid apoptosis are acquired by accumulation of random genetic mutations, and cancer is therefore defined as a genetic disease (Vogelstein & Kinzler, 2004). Because a single mutation is normally not sufficient enough to cause a cell to become cancerous, it is best to not think of mutated genes as a cause of cancer, but more of as a contribution to the genesis of it (Vogelstein & Kinzler, 2004). Cells have numerous safeguard mechanisms in place in order to repair and correct for mutations however, it takes a long time before there are sufficient mutations accumulated in the cell to cause a cell to divide uncontrollably. Therefore cancer is an illness which usually shows later in life. Alterations in three types of genes are known to cause tumour genesis: oncogenes, tumour-suppressor genes and stability genes (Sarkar et al., 2013).

COUPLING CANCER AND CIRCADIAN RHYTHMICITY: DE-REGULATED TEMPORAL TRANSCRIPTION AND OUTPUT

Understanding the molecular basis of the cellular circadian clock and the means by which uncontrolled cell growth is caused is fundamental in understanding the significant role that the circadian clock plays in the genesis and treatment of cancer. If cells show circadian rhythmicity in the expression of over 10% of its genes (Innominato et al., 2010), it is safe to assume that when cells become cancerous and show deregulated gene expression the circadian molecular clock is likely to be involved one way or the other (Filipski et al., 2004). Like previously described, individual cells can show uncontrolled cell growth by means of evading apoptosis, being independent of growth signals and even avoid anti-growth signals. In this thesis it is hypothesized that uncontrolled cell growth can have its origin both from within the cell by disruption the molecular clock gene components and by influence of circadian driven changes in physiology. Both topics are individually discussed in this thesis, though one must understand that each of these systems does not operate independently, but influence each other through various output mechanisms and pathways, making it a complex interplay between physiological parameters and endogenous genetic output.

3 ways of affecting the molecular clock and output

Fundamental in understanding the circadian molecular clock is that its output and input, which lead to tumour genesis, can be disrupted in various ways. Though not all of them are explained in detail in this thesis, they are briefly summarized. The first way in which the clock can contribute to the genesis of cancer is that cells can have a properly working molecular clock, though their output is misinterpreted. The clock itself is therefore not the cause of the tumour, but does contribute to it. Sometimes the molecular clock is not working properly anymore though. The output of the clock is off balance and the overexpression or absence of certain components can lead to trigger the genesis

of cancers. The final option is the one in which the molecular clock is completely obliterated and all circadian rhythmicity of the cell regarding the clock is lost, therefore leading to loss of control of cell division. The final option is not seen very often however (Innominato et al., 2010).

Because one can understand that the genesis of uncontrolled cell growth and division is likely to be affected by the circadian clock because of transcriptional and translational end products, it is not hard to imagine that tumorous cells show circadian variation by which chemotherapy is effective in treating them. Indeed, when proteins like membrane pumps and kinases, which directly influence the effectiveness by which chemotherapy act, are more active at a certain time point during the day it is safe to say that it is likely that the circadian clock is involved in the effectiveness by which cytotoxins and radiation therapy act. This is the third and final topic to be discussed in this thesis.

MECHANISMS

MOLECULAR CLOCK GENE COUPLED TUMOURIGENESIS

All rhythmical and temporally controlled functions of the cell, like cell division and genome replication, are controlled and driven by pacemakers originating both internally and externally. Like previously described, the external pacemaker signals are driven by the SCN and the internal pacemaker is driven by the output of the molecular clock genes. When these clock genes are malfunctioning, damaged or their transcription is deregulated, it will cause the endogenous clock to lose control of temporally regulated replication and division. Indeed, core clock genes have proven to play a significant role in the tumourigenesis of cancers like breast- and colon cancer (Pleasance et al., 2009). Malfunctioning output by the core molecular clock is caused by either de-synchronisation through in example shift work or jet lag, or by genetic mutations like SNP's or chromosomal shifts (Kelleher et al., 2014). The impact de-synchronisation of the molecular clock has on the increased risk of cancer by for example shift work is demonstrated by the fact that the International Agency of for Research on Cancer and Epidemiologic Studies put shift work in the Group2A of carcinogens (Kelleher et al., 2014). Furthermore, studies show that sleep deprivation, potentially leading to asynchronous central and peripheral clocks, has an astonishing effect on the balance of the immune system, increasing T-helper-1 and T-helper2 counts but suppressing Natural Killer cell activity. This ultimately leads to a higher risk of developing cancer (Irwin et al., 1996). Next to the important role of a-synchronisation to the external environment, it is shown that physiological mitotic processes are controlled by genes and proteins like P21, Wee-1 and thymidilate synthase, and they are on their turn controlled by clock genes (Wood et al., 2008).

These examples illustrate the potential importance and relevance of core clock genes in the genesis of cancer. But how do they specifically affect the cell cycle and what processes do they control that make them such a dangerous adversary when mutated? The circadian system plays in controlling the cell cycle, temporal growth and division. By way of clock controlled genes that have either ROR-elements or E-boxes in their promoters the circadian clock has been found to play a role in the cell cycle (Kelleher et al., 2014). E-boxes are specific nucleotide sequences which are bound by enzymes and which promote gene transcription of the genetic region upstream of the promoter. By way of binding to these E-boxes the molecular clock genes temporally control the expression of genes that control cell cycle. Genes that are affected by this way of genetic regulation are cell cycle controlling proteins and factors like the tumour suppressor gene p53, c-MYC, caspases, cyclins and

MDM2. MDM2 in turn is a protein that inhibits the transcription of p53, one of the most important controllers and regulators of cell division and proliferation. Because of the direct effect the molecular clock genes have on these regulatory elements, circadian disruption by either environmental cues or internal genetic malfunctioning can lead to interference of the cell cycle and ultimately even lead to defects in proliferation. An overview of the pathways involved in controlling and regulating gene expression and cell proliferation is given below in Figure 3.

