

Review

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Circadian angiogenesis

Abstract: Daily rhythms of light/darkness, activity/rest and feeding/fasting are important in human physiology and their disruption (for example by frequent changes between day and night shifts) increases the risk of disease. Many of the diseases found to be associated with such disrupted circadian lifestyles, including cancer, cardiovascular diseases, metabolic disorders and neurological diseases, depend on pathological de-regulation of angiogenesis, suggesting that disrupting the circadian clock will impair the physiological regulation of angiogenesis leading to development and progression of these diseases. Today there is little known regarding circadian regulation of pathological angiogenesis but there is some evidence that supports both direct and indirect regulation of angiogenic factors by the cellular circadian clock machinery, as well as by circulating circadian factors, important for coordinating circadian rhythms in the organism. Through highlighting recent advances both in pre-clinical and clinical research on various diseases including cancer, cardiovascular disorders and obesity, we will here present an overview of the available knowledge on the importance of circadian regulation of angiogenesis and discuss how the circadian clock may provide alternative targets for pro- or anti-angiogenic therapy in the future.

Keywords: angiogenesis; cancer; circadian; vascular endothelial growth factor (VEGF); zebrafish.

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Introduction

Daily cycles of light and darkness on Earth have led to the development of highly conserved anticipatory signalling

processes, which are crucial to prepare most organisms from bacteria to human beings for the coming of the day and the night (1). These processes couple environmental light/darkness to biological functions and are naturally oscillating with a period of close to 24 h, thus collectively known as circadian rhythms (circa: about; diem: a day). Multiple aspects of mammalian physiology are under circadian regulation. The most obvious circadian rhythms in humans are perhaps those of activity/rest (2) and feeding/fasting (3). However, these rhythms are tightly coupled to a number of enabling physiological processes, such as regulation of blood pressure (4), heart rate (5), ventilation rate (6), metabolism (3), kidney and intestinal activity (7) and production of hormones that modulate these processes (8).

The importance of circadian signalling for maintaining our health is underscored by increased disease risk in people who frequently change their activity pattern from being awake during the day or during the night, such as people engaged in shift-work (9). Because of increased globalization and the 24-h lifestyle found in most major cities today, we are experiencing a drastic increase in the number of people on shifting working schedules submitting themselves to disrupted circadian rhythms (10). Epidemiologic studies have shown that such disruptions are coupled to an increased risk of cancer, including breast (11), prostate (12) and colorectal cancer (13), metabolic disorders including obesity (14), diabetes (15) and cardiovascular disorder (16) as well as psychiatric disorders including depression and various other diseases (17, 18). These diseases are for the most part driven by pathological changes to the vasculature (19–23) – in particular pathological angiogenesis, i.e., the growth of new blood vessels from an existing vasculature – which therefore has become a major research focus of the medical industry in recent years (24). In adults the healthy vasculature in most tissues is quiescent, presumably owing to the presence of high levels of endogenous angiogenesis inhibitors relative to pro-angiogenic factors. However, this intimate balance can easily be tipped in favor of angiogenesis – a process known as the angiogenic switch – which often will lead to rapidly progressive disease (21). As such, angiogenesis is crucial for solid tumor growth

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(22), obesity (25–27), arteriosclerotic plaque growth and instability (28), regeneration of heart or brain tissue following myocardial infarction (28) or stroke (29), as well as for chronic inflammatory diseases such as rheumatoid arthritis (19) and neurodegenerative diseases (30) and retinopathies, including age-related macular degeneration (31) and diabetic retinopathy (32). This realization has led to a surge in the clinical development and use of drugs targeting the angiogenic switch and in particular the factor vascular endothelial growth factor (VEGF) as an anti-angiogenic therapy in cancer and retinopathies (31, 33, 34). While such an approach has led to some progress in the management of these diseases, it is clear that more targets are needed in order to overcome resistance and increase the response to anti-angiogenic therapy (35). The angiogenic switch is regulated at multiple levels, and as we will discuss in more detail below, also by circadian clock factors. It is therefore pertinent to elucidate how circadian rhythms may influence angiogenesis and how this process could be targeted in order to prevent development and progression of angiogenesis-dependent diseases.

