REVIEW

Role of circadian rhythm disorders on EMT and tumour-immune interactions in endocrine-related cancers

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Abstract

The circadian rhythm is a major environmental regulator of plants and animal physiology. The alternation of days and nights is translated at the cell and tissue level thanks to a molecular machinery, called the circadian clock. This clock controls in particular numerous endocrine functions, and its imbalances can have serious consequences on homeostasis. This is particularly true for the development of endocrine-related cancers, like breast, ovarian and prostate cancer. Circadian rhythm disorder (CRD) not only affects key hormone levels (including oestrogen, melatonin, insulin, glucagon, cortisol) but also favours a pro-inflammatory and immunosuppressive phenotype in the tumour microenvironment. This particular aspect is conducive to epithelial-mesenchymal transition (EMT) of solid epithelial tumours and cancer cell dissemination. It also favours resistance to chemo- and immunotherapy. Here, we discuss the current knowledge on this crosstalk between CRD, EMT and the immune microenvironment in endocrine-related cancers and its consequences for the development of efficient therapies.

Key Words

- circadian clock
- EMT
- tumour immune microenvironment
- immunotherapy

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Introduction

Circadian clock machinery

Many living organisms developed an intrinsic 24 h biological clock to adapt their physiology and behaviour to day–night cycles (Panda 2016). This endogenous time-tracking system is driven by environmental cues such as light, which is a major *Zeitgeber* ('time-giver') and feeding and physical activities. This sum of external stimuli is translated into molecular information by the CNS, and is required to synchronize and to entrain the central circadian clock and the peripheral circadian clocks. The mammalian circadian clock consists of a central oscillator, located in the suprachiasmatic

nucleus (SCN) of the hypothalamus, and peripheral oscillators present in all tissues and cell types. Light activates the pacemaker of the central circadian clock in the SCN through the retino-hypothalamic tract and together with non-photic *Zeitgebers* (e.g. meals, physical and social activities), synchronize and entrain peripheral circadian oscillators via neural and endocrine pathways. Altogether, these interconnected oscillatory networks maintain the synchrony between central and peripheral clocks.

Central and peripheral circadian clock is regulated at the molecular level by interconnected auto-regulatory transcriptional/translational feedback loops of positive

and *negative transcriptional regulators* (Lowrey & Takahashi 2011). The core positive regulator of this cellular clock is the BMAL1(ARNTL)/CLOCK complex that upon transactivation in the nucleus induces the transcription of target genes containing E-box elements in their promoters, including their principal negative regulators *PER* and *CRY* genes. These negative regulators form multimeric complexes in the cytoplasm that translocate to the nucleus and inhibit the activity of the BMAL1/CLOCK complex, creating the core loop of the molecular clock machinery. BMAL1/CLOCK complex targets other regulatory molecules like REVERBs and RORs, which repress or activate BMAL1 transcription, respectively, functioning as secondary stabilizing loop. Furthermore, the core complex initiates a rhythmic expression of clock-controlled genes resulting in a cell/tissue specific circadian output and consequently rhythmic biological processes. This molecular clock has been found to control several cancer hallmarks (Fig. 1) (Sulli *et al.* 2019). Key mechanisms include cellular metabolism (Sahar & Sassone-Corsi 2009), cell migration (Druzd *et al.* 2017, He *et al.* 2018), inflammation and immune responses (Castanon-Cervantes *et al.* 2010, Scheiermann *et al.* 2018), proliferation, differentiation and cell survival (Janich *et al.* 2013, Beker *et al.* 2019). Furthermore, recent human and mouse studies linked molecular clock defects to the activation of stemness and epithelial-to-mesenchymal transition (EMT) (Janich *et al.* 2014, Matsu-ura *et al.* 2016, Papagiannakopoulos *et al.* 2016).

Circadian rhythm disruption and endocrine related cancers

The adaptation of animal physiology to specific patterns of rest and activities relies on accurate regulation of cells

