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Circadian rhythm–related genes: implication in autoimmunity and type 1 diabetes

B. Lebailly[1,2], C. Boitard[1] & U. C. Rogner[1]

[1] *Institut Cochin (INSERM U1016, CNRS UMR-S8104, Département "Endocrinologie, Métabolisme et Diabètes), Paris, France*
[2] *Cellule Pasteur, University Pierre and Marie Curie, Paris, France*

Recent gene association and functional studies have proven the implication of several circadian rhythm-related genes in diabetes. Diabetes has been related to variation in central circadian regulation and peripheral oscillation. Different transcriptional regulators have been identified. Circadian genes are clearly implicated in metabolic pathways, pancreatic function and in type 2 diabetes. Much less evidence has been shown for the link between circadian regulation and type 1 diabetes. The hypothesis that circadian genes are involved in type 1 diabetes is reinforced by findings that the immune system undergoes circadian variation and that several autoimmune diseases are associated with circadian genes. Recent findings in the non-obese diabetic mouse model pinpoint to specific mechanisms controlling type 1 diabetes by the clock-related gene *Arntl2* in the immune system.
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Circadian Genes and Diabetes

There is growing evidence about the implication of the circadian rhythm in diabetes development [1]. Studies in mice have shown that the disruption of circadian rhythms can accelerate diabetes and β-cell loss [2]. In humans a link between the central circadian rhythm regulation and glucose homeostasis has been suggested by findings such as the polymorphism in *MTNR1B*, encoding the melatonin receptor 1B, that increases the risk for type 2 diabetes [3].

Transcription and translation of core clock components circadian locomotor output cycles kaput (CLOCK), aryl hydrocarbon receptor nuclear translocator-like 1 (ARNTL1), aryl hydrocarbon receptor nuclear translocator-like 2 (ARNTL2), period circadian proteins (PER1, PER2, PER3) and Cryptochromes (CRY1 and CRY2) play a pivotal role in rhythm generation in the suprachiasmatic nucleus, which is the site of the master circadian oscillator in mammals, but also in the control of peripheral oscillations.

The direct relation of *CLOCK*-related genes in diabetes has already been shown [4]. *ARNTL1*, which is also called brain and muscle ARNT-like 1 (*BMAL1*) or member of PAS superfamily 3 (*MOP3*), has been genetically linked to hypertension and type 2 diabetes in humans [5]. Knockout mice for *Arntl1* and *Clock* exhibited a role for the β-cell clock in coordinating insulin secretion with the sleep-wake cycle, and revealed that ablation of the pancreatic clock can trigger the onset of diabetes mellitus [6]. The major circadian pacemaker *ARNTL1* has in addition been associated with susceptibility to gestational diabetes mellitus [7]. CRY, another component of the core clock, is required for the regulation of inflammatory cytokines via the NF-kappaB pathway [8]. Absence of this key circadian clock component leads to the activation of this signalling system and elevated levels of inflammatory molecules in the body. Low-grade constant inflammation could be the underlying cause of chronic diseases such as diabetes. Further examples of the involvement of *CLOCK*-related genes include the *PER3* length polymorphism discovered in patients with type 2 diabetes mellitus [9].

Other homologous genes of the family of basic helix-loop-helix transcription factors, such as the dioxin-receptor encoding gene *AHR* [10], *ARNT* [11] have been associated with diabetes. The Aryl Hydrocarbon Receptor (AHR) is a cytosolic transcription factor that is normally inactive, bound to several co-chaperones. Upon ligand binding to chemicals such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the chaperones dissociate resulting in AHR translocating into the nucleus and dimerizing with ARNT (AhR Nuclear Translocator), leading to changes in gene transcription. Activation of the AHR has long been known to cause immunotoxicity, including thymic involution. More recent data suggested a role for the AHR in regulatory T-cell (Treg) and T-helper 17 (Th17) cell development [12,13]. Activation of the aryl hydrocarbon receptor by the dioxin TCDD prevents diabetes in NOD mice and increases Foxp3$^+$ regulatory T cells in pancreatic lymph nodes [10].