Organization of the circadian clock

Light and food are the principal agents responsible for coordinating circadian rhythms (36). Light is detected by non-vision forming retinal ganglion cells in the retina, which convey such day/night information to the suprachiasmatic nucleus (SCN) via the retino-hypothalamic tract (37). In the SCN, the signals are amplified and coordinated, and from here neuronal and humoral cues are generated which sets the pace for the coordinated rhythmic functions of the rest of the organism (38). Thus the SCN is considered to be the master clock and pacemaker. Food, the second major circadian timing factor or zeitgeber, is crucial for rhythm generation in the liver, which in turn regulate metabolic activities in the rest of our organism (39). Interestingly, many recent studies have also found circadian clocks in many other cell types, which may be regulated both by SCN- or liver-derived signals as well as other circadian mediators (40, 41). While, the cellular clocks within each cell of a tissue are usually coordinated, they may be out of sync with clocks in other tissues if timing cues are not coordinated with each other. As such, the vascular clock may be regulated differently by central and peripheral circadian clocks in different vasculatures, for example as a result of pathological disruption of blood pressure rhythms (42), differences in sympathetic innervation (43) or expression of receptors for endocrine circadian

modulators (44), due to disrupted rhythms of blood sugar levels (45, 46) or in other ways, which all could be important in causing diseases.

Regardless of the input (light, endocrine entraining factor or other) the molecular clock-work of all cells is organized in a very similar fashion (47) and build on a remarkably simple transcription-translation feedback loop (see Figure 1). Bmal1 is a key element of the positive limb of the loop. Bmal1, a member of the basic helix-loop-helix, PAS-domain containing family of transcription factors interacts with other members of this family, usually CLOCK or Npas2 to form a heterodimeric transcriptional activator, which drives transcription through binding to E-boxes in the promoters of target genes (48). Among these are members of the Period and Cryptochrome families (49, 50), which act as transcriptional repressors, inhibiting transcription at both their own promoters as well as those of other circadian output genes. This simple organization is referred to as the core loop, but it is regulated by a number of associated pathways that strengthen the system (38). These include ROR/Rev-Erb factors, D-box and F-box binding factors, protein kinases, ubiquitin ligases and multiple co-activators or -repressors, etc., factors that are important for conferring the right timing on the system, but are not involved in generating rhythmicity *per se*.

Mechanisms of angiogenesis

The development and growth of the vascular system is mainly achieved through angiogenesis – the sprouting and growth of new blood vessels from an existing vasculature (23), as opposed to vasculogenesis, which refers to the *de novo* formation of blood vessels and which is principally involved in formation of the first major vessels during early development (51). Angiogenesis is also important in adults during the female reproductive cycle (52), in wound healing/regeneration (53, 54) and in tissue (i.e. adipose or muscle) growth (55). However, in most adult tissues, the vasculature is quiescent and non-growing, but can be induced to grow in response to, for example, local tissue inflammation (56), hypoxia (21, 57–59) or other cues that induce the production of angiogenic factors. Angiogenesis is a multi-step process (60), starting with the destabilization of the vascular wall by degradation of the basement membrane and detachment of vascular mural cells such as smooth muscle cells and pericytes. This exposes the abluminal side of the endothelium on which a few leading tip cells emerge, start to move toward the angiogenic

