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A Tibetan Allele of *EPAS1* as a Model for Investigating the Role of HIF-2α in Ewing Sarcoma

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Abstract

Hypoxia is a hallmark symptom of solid tumors, including Ewing Sarcoma (EwS), which promotes tumor progression through a variety of complex biochemical and genetic pathways. The development of hypoxic tissues in tumors activates hypoxia inducible factors (HIF) which are believed to increase therapy resistance and metastasis. Endothelial Per-Arnt-Sim (PAS) domain-containing protein 1 (*EPAS1*) is known to encode an understudied HIF subunit called HIF-2α which may be a potential target for future therapies. A Tibetan population living in the Himalayan belt at high altitude has been shown to possess a Denisovan variation of the *EPAS1* gene with reduced expression that contributes to their population's hypoxia resistance. The relationship between thriving under hypoxic conditions and reduced expression of *EPAS1* suggests this gene may be significantly involved in progressing hypoxic cancers. The objective of this study is to investigate the function of the *EPAS1* gene under hypoxic conditions and its role in promoting cancer using the Tibetan allele as a convenient model for reduced expression. Three phases of experiments investigating tumor growth, therapy resistance, and metastasis will be performed using spheroid models and scratch wound analysis on EwS cells transfected with the Tibetan allele of *EPAS1* (*EPAS1t*). It is expected that reduced *EPAS1* expression will correlate to increased tumor growth, therapy resistance, and cell migration. These results would highlight *EPAS1*, and consequentially HIF-2α, as a biomarker for patient prognosis and a potential target for future therapies. Future studies may wish to expand this experiment by repeating the study with alternative cell lines, using animal models, or investigating the impacts of upregulated *EPAS1* in hypoxic tumors.

Keywords: hypoxia; Ewing sarcoma; HIF-2α; *EPAS1*; solid tumors; Tibetan *EPAS1*; chemotherapy resistance; metastasis

Introduction

Ewing Sarcoma (EwS) is an aggressive pediatric bone cancer which commonly exhibits hypoxic regions [1]. Considering that a quarter of EwS patients relapse after treatment, and that few options exist for treating metastatic disease, investigating the mechanisms progressing this disease is clearly necessary to improve patient outcome [2]. Hypoxic microenvironments are characteristic of solid tumors and are the consequence of irregular vascularization from excessive cell proliferation [3–5]. Hypoxia is a marker for poor prognosis in EwS patients as it increases therapy resistance and metastasis by reducing anti-tumor immunity and modifying tumor metabolism [1, 4, 5]. As distance from functioning vessels increases, the tissue becomes increasingly hypoxic [4]. Established hypoxic conditions in the tumor microenvironment (TME) activates hypoxia inducible factors (HIF) which help tumors evade the immune system, resist treatment, alter metabolism, and metastasize [3, 6]. HIFs are heterodimeric proteins made of two subunits, alpha and beta, which regulate hundreds of genes involved in an array of cellular and physiological processes [6]. The alpha subunit is one of three different proteins, HIF-1α, HIF-2α, or HIF-3α, whereas the beta subunit is one constitutively expressed protein, HIF-1β [4]. In tumorous tissues, HIFs play an extensive role in allowing cancer cells to persist under hypoxic conditions [4]. Under normoxic conditions, HIF-α subunits are quickly degraded by prolyl hydroxylase domain containing enzymes (PHDs) via hydroxylation [5]. Hydroxylation prevents dimerization and causes the HIF-α subunit to bind with Von Hippel-Lindau (VHL) proteins, signaling for their degradation by proteasomes [5, 6]. Under hypoxia, hydroxylation is not possible and the HIF-α subunit enters the nucleus, binding with HIF-1β before acting on HIF dependent genes [6]. High levels of HIF-1α is known to increase cancer progression, mortality, and therapy resistance [5, 6, 8]. While the literature extensively describes the role of HIF-1α, little is reported on the role of HIF-2α in hypoxic tumors [5, 6, 8]. Endothelial Per-Arnt-Sim (PAS) domain-containing protein 1 (*EPAS1*) encodes for HIF-2α and may be key in understanding the effect of hypoxia on EwS [7]. As such, studies investigating the expression of *EPAS1* would be valuable to explore HIF-2α's impact in hypoxic tumor progression.