The aryl hydrocarbon receptor nuclear translocator (*ARNT*) gene is a positional and functional candidate for type 2 diabetes [14]. Using oligonucleotide microarrays and real-time PCR of pancreatic islets isolated from humans with type 2 diabetes versus normal glucose-tolerant controls, a 90% decrease in expression of the transcription factor ARNT, also called Hypoxia Inducible Factor 1 β (HIF1β), has been identified. ARNT knockout mice exhibited abnormal glucose tolerance, impaired insulin secretion, and changes in islet gene expression that mimicked those in human diabetic islets. These data

Correspondence to: U. C. Rogner, INSERM U1016, CNRS UMR 8104, Institut Cochin, 123, boulevard de Port-Royal, 75014 Paris, France.
E-mail: ute.rogner@inserm.fr

suggested an important role for decreased ARNT and altered gene expression in the impaired islet function of human type 2 diabetes. ARNT regulates many β-cell genes, insulin secretion and glucose tolerance [11].

Taken together, the present data suggest a strong link between diabetes and the circadian rhythm via a number of different gene pathways, including those that implicate insulin metabolism and immune regulation.

> "Present data suggest a strong link between the circadian rhythm and diabetes. Studies performed in the 1960s and 1970s indicated that the immune system undergoes circadian variation, and many other examples have been published since."

Circadian Genes and the Immune System

Studies performed in the 1960s and 1970s had already indicated that the reaction of the immune system to pathogens undergoes circadian variation (reviewed in Ref. [15]). These early studies also showed circadian variation of specific immune cell types and their activity, for example the variation of mitotic activity and cell degeneration in the mouse thymus over a period of 24 h [16,17]. Many other examples have been published since, some of which concern highly specific immune functions. Recent studies showed for example that Th17 differentiation is controlled by the circadian clock [18], that the circadian gene *Bmal1* regulates diurnal oscillations of Ly6C(hi) inflammatory monocytes [19], or that the circadian clock protein CRY regulates the expression of proinflammatory cytokines [8].

In this context, it does not seem surprising that autoimmune disease is influenced by the circadian rhythm. This observation has particularly been made in rheumatoid arthritis, where patients suffer more from stiffness and pain in the morning than during other times of the day. This phenomenon has been attributed to the circadian regulation of cytokines (reviewed in Ref. [20]). Although the link has not been made as strongly for human type 1 diabetes, the hypothesis of circadian genes being involved in the disease appears rather justified.

The *ARNTL2* Gene

The *ARNTL2* (aryl hydrocarbon receptor nuclear translocator-like 2) gene is also known as *BMAL2* (brain and muscle ARNT-like 2), *PASD9* (PAS domain-containing protein 9), *MOP9* (member of PAS protein 9) or *CLIF* (Cycle-like factor). The cloning of *ARNTL2* has been described by Hogenesch et al. [21] under the name *MOP9*, by Maemura et al. [22] under the name *CLIF*, and by Okano et al. [23] and Ikeda et al. [24] under the name *BMAL2*.

By searching a human EST database for sequences similar to *BMAL1* (*ARNTL*), followed by PCR performed on foetal brain cDNA and RACE using adult brain cDNA, Ikeda et al. [24] cloned the *ARNTL2* gene, which they called *BMAL2*. The deduced 551-amino acid protein had an N-terminal bHLH domain, followed by PASA and PASB domains and a long C-terminal sequence. Ikeda et al. identified two putative nuclear localization signals near the N terminus. BMAL2 shared 52% amino acid identity with zebrafish Bmal2 and 49% identity with human BMAL1. By Northern blot analysis *BMAL2* transcripts of 7.0 and 8.0 kb were detected in adult liver and foetal brain. A 2.2-kb *BMAL2* transcript was also detected in adult liver. The authors detected no expression in adult heart, brain, pancreas, placenta, lung, liver and kidney. A fluorescence-tagged BMAL2 protein located predominantly in the nucleus of transfected HEK293 cells.

Hogenesch [21] mapped the gene to human chromosome 12p11.22–11.23. They described the fact that MOP9 displays significant homology to the Drosophila circadian factor CYCLE and the mammalian ortholog MOP3/BMAL1. MOP9 was found to form a transcriptionally active heterodimer with the circadian CLOCK protein, the structurally related MOP4, and hypoxia-inducible factors, such as HIF1alpha. They confirmed that *MOP9* was expressed in several brain regions such as the thalamus, hypothalamus and amygdala. Like *CLOCK*, *MOP9* expression was found in the suprachiasmatic nucleus.

