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RESEARCH

Hypoxia-Inducible Factor-1α and its Role in the Proliferation of Retinoblastoma Cells

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Abstract In order to better understand the role of HIF-1α in the proliferation of the retinoblastoma cells, a siRNA knockdown of HIF-1α followed by a proliferation assay was performed. Further sequencing was then carried out in order to assess knockdown efficiency and expression of HIF-1α. Up-regulation of HIF-1α gene expression in $CoCl_2$-treated retinoblastoma cells was demonstrated via melting curve analysis from PCR tests and was further analyzed using western blot and densitometry analysis. Reduction of HIF-1α expression in retinoblastoma, post HIF-1α knockdown, was observed after siRNA transfection into Y-79 cells. Knockdown of HIF-1α resulted in a significant decrease in proliferation thereby demonstrating that HIF-1α is involved in promoting survival and proliferation in retinoblastoma cells. Stabilization of HIF-1α in retinoblastoma cells using $CoCl_2$ was unsuccessful.

Keywords Hypoxic · Hypoxic-inducible factor · Retinoblastoma · Knockdown · Proliferation

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Introduction

Retinoblastoma is the most common intraocular malignancy in children [1]. Cells in the periphery of the tumor are inevitably hypoxic when the tumor is growing [2]. More importantly, sustained hypoxic environments have shown to lead to cellular changes that result in more aggressive tumors [3]. Overall, solid tumor hypoxia is strongly associated with tumor propagation, malignant progression, and resistance to therapy [3, 4]. Additionally, it has been shown that tumor cells are capable of resisting hypoxia-induced apoptosis, allowing them to continue to survive and proliferate [5, 6]. Therefore, hypoxic responses are critically important for understanding tumor progression and cancer prognosis.

The effects of oxygen deprivation on tumor growth often appear paradoxical. Although low oxygen can have negative effects on cell growth, tumor cells acquire adaptations that curtail these effects. A significant number of these adaptations are mediated through the Hypoxia Inducible Factor (HIF), a transcription factor made up of an oxygen-regulated α-subunit and a constitutively expressed β-unit [7]. Under hypoxic conditions, the HIF-1α protein accumulates and translocates to the nucleus where it heterodimerizes with HIF-1β to form an active transcription factor [7]. In response to low oxygen, HIF activates the transcription of various target genes involved in the promotion of cell survival, glucose metabolism, tumor angiogenesis, and invasion [8]. During normoxia, HIF-1α is hydroxylated at its oxygen-dependent degradation domain (ODDD), which promotes its interaction with the von Hippel-Lindau (pVHL) ubiquitin ligase and, consequently, becomes marked for degradation [7]. During hypoxia, however, hydroxylase activity is inhibited, allowing HIF-1α to be stabilized [9].

In 2010, Mendez et al. performed an in vitro knockdown of HIF-1α in glioma cells using RNA interference [10]. They found that treated cells, compared to control cells, had a reduced ability to migrate and that they formed fewer and

smaller tumor spheres. The results of these experiments suggest that HIF-1α has a positive effect on the proliferation of tumor cells.

Additional studies have indicated that hypoxic regions are present in retinoblastoma, though the role of HIF-1α in the progression of this tumor has yet to be studied [2, 11]. The fact that tumor cells in retinoblastoma continue to survive despite hypoxic conditions necessitates research into what factors mediate their adaptive capabilities. Modern retinoblastoma treatment includes radiation, chemotherapy, and in more severe cases, enucleation [12]. Due to the fact that tumor hypoxia is associated with resistance to therapy it has been intensely investigated, and efforts are underway to test HIF-1 inhibitors as potential anticancer therapeutics [3, 4, 7]. The primary objective of this study was to characterize the role of HIF-1α in the proliferation of retinoblastoma cells in a simulated hypoxic environment.

Materials and Methods

Cell Culture

The human retinoblastoma cell line, Y-79 (American Type Culture Collection, Manassas, VA), was cultured in 21 % O_2 (normoxia), 5 % CO_2, and 95 % humidified atmosphere air at 37 °C in RPMI-1640 medium (Invitrogen Life Technologies, Burlington, ON), supplemented with 10 % fetal bovine serum (FBS; Invitrogen), 1,250 μg of fungizone (Invitrogen), and 50,000 U of penicillin–streptomycin (Invitrogen).

