Cobalt Chloride-induced Hypoxia Can Lead SKBR3 and HEK293T Cell Lines toward Epithelial-mesenchymal Transition

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ABSTRACT

Hypoxia is a common characteristic of the tumor microenvironment. In response to hypoxia, expression of the hypoxia-inducible factor (HIF) can lead to activation of downstream molecular events such as epithelial-mesenchymal transition (EMT), invasion, and angiogenesis. In this study, CoCl2 was used to simulate hypoxia in SKBR3 and HEK293T cell lines to investigate whether this treatment can induce hypoxia-associated EMT and invasion in the studied cells. SKBR3 and HEK293T cells were treated with different concentrations of $CoCl_2$ at different exposure times and their viability was analyzed. To confirm successful hypoxia induction, the expression levels of *HIF1a* and *vascular endothelial growth factor A (VEGFA)* mRNA were assessed.

Additionally, the expression of EMT-associated markers including snail, E-cadherin, N-cadherin, and vimentin, as well as invasion-related genes including *matrix metalloproteinase-2 (MMP2)* and *MMP9* was measured.

We found that cell viability in $CoCl_2$-treated cells was concentration-dependent and was not affected at low doses. While the expression of *HIF* and *VEGFA* genes was upregulated following hypoxia induction. E-cadherin expression was significantly downregulated in HEK293T cells; while, N-cadherin and snail were upregulated in both cell lines. Moreover, an increment of *MMP* expression was only observed in SKBR3 cells.

Taken together, the findings indicated that $CoCl_2$ can mimic hypoxia in both cell lines, but EMT was triggered in SKBR3 cells more effectively than in HEK293T cells, and invasion was only stimulated in SKBR3 cells. In conclusion, SKBR3 cancer cells can be used as an EMT model to better understand its control and manipulation mechanisms and to investigate new therapeutic targets for the suppression of tumor metastasis.

Keywords: Cobaltous chloride; Epithelial-mesenchymal transition; Hypoxia; Hypoxia-inducible factor 1

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INTRODUCTION

Hypoxia is a key feature of the tumor microenvironment (TME) and refers to a reduction of the proportion of oxygen in the target tissue, and is capable to facilitate and speed up the growth of tumor cells. Most solid tumors are exposed to mild (<0.5 mmHg) or moderate (0.5-20 mmHg) hypoxia, a situation that can stop the growth of normal cells but allows growth and propagation of cancer cells through adaptation mechanisms, provided by the expression of the hypoxia-inducible factor (HIF).[1-3] HIF is a heterodimer made up of the oxygen-sensitive HIF1α and the oxygen-persistent HIF1β molecules. In normoxia conditions, the oxygen labile HIF1α molecule is hydroxylated by HIF prolyl hydroxylase (PDH) before recognition by von Hippel Lindau tumor suppressor protein (pVHL) and degradation by proteasome-based mechanisms. In low oxygen tension conditions, the PDH enzyme is inactivated, thus the HIF1α segment will be stable and can translocate into the nucleus to dimerize with HIF1β and create the active HIF complex. HIF can bind to the hypoxia response element (HRE) and activate their promoter sequences in the target genes and consequently initiate their expression.[4-7] Under hypoxia, the expression of more than 100 genes is stimulated, which orchestrates several cellular processes such as proliferation, angiogenesis, and glycolysis, which help tumor cell survival in a low-oxygen and low-nutrient environment.[8-9] Hypoxia is also a well-known epithelial-mesenchymal transition (EMT) activator in a variety of cancer cells. EMT, a primary driver of metastasis, is a reversible multi-step process in which epithelial cells transform into mesenchymal cells, allowing cancer cells to obtain motility and migratory potential.[10-11] HIF binds to the HRE sites in the snail's minimal promoter, which is an E-box-binding protein that serves as an EMT-activating transcription factor, and induces snail expression in response to hypoxia.[12] The key molecular event during EMT is inhibition of *E-cadherin* gene expression, which is a crucial cell adhesion protein while up-regulating several genes associated with mesenchymal characteristics such as *N-cadherin* and *vimentin*.[13-14]

