LEVELS OF MIR-374 INCREASE IN BEWO B30 CELLS EXPOSED TO HYPOXIA

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In humans, trophoblast hypoxia during placental development can be a cause of serious pregnancy complications, such as preeclampsia and fetal growth restriction. The pathogenesis of these conditions is not fully clear and may be associated with changed expression of some genes and regulatory molecules, including miRNA, in trophoblast cells. The aim of this study was to analyze miRNA profiles and measure the expression of their target genes in a model of trophoblast hypoxia. Human choriocarcinoma BeWo b30 cells were used as a trophoblast model. Hypoxia was induced by cobalt chloride ($CoCl_2$) and an oxyquinoline derivative. MRNA and miRNA expression profiles were evaluated by means of next generation sequencing (NGS); the expression of individual genes was analyzed by PCR. We studied the secondary structure of mRNAs of target genes for those miRNAs whose expression had changed significantly and analyzed potential competition between these miRNAs for the binding site. The observed changes in the expression of the key genes involved in the response to hypoxia confirmed the feasibility of using $CoCl_2$ and the oxyquinoline derivative as hypoxia inducers. The analysis revealed an increase in miR-374 levels following the activation of the hypoxia pathway in our trophoblast model. The changes were accompanied by a reduction in *FOXM1* mRNA expression; this mRNA is a target for hsa-miR-374a-5p and hsa-miR-374b-5p, which can compete with hsa-miR-21-5p for the binding sites on *FOXM1* mRNA. The involvement of *FOXM1* in the regulation of the invasive cell potential suggests the role of miR-374 and *FOXM1* in the pathogenesis of disrupted trophoblast invasion during placental development as predisposing for fetal growth restriction and preeclampsia.

Keywords: placenta, choriocarcinoma, BeWo, hypoxia, cobalt, oxyquinoline, *FOXM1*, microRNA, miR-374a-5p, miR-374b-5p

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УРОВЕНЬ микроРНК MIR-374 ПОВЫШАЕТСЯ В КЛЕТКАХ BEWO B30 ПРИ ГИПОКСИИ

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Воздействие гипоксии на клетки трофобласта в процессе формирования плаценты человека может приводить к развитию ряда патологий беременности, таких как преэклампсия и задержка роста плода. Патогенез данных состояний не до конца раскрыт и может быть связан с изменением экспрессии в трофобласте ряда генов и регуляторных молекул, включая микроРНК. Целью данного исследования было изучить профили микроРНК и экспрессии соответствующих генов-мишеней в модели гипоксии трофобласта. Моделирование трофобласта проводили с использованием клеточной линии хориокарциномы человека BeWo b30. В качестве индукторов гипоксического ответа использовали хлорид кобальта ($CoCl_2$) и производное оксихинолина. Анализировали уровень мРНК и микроРНК с помощью секвенирования следующего поколения (NGS) с подтверждением экспрессии отдельных генов ПЦР. Анализировали вторичную структуру мРНК гена-мишени значимо изменившихся микроРНК и возможную конкуренцию за места связывания. Изменение экспрессии ключевых генов ответа на гипоксию подтвердило релевантность $CoCl_2$ и производного оксихинолина в качестве индукторов гипоксии. Выявлено повышение уровня микроРНК семейства miR-374 при индукции гипоксического пути в модели трофобласта. Наблюдаемые изменения сопровождались снижением экспрессии мРНК гена *FOXM1*, которая служит мишенью для hsa-miR-374a-5p и hsa-miR-374b-5p. Данные микроРНК могут конкурировать за места связывания в мРНК *FOXM1* с hsa-miR-21-5p. Участие гена *FOXM1* в регуляции инвазивного потенциала клеток позволяет предположить роль микроРНК miR-374 и *FOXM1* в патогенезе нарушения инвазии трофобласта при формировании плаценты как предпосылки к развитию задержки роста плода и преэклампсии.

Ключевые слова: плацента, хориокарцинома, BeWo, гипоксия, кобальт, оксихинолин, *FOXM1*, микроРНК, miR-374a-5p, miR-374b-5p

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The maintenance of homeostasis during fetal development is essential for a healthy pregnancy. In pregnancy, trophoblast cells replace the endothelial lining of uterine spiral arteries, making them insensitive to systemic tone regulators and thus ensuring a constant blood flow to the placenta and the fetus. Inflammation and severe hypoxia impair trophoblast invasion [1], which is believed to be one of the major causes of preeclampsia, a severe pregnancy complication. MicroRNA (miRNA) secreted by trophoblast cells in the setting of hypoxia may be markers of impending preeclampsia and, at the same time, a pathogenic component of this condition due to their role as regulators of gene expression [2–4].