Cobalt Chloride Treatment

A hypoxic environment was simulated using the chemical agent, Cobalt Chloride ($CoCl_2$). $CoCl_2$ is able to stabilize HIF-1α by inhibiting its hydroxylation and interaction with pVHL, thereby preventing its degradation [13]. In order to stabilize HIF-1α in the Y-79 cells, $CoCl_2$ (Sigma-Aldrich, St. Louis, MO) was dissolved directly into culture medium to produce a final concentration of 50 μM $CoCl_2$. A 50 μM concentration of $CoCl_2$was chosen based on a previous study[14] that demonstrated the greatest HIF-1α expression using this concentration. The cells were left to incubate for 24 h in a humidified atmosphere incubator (21 % O_2, 5 % CO_2 at 37 °C) prior to siRNA transfection.

siRNA Transfection

siRNA knockdown against HIF-1α was carried out in cells treated with and without $CoCl_2$, in order to evaluate the effect of HIF-1α in both a normoxic and hypoxic-like situation. ON-TARGET plus SMARTpool siRNA against HIF-1α (Dharmacon, Lafayette, CO) were transfected at a final concentration of 50nM. A non-targeting negative control siRNA was also applied. The siRNA was transfected using Lipofectamine RNAiMax reagent (Invitrogen) according to the manufacturer's protocol. siRNA was transfected in both $CoCl_2$-treated and non-treated cells in 24-well plates. The pool of siRNA directed against HIF-1α include the following four sequences: CAAGUAGCCUCUUUCACAA, GAUGGAAG CACUAGACAAA, AGAAUGAAGUGUACCUAA, and GAACAAAUACAUG-GGAUUA. The cells were then incubated at 37 °C in 5 % CO_2 for 48 h.

Reverse Transcription—PCR

For RT-PCR analysis, total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen Inc., Toronto, Ontario) according the manufacturer's protocol. cDNA synthesis was carried out using the iScript Reverse Transcription Supermix (Bio-Rad, Hercules, CA) and PCR was performed using QuantiTect SYBr Green RT-PCR Master Mix and primer assay pairs for HIF-1α and GAPDH, which was used as a reference gene. A Chromo4 thermocycler (MJ Research, Waltham, MA) was used and all results were analysed using the GeneEx software.

Western Blot Analysis

Protein samples were extracted and homogenized in lysis buffer. Samples were separated using 8 % SDS-Page gel and then transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was probed overnight with the following primary antibodies: anti-HIF-1α (1:500; Novus Biologicals, Oakville, ON) and anti-actin (1:250; Santa Cruz Biotechnology, Santa Cruz, CA). The primary antibodies were probed with horseradish peroxidase-conjugated anti-mouse (1:2,000) and anti-rabbit (1:5,000) secondary antibodies (Santa Cruz Biotechnology). The proteins were visualized by enhanced chemiluminscence with autoradiographic film (Hyperfilm ECL; GE Healthcare, Uppsala, SE).

Cell Proliferation Assay

The cell proliferation assay was carried out using an MTT assay[1]. Retinoblastoma cells were seeded in a 96-well plate at a density of 5×10^3 cells/well in 100 μL of culture medium.

[1] The MTT Assay

The tetrazolium salt (MTT) is reduced in metabolically active cells by a mitochondrial dehydrogenase to form insoluble purple formazan crystals, which are solubilized by the addition of an acidified isopropanol solution. The color can then be quantified by a spectrophotometer. Because tetrazolium salts are reduced only by metabolically active cells, this assay exclusively detects viable cells. Because proliferating cell are more metabolically active, absorbance values that are higher than the control cells indicate an increase in the rate of cell proliferation. Conversely, a lower absorbance value indicates a decrease in cell proliferation.

Cells were treated with or without $CoCl_2$, followed by siRNA transfection. The cells were then treated with 10 μL of MTT reagent (Sigma-Aldrich) for 4 h in 5 % CO_2 at 37 °C. After incubation, the resulting formazan crystals were dissolved using the MTT Solubilization Solution (Sigma-Aldrich). Absorbance was spectrophotometrically measured at a wavelength of 570 nm.

