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Evidence for circadian rhythms in human trophoblast cell line that persist in hypoxia

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ABSTRACT

Circadian clock governs daily rhythmicity of a number of physiological processes such as reproductive functions. The existence of circadian clocks in the placenta is not clearly established. In order to investigate whether human placenta may function as circadian oscillator, we utilized HTR-8/SVneo cells derived from human first-trimester trophoblast. In serum-shocked cells we found circadian expressions for the clock genes *Per2* and *Dec1* as well as for *Dbp*, a canonical clock-controlled gene. We obtained similar results for *Vegf*, a circadian output involved in the control of placental vasculogenesis and trophoblast functions. Interestingly, circadian oscillations persisted and even enhanced in cells experimentally rendered hypoxic with $CoCl_2$. These results could be explained since the hypoxic milieu of the first-trimester placenta is considered the optimal condition for normal placentation. These data collectively support a possible role for the differential rhythmic expression of *Vegf*, influenced by circadian clock, in the adjustment of placental vascularization and trophoblast functions to the specific requirements of the different gestational ages.

Circadian clock governs daily rhythmicity of a number of physiological processes. The basic circadian molecular clockworks consist of interacting transcriptional/translational feedback loops [1]. Briefly, a positive loop includes CLOCK-BMAL heterodimers, transcriptional activators that bind to E-box elements located in the promoter of negative elements such as *Period* (*Pers*), *Cryptochrome* (*Crys*), and *Differentiated embryo-chondrocyte* (*Decs*) genes [1,2]. The negative limb of the feedback loop represses their own transcription by inhibiting CLOCK-BMAL activity. Oscillations in gene expression resulting from these feedback loops have period lengths of approximately 24 h, thus giving rise to overt circadian rhythms [1].

In mammals, a master clock generating circadian rhythms is located in the suprachiasmatic nucleus of the hypothalamus. Circadian oscillators have been also identified in several peripheral tissues and cell lines [1]. In the periphery, circadian clock may control different processes such as hormone secretion, timing of cell division and differentiation during development [3].

Animal reproduction is deeply affected by the circadian clock. A support to circadian rhythm influences on reproductive functions comes from studies on the reproductive biology of animals with a disrupted circadian timing system [4,5]. However, the mechanism underlying the temporal control of reproduction has not yet been completely explained. Hormonal activity may be involved, since a circadian control of reproductive hormone levels has been evidenced in animals and humans [4,6].

The presence of clock and clock-controlled genes rhythmically expressed over 24 h has been demonstrated in mammalian reproductive tissues, such as oviducts and uterus [4,7]. In mice testis instead clock genes have been evidenced, but their inability to cycle suggests that clock proteins have not circadian functions in spermatogenesis [8].

A fetus biological clock entrainable by the mother has been evidenced several years ago [9]; afterwards a rhythmic expression of clock and clock-controlled genes has been demonstrated in rat SCN during fetal life [10–12]. Recently clock genes rhythmically expressed have been identified in non-human primate fetus [13]. Moreover, an interplay between maternal and fetal rhythms via the placenta has been demonstrated. Disruption of fetal-maternal interaction during gestation leads to several disturbances among which a too short or too long gestational period [14].

A circadian clock in the placenta is not yet demonstrated, although its existence may be suggested by the observation that some placental hormones are released in a circadian fashion [15,16]. In order to investigate whether human placenta may function as circadian oscillator, we utilized cells derived from the trophoblast, i.e. the main component of placenta contributing to the formation of chorionic villi that reach their final organization at the end of first trimester of pregnancy. There are two sets of chorionic villi, the floating villi and the anchoring villi, in which an in-

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ner proliferative cytotrophoblast (CT) and an outer non-proliferative syncytiotrophoblast (ST) layers are observed. Floating villi are devoted to fetal-maternal exchanges, whereas anchoring villi serve to maintain the attachment of the placenta to the uterine wall by means of CT cell columns separated from their basement membrane. Extravillous trophoblast (EVT) cells, arising from the tips of columns, migrate and invade the uterine wall: some remain dispersed in the decidua as interstitial trophoblasts, some fuse to form placental bed giant cells, and others invade the endometrial as well as a myometrial portion of the maternal spiral arterioles [17]. EVT cell functions are tightly regulated by multiple factors among which hormones, local messengers [18,19] and hypoxia, the physiological milieu of the first ten weeks of pregnancy. Indeed hypoxia is essential for blastocyst implantation and for the correct EVT cell proliferation and differentiation along an invasive phenotype needed for a successful human pregnancy [20].