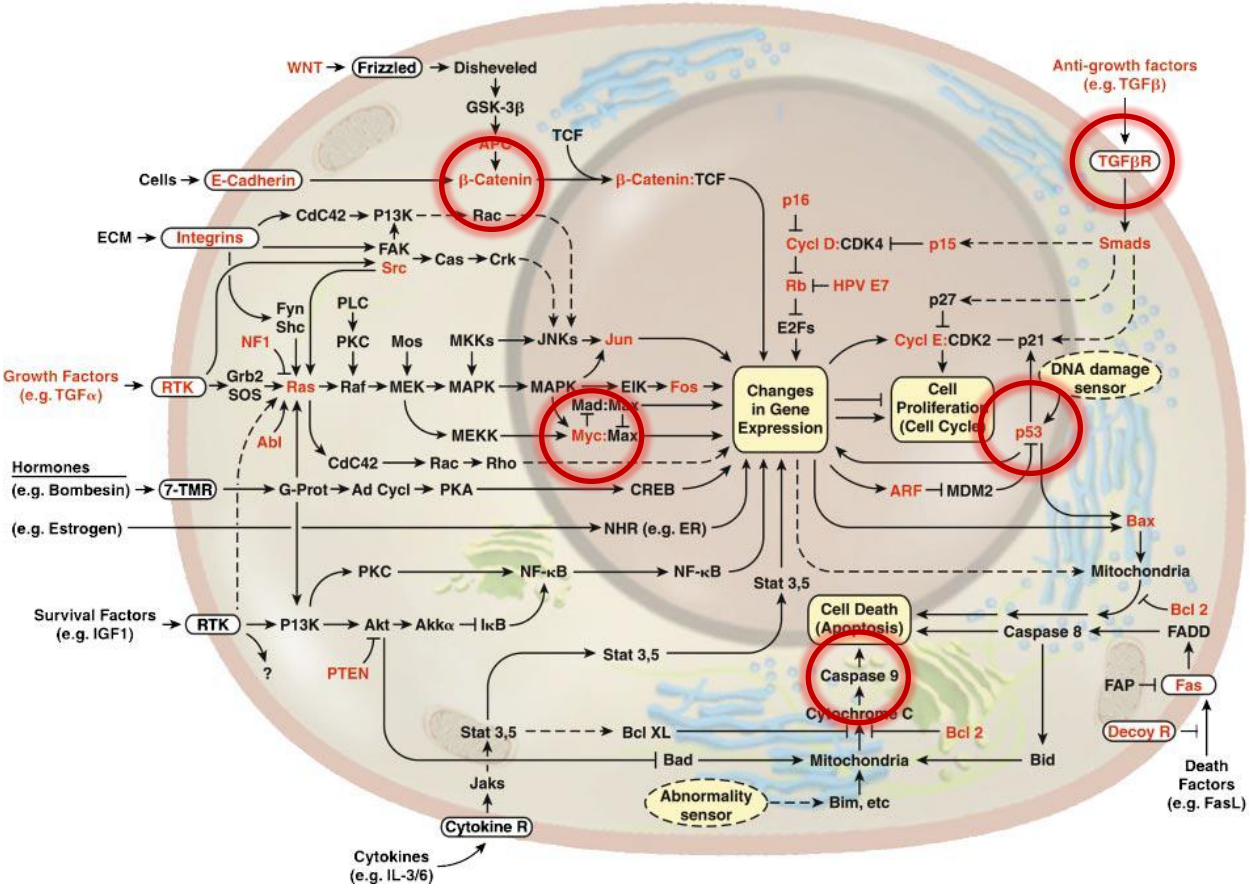

FIGURE 3 An overview of the molecular pathways involved in controlling gene expression and cell proliferation. These genes and elements are the safe keepers that guard the cell from uncontrolled cell division. Disruption in these pathways can lead to self-sufficiency in growth signals, insensitivity to ant-growth signals and evading apoptosis. Highlighted here are the genes involved in regulating the cell cycle and are influenced by core clock gene expression. Like previously stated in the text, p53, a tumour repressor gene, is an important element in controlling the cell cycle, it is a DNA damage sensor that inhibits mitosis in the event that the DNA is not properly replicated and therefore damaged. MYC, an oncogene, is a gene involved in regulating transcription of countless other genes by binding of specific binding sites. The expression of both P53 and MYC are temporally controlled by the core clock genes like BMAL1 and CLOCK. Other factors like hormone levels and receptor activity bounded by ligases do control the expression of these regulatory genes as well. What should be noted however is that the expression of these hormones and receptors are likely to be also temporally controlled. Image taken from *The Hallmarks of Cancer* by Hanahan & Weinberg (2000)

The expression of core clock genes in cancerous cells has been intensively studied and in almost all cases the expression of these core clock genes differed from the expression of that in normal healthy cells (Levi et al., 2007). The changes in gene expression were corrected for expected changes visible in core clock gene expression due to different circadian phases and it was concluded

that indeed the expression of the clock genes itself was abnormal. The mechanism by which the individual core clock genes regulate the cell cycle and proliferation and by what ways they are involved in tumour genesis is outlined below.

Bmal1 and Clock

Bmal1 and clock have important regulatory functions in controlling gene expression. When the transcripts of Bmal1 and clock are translated their proteins translocate to the cytoplasm where they heterodimerize and form a complex that can bind E-boxes in promoter regions of cell cycle regulatory genes that enhance transcription and thereby encourage cell cycle progression (Kelleher et al., 2014). Furthermore, CLOCK, the translated protein of the clock gene transcript, has proven to have important acetyltransferase functions (Doi et al., 2006). Acetyltransferase is an important mechanism of epigenetic gene expression control. By acetylating histones, elements around which DNA strands are bound; CLOCK generally leads to an increasing in the expression of the genes involved in those regions. Next to these direct genetic regulatory functions, CLOCK has been identified as a possible microsatellite instability gene, not directly affecting cell growth but altering cellular responses to DNA damage, implying a safe keeper role for the gene as well (Kinzler & Vogelstein, 1997).

Next to the regulatory effects on gene expression, the Bmal1 gene is an important regulator of the p53 pathway (Gery et al., 2006). Cells which have proven to have a lower expression of the Bmal1 gene have difficulty in activating the p21 protein, a protein that is activated by p53. This inability of p53 activating p21 causes the cell to be irresponsive to DNA damage and ultimately leads to cell cycle progression even in the presence of DNA damage. P53 and P21 are considered important anti-growth signals, and like previously stated the ability of the cell to ignore these signals is an important step to becoming cancerous.

In addition of controlling the p53 cell cycle controlling pathway, Bmal1 also plays an important role in targeting the proto-oncogene c-Myc by means of binding to E-boxes located in the promoter sequences of this gene (Fu et al., 2005). Myc is an important gene in regulating gene expression by binding to specific sequences on targeted genes, often growth factors, thereby enhancing their transcription. Overexpression of Myc by binding of Bmal1therefore leads to increased levels of growth signals. Abnormally high levels of Bmal1 are likely to cause these events to happen (Kelleher et al., 2014), staging the set of the cell to become self-sufficient in these signals when not properly controlled. Both RORα and REV-erbβ have also proven to play a role in regulating Myc expression (Dissualt & Giguere, 1997).