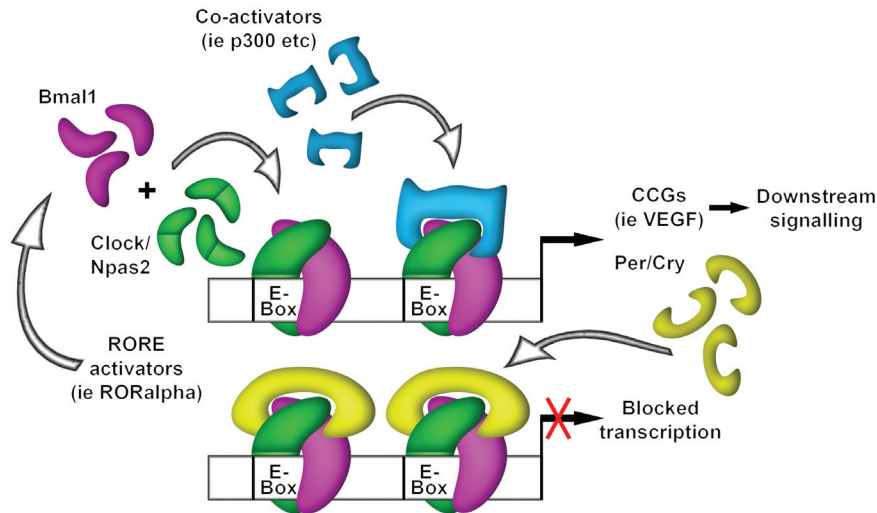


Figure 1 Scheme indicating the key players in the core circadian transcription-translation negative feedback loop. Following induction by RORE-binding factors such as ROR-alpha, Bmal1 (shown in purple) will accumulate, enter into the nucleus and bind the co-factors Clock or Npas2 (shown in green). The heterodimeric complexes bind to E-boxes in the promoters of target genes, and recruit co-factors such as p300 (shown in turquoise) to induce transcription of clock controlled genes (CCGs) such as VEGF, which elicit further downstream signalling important for angiogenesis, as well as members of the Period (Per) and Cryptochrome (Cry) families (shown in yellow). The latter will negatively feedback onto the Bmal1/Clock/Npas2 complex by repressing their transcriptional activation, thus creating diurnal oscillations in CCG mRNA levels.

signal and thus form a sprout. Cells located behind the tip-cell and thus preserving the connection to the original vessel, also known as stalk cells, proliferate, form a lumen and start to mature by recruiting new vascular mural cells and make the new vessel ready for perfusion once the tip-cell has found and anastomosed with a second existing or new vessel and thereby established a circulation loop (60, 61). Each of these steps is regulated by various angiogenic or vascular maturation factors. For example, basement membrane degradation is achieved by production and secretion of matrix metallo-proteinases (62), whereas VEGF and Dll4/Notch signalling are important for regulating organized sprouting (63–65). Patterning factors such as netrins and plexins are important for the guidance of the growing vessels (66, 67) and PDGF-B is considered a major factor involved in vessel maturation and stabilization by recruiting new mural cells to the endothelium (68). There are however many other angiogenic and vascular maturation factors that are important [see Cao et al. (24) for a recent excellent review on this subject].

Circadian control of angiogenesis in zebrafish

Binding sites for circadian transcription factors including E-boxes (the most important), D-boxes, F-boxes and ROREs

are present in the promoters of many different angiogenic factors, receptors or guidance molecules. Thus the circadian clock could potentially be important for regulating their production and thus for induction and guidance of blood vessel growth. If such regulation does in fact occur *in vivo* has, however, not been investigated for the majority of these factors to date. In our group, we have recently uncovered a mechanism by which circadian light/dark (LD) cycles regulate angiogenesis via production of VEGF primarily during the dark phases during zebrafish development (69, 70). As the developing zebrafish embryo is transparent, and develops outside the womb and thus is exposed to environmental LD cycles, all cells including endothelial cells and VEGF-expressing myocytes are directly responsive to light. We found that exposing zebrafish embryos to constant light (LL) from immediately after the egg was fertilized and onwards, led to significantly inhibited developmental angiogenesis compared to embryos exposed to regular LD or constant dark (DD) cycles (70). This finding indicate that zebrafish provide an excellent system to study circadian regulation of angiogenesis by LD cycles. The reduced angiogenesis phenotype in LL was recapitulated in embryos lacking Bmal1, and rescued in LL-exposed embryos lacking Period2, indicating that LL may down-regulate Bmal1 while up-regulating Period2 (70). Indeed we found that the promoter activity of Bmal1 was significantly lower in LL while that of Period2 was significantly higher, and the same was true

for the mRNA transcript levels of these genes (70). Using *in silico* analysis, we identified several putative E-boxes that could be a substrate for Bmal1, in the promoter regions of VEGF from various species including zebrafish, mouse and humans, indicating that Bmal1 may directly regulate VEGF. Indeed, we found that Bmal1 do bind to these E-boxes in the VEGF promoter, and that binding to each of these E-boxes contributes to VEGF production as specific deletion of the E-boxes led to a near-complete block of VEGF production during development (70). These findings proved that Bmal1 positively regulates VEGF production via E-boxes in the promoter region and that this regulation is disrupted in LL as a consequence of reduced Bmal1 levels. We further found that both zebrafish Bmal1 and VEGF cycles with a circadian rhythm in LD, at least during the first 6 days of zebrafish development, with VEGF levels being elevated during the dark-phase (70). As expected, the night-time peak in VEGF levels was abrogated in LL conditions (70), lending mechanistic insight into how disruption of circadian rhythms by LL could lead to reduced angiogenesis.