Aim of the present study was to verify whether clock genes are rhythmically expressed in HTR-8/SVneo cells, a well characterized model of human first-trimester EVT. Moreover, in order to evaluate the influences of oxygen levels, we compared temporal clock and clock-controlled gene expression in normoxic conditions with those observed in chemically induced hypoxia.

Materials and methods

Cell culture and serum-shock procedures. The HTR-8/SVneo trophoblast cell line was kindly provided by Dr. CH Graham, Queen's University, Kingston, Ontario (Canada). Cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Milan, Italy) at 37 °C in 5% CO_2. For extraction of cellular total RNA, cells were seeded in 24-well plates to reach confluence after about 2 days. Serum-shock was performed as previously described [21]. For mimicking hypoxia, 200 μM $CoCl_2$ was added from 0 to 54 h after serum-shock [22]. At various times indicated in the Figure legends, cells were harvested in 250 μl of TRIzol reagent (Invitrogen, Milan, Italy), frozen and stored at −80 °C. RNA was later isolated according to the manufacturer's protocol. RNA isolation has been repeated in three independent experiments.

qRT-PCR. Total RNA (1 μg) from cells was used to perform cDNA synthesis (iScript™ cDNA synthesis kit, Biorad, Milan, Italy). Aliquots of diluted first-strand cDNA was PCR amplified with a Chromo4 Real-Time PCR Detection System (Biorad, Milan, Italy) using iQ™ SYBR® Green Supermix (Biorad), according to the manufacturer's recommendations. The following primers were used: *Per2*, 5′-GGCTTCACCATGCCTGTTGT-3′ and 5′-GGAGTTATTTCGGAGGCA AGTGT-3′; *D-albumin site binding protein (Dbp)*, 5′-GTTGATGA CCTTTGAACCCGA-3′ and 5′- CCTCCGGCACCTGGATTTTT-3′; *Dec1*, 5′-GAAAGGATCGGCGCAATTAA-3′ and 5′-CATCATCCGAAAGCTGC ATC-3′; *Vascular endothelial growth factor (Vegf)*, 5′-TGCAGATT ATGCGGATCAAACC-3′ and 5′-TGCATTCACATTTGTTGTGCTGTAG-3′. The relative levels of each RNA were calculated by the $2^{-\Delta\Delta CT}$ method (CT standing for the cycle number at which the signal reaches the threshold of detection); 18s rRNA (5′-GTAAC CCGTTGAACCCCATT-3′ and 5′-CCATCCAATCGGTAGTAGCG-3′) was used as housekeeping gene. Each CT value used for these calculations is the mean of three replicates of the same reaction. To ascertain that the apparent rhythmic expression of clock- and clock-controlled genes mRNA was not an artefacts of normalizing to *18s* housekeeping gene, control experiment was conducted normalizing with another reference gene, i.e. *Tbp* (5′-CACGA ACCACGGCACTGATT-3′ and 5′-TTTTCTTGCTGCCAGTCTGGAC-3′). Nearly identical circadian rhythms were observed in normoxia and hypoxia when clock- and clock- controlled genes transcript levels were normalized to *18s* rRNA or *Tbp* mRNA.

Statistical analysis. All the results were expressed as means ± SEM. Treatments, sampling time and their interaction were tested by one- or two-way ANOVA. *P*-values <0.05 were considered statistically significant. Bonferroni's test was applied for post hoc comparison. Data were analyzed using the software GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA). To evaluate the period length of genes' expression, we measured the time span between two consecutive peaks. To compare overall levels of genes' expression in the two different treatments, mean expression levels were calculated.

Results and discussion

EVT cells exert a crucial role during human placentation and they have to proliferate and invade uterine wall in tightly regulated spatially and temporally manner for a successfully pregnancy [17]. Considering the existence of a cross-talk between cell proliferation and circadian system [23], we have hypothesized that trophoblast cells may be a useful model to study the circadian clock of the placenta. Taking into account the difficulty to obtain first-trimester human trophoblast primary cultures, we utilized the HTR-8/SVneo cell line, that derives from human first-trimester EVT [24] and represents a highly useful model to study the mechanisms that regulate normal human trophoblast cell growth as well as the control of EVT cell proliferation, migration and invasiveness operated by several modulators [18,19,25]. These cells were treated with serum-rich medium to verify the presence of circadian oscillators. Previous studies demonstrated that serum-shock synchronizes quiescent cell culture [21,26]. Using qRT-PCR approach, we investigated the expression of the clock genes *Per2* and *Dec1* as well as of *Dbp*, a canonical clock-controlled gene. Moreover, we analyzed the expression of *Vegf*, a circadian output [27] involved in both placental vasculogenesis and angiogenesis [28], as well as in trophoblast function regulation [29,30].