Results

Up Regulation of HIF-1α Gene Expression in $CoCl_2$-Treated Retinoblastoma Cells

Because HIF-1α is degraded under normoxic conditions, Y-79 cells were treated with $CoCl_2$, a compound that stabilizes the HIF-1α protein [6, 13, 14]. PCR results were analyzed using ΔC_T[2], a method for quantifying relative changes in gene expression [15, 16]. HIF-1α RNA levels were increased in all $CoCl_2$-treated cells (Fig. 1), suggesting that $CoCl_2$ is involved in inducing HIF-1α gene expression. Melting curves (Figs. 2 and 3) revealed consistent melting points for the HIF-1α and GAPDH PCR products. The absence of additional peaks elsewhere is indicative of minimal contamination and/or priming defects [15].

Western blot analysis revealed that $CoCl_2$-treatment resulted in no significant changes in HIF-1α protein expression (Fig. 4). Densitometry analysis[3] of the HIF-1α bands (Fig. 5) revealed only a slight increase in optical density with $CoCl_2$ treatment in the negative controls. Although $CoCl_2$ should theoretically stabilize the HIF-1α protein by inhibiting its degradation [6, 13, 14], the results provided by this study do not appear to support this hypothesis.

HIF-1α Knockdown Reduces HIF-1α Expression in Retinoblastoma Cells

To better understand the role of HIF-1α in proliferation, HIF-1α-specific siRNA was transfected into Y-79 cells. RNA and protein samples were analyzed from knockdown and control cells in order to examine the knockdown efficiency of the siRNA. PCR and western blot data indicated that HIF-1α knockdown cells had reduced levels of HIF-1α.

In these cells that were treated with HIF-1α siRNA, comparative quantification of PCR results using the $\Delta\Delta C_T$ method indicated an approximately 50 % knockdown of HIF-1α RNA in $CoCl_2$-treated cells and about a 45 % knockdown of HIF-1α RNA in untreated cells (Fig. 2), both of which demonstrate changes in gene expression. These results suggest that the given pool of siRNA sequences was able to successfully target HIF-1α RNA and silence the gene [17].

Western blot analysis revealed decreased expression of HIF-1α protein in $CoCl_2$-treated knockdown cells (Fig. 4). However, the knockdown in untreated cells also caused a marginal decrease in HIF-1α. According to densitometry analysis (Fig. 5), a knockdown in $CoCl_2$-treated cells caused the greatest decrease in HIF-1α protein levels.

HIF-1α Knockdown Reduces Proliferation of Retinoblastoma Cells

In order to assess the proliferation potential of the cells, MTT assay results were compared from control, HIF-1α RNA knockdown, $CoCl_2$-treated, and $CoCl_2$-treated with HIF-1α RNA. The spectrophotometer absorbance value was used as an indicator of the amount of viable cells. Absorbance values that are higher or lower than controls are interpreted as increases or decreases in proliferation respectively.

There was a significant decrease in proliferation after a knockdown of HIF-1α in both untreated ($p < 0.001$) and $CoCl_2$-treated ($p < 0.001$) cells (Fig. 6). These results suggest that HIF-1α is involved in the proliferation of Y-79 retinoblastoma cells. $CoCl_2$-treatment did not cause an increase in proliferation. With respect to the negative controls, no significant ($p = 0.50$) difference in proliferation between untreated and $CoCl_2$-treated cells (Fig. 6) was observed.

Discussion

Under normal conditions, the retina is highly oxygenated due to extensive vascularization [18]. In most solid tumors, including retinoblastoma, there exists a stage-wise progression of hypoxia by which oxygen levels can drop to as low as 1.5 %, which is low enough to activate of HIF-1α [11, 19]. HIF-1α, is considered to be a mediator of cell-survival responses, and it is highly expressed during hypoxia or when mutation of oncogenes or tumor suppressors occurs [1]. Retinoblastoma is caused by a critical mutation in the associated tumor suppressor, the retinoblastoma protein (pRb), and pRb functions as a tumor suppressor by inhibiting cell cycle progression from G1- to S-phase[1, 20]. Retinoblastoma is a tumor that typically displays hypoxic regions [2, 11]. Due to the fact that tumor hypoxia and, consequently, the overexpression of HIF-1α are associated with poor patient prognosis, the

[2] Relative and Comparative C_T Methods

Relative quantitation (ΔC_T) compares transcript abundance across multiple samples, using a co-amplified internal control (in our case, GAPDH) for sample normalization. Comparative quantitation ($\Delta\Delta C_T$) compares the C_T values of the samples of interest (in our case, the knockdowns) with a control. The C_T value is the cycle number at which fluorescence crosses the threshold (i.e. exceeds background level).

