The Effects of Hypoxia on the Expression of Hypoxia-Inducible Factor 1α (HIF-1α) and Carbonic Anhydrase 9 (CA9) in Various Breast Cancer Cell Lines

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Introduction

Studies show that 1 in 8 women will develop an invasive type of breast cancer in the United States. Impacting 2.1 million women annually, breast cancer is the leading cancer in women. Typically, tumors in the body stimulate low oxygen conditions, otherwise known as hypoxic conditions. Hypoxia is considered a stress factor that causes gene instability and alters the signal transduction pathway. Cobalt(II) Chloride ($CoCl_2$.) is commonly used to mimic hypoxia in vitro by directly inducing protein expression of hypoxia-induced factor (HIF-1α). Three breast cancer cell lines were used in this study: MDA-MB-231, BT-474 and MCF-7. MDA-MB-231 is an epithelial human breast cancer cell line that was extracted from a female with metastatic adenocarcinoma. BT-474 is also an epithelial cell line that is extracted from a female with ductal carcinoma. MCF-7 is an epithelial-like cell line that is extracted from a female with ductal carcinoma. The expression of HIF-1α, a heterodimer that regulates cellular homeostatic responses, and its downstream target carbonic anhydrase 9 (CA9) were measured. CA9 is a transmembrane zinc metalloenzyme that catalyzes the reversible hydration of CO2. Hypoxia typically causes an overexpression of HIF-1a and CA9 both of which play a crucial role in tumor angiogenesis.

Literature Review

Bando, from Tokyo Metropolitan Cancer and Infectious Diseases Center, conducted a study in 2003 that aimed to identify the genes upregulated by hypoxia in human breast cancer cell lines, a hormone-dependent MCF-7 and a hormone-independent MDA-MB-231, using microarray analysis(Bando 2003). Relda Cailleau, author of the novel *In Vitro Cellular & Developmental Biology - Plant*, studied the effects of Hypoxia on solely the expression of HIF1α

(Cailleau 1978). This study indicated an upregulation in the expression of HIF1α . Another study by the National Institute of Health tested for the link between hypoxia and DNA damage in multicellular tumor spheroids(Riffle 2017). This study concluded that in these microenvironments, DNA damage was observed in hypoxic tumor microenvironments, suggesting that hypoxia is detrimental to a cell's health. A study completed in 2014 by Deb, et. al tested the differences in expression of HIF1α and CA9 in male and female breast cancer patients and found that in hypoxic conditions both expressed approximately the same percentage (16%). Another study wanted to understand the molecular pathogenesis pathways that cause metastasis and BCa relapse (Zhou 2019).

Given these contradictory findings, the purpose of this experiment was to test the effects of hypoxia on the expression of HIF1α gene and the downstream target CA9 on various breast cancer cell lines. It was hypothesized that an increase in concentration of $CoCl_2$ is correlated with a downregulation in HIF1α and an upregulation on CA9.

Methodology

Three types of cell lines: MDA-MB-231, BT-474 and MCF-7 were used for determining the effects of hypoxic conditions on HIF1α and CA9. MDA-MB-231 is an epithelial human breast cancer cell line that was extracted from a female with metastatic adenocarcinoma. BT-474 is also an epithelial cell line that is extracted from a female with ductal carcinoma. MCF-7 is an epithelial-like cell line that is extracted from a female with ductal carcinoma. These measured the expression of HIF-1α and its downstream target carbonic anhydrase 9 (CA9). Hypoxia

typically causes an overexpression of HIF-1α and CA9 both of which play a crucial role in tumor angiogenesis.

Cell Culture

To provide nutrients to the cells, the media was changed every other day. To change the media, a vacuboy was used. Then, using a 10 ml pipe, 10ml of media were placed into each petri dish. The next method utilized was splitting the cells. Before any data collection analysis technique the cells had to split. To be split, 4 uL of Trypsin were added to each plate, this was to break the clusters of cells from the bottom of the plate and allow them to be loose in the solution. These cells were incubated at 37°C in the incubator for no more than 10 minutes. The cells were then observed under the microscope, to confirm that the cells had been broken from their clusters. 10mL of media was added to stop the trypsin's effect on the cells, as long periods of time in trypsin leads to degradation of the cell. This mixture was transferred to sterile 15 ml conical tube and centrifuged at 491rpm for 5 min. The suspended cells were then broken by plunging repeatedly. Using a 10uL surgical pipette and a vacuboy, media was extracted and put into new petri dishes.

