Construction of a Fluorescent Reporter Gene for the Analysis of
OGDH2 Protein Stability in Hypoxia

THESIS

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Abstract

Hypoxia is a frequent feature of the tumor microenvironment and produces a variety of cellular metabolic adaptations. Recently, the ability of cancer cells to proliferate in hypoxia was found to depend on critical regulatory changes in mitochondrial glutamine metabolism. Hypoxic glutamine metabolism is regulated in part by activation of the transcription factor hypoxia inducible factor 1 α (HIF1 α), and induction the ubiquitin ligase seven in absentia homolog 2 (SIAH2) which marks a key splice variant of the E1 subunit of the α -ketoglutarate dehydrogenase complex (OGDH2) for proteolysis. The SIAH2-mediated destruction of OGDH2 redirects glutamine metabolism toward a reductive pathway, which generates citrate and lipids to support cellular proliferation.

While OGDH2 has emerged as a critical factor in hypoxic tumor growth, the signaling pathways that regulate its destruction are poorly understood. To study OGDH2 regulation, we constructed a fluorescent reporter gene, combining a ruby red fluorescent gene with either wild type OGDH2 or a hypoxia-stable mutant of OGDH2 created by point mutagenesis of the ubiquitinated lysine residue. We stably expressed these fusion proteins in human colorectal and renal cell carcinoma cell lines. Fluorescence microscopy and Western Blot analysis confirmed the expression of the fusion protein, its mitochondrial localization, and the cytoplasmic distribution of the unmodified ruby

protein. This fluorescent reporter protein will be used to follow OGDH2 protein cellular localization and stability in hypoxia. This fusion protein will be used in an shRNA screen to identify genes required for OGDH2 destruction. Regulation of hypoxic glutamine metabolism through OGDH2 may provide additional molecular targets for novel anticancer strategies.

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Chapter 1: Introduction

Cancer is an incredibly complex genetic disease. Recent advances in genomic technologies have enabled the identification of thousands of mutational events that influence tumor development and progression (Cairns, Harris, & Mak, 2011). These genetic derangements accumulate in diverse combinations and generate a unique mutational profile for each tumor. Patterns of gene expression also vary, giving rise to tremendous phenotypic heterogeneity both among and within tumors (Burrell, McGranahan, Bartek, & Swanton, 2013).

Phenotypic variation promotes tumor evolution through natural selection. Individual clonal subpopulations differ in a multitude of features, including morphology, expression of cell surface markers and growth factor receptors, metabolism, and potential for proliferation, angiogenesis, and metastasis (Dick, 2008; Fidler & Hart, 1982; Heppner, 1984; Marusyk & Polyak, 2010; Nicolson, 1984). Given a broad assortment of morphologically and functionally diverse clones, some cells will be better equipped for survival and growth in the given microenvironmental setting. In such a scenario, the outgrowth of the fittest subpopulations is inevitable. Tumors are thus highly adaptable to their microenvironmental conditions and readily develop resistance to cancer therapies.

Microenvironmental conditions powerfully shape the selection landscape that directs tumor evolution. Malignant tissue is characterized by aberrant vasculogenesis,

which often fails to keep pace with tumor growth (Brown & Giaccia, 1998). As a result, the poorly formed vasculature is unable to uniformly supply tumor tissue with adequate levels of oxygen and cellular fuels. Additionally, inadequate removal of cellular wastes leads to the accumulation of lactate in the extracellular space (Dasu, Toma-Dasu, & Karlsson, 2003). Consequently, malignant tissue is frequently hypoxic, acidotic, and nutrient-poor (Brown & Giaccia, 1998; Milosevic, Fyles, Hedley, & Hill, 2004). These adverse microenvironmental conditions exert strong selection pressures, leading to the emergence of an adaptive phenotype.

Hypoxia is an especially pervasive feature of the tumor microenvironment, and arises when the cellular demand for oxygen exceeds levels supplied by the circulation (Nordsmark, Overgaard, & Overgaard, 1996; Overgaard & Horsman, 1996). This is particularly common in malignant tumors, since unrestrained proliferation increases cellular energetic demands, while the oxygen supply is reduced by an aberrant vasculature (Brown & Giaccia, 1998). When oxygen levels are low, cancer cells must undergo significant compensatory metabolic changes in order to survive and proliferate.