[3] Densitometry

Densitometry analysis was performed using ImageJ, a java based imaging program developed at the National Institute of Health (http://rsbweb.nih.gov/ij/).

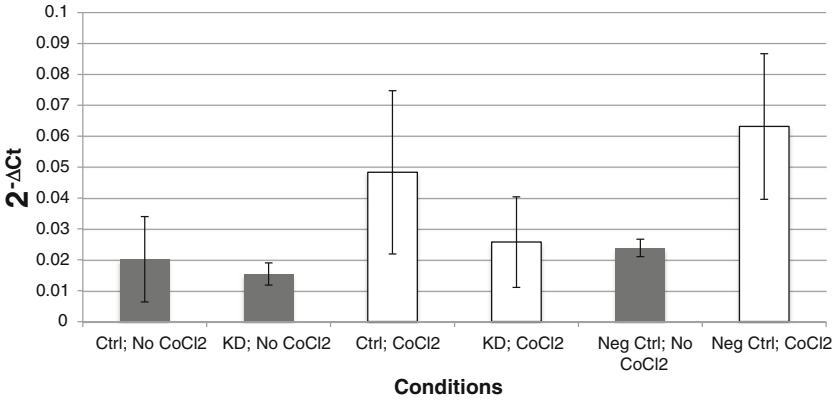

Fig. 1 PCR quantification showing relative values of HIF-1α gene expression in controls (Ctrl*), knockdowns (KD) and negative controls (Neg Ctrl**). Values have been normalized to GAPDH. Grey columns represent cells that were not treated with $CoCl_2$, while white represents those treated with $CoCl_2$. Error bars represent standard deviations. *Untreated Y-79 cells

protein is currently being been investigated as a potential target for anti-cancer therapy.

In order to study the effects HIF-1α, the expression of this particular subunit needed first to be stabilized. $CoCl_2$ was selected as it reportedly induces a biochemical and molecular response similar to what happens under hypoxic conditions and it has been widely used as a hypoxic-mimetic agent both in vivo and in vitro [14, 21] 23. Our PCR results indicate that $CoCl_2$ increases HIF-1α gene expression (Fig. 1), which was previously indicated in another study [22]. However, in terms of protein levels, $CoCl_2$-treatment did not result in greater HIF-1α expression (Fig. 4) suggesting that $CoCl_2$ was not stabilizing the protein. It is possible that the concentration of $CoCl_2$ was not high enough to interfere with HIF-1α degradation. An earlier study by M.S. Al Okail exposed human glioblastoma cells to a range of concentrations of $CoCl_2$ and reported that 50uM resulted in highest HIF-1α protein expression. Retinoblastoma and glioblastoma are similar tumors in

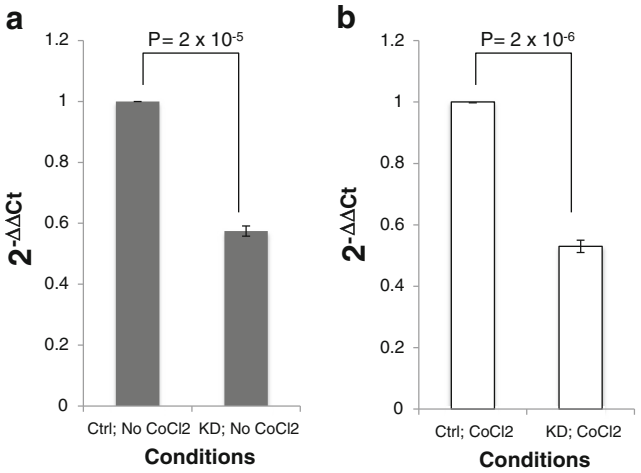

Fig. 2 PCR quantification analysis using the $2^{-\Delta\Delta CT}$ method. The graphs show relative changes in gene expression of HIF-1α between controls (Ctrl) and knockdowns (KD). Controls have been normalized to 1. Error bars indicate standard deviation. **a** HIF-1α expression is decreased after a knockdown in cells without $CoCl_2$. **b** HIF-1α expression is decreased after a knockdown in $CoCl_2$-treated cells. (*P* values measured with student's t-test.)

that they arise from neuroepithelial tissue and, as such, the same concentration was used in order to gauge its effectiveness [23]. This concentration, however, did not have a significant effect on the HIF-1α protein levels in our retinoblastoma cells. Future works that are to be performed should include treatment optimized with a dose-response experiment to find the optimal $CoCl_2$ concentration for maximal HIF-1α protein expression in retinoblastoma.