Cell Counting and Confluency

Using cell counting techniques, approximate amount of cells are measured into these petri dishes to attain efficiency for the analysis techniques such as qPCR and Western Blot analysis. This was obtained after splitting the cells and instead of using a surgical pipette to place the media into new petri dishes, 10uL of the mixture were placed on a microscope slide along with 10uL of Trypan Blue. In the 4 boxes that were depicted in the microscope, the cells were counted and multiplied by the desired amount for the trial that was being conducted.

qPCR Analysis

qPCR analyzes the RNA, creating a large sample to provide a trend of the expression of HIF-1α and CA9 as the concentration of $CoCl_2$. To obtain pure RNA, media was extracted from each set of the petri dishes. Using TRIZOL, an acid-guanidinium-phenol based reagent, to break the cellular membrane, contents were extracted and dispensed, as the RNA was the only part of the mixture needed.When all the contents are released and only the RNA remained at the bottom of the tube, the tubes were centrifuged for periods of 5 and 10 minutes according to Albumin standards.

For the RNA to be analyzed, each well on the PCR plate was mixed with SYBR- Green, nuclease-free H_2O and the gene primers (HIF1α and CA9). For the mixture, 12.5uL of SYBR-Green per well were added. 0.5uL of nuclease-free H_2O per well was added. Finally 1uL of the forward primer and backwards primer per well was added. This was calculated by multiplying all these quantities by the amount of wells. Utilizing a 20uL surgical pipette, 15uL were extracted from the combined mixture to put into each well. Next dilutions of the cDNA in a 1:10 ratio of cDNA to nuclease-free water were utilized accordingly to the Albumin standards. 10uL of the RNA sample was then added to each well corresponding to the level of concentration of $CoCl_2$. Subsequently the PCR plate was sealed with PCR plastic wrap and centrifuged at 4000 rpm for 3minutes. The plate was inspected for air pockets which could skew the results of the analysis. After the air pockets were removed, the software Quantstudio was used to set the guidelines of the PCR plate. The PCR plate was placed in the qPCR machine and the analysis was started.

After the analysis was complete, the data from the computer was put into tables and T-tests and an ANOVA test was conducted to find the significance of the data. This was repeated for optimal results.

Western Blot Analysis

Western Blot analysis was utilized as a method of analyzing the expression of HIF1α and CA9 in proteins. To normalize the proteins, Albumin standards were prepared. BCA Protein was created by combining Reagent A and Reagent B from the Pierce BCA Protein Assay in a ratio of 50:1. Next 20 tubes with the volume capacity of 2mL were prepared and 200uL of the BCA working reagent was added into each tube. Since proteins come in high concentrations, sterile water was added to dilute the sets in the Albumin standards of 1:5 in a different set of 10 tubes. 10uL of Albumin standards were added to the first set of tubes containing the BCA working reagent. This was repeated for each tube of the set.

10uL of the diluted protein sample was then added to the second set of tubes (1 set is 10 tubes). Each sample was incubated to promote the homogenous mixing of its contents. These tubes, containing the diluted protein, were incubated at 37°C for 10 minutes, these tubes were cooled down to room temperature to prevent protein degradation. During the waiting period of the protein cooling down, the Nanodrop spectrophotometer was set up. This device analyzed the protein to detect the concentration and purity of each sample. After the protein is cooled down, the protein was measured in the spectrophotometer by adding 1uL to the machine and testing the levels of concentration of the protein and repeated for each sample.

This data was uploaded onto the computer and the samples were normalized to 20uL using the concentrations provided by the spectrophotometer. After normalization, 16uL of the diluted

protein samples and 4uL of the 4 times concentrated LDS Sample Buffer were mixed. The buffer & protein solution was incubated at 95°C for 10 minutes. Finally, gel electrophoresis was conducted to get the images of the protein.

Gel Electrophoresis(used in preparing the western blot)

A gel electrophoresis analysis was conducted. The materials utilized for gel electrophoresis were a 250 mL flask, volumetric cylinder, spatula, gel casting tray, gel combs, tape, electrophoresis tank, power supply, and cables. The gel electrophoresis was prepared for an initial run without any genes being tested. After the initial run was completed, running buffer was added until the entire tank was filled. The gel plates were placed into the tank containing the running buffer. 5uL of the ladder solution was then added to the first well and 20uL of the sample was added to each of the other wells.