Though a variety of microenvironmental factors may influence malignant progression, hypoxia merits special consideration, as it has consistently proven to be an adverse prognostic indicator across a variety of tumor types (Nordsmark et al., 1996). The reasons for this relate to hypoxic effects both on malignant progression and on tumor sensitivity to therapeutic interventions.

Hypoxia may impair the effectiveness of both radiation and chemotherapeutic treatments. Molecular oxygen is believed to enhance the damage induced by ionizing

radiation through what is known as the oxygen fixation hypothesis (Alexander & Charlesby, 1954; Johansen & Howard-Flanders, 1965). This hypothesis purports that x-rays damage DNA indirectly by producing hydroxyl radicals upon interaction with water molecules. The interaction between these hydroxyl radicals and DNA subsequently generates DNA-derived free radicals, which can be reduced by sulfhydryl compounds relatively easily in the absence of molecular oxygen. Upon interaction with molecular oxygen, however, these DNA-derived radicals are converted to peroxides which represent a more durable form of DNA damage (Liu, Lin, & Yun, 2015). Low oxygen conditions may therefore diminish the clinical response. Some chemotherapeutic agents also require molecular oxygen for maximal effectiveness. Bleomycin, for example, is less effective in hypoxic conditions due to the reduced production of reactive oxygen species (Cunningham, Ringrose, & Lokesh, 1984; Shannon, Bouchier-Hayes, Condrón, & Toomey, 2003).

Hypoxia induces apoptosis in tumor cells in vitro and may select for cells with lower apoptotic potential (Graeber et al., 1996). Cells may be selected for loss of p53, a positive regulator of apoptosis, or for overexpression of anti-apoptotic genes like bcl-2 (Graeber et al., 1996). Tumors exposed to such selection pressures may become more resistant to treatment measures which would normally induce apoptosis, including radiotherapy and various chemotherapeutic agents.

Hypoxia also contributes to a poorer clinical outcome by promoting a more aggressive tumor phenotype. HIF1 has been identified as the major transcription factor which mediates the cellular response to hypoxia (Iyer, 1998) and its levels have been

associated with increased mortality in a wide variety of cancer types, including those of the brain, breast, colon, esophagus, head and neck, oropharynx, liver, lung, skin, stomach, uterus, as well as in acute leukemias (G. L. Semenza, 2009). Its numerous downstream targets influence a variety of signaling pathways, which mediate tumor angiogenesis, invasiveness, stemness, cellular differentiation, and metabolism (Li, Zhou, Xu, & Xiao, 2013; Semenza, 2013). While many aspects of the complex interplay among these various signaling pathways remain to be elucidated, hypoxic regulation of HIF1 is relatively well understood. HIF1 is a heterodimer composed of an oxygen-responsive alpha subunit and a constitutively active beta subunit (M. Ivan, 2001; Jaakkola et al., 2001). In normoxic conditions, the alpha subunit is destroyed through ubiquitination and proteolysis (Jiang, Semenza, Bauer, & Marti, 1996). These processes are mediated by prolyl hydroxylases, which hydroxylate prolines 402 and 564 (Bruick & McKnight, 2001; Epstein et al., 2001; Mircea Ivan et al., 2001; Jaakkola et al., 2001; Kondo, Klc, Nakamura, Lechpammer, & Kaelin Jr, 2002; Stiehl et al., 2006). Since molecular oxygen is a low affinity substrate for the prolyl hydroxylases, low oxygen conditions impair hydroxylation and lead to stabilization of HIF1 α (Stiehl et al., 2006).

The ubiquitination and proteolytic degradation of HIF1 α is achieved by a complex comprised of von Hippel Lindau protein (VHL), elongins B and C, cullin2, and ringbox1 (Gregg L. Semenza, 2009). The VHL complex recognizes the hydroxylated prolines, and functions as an E3 ubiquitin ligase, resulting in HIF1 α 's proteolytic destruction (Stickle et al., 2004). VHL functions as a tumor suppressor. In renal cell carcinomas, loss of VHL results in constitutive activation of HIF1 α and its target genes (Kondo et al., 2002).