Regulation of angiogenesis by circadian factors in mice

In mice, light has recently been shown to reduce VEGF-A levels in the prenatal retina. Interestingly, mice reared in constant darkness from embryonic day 15–16 (3–4 days before birth) had too much VEGF, and thus impaired hyaloid vessel regression coupled with an overgrowth of the retinal vasculature at post natal stages (71). While the role of circadian transcription factors in this process was not investigated, others have shown that Bmal1 positively and Period2, Cryptochrome1 and Dec2 negatively regulate hypoxia-induced tumor-cell derived VEGF-A in mice (72, 73), providing support for the idea that the mechanism behind circadian regulation of VEGF in zebrafish could be conserved in mammals. As mice, in contrast to humans and zebrafish have elevated production of Bmal1 during the day, tumor- and cartilage-derived VEGF was found to be significantly elevated shortly after light-onset compared to during the night (72, 73); a finding that has been corroborated in other studies and linked to increased sensitivity of anti-angiogenic drugs when these are delivered during the day, and decreased effects combined with increased side-effects when treatment was given during the night (74). Disruption of circadian rhythms in tumor-bearing mice by exposure to constant light, which in zebrafish led to chronically elevated Period2

levels coupled to a reduction in VEGF production (70), has been reported to only slightly decrease the levels of tumor VEGF-A (75), although in this particular study LL did not lead to inhibited angiogenesis because of a compensatory up-regulation of other pro-angiogenic factors. Interestingly, in a carcinogen-induced mouse sarcoma model, tumor VEGF levels exhibited a larger peak in expression level during the night than during the day (76), which could indicate that circadian control of VEGF and angiogenesis is context-dependent and differs between different tumor types and models. Other studies implicate Period2 as an important negative regulator of tumor angiogenesis. Period2-overexpressing tumor cells grow slower when implanted in mice (77) and period2 knockout mice are prone to develop teratomas following irradiation (78). As tumor growth is an angiogenesis-dependent process, these findings seem to indicate that Period2 may inhibit angiogenesis in mice.

An important regulator of Bmal1 – and therefore the positive limb in the core circadian transcriptional regulatory network – is retinoic acid receptor-related orphan receptor (ROR)-alpha (79). Staggerer mice, which are deficient in ROR-alpha, exhibit elevated induction of angiogenesis following tissue ischemia (80). However is not clear if the exaggerated ischemia-induced angiogenesis in these mice is caused by disruption of Bmal1 signalling or by other effects of ROR-alpha.

Also, in cell lines, Bmal1, Bmal2 and Clock have been found to be important for regulation of VEGF levels and for circadian oscillations in VEGF production leading to elevated production of VEGF during the subjective night in a human cell line (81, 82). In contrast to these findings, Period2 has recently been implicated as a pro-angiogenic gene, as mice exhibiting a homozygous null mutation in the period2 gene, exhibit signs of vascular senescence including inhibited VEGF-induced angiogenesis into implanted matrigel plugs as well as impaired development of collateral arteries in a hind limb ischemia model (83). However, this phenotype was not well described from a molecular point of view, and could be associated with other aspects of circadian disruption including changes in eNOS activity (see below), which is known to be highly important for induction of senescence (84–86).