Care was taken to adding the sample to each well because if done improperly, it would result in an overexpressed image of protein in the western blot images or vice versa. The electrophoresis chamber was run at 180 Volts and 120mA for 30-45 minutes. Once completed, the gel plate was opened carefully in order to prevent fracture of the gel. Next, the gel was trimmed, cutting the loading area. Then, using Albumin standards, the transfer housing was prepared and all the components were soaked with transfer buffer. The tank was filled with transfer buffer and run at 10 volts and 200mA for 1 hour. After the second run was completed, the membrane was removed from the transfer housing, washed with water and placed in blocking buffer. The plate containing the blocking buffer was incubated for 5 minutes. Subsequently, 10mL of the primary antibody (GAPDH) was prepared by diluting the primary antibody (GAPDH) in blocking buffer in the standards of 1:1000. The membrane was placed in the

primary antibody and was left in the cycler for 2 hours. The cycler spun the tube so the primary antibody would evenly spread throughout the membrane.

The membrane was taken out and chemiluminescent was sprayed on the membrane. The chemiluminescent solution is light sensitive therefore the membrane had to be sprayed in a dark room. The western blot machine was set up and after 5 minutes of the membrane in the solution, it was placed for imaging. Periods of 10, 15 and 60 seconds were utilized to process the image. This process was repeated numerous times for optimal results.

Statistical Analysis and Graphing

Statistical analyses were conducted. For the T-test, ANOVA, and determining the level of significance, a software called Graphpad was used. The T-test was used to compare the control to each experimental group. The ANOVA test analyzed the variance of the p-values in each set of the data.

Results

qPCR Results

qPCR analyzes RNA, creating a large sample to provide a trend of the expression of HIF-1α and CA9 as the concentration of $CoCl_2$. The expression of HIF1 and CA9 are tested in four experimental groups and one control group. The means of the control and each sample were compared in all trials using an independent sample student t-test where p-values are displayed with a $p<.0.05$ indicating significance. Smaller p-values, indicated by asterisks, results in higher significance.

Table 1. HIF1α expression in a 24 hour trial under varying $CoCl_2$ concentrations.

HIF							
	24h						
Cell Line	Control	50 µM	100 µM	200 µM	300 µM	ANOVA	
MDA-MB-231	1.022	0.468	0.324	0.225	0.200		mean
	0.0127	0.0556	0.0438	0.0424	0.0396		SEM
		<0.0001	<0.0001	<0.001	<0.001	<0.0001	p value
		****	****	****	****	****	level of significance /4
MCF-7	1.013	0.512	0.459	0.330	0.296		mean
	0.008	0.052	0.062	0.036	0.043		SEM
		0.0005	0.0005	<0.0001	<0.0001	<0.0001	p value
		***	***	****	****	****	level of significance /4
BT-474	1.003	0.747	0.619	0.453	0.318		mean
	0.001	0.076	0.048	0.012	0.070		SEM
		0.0285	0.0013	<0.0001	0.0006	<0.0001	p value
		*	**	****	***	****	level of significance /4

Table1 depicts a 24 hour trial in which levels of $CoCl_2$ are increased from 50 to 100 microMolar. As seen in each breast cancer cell line, as the concentration of $CoCl_2$ increases there results a statistically significant downregulation in the expression of HIF1α. This suggests that CoCl2 is concentration dependent on the expression of both genes and the increase in concentration of CoCl2 up till 100uM provides a significant decrease in HIF1α.

Table 2. HIF1α expression in a 48 hour trial under varying $CoCl_2$ concentrations.

HIF							
	48h						
Cell Line	Control	50 µM	100 µM	200 µM	300 µM	ANOVA	
MDA-MB-231	1.003	0.570	0.428	0.191	0.161		mean
	0.0012	0.0592	0.0805	0.0448	0.0314		SEM
		0.0019	0.0020	<0.0001	<0.0001	<0.0001	p value
		**	**	****	****	****	level of significance /4
MCF-7	1.013	0.490	0.314	1.013	0.141		mean
	0.013	0.087	0.014	0.013	0.002		SEM
		0.0034	<0.0001	0.0001	<0.0001	<0.0001	p value
		**	****	***	****	****	level of significance /4
BT-474	1.024	0.799	0.686	0.400	0.299		mean
	0.017	0.048	0.055	0.092	0.126		SEM
		0.0116	0.0043	0.0027	0.0047	0.0339	p value
		*	**	**	**	*	level of significance /4

Table 2 depicts a 48 hour trial in which levels of $CoCl_2$ are increased from 50 to 100 microMolar. Once again, as the concentration of $CoCl_2$ increases a statistically significant downregulation in the expression of HIF1α is observed in each of the breast cancer cell lines.