The interactions among hypoxia, HIF1 signaling, and metabolic signaling pathways are complex and only beginning to be unraveled. It is well established that hypoxia activates HIF1 α , and is a predictable consequence of rapid tumor growth that outpaces new vessel formation. However, HIF1 activation is often observed in cancer cells even under normoxic conditions (Lum, 2007).

HIF1 may be induced in normoxia by the activation of oncogenes, such as Ras or phosphoinositide 3-kinase (PI3K), or by loss of tumour suppressors such as VHL or phosphatase and tensin homolog (PTEN) (Bárdos & Ashcroft, 2004; Mazure, Chen, Yeh, Laderoute, & Giaccia, 1996). It may also be activated by the accumulation of TCA cycle metabolites (succinate and fumarate) or reactive oxygen species (superoxide) (Gottlieb & Tomlinson, 2005). The adaptive benefit of HIF1 signaling to normoxic cancer cells is uncertain and how hypoxia may contribute to malignant progression if HIF1 signaling occurs in normoxic tumor cells is also unclear.

HIF1 activation induces critical changes in cellular metabolism, with a shift toward reliance on glycolytic ATP production and repressed mitochondrial function (Iyer, 1998). HIF1 activation stimulates glycolysis through the induction of glycolytic genes and suppresses mitochondrial function by inducing pyruvate dehydrogenase kinase 1 (PDK1) (Kim, Tchernyshyov, Semenza, & Dang, 2006; Papandreou, Cairns, Fontana, Lim, & Denko, 2006). PDK1 inhibits pyruvate dehydrogenase through phosphorylation, and blocks pyruvate entry into the TCA cycle (Patel & Korotchikina, 2001; Roche, 2001). This HIF1-dependent activation of PDK1 and subsequent inhibition of pyruvate

dehydrogenase and mitochondrial glucose oxidation are characteristic of malignant tumors and essential for their growth (Papandreou et al., 2006).

As noted above, reliance on glycolysis is a key feature of the malignant metabolic phenotype and was first described almost a century ago by Otto Warburg (Warburg, 1956). Increased glycolytic activity is advantageous in oxygen-deficient conditions, since it offers a means of adequate energy production when oxygen levels are too low to support oxidative phosphorylation. Additionally, reducing mitochondrial function may decrease oxygen consumption to levels that are compatible with oxygen supply.

Increased glycolytic activity is accompanied by changes in cellular utilization of fuels. Cancer cells will consume greater quantities of glucose in order to compensate for the much lower efficiency of glycolytic ATP production. Mitochondrial glucose oxidation generates up to 36 molecules of ATP for every two produced through glycolysis.

Evidence has emerged that cancer cells also exhibit critical alterations in glutamine metabolism (DeBerardinis & Cheng, 2010; Wise & Thompson, 2010). In the setting of impaired mitochondrial glucose oxidation, glutamine can be used to replenish TCA cycle substrates, such as alpha-ketoglutarate (Sun & Denko, 2014). Recent work has demonstrated that glutamine-derived alpha-ketoglutarate may be reductively carboxylated by isocitrate dehydrogenase to generate citrate (Sun & Denko, 2014). The glutamine-derived citrate can then be shuttled to the cytoplasm and used to support *de novo* lipogenesis, and ultimately, cellular proliferation (Sun & Denko, 2014).

Experimental evidence has shown that HIF1 activation is responsible for shifting glutamine metabolism from oxidation to reductive carboxylation (Metallo et al., 2012; Sun & Denko, 2014). A mechanism for this phenomenon has been proposed, whereby HIF1 activation promotes the destruction of a key component of alpha-ketoglutarate dehydrogenase complex (α -KGDH) by the ubiquitin ligase, SIAH2 (Sun & Denko, 2014). The destruction of this α -KGDH component blocks the oxidation of α -KG and forces glutamine metabolism in the reductive direction (Sun & Denko, 2014). These events are outlined in Figure 1.

Figure 1 A model depicting how hypoxic destruction of OGDH2 may promote the reductive carboxylation of α -KG (Sun & Denko, 2014).