HIFs help tumors evade anti-tumor immune responses and respond to oxidative stress. The mechanism by which hypoxic tumors alter the immune system is complicated, allowing the tumor to evade immune responses and increase therapy resistance [5]. HIFs influence the activity of immune system components including natural killer cells (NK), Tregs, dendritic cells (DC), B cells, and more [5, 6]. NK cells are excluded from hypoxic TME whilst the number of Tregs is increased [5]. This increase in immunosuppressant Treg cells has shown to have a positive and necessary relationship with HIF-2α expression [5]. HIF-1α creates tolerogenic dendritic cells and can promote B cell immaturity which contributes to the suppression of the immune response in tumors [5]. Ultimately, the increased expression of HIFs under hypoxic tumor conditions is correlated with an impaired immune response, allowing the cancer cells to evade the immune system and progress [5, 6]. Not only can cancer avoid the immune system, using HIF's it converts the cell's primary metabolism for survival under hypoxic conditions [5, 6, 8]. HIF-1 mediates the change from oxidative to glycolytic metabolism to prevent buildup of reactive oxygen species (ROS) from oxidative phosphorylation in the mitochondria under hypoxia [6, 8]. HIF-1 induced regulation factors prevent the conversion of pyruvate to acetyl coenzyme A for the citric acid cycle and instead convert pyruvate to lactate to enter the lactic acid cycle [6, 8]. Changes in mitochondrial morphology have also been observed in hypoxic TME, further reducing the ability of tumor cells to perform oxidative phosphorylation [6, 8, 9]. Due to the high rate of proliferation, cancer cells tend to consume large amounts of glucose and consequentially will produce excess lactate and H+ [6]. These products are excreted into the extracellular matrix which contributes to immunosuppression and therapy resistance [6]. Ultimately, the ability of HIFs to modify metabolic processes contributes to cancers ability to persist under hypoxia.

HIFs play a large role in radiation and chemotherapy resistance. Hypoxic tumor cells are 2-3x more likely to survive ionizing radiation than normoxic tumor cells [10]. While radiation is capable of directly breaking DNA, it more often relies on water radiolysis to create ROS that are more effective at killing cells [9, 10]. The reduction of ROS due to HIF mediated factors hinders the efficiency of radio and chemotherapies [10, 11]. Furthermore, due to the hypoxia mediated change in metabolism, glycolysis associated pathways such as the pentose phosphate pathway (PPP) are activated [9]. PPP promotes increased regeneration of glutathione (GSH), a natural antioxidant, further reducing the deadliness of ROS [9]. Additionally, DNA that is damaged under normoxia conditions can be stabilized, leading to cell death [9]. Under hypoxia this stabilization is not possible, and spontaneous repair is more probable, making DNA damage less lethal [9]. Similarly to radiation, chemotherapeutic agents depend on oxygen for optimal performance [11]. HIF-1α has been shown to facilitate drug resistance in many cancers through drug efflux, autophagy induction, apoptosis inhibition, and more [11, 12]. While an abundance of literature explores the genetic and biochemical pathways mediated by HIF-1α impacting therapy resistance, little is reported on the role of HIF-2α in this context [11, 12]. Considering negative impacts of HIF-1 and related pathways on treatment efficiency, they seem to be an obvious target for new treatments to improve patient outcomes [6]. However, very few HIF-1 inhibiting treatments have been successful due to their high level of toxicity for humans or failure in human trials [6]. As such there is a need to further investigate the impact of HIF expression, with particular interest in HIF-2α, in cancer progression, therapy resistance, and metastasis.