What is evident from these mechanisms is the important role that both Bmal1 and Clock play in controlling the cell cycle, whereas an unusual high or low expression of either gene can easily shift the cell into uncontrolled cell growth. An accumulation of mutations in either of these genes readily affect one another, as the products of both genes heterodimerize in the cytoplasm. Malfunctioning pathways controlled by these genes should not to be underestimated, as they directly affect cell division control and possibly cause the cell to become self-sufficient in growth signals and/or become irresponsive to anti-growth signals, thereby already reaching two of the classical hallmarks of cancer as described by Hanahan and Weinberg.

Period and Cryptochrome

Studies have shown that both Per1 and Per2 mRNA's are often down-regulated in several cancerous tissues, including breast, lung, colorectal, pancreatic, endometrial and myeloid cancers (Innominato et al., 2010)(Lévi et al., 2007). These down-regulated levels of Per1 and Per2 transcript in cancerous tissue imply a tumor repressor activity in healthy cells, which is confirmed in several studies (Gery et al., 2006)(Fu et al., 2002). This tumour repressor activity is illustrated by the fact that differing levels of Per2 protein proved to be a powerful prognostic tool to predict survivability in patients with metastatic colorectal cancer, where decreased levels almost always implicates a lower survival rate (Innominato et al., 2010). In a particular study where 67 colorectal cancer patients had positively labelled Per2 in more than 60% of their tumorous tissue, their survival chances increased with 42% with respect to 66 patients in which only 10% of their tumorous tissue was positively stained with Per2 (Innominato et al., 2010). Furthermore, clinical findings suggest and confirm a major role for Per2 and its tumour suppressor activity as Per2 is the component most often mutated of the molecular clock machinery in colorectal cancers (Sjoblom et al., 2006).

In contrast to the tumour suppressor activity of Per2, higher expression of Cryptochrome appears to be causing an increased risk of tumour genesis (Innominato et al., 2010). Though both part of the negative arm of the transcriptional-translational negative feedback loop of the molecular circadian clock, their effects on tumourigenesis are not quite the same. It appears that high levels of circulating Cry mRNA molecules are a very good predictor of poorer survival chances in several cancers, even more so when these high levels of CRY contrast low levels of Per2 (Eisele et al., 2009). In patients that have a very low Per2/Cry ratio, survival chances dropped with up to 223% when compared to patients with high Per2/Cry ratio's (Eisele et al., 2009), illustrating not only the malignant effects of high levels of Cry alone, but the dangerous synergy that is created when these malfunctioning levels of Cry are coupled to low levels of Per2.

These data and studies show the important effect the circadian molecular clock genes Period and Cryptocrome have on influencing cell cycle and proliferation and thereby effecting tumourigenesis. The mechanism underlying this tumour suppressor function of the Per2 gene is quite similar to that of Bmal1, both ultimately acting on- and activating the cell regulatory element p53.

The precise mechanism is that Period interacts with an Ataxia Telangiectasia mutated gene, which is a serine/threonine protein kinase. This usually leads to cell cycle arrest when DNA doubled stranded bonds are broken during or before the onset of mitosis, and is mediated by targeting of tumour suppressor genes like p53 and CHK2 (Gery et al., 2006). Also, as one of the two actors of the negative limbs of the transcriptional-translational negative feedback loop, exceptionally high levels of the Per protein causes inhibition of the expression of the Bmal1 and Clock genes, thereby lowering the activating action of p53 on p21, which is orchestrated by Bmal1. In harmony with the tumour promoting properties of exceptionally high levels of Per, exceptionally low levels of Per2 cause an abnormal increase in the expression of Bmal1, thereby causing it to bind to the proto-oncogene Myc and ultimately leading to a total increased gene expression throughout the cell.

Next to these functions, the Per2 gene is also linked to play a major role in regulating cyclin D and β-catenin protein (Kelleher et al., 2014). Both cyclin D and β-catenin are major players in the regulation of the cell cycle and proliferation, as is previously described and illustrated in Figure 3. Lower levels of circulating Per2 are associated with increased levels of cyclin D and β-catenin and therefore can cause uncontrolled cell growth. Studies confirmed that the Per2 gene product acts by

way of a tumour suppressor element through de-activation of cyclin D and β-catenin, and thereby lowering the risk of uncontrolled cell growth (Wood et al., 2008).

It is evident from these illustrations and mechanisms that a complex and delicate balance exist between the circadian clock genes and their actors in the control of expression of genes required for controlled replication and proliferation. Abnormal higher levels of one the molecular clock genes usually leads to the repression of others, as is the case when the transcriptional-translational negative feedback loop is functioning properly, and where as the effect of high levels of the first gene might be cancerous, the repression of the second gene might have beneficiary tumour suppressor effects.

Each of the molecular clock genes and their function and action on the cell division regulatory components are illustrated in Figure 4.