and tissues functions by endocrine factors. Circadian and endocrine systems are highly interconnected, and circulating levels of many endocrine factors are time-of-day regulated by central and peripheral circadian clocks (Gamble *et al.* 2014). This is the case of metabolic hormones (gherlin, leptin, melatonin, insulin, adiponectin, cortisol), sex hormones and thyroid stimulating hormone (TSH), and circadian disruption is associated with endocrine-related pathologies like metabolic and cardiovascular disease and type 2 diabetes mellitus. Circadian rhythm disorder or circadian rhythm disruption (CRD), which is a desynchronization between the internal sleep-wake rhythm and the day/night cycles or misalignment of tissue clocks, is also known to have a significant impact on the development of endocrine tumours (Urbanski 2011, Angelousi *et al.* 2019, Ikegami *et al.* 2019, Reinke & Asher 2019). In fact, six out of the ten most frequent cancers are endocrine tumours, including breast, prostate, cervical, uterus, thyroid and ovarian cancers (Globocan) (Bray *et al.* 2018), they count for ca. 55% of all cancers and represent ca. 22% of deaths from cancer worldwide. Epidemiology studies linked circadian disorders to increased risk of cancer in a wide range of organs, including endocrine tissues (Straif *et al.* 2007, Bhatti *et al.* 2013, Papantoniou *et al.* 2015, Hansen 2017, Samuelsson *et al.* 2018). But this fact is still controversial, potentially due to the high variability of datasets/cohorts, differences in the characterization of night shift work (e.g. exposure lengths, intensity, frequency or duration of night work period etc.). Furthermore, inter individual circadian differences (e.g. chronotype) are not taken into consideration. Consequently, all these factors strongly affect the statistical outcomes of epidemiological studies (Cordina-Duverger *et al.* 2018). Despite these discrepancies, epidemiological studies are well supported by genome-wide association

Figure 1

Circadian regulation of hallmarks of cancer. Most of the biological functions known to be hallmarks of cancer (31) are directly regulated by the circadian clock. Specific biological processes under circadian regulation are specified in red.

studies (GWAS) showing either a significant association between genetic variations located in circadian genes, and the risk of breast cancer or between circadian clock gene expression and tumour grade and metastatic-free survival (Benna *et al.* 2017, Mocellin *et al.* 2018, Morales-Santana *et al.* 2019). Consequently, the International Agency for Cancer Research confirmed the classification of night shift work as probably carcinogenic to humans (Group 2A) in 2019 (Ward *et al.* 2019). This classification, based on limited evidence from epidemiological studies and on strong experimental and mechanistic evidence from animal studies, highlights specific risks for breast, prostate and colorectal cancer in humans (Ward *et al.* 2019). However, additional experimental studies strongly suggest that a wider range of cancer types can be favoured by CRD. Our recent knowledge on the involvement of clock genes in endocrine cancer development is reviewed elsewhere (Angelousi *et al.* 2019).

Circadian disruption not only affects the proper cancer cells but also the surrounding tissues and the organism homeostasis. Indeed, the dynamic interaction between cancer cells and immune cells, furthermore a continuous cross-talks with other stromal components, affects every aspect of tumour development, from initiation to metastatic colonization (El-Kenawi *et al.* 2020). Recently, an increasing number of studies suggest a critical role of circadian regulation in shaping the tumour-immune interplay. Here we aim to provide a comprehensive summary of our current knowledge on the link between circadian disruption and inflammation, EMT and tumour immunity, which are critical features of both metastasis and therapeutic responsiveness.

CRD crosstalk with EMT and its link with inflammation

EMT and inflammation

Within the hallmarks of cancer, inflammation and cancer cell invasion and dissemination are two crucial processes, the link of which is not obvious at first glance (Hanahan & Weinberg 2011). During the last two decades, the epithelial- to- mesenchymal transition (EMT) and its reciprocal mesenchymal-epithelial transition (MET) have been in the spotlight to explain how cancer cells disseminate from primary tumours to give rise later to metastasis (Thiery *et al.* 2009, Nieto *et al.* 2016). During EMT, cellular organization switches from the well-defined epithelial state (with core features like apico-basal

polarity, a basement membrane, an extracellular matrix and numerous epithelial cell junctions) towards the mesenchymal state (including core features like back-front polarity, interstitial matrix, vimentin-based intermediate filaments, absence of cell-cell junctions) (Yang *et al.* 2020). EMT is a potent modulator of the cellular phenotype, and E/M transiting cells present a continuum of successive intermediate states covering the epithelial-mesenchymal spectrum (epithelial, early hybrid EMT, hybrid EMT, late hybrid EMT and mesenchymal) (Pastushenko & Blanpain 2019). These E/M intermediate phenotypes are associated with specific properties of cell survival, stemness, migration, invasion and metastatic potential, placing EMT as an integral component of the progression of most types of carcinoma (Blanco *et al.* 2002, Rhim *et al.* 2012, Ye *et al.* 2015, Dongre & Weinberg 2019).

Inflammation is one of the key players that facilitates EMT within the primary tumours, and directly or indirectly induces the transcriptional programme leading to the drastic changes observed at the cellular level during EMT (López-Novoa & Nieto 2009). Local inflammation at the primary tumours leads to the production of pro-EMT cytokines and chemokines like IL1- β , IL6, TGF- β and TNF- α by the tumour microenvironment but also by the proper tumour cells (Suarez-Carmona *et al.* 2017). These cytokines and growth factors are the main known EMT inducers and can act synergistically to induce EMT (Davaine *et al.* 2020). They activate canonical signalling pathways through their respective receptors, leading to the expression of pro-EMT transcription factors (EMT-TFs), mainly *SNAI* and *ZEB* genes.