A discrepancy between the patterns of HIF-1α expression at the RNA (Fig. 1) and protein levels (Fig. 4) was noted. In the presence of oxygen, the HIF-1α protein is tightly regulated and has a half-life of less than five minutes because it is continually targeted for degradation [24]. Therefore, the levels of protein expression that are shown in the western blot may vary depending on the extent to which HIF-1α has been cleared. When comparing the HIF-1α protein levels of the control to its corresponding non-targeting negative control siRNA (Fig. 4), we expected them to be the same because HIF-1α was not being knocked down in these cells; however, it was found that there are lower levels of HIF-1α in the negative controls. It is possible that the negative control cells had more of their HIF-1α protein degraded at the time of protein extraction.

Retinoblastoma cells are known to be difficult to transfect because they are non-adherent. The cells were transfected using lipofectamine, a lipid reagent [18]. The negatively charged nucleic acids of the siRNA bind to the cationic lipid, forming complexes that deliver siRNA into cells via endosomal entry [17]. Non-adherent cells tend to be suspended in culture media, making it harder for them to come into contact with these complexes. Despite this, the experiment was still able to achieve about a 50 % knockdown of the target. Data revealed that a knockdown of HIF-1α in $CoCl_2$-treated and untreated cells resulted in a significant decrease in proliferation (Fig. 6), presumably due to lower levels of HIF-1α. Based on these results, one can infer that HIF-1α is involved in the promotion of cell survival and proliferation. On the other hand, it is possible that $CoCl_2$ would cause an increase in proliferation because it is known to stabilize HIF-