The role of the circadian clock in human tumor angiogenesis

Disruption of circadian rhythms during cancer treatment is clinically relevant. Approximately 50% of colorectal cancer

patients, for instance, experience disruption of circadian rhythms during chemotherapy treatment. Patients with disrupted circadian rhythms as measured by actigraphs (wristwatch-style motion detectors) had significantly shorter survival times than those with normal circadian rhythms (87). Fatigue and weight loss were also higher in patients with disrupted circadian rhythms. In another study, colorectal cancer patients receiving chemotherapy who had good performance status and normal circadian rhythms had better survival and response to treatment, as well as less fatigue and better quality of life (88). Lifestyle adaptations may help patients to entrain circadian rhythms of sleep and food intake to normal 24-h cycles; these include timing and composition of meals; regulation of consumption of herbal sedatives and stimulants and alcohol; timing of exercise and morning exposure to sunlight; and mind-body programs that diminish sleep-disturbing stress, as well as therapies like cognitive-behavioral treatment for insomnia that promote sleep (89, 90).

From a molecular point of view, and in agreement with the findings from pre-clinical models, CLOCK was reported to be significantly up-regulated and Per2 was down-regulated in human colorectal cancer tumors compared to adjacent healthy tissue and the levels of CLOCK from different patients strongly correlated with the level of VEGF detected in their tumor biopsies as well as degree of metastatic dissemination of tumor cells and poor prognosis (91, 92). In addition, other angiogenic factors have been found to oscillate in a circadian fashion, including bFGF, EGF and IGFBP in breast cancer patients, where peak plasma levels are generally found during the day and low levels in the night (93). Even in non-malignant disorders, VEGF levels have been found to oscillate in a similar manner as in cancer patients (94). As such, plasma VEGF levels in a patient with POEMS exhibited circadian oscillations with the highest levels found at night, resulting in night-time peripheral oedema, which subsided during the day, when VEGF levels had normalized (94). Interestingly, in the non-vascularized cornea, anti-angiogenic angiostatin is increasing during the dark-phase, in which the eye is closed and therefore particularly sensitive to hypoxia-induced angiogenesis (95). This may in such tissues be an intrinsic mechanism to prevent angiogenesis during the night.

Circadian rhythms and NO synthesis

Nitric oxide (NO), produced by endothelial cell nitric oxide synthase (eNOS), is among the most potent and important vaso-active molecules and is also important

for regulation of angiogenesis (96). The endothelial cell clock, and endothelial cell Bmal1 in particular is critical for maintaining physiological activity of eNOS. Without Bmal1, such as in Bmal1-KO mice, or if Bmal1 signalling is deregulated such as in Clock-mutant mice, NO production is reduced, indicating inhibited activity alternatively that eNOS is uncoupled and produce increased amounts of superoxide rather than NO (97, 98). Conversely, NO is an important mediator of circadian rhythms in the endothelium as age-related decline in eNOS activity lead to a dysfunctional EC circadian clock, which could be phenocopied in younger mice by eNOS inhibition and rescued in older mice by administering an NO-donor (99). In addition to Bmal1/Clock, the negative circadian regulator Period2 may play an important role in maintaining the size and function of the endothelial progenitor cell pool in the bone marrow, which in turn is important for physiological angiogenic responses to ischemic insults (83). Period2 mutation also caused reduced NO production (although eNOS levels were not changed) as well as increased levels of COX-1-derived vasoconstrictors (83, 100), indicating that disruption of Bmal1 and Period2 both lead to similar changes in regulation of vascular tone despite the fact that they exhibit opposite regulation of the circadian clock. However, both proteins are crucial for EC clock function in general so the deregulated NO production may be a result of an impaired clock, and thus related to deregulation of clock-output genes, rather than Bmal1 or Period2 directly changing eNOS function or activity. In line with this hypothesis, both Bmal1 or period1–3 triple knockout lead to remodelling and toughening of the vascular wall (101), which in turn led to development of atherosclerosis in the circadian factor-deficient vessels even when these were implanted into wild type mice (102) that exhibited normal blood lipid levels and overall circadian rhythms.

Angiogenic functions of circadian factors or output molecules

While genetic studies showing direct involvement of circadian transcription factors in regulation of angiogenesis are intriguing, disrupted circadian rhythms in patients – who are usually not harbouring mutations in circadian clock genes – would probably be brought about through deregulated secretion and functions of output molecules, such as melatonin or cortisol. Melatonin may either promote (103) or inhibit (104) angiogenesis depending on the pathological situation. Melatonin inhibits tumor angiogenesis by lowering both basal and hypoxia-induced tumor-cell VEGF

production (104–106). However, melatonin also promotes beneficial angiogenesis in ulcers (103) as well as during wound healing (107) and bone repair (108). Furthermore, melatonin inhibit blood-retinal barrier breakdown in response to hypoxia during progression of proliferative retinopathy (109). Melatonin may have both unspecific effects as an anti-oxidant, as well as elicit specific signals through melatonin receptors, which may be the underlying reason for the divergent role of melatonin in different contexts. The exact mechanism by which melatonin influence hypoxia-induced VEGF production and angiogenesis in malignant versus non-malignant cells still remain obscure.