Hypoxia also stimulates the expression of some other key EMT regulators, including transcription factors such as Zeb1 and Twist, which are responsible for the suppression of epithelial-associated proteins.[15-16] Hypoxia also upregulates the production of inflammatory cytokines such as IL-1, IL-6, and TNFα in TME which can promote EMT through inflammatory mechanisms.[17] Triggering the signaling pathways is another mechanism that links HIF to EMT.[18] In addition, hypoxia-mediated activation of angiogenesis factors and expression of matrix metalloproteinases (MMPs) leads to angiogenesis and extracellular matrix (ECM) breakdown, which is linked to tumor development and metastasis.[19-21]

In cancer biology studies, cobalt chloride ($CoCl_2$) is commonly applied for in vitro induction of hypoxia. Treatment with $CoCl_2$ greatly enhances *HIF1* expression and imitates a hypoxia-like response, regardless of oxygen levels.[22] Since hypoxia plays an essential role in cancer cell growth and metastasis, therefore, developing a simple in vitro model of hypoxia-induced EMT and studying the cellular and molecular changes that occur as a result of HIF1 activation may aid in the development and validation of medications that target hypoxia-induced metastasis in cancer. In this study, the alteration of the expression level of *HIF1* and other markers involved in angiogenesis, invasion, and EMT were studied in both cancerous and non-cancerous cell lines following treatment with various concentrations of $CoCl_2$. We aimed to see whether hypoxia could induce EMT in those cell lines and to investigate if these cell lines could be used as an in vitro EMT model.

MATERIALS AND METHODS

Cell Culture and Hypoxia Induction

SKBR3 breast cancer cell line and HEK293T non-cancerous cell line were purchased from the Stem Cell Technology Research Center of Iran and incubated at 37°C with 5% CO^2 in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, USA). Cells at their exponential phase of growth were employed for hypoxia induction using $CoCl_2$ (Sigma-Aldrich, USA). For this purpose, cells were seeded on 6-well plates and cultured for 24h to facilitate cell adhesion before exposure to different doses of $CoCl_2$ (0-500 µM).

All methods were approved by the ethics committee of the Iran University of Medical Sciences (ethics code: IR.IUMS.FMD.REC 1396.9321126004).

Cell Viability Assay

Approximately 4×10^3 cells from each cell line were seeded in 96-well plates and after overnight incubation, hypoxia was induced by treatment with various concentrations of CoCl₂ (0–500 µM) at three-time points (24, 48, and 72 h). After the incubation period, the medium was refreshed and 10 µL of 5 mg/mL MTT (Sigma-Aldrich, St. Louis, MO, USA) was added to each well, and plates were kept at 37°C for 3h. The content was then removed and 100 µL DMSO (Sigma-Aldrich, USA) was added to dissolve the formazan crystals within 15 min at 37°C. The optical density (OD) of the wells was measured by an ELISA plate reader (Biohit, Finland) at 570 nm versus the 630 nm reference filter and the viability percentage was determined.

Gene Expression Analysis

For gene expression analysis, cells were treated with 150 and 200 µM of CoCl₂ for 48h, and an equal volume of phosphate-buffered saline (PBS) was added to the control group. Following RNA extraction (Gene-All, South Korea), 1 µg of the purified RNA was used for cDNA synthesis according to the manufacturer's suggested method (Takara, Japan). In 20 µL reaction volumes, 10 µL of 2X SYBR-Green master mix (BioFact, South Korea), 1 µL cDNA, and 0.5 µl of both reverse and forward primers were used for real-time PCR. Real-time PCR was done through the following protocol: denaturation (95°C for 10 min), 40 cycles of amplification and quantification (95°C for 15 s, 60°C for 1 min, 72°C for 30 s with a single fluorescence measurement), melting curve analysis (60–95°C with a heating rate of 0.1°C/second and a continuous fluorescence measurement) and finally a cooling step to 4°C. Table 1 shows the sequence of the primers as well as their annealing temperature and product size (Supplementary Table 1). Hypoxanthine phosphoribosyl transferase 1 (HPRT1) was used as the housekeeping gene and the relative expression value was computed using the $2^{-\Delta\Delta CT}$ formula.

Statistical Analysis

The experiments were performed in a triplicate manner and the data from at least three sets of experiments were presented as the median±interquartile range (IQR). Graph Pad Prism version 8.0 (GraphPad Prism Software, San Diego, CA, USA) was used for statistical analysis. Kruskal-Wallis analysis followed by Dunn's post-test and Mann–Whitney were used for assessment of the differences between groups. Statistical significance was defined as a $p<0.05$.