Tibetan populations living in the Himalayan belt at high altitudes show greater hypoxia resistance than low altitude populations [13]. This resistance is attributed to a unique allele of *EPAS1*, believed to be inherited through past hybridization with Denisovan populations [13]. This advantageous variation shows reduced expression of *EPAS1* which reduces hemoglobin levels and increases resistance to polycythemia, the overproduction of red blood cells as a response to hypoxic stress [14]. This trend of thriving under hypoxic conditions and reduced expression of *EPAS1* suggests this gene may be involved in the progression of hypoxic cancers [14]. In non-small cell lung cancer (NSCLCs), *EPAS1* was found to be down regulated through a negative feedback loop [7]. The relationship between down regulation of *EPAS1* and hypoxia resistance was confirmed using transgenic mice [14]. In some lung cancers, *EPAS1* is downregulated through the methylation of a GC rich region in the promoter region by DNA methyltransferases (DNMT1) [7]. The Tibetan allele of *EPAS1* is known to be downregulated through the disrupted activity of four enhancers including enigma homolog (ENH) 4, ENH5, ENH6, and ENH8 [15]. The reduced activity of *EPAS1* through transcriptional control and its contribution to hypoxic resistance highlights corrective transcriptional activity to be a possible avenue for future therapies. While the role of HIF-1α in hypoxic TME has been extensively investigated, HIF-2α remains elusive regarding its effect on patient outcome due to its contradicting impact on anti-tumor immunity and hypoxia resistance [7]. While some literature suggests that the overexpression of HIF-2α may help promote tumor progression through its positive relationship with impaired immune response, other evidence implies that reduced HIF-2α is favorable for tumor growth under hypoxic conditions [5–7]. The purpose of this study is to address this contradiction by investigating the effect of the *EPAS1* gene under hypoxic conditions and its role in cell proliferation, therapy resistance, and metastasis by using the Tibetan *EPAS1* (*EPAS1t*) allele as a model for reduced expression. It is expected that reduced expression of HIF-2α should increase the tumor's ability to progress under hypoxic conditions and decrease its sensitivity to treatment.

If a correlation between tumor progression and reduced HIF-2α is found, then the Tibetan model may provide a convenient mechanism for exploring the impact of reduced *EPAS1* expression on genetic and biochemical pathways in hypoxic tumors.

Methods

Producing Tibetan *EPAS1* Tumor Cell Lines

Homologous recombination will be used to replace *EPAS1* alleles in SK-ES-1 Ewing sarcoma (EwS) cells with the Tibetan allele (*EPAS1t*) [16]. Upstream and downstream homologous regions, *EPAS1t*, SacB (a counter selection gene) and BleoR (an antibiotic resistance gene) will be amplified independently using polymerase chain reactions (PCR) with taq polymerase (10342053, Invitrogen™) [17, 18]. All genetic material can be purchased from Invitrogen and the cell line will be purchased from the American Type Culture Collection (ATCC). A negative control will be used to ensure selective amplification of the desired genetic material from the PCR. A negative control will contain all the components of the PCR reaction, except the template cDNA, denoting it as a non-template control. The identity of each amplified product will be confirmed using gel electrophoresis before purification. After the DNA products are cleaned, the ends of each product will be digested with restriction enzymes to create overhangs which, once ligated, will produce the desired plasmid as seen in Figure 1. SK-ES-1 EwS cell line (wild type EwS cells) will be transfected with the produced plasmid via heat shock at 42°C for 30 seconds before overnight incubation at 37°C [19]. The transfected cells will be grown on agar plates in the presence of an antibiotic, zeocin, and glucose to select for positive clones and establish a cell library. Optimum antibiotic concentration may be determined by a titration panel, testing three concentrations of 100 µg/mL, 200 µg/mL, and 300 µg/mL for 1 week. The inclusion of SacB gene as a counter selector on the plasmid will help ensure selection for two recombination events and the replacement of the wild type *EPAS1* gene with *EPAS1t* [18]. To check the efficiency of the antibiotic, wild type EwS cell lines will be grown in the presence of the antibiotic for two weeks as an additional control. To confirm successful replacement of *EPAS1*, some cells from the positive recombinants will be lysed with the freeze thaw method and their DNA isolated. PCR will be used to selectively amplify the *EPAS1t* and BleoR region by placing primers in the flanking homologous regions. The amplified products will be run on gel electrophoresis to check for positive recombination by comparison to *EPAS1* from wild type cells which will be amplified by the same primers.

