Research Plan

Title: The Effects of Hypoxia on hypoxia-inducible factor 1α (HIF-1α) and carbonic anhydrase 9 (CA9) expression in various breast cancer cell lines

Category: Biomedical and Health Sciences

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A. **Rationale:** Breast Cancer is a disease which affects 245,000 women and 2,200 men in the United States yearly. Hypoxia is a condition of low oxygen and is known to promote cancer growth and metastasis. Hypoxia can be artificially induced by exposing Breast Cancer cells to $CoCl_2$, a chemical that directly induces the expression of hypoxia-inducible factor 1α (HIF-1α). The aim of this study is to investigate the optimal conditions for $CoCl_2$ treatment of breast cancer cells in vitro. Using quantitative RT-qPCR and Western blot analysis, mRNA will be measured as well as protein expression levels of HIF-1α and its downstream target carbonic anhydrase 9 (CA9) in various breast cancer cells (MCF-7, MDA-MB-231 and BT-474). During the trials, breast cancer cells will be treated with various concentrations of $CoCl_2$ (50, 100, 200, and 300 μM) for a period of 24 and 48 hours. Results showed that $CoCl_2$ in concentration range 50 to 100 μM for 24 hours modulates HIF-1α and CA9 expression.The results will depict that $CoCl_2$ in the tested concentrations (50-300 μM) downregulated HIF1α mRNA expression, but upregulated HIF1α protein levels. This research will help better understand the general role of hypoxia on two specific genes —HIF1α and CA9 and can potentially lead to the possibility of using target gene therapy to treat cancer.

B. **Research Question:** What role does the effects of cobalt chloride induced hypoxia on various breast cancer cells play on the expression of HIF1a and CA9 genes?
Hypothesis: As the concentration of CoCl2 increases in MDA-MB-231,MCF-7, BT-474, the expression of HIF1a should increase and the expression of CA9 should decrease.

C. Procedure: Three types of cell lines: MDA-MB-231, BT-474 and MCF-7 will be used for determining the effects of hypoxic conditions on HIF1α and CA9. MDA-MB-231 is an epithelial human breast cancer cell line that will be 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 will measure the expression of HIF-1α and its downstream target carbonic anhydrase 9 (CA9). These tissues will be given by the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University in Baltimore, Maryland as well as purchased from ATCC. 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 will be changed every other day. To change the media, a vacuboy will be used. Then, using a 10 ml pipe, 10ml of media will be placed into each petri dish. The next method utilized will be splitting the cells. Before any data collection analysis technique the cells had to split. To be split, 4 uL of Trypsin will be added to each plate, this will be to break the clusters of cells from the bottom of the plate and allow them to be loose in the solution. These cells will be incubated at 37°C

in the incubator for no more than 10 minutes. The cells will be then observed under the microscope, to confirm that the cells had been broken from their clusters. 10mL of media will be 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 will be transferred to sterile 15 ml conical tube and centrifuged at 491rpm for 5 min. The suspended cells will be then broken by plunging repeatedly. Using a 10uL surgical pipette and a vacuboy, media will be 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 will be obtained after splitting the cells and instead of using a surgical pipette to place the media into new petri dishes, 10uL of the mixture will be placed on a microscope slide along with 10uL of Trypan Blue. In the 4 boxes that will be depicted in the microscope, the cells will be counted and multiplied by the desired amount for the trial that will be conducted.

qPCR Analysis

1. 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 will be extracted from each set of the petri dishes. Using TRIZOL, an acid-guanidinium-phenol based reagent, to break the cellular membrane, contents will be extracted and dispensed, as the RNA will be 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 will be centrifuged for periods of 5 and 10 minutes according to Albumin standards.
2. For the RNA to be analyzed, each well on the PCR plate will be 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 will be added. 0.5uL of nuclease-free H_2O per well will be added. Finally 1uL of the forward primer and backwards primer per well will be added. This will be calculated by multiplying all these quantities by the amount of wells. Utilizing a 20uL surgical pipette, 15uL will be 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 will be utilized accordingly to the Albumin standards. 10uL of the RNA sample will be then added to each well corresponding to the level of concentration of $CoCl_2$. Subsequently the PCR plate will be sealed with PCR plastic wrap and centrifuged at 4000 rpm for 3minutes. The plate will be inspected for air pockets which could skew the results of the analysis. After the air pockets will be removed, the software Quantstudio will be used to set the guidelines of the PCR plate. The PCR plate will be placed in the qPCR machine and the analysis will be started.
3. After the analysis is complete, the data from the computer will be put into tables and T-tests and an ANOVA test will be conducted to find the significance of the data. This will be repeated for optimal results.

Western Blot Analysis

Western Blot analysis will be utilized as a method of analyzing the expression of HIF1α and CA9 in proteins. To normalize the proteins, Albumin standards will be prepared. BCA Protein will be 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 will be prepared and 200uL of the BCA working reagent will be added into each tube. Since proteins come in high concentrations, sterile water will be added to dilute the sets in the Albumin standards of 1:5 in a different set of 10 tubes. 10uL of Albumin standards will be added to the first set of tubes containing the BCA working reagent. This will be repeated for each tube of the set.