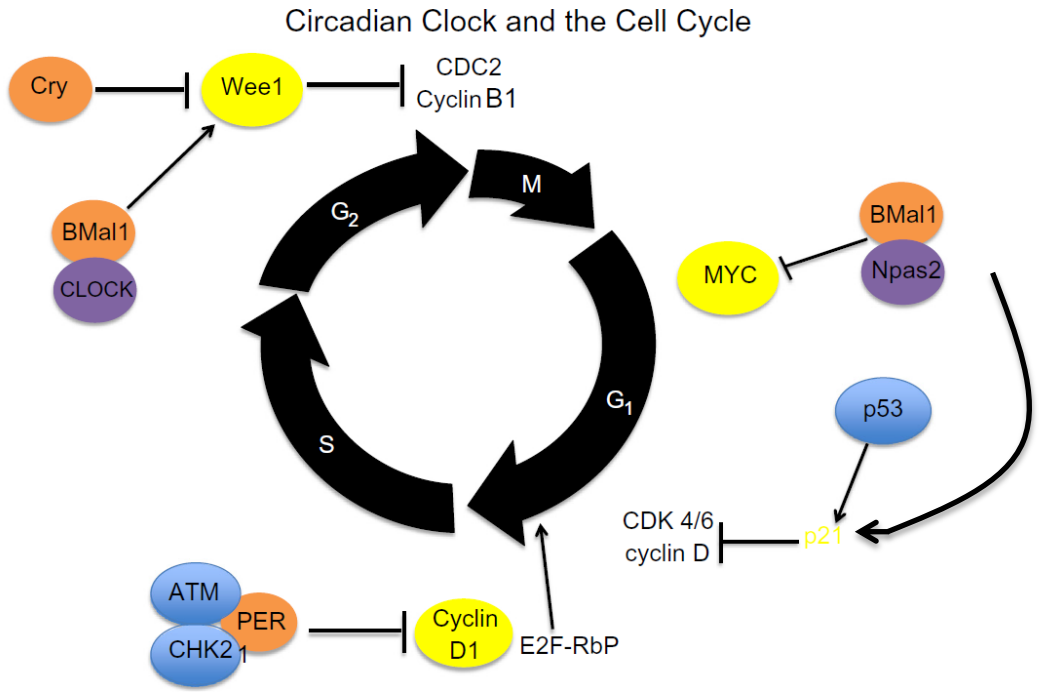

FIGURE 4 Depicted here is the cell cycle and the actors which control and influence its progression. The cell cycle consists of four different phases, the G1, S, G2 and M phase. The G1, S and G2 together are called the interphase, as during the M phase the cell divides into two daughter cells, and not much is visible on the outside during the other three. During the G1 phase however the cell enlarges and prepares for genetic replication. If the cell is large enough and conditions are right the real replication of the genetic material takes place during the S phase. When genetic replication is completed the cell enters the G2 phase, where the cell enlargers even further and checks if the DNA is replicated correctly before it can initiate the final mitosis of the cell. During this cycle the cell has to pass numerous roadblocks and checks in order to progress into the next phase of the cell cycle. These roadblocks are depicted here in yellow and consist normally out of regulatory proteins and cyclins, which, as the name already tells us, show a cyclic pattern and are controlled directly by numerous molecular clock components and elements and indirectly through regulatory elements like p53. Image taken from *Circadian molecular clocks and cancer* by Kelleher et al. (2014).

The cell cycle and molecular clock genes

It is clear from the previous examples that molecular clock gene components play a crucial role in the regulation of cell division. Where each and every one of these components interacts within this cycle is however rather complicated and subject to much variation, both circadian and non-circadian. A simplified version and most accepted view of this complex interaction is shown in Figure 4 (Kelleher et al., 2014). The cell cycle consists of four different and distinct phases. During the first phase, deemed G1, the cell enlarges and prepares for replication of the genome. When the cell is ready it progresses after checking by regulatory elements into the S phase, during which genetic material is synthesized and replicated (Nagoshi et al., 2004). The regulatory elements that check if the cell is ready to progress to the next phase are cyclins and cyclin-dependent kinases (CDKs)(Kelleher et al., 2014). Cyclins attach to and active cycling-dependent kinases, which active cell cycle progression and allow the cell to cycle from phase to phase. Cyclin-dependent kinase levels oscillate during the cell cycle, which is due to the cyclic variation in activity of cylcins, their name deriving from this characteristic. Gating of cell division by these cyclins and CDK's is circadian controlled (Nagoshi et al., 2004). Genes which are affected by these circadian molecular clock genes include Myc, Wee1, Cyclin D and P21, as depicted in Figure 4.

Myc

Myc controls 15-20% of the functional genes of the genome. Elevated levels of Myc cause deregulated cell progression from the G1 into the S phase, by way of expression of CDK4 and cyclin 2. Myc also causes overexpression of certain transcription factors like E2F1 and E2F. The expression of Myc is controlled by circadian genes Bmal1 and Npas2 (Kelleher et al., 2014).

Wee1

Wee1 is an important gatekeeper that controls cell progression from the G2 phase into the M phase by inhibition of cyclin B1 and CDK2. Cyclin B and CK2 are important elements required to be present in high numbers in order for the cell to progress to the M phase. Wee1 is a cell cycle inhibitory element therefore, and decreased levels of Wee1 are often inversely related with increased recurrence of certain types of cancers (Beck et al., 2010). Levels of Wee1 are regulated circadianly by the Clock-Bmal1 heterodimere, which cause an increase in Wee1 levels, and by the Cry protein, which causes inhibition of the Wee1 complex. One way of controlling the levels of Wee1 is by regulating the transcription of mi-RNA195, which is a silencing RNA which attaches to the Wee1 promoter site and therefore disables transcription of the Wee1 gene. The circadian molecular clock genes are thought to control the levels of mi-RNA95 and thereby control the levels circulating Wee1 and ultimately regulate the progression of cells into the next phase of the cell cycle (Savvidis & Koutsilieris, 2012).

Cyclin D

Cyclin D affects the cell cycle by binding and activation of CDK4 and CDK6. The activation of these CKDs cause the cell to progress from the G1 in to the M phase. Increased activity of cyclin D therefore causes increased speed and acceleration of cell division (Kelleher et al., 2014). The inverse effect is demonstrated in situations where decreased levels of cyclin D are associated with increased apoptosis of tumorous lymphocytic tissues (Savvidis & Koutsilieris, 2012). Furthermore, ablation of the Cyclin D gene in breast cancer cultures caused senescence and degradation of the affected tissues. Activity of Cyclin D is circdianly controlled by the molulor clock gene Per. Per is an inhibitory element in the transcription of cyclin D and can therefore be seen as a tumour suppressing element.

P21

The last element to be explained here is p21. P21 is activated by p53 and causes inhibition of cyclin D, which like previously described causes cell cycle progression. Therefore, activation of p21 by p53 causes cell cycle arrest. The activation of p21 by p53 is facilitated by the presence of the molecular clock gene protein product of Bmal1, and is thereby circadianly regulated. Paradoxically to its inhibitory effects on tumourigenesis when activated by p53, p21 when acting alone has oncogenic properties (Savvidis & Koutsilieris, 2012). Its effects include gene transcription, regulation apoptosis and modulating DNA repair (Kelleher et al., 2014).

It is evident that the cell cycle is a carefully and fragile orchestrated machinery which is driven by multiple effectors including the circadian molecular clock. The importance of molecular clock genes on tumourigenesis are not the be underestimated and its clinical significance is showing to be more and more promising.