RESULTS

The Impact of Cobalt Chloride on Cell Viability

First of all, the CoCl₂ effect on cell viability was assessed. Different quantities of CoCl₂ (0–500 µM) showed minimal inhibitory effect on cell survival at 24 h. Also, following 48 and 72 h, CoCl₂ concentrations up to 150 and 200 µM did not show any significant influence on cell viability in SKBR3 and HEK293T cells, respectively. The addition of higher concentrations of CoCl₂ gradually resulted in a decline in cell viability in a time and concentration-dependent manner. Therefore, 150 and 200 µM concentrations of CoCl₂ were used for induction of hypoxia in SKBR3 and HEK293T cells in all experiments (Figure 1).

Effects of Cobalt Chloride on Expression of Hypoxia Related Genes

The expression levels of *HIF1α* and *vascular endothelial growth factor A (VEGFA)* genes were measured within 24-72 h post CoCl₂ exposure to assess the efficacy of hypoxia induction. In the HEK293T cell line, CoCl₂ administration promoted *HIF1α* expression in a time-dependent manner. However, CoCl₂-treated SKBR3 cells displayed higher *HIF1α* mRNA expression, with the greatest expression at about 48h post-treatment; a longer incubation period did not increase *HIF1α* expression. Similarly, in both cell lines, *VEGFA* expression steadily rose after CoCl₂ treatment until 48h but longer incubation times did not increase *VEGFA* expression and even a decrease was observed. Therefore, 48h treatment with CoCl₂ was chosen for additional tests (Figure 2).

CoCl₂ Induces EMT-related Molecular Changes in SKBR3 and HEK293T Cell lines

HEK293T cells showed a considerable drop in *E-cadherin* expression as the epithelial marker, as well as an increase in *N-cadherin* expression as the indicator marker of mesenchymal cells, but no change in *vimentin* expression was observed. SKBR3 cells did not undergo full EMT following hypoxia and showed a minor drop in E-cadherin as well. Additionally, they showed a significant rise in mesenchymal phenotypic markers such as vimentin and N-cadherin in response to the

hypoxic environment. In both cell lines, snail as a sensitive transcription factor and major inducer of EMT was upregulated under hypoxia compared to normoxia condition (Figure 3).
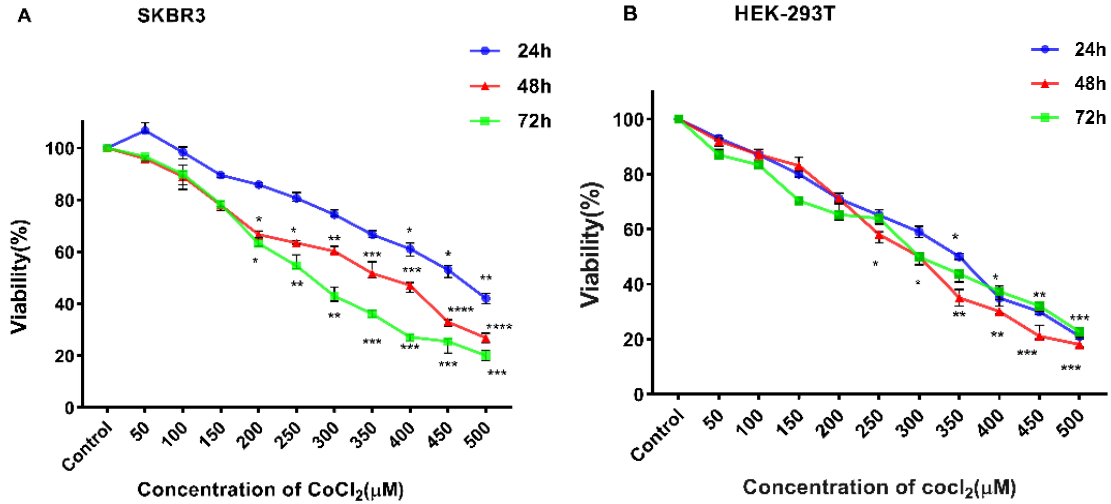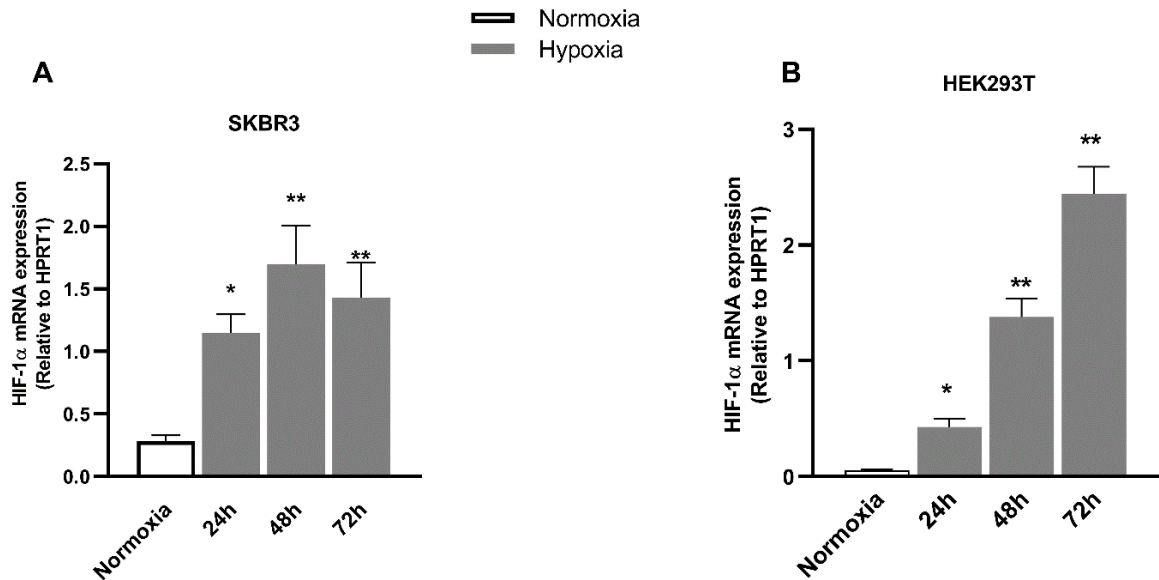