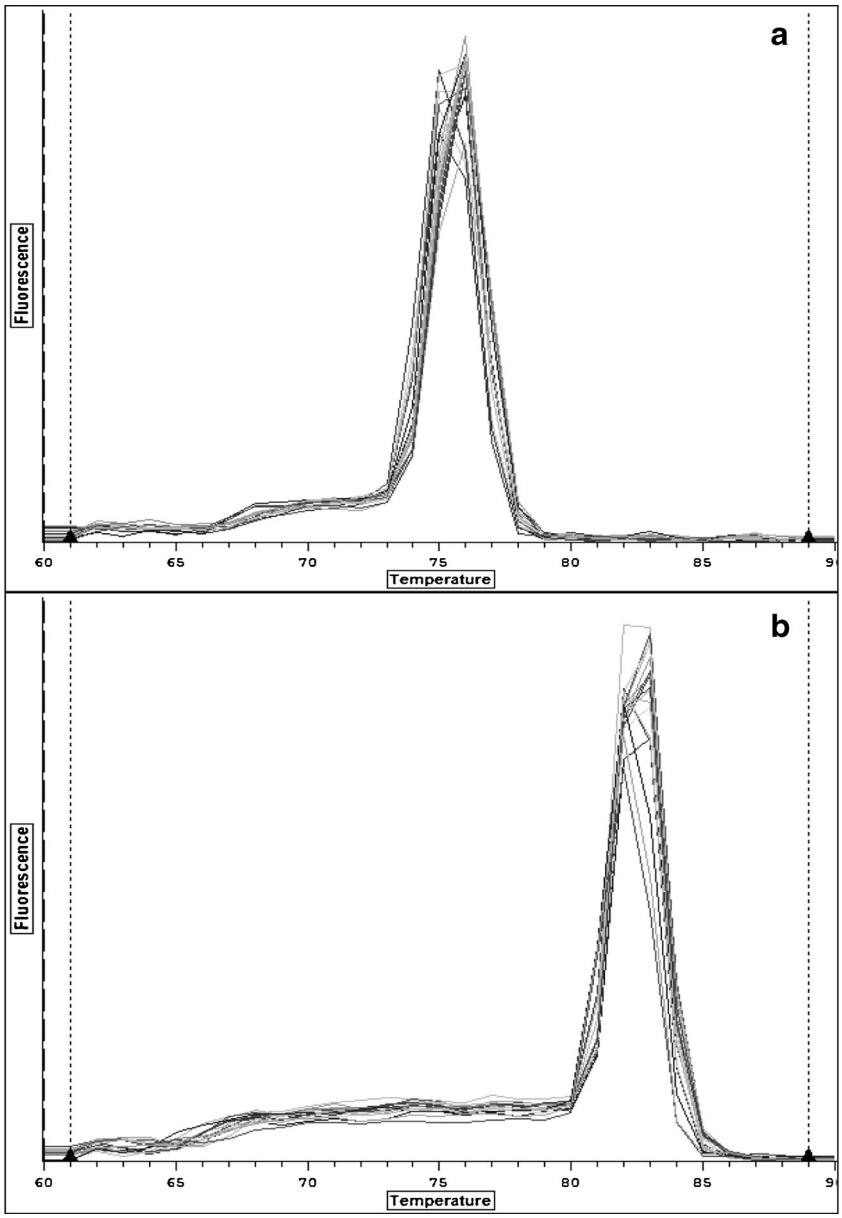

Fig. 3 Melting curves for the following PCR products: **a** HIF-1α and **b** GAPDH. Consistent melting points are an indication of minimal PCR contamination and priming defects

1α. The later scenario, however, did not occur within our given experiment (Fig. 6). As previously mentioned, the $CoCl_2$ concentration may not have been high enough to interfere with HIF-1α degradation. Furthermore, verification was needed in order to ensure that the siRNA knockdown was not causing cell death and consequently causing the decrease in absorbance values. Based on observations of cell morphology after the transfection, cell death did not occur. Future experiments should verify this finding by using flow cytometry. If HIF-1α is indeed the reason for higher levels of proliferation seen in the controls (Fig. 6), then this suggests that HIF-1α can be activated in normoxia, as the cells were never exposed to a hypoxic environment. This raises the question of whether or not HIF activity is dependent on oxygen sensing. Earlier studies have examined further levels of HIF regulation such as protein phosphorylation [25–27]. In

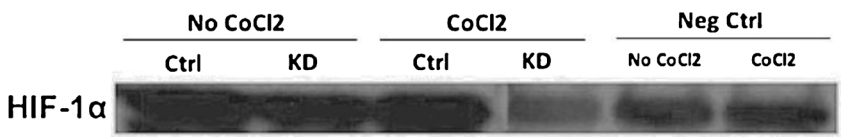

Fig. 4 Western blot revealing protein levels of HIF-1α in various conditions. Lower levels of HIF-1α are found in the knockdowns (KD). Negative controls show slightly higher levels of HIF-1α with $CoCl_2$ treatment. *Ctrl: Untreated Y-79 cells

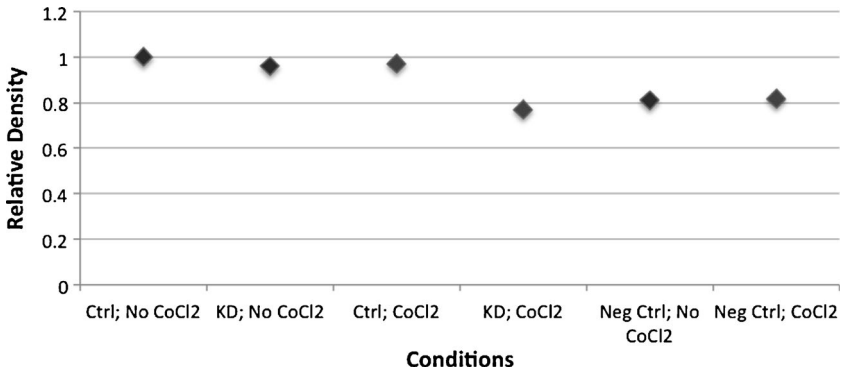

Fig. 5 Plot showing relative densities of protein bands from the western blot in Fig. 4. The greatest decrease in HIF-1α proteins levels occurred with a knockdown in $CoCl_2$-treated cells. *Ctrl: Untreated Y-79 cells **Neg Ctrl: Y-79 cells treated with a non-targeting siRNA

hamster fibroblasts, it was found that HIF-1α is phosphorylated under low oxygen conditions; this phosphorylation led to enhanced HIF-1-dependent transcriptional activation of the VEGF gene [28, 29]. Therefore, we note that there are additional factors involved in HIF regulation that must be examined.

