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Circadian proteins and reproduction: An update

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Abstract

Endogenous circadian rhythms play an important role in effective functioning of complex organisms to enhance their survival. The master clock or suprachiasmatic nucleus in the ventral hypothalamus helps synchronize all independent peripheral clocks for temporal regulation of all physiological activities. The important clock proteins are Circadian Locomotor Output Cycle Kaput (CLOCK), Brain-Muscle-Arnt-Like protein 1 (BMAL1), Cryptochrome (CRY) and Period (PER). These proteins have a role in reproduction and functioning of gonads. The expression of these proteins is controlled by a histone deacetylase Sirtuin1 (SIRT1). The presence, role and functioning of these compounds in the reproductive tissues is discussed.

INTRODUCTION

Survival of an organism depends on two factors i.e. food availability and its reproductive ability. For effective reproduction, the organism should respond to the environmental cues appropriately. Animals inhabiting outside the tropics, adapt to seasonal changes in environment by altering their physiology and behavior including reproduction, migration, hibernation, molting, immune function and body weight. Seasonal regulation of reproductive activity, in particular is important to ensure that the offspring have sufficient food for survival. Though temperature, rainfall and humidity change during the four seasons, change in day length (photoperiod) has been used as the initial predictive factor for seasonal time measurement in many organisms, including plants, insects and vertebrates [1]. Circadian rhythms are endogenous biological rhythms with periods near 24 hours which play a fundamental role in the effective functioning of complex organisms by allowing the body to anticipate changing environments in ways that enhance survival [2, 3]. One of the most obvious functions of circadian rhythms is to provide an organism with the sense of time of day to ensure that physiological and behavioural events coincide [4]. The circadian clock is cell-autonomous and self-sustained by an elaborate cooperation of genetic components, but it is also entrainable by external time cues, called '*zeitgebers*' meaning 'time givers'. The suprachiasmatic nucleus (SCN) located in the ventral hypothalamus serves as the central pacemaker, which is known as the 'central or master clock' in mammals. Peripheral tissues also contain independent clocks. The SCN receives the environmental time information in the form of light through retino-ganglionic pathway to adjust or entrain its phase and then synchronizes other clocks to exhibit overt circadian rhythms such as the rest-activity cycle, periodic daily variations in metabolism, body temperature and the rhythmic regulation of endocrine axes [5, 6].

The molecular circuitry of circadian clock

The self-sustainable and autonomous function of circadian timing system depends upon a molecular mechanism. The endogenous circadian rhythms are controlled by different genes via transcription-translation auto-regulatory feedback loops. Some of these genes are Neuronal PAS (*Period-Arntl-Sim*) domain protein 2 (*Npas2*), Casein kinase1e (*Csnk1e*), Cryptochrome(*Cry1, Cry2*), Period (*Per1, Per2, Per3*), Clock homologue (mouse) (*Clock;* Circadian Locomotor Output Cycle Kaput), and Aryl hydrocarbon Receptor Nuclear Translocator-like (*Arntl*) [7-9].

These proteins include Circadian Locomotor Output Cycle Kaput (CLOCK) and Brain-Muscle-Arnt-Like protein 1 (BMAL1). These two proteins are core circadian proteins and belong to the basic Helix-Loop-HelixPeriod-ARNT-SIM (bHLHPAS) transcription factor superfamily. CLOCK and BMAL1 form heterodimers so as to recognize E-box elements in the cis-regulatory regions of their downstream genes so as to activate transcription of these targets [10-12]. The negative regulators of these genes i.e. PER (period) proteins (PER1, PER2, PER3) and CRY (cryptochrome) proteins (CRY1, CRY2) inhibit their own gene expression and that of other target genes by repressing the E-box-mediated transcriptional activity of the CLOCK/BMAL1 heterodimer and thus form the core feedback loop (Figure1).Such target genes include their negative regulators, such as the *Period* genes (*per1, per2, per3*) and *Cryptochromes* (*cry1* and *cry2*). During the day as in figure 1, the basic helix-loop-helix PAS-domain containing transcription factor CLOCK (or NPAS2) interact with BMAL1 to activate transcription of the *Per* and *Cry* genes, resulting in high levels of these transcripts. During the night, the PERCRY repressor complex is degraded, and CLOCKBMAL1 can then activate a new cycle of transcription [13].