Figure 1. The effect of cobalt chloride (CoCl^2) treatment on the viability of SKBR3 and HEK293T cells
(A). SKBR3 and (B) HEK293T cells were treated with various concentrations (0-500 μM) of CoCl$_2$ for 24, 48, and 72 h, and their viability was assessed using an MTT assay. Data shows the median (±IQR) of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, and**$p<0.0001$**

C
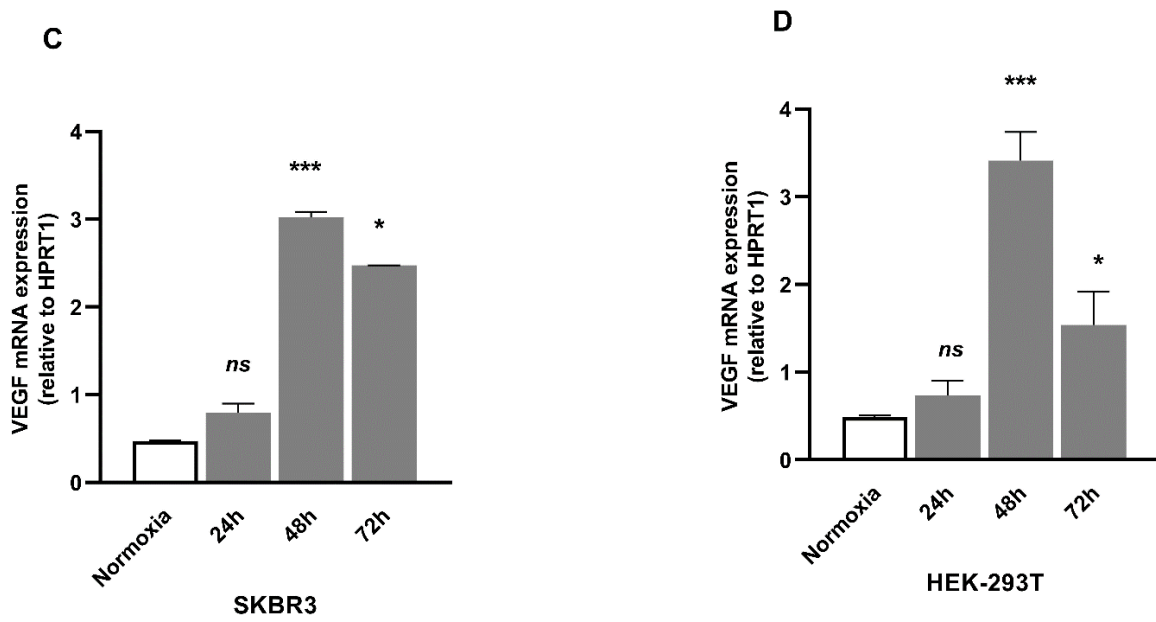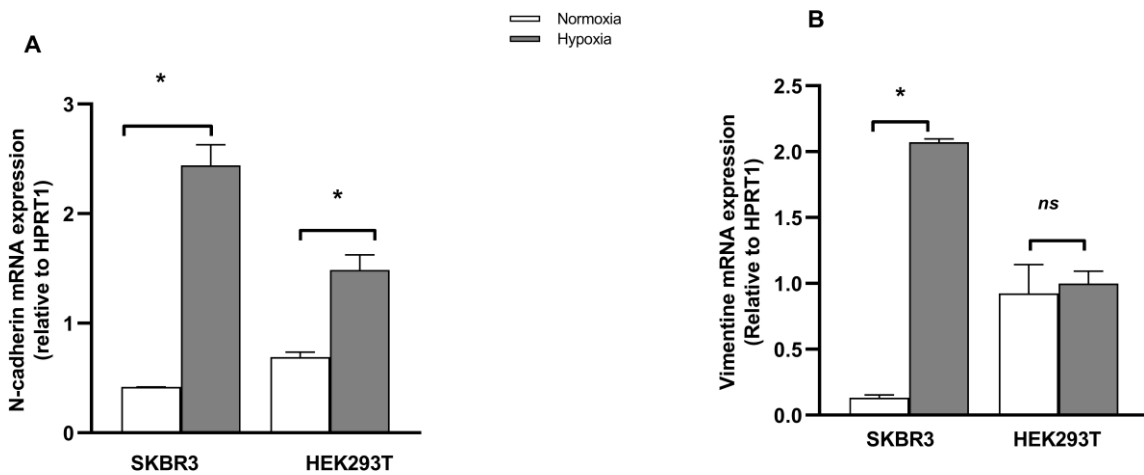