Circadian clock genes modulate EMT induction and inflammation

Core transcription factors (TFs) from the circadian clocks can favour or repress EMT induction. PER2 downregulation in breast epithelial and tumour cell lines is associated with EMT-TFs expression, increased stemness and expression of EMT markers together with EMT-specific cellular properties (Hwang-Verslues *et al.* 2013). Oppositely, BMAL1 downregulation reinforces the epithelial state of colorectal cancer cells with a decrease in migration, invasion and drug resistance (Zhang 2019). In addition, decreased melatonin level induced by circadian rhythm disruption in rats is associated with EMT, and increased metastatic spreading of grafted breast tumour cells through the upregulation of RSK2 (Mao *et al.* 2016). Altogether these results suggest that the diurnal phase of the circadian rhythm could be more permissive than

the nocturnal one for EMT induction. The fact that core circadian clock TFs and core EMT-TFs bind similar response elements on DNA (E-boxes) also suggest that these TFs can either compete for promoter and enhancer occupancies or synergize to activate or repress the expression of common target genes.

Core clock TFs can also modulate inflammation, as demonstrated by murine knockout studies. *Bmal1*^{-/-} mice respond to an endotoxic shock by a severe proinflammatory response (Curtis *et al.* 2015) while *Per2*^{-/-} and *Clock*^{-/-} mice show a reduced inflammatory response with little or no IL1- β and IFN- γ production (Liu *et al.* 2006, Bellet *et al.* 2013). It is obvious that *Clock* and *Bmal1* are transcription factors with pleiotropic effects, and that some of the phenotypes observed in the mutants are not always related with circadian rhythm. It is therefore necessary to be able to confirm the functional link between a phenotype and the circadian rhythm by other experimental approaches. Circadian disruption through environmental stress like chronic jetlag also increases the circulating levels of pro-inflammatory cytokines and growth factors (Castanon-Cervantes *et al.* 2010). Regarding inflammatory diseases, the daily variation in the intensity of symptoms such as joint pain or inflammation of the airways is also well known and described (Jarjour 1999, Straub & Cutolo 2007).

Altogether it is clear that circadian rhythm disruption can be associated with EMT induction and favour a pro-inflammatory context at the systemic level and probably in the tumour microenvironment (TME). However, the functional hierarchy between the three processes remains unexplored (Fig. 2).

Our recent study on CRD during mammary cancer progression in mice may help to understand the interconnection between CRD, inflammation, EMT and tumour malignancy. We used mice that spontaneously developed mammary tumours from puberty (line MMTV-PyMT (Guy *et al.* 1992)) but in a genetic background (FVB/N $\times$ C57bl/6) where the development of tumours was not too fast and extended over more than 10 weeks. It was interesting for us to be able to induce circadian stress over a long period, thus mimicking the long-term effects of night shift work observed in nurses, where long exposure to shift work significantly increases the risk of breast cancer (Cordina-Duverger *et al.* 2018). The MMTV-PyMT model was also interesting because it reproduces the different phases of tumour progression, starting at puberty, when the MMTV promoter becomes active. We exposed the mice either to 12 h light:12 h darkness conditions or to a chronic jetlag (JL) lasting for 10 weeks (Hadadi *et al.* 2020). In JL mice we observed ca. 30 % more high-grade mammary carcinoma, two to three times more disseminated cancer cells and two times more mice with lung metastasis compared to LD mice. This aggressive phenotype was associated with an increase in *Il1*- β expression and an increase in EMT markers expression like *Inha*, *Zeb2* and *Cdh2* in tumour cells together with a decrease of *Per* genes expression. The proportion of cancer cells positive for mammary stem cells markers was also increased in agreement with previous studies showing that EMT confers stem properties to mammary tumour cells (Mani *et al.* 2008). These results confirm, in a physiological context, that CRD may link together EMT and inflammation to favour cancer cell dissemination. One hypothesis would be that CRD first favours

Figure 2

CRD crosstalk with EMT and its link with inflammation. Circadian rhythm dysregulation leads to an increase of pro-inflammatory signals in the TME. It includes cytokines and chemokines known to induce the expression of EMT-TFs. Activation of ZEB and SNAIL transcription factors leads to the start of the EMT programme. In addition, EMT-TFs bind to E-box like CLOCK and BMAL1 and may compete for promoter occupancy at circadian controlled genes, modifying the circadian regulation of such genes. Activation of the EMT programme leads to an increase of CSCs (dark blue cells), favours cancer cells (blue cells) migration and invasion thanks to changes in cancer cell properties and TME (increased production of fibronectin fibres (violet) by CAFs (brown cells), distinct immune context (yellow and green cells) and eventually increases the number of disseminated cancer cells.