Table 3. CA9 expression in a 24 hour trial under varying $CoCl_2$ concentrations.

CA9							
	24h						
Cell Line	Control	50 µM	100 µM	200 µM	300 µM	ANOVA	
MDA-MB-231	1.045	1.802	2.985	3.371	3.080		mean
	0.038	0.182	0.393	0.302	0.233		SEM
		0.0152	0.0079	0.0016	0.0010	0.0003	p value
		*	**	**	***	***	level of significance /4
MCF-7	1.104	17.290	32.545	60.370	70.830		mean
	0.033	3.772	10.415	17.876	21.353		SEM
		0.0104	0.0271	0.0295	0.0309	0.0460	p value
		*	*	*	*	*	level of significance /4
BT-474	1.034	9.488	42.579	50.813	65.055		mean
	0.012	0.628	8.360	5.164	6.654		SEM
		0.0002	0.0077	0.0006	0.0007	<0.0001	p value
		***	**	***	***	****	level of significance /4

Table 3 depicts a 24 hour trial in which levels of $CoCl_2$ are increased from 50 to 100 microMolar. Once again, as the concentration of $CoCl_2$ increases a statistically significant downregulation in the expression of CA9 results in each of the breast cancer cell lines. This suggests that when metastasis occurs CA9 expression is decreased indicating an inverse relationship between increased hypoxic conditions and the expression of CA9.

Table 4 CA9 expression in a 48 hour trial under varying $CoCl_2$ concentrations.

CA9							
	48h						
Cell Line	Control	50 µM	100 µM	200 µM	300 µM	ANOVA	
MDA-MB-231	1.003	0.570	0.428	0.191	0.161		mean
	0.0012	0.0592	0.0805	0.0448	0.0314		SEM
		0.0019	0.0020	<0.0001	<0.0001	<0.0001	p value
		**	**	****	****	****	level of significance /4
MCF-7	1.013	0.490	0.314	1.013	0.141		mean
	0.013	0.087	0.014	0.013	0.002		SEM
		0.0034	<0.0001	0.0001	<0.0001	<0.0001	p value
		**	****	***	****	****	level of significance /4
BT-474	1.024	0.799	0.686	0.400	0.299		mean
	0.017	0.048	0.055	0.092	0.126		SEM
		0.0116	0.0043	0.0027	0.0047	0.0339	p value
		*	**	**	**	*	level of significance /4

Table 4 depicts the 48 hour trial in which levels of $CoCl_2$ are increased from 50 to 100 micromolar. Once again, as the concentration of $CoCl_2$ increases a statistically significant downregulation in the expression of CA9 results in each of the breast cancer cell lines. This suggests that the expression of CA9 in these cell lines are very accurate.

Figure 1: HIF1α and CA9 Expression in MDA-MB-231.