SKBR3

D

HEK-293T

Figure 2. Cobalt chloride induces *hypoxia-inducible factor1α* (*HIF1α*) mRNA expression in SKBR3 and HEK293T cells
The expression of hypoxia-related genes including *HIF1α* and *vascular endothelial growth factor A* (VEGFA*) were studied in SKBR3 and HEK293T cell lines following their chemical treatment with 150 and 200 µM concentrations of CoCl$_2$. (A&B) *HIF1α* mRNA expression was higher in CoCl$_2$ treated than in control cells in normoxia condition. (C&D) Both CoCl$_2$ treated SKBR3 and HEK293T cells showed increased expression of *VEGFA*. Data were shown as the median (±IQR) of three independent experiments. *$p < 0.05$, **$p<0.01$, ***$p<0.001$, ns: not significant
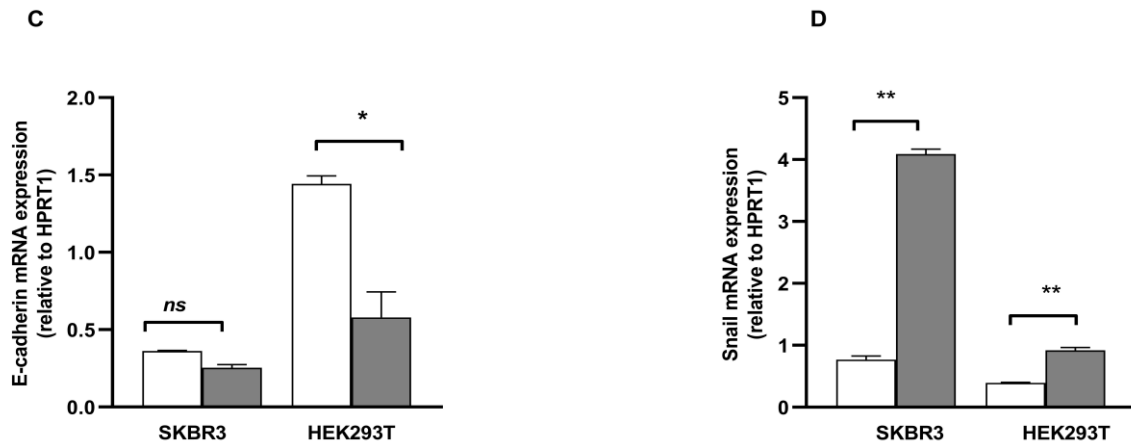
A