inflammation in the vicinity of primary tumours, leading to an increase of pro-inflammatory cytokines directly acting on cancer cells. As a consequence, tumour cells activate the EMT transcriptional programme, modifying their intrinsic cell properties together with the TME and facilitating finally their dissemination in the organism. Additional experiments using orthotopic graft of tumours from JL mice to a LD host and its reciprocal (tumours from LD mice in a JL host) will help to answer such hypothesis and clarify in which measure the pro-inflammatory context resulting from CRD is one of the causes leading to EMT in primary tumours. The fine characterization of E/M intermediate phenotypes in primary tumours is also crucial to better understand the mechanisms leading to cancer cell dissemination in this experimental system. EMT is also known to modulate the immune response by its action on stromal cells and more specifically of various subtypes of innate and adaptive immune cells (Kudo-Saito *et al.* 2009, Dongre & Weinberg 2019). The interplay between CRD, EMT and tumour immunity is crucial during solid tumour progression and will be discussed subsequently.

CRD in tumour immunity

Most immune cell types have intrinsic circadian clock, and several of their characteristics and functions are under circadian regulation. The circadian aspect of many immune features including leukocyte homeostasis, trafficking and immune responses are well documented in the contexts of infection and inflammation (Labrecque & Cermakian 2015, Carter *et al.* 2016, Scheiermann *et al.* 2018). However, the effect of circadian rhythms on tumour immune microenvironment is less fully discovered yet.

CRD in leukocyte trafficking and function

NK cells

First, the innate immune system component NK cells, which also has an essential role in immunosurveillance and tumour cell killing, express circadian functional activities. Chronic CRD altered circadian expression of IFN γ , perforin and granzyme b resulting in reduced gene and protein levels of these factors (Logan *et al.* 2012). Furthermore, NK cells from jet-lagged mice showed reduced cytolytic activity upon tumour challenge. Studies identified *Per1* and *Per2* genes as potential regulators of NK cell function (Arjona & Sarkar 2006, Logan *et al.* 2013).

Macrophages

The diurnal rhythmic circulation/migration/homing of leukocytes is driven by both their intrinsic clock and the hormonal and metabolic clues modulating the chemokine system (He *et al.* 2018, Scheiermann *et al.* 2018, Beam *et al.* 2020). Rhythmic circadian machinery has been described in the majority of immune cells, including key players of TME (monocytes (Nguyen *et al.* 2013), macrophages (Keller *et al.* 2009), dendritic cells (Silver *et al.* 2012), neutrophils (Ella *et al.* 2016), CD4⁺ T (Bollinger *et al.* 2011) and CD8⁺ T cells (Nobis *et al.* 2019), NK (Arjona & Sarkar 2005) and B cells (Silver *et al.* 2012)). In solid tumours, one of the most abundant immune type is tumour associated macrophages (TAM). TAMs show high heterogeneity, and many of their phenotypes are actively contributing to immunosuppression, tumour growth and metastasis (Laoui *et al.* 2011). Monocytes/macrophages have a robust circadian rhythm and around 8–15% of their transcriptional/translational output is under circadian regulation (Keller *et al.* 2009, Trott & Menet 2018). Their biology is indeed controlled by their circadian rhythms, and dysregulation of their internal circadian clocks can have consequences on cancer biology. Bmal1-deficiency in myeloid cells leads to increased myeloid infiltration and enhanced inflammatory responses including several tumour promoting chemokines/cytokines for example, Ccl2, Ccl8, Il1b, Il6 or Tnfa (Nguyen *et al.* 2013, Oishi *et al.* 2017, Early *et al.* 2018). In addition, in Bmal1^{-/-} macrophages, the reduced *Reverb* expression leads to increased CX3CR1 and Mmp9 expression (Oishi *et al.* 2017), both favouring a pro-tumour TME. CX3CR1⁺ macrophages have been described to drive the expansion of CD4⁺ FoxP3⁺ regulatory T (Treg) cells in murine colon cancer model favouring a pro-tumour TME (Gu *et al.* 2019) and Mmp9 production by macrophages/myeloid derived suppressor cells (MDSCs) was associated with the development of a pre-metastatic niche in the lungs (Yan *et al.* 2010, Owyong *et al.* 2019). CRD can also indirectly affect myeloid cells through its effect on adjacent tissues. For example, Gibbs *et al.* showed that CRD in lung epithelial club cells leads to elevated CXCL5 and consequently increases the recruitment of CXCR2⁺ myeloid cells (neutrophils, MDSCs) to the lungs (Gibbs *et al.* 2014). This favours metastasis, since the accumulation of neutrophils (Jablonska *et al.* 2017)/MDSCs (Wang *et al.* 2019) and the CXCL5/CXCR2 axis are known to play critical role in pre-metastatic niche formation and metastatic dissemination/colonization (Halpern *et al.* 2011, Steele *et al.* 2016, Romero-Moreno *et al.* 2019, Sano *et al.* 2019, Zhang *et al.* 2020).