In vitro trophoblast models employ human choriocarcinoma cell lines, like BeWo b30: they are not susceptible to contact inhibition, can form confluent cell layers, have high motility [5], and therefore can be used as both villous or extravillous trophoblast models. The use of microfluidic platforms allows researchers to bring a trophoblast model closer to the actual conditions inside the organism [6, 7].

The activation of the hypoxia signaling pathway is associated with the transcription of hypoxia-inducible factor (HIF) targets. One of the classical ways to model chemical hypoxia is through exposure of the cell to cobalt (II) chloride, a compound that causes an elevation of HIF levels in the cell [8]. Derivatives of 8-oxyquinoline (OD), too, are capable of activating the hypoxia pathway by stabilizing HIF in the cell [9]. OD effects on BeWo b30 cells imitate the effects of hypoxia in the placental trophoblast [10].

The aim of this study was to analyze miRNA profiles and the expression of miRNA target genes in a model of trophoblast hypoxia.

METHODS

BeWo b30 cells were kindly provided by Prof. Dr. Christiane Albrecht (University of Bern, Switzerland) with permission from Prof. Dr. Alan Schwartz (Washington University in St. Louis, USA). The obtained cells were grown in 6-well plates, growth area 9.6 cm^2 (Corning; USA) in Gibco DMEM, high glucose (Thermo Fisher Scientific; USA) supplemented with 10% Gibco FBS One Shot (Thermo Fisher Scientific; USA), 1% Gibco MEM NEAA (100X) and 1% Gibco Pen Strep (100X). Upon reaching 80% confluence, the cells were transferred to a fresh medium that did not contain any chemical inducers of hypoxia or to a medium supplemented with 5 μM OD 4896-3212 (ChemRar High-Tech Center; Russia) or with 300 μM cobalt (II) chloride (Sigma-Aldrich; USA). After 24 h, the cells were lysed in a Qiazol Lysis Reagent (Qiagen; Germany), and RNA was extracted by means of phenol-chloroform extraction [11] using a miRNeasy Mini Kit (Qiagen; Germany). RNA concentrations were measured with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific; USA). RNA quality control was performed in an Experion automated electrophoresis system (Bio-Rad; USA). RNA Quality Indicator (RQI) was at least 9 for all the trialed samples.

Libraries for next generation sequencing (NGS) were prepared using an NEBNext Multiplex Small RNA Library Prep Kit for Illumina (New England Biolabs; USA) and Illumina Stranded mRNA Library Prep Kit (Illumina; USA). The libraries were then sequenced in a NextSeq 500 System (Illumina; USA).

To analyze the expression of individual genes, 500 ng total RNA was reverse-transcribed using an M-MLV RT Kit (Evrogen; Russia). The obtained DNA was amplified by real-time PCR using qPCRmix-HS SYBR reagents (Evrogen; Russia). Differences in mRNA and miRNA expression between BeWo b30 cultures exposed and unexposed to hypoxia inducers were analyzed using Student's *t*-test and the Benjamini–Hochberg procedure for multiple comparisons to control the False Discovery Rate (FDR). Differential expression analysis was conducted using DESeq2 v1.28.1 [12] Differences in the expression of highly represented miRNA and mRNA were considered significant at FDR-$p < 0.05$ and log2 fold change not less than 0.4 [13].

RESULTS

The analysis of publications investigating the effects of hypoxia on the transcriptome of various cells allowed to identify a number of key genes involved in the cell response to hypoxia [14]. The expression of these genes in BeWo b30 cells exposed to OD and $CoCl_2$ was estimated from NGS data (Table 1). The expression of all genes listed in Table 1 changed significantly ($p < 0.05$), except for the genes *CDKN1A* and *ENO1* in the cells exposed to OD and the genes *SLC2A1* and *TMEM45A* in the cells exposed to $CoCl_2$. Interestingly, the expression of the *EPO* gene, which encodes erythropoietin, decreased in BeWo b30 cells exposed to cobalt, whereas under true hypoxia with low oxygen levels its expression increases [14], just like in BeWo b30 cells exposed to OD.