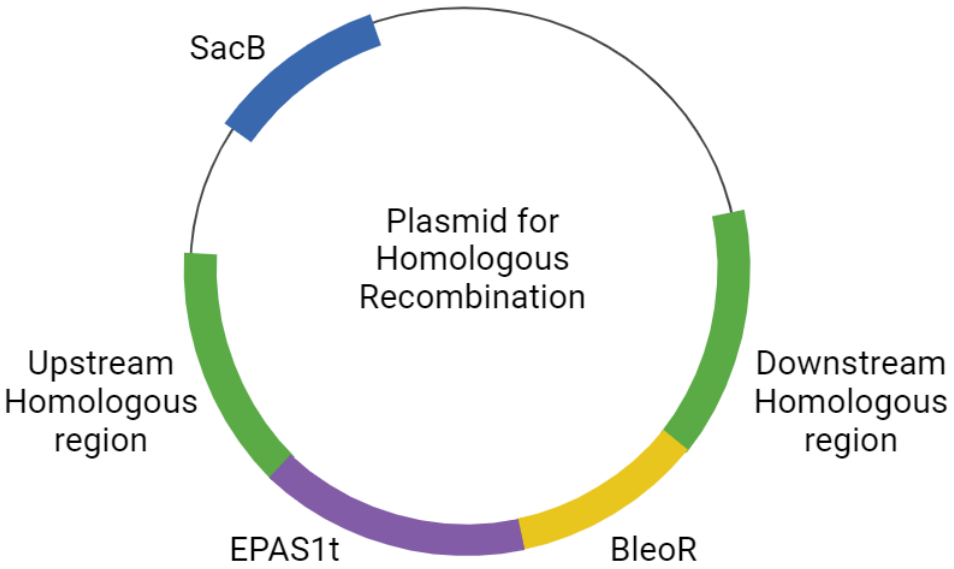

Figure 1. Plasmid for HR in SK-ES-1 cells to replace *EPAS1* with *EPAS1t* and BleoR. Figure created on BioRender.com.

Confirming *EPAS1* Expression in Cell Libraries

Expression levels of *EPAS1* in transfected cells will be compared to wild type cells using qPCR [20]. EwS cells from the transformed and wild type cell lines will be lysed with a freeze thaw method before isolating the RNA with Invitrogen TRIzol reagent and PhasemakerTM Tubes [21, 22]. The quantity of RNA will be checked using Nanodrop before the addition of 1 µL DNase I (AM2222, Invitrogen™) for up to 10 µg RNA in a 50 µL reaction followed by incubation at 37°C to clean the RNA of any residual DNA. The isolated RNA will be reverse transcribed using a reverse transcriptase enzyme to produce cDNA before the addition of *EPAS1* specific primers. Primers were designed according to Li et al., 2019 [23]. Primers for a reference gene, such as Beta-actin, will also be included. The cDNA will then be amplified using a High-Capacity cDNA Reverse Transcription Kit (4368814, ThermoFisher Scientific)

with the addition of sybrgreen (S7563, Invitrogen™) and the fluorescence will be monitored. The time to reach the threshold of fluorescence will be recorded as the cycle threshold (CT) value which has an inverse relationship with *EPAS1* expression [21].

Producing Spheroids and Observing Growth Rates

Spheroids will be grown using the Liquid Overlay Technique (LOT) [24, 25]. Both transfected and wild type cells will be collected and cultured in low adhesion 96-multiwell plates for 24 hours at an initial concentration of 20,000 cells per 100µl [24, 26]. After culturing is complete, 50µl of complete media will be added. For maintenance, 2/3 of the media volume will be replaced every 2-3 days [26]. Growth of transfected and wild type cells will be monitored using transmitted light microscopy and MetaXpress software from ImageXpress Pico system to quantify spheroid size and morphology [24-27]. Images of the cells should be taken every 3 hours for the first 168 hours which can be done automatically by the MetaXpress software. Differences in growth rates between the transfected and wild type EwS cells will be recorded.