In our recent study, we confirmed the deleterious effects of CRD on TME in an *in vivo* model of breast cancer progression. Long-term exposure to chronic jetlag of mice developing spontaneous mammary tumours (MMTV-PyMT) enhances metastatic processes and alters the TME (Hadadi *et al.* 2020). We observed that the primary tumours from jet-lagged mice showed an enrichment of pro-tumour TAM population which was partially driven by the upregulation of the CXCL5/CXCR2 axis. In parallel, the infiltration of anti-tumour cytotoxic CD8+ T cells decreased while the suppressive Foxp3+ Treg cell population increased in these tumours. As a consequence of CRD, we observed a general dysregulation of the cytokine/chemokine network potentially contributing to the development of immune-suppressive microenvironment. In line with our observations, using syngeneic tumour model, Ramos *et al.* showed that the loss of Bmal1 in tumour cells leads to enhanced infiltration of macrophages in mice under high fat diet (Ramos *et al.* 2020). Likewise, Alexander *et al.* reported that Bmal1^{-/-} macrophages, when co-injected with B17-F10 melanoma cells, promote tumour growth and reduce CD8+T cell infiltration, as well as the proportion of infiltrated NK and IFN γ + CD8+T cells, thus decreasing the immune anti-tumour activity (Alexander *et al.* 2020). Both studies identified Bmal1-driven metabolic changes as underlying regulatory mechanisms.

These studies suggest that the high interconnection between intrinsic circadian clock and cellular metabolism in the macrophages may play a critical role in shaping the tumour immune microenvironment.

Neutrophils/granulocytic MDSCs

Besides macrophages, tumour associated neutrophils/granulocytic MDSCs are also contributing to tumour progression by promoting angiogenesis, metastasis and by attenuating anti-tumour immunity (Masucci *et al.* 2019). Neutrophils are also affected by the CXCR2-signalling, and several of their key functions have diurnal rhythmicity (Aroca-Crevillén *et al.* 2020). Adrover *et al.* reported that neutrophils exhibit a diurnal ageing pattern driven by a Bmal1-regulated CXCL2/CXCR2 axis (Adrover *et al.* 2019). They showed that this mechanism regulates the switch between the highly migratory/inflammatory ('younger') and the aged/homeostatic phenotype. Specific deletion of Bmal1 in neutrophils resulted in impaired homeostatic neutrophil clearance and enhanced migration to inflamed tissues (Adrover *et al.* 2019). In addition, this ageing phenotype is associated with degranulation and reduced neutrophil

extracellular traps (NETs) forming capacity (Adrover *et al.* 2020), suggesting a critical role of Bmal1/circadian machinery for the control of neutrophil immune responses. In the context of tumour microenvironment, these findings suggest that circadian disruption potentially boosts neutrophil driven inflammation and besides may increase NETs formation, which recently has been shown to interfere with anti-tumour T and NK cell cytotoxicity (Teijeira *et al.* 2020).

Dendritic cells and T cells

Another myeloid population, dendritic cells (DCs) are heterogenous antigen-presenting cells (APCs) with a critical role in anti-tumour immunity through the activation of T and NK cell cytotoxicity. However, certain tumour infiltrating DC populations can also initiate immunotolerance and suppressive immune responses (Murgaski *et al.* 2019). Until now, the role of the clock machinery in dendritic cell functions is less understood. Hopwood *et al.* revealed that the time of day that mice are infected with parasitic worms is a critical component of an effective immune response. This is due at least by the Bmal1-dependent modulation of the ability of DCs to initiate specific T cell polarization and responses (Hopwood *et al.* 2018). Thus, Bmal1^{-/-} DCs had impaired capacity to induce Th 1 mediated immune responses due to the downregulation of the promoting cytokines/signalling pathways, including IL-12, IL-23 or IL-27 mediated pathways (Hopwood *et al.* 2018). It has also been shown that Bmal1 depletion in DCs reduced their migration into the spleen, resulting in significantly reduced CD8 T cell expansion (Nobis *et al.* 2019). Interestingly, in this study using the DC-OVA vaccination model, the circadian expansion and activation of OVA-specific CD8 T cells were regulated by the intrinsic clock of CD8 T cells rather than that of the DCs, which was dispensable. These results suggest that circadian disruption may alter DC-induced T cell responses either through the alteration of their cytokine production or through their migratory capacities.