The α -KGDH complex is comprised of three subunits: E1 (oxoglutarate dehydrogenase, OGDH), E2 (dihydrolipoamide s-succinyltransferase, DLST), and E3 (dihydrolipoamide dehydrogenase, DLDH) (Patel & Harris, 1995). Collectively, these enzymes catalyze the forward, oxidative, TCA cycle reaction converting alpha-ketoglutarate to succinyl CoA and NADH (Patel & Harris, 1995).

The E1 subunit is known to have three distinct splice variants, which differ in size (Sun & Denko, 2014). OGDH1 and OGDH3 are 114 kDa proteins, each containing a thymine pyrophosphate binding domain and a transketolase-like domain (Sun & Denko, 2014). OGDH2 is just 48 kDa and due to a premature stop codon lacks the transketolase-like domain (Sun & Denko, 2014). It is the only splice variant which is destroyed in hypoxia, and its destruction appears to be key to directing glutamine metabolism toward the reductive pathway (Sun & Denko, 2014). The OGDH2 protein is ubiquitinated by SIAH2 at the lysine residue in the 336 position (Sun & Denko, 2014). Its stabilization, by substituting an alanine for the ubiquitinated lysine, has been shown to abrogate the hypoxic reduction in alpha-KGDH activity and reduce glutamine dependent lipid synthesis by hypoxic cells in vitro (Sun & Denko, 2014). OGDH2 stabilization has also been shown to block tumor growth in model systems (Sun & Denko, 2014).

Targeting cancer cell metabolism may represent a promising therapeutic strategy. Despite the genetic and phenotypic idiosyncrasies of individual tumors and tumor subpopulations, cancer cells share a number of key functional and metabolic features. Unrestricted proliferative activity distinguishes cancer cells from their normal tissue counterparts and increases cellular biosynthetic demands. Some non-cancer cell types,

such as epithelial cells, also exhibit high rates of proliferation; however, cancer cells face the added challenge of meeting their energetic needs in uniquely hostile conditions. Cancer cell proliferation, therefore, depends upon fundamental compensatory metabolic changes. The malignant metabolic phenotype that emerges may be viewed as a hallmark of cancer and may prove to be the disease's Achilles heel.

While the interaction between hypoxic and normoxic cancer cells is poorly understood, it was recently shown that normoxic cancer cells can metabolize lactate produced by hypoxic cells and use it to support their own biosynthetic and energetic needs (Guillaumond et al., 2013). The glycolytic phenotype induced by HIF1 activation in hypoxic cells may thus promote the proliferation of normoxic cancer cells in their vicinity.

A number of important questions remain as to how OGDH2 stabilization blocks tumor growth. The metabolic effects of OGDH2 stabilization in hypoxic cells have been demonstrated; however the hypoxic cells represent only a fraction of the tumor mass. One would expect that normoxic cancer cell proliferation must also be suppressed in order for tumor growth to cease. How OGDH2 stabilization in hypoxic cells might affect normoxic cancer cell proliferation is not known. Given the recent findings of Guillaumond and colleagues, one might speculate that OGDH2 stabilization could affect glycolytic activity in hypoxic cells (Guillaumond et al., 2013). Further study is also needed to clarify the mechanistic details of OGDH2 destruction in hypoxia and its function in normoxia. It is presumed that OGDH2 ubiquitination and proteolysis occurs in the cytoplasm, but this has not been confirmed experimentally.

The tremendous complexity of cancer genetics and relevant signaling pathways poses a challenge to cancer therapeutics. The phenotypic heterogeneity and mutability of tumors confers a remarkable ability to adapt to almost any treatment modality. Nevertheless, the interface between hypoxic and metabolic signaling pathways holds promise for the development of new anti-cancer agents with improved effectiveness. The classic metabolic changes observed in cancer cells are essential for tumor growth and may provide therapeutic opportunities to undermine cancer cells' own strategies for coping with their microenvironmental challenges. The HIF1 dependency of these metabolic pathways also has therapeutic relevance. Since HIF1 activation is relatively rare in normal tissues, therapies targeting this pathway should possess specificity for malignant tissue with minimal adverse effects.

As tumor metabolism represents a promising therapeutic target and OGDH2 plays a fundamental but poorly understood regulatory role, a tool to facilitate research in this area would be valuable. The present project aimed to construct a fluorescent reporter gene combining OGDH2 with a ruby red fluorescent protein. Following validation, the reporter gene construct may provide unique insight into the regulation and function of OGDH2 in cancer cell metabolism.