To verify the results of sequencing and confirm the activation of key hypoxia-responsive genes, the expression of *BNIP3*, *SLC2A3*, *PDK1* and *VEGFA* in BeWo b30 cells was evaluated by PCR. *ACTB* and *GUSB* were used as reference genes. PCR confirmed that the expression of the listed genes had been activated (Table 2).

Among highly expressed miRNAs accounting for over 95% of all BeWo b30 miRNAs, those miRNAs were identified whose level had changed significantly in the setting of chemically induced hypoxia. Those included 7 miRNAs expressed in BeWo b30 cells exposed to OD (Table 3) and 16 miRNAs expressed in BeWo b30 cells exposed to $CoCl_2$ (Table 4); only 2 miRNAs (hsa-miR-374a-5p and hsa-miR-374b-5p) occurred in both lists.

We analyzed the expression of hsa-miR-374a-5p and hsa-miR-374b-5p targets in BeWo b30 cells exposed to OD and $CoCl_2$. An earlier study reports that hsa-miR-374b-5p can regulate *FOXM1* expression in SiHa cervical cancer cells [15]. In our study, *FOXM1* expression in BeWo b30 cells decreased significantly after exposure to OD and $CoCl_2$ (1.7 and 2.6-fold, respectively). Previously, it was shown that hsa-miR-21-5p can caused a decline in *FOXM1* expression [16]; so, we analyzed the seed regions of hsa-miR-21-5p, hsa-miR-374a-5p and hsa-miR-374b-5p, to reveal that each of these 3 miRNAs has only one binding site in the 3'-untranslated FOXM1 mRNA region (see the Figure).

DISCUSSION

Cell responses to hypoxia may vary, which shows in the activation of different genes and in the varying degrees of such activation. An earlier literature analysis identified a number of key genes activated in all cells exposed to hypoxia [14]. The expression of those genes was also elevated in our BeWo b30 cells exposed to OD and $CoCl_2$, suggesting the activation of the HIF pathway. There are reports that BeWo cells exposed to $CoCl_2$ overexpress glucose transporter 1 (GLUT1) encoded by the *SLC2A1* gene [17–19]. These reports are consistent with our findings. Interestingly, the expression of the *EPO* gene, which encodes erythropoietin, declined in BeWo b30 cells exposed to $CoCl_2$, whereas true hypoxia with low oxygen levels causes an increase in *EPO* expression [14], just like in

Table 1. Expression of key genes involved in response to hypoxia in BeWo b30 cells (based on sequencing data)

Gene	log_2 fold change	
	Exposure to OD	Exposure to $CoCl_2$
DDIT4	3.5	3.6
KDM3A	1.8	3.6
BNIP3	2.3	3.3
NDRG1	3.4	3.2
SLC2A3	3.1	2.8
BHLHE40	2.8	2.6
P4HA1	2.9	2.5
PDK1	1.7	2.3
ANKRD37	3.2	2
VEGFA	1.6	1.9
ERO1A	1.7	1.9
ALDOC	4.4	1.8
CDKN1A	0.6*	1.3
STC2	1.3	1.1
ENO1	0.6*	1
SLC2A1	1.4	0.7*
TMEM45A	2	0.1*
EPO	1.4	–1.5

Note: * — designates FDR-p > 0.05; for other genes, FDR-p < 0,05.

BeWo b30 cells exposed to OD. It was shown previously that HIF can directly increase *EPO* expression in BeWo cells [20]. In another study, the level of *EPO* expression in BeWo cells was undetectable, which did not allow the researchers to assess how $CoCl_2$ and hypoxia affected its expression [21]. Cobalt is known to stimulate erythropoietin expression in the kidneys [22]. *EPO* expression is controlled by HIF-2α, and though cobalt generally induces both HIF-1α, and HIF-2α, it did not affect *EPO* expression in liver cancer cell lines Huh7 and HepG2 [23]. Perhaps, the expression of this gene may be dependent not only on the activation of the HIF signaling pathway but also on other tissue-specific factors.