The circadian control of DC migration into peripheral tissues (to collect antigenic materials) has not yet been investigated but Druzdz *et al.* reported that DCs trafficking into secondary lymphoid organs had diurnal rhythmicity driven by the oscillatory expression of CCR7 (Druzdz *et al.* 2017). Interestingly, DCs and lymphocytes trafficking peak in phase (Druzdz *et al.* 2017). The principle results of this study showed that both T and B lymphocyte numbers oscillate in a circadian fashion, and their homing to/emergence from the lymph nodes are regulated by the

rhythmic expression of CCR7 and S1PR1, respectively. Lineage specific Bmal1 depletion confirms that the intrinsic molecular clock of these cells drove the diurnal expression of CCR7 and S1PR1. Furthermore, in line with the previously mentioned DC vaccination study (Nobis *et al.* 2019) the level of adaptive immune responses (T cell activation and cytokine production) is also time-of-day dependent. An earlier study showed the circadian control of T cell proliferation through the oscillatory variation of ZAP70 (TCR associated kinase) levels (Fortier *et al.* 2011). Secretion of several cytokines including IL-2, IFN- γ , TNF- α and IL-10 by CD4+ T cells has also been shown to follow diurnal rhythms (Bollinger *et al.* 2011). Recently Treg cells were shown to exhibit diurnal activity; however, they do not seem to have a functional circadian machinery (Hand *et al.* 2020). These observations suggest that the daily variation in Treg functionality is driven by external signals acting on their recruitment or retention within tissues.

Based on these observations we can hypothesize that CRD may significantly impair the adaptive anti-tumour immune responses through (i) the attenuation of T cell homing to the lymph nodes, (ii) the limitation of APC – T cell interaction and consequently (3) reduced level of T cell activation.

Altogether the existing data support the role of circadian mechanisms in the regulation of tumour immunity and the shaping of tumour immune microenvironment (Fig. 3).

EMT and immunosuppression at TME a putative interconnection with CRD?

The first study showing that EMT can be linked with immunosuppression was published 10 years ago (Kudo-Saito *et al.* 2009). The authors overexpressed Snail1 in mice melanoma cell lines and consequently observed a clear immunosuppression when co-culture with splenic cells, with an increased number of Treg CD4+ Foxp3+ cells. The mechanism proposed by the authors to explain this increase relies on thrombospondin-1 production by Snail1+ melanoma cells that generate immunosuppressive DCs which, in turn, induce Treg cells. However, it remains unclear whether this *in vitro* phenotype can occur under physiological conditions. A more recent study addresses this question in a spontaneous model of mammary carcinogenesis in mice (Dongre *et al.* 2017). They sorted epithelial or mesenchymal mammary cancer cells from primary tumours and performed orthotopic grafts in syngeneic immunocompetent mice. The comparison of TME between conditions highlights a clear immunosuppressive TME when grafted cancer cells were mesenchymal, with more Treg cells, M2 (protumour) macrophages and few or no cytotoxic CD8+ T cells. Interestingly, the phenotypic context of cancer cells also affects their intrinsic properties regarding the immune response. Mesenchymal mammary cancer cells expressed low levels of MHC-I and high level of PD-L1 while epithelial cancer cells presented an opposite phenotype.

Figure 3

Impact of circadian dysregulation in the tumour immune microenvironment. Circadian dysregulation promotes the development of a suppressive immune microenvironment by affecting different immune cell populations. Circadian disruption alters the cytokine/chemokine production in the tumour leading to increased recruitment and enrichment of myeloid cell types promoting tumour progression (e.g. neutrophils, monocytes/TAMs). The accumulation of these cells and their immunosuppressive actions result in reduced CD8 T cell infiltration and regulatory T cell enrichment (Treg). Dysregulated circadian mechanism also impairs the activation and function of anti-tumour immune cells (e.g. NK or CD8 T cells). In addition, essential anti-tumour DC functions (e.g. migration to the lymph nodes, antigen presentation, T cell activation) are also negatively affected. DC, dendritic cell, TAM, tumour-associated macrophage, NK, natural killer cell, hypothesized effect not yet confirmed in tumours.

These two studies propose that immunosuppression results from the E/M phenotypes of cancer cells, placing EMT upstream of the immunosuppressive context in TME. However, other studies propose that the immunosuppressive context in TME is a prerequisite for EMT in epithelial tumours. A first study used a spontaneous model of melanoma and showed that CXCL5 attracts MDSCs in TME which, in turn, secrete cytokine and growth factors known to induce EMT (Toh *et al.* 2011). A second study used a spontaneous model of mammary carcinoma in mice and showed the crucial role of SPARC to favour the formation of an immunosuppressive TEM with more Treg and MDSCs (Sangaletti *et al.* 2016). Surprisingly, SPARC overexpression in tumour cells also induces EMT but this induction depends on the presence of MDSCs at the vicinity of the tumour cells. The authors propose that SPARC expression by tumour cells promote the expression of COX-2, which in turn induced GM-CSF, IL-6 and the CXCL12–CXCR4 axis, favouring the recruitment of MDSCs at TME and consequently the activation of the EMT programme in cancer cells. How MDSCs favour EMT in a non-cell-autonomous way remains to be clarified.