The *Bmal1* activation and concentration is governed by ROR and REV-ERB proteins which are members of the *r*etinoic acid-related *o*rphan *r*eceptor (ROR) family with DNA binding domains that directly interact with ROR (RORE consensus sequence, A[A/T]NT[A/G]GGTCA, where N is any nucleotide) elements [13,14]. The expression of these nuclear receptors is directed by CLOCK/BMAL1 heterodimer and REV-ERBs and RORs subsequently compete with each other to bind the ROR-responsive elements (ROREs) present in the 5' flanking region of the *Bmal1* gene. The function of ROR α and REV-ERB α/β is opposite to each other i.e. ROR α induces *Bmal1* expression while REV-ERB α/β suppresses it, thus regulating the cyclic expression of *Bmal1* [13, 15].

Post-translational modification and degradation of circadian

clock proteins are needed and crucial steps for determining circadian periodicity of the clock. PER1 and PER2 proteins are progressively phosphorylated as they accumulate during the late afternoon and night. *Casein kinase 1 delta* (CSNK1δ) and CSNK1ε are involved in PER and CRY phosphorylation [16]. One of the roles for phosphorylation of clock proteins is to target them for polyubiquitylation and degradation by the 26S proteosomal pathway.

Circadian transcription of *Clock Bmal1* target genes is accompanied by circadian changes in histone H3 acetylation and chromatin remodeling [17].

The CLOCK protein has been shown to possess histone acetyltransferase activity and can acetylate its partner, BMAL1, on lysine 537 [18, 19]. Similar to other HATs it acetylates other proteins [20], in particular its own dimerization partner BMAL1 [19]. Thus CLOCK establishes connections with various metabolic pathways. This shows that CLOCK serves as a link between epigenetic control and circadian clock.

Circadian rhythms and SIRT1

The metabolic control in an organism is related to the circadian rhythm. This relationship between metabolic control and circadian rhythm is dependent on the transcriptional regulation of clock-controlled genes (CCGs) i.e. *per* and *cry* by the clock machinery. Histone deacetylase (HDAC) mediated deacetylation of histones results in gene silencing. Deacetylation of the histones leads to chromatin condensation and silenced gene expression [21, 22]. Out of four classes of mammalian HDACs, the third class comprises of seven mammalian enzymes which are homologs of yeast *sir2* (silencing information regulator) and are known as SIRT1 to SIRT7 [23, 24].

The histone deacetylase *sirtuin1* (SIRT1), a nicotinamide adenine dinucleotide (NAD^+)-dependent *sirtuin* affects clock gene expression by interacting with CLOCK protein and then deacetylating lysine 537 of BMAL1 as well as lysine 9 and 14 of histone H3 [25]. SIRT1 has also been found to be required for transcription of several core clock genes, including *Bmal*1, *Ror*γ, *Per*2, and *Cry*1 [26]. A couple of recent studies have shown that intracellular NAD^+ levels show circadian oscillations driven by the circadian clock [27, 28].

SIRT1 has been found to be required for testosterone synthesis and deactivation of SIRT1 reduces LH, FSH secretion [29]. Thus circadian physiology involves the complex program of gene expression which is responsible for chromatin transitions. These remodeling events ensure proper timing and extent of circadian regulation. SIRT1 appears to modulate circadian gene expression by repressing transcription through its HDAC activity in a timedependent manner, presumably by enhancing localized chromatin condensation [25]. Also, SIRT1 binds CLOCK-BMAL1 in a circadian manner and promotes the deacetylation and degradation of PER2 [26].