Using the endothelial PAS domain-containing protein 1 (EPAS1) as bait in a yeast 2-hybrid screen of a human umbilical vein endothelial cell cDNA library, Maemura et al. [22] also cloned *ARNTL2*, which they called *CLIF*. The deduced 602-amino acid protein contained a bHLH/PAS domain and shares 44.1% identity with drosophila cycle and 44.9% identity with human BMAL1. It differed from the BMAL2 protein reported by Ikeda et al. [24] at its N terminus. Northern blot analysis detected widespread and variable expression of *CLIF* transcripts of 8, 6, 2.4 and 2.2 kb. Highest *CLIF* expression was detected in brain and placenta. *In situ* hybridization revealed expression of *CLIF* transcripts in endothelial cells of human heart, lung and kidney and in endothelial cells and neurons of brain.

The plasminogen activator inhibitor-1 (PAI-1) is the major physiologic inhibitor of tissue-type plasminogen activator in plasma, and is elevated in a variety of clinical situations that are associated with increased risk of ischemic cardiovascular events. Maemura et al. [22] showed that in endothelial cells, CLIF formed a heterodimer with CLOCK and upregulated the plasminogen activator inhibitor-1 (*PAI-1*) gene through E-box sites. PER2 and CRY1 inhibited the *PAI-1* promoter activation by the CLOCK:CLIF heterodimer. These results suggested that CLIF regulates the circadian oscillation of *PAI-1* gene expression in endothelial cells. Schoenhard et al. further studied this interaction [25]. They described that the CLOCK:BMAL1 and CLOCK:BMAL2 heterodimers made additive contributions to *PAI-1* gene transcription. The abilities of these heterodimers to activate gene expression differed by twofold. The susceptibilities of these circadian activators to inhibition by PER and CRY proteins were, however, found to be equivalent and redox independent. Schoenhard et al. hypothesized that the different BMAL1 and BMAL2 spatiotemporal distributions allowed intrinsic circadian clocks to modulate the amplitudes of their oscillators while they maintained circadian periodicity. In this way, the fundamental circadian clock components were thought to drive circadian variation in *PAI-1* expression, which in turn

could then influence the pathogenesis of acute atherothrombotic events.

Takeda et al. [26] described the regulation of thrombomodulin (TM) by the CLOCK:BMAL2 dimer. Again, this confirmed that cardiovascular diseases are closely related to circadian rhythm under control of an internal biological clock mechanism.

The studies showed that the human *ARNTL2* gene encodes a basic helix-loop-helix (bHLH)/PAS domain transcription factor with a probably ubiquitous expression pattern and with strong expression in the brain. Strikingly, the studies showed multiple splice variants of the gene transcript [27].

> "The human clock-related *ARNTL2* gene encodes a basic helix-loop-helix (bHLH)/PAS domain transcription factor and its transcript exhibits an ubiquitous expression pattern with multiple splice variants."

Apart from being potentially involved in transcriptional regulation, ARNTL2 has been found to control cellular proliferation. Overexpression of antisense *ARNTL2* RNA in human 293EBNA cells resulted in reduced cell cycle time, increased plating efficiency in soft agar, diminished TNF-alpha-induced increment of CPP32/caspase-3 activity, and a reduced proportion of cells in the G2 phase with a concomitantly increased proportion of cells in the S phase [28]. The authors concluded that the frequent downregulation of *ARNTL2* is related to hepatocellular carcinoma (HCC).

Okano et al. [23] cloned cDNAs encoding mouse and rat *Bmal2* (*mBmal2* and *rBmal2*) from mouse midbrain and rat-1 fibroblast cells, respectively. Their phylogenetic analysis suggested that vertebrate *Bmal1* and *Bmal2* genes were generated by a single gene duplication of an ancestral *Bmal* gene, a vertebrate ortholog of *dCyc* gene. The authors described that BMAL2 proteins have diverged about 20-fold more rapidly than BMAL1 proteins after the duplication, suggesting an as-yet-unidentified function conserved in BMAL1 but not in BMAL2. *mBmal2* mRNA was constitutively expressed throughout the day under light–dark cycle in the mouse hypothalamus containing suprachiasmatic nucleus. Sasaki et al. [29] used murine NIH3T3 cells and showed that, like in humans, [25] the BMAL2-CLOCK activity was inhibited by PER2 pointing to a negative and positive role of BMAL2 in circadian transcription.