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Figure 3. Cobalt chloride-induced hypoxia, changes Epithelial-Mesenchymal Transition (EMT) related genes expression in SKBR3 and HEK293T cells. The expression of EMT-related genes including Snail, E-cadherin, N-cadherin, and vimentin were studied in SKBR3 and HEK293T cell lines following their chemical treatment with 150 and 200 μM concentrations of CoCl₂. (A) N-cadherin and (D) snail were upregulated in both cell lines. (B) Vimentin expression was significantly upregulated in SKBR3 cells but not in HEK-293T. (C) E-cadherin expression was significantly downregulated in HEK293T cells but not in SKBR3; *Data* were shown as the median (± IQR) of three independent experiments. *p<0.05, **p<0.01, ns: not significant

CoCl₂ Induces Expression of *MMP* Genes

Hypoxia enhanced *MMP2* and *MMP9* expression in SKBR3 cells as compared to cells under normoxic conditions, suggesting that *MMP expression* facilitates tumor invasion. On the other hand, induction of hypoxia did not influence the expression of *MMPs* in HEK293T cells (Figure 4).
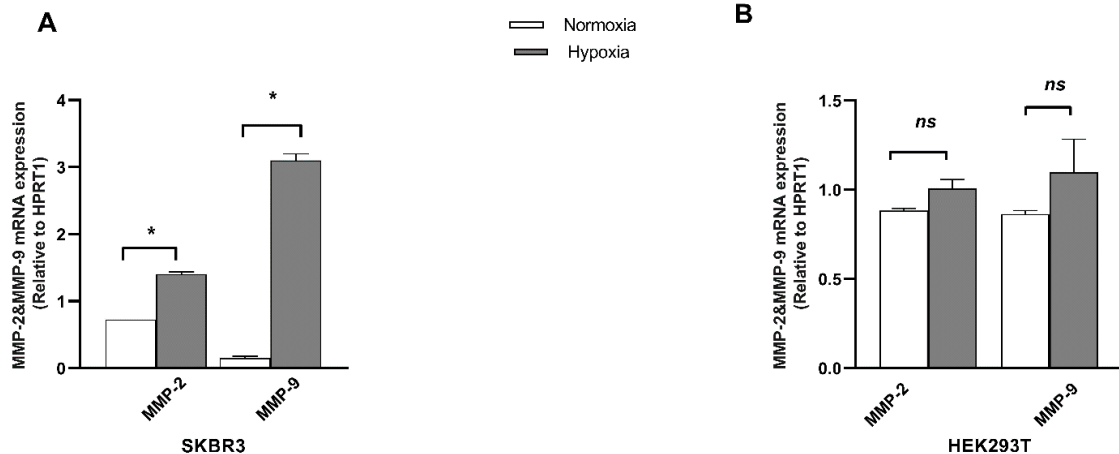
A

B

Figure 4. Impact of Cobalt chloride treatment on expression of invasion-related genes. (A) Cobalt chloride (CoCl²) treated SKBR3 cells showed a significant increase in the expression of Matrix Metalloproteinase (MMP2 and MMP9). (B) There was no significant change in the expression of MMP2 and MMP9 in HEK-293. *p*<0.05, ns: not significant

DISCUSSION

Hypoxia is a typical hallmark of malignant solid tumors and it has been linked to increased invasion and metastasis.[23] To build a strategy to prevent hypoxia development and management of its consequences, understanding the vital pathways triggered by hypoxia and considering the expression pattern of hypoxia-related markers is helpful. CoCl$_2$ treatment is a simple chemical method to imitate hypoxia and investigate its consequences. Co^{2+} is an iron-chelating molecule that can replace Fe^{2+} in the heme-based oxygen sensor enzymes, preventing them from interacting with oxygen. The tumor cells would then experience a lack of oxygen in normoxic conditions and upregulate the expression of hypoxia-inducing factors as a result.[24]

In this study, following CoCl$_2$ treatment, the levels of *HIF1α* and *VEGFA* mRNA, which are the key downstream genes of HIF, were significantly increased in both cell lines, indicating that CoCl$_2$ could successfully induce hypoxia-related events in HEK293T and SKBR3 cell lines. HIF1α and VEGFA promote tumor development, dissemination, as well as metastasis and in this study, their expression levels increased steadily as hypoxia was prolonged. These findings were in line with Zhang et al findings that showed that 786-0, HK2, and HMEC-1 promote HIF1 up-regulation following exposure to low concentration (150 µM) of CoCl$_2$.[25] In breast cancer cells, including MCF7 and MDA-MB231, a certain concentration of CoCl$_2$ (150 and 25 µM, respectively) enhances *HIF1α* expression, as well as genes involved in angiogenesis and apoptosis.[26] HIF mRNA and protein levels were also elevated in other cancerous cells such as the human pancreatic cancer cell line PC-2 after treatment with CoCl$_2$.[27]