Here, we presented clear scientific results linking CRD or EMT to immunosuppression. We also detailed the current knowledge on the consequences of CRD on EMT induction. Our last study also confirmed a potential interconnection between CRD, EMT and immunosuppression. In our system, we observed that chronic CRD increases CXCL5 and CXCR4 expression in tumour cells and CXCL12 and CXCR2 expression in BM cells, together with the activation of EMT markers in tumour cells. This led to the recruitment of pro-tumour macrophages in TME, to an increased number of CSCs in primary tumours and to an increased spread of cancer cells. The use of an CXCR2 inhibitor reverses

the phenotype, supporting the fact that blocking the recruitment of pro-tumour myeloid cells also decreases EMT induction and cancer-cell dissemination. Our results confirmed those of Sangaletti *et al.* who also observed a reversion of the EMT phenotype in mammary tumours of mice treated with zoledronic acid (ZA), a drug known to interfere with MDSC expansion and differentiation (Sangaletti *et al.* 2016).

Altogether these studies help to define a framework linking CRD to inflammation, EMT and immunosuppression. First, CRD favours metabolic disorders with fatty acid and glucose metabolism dysregulation in liver and adipose tissues, leading to a global pro-inflammatory context. Concomitantly with primary tumour growth, this context promotes the expansion of pro-tumour myeloid populations as well as a pro-EMT situation at TME. The recruited myeloid cells are promoting an immunosuppressive environment and EMT in cancer cells. EMT occurring in cancer cells increases the proportion of CSC and facilitates their dissemination but also positively feeds the regulatory loop maintaining immunosuppression. Finally, it also affects the efficiency of therapy because it is now clear that specific E/M states in cancer cells exhibit different levels of chemo and immunotherapy resistance (Fig. 4). To tone down this dynamic, drugs able to restore a functional circadian rhythm may be good targets to improve chemo and immunotherapies efficiencies.

Targeting circadian genes to overcome therapy resistance

Mutations in several components of the molecular clock have been linked to chemoresistance (Fang *et al.* 2015,

Figure 4

Conceptual framework linking CRD, EMT and immunosuppression. Somatic or germline mutations, ageing, environmental factors can cause CRD, leading to an alteration of homeostasis. Resulting metabolic and endocrine disorders together with genomic instability favour the onset and development of endocrine-related cancer. In particular, metabolic dysregulation accelerates tumour growth and modifies immunity. This leads to a modification of the tumour microenvironment, towards a more immunosuppressive and proinflammatory context favouring EMT. EMT in turn leads to an increase in CSCs and facilitates the invasion and spread of cancer cells while promoting resistance to therapy.

Katamune *et al.* 2019). In addition, chronotherapy studies (Dallmann *et al.* 2016) and our observations (CRD induced stemness and pro-EMT programme) suggest the involvement of CRD in the development of chemoresistance. Several strategies are under investigation to overcome therapy resistance including targeted immunotherapy. However, many endocrine cancers are considered difficult to treat with immunotherapy as majority of them are immunologically 'cold', and currently there is extensive research to improve the immunotherapy outcome for these patients. There are increasing number of approved immunomodulators available (Pembrolizumab (anti-PD-1) for ovarian, cervical, endometrial and prostate cancer, Atezolizumab (anti-PD-L1) for breast cancer) in combination therapies but despite the improvements, the success rate is still relatively modest. The existing literature strongly promotes the importance of the circadian machinery in anti-tumour immunity, and seemingly circadian disruption contributes to the development of 'cold' tumours through the accumulation of immune-suppressive cells in the microenvironment (Alexander *et al.* 2020, Hadadi *et al.* 2020, Ramos *et al.* 2020). A recent study reported that BMAL1 regulates PD-L1 expression in macrophages via metabolic reprogramming (Deng *et al.* 2018). In line, meta-analyses revealed that dysregulated circadian clock is associated with T cell exhaustion, alteration in immune infiltrate and upregulation of immune inhibitory molecules (e.g. PD-L1 and CTLA-4) (Wu *et al.* 2019, Zhou *et al.* 2020). All these data suggest that circadian clock components also affect tumour escape mechanisms. Interestingly, EMT has been also linked to tumour immune evasion, immune exclusion and immune checkpoint inhibitor resistance (Dongre *et al.* 2017, Terry *et al.* 2017, Chae *et al.* 2018, Soundararajan *et al.* 2019, Thompson *et al.* 2020) which further supports the importance of circadian regulation in therapy responsiveness. Thus, we hypothesize that using pharmacological modulators (Miller & Hirota 2020) of circadian genes is a suitable approach to reprogramme the tumour microenvironment, consequently promote anti-tumour immunity and potentially enhance the effectiveness of therapeutic agents.