Results obtained showed that *Per2* and *Dec1* mRNAs were rhythmically expressed in HTR-8/SVneo cells treated with serum-shock (Fig. 1; $p < 0.0001$; one-way ANOVA) with period length close to the 24 h. Rhythmic expressions were also found for *Dbp* and *Vegf* (Fig. 1; $p < 0.0001$; one-way ANOVA). The period length of *Dbp* mRNA oscillation was close to 20 h, whereas *Vegf* displayed a temporal fluctuation only 30 h after serum shock. The phase relationships between the circadian gene expression rhythms were in accordance with those observed in other mammalian cell lines [27,31].

Physiologically, EVT cell proliferation and differentiation along an invasive phenotype occur in a hypoxic environment [20]. It has been reported that before the 10th week of gestation, a time of maximal placental invasion of the uterus, oxygen levels in the placental tissue are much lower than in the surrounding endometrium. After the first trimester of pregnancy, when placental invasion is largely complete, oxygen concentrations increase. Low-oxygen environment of the placenta in the first trimester may provide the optimal condition for adequate trophoblast invasion and normal placentation [25].

Hypoxia has been shown to modify the expression of several genes that in turn are regulated by hypoxia inducible factor (HIF)-1, a master regulator of oxygen homeostasis. HIF-1, a basic helix-loop-helix PAS (bHLH-PAS) transcription factor, binds to HBS (HIF Binding Site, A/GCGTG) motifs identified in the promoter of hypoxia-induced genes such as *Vegf*. HIF-1 binds DNA as a heteromeric complex composed of two subunits, the constitutively expressed HIF-1β and the inducible HIF-1α [32]. HIF-1α is present at low oxygen tension, but is rapidly ubiquitinated and degraded by the proteasome under normoxic conditions. It has been reported that HIF-1α is expressed at high levels in placental trophoblast between 5th–8th week of pregnancy and dramatically decreases after 10–12 weeks, when O_2 levels increase [33]. These