Testing Therapy Resistance

Spheroids grown in the previous method will be used to compare therapy resistance between transfected and wild type EwS cells. Spheroids of wild type and transfected EwS cells will be divided into three groups and treated with Cyclophosphamide, a chemotherapy used to treat EwS, of increasing concentration for 72h [28]. The first group will be treated with 10µM to mimic more physiologically relevant doses. The second group will be treated with 50µM to observe more noticeable cytotoxic responses. The final group will be treated with 100µM to possibly induce more significant cytotoxic responses. Cell morphology and viability will be monitored using transmitted light microscopy and MetaXpress software from ImageXpress Pico Systems [24-26]. The rate of cell death for each respective cell type and treatment concentration will be observed using a Caspase assay (C10723, Invitrogen™) per the manufacturer's instructions and reported as a marker for therapy resistance.

Scratch Wound Assay

A Scratch Wound Assay will be used to measure and compare the migration of transfected to wild type EwS cells [29]. 60mm dishes are prepared by coating with extra cellular matrix (ECM) substrates before incubation overnight at 4°C [29]. Unbound ECM will be removed using a PBS wash [29]. Trypsin will be used to detach transfected and wild type EwS cells from their original culture plates before replating these cells onto the prepared dishes [29]. These cells must be plated and incubated to create a confluent monolayer [29]. After the monolayer is produced, a straight line will be scratched through each dish with a pipette tip [29]. Detached cells are washed away with PBS before replenishing the ECM matrix [29]. The cells are then incubated at 37°C for 24h hours to allow the cells to migrate [29]. The cells will be washed again with PBS before fixation with 3.7% paraformaldehyde for 15 minutes [29]. The cells will then be stained using 1% crystal violet in 50% ethanol for 10 minutes before imaging under a phase contrast microscope [29]. Computer software such as ImageJ can be used to compare images of the cells at time zero to the final time to determine the rate of migration (Rm) and the percentage of wound closure [29]. Rm and percentage of wound closure for each cell type will be recorded. Figure 2 visualizes the experimental workflow.