CIRCADIAN DRIVEN CHANGES IN PHYSIOLOGY AND CANCER SUSCEPTIBILITY

The immune system

Though the molecular clock genes can directly interact with the cell division machinery, its output is often in forms of circadian oscillations of hormones, neurotransmitters which on their turn can interact with and regulate cell division and proliferation. One of the systems influenced by these oscillations is the immune system. The immune system consists of two main components, the innate and the adaptive immune system. The innate immune system can be seen as the first line of defence and the adaptive immune system as the immune system that is activated when specific action against specific pathogens is required. The adaptive immune system is therefore also called the specific immune system. The innate immune system is not so specific however, and scavenges for pathogens and cellular malfunctions throughout the body constantly. One of the main components of the innate immune system are the natural killer cells. Natural killer cells are immune cells that scan healthy and unhealthy cells for signs of infection and damage and induce apoptosis when the subjected cell is deemed infected or damaged. One of the main functions of natural killer cells is search & destroy of tumorous cells by scanning cell membrane surface molecules. Natural killer cells are therefore important actors in the defence against and treatment of cancers. However, like most immune cells, natural killer cells levels and activity are subjected to circadian oscillations and rhythmicity (Irwin et al., 2009). It is shown that sleep deprivation studies which affect the internal molecular clock cycle negatively impact circulating natural killer cells levels and can even leads to immunodeficiency (Kelleher et al., 2014). Like natural killer cells, T-helper 1 and T-helper 2 cytokine production is also affected by the state of the molecular clock (Kelleher et al., 2014). Both natural killer cells and T-helper 2/1 cytokines are components of the cell specific immunity, and are therefore important in the body's defence mechanisms against tumourigenesis. Overcoming the risk of being killed by apoptosis is one of the hallmarks of cancer, and disruptions of the endogenous circadian system can lead ultimately to chronic lower levels of the immune components with increased risk of cancer as a result.

Melatonin

The circadian system controls many bodily functions throughout the body such as physiological parameters like core body temperature, endocrine functions and the wake/sleep cycle. Though its impact is great and felt throughout the entire body, the hormone melatonin is one of the few known parameters that also can be measured in humans which is directly under the control of the SCN. Melatonin is synthesized in the pineal gland and its secretion forms an important nocturnal output signal (Blask et al., 2005). Melatonin secretion is inhibited during the light phase, when blue light

reaches and excites the retina and afferent neurons project to higher brain areas where this information is integrated and ultimately results in inhibition of melatonin secretion. When light diminishes the inhibitory signals to the melatonin production stop and the pineal gland starts to produce melatonin, which cumulates during the dark phase of the 24 hour day and acts as a potent sleep inducing hormone. Melatonin can therefore be seen as the internal hormonal endogenous representation of the outside light dark cycle.

Next to being the only endogenous hormonal output signal of the circadian clock, melatonin is also the only chronobiotic neurotransmitter which regulates tumour growth and development and also inhibits the proliferation of human cancer cells in vitro at normal nocturnally induced levels (Blask et al., 2005). Studies and clinical trials show that when administered in combination with traditional chemotherapies, melatonin can raise the therapeutic index of the patient's survival drastically and induce apoptotic cell death on cancer cells (Blask, 2001). Also when administered as the only acting drug on tumorous cultures, melatonin proved to have a potent anti-cancer and anti-tumour genesis effect (Blask et al., 2002).

Next to being produced nocturnally by the pineal gland under the absence of light, a major source of melatonin comes from dietary sources such as plants and consumption of these melatonin containing plants appears to be of significant quantities (Blask et al., 2005). Blood levels of circulating melatonin can reach peaks as high as or even higher than those reached during the night (Dubbels et al., 1995). Also, more and more people take dietary supplements containing neurotransmitters and sleep inducers like melatonin to correct for and anticipate the consequences of deregulated sleeping patterns as caused by shift work or jet lag (Lerner, 1999). Because of these findings a dietary experiment was designed by Blask et al. (2005) in which rats were given access to either a 5% corn oil diet, a low melatonin concentration containing diet and high melatonin concentration containing diet, the last two also tested again in the presence of non-selective melatonin receptor antagonists, as depicted in Figure 5. When rats were kept on high dosage melatonin containing diets, hepatoma growth onset decreased significantly as well as the rate of tumour growth. Because in normal lab conditions rats are nocturnal animals and they feed during the night, in this experiment where there was a 12:12 LD cycle, it is likely that the consumption of the melatonin containing food coincided with the natural production of melatonin and that in effect the dietary administered melatonin reinforced the natural anti-cancer working mechanisms of the endogenous melatonin signal. What is also evident from these results is that melatonin exerts its inhibitory effects on cancer growth through the melatonin receptors MT1

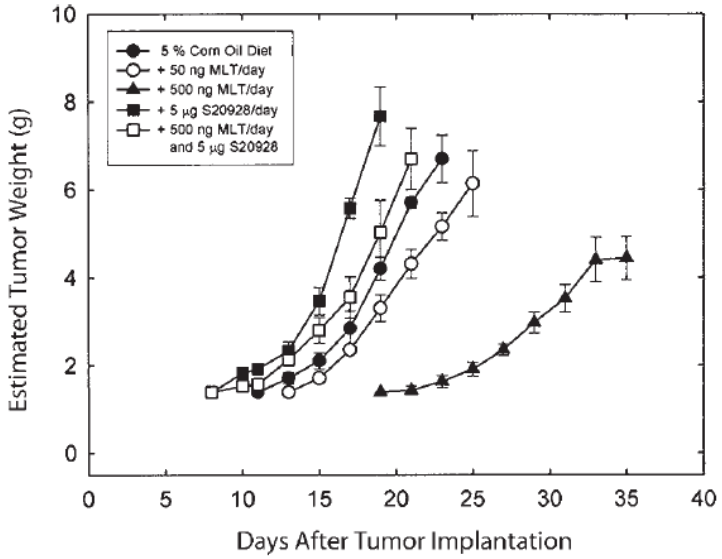

FIGURE 5 The effects of dietary melatonin on tumour weight in the presence or absence of non-selective antagonists for the melatonin receptors (From Blask et al. 2005).

and/or MT2, as non-selective antagonists completely blocked the effects induced by the dietary melatonin, as well as the effects naturally caused by naturally circulating melatonin levels.

The melatonin induced inhibitory pathway

What is clear is that melatonin has an inhibitory effect on tumourigenesis and even slows the rate at which various tumours grow. Melatonin does this by blocking the cell cycle and stopping progression from the G1 phase to the successive S phase. Both the MT1 and the MT2 receptors are G-protein coupled inhibitory receptors and when melatonin ligands bind to the protruding cell membrane surface part of the receptor, the intracellular alpha subunit of the receptor leaves the other subunits and deactivates adenylyl cyclase. Because adynyl cyclase activity is necessary for the formation of intracellular cAMP, inhibition by the alpha subunit causes an intracellular decrease in cAMP concentration. This again leads to lowered PKA activity. The entire intracellular cascade ultimately leads to the closing of so called LA receptors by lowered PKA activity and cause a lowered LA uptake. LA, or linoleic acid, is an essential omega-6 polyunsaturated acid and is a potent promoter of tumourigenesis, cell cycle progression and cell proliferation. Intracellular LA causes expression of the oestrogen receptor, cell cycle progression by activation cell cycle regulatory elements, and the mitogen-activated protein kinase (MAPK) growth cascade. Furthermore, when LA is transported from the interstitium to the internal cellular environment it is rapidly metabolized into 13-HODE, which is a potent activator of the EGFR/MEK/ERK1/2 cascade. Activation of this cascade by 13-HODE causes mitotic cell growth, activation of transcription factors and leads to tumourigenesis. This complex interaction between melatonin and its inhibitory actions on tumour growth during the night is illustrated in Figure 6.

