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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Factors Involved in miRNA Processing Change Its Expression Level during Imitation of Hypoxia in BeWo b30 Cells

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Abstract—One of the main complications of pregnancy and causes of maternal and perinatal mortality is preeclampsia. The pathophysiology of preeclampsia is associated with the development of placenta and fetal hypoxia and secretion of a number of effective molecules. The human choriocarcinoma cell line BeWo b30 is often used as a model of the placental barrier. It was shown that oxyquinoline derivatives can mimic hypoxia by suppressing HIF-prolyl hydroxylases and the accumulation of HIF-1α. This effect also leads to a change in the expression of microRNAs and their target genes. However, with hypoxia in cells, not only the level of individual miRNAs but also the ratio of miRNA isoforms (isomiRs) can change, presumably due to inaccuracies in the work of the Drosha and Dicer enzymes. In this work, we showed a change in the expression of the factors involved in the maturation of miRNAs when simulating hypoxia in BeWo b30 cells with an oxyquinoline derivative, which may be one of the causes for the change in the ratio of miRNA isoforms.

Keywords: Drosha, Dicer, DGCR8, TARBP2, placenta, choriocarcinoma, BeWo, miRNA, miRNA isoforms, isomiRs

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One of the main complications of pregnancy and the causes of maternal and perinatal mortality is preeclampsia, a pathology whose pathogenesis is associated with disturbance of the utero-placental blood flow and the development of placental and fetal hypoxia. According to one theory, placental hypoxia is the primary factor leading to the development of this disease: apparently, at the trophoblast hypoxia, various effector molecules that trigger pathological changes can be released into the bloodstream of the mother and the fetus [3, 4]. One of these classes of molecules can be microRNAs—short noncoding RNAs that regulate gene expression at the posttranscriptional level [5]. It is known that the miRNA sequence is not always canonical: miRNA isoforms that can be shorter or longer than the canonical form by several nucleotides at the 3'- or 5'-end of the sequence were found. Such changes can significantly alter the regulatory targets of the molecule [6]. When simulating hypoxia in BeWo b30 cells, a change was shown in the expression profile of miRNA isoforms and their target genes in the cell [7], including in the endothelial cells [8], which are also an integral part of the placental barrier. The molecular mechanisms underlying the formation of miRNA isoforms remain little studied.

The mechanism of miRNA maturation includes several sequential steps. Initially, in the course of transcription, a pri-miRNA molecule in the form of a hairpin is formed in the cell nucleus. Next, pri-miRNA undergoes intranuclear processing, the purpose of which is to remove the unpaired ends of the molecule. At this stage, the main role is played by the complex consisting of the double-stranded RNA (dsRNA) of the specific endoribonuclease Drosha and the dsRNA-binding protein DGCR8 [9]. The resulting pre-miRNA molecule is then transported by the XPO5/RAN-GTP proteins to the cytoplasm, where the second processing stage is performed by the com-

Abbreviations: dsRNA, double-stranded RNA, FDR, false discovery rate (level of false positive results), HIF, hypoxia-induced factor, DMSO, dimethyl sulfoxide, FITC, fluorescein isothiocyanate.

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Table 1. List of genes involved in miRNA maturation

Functional group	Genes
Regulation of activity of the Drosha/DGCR8 complex	DROSHA, DGCR8, DDX5, SNIP1, HNRNPA1, SMAD7, SMAD3, SMAD5, SRSF1, SMAD1, SMAD9, SMAD2, DDX17, SMAD6, SMAD4
Pre-miRNA transport	RAN, XPO5
Regulation of pre-miRNA maturation	LIN28B, LIN28A, KHSRP, TP53
Regulation of activity of the Dicer/TARBP2 complex	DICER1, PRKRA, TARBP2

plex of Dicer and TARBP2 proteins, which results in the cutting of the pre-microRNA hairpin loop. As a result, the RNA duplex is formed, which is further decomposed into two mature miRNA chains [10]. It is known that the work of the Drosha/DGCR8 and Dicer/TARBP2 complexes often occurs with shifts at the hairpin cutting positions, which ultimately leads to the formation of various microRNA isoforms, the nucleotide sequences of which differ from each other in several positions at the ends of the molecules [6]. Moreover, a number of other proteins can strongly affect the work of these complexes, which may lead to a change in the miRNA isoform profile [11]. For example, the families of the RNA-binding proteins DDX5 and DDX17 function as mediators between the Drosha/DGCR8 complex and SMAD, SRSF1, SNIP1, and TP53 proteins, which have a stimulatory effect on intranuclear processing of miRNAs. An example of another regulatory mechanism is the work of LIN28, KHSRP, HNRNPA1, and PACT proteins, which control both intranuclear and cytoplasmic processing of the clearly expressed let-7 miRNA family.