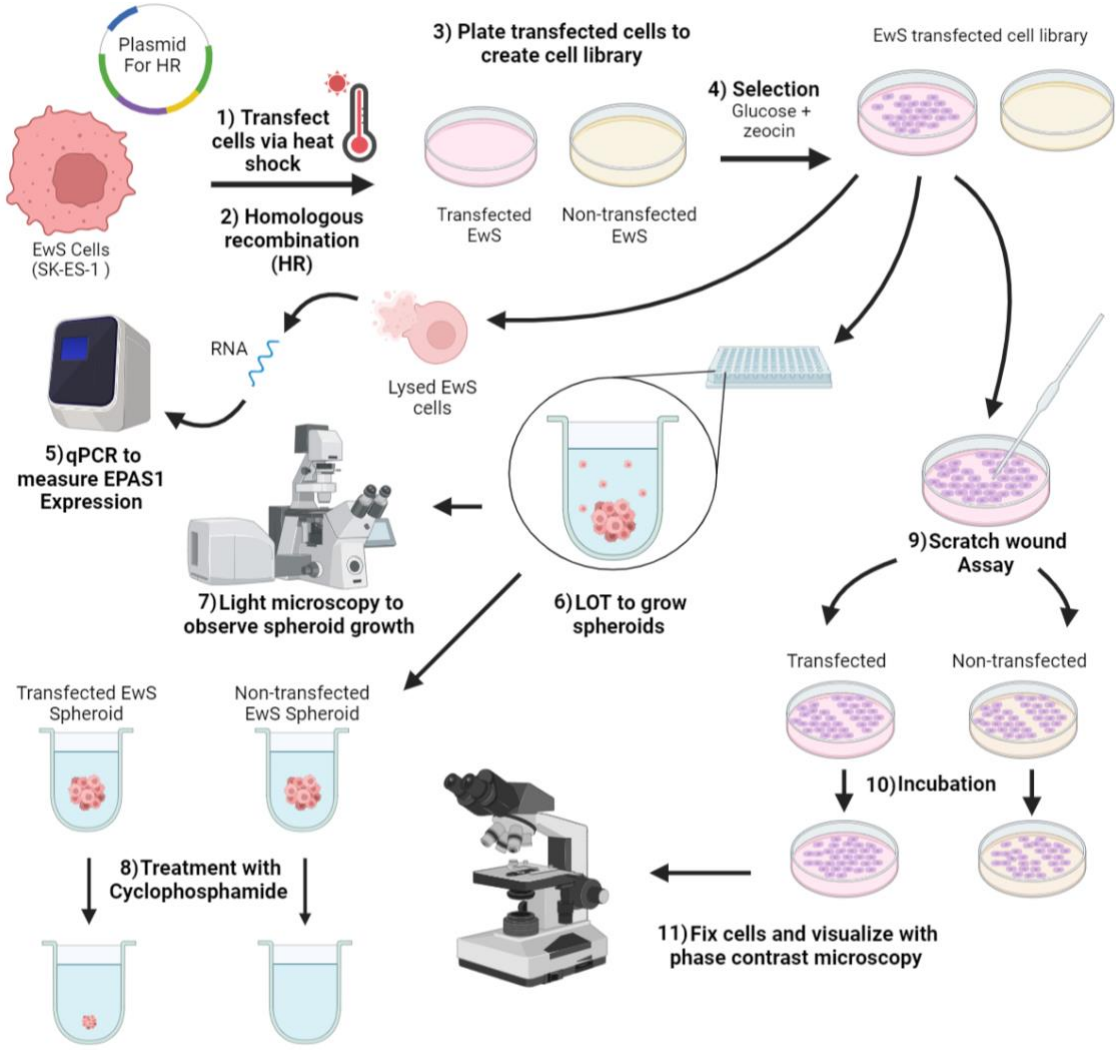

Figure 2. Visual summary of experimental workflow to produce *EPAS1t* transfected EWS spheroids (1-5) and compare their growth (6-7), therapy resistance (8), and cellular migration (9-11) to wild type EWS. Figure created on BioRender.com.

Results

Producing Tumor Cell Lines

Due to the presence of zeocin and glucose on the agar plates, only transfected cells that have undergone two successful recombination events to replace *EPAS1* with *EPAS1t* and BleoR will survive. Cells that have not undergone any recombination events will die as they have no antibiotic resistance to zeocin from BleoR [17]. Cells which have only undergone one recombination event will die due to the presence of glucose and SacB, which transcribes enzymes that convert glucose to a toxin [18]. As such we expect to see colonies present on the plate with transfected EwS cells and no colonies present on the control plate with wild type EwS cells. DNA extracted and amplified from the successfully transfected colonies will be used to confirm positive recombination. It is expected that the amplified product from the transfected cells, which will include both *EPAS1t* and BleoR, will be longer than the amplified region from wild type EwS cells as it will only include *EPAS1*. This difference in size can be observed when the amplified products are run on gel electrophoresis.

Confirming *EPAS1* Expression in Cell Libraries

It is expected that the transfected EwS cells will have higher CT values than wild type EwS cells as it will take longer for the fluorescence to reach the threshold level when starting with reduced mRNA. The CT values will confirm that *EPAS1t* is a successful and convenient model for reduced expression of *EPAS1* as it will indicate lower mRNA content in the transfected cells than the wild type EwS cells.

Spheroid Growth Rates

It is expected that the transfected EwS spheroids with *EPAS1t* will have higher growth rates than wild type EwS cells. This will be observed by higher rates of diameter growth in the transfected spheroids than wild type spheroids.