In this study, the Y-79 cell line was cultured at 21 % oxygen, which is typical of most cell culture methods. While atmospheric air that humans breathe is about 21 % oxygen, this is several-folds higher than what is encountered by cells in the body or about 2–5 % oxygen for cells in tissue [28]. As a result, cells were cultured in an environment that is too unnaturally rich in oxygen [28]. Cell cultures should attempt to approximate true physiological conditions such that additional studies in this domain need to reconsider the levels of oxygen exposure. This would allow an increased ability to observe the proliferation potential of tumor cells in conditions more similar to those in vivo.

Conclusion

By first stabilizing HIF-1α with $CoCl_2$ then knocking it down, the experiment effectively simulated a clinical scenario in which hypoxic tumor cells were targeted with anti-HIF therapeutics. This study was able to show that a knockdown of HIF-1α results in a decrease in proliferation. This provides compelling evidence that the protein is involved in survival and proliferation of retinoblastoma cells. Stabilization, however, of the HIF-1α protein was not achieved in this study; therefore, it remains undetermined whether or not stabilization increases proliferation in our cell line. The experiments performed herein should be repeated using either a greater concentration of $CoCl_2$ or a different chemical agent that can better stabilize HIF-1α. If HIF-1α increases proliferation, specific downstream targets that mediate this process need to be identified to fully illustrate the pathway. A worth-while follow-up experiment would be to evaluate the expression of

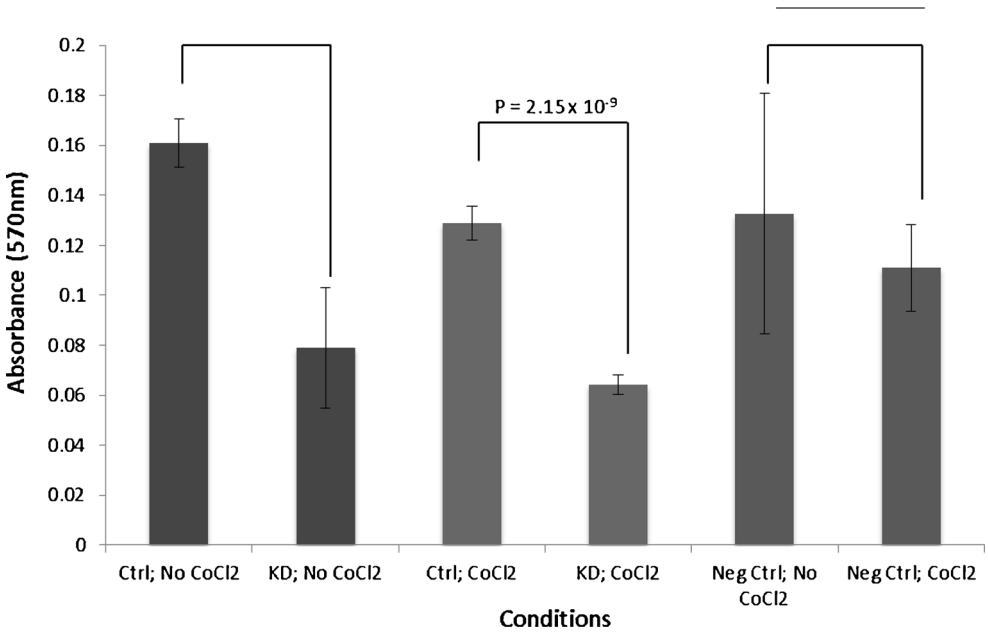

Fig. 6 Graph indicating absorbance values from the MTT assay. Values varying from controls are interpreted as increases or decreases in proliferation. HIF-1α knockdown resulted in a significant decrease in proliferation in both $CoCl_2$-treated and untreated cells. There was no significant difference in proliferation in negative controls. (*p* values were measured by student's t-test.) *Ctrl: Untreated Y-79 cells **Neg Ctrl: Y-79 cells treated with a non-targeting siRNA

known HIF targets, such as glucose transporter-1 (GLUT-1), VEGF, and erythropoietin (EPO) [10, 27, 29], all of which could aid in stimulating proliferation.

Competing Interests The authors declare that they have no competing interests.

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