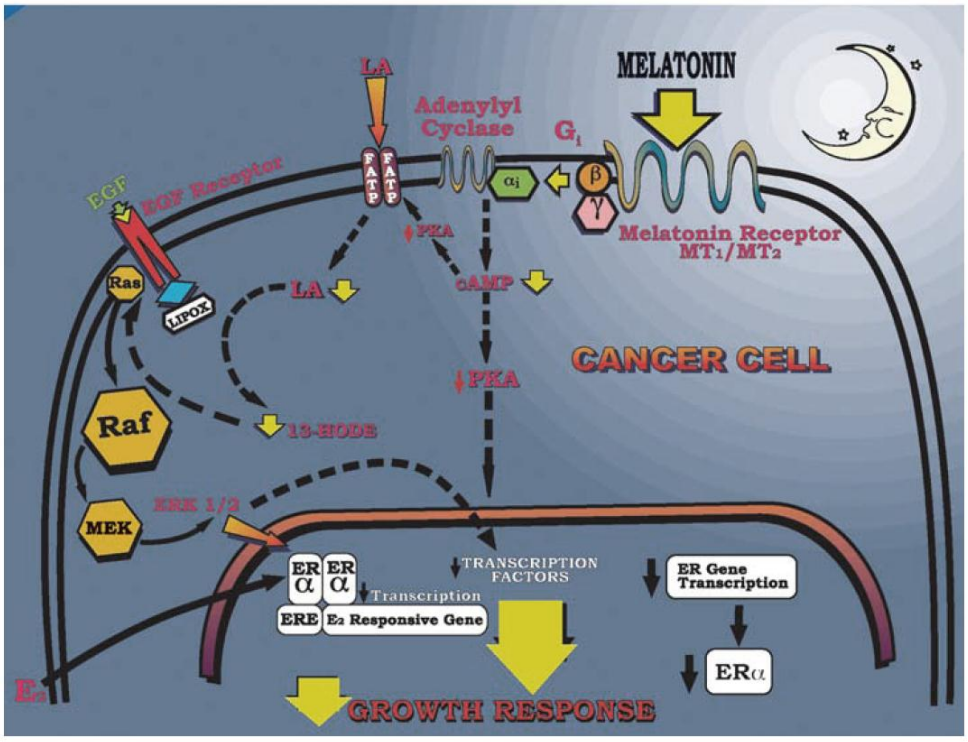

FIGURE 6 Illustrated in this diagram is the signal transduction and metabolic pathway mediated by melatonin leading to decreased LA uptake. The decreased LA uptake in turn leads to lower 13-HODE levels which have inhibitory effects on mitotic pathways, transcription factors and expression of growth signal receptors (Innominato et al. 2010).

That melatonin is a potent anti-cancer hormone is reinforced by the fact that tumours appear to be developing mechanisms to absorb melatonin into the cell, thereby denying it the change to bind to the extracellular MT receptors (Innominato et al., 2010). This may possibly represent a strategy by which tumours develop a resistance to this potent anti-cancerous drug.

CIRCADIAN VARIATION IN CHEMOTHERAPY RESISTANCE: CHRONOTHERAPY

Just like the output of the circadian molecular clock has its influence on the cell cycle, cell proliferation and when not properly balanced even the genesis of tumours, so it too has tremendous effects on the resistance of tumour cells to chemotherapies. When almost 10 to 20% of the total translational genome is circadian controlled (Innominato et al., 2010), it is likely that for example the expression of transport receptors by which chemo toxins enter the cell are expressed with oscillating density of the cell membrane surface, thereby directly influencing the effect of the chemotherapy when administered at a certain time. In other words, one could say there is circadian variation in chemotherapy resistance. For these reasons the field of chronotherapy was developed. The field of chronotherapy uses chronotherapeutics to apply the circadian variation in chemotherapy resistance to optimize the effectiveness of drug delivery treatments while minimizing its side effects (Librodo et al., 2012).

Circadian time-dependent therapeutic index

The circadian clock of the human body temporally controls physiological parameters that drive drug metabolism at whole body and cellular level and thereby influence pharmacological determinants. This is illustrated in Figure 7. The circadian system drives both circadian physiology and molecular clocks, which in turn influence the rhythm of circadian dependant physiological parameters. The output of the circadian variation in physiology has its effect throughout the whole body where it affects both pharmacokinetics as pharmacodynamics. The output of the cellular molecular clock is understandably only sensible on a cellular level, it too however has its effect on cellular pharmacokinetics and pharmacodynamics. Pharmacokinetics can be interpreted as the fate of drugs administered externally to an organism which describes its changing concentration due to the hosts' drug metabolism. Pharmacodynamics describe the effects that a certain drug has at a certain concentration, it also describes for example what concentration levels are needed to have an effect at all and at what levels you see receptor saturation. The mechanisms underlying these changes in drug mechanism as driven by the circadian clock are specific to each individual drug, rather than a class of drugs or set of drugs (Innominato et al., 2010). An example can be given by the therapeutic index of the drug 5-fluorouracil (5-FU). 5-fluorouracil is an important anti-cancer drug used in the treatment of a great variety of tumours and malignity's. It acts through inhibition of thymidylate synthase after being activated intracellular. Thymidylate synthase is essential in the synthesis and repair of DNA, therefore 5-fluorouracil induces lethal DNA damage and disrupts RNA transcription, making it a potent anti-cancer drug.