10uL of the diluted protein sample will be then added to the second set of tubes (1 set is 10 tubes). Each sample will be incubated to promote the homogenous mixing of its contents. These tubes, containing the diluted protein, will be incubated at 37°C for 10 minutes, these tubes will be cooled down to room temperature to prevent protein degradation. During the waiting period of the protein cooling down, the Nanodrop spectrophotometer will be set up. This device analyzed the protein to detect the concentration and purity of each sample. After the protein is cooled down, the protein will be 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 will be uploaded onto the computer and the samples will be 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 will be mixed. The buffer & protein solution will be incubated at 95°C for 10 minutes. Finally, gel electrophoresis will be conducted to get the images of the protein.

- **Gel Electrophoresis(used in preparing the western blot)**

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

Care will be 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 will be run at 180 Volts and 120mA for 30-45 minutes. Once completed, the gel plate will be opened carefully in order to prevent fracture of the gel. Next, the gel will be trimmed, cutting the loading area. Then, using Albumin standards, the transfer housing will be prepared and all the components will be soaked with transfer buffer. The tank will be filled with transfer buffer and run at 10 volts and 200mA for 1 hour. After the second run will be completed, the membrane will be removed from the transfer housing, will behed with water and placed in blocking buffer.

The plate containing the blocking buffer will be incubated for 5 minutes. Subsequently, 10mL of the primary antibody (GAPDH) will be prepared by diluting the primary antibody (GAPDH) in blocking buffer in the standards of 1:1000. The membrane will be placed in the primary antibody and will be left in the cycler for 2 hours. The cycler spun the tube so the primary antibody would evenly spread throughout the membrane.

The membrane will be taken out and chemiluminescent will be 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 will be set up and after 5 minutes of the membrane in the solution, it will be placed for imaging. Periods of 10, 15 and 60 seconds will be utilized to process the image. This process will be repeated numerous times for optimal results.

Statistical Analysis and Graphing:
Statistical analyses will be conducted. For the T-test, ANOVA, and determining the level of significance, a software called Graphpad will be used. The T-test will be 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.

Data Analysis: For this data analysis, qPCR, western Blots. For qPCR analysis, RNA will be extracted by TRIzol reagent and chloroform and precipitated by iso-propanol. Quantitative PCR investigations will be performed by utilizing PowerUp SYBR Green Master Mix and QuantStudio 3 Continuous PCR System (Applied Biosystems).
Statistical analysis: Statistical analysis will be performed using GraphPad Prism 7 software (GraphPad). Statistical analyses will be performed by one-way ANOVA and t-test. Results will be accepted as statistically significant if the p value will be less than 0.05. Each test will be performed multiple times in duplicates, and for accuracy, each example will be run twice.

Risk and Safety: There will be many risks involved since the experiment used potentially hazardous biological agents. For example, Cobalt chloride is a carcinogenic chemical used to induce artificial hypoxia, and whenever it will be utilized gloves had to be worn. Another example is trizol which is a caustic chemical used to break the nuclear membrane of cells. For utilizing that chemical proper equipment and gloves will be used.

D. Bibliography:

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3. Potentially Hazardous biological agents research:

1. The human breast cancer (MCF-7, MDA-MB-231, BT-474, and T-47D) cell lines used for this research are categorized under biosafety level 1 (BSL-1). These cell lines will be obtained from the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins University in Baltimore, Maryland and will also be purchased from ATCC. The risk assessment process aided in the determination of the biosafety level 1, as these cells are not known to cause diseases in adults and children. Thus these cells present minimal potential hazards which falls under the BSL-1 containment.
2. For this research, all potentially hazardous biological agents will be utilized under a ventilated bio-safety cabinet as well as the utilization of proper personal protective equipment (PPE) (lab coat, bio-safety and chemical grade gloves) and for all hazardous waste, it will be properly disposed of in a PK Impex hazardous will bete container.

4. Hazardous chemicals, activities & devices:

1. The risk assessment process concluded that these cells are not dangerous to humans and therefore can be deemed as Biosafety-Level-1**.** Some of the chemicals that will be used are chloroform, trizol, methanol, ethanol, DMSO, and Cobalt Chloride. During the utilization of potentially hazardous biological agents, supervision will be always present along with safety procedures to prevent any

accidents or injuries. Methods of disposal include but are not limited to when handling all cell lines, such as working in biosafety cabinet (cell culture hood), using proper personal protective equipment (PPE) (lab coat, bio-safety and chemical grade gloves), and proper decontamination and disposal procedures (chemical disinfection of all working surface and glassware) and proper disposal of biological materials according to the laboratory regulations. All experiments will be performed under the Laboratory Director supervision.

- No changes were made to the experiment.