Circadian Proteins and SIRT1 in Testis

Report of clock genes is a recent development and first clock gene to be reported in testis was *Per1* followed by*Per2*, *Clock*, *Per3*, *Cry1* and *Cry2* [30-35].

It has been found that *Per1* gene expression in testis is not driven by CLOCK/BMAL1 dimers and suggested that the testis might represent the first described circadian clock free tissue in mammals [36]. They also demonstrated that circadian clock genes are rhythmically expressed in mammalian testis and in some accessary reproductive tissues. The clock gene mRNA but not the protein has been found to oscillate in the testis and extratesticular tissues [37]. No alteration in testicular expression of *Cry1*and *Per1* mRNA was found in *Cry2* knockout mice [35]. At first it was suggested that the *mPer1* and *mPer2* may be expressed rhythmically in testes [33]. But then subsequent studies have shown that expression of *mPer1*, *mCry1* and *Clock* genes in testes is non-rhythmic but depends on the developmental stages [35-36, 38-39]. In *Bmal1* knockout mice the reduction in sperm count, shrunken seminal vesicles and decreased serum testosterone concentration has been observed [40]. *StAR (Steroidogenic Acute Regulatory)* protein expression in the testis was also dramatically reduced in *Bmal1* KO testis as compared with wild-type littermates [40].

It has previously been shown that SIRT1 is expressed in male germ cells, principally in spermatogonial stem cells and round spermatids [41]. One study suggested that SIRT1 regulates spermatogenesis at postnatal stages by controlling hypothalamus-pituitary gonadotropin (HPG) signaling [29]. They found that spermatogenesis was arrested in late-meiotic prophase with degenerating or dying spermatocytes. The reduced expression of *StAR* protein in SIRT1 mutant mice was also observed. They suggested that SIRT1 is required for post natal testis development. In vitro studies have suggested that SIRT1 induces specific deacetylation of BMAL1. SIRT1 controlled acetylation thus may constitute a critical regulatory step in the control of BMAL1 protein stability [25-26]. SIRT1 has also been found to regulate the deacetylation of PER2. In the absence of SIRT1, high protein levels of PER2 may lead to the repression of *Per1*, *Per2*, *Cry1* mRNA expression. Thus, SIRT1 acts as a regulator of circadian gene expression in gene specific manner [26].

Circadian Proteinsand SIRT1 in Ovaries:

The earliest studies suggested the noncyclic expression of *Per* and cytoplasmic localization of PER protein in the *Drosophila* ovary [42-44]. Further studies on rat ovaries suggested the rhythmic expression of *Per1* and *Per2* with *Per1* reaching its peak at ZT12014 whereas *Per2* at ZT 17-18 [45]. In a study on mice, it was found that *Arntl* transcripts were elevated in granulosa cells, and particularly in theca layers of growing and antral follicles at ZT 0 as compared to ZT 15. *Per2* transcripts were higher in granulosa and theca cells of growing follicles at ZT 15 [46]. They found that Rhythmic expression patterns of *Arntl* and *Per2* transcripts and protein products were out of phase with respect to SCN suggesting that a molecular clock is present in the ovary like other peripheral oscillators. The expression of *Arntl* was significantly increased after the LH surge on the day of proestrus. This study suggested the presence of LH sensitive ovarian oscillators.LH and FSH have been found to act as potential endocrine signals to entrain the ovarian clock. LH and FSH were found to cause large shifts in phase of *Per1-Luc* when applied to cultured granulosa cells [47]. Clock gene expression has also been described in ruminants, Japanese quail and domestic hens with the rhythmic expression of *Per2* and *Per3* mRNA has been observed in granulosa and theca cells of quail ovary [48-50].