Pharmacokinetics

Even when intravenously administered continuously for 24 hours, the plasma concentration of 5-fluorouracil shows a 24 hour oscillating pattern where plasma concentrations peak during the light phase of the day and show the lowest levels during the night (Milano et al., 2002). A circadian pattern in plasma concentrations of 5-fluorouracil was also observed in patients in which 5-fluorouracil was continuously administered for over 2 weeks, suggesting that a mechanisms which is subject to an internal pacemaker drives the oscillations in 5-fluorouracil concentrations (Milano et al., 2002). 5-fluorouracil is catabolized by dihydropyrimidine dehydrogenase in the blood

plasma, and the activity of dihydropyrimidine dehydrogenase perfectly inversely matches the plasma concentrations of 5-fluorouracil. Dihydropyrimidine dehydrogenase is the most active during the night, thereby lowering subjective 5-fluorouracil blood plasma concentrations, and it is less active during the day, explaining the 24 hour 5-fluorouracil blood plasma pattern circadian pattern observed in the blood (Lévi et al., 2010). Intracellular concentrations of 5-fluorouracil are also controlled by dihydropyrimidine dehydrogenase, this version of dihydropyrimidine dehydrogenase is only present inside the cell however. The pharmacokinetics of the intracellular environment are therefore measured separately from that of the outside environment; the blood plasma and interstitial fluid. Intracellular dihydropyrimidine dehydrogenase also has its peak activity during the subjective night phase, thus lowering intracellular 5-fluorouracil concentrations when extracellular concentrations are already at their lowest due to the extracellular dehydrogenase activity. The example of 5-fluorouracil pharmacokinetics only shows the importance that the circadian system has on controlling chemotherapeutics and its proving to become more significant in the clinic by the day (Innominato et al., 2010).

Pharmacodynamics

The example of 5-fluorouracil and the effect pharmacokinetics has on circulating concentrations can also be used when describing the effect that pharmacodynamics have on the effect of 5-fluorouracil. DNA synthesis and repair are the main targets of 5-fluorouracil, where DNA synthesis in normal healthy cells peaks during the day and is lowest during the night. Because tumours almost always have ablated circadian rhythms which no longer function properly, the effect that 5-fluorouracil has should be primarily focused on healthy cells. Because during the night the whole-body concentrations of the host are lowest and DNA synthesis and repair in normal cells is lowest the proportion of healthy cells damaged by 5-fluorouracil is significantly lower when the drug is administered during the night (Innominato et al., 2010). Because of these mechanisms a more personalized chronobiological profile was designed in which patients received 5-fluorouracil during the night phase of the day and toxicity to normal healthy cells decreased significantly (Lévi et al., 2010)

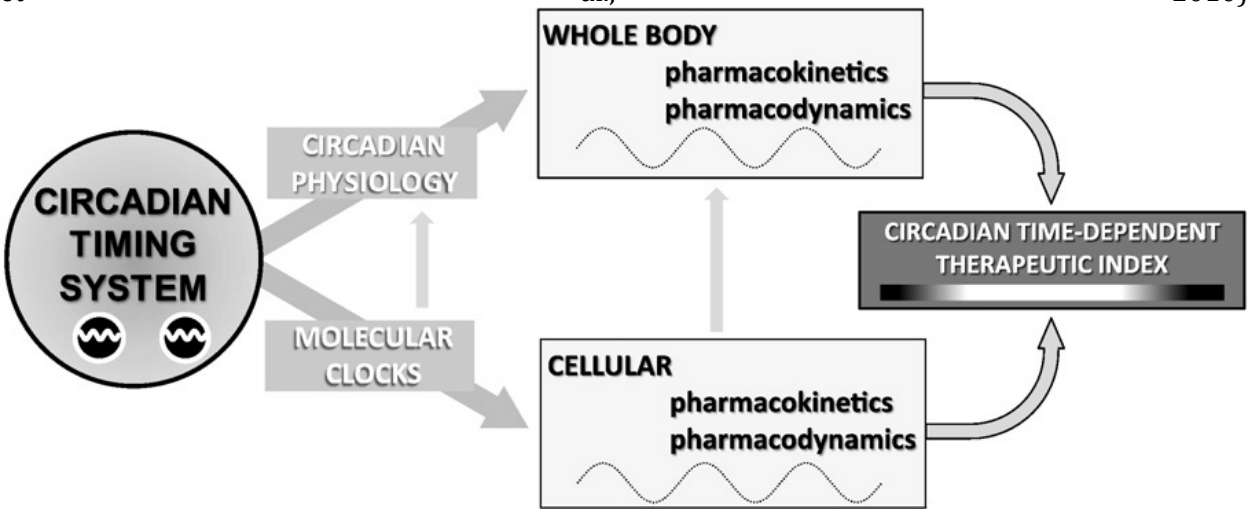

FIGURE 7 The mechanism by which chronotherapy works as described by Innominato et al. (2010). The circadian system drives both circadian physiology and molecular clocks, which in turn influence the rhythm of circadian dependant physiological parameters. The output of the circadian variation in physiology has its effect throughout the whole body where it affects both pharmacokinetics as pharmacodynamics. The output of the cellular molecular clock is understandably only sensible on a cellular level, it too however has its effect on cellular pharmacokinetics and pharmacodynamics. Pharmacokinetics can be interpreted as the fate of drugs administered externally to an organism which describes its changing concentration due to the hosts' drug metabolism. Pharmacodynamics describe the effects that a certain drug has at a certain concentration, it also

describes for example what concentration levels are needed to have an effect at all and at what levels you see receptor saturation. Both pharmacokinetics as pharmacodynamics ultimately influence the circadian time-dependant index of a circadianly administered cytotoxin. As mentioned before, significant results have been shown when the field of chronotherapy is applied in the on-going battle against cancers.

FUTURE PERSPECTIVES

It is daunting to know the influence the circadian clock has on an astonishing variety of biological functions like circulating hormone levels, cell cycle progression and proliferation of cells. When not properly controlled or with parts of the system failing it is easy for a cell to shift to uncontrolled cell growth and proliferation, setting the steps of acquiring the classical hallmarks of cancer. It is therefore likely that when tumours arise, that the lack of circadian organisation has no part in the genesis of it. Even if the circadian system is not intrinsically malfunctioning, it may still be involved in tumours genesis through de-synchronisation between the external environment and the internal circadian system.

That the circadian clock not only has its influence on the genesis of cancer but can also be applied in the treatment of is indicated by success in the field of Chronotherapy, though sometimes limited. Here results show an initial improvement in patients subjected to chronotherapy (Innominato et al., 2010). People should be individually handled and a chronobiological profile is required however, as different chronotypes could have impacting results on the outcome of the treatment. Not much research on other diseases with respect to circadian control is done in this light however, as the field of chronotherapy is rather new and researchers are waiting for more clinical results from the cancer trials.

Though many studies look promising it should be noted however that much research is done in the lab on nocturnal animals. Nocturnal animals have an activity phase which is usually 12 hours different of that on humans. When certain mechanisms and cascades are explored in nocturnal animals, it is not safe to assume that the same mechanisms and cascades will work the same way in humans. This really important difference is not often mentioned in literature however, but it should be taken into high regard when applying new chronobiotic knowledge.