The BeWo human choriocarcinoma cell line (in particular, its BeWo b30 clone) is often used to simulate the placental barrier in vitro, since it mimics the properties of trophoblast cells. This cellular model makes it possible to study the transport, barrier, secretory, and other functions of the placental barrier and simulate various pathological conditions [1]. For example, using an oxyquinoline derivative that inhibits enzymes HIF prolyl hydroxylase, hypoxia can be simulated due to accumulation of HIF-1α and regulation of expression of the target genes associated with the cell response to hypoxia [2].

The aim of this study was to investigate the changes in the expression of the protein factors that are involved in the miRNA maturation process when simulating hypoxia in BeWo b30 cells.

Cells were cultured on Corning Transwell permeable polyester inserts with an area of 0.143 cm^2 and a pore size of 1.0 μm. After reaching the monolayer confluence, which was monitored by impedance spectroscopy [12–14], and permeability for 70-kDa FITC-dextran, the cells were incubated for 24 h in the presence of the oxyquinoline derivative D014-0021 at a concentration of 10 μM as described previously [15]. Since D014-0021 is dissolved in dimethyl sulfoxide (DMSO) before its addition to the culture medium, the medium in the control experiment was supplemented with DMSO to a concentration of 2 vol %. After a 24-h incubation, the cells were lysed with the Qiazol Lysis Reagent for subsequent extraction of RNA using the Qiagen miRNeasy Micro Kit reagent kit. Then, a genome-wide transcriptome analysis was performed using the Affymetrix GeneChip Human Transcriptome Array 2.0 microarrays. To search for the differentially represented genes in BeWo b30 cells under the control conditions and in simulated hypoxia, we used Student's *t* test with the Benjamin–Hochberg correction for multiple comparisons by calculating the false discovery rate (FDR) values. The probabilities associated with the analysis of enrichment of regulatory pathways were calculated by the Monte Carlo method with the number of repetitions of 100000. As a result of the literature analysis, 24 genes encoding the proteins involved in miRNA maturation were established (Table 1).

When comparing the results of the transcriptome analysis of the samples with hypoxia relative to the control group, 7637 out of 25683 possible differentially expressed genes were identified (FDR < 0.05). The expression of 17 out of the 24 genes associated with miRNA processing statistically significantly differed in hypoxia and under the normal conditions. Table 2 shows six genes with the greatest difference in expression. The *DICER1* and *DGCR8* genes were found to be differentially expressed, which means a direct change in the activity of the complexes that perform miRNA processing. Moreover, the majority of the RNA-binding proteins that regulate the activity of the Drosha/DGCR8 and Dicer/TARBP2 complexes were also significantly differentially represented at the transcriptome level. To additionally validate the significance of the found changes, we estimated the probability of finding at least 17 genes with a significantly changed expression level at a random selection of 24 out of 25683 transcripts, the expression of 7637 of which statistically significantly changed. The calculated probability was less than 1.6×10^{-5}, which proves the nonrandomness of the found enrichment of the gene list.

Thus, it was shown that, in the placental barrier model based on the BeWo b30 cell line, the simulation

Table 2. Changes in the expression level of the genes involved in miRNA maturation in BeWo b30 cells in hypoxia and under normal conditions

Gene name	Mean expression level in cells subjected to hypoxia, $\log_2$ (a.u.)	Average level of expression in cells under control conditions, $\log_2$ (c.u.)	Multiplicity of change, times	FDR p-value
SMAD7	9.3	6.7	6	1.98×10^{-3}
SNIP1	11.5	9.1	5.3	5.01×10^{-4}
Dicer1	12.0	13.0	2	2.38×10^{-4}
DGCR8	7.0	7.9	1.9	1.08×10^{-3}
TP53	11.6	10.7	1.9	2.84×10^{-3}
DDX5	15.3	16.0	1.6	2.86×10^{-4}

of hypoxia with an oxyquinoline derivative was accompanied by a significant change in the expression level of the majority of genes encoding the proteins involved in the molecular mechanism of miRNA processing. The group of these proteins included both the enzymes directly involved in miRNA processing mechanisms and the proteins that indirectly regulate this mechanism by affecting the key components. These results are in good agreement and provide theoretical justification for the change in the expression profile of miRNA isoforms observed in hypoxia.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

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