Chapter 2: Materials and Methods

OGDH2 and mRuby2 genes were acquired from Origene (pCMV6-OGDH2) and Addgene (pCDNA3-mRuby2), respectively. An hypoxia stable OGDH2 mutant was created through site-directed mutagenesis, which converted the ubiquitinated lysine residue of OGDH2 to an alanine (336 KA). This was accomplished with Agilent Technologies QuickChange II Site-Directed Mutagenesis Kit, following the manufacturer's instructions. The mutated protein demonstrated increased resistance to hypoxic degradation compared to the wild-type protein in stably transfected RKO cells.

The OGDH2 genes (both mutant and wild type) were amplified using custom primers, which added HindIII and NheI restriction sites on the forward and reverse primers, respectively. The upstream primer sequence was 5'-ATCGAAAGCTTGAGGAGATCTGCCGCCGCGATCGCC-3' and the downstream primer was 5'-ATATAGCTAGCTGTTGGTGAGCGGAACTCATGCTGGA-3'. Forward and reverse primers are depicted alongside the corresponding portions of the OGDH2 sequence in Figure 2.

5'-ATCGAAAGCTT**GAGGAGATCTGCCGCCGCGATCGCC**-3'

.....**GAGGAGATCTGCCGCCGCGATCGCC**ATGTTT
 CATTTAAGGACTTGTGCTGCTAAGTTGAGGCCATTGACGGCTTC
 CCAGACTGTTAAGAC....**[OGDH2]**....GAAGGGAAAAAGGTAA
 GGCCCAGAGAGAGGCGTGCAAGGCAGATCGTCAAGGCCCAT
 GT**TCCAGCATGGAGTTCGCTACCAACA**.....

3'-**AGGTCGTACCTCAAGGCGAGTGGTTGT**CGATCGATATA-5'

Figure 2 Primer sequences

The forward primer is identical to the upstream portion of the OGDH2 sequence and the reverse primer is the reverse complement of the downstream portion. The genes were amplified by polymerase chain reaction (PCR), using a Taq polymerase with 30 cycles of denaturation, annealing, and extension steps at 94°C, 68°C, and 72°C, respectively. Both OGDH2 and mRuby2 plasmids were subsequently digested with HindIII and NheI restriction enzymes (New England Biolabs) according to the manufacturer's protocol, but with incubation for 3.5 hours at 37°C. The OGDH2 genes and the cut mRuby2 vectors were isolated through gel extraction (Qiagen gel extraction kit). The OGDH2 genes were then ligated into the mRuby2 backbone using a T4 DNA ligase (Invitrogen) in a total volume of 50 ul, with a vector to insert ratio of approximately 1:5. The products of the ligation were used to transform a strain of *Escherichia coli* (Chem-Agilent XL1-Blue) following the provided protocol, but without the addition of β -mercaptoethanol.

Transformed bacteria were plated on Luria Broth agar containing 100 mg/ml ampicillin and incubated overnight at 37°C. More than a dozen colonies were randomly selected for analysis. Single colonies were used to inoculate aliquots of Luria broth which were incubated with agitation overnight at 37°C. The broth was subsequently centrifuged and plasmid DNA was isolated from the bacterial cells using a DNA purification kit (Epoch Life Sciences).

Purified DNA was then digested with various restriction enzymes to confirm the incorporation of the OGDH2 gene. Interestingly, it was noted that only the successful transformants produced visibly pink pellets at the centrifugation step just prior to DNA isolation. Colonies producing white-beige pellets at this step proved to lack the plasmid construct on analysis. These bacterial cells also demonstrated red fluorescence upon microscopic examination, suggesting a quick means of identifying colonies of successful transformants. Sequence analysis was performed at the Ohio State University Comprehensive Cancer Center genomic core facility to confirm the nucleotide sequences and the orientations of the fusion genes in the vector constructs.