Melatonin may be a potential candidate to reach such objectives. Anti-tumour effects of melatonin on hormone-dependent cancer have been described mostly for breast and prostate cancer but some studies also highlight anti-proliferative and pro-apoptotic effects on ovarian and cervical cancer cell lines (Li *et al.* 2017). This hormone is known for its oncostatic properties since decades and many experimental studies have demonstrated *in vitro* and

in vivo its anti-tumour activities like anti-inflammatory, anti-oxidative, anti-angiogenic, anti-proliferative and pro-apoptotic properties (reviewed in Hill *et al.* 2015). Melatonin is also able to disrupt oestrogen-dependent cell signalling and has also been shown to slow down EMT induction in breast cancer cell lines (Mao *et al.* 2012, Gonçalves *et al.* 2016). Together with its anti-inflammatory properties, melatonin may help to disrupt the vicious circle linking CRD, inflammation, EMT and immunosuppression. Currently some clinical trials are underway testing melatonin in cancer neoadjuvant and adjuvant therapies, most of them in order to correct side effects of chemotherapy, surgery and radiation like cognition loss, depressive symptoms and decreased sleep quality (source: clinicaltrials.gov). But to date, there is not enough evidence from available clinical studies to conclude that melatonin confers positive effects for endocrine-related cancer patients (Li *et al.* 2017).

Other good candidates can be ROR γ agonists. A recent phase 1 clinical trial (NCT02929862, NCT03396497) reported promising preliminary results on the anti-tumour activity of the small-molecule ROR γ agonist LYC-55716 (Cintirorgon) in combination with Pembrolizumab (Mahalingam *et al.* 2019). However, its direct effects on the circadian machinery have not yet been investigated, similarly to many other molecules (Sulli *et al.* 2019).

Lastly, considering the involvement of circadian regulation in immune cell trafficking and immune activation, a chronotherapeutic approach of vaccinations or adoptive cell transfer seems to be a wise strategy to significantly improve therapy outcomes.

Conclusions

In conclusion, all the existing data provide solid bases on the existing crosstalks between CRD, EMT and the immune system in primary tumours and open new questions about the role of circadian regulation in the tumour microenvironment. We need systematic mechanistic studies to deepen our understanding of the interdependencies and functional hierarchy between these important biological processes, in physiology and pathology. We hope that in the near future, there will be more and more studies addressing the following important questions.

One main question relies on the contribution of local and global effects of circadian clock dysregulation at the level of the tumour, of the TME and of the whole organism. We actually know that CRD increases the

incidence of endocrine-related cancers but it is not clear whether CRD favours the initiation steps of tumour formation (genomic instability, DNA replication) or rather favours early cancer cell dissemination and tumour growth through its endocrine and metabolic action on cancer and immune cells. The loss of circadian clock in cancer cells has been frequently described and impacts on the biology of neighbouring non cancer cells but also on distant cells and organs. Indeed, tumour induced circadian disruption/reprogramming of rhythmic metabolism has been reported (Masri *et al.* 2016, Hojo *et al.* 2017) together with dysregulated cytokine signalling in peripheral blood cells of breast cancer patients (Wang *et al.* 2020). Studies based on pharmacological modulators of circadian genes may help to better understand and correct such deleterious effects. We and others also showed that a systemic dysregulation of the circadian clock in cancer models globally affect the immune system and increase cancer cell dissemination and metastatic potential. It remains to clarify however in which measure CRD directly contributes to such phenotypes through a cell-autonomous effect of clock genes in these cells or indirectly through altered levels of hormones and other circulating molecules acting on cancer and immune cells. We can expect that the emerging research field of chrono-tumour immunology will target these questions. Furthermore, in the future, we still need to confirm the direct link between TME and EMT and perform mechanistic studies to reveal the underlying interconnecting pathways between the different stromal components and tumour cells. To understand the complex role of circadian rhythm in tumours we should perform systematic investigation of the effect of circadian clock loss by comparing models where the circadian machinery is omitted either in (i) tumour cells, (ii) in different stromal components or (iii) in the whole organisms.

In general, the connection between circadian rhythm and therapeutic effect is only investigated in the view of chronotherapy with the aim to improve drug efficiency and reduce adverse toxicity effects by administering chemotherapy drugs at the appropriate time of the day. Therefore, it would be exciting to investigate whether the use of circadian modulators in combination with existing therapies could enhance therapy outcomes but also to clarify the impact of circadian modulators on the efficiency/toxicity of immunotherapies.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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