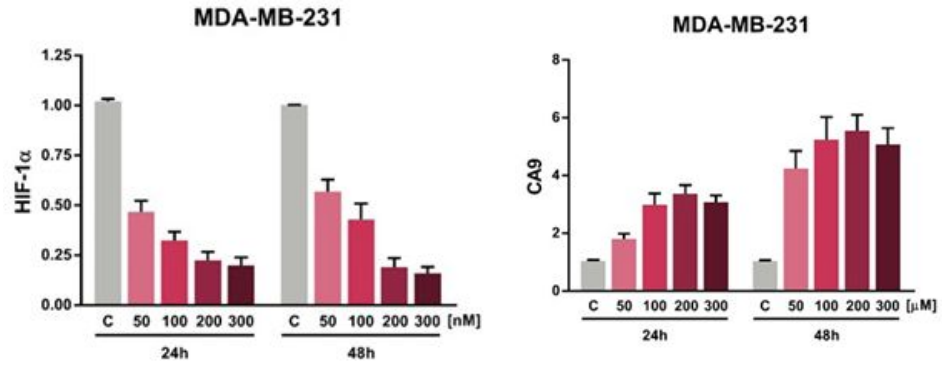
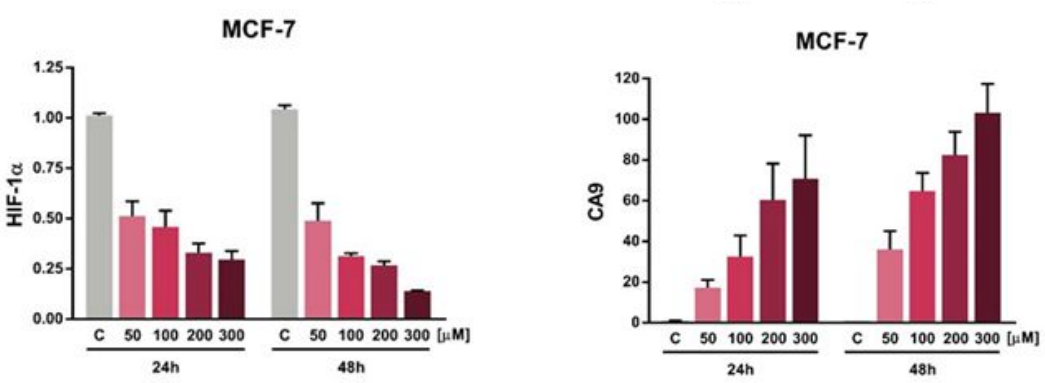


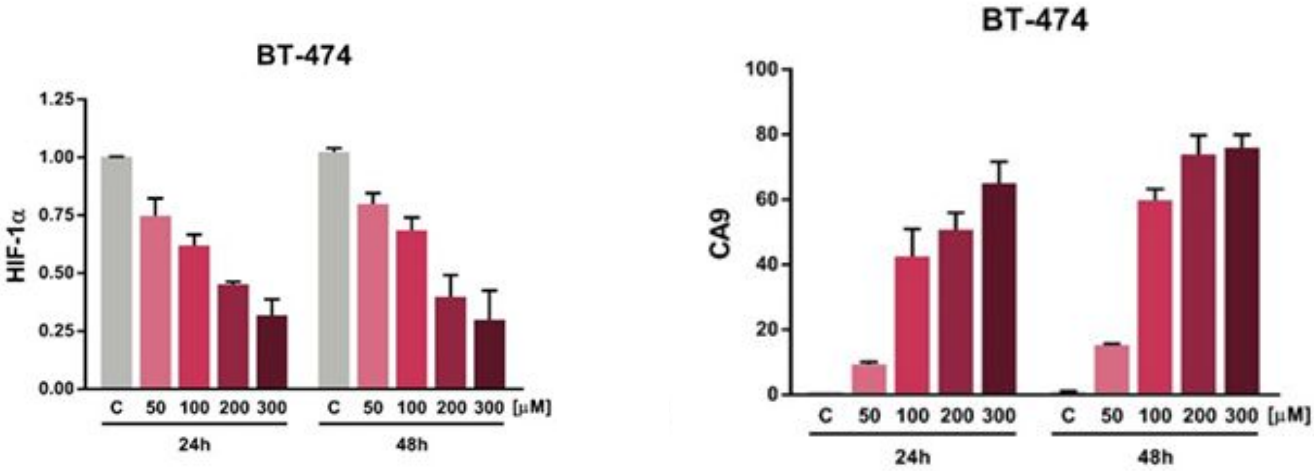
Figure 1 contains two graphs that illustrate protein expression of HIF1α and CA9 in the cell line MDA-MB-231. As $CoCl_2$ concentration is increased, the expression of HIF1α decreases and CA9 increases. The colors of each bar represented in the graphs depict the actual color of each group with their respective concentration of $CoCl_2$. There is minimal CA9 activity of the control during the 24 and 48 hour trial this is due to ample amounts of oxygen and therefore no hypoxic conditions, since CA9 is upregulated as hypoxia levels increase-decreasing the levels of oxygen. The human breast cancer line, MCF-7 was derived from the pleural effusion from a 69 year old female suffering from invasive lobular carcinoma.