Arntl2 is considered as a paralog to *Arntl*, and both are homologs of the *Drosophila* gene *Cycle*. Further homologs were isolated in several other species such as fish [30] and birds [23,31,32]. Large-scale sequencing projects identified *Arntl2* homologs in lizards (*Anolis carolinensis*) and the tropical clawed frog (*Xenopus tropicalis*).

ARNTL2 Expression and Disease

Genetic drifts in *ARNTL2* polymorphisms have been described in the human population leading to variation in the circadian rhythm regulation [33]. The murine ARNTL2 can rescue many ARNTL1 functions in the suprachiasmatic nucleus [34], but also essential functions of ARNTL1 in controlling obesity and type 2 diabetes [35]. The *ARNTL2* gene has been associated with a number of human diseases. *ARNTL2* is a candidate for many human brain disorders including anxiety disorders [36], Parkinson disease [37], alcohol use disorders [38] and bipolar disorder [39]. The gene has been described as a candidate in cancer, for example kidney cancer [40], colorectal cancer [41] and HCC [28]. Finally, *ARNTL2* has also been described as being related to rheumatoid arthritis [42]. The study showed that throughout the *ex vivo* experiments *ARNTL2* and *NPAS2* were the most affected clock genes in human immune-inflammatory conditions and that the molecular machinery controlling the circadian rhythm is disturbed in rheumatoid arthritis patients.

Arntl2 is a Candidate Gene for Type 1 Diabetes

The *Arntl2* gene has been discovered as a candidate gene for type 1 diabetes within the *Idd6* locus of the non-obese diabetic (NOD) mouse [43]. The gene is downregulated in NOD mice compared to that of other non-diabetic mouse strains, and numerous polymorphisms between these strains have been discovered [44]. Further functional studies confirmed the candidate gene and showed that ARNTL2 controls proliferation of peripheral CD4(+) T cells and diabetes development [45,46]. It has also been shown that ARNTL2 binds to the promoter of the interleukin-21 (*Il-21*) gene, which itself controls the proliferation of immune cells [47]. *Il-21* is located in the type 1 diabetes locus *Idd3* [48]. The interaction is absent in the NOD mouse but present in non-diabetic mouse strains (C57BL/6, C3H/HeJ). This leads to an upregulation of *Il-21* in the NOD mouse. ARNTL2 appears to control *Il-21* expression without interaction with other circadian factors such as CLOCK or BMAL1 (Figure 1) [49]. These data point to a novel mechanism controlling type 1 diabetes development independent of other known regulatory pathways. Interestingly, *IL-21* has also been associated to human type 1 diabetes [50], and recent findings confirmed that IL-21 producing cells [51] are increased in type 1

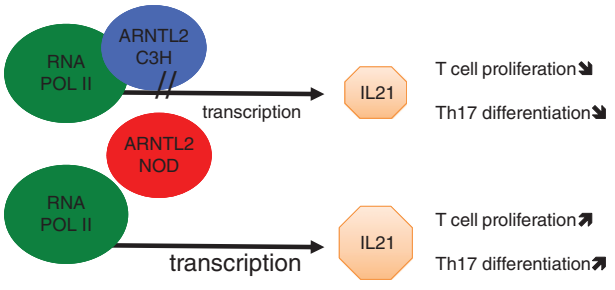

Figure 1. Current model for the *Il-21* regulation by ARNTL2. ARNTL2 expressed from C3H alleles can bind to the *Il-21* promoter and inhibits transcription but not RNA polymerase II binding. The NOD-derived protein cannot bind, leading to high transcriptional levels of *Il-21*, increased numbers of IL-21 producing cells, and finally to increased numbers of T cells and Th17 cells.

diabetes patients. These data provide some indications that the ARNTL2 pathway could be conserved between the two species.

> "The *Arntl2* gene has been discovered as a candidate gene for type 1 diabetes within the *Idd6* locus of the non-obese diabetic (NOD) mouse. ARNTL2 regulates interleukin-21 transcription in $CD4^+$ T cells. IL-21 is a cytokine involved in type 1 diabetes development."

Conflict of Interest

The authors declare no conflicts of interest.

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