Human cancer cell lines were stably transfected with the empty vector, wild type, and mutant reporter gene plasmids using lipofectamine 2000. The cell lines used were RKO, a human colorectal carcinoma cell line (ATCC), SAS, a base of tongue squamous cell carcinoma, and RCC4, a renal cell carcinoma. The RCC4 cell line lacks VHL, resulting in constitutive HIF1 activation, and it was used in parallel with RCC4 cells with restored VHL function. The SAS cells and both RCC4 cell lines were generously provided by Q. Le and A. Giaccia of Stanford University. All cell lines were maintained

at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25 mM glucose, and 4 mM L-glutamine. Geneticin (G418) was used as a selectable marker for cells containing either the empty vector or the construct. All cell lines were sorted with fluorescence activated cell sorting (FACS) several weeks following transfection at the Ohio State University Comprehensive Cancer Center Analytical Cytometry Facility.

To confirm the hypoxic destruction of the wild type OGDH2 fusion protein and the hypoxia resistance of the stable mutant fusion protein, Western blotting was performed for wild type and mutant RKO transfectants, with lysis following completion of four treatment conditions: normoxia, normoxia with 0.5 mM DMOG (dimethyloxalylglycine, a HIF-stabilizing prolyl hydroxylase inhibitor which activates HIF under normoxic conditions), hypoxia (1% O₂) with immediate lysis, and hypoxia with 1 hour of reoxygenation. The cells were kept in their respective normoxic or hypoxic conditions for 24 hours prior to immediate lysis or reoxygenation. Hypoxic conditions were generated and maintained in a HypOxygen H35 workstation.

Chapter 3: Results

Verification of the construct

Sequencing confirmed that the OGDH2 and mruby2 sequences were present in the plasmid in the desired positions and in frame with one another. The presence of the xpress epitope near the amino terminus of the mruby2 sequence was also verified. In addition, substitution of an alanine residue for lysine at the 336 position was confirmed for the hypoxia stable mutant. No other mutations were observed. The constructed reporter gene is depicted in Figure 3.

Figure 3 The OGDH2-mRuby2 reporter gene construct.

Transfection of human cancer cell lines

Successful stable transfection of cell lines derived from a colorectal carcinoma (RKO), an oral squamous cell carcinoma (SAS), and a renal cell carcinoma (RCC4) was verified with Western blotting, flow cytometry, and fluorescence microscopy. Western blotting results are shown in Figure 4.

Figure 4 Western blot demonstrating the expression of mRuby2 and OGDH2-mRuby2 fusion proteins.

Both the xpress antibody (left) and the α KGDH antibody (right) demonstrated the expression of the fusion proteins. Each blot shows the untransfected parent on the far left, with the plain ruby vector beside it, followed by WT and mutant fusion proteins. The xpress antibody shows the ruby protein at about 30 KDa, while the α KGDH antibody reveals the endogenous OGDH2 at approximately 40 KDa. Fusion proteins on both blots

appear at approximately 70 KDa, where they would be expected based on the combined weights of the component proteins. The xpress antibody also shows nonspecific bands at approximately 115 KDa, which suggests relatively even loading of the samples.

Cellular localization patterns of the construct and empty vector

Fluorescence microscopy similarly provided confirmation of expression of the fusion protein. Microscopy revealed a diffuse cytoplasmic distribution of the mRuby2 protein, while the OGDH2-mRuby2 fusion protein appeared to be organized in a punctate pattern of numerous discrete structures, strongly suggestive of mitochondrial localization (Figure 5).

Figure 5 Distribution patterns of OGDH2-mRuby2 (left) and mRuby2 (right) proteins in RKO cells.

Confirmation of the mitochondrial localization is planned, by co-localizing the fusion proteins with a mitochondrial membrane protein under immunofluorescence imaging.

FACS analysis of parent and transfected cell lines

The expression of the fluorescent protein was also confirmed by flow cytometric analysis, with subsequent collection of the fluorescent cells. Figure 6 shows a scatter plot of untransfected RKO cells, which did not exhibit red fluorescence.

Figure 6 Flow cytometric analysis of mRuby2 protein expression in RKO cells.

Substantial populations of red fluorescent cells were observed in RKO cells transfected with mRuby2, mRuby2-OGDH2, and mRuby2-mutant OGDH2 RKO plasmids (Figure 7).

Figure 7 Flow cytometric analysis of ruby red fluorescence in mRuby2, mRuby2-OGDH2, and mRuby2-mutant OGDH2