Therapy Resistance

For both the transfected and wild type EwS spheroids it is expected that the rate of cell death will increase as Cyclophosphamide concentration increases. Due to the reported trends of increased therapy resistance under hypoxic conditions and increased hypoxia resistance with reduced *EPAS1* expression, it is expected that the transfected spheroids will exhibit greater therapy resistance than wild type spheroids [9, 10]. This will be represented by higher cell death, as indicated by the intensity of floral signals from the caspase assay, in wild type spheroids in comparison to transfected spheroids at equal concentrations of Cyclophosphamide. It is furthermore expected that higher cell death will be observed in spheroids exposed to higher concentrations of Cyclophosphamide for both transfected and wild type spheroids. The expected trends are illustrated in Figure 3.

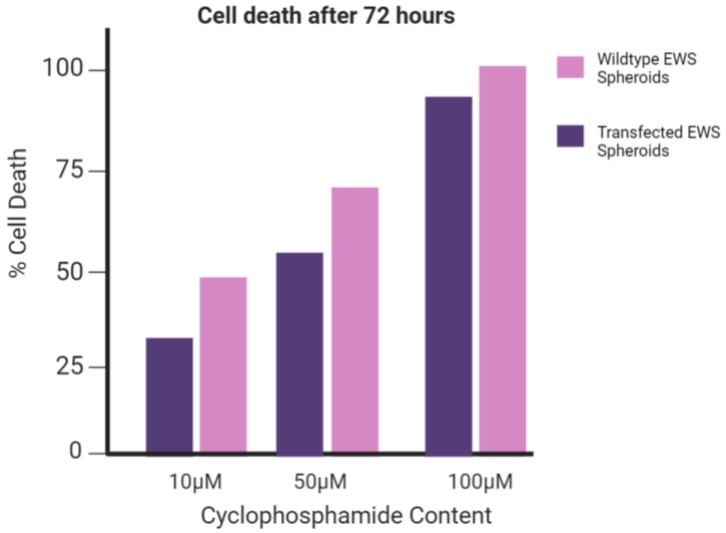

Figure 3. Expected differences in cell death between wild type and transfected spheroids at different concentrations of Cyclophosphamide exposure after 72h. Figure created on BioRender.com.

Scratch Wound Assay

Considering the correlation between reduced *EPAS1* and hypoxia resistance, it is expected that the transfected EwS cells will showcase higher RM and greater wound closure in comparison to the wild type EwS cells.

Discussion

EwS is an aggressive childhood bone cancer in which its solid tumors typically contain hypoxic microenvironments which elicits the expression of HIF's [1]. While the role of HIF-1α in tumor progression and therapy resistance has been substantially investigated, the role of HIF-2α remains elusive [1, 11, 12]. The goal of this study is to investigate the effects of HIF-2α in hypoxic tumors using a Tibetan allele of *EPAS1* as a model for reduced HIF-2α expression. While increased HIF expression is considered favorable for tumor growth, the Tibetan populations hypoxia resistance suggests that reduced HIF-2α may promote tumor progression under hypoxic conditions [6, 7, 9, 10, 14]. This experiment aims to resolve this contradiction and establish the influence of HIF-2α expression on tumor growth, therapy resistance, and metastasis in EwS by comparing spheroid growth rates, therapy resistance, and cell migration between wild type EwS cells and EwS cells transfected with *EPAS1t*.

Spheroids are an excellent model for investigating solid tumors as they closely mimic the hypoxic TME and are suitable for high throughput analysis in drug screening [25]. Comparing spheroid growth rates of transfected and wild type EwS cells is indicative of the effect of *EPAS1* expression on tumor growth. If the expected results would indicate that tumors with reduced *EPAS1* expression, and lower HIF-2α, are more aggressive. These results highlight HIF-2α as a poor indicator for patient prognosis and further identifies *EPAS1* as a possible target for upregulation to slow the advancement of EwS in future therapies.