Cortisol (dexamethasone), which is high in the early morning, elevated in stressful constant light conditions (110) but reported to be both elevated (111) and reduced (112) in the morning in shift-workers, possibly depending on the degree of experienced stress in the individual (111), is a more clear-cut inhibitor of angiogenesis. Cortisol inhibits pathological VEGF-A production in tumors (113) as well as physiological VEGF-A production in growth plate chondrocytes (114) and vascular smooth muscle cells (115). Cortisol however also inhibits angiogenesis via anti-inflammatory effects on leucocytes that are often a source of multiple angiogenic factors, including VEGF (116). Fibromodulin was recently found to be a potent angiogenic factor produced by melanocytes and important for pathological angiogenesis in the eye, which could be related to inflammation (117). The importance of cortisol and fibromodulin in circadian regulation of angiogenesis has so far not been investigated.

Prokineticin1 and -2 are important secreted regulators of circadian synchronization within the SCN, where they inhibit sleepiness and potentiate the light-induced output (118). Prokineticin1 is also known as EG-VEGF and is a potent angiogenic and vascular permeability factor in the adrenal medulla as well as potentially other fenestrated vascular beds (119). Even Prokineticin2 – also known as Bv8 – is an important ‘alternative’ angiogenic molecule in tumors, where it has been found to mediate resistance to anti-VEGF therapy (120, 121). More studies are however needed to establish if disturbed circadian rhythms may lead to deregulated production of prokineticins and if this translates to differences in plasma levels of this cytokine, which could therefore be important for induction of pathological angiogenesis in disease.

Conclusions and perspectives

Angiogenesis is one of the most important processes for disease progression and therefore tremendous interest

has been placed on attempting to modulate angiogenesis therapeutically either by anti-angiogenic therapy in cancer, retinopathies, metabolic diseases or chronic inflammatory diseases alternatively by pro-angiogenic therapy in, for example, neurodegenerative disorders, myocardial infarction, stroke or diabetic peripheral vascular disorders. Unfortunately some previous efforts to target angiogenesis by focusing on blocking or delivering VEGF or VEGF-receptors have not provided the significant clinical benefits that researchers and patients were hoping for, probably because the complexity of the angiogenic process cannot be accurately modulated by targeting only a single pathway (35, 122). As such, other methods or different targets with broader actions need to be identified. Recently the circadian clock has emerged as a potentially important regulator of angiogenesis in disease (69). Therefore, the modern changes in lifestyle, which encompass a frequent disruption in these rhythms for a growing number of people including shift-workers (10), may explain why angiogenesis-dependent diseases including cancer, cardiovascular disorders, metabolic disorders and chronic inflammatory disorders are on the rise (see Figure 2). Circadian rhythms may affect angiogenesis directly by regulation of pro- or anti-angiogenic factors, which has been discussed in detail in this review. However, many indirect modes of regulation may be as – or even more – important, for example circadian regulation of blood pressure and perfusion may cause circadian changes in tissue oxygenation/hypoxia (123–125), which could affect the vasculature. Also circadian changes in core body temperature have been shown to have pronounced effects on cold-regulated signalling factors (126), which are important for healthy physiological processes, and perhaps also for regulation of angiogenesis, during the night. Finally, disruption of circadian changes in blood sugar levels could play a major role in vascular pathologies, including induction of angiogenesis in diabetic patients, as high blood sugar levels have to be coupled with high levels of intracellular ROS-scavengers (127), which exhibit circadian regulation (128). Shift-work commonly leads to uncoupling of activity/rest cycles from the LD period, potentially leading to circadian disruption of the organism. However, there are also other, more subtle ways in which circadian rhythms can become deregulated. In patients with sleep apnea for example, the quality of sleep may be insufficient to completely reset the clock and prepare the person for the new day, once that person wakes up (129). Also other types of sleep disorders as well as stress and worries brought about increasing demands from the society on our personal performance may influence the circadian clock and cause disease (130). Furthermore, genetic disruption of