The topics described in this thesis show the fragile relationship of the circadian molecular clock and its control and effect on cell division and proliferation. A vast amount of genes, proteins and other elements are part of this complex system. Some molecular output genes have tumour-suppressor functions while others are oncogenic, some are even both. What is most important however in these regards is the balance and oscillations that exist in healthy cells that prevent the genesis of cancer.

REFERENCES

1. Beck H, Nashe V, Larsen MS et al. (2010) Regulators of cyclin-dependant kinases are crucial for maintaining genome integrity in S phase. Journal of Cellular Biology 118: 629-638
2. Blask DE, Bartsch C, Bartsch H et al. (2001) In: The pineal Gland and cancer. Springer Verslag 309-342
3. Blask DE, Dauchy RT, Sauer LA (2005) Putting cancer to sleep at night: The neuroendocrine/circadian melatonin signal. Endocrine 27-2: 179-188
4. Blask DE, Sauer L, Dauchy RT, (2002) Curriculum of topics of medical chemistry. 2 113-132
5. Doi M, Hirayama J, Sassone-Corsi P (2006) Circadian regulator CLOCK is a histone acetyltransferase. Cell 125: 497-508
6. Dubbels R, Reiter RJ, Klenke E et al. (1995) Journal of Pineal Reseach 18: 28-31
7. Eisele L, Prinz R, Klein-Hitpass et al. (2004) Combined Per2 and Cry1 expression predicts outcome in chronic lymphocytic leukemia. European Journal of Haematology 83: 320-327
8. Fedi P, Tronick SR, Aaronson SA (1997) Growth Factors. Cancer Medicine. Baltimore. 41-64
9. Filipski E, Delaunay F, King VM, Wu MW, Claustrat B, Grechez-Cassiau A, et al. (2004) Effects of chronic jet lag on tumor progression in mice. Cancer Research 64: 7879-7885
10. Fu L. Pelicano H. Liu J, Huang P, Lee C (2002) The circadian gene Period2 plays an important role in tumor suppression and DNA damage response in vivo. Cell 111: 41-50
11. Gery S, Komatsu N, Baldjyan L, Yu A, Koo D, Koeffler HP (2006) The circadian gene per1 plays an important role in cell growth and DNA damage control in human cancer cells. Molecular Cell 22: 375-382
12. Hanahan D, Weinberg R A (2000) The Hallmarks of Cancer, The Cell: 100, pp57-70
13. Innominato PF, Lévi FA, Bjarnason GA (2010) Chronotherapy and the molecular clock: Clinical implications in oncology. Advanced Drug Delivery Reviews 62: 979-1001
14. Irwin M, McClintick J, Costlow C et al. (1996) Partial Night sleep deprivation reduces natural killer and cellular immune responses in humans. Faseb. Journal 10: 643-653
15. Kelleher FC, Rao A, Maguir A (2014) Circadian Molecular Clocks and Cancer. Cancer Letters 342: 9-18
16. Keller M, et al. (2009) A circadian clock in macrophages controls inflammatory immune responses. Proc. Natl. Acadamic of Science 106: 21407-21412
17. Kinzler KW, Vogelstein B (1997) Cancer-susceptibility genes. Gatekeepers and caretakers. Nature 386: 761-763
18. Lerner AB, Olcese J (1999) Melatonin after four decades, advances in experimental medicine and biology. Kluwer Academical Publications 460: 1-3
19. Lévi F, Focan A, Karaboue A, de la Valette V, Focan-herard BF, Baron B, Kreutz F, Biacchetti S (2007) Implications of circadian clock for the rhythmic delivery of cancer therapeutics. Advanced Drug Delivery Reviews 59: 1015-1035
20. Lévi F, Okyar A, Dulong S, Innominato PF, Clairambault J (2010) Circadian timing in cancer treatments, Annual Revenue of Pharmacological Toxicology 50: 377-421
21. Lévi F, Schibler U (2007) Circadian Rhythms: Mechanisms and therapeutic implications. Annual Revenue of Pharmacological Toxicology 47: 593-628
22. Librodo P, Buckley M, Luk M, Bisso A (2012) Chronotherapeutic Drug Delivery. The Art and Science of Infusion Nursing. 35:5
23. Lowrey PL, Shimomura K, Antoch MP, Yamazaki S, Zemenides PD, Ralph MR, Menaker M., and Takahashi JS, (2000) Positional syntenic cloning and functional characterization of the mammalian circadian mutation tau. Sience. New York, N.Y. 288: 483-491

24. Milano G, Chamorey AL (2002) Clinical pharmacokinetics of 5-fluorouracil with consideration of chronopharmacokinetics. Chronobiological institute 19: 177-189
25. Nagoshi E, Saini C, Bauer C et al. (2004) Circadian gene expression in individual fibroblasts: cell autonomous and self-sustained oscillators pass time to daughter cells. Cell 119: 693-705
26. Pleasance ED, Cheetham RK, Stehpens PJ et al. (2010) A Comprehensive catalogue of somatic mutations from a human cancer genome. Nature 458: 719-724
27. Sarkar S, Horn G, Moulton K et al. (2013) Cancer Development, Progression, and Therapy: An Epigenetic Overview, International Journal of Molecular Sience, 14: 21087-21113
28. Savvidis C, Koutsilieris M (2012) Circadian Rhythm Disruption in Cancer Biology. Molecular Medicine 18: 1249-1260
29. Sjoblom T, Jones S, Wood LD, Parsons DW et al. (2006) The consensus coding sequence of human breast and colorectal cancers. Science 314: 268-274
30. Takashi JS, Hong HK, Ko CH, McDearmon EL (2008) The genetics of mammalian circadian order and disorder: implications for physiology and disease. National Revenue of Genetics 9: 764-775
31. Vogelstein B, Kinzler K W (2004) Cancer Genes and the Pathways They Control, Nature Medicine, 10, 789-799
32. Wood Pa, Yang X, Taber A et al. (2008) Period 2 mutation accelerates ApcMin/+ tumourigenesis. Molecular Cancer Research 6: 1786-1793
33. Wyllie AH, Kerr JF, Currie AR (1980). Cell Death: The significance of Apoptosis. International Revenue of Cytol. 64: 251-306
34. Zuo L, Weger J, Yang Q, Goldstein AM, Tucker MA, Walker GJ, Hayward N, Dracopoli NC (1996) Germline Mutations in the p16 binding domain of CDK4 in familial melanoma. Nature Genetics 12: 97-99