Monitoring EMT markers showed that after treatment with CoCl$_2$, the transcription factor and the signature components of mesenchymal phenotypic markers including snail, vimentin, and N-cadherin were upregulated in SKBR3 cells. Also, the expression of E-cadherin as the epithelial marker was reduced, confirming the switch from an epithelial to a mesenchymal phenotype, but this reduction was not significant in comparison to normoxia. Similarly, hypoxia caused HEK293T cells to up-regulate snail as an EMT transcription factor and inhibit *E-cadherin* expression. Although there has been a significant shift in N-cadherin as a mesenchymal marker, CoCl$_2$-induced

hypoxia did not affect vimentin levels in the HEK293 cell line.

Aside from alterations in the expression of molecular markers, mesenchymal cells are known for their migration and invasion capacity. Significant changes in the expression of migration-related genes such as *MMP2* and *MMP9* were observed in SKBR3, but not in HEK293T cells. Several studies have reported induction of EMT under CoCl2-induced hypoxia, which is consistent with our findings. HIF overexpression in response to CoCl$_2$-induced hypoxia can promote EMT and metastasis in hepatocellular carcinoma (HCC) cells, too; pointing out to the important role of HIF in the EMT phenomenon.[28] CoCl$_2$ treatment also induces EMT in renal cell carcinoma (RCC) cell lines and is associated with increased expression of fibronectin as a mesenchymal marker and decreased expression of *E-cadherin* in these cells.[29] Morphological changes in HT29 human colorectal cancer cells exposed to 50 µM CoCl$_2$ point out to EMT occurrence and their increased motility and invasiveness.[30]

We observed a successful EMT following CoCl2-induced hypoxia in SKBR3 cells, while HEK293T cells failed to undergo a full EMT. These characteristics confer the ability of invasion and migration to SKBR3 cells.[31]We found that exposure of HEK293T cells to CoCl$_2$ can up-regulate the expression of snail, the major inducer of EMT that shifts the cells from epithelial to mesenchymal characteristics. However, the expression of *vimentin*, which regulate motility in many cells; and the proteolytic characteristics of MMPs, which promote invasion through ECM degradation, did not increase in these cells. So, it seems that the increased expression of snail was insufficient to establish a migratory phenotype in HEK293T cells. Research on breast cancer cells found that up-regulated snail expression under hypoxia caused an increased expression of vimentin as well as decreased expression of E-cadherin, which are typical indicators of EMT induction but had no influence on their migration. The migration capacity of these cells was stimulated by prominent expression of snail via snail vector, which was not triggered even at moderate levels of enhanced expression of snail.[32] One of the limitations of this study is that we did not evaluate the alterations related to EMT at the protein level to validate hypoxia-induced EMT and also did not examine the enzymatic activity of MMPs during hypoxia.

Furthermore, according to the findings of Vaapil et al, EMT did not occur in the MCF10A cell line, which

is a non-tumorigenic epithelial cell line. In brief, EMT is associated with the invasiveness of cancer cells and their metastasis.[33-34] HEK293T, like MCF10A, is a non-malignant cell line that does not grow aggressively, which might explain why HEK293T behaved differently in terms of migration and expression of the invasion-related genes in response to cobalt chloride-induced hypoxia in comparison to SKBR3 cells.

In brief, establishing a standard and robust HIF inducible-EMT model and analyzing the gene expression changes and signaling cascades in the hypoxic cells is regarded as a fundamental step in cancer research that enables us to assess the recently developed techniques and hinder cancer growth and metastasis.

In conclusion, EMT is better triggered in SKBR3 than in HEK293T cell line and finally, invasion can only be stimulated in SKBR3 cells. So, the SKBR3 cancer cell line can be used as an EMT model but the usage of HEK293T as a model for cancer-related EMT may not reflect all critical components of the EMT process. This information can aid in future research into the processes of EMT as well as the development of new treatment targets to prevent tumor invasion and metastasis.

CONFLICT OF INTEREST

The authors declare no conflict of interest for this publication.

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