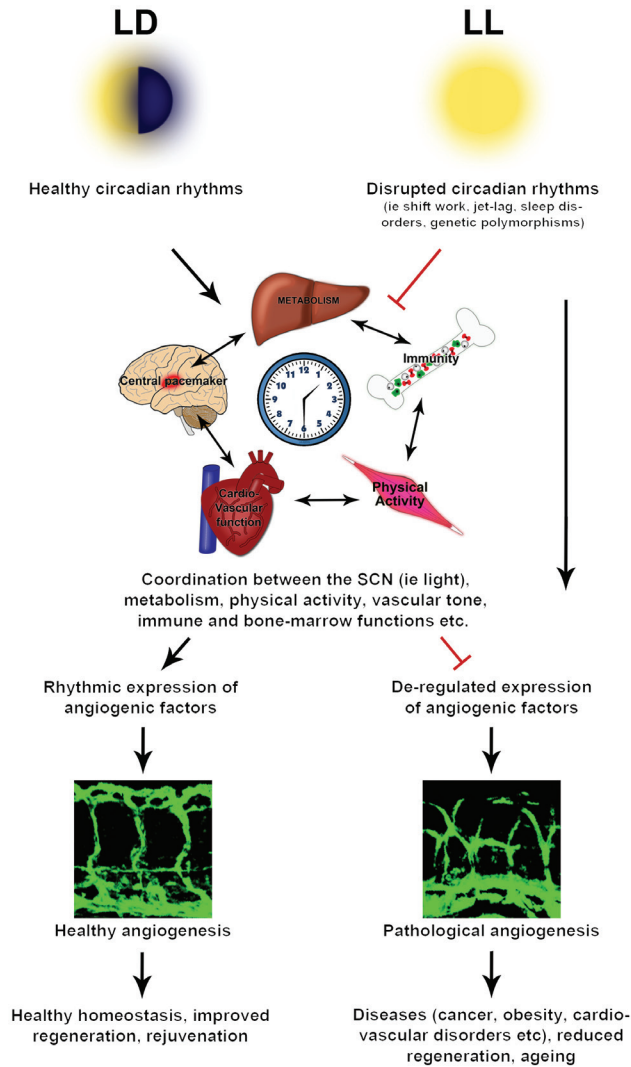


Figure 2 Scheme indicating our hypothesis on how disruptions in circadian rhythmicity increase the risk of disease, alternatively poorer response to treatment. Various types of circadian disruption such as shift-work, jet-lag, sleep disorders or genetic polymorphisms negatively affect important physiological functions, including rhythm generation by the central pacemaker in the SCN, metabolism, immune function, physical activity, cardiovascular functions and intestinal functions. This in turn may lead to pathological deregulation of angiogenic factors, which may also be directly deregulated by disrupted rhythms of circadian transcription factor levels (right). This, in turn, leads to pathologic angiogenesis and increased risk of angiogenesis-dependent diseases in people with disrupted circadian rhythms. The micrographs in the image are of healthy (left) or disease (right) blood vessels (shown in green) in 3-day-old zebrafish embryos.

the circadian signalling pathways may be a factor behind disease-development in humans (131–133). Particularly in cancer cells, which have an unstable genome, mutations in the circadian clock genes are not uncommon (133, 134), and therefore the circadian clock could be locally

disturbed, giving the tumor a metabolic benefit to acquire more nutrients at times when the organism in general is metabolically inactive (134).

Regardless of whether angiogenesis is directly or indirectly regulated by circadian rhythms, it seems important that we learn more about how to identify and target disruptions of the circadian rhythm in blood vessels in humans as a preventive or therapeutic strategy in the future (89). Alternatively, manipulation of the circadian clock in vascular cells (endothelial or perivascular cells) by circulating signals such as melatonin or cortisol may be a promising strategy for pro- or anti-angiogenic treatment as the vasculature is more exposed to circulating factors and drugs compared to other cells.

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