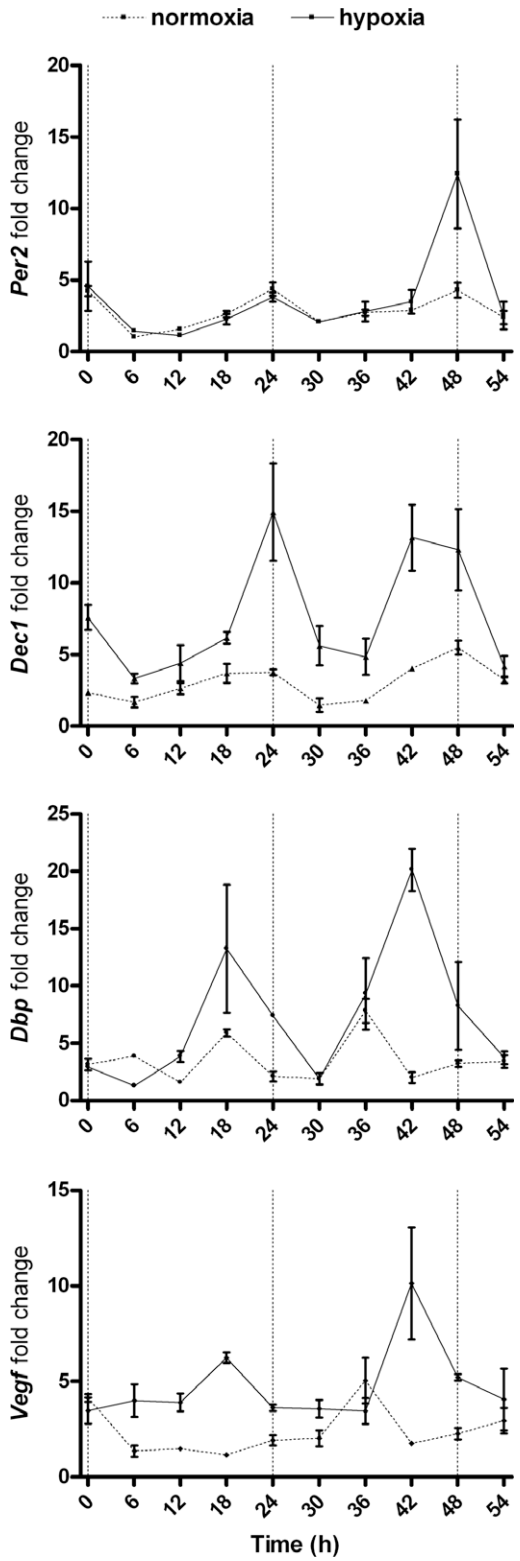

Fig. 1. Chemically-induced hypoxia enhances circadian gene expression in serum-shocked HTR-8/SVneo cells. mRNA levels of *Per2, Dec1, Dbp* and *Vegf* were determined using qRT-PCR in serum-shocked cells grown in the absence (dotted lines) or presence (solid lines) of 200 μM $CoCl_2$. For all genes, the constitutively expressed 18S rRNA was used to normalization. For each time point the mean ± SEM of three independent experiments is shown.

data prompted us to investigate the influence of hypoxia in our in vitro model. We mimicked hypoxic environment adding $CoCl_2$ to the culture medium of cells previously serum-shocked. $CoCl_2$ is a compound widely used to induce a hypoxia response in many cellular models, including HTR-8/SVneo cells [22,34].

Circadian oscillations persisted (*Per2*: $p < 0.002$; *Dec1*: $p < 0.004$; *Dbp*: $p < 0.003$; *Vegf*: $p < 0.0001$; one-way ANOVA, Fig. 1) in HTR-8/SVneo cells experimentally rendered hypoxic. For all genes the period length of mRNA expression was close to the 24 h. Moreover, gene expression levels in hypoxia displayed the same temporal profile and phase relationship that we observed in normoxic condition, except for *Vegf* mRNA (Fig. 1). *Vegf* circadian oscillation is already present during the first 30 h, probably due to the combined effects of both circadian and hypoxic systems.

The persistence of circadian gene expression in cells experimentally rendered hypoxic may be surprising, since it has not been shown in other cell types as fibroblasts [22]. This discrepancy could be justify considering that EVT cells share some properties with tumor cells [25] and that recent investigations reported mRNA circadian profiles of cardinal clock genes and outputs in hypoxic tumor cells [27,35].

We found interesting results comparing mean expression levels in both normoxic and hypoxic conditions. A strong increase (ranging from +80% to +150%) of mean expression levels for *Dec1*, *Dbp* and *Vegf* was clearly evident in cells chemically rendered hypoxic (*Dec1*: $p < 0.001$; *Dbp*: $p < 0.02$; *Vegf*: $p < 0.0004$; two-way ANOVA, Fig. 1). The effect of hypoxic treatment on *Per2* expression level started from the second circadian cycle around 36 h after serum-shock (+47%, Fig. 1). In fact for the first 24 h, *Per2* mRNA levels in normoxic and hypoxic milieu overlapped. *Per2* delay in response to $CoCl_2$ could be explained by the absence of hypoxia responsive elements (HBS) on its promoter. Conversely, *Dec1* and *Vegf*, that posses these elements [27,36], rapidly responded to the treatment (+158% and +113% in the first circadian cycle, respectively). The magnitude of *Dbp* rhythm increased in the first 24 h after serum-shock (+71%) most likely under the influence of both circadian and hypoxic pathways. Also in the second circadian cycle, the increase of mean expression level was marked for *Dbp*, *Dec1*, and *Vegf* (+135%, +159%, and +68%, respectively).

Here, we reported for the first time the existence of circadian oscillators in human EVT cell line. Interestingly, circadian rhythms are not only maintained but even significantly amplified in chemically induced hypoxia. In particular, we demonstrated an enhancement of *Vegf* rhythmic expression in hypoxic condition. An increase of *Vegf* mRNA expression in placental trophoblast under hypoxia was already demonstrated by Li et al. [37]. Although caution is needed when extrapolating results obtained in trophoblast-derived cell lines to normal trophoblast cells, we could hypothesize that during the first trimester of pregnancy when the oxygen levels are very low, the trophoblast circadian clockwork oscillates with a robust rhythm. Conversely, starting from the 10th week of gestation, when the increase of maternal blood flow allows the establishment of a normoxic placental environment, the circadian clock slows down. The consequent change of the amount and intensity of the *Vegf* stimulus may contribute to adjust placental vascularization and trophoblast functions to the specific requirements of the different gestational ages.

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