Spheroids will be treated with Cyclophosphamide, a common chemotherapy used to treat EwS, to assess the difference in therapy resistance between transfected and wild type EwS cells [28]. EwS tumors typically showcase high levels of therapy resistance with approximately quarter of patients relapsing post treatment [2, 3, 10]. If *EPAS1t* transfected spheroids exhibit higher therapy resistance, then low HIF-2α expression may again be a marker for poor prognosis in EwS patients. Additionally, the results of this experiment will provide insight into the role of HIF-2α in therapy resistance specifically. While the effect of HIF-2α is reported to have a positive and necessary relationship with Treg production, further studies in this area may seek to expand upon the effect of HIF-2α on anti-tumor immunity and its impact in chemotherapy resistance [5]. If

this study highlights a correlation between reduced HIF-2α and increased therapy resistance, this suggests that HIF-2α expression may have a multifaceted impact on tumor therapy. Further investigation into the interaction of HIF-2α and anti-tumor immunity may be required to support the inhibition or activation of HIF-2α as a possible treatment for hypoxic tumors as the spheroid model does not account for immunological factors. Future studies may wish to investigate the effect of reduced HIF-2α on anti-tumor immunity using animal models or may knock out EPAS1 all together to determine if the gene is necessary for cellular functions related to the progression of cancer and therapy resistance.

The third phase of experimentation aims to establish the effect of reduced *EPAS1* expression on metastasis using a scratch wound assay to compare the rate of cell migration between transfected and wild type EwS cells. The correlation of reduced *EPAS1* and hypoxic resistance in some cancers and the Tibetan population suggests that the *EPAS1t* transfected cells will have increased rates of migration and wound closure [13, 14]. These results would be indicative of greater rates of metastasis for EwS tumors with lower HIF-2α. Consequentially, increased cell migration would further suggest that low HIF-2α is an indicator of increased tumor aggression and poor prognosis for EwS patients and may identify *EPAS1* and HIF-2α as a target for new treatments in metastasis prevention.

Due to the previously described contradictions surrounding HIF-2α and its impact on hypoxic tumors, it is not obvious that the described expected results will be observed. Opposite trends, in which reduced *EPAS1* expression elicits reduced tumor growth, increased sensitivity to chemotherapy, and reduced cell migration or wound closure, would indicate that *EPAS1* may be a target for downregulation, rather than upregulation, in future cancer therapies. As several mechanisms of *EPAS1* downregulation have been described, in the Tibetan population and NSCLC, it may be possible to explore these mechanisms of down regulation for therapy development in future studies [7, 14, 15]. When considering the results of this study and current contradictions in the literature, general dysregulation of *EPAS1* may play a significant role in the progression of hypoxic tumors rather than reduced or over expression explicitly. In this case, this experiment will provide useful information for identifying the effects of reduced *EPAS1* expression on tumor progression. Future studies may develop a model to study the overexpression of *EPAS1* in EwS to explore this hypothesis and to complement the proposed experiment. Additionally, due to the use of a single cell line, the results of this experiment may fail to be widely applicable to other cancer cell lines or types. To resolve this, future studies may wish to repeat this experiment using alternative hypoxic tumor cell lines.

Conclusions

HIF signaling pathways are known to play a major role in the progression of EwS through tumor growth, therapy resistance, and metastasis [1]. Investigating reduced *EPAS1* expression levels in EwS tumors may help describe the impact of HIF-2a on patient prognosis and identify *EPAS1* as a potential target for new therapies. Future research should continue to explore the impacts of dysregulation of *EPAS1* on tumor progression, possibly exploring the impact of over expression.

List of Abbreviations

DC: dendritic cells
DNMT1: DNA methyltransferases 1
ENH: Enigma homolog
EPAS1: endothelial PAS domain-containing protein 1
EPAS1t: endothelial PAS domain-containing protein 1 Tibetan allele
EwS: Ewing sarcoma
GSH: glutathione
HIF: hypoxia inducible factor
NK: natural killer
NSCLC: non-small cell lung cancer
PHD: prolyl hydroxylase domain
PPP: pentose phosphate pathway
ROS: reactive oxygen species
TME: tumor microenvironment
VHL: Von Hippel-Lindau

Conflicts of Interest

The author declares that they have no conflict of interest.

Ethics Approval and/or Participant Consent

Ethics approval is not required as cell lines purchased from the ATCC will be used. No human or animal participants will be involved.

Authors' Contributions

JES: Designed the study, conducted background research, drafted and edited the manuscript, and approved the final version to be published.

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