Bmal1$^{-/-}$ female mice were found to exhibit prolonged estrous cycle and they were infertile when mated to *Bmal1*$^{+/+}$ males. The progesterone hormone secretion as well as *StAR* protein expression was decreased in *Bmal1*$^{-/-}$mice at d 3.5 [51]. A reduced ovary weight in *Bmal1* null mice as well as decreased progesterone secretion in non-cyclic *Bmal1* null mice was

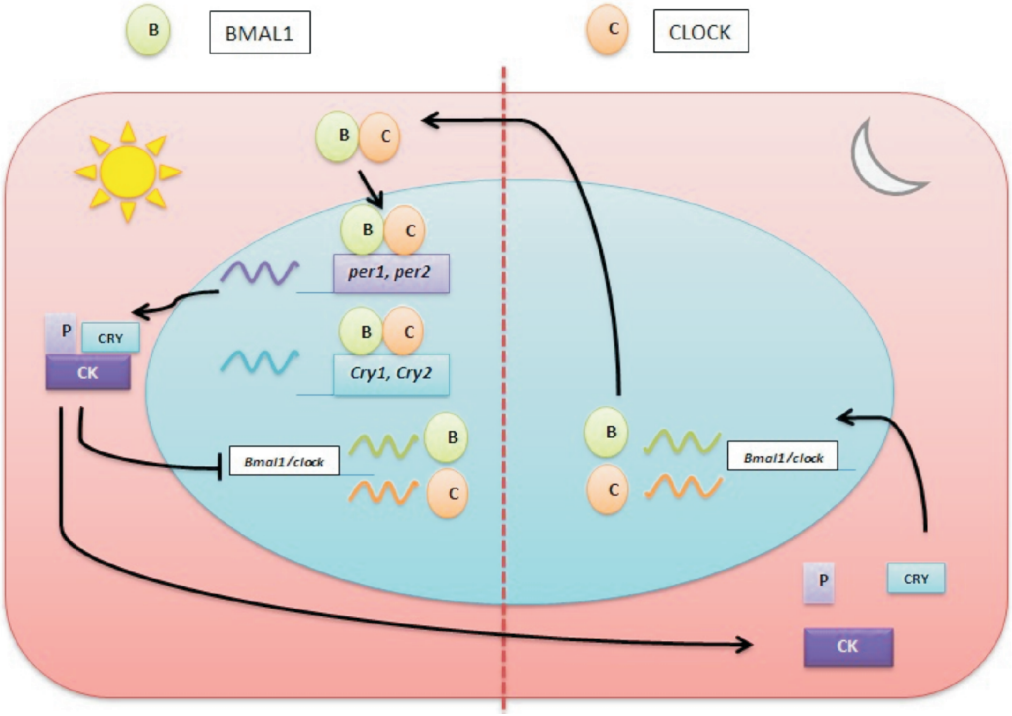

Fig. 1 The figure shows the molecular framework of the circadian clock. During day time the BMAL1 (B) and CLOCK (C) heterodimer in nucleus binds to the E-boxes of Per and Cry genes, thus regulating their expression. The PER (P) and CRY heterodimer in the cytoplasm binds to the Casein Kinase δ/□ (CK) and as the concentration of PER and CRY reaches at their optimum the PER, CRY and CK heterotrimer blocks the expression of BMAL1 and CLOCK proteins. During night this heterotrimer is degraded unblocking Bmal1 and Clock gene expression.

observed. Beside this a reduced number of follicles and corpora lutea per section at estrous and metestrouswere found. Non-cycling *Bmal1* null mice had ovarian morphology similar to mice in the estrus-to-metestrus transition, showing large antral follicles, in conjunction with reduced luteal and increased stromal tissue [52].

Rat oviduct has also been found to express clock genes rhythmically with a putative clock controlled gene, plasminogen activator inhibitor 1 which has been implicated in embryo development [53]. The non-pregnant and pregnant mice uterus also expresses clock genes rhythmically [54-55]. Rat placenta of mid and late gestation was also found to express *Per1* mRNA in all the layers, although only the maternally derived decidua expressed this transcript rhythmically [56].