Figure 2: HIF1α and CA9 Expression in MCF-7



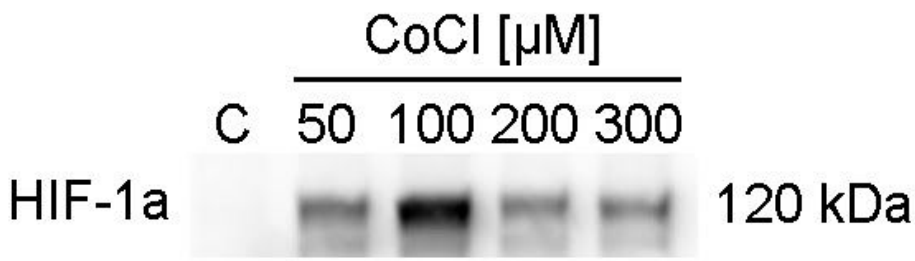
These two graphs illustrate the expression of HIF1α and CA9 in the cell line MCF-7 and shows that as concentration of $CoCl_2$ is increased, the expression of HIF1α decreases and CA9 increases. This further confirms the relationship found in MDA-MB-231.

Figure 3: HIF1α and CA9 Expression in BT-474



These two graphs indicate the expression of HIF1α and CA9 in the cell line BT-474. Once again we observe that as $CoCl_2$ is increased, the expression of HIF1α decreases and CA9 increases. The control group in CA9 had little to no expression between the two 24 and 48 hour trials.

Figure 4. Western Blot of the protein expression of HIF1α in the MDA-MB-231



This Western Blot indicates an upregulation in proteins in MDA-MB-231. At 100μM there is a large amount of protein that is expressed HIF1α therefore depicting the darker shade. This indicates that the proteins depicted the opposite trend from the qPCR which depicted a downward trend for the expression of HIF1α.

Discussion

The purpose of this experiment was to observe the effects of $CoCl_2$ induced hypoxia on the expression of HIF1α gene and the downstream marker CA9 on MDA-MB-231, MCF-7 and BT-474 breast cancer cell lines. The hypothesis stated that as the concentration of $CoCl_2$ increases, the expression of HIF1α will decrease and there would be an upregulation on CA9. This research will help better understand the general role of hypoxia on these two specific genes —HIF1α and CA9 and how these genes affect tumor angiogenesis.

These results were supported by other scientists who found similar results for different types of experiments. One such study, conducted by the international journal of radiation biology, found that there was an upregulation of CA9 and a downregulation of HIF1α while testing for role of HIF1α, HIF2α and CA9 in HNC resistance to accelerated and hypofractionated radiotherapy (Koukourakis 2009). Another study conducted in the novel *Current Cancer Drug Targets*, analyzed the expression of CA9, CA12, HIF1α,, EPAS1, SCL2A1 and VEGF genes in hypoxia and normoxia (Cruzeiro 2018). The study concluded an upregulation in CA9.

There were numerous complications that occurred during this experiment. During the distribution of specific and small quantities, fewer quantities or more quantities may have been added, possibly altering the results. Another limitation, is a small number of trials, and resulted in no significant trend and therefore the trials had to be repeated multiple times to provide for a significant trend.

Results indicated that $CoCl_2$ in the tested concentrations (50-300 μM) downregulated HIF1α mRNA expression, but upregulated HIF1α protein levels. This study found that $CoCl_2$ significantly upregulates CA9 mRNA expression in a concentration-dependent manner. Further

analysis demonstrated that CoCl2 at concentrations between 50 to 100 μM is the most optimal as it provides the most expression of both CA9 and HIF1α, thus providing the evidence for conducting future experiments using these concentrations and time intervals. The significance of this study lies in the fact that the tumor microenvironment of these cell lines are optimal between the concentrations 50 to 100 μM. This can aid in future studies, in discovering a contributing factor to the onset of cancer and how it can be treated as both genes play a role in tumor angiogenesis. The results of this experiment can aid in understanding the role of hypoxia in cancer metastasis and also provide for a mechanism, in future studies to reverse the hypoxic conditions of patients who have cancer.

Conclusion

The results indicate that in human breast cancer cells, concentrations of 50μM and 100μM of $CoCl_2$ significantly induced HIF1α expression.The expression of HIF1α decreased as the expression of CA9 increased as the concentration of CoCl2 increased. Thus, the expression of both genes were $CoCl_2$ concentration dependent.

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