Exposure of trophoblast cells to hypoxia can induce the release of certain molecules, including hypoxia-associated miRNA [24]. MiRNA released by the cell can affect the neighboring cells; shifts in miRNA expression may determine the scope of miRNA effects. However, microRNA concentrations in the producing cell have to be sufficiently for such effects to occur. Therefore, we selected 10% of miRNAs that were present in the highest concentrations in the BeWo b30 culture and then identified those whose expression had changed significantly following BeWo b30 exposure to OD or $CoCl_2$. Interestingly, of all miRNAs whose expression had significantly changed after exposure to OD or $CoCl_2$ (7 and 16 miRNAs respectively) only 2 (hsa-miR-374a-5p and hsa-miR-374b-5p) responded with overexpression to both hypoxia inducers. These 2 miRNAs are encoded in the X-chromosome in the introns of the *FTX* gene that codes for the long non-coding RNA participating in the inactivation of the X-chromosome. The miR-374 family members participate in the regulation of cell

Table 2. Expression of key genes involved in response to hypoxia in BeWo b30 cells (based on PCR data)

Gene	Linear fold change*	
	Exposure to OD	Exposure to $CoCl_2$
BNIP3	3.7	4
SLC2A3	2.2	3.6
PDK1	1.7	2.4
VEGFA	1.6	3.1

Note: * — FDR-p < 0,05.

Table 3. MiRNA with significantly changed expression after exposure to OD

miRNA	log_2 fold change	FDR-p
hsa-miR-96-5p	↓0.5	1.8×10^{-3}
hsa-miR-21-5p	↑0.4	2.7×10^{-2}
hsa-miR-429	↑0.5	4.2×10^{-4}
hsa-miR-374b-5p	↑0.5	6.6×10^{-3}
hsa-miR-374a-5p	↑0.5	1.4×10^{-3}
hsa-miR-26b-5p	↑0.6	8.0×10^{-8}
hsa-miR-181a-2-3p	↑0.7	3.8×10^{-7}

Table 4. MiRNA with significantly changed expression after exposure to $CoCl_2$

miRNA	log_2 fold change	FDR-*p*
hsa-miR-1260b	↓0.7	9.8×10^{-9}
hsa-miR-4521	↓0.7	1.6×10^{-11}
hsa-miR-148a-3p	↓0.7	8.5×10^{-8}
hsa-miR-425-5p	↓0.5	4.1×10^{-12}
hsa-miR-378i	↓0.5	6.6×10^{-10}
hsa-miR-32-5p	↓0.4	2.5×10^{-4}
hsa-miR-151a-3p	↓0.4	2.1×10^{-5}
hsa-miR-200a-3p	↑0.4	2.5×10^{-5}
hsa-miR-126-3p	↑0.4	4.0×10^{-7}
hsa-miR-484	↑0.4	9.5×10^{-8}
hsa-miR-181a-5p	↑0.5	5.2×10^{-11}
hsa-miR-25-3p	↑0.5	7.8×10^{-11}
hsa-miR-27a-3p	↑0.5	1.2×10^{-12}
hsa-miR-320a-3p	↑0.5	7.0×10^{-11}
hsa-miR-374a-5p	↑0.6	6.1×10^{-6}
hsa-miR-374b-5p	↑0.6	5.1×10^{-7}

proliferation and differentiation, growth and carcinogenesis [25]. Hsa-miR-374a-5p and hsa-miR-374b-5p have a very similar sequence; the seed-regions of these molecules are the same, suggesting that they target the same genes. It was reported that hsa-miR-374a-5p concentrations were elevated in the blood of women who delivered prematurely [26] or gave birth to babies with small gestational weight [27]; this might indicate a potential association between hypoxia and placental pathology. Under hypoxic conditions, villous trophoblast changes its metabolism from aerobic to anaerobic; this reduces oxygen consumption but increases the need for glucose. As a result, the fetus receives more oxygen but less nutrition, which might lead to intrauterine growth restriction and premature delivery [28]. A similar effect was observed in BeWo b30 cells exposed to another OD [10].

FOXM1 was identified as an hsa-miR-374a-5p and hsa-miR-374b-5p gene target; this gene encodes a transcriptional factor. The levels of its mRNA in BeWo b30 cells declined following exposure to OD and $CoCl_2$. An earlier study demonstrated that hsa-miR-21-5p was capable of reducing *FOXM1* expression