The presence of SIRT1 protein has been reported in granulosa cells in human ovary [57]. Resveratrol a phytoestrogen known to bind equally to estrogen receptors α and β, and structurally similar to synthetic estrogens, such as DES and 17 β-estradiol benzoate has been found to cause an increase in SIRT1 mRNA levels as well as the stimulation of the deacetylating function of SIRT1 [57-59]. The role of SIRT1 in promotion of luteinization of the ovary has also been suggested and it wasfound that SIRT1 inhibition in human granulosa cells results in impaired cell-cycle progression (figure 2) [57, 60]. It might be speculated from these studies that the hormonal stimulation affects the expression of SIRT1 in circadian fashion and thus, regulates the luteinization.

Circadian proteins, SIRT1 and sex hormones

The female rats have been found to show rhythmicity in the plasma corticosterone levels during each day of the estrous cycle, with maximum values around 08:00 pm. The preovulatory luteinizing hormone (LH) surge was also observed at 08:00 pm on the day of proestrous [61]. In *Bmal1* null mice the decreased secretion of progesterone has been reported but the level of estradiol was found to be normal [51]. Wild-type mice of 6 months age were reported to have normal estrus cycle, *Bmal1* null mice were acyclic and had significantly lower progesterone levels than the wild-type mice [52]. LH surge causes ovulation and it occurs on the afternoon of proestrus, when a timed circadian signal manifests concomitant with an appropriate steroidal environment [62]. The daily expression of an LH surge in ovariectomized animals with chronic estrogen replacement shows a circadian rhythmicity in this phenomenon [62].

LH has been found to play a role in regulating the expression of *Arntl*. The increased expression of *Arntl*at ZT18 on the day of proestrus suggests that LH regulates the expression of this transcript. hCG treatment in juvenile primed with eCG resulted in the altered expression of *Arntl* and *Per2* as compared to the control animals treated with eCG alone [46].

In mice uterus estrogen has been found to have marked effects on the rhythm of PER2 expression as in uterine explants from *Per2::Luc*mice E_2 shortened the period of *Per2::Luc*expression [63]. *Per2* has been suggested to link the circadian cycle to the estrogen receptor ERα signaling network. Binding of PER2 enhances ERα degradation, while suppression ofPER2 levels leads to ERα stabilization [64].

SIRT1 has been found to be required for testosterone synthesis and deactivation of SIRT1 reduces LH, FSH secretion [29]. Thus circadian physiology involves the complex program of

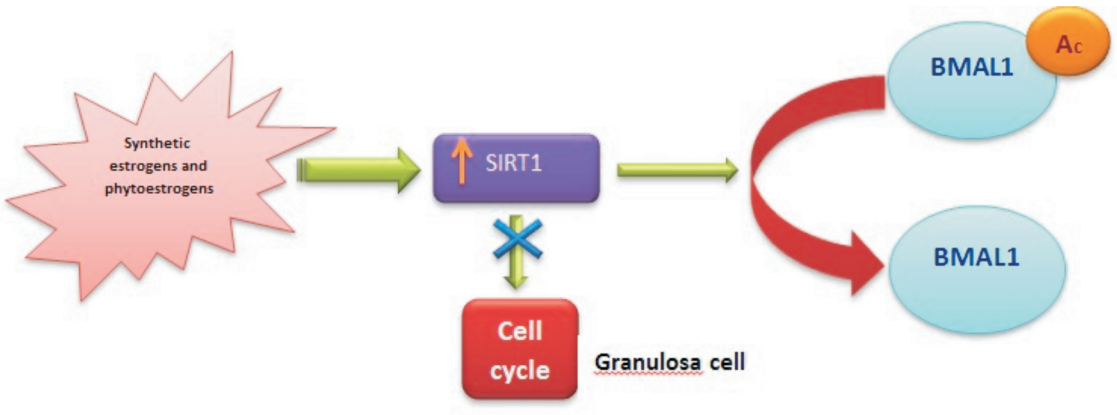

Fig.2 Synthetic estrogens or phytoestrogens results in the increased SIRT1 expression leading to increased deacetylating function of SIRT1. Also in human granulosa cells the inhibition of SIRT1 results in inhibition of cell cycle.

gene expression which is responsible for chromatin transitions. These remodeling events ensure proper timing and extent of circadian regulation.SIRT1 inactivation in mice leads to a significant 2-fold reduction in hypothalamic GnRH expression and FSH levels, and leads to an almost complete loss of circulating LH [29].

Clock disruption and reproduction

Per: Disruption in *per* gene decreases male fecundity in *Drosophila melanogaster.* The number of spermatozoa released after 2 days of adult emergence in *D. melanogaster* was found to be significantly lower in *per* mutants [65]. Also, reduced fertility was found in *per1*$^{-/-}$ and *per2*$^{-/-}$ middle aged female mice.Though a normal trend in pregnancy was observed in both *per1*$^{-/-}$, *per2*$^{-/-}$ and control mice, but successful parturition and weaning of pups was reduced in mutant mice [66]. In *Per1*KO mice, *StAR* expression has been found to be increased but in *Per2* KO mice and*Per1* KO/*Per2*KO mice, *StAR* expression has been found to be decreased.

***Cry*:** The *Cry1* knockout mice exhibits free running activity in constant darkness with an activity period of less than 1 hour [67]. Also, the *Cry1*/*Cry2* double knockout shows a loss of rhythmic *per1* mRNA expressionin the SCN. Expression of any clock genes such as *dec2* and *per1* in liver were found to be arrhythmic [68].

***Bmal1*:** In *Bmal1* null mice at the age of 2 months a decreased ductal length during mammary gland development was observed. Decreased ovarian weight, reduced progesterone secretionacross the estrous cycle, decreased number of follicles, corpora lutea and reduction in the number of pups delivered was observed in *Bmal1* null mice [40, 51-52]. Abnormal steroidogenesis with decreased progesterone secretion has been observed both in *Bmal1*$^{-/-}$ males as well as in females [51]. Though the ova produced by these animals were viable but the incidence of implantation failure was more in*Bmal1*$^{-/-}$ mice [51]. In *Bmal1*$^{-/-}$ mice testes a significantly reduced level of testosterone and follicle-stimulating hormone (FSH) with reduced steroidogenic enzymes (3-β-hydroxysteroid dehydrogenase, 17-α-hydroxylase) and steroidogenic acute regulatory protein (*StAR*) has been found [40].

***Clock*:** The first study on animals with genetically altered rhythmicity was conducted on *Clock* mutant mice [67]. In *Clock* mutants the levels of estradiol and progesterone at full term pregnancy were reduced than wild type. The level of progesterone was also reduced. High rate of fetal reabsorption and pregnancy failure was found in *Clock* mutant mice. Many studies suggest that *Clock* gene disruption affects fertility [52, 69-71].Animals having *Clock*$^{\Delta 19}$ *mutation exhibited prolonged estrous cycle* [70-71].

CONCLUSION

This review has focused on the epigenetic control of circadian proteins and their role in mammalian reproduction with special emphasis in gonads. The rhythmic expression of circadian proteins during different developmental stages as well as various phases of an organism's reproductive cycle has been discussed in detail. SIRT1 has been found to regulate metabolic functions via regulating circadian gene expression. Many researchers have identified the metabolic testicular functions of SIRT1 via regulating circadian gene expression, but scant reports are available in ovarian metabolism. SIRT1 regulated circadian rhythmicity in reproduction both in males and females shows the involvement of chromatin remodeling machinery. Though a great deal of research is available on circadian rhythmicity in female reproductive system, investigations on the interaction of SIRT1 with circadian ovarian clock are needed. Further studies are required to decipher the epigenetic control on clock machinery involved in ovarian physiology, primarily oocyte maturation and ovulation, so as to mitigate the reproductive failures and improve reproductive fitness to increase livestock production. This would help in sustainable biodiversity and much required to ensure basic parameters of livelihood security.

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