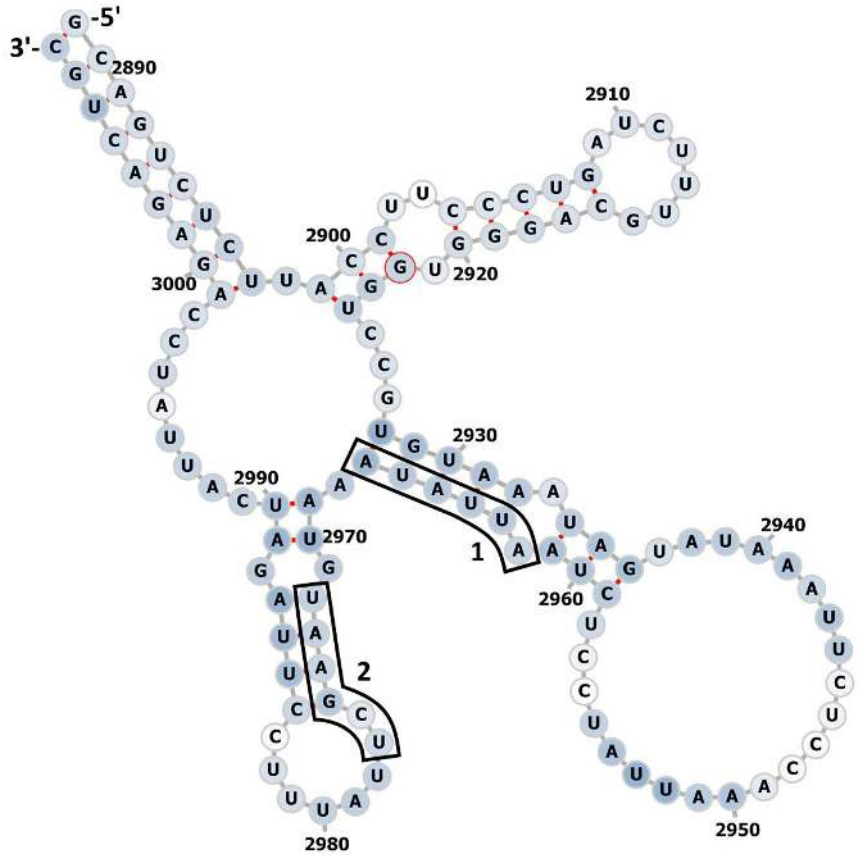

Fig. Positions of microRNA binding sites on a fragment of a 3'-untranslated region of *FOXM1* mRNA (transcription variant 2). Nucleotide numbers are represented by 4-digit numbers. 1 — binding sites for hsa-miR-374a-5p and hsa-miR-374b-5p; 2 — a binding site for hsa-miR-21-5p

and proliferation of HTR8/SVneo choriocarcinoma cells; in the setting of preeclampsia, hsa-miR-21-5p levels were increased and the expression of *FOXM1* was decreased in the placenta [16]. These reports are in good agreement with our findings. At the same time, a significant elevation of hsa-miR-21-5p concentrations in BeWo b30 cells was observed only after exposure to OD, as opposed to $CoCl_2$, suggesting the involvement of hsa-miR-374a-5p and hsa-miR-374b-5p in the regulation of *FOXM1* expression in our trophoblast model. According to the spatial principle, the proximity of binding sites implies competition between hsa-miR-21-5p and hsa-miR-374a-5p/hsa-miR-374b-5p, because in order for the complex of argonaute proteins with these miRNAs to interact with *FOXM1* mRNA, a significant steric strain is needed in the target mRNA sequence (see the Figure).

Impaired trophoblast invasion of the uterine wall and spiral arteries in the setting of preeclampsia might be explained by the reduced expression of *FOXM1* governed by hsa-miR-374b-5p, as was previously demonstrated for SiHa cervical cancer cells [15]. At < 3% oxygen level simulating physiological hypoxia, *FOXM1* expression in JEG-3 choriocarcinoma cells was initially high. But as the level of oxygen was falling, so was the expression of the gene. *FOXM1* knockdown suppressed JEG-3 cell migration, and the culture medium in which the cells had been grown inhibited angiogenesis in the culture of endothelial cells (HUVEC) [29].

CONCLUSIONS

Our study demonstrates that exposure of BeWo b30 cells to oxyquinoline derivatives and cobalt (II) chloride may be used as a trophoblast hypoxia model. This was confirmed by the activation of key hypoxia-responsive genes. At the same time, the response of BeWo b30 cells to hypoxia manifesting in the changed miRNA expression varied significantly depending on the compound used to induce hypoxia. Both cobalt and the oxyquinoline derivative caused an increase in the expression of miR-374, suggesting its participation in response to hypoxia. The reduced expression of *FOXM1*, the gene target for the miR-374 family, suggests the role of miR-374 and *FOXM1* in the pathogenesis of impaired trophoblast invasion during placental development as a prerequisite for intrauterine growth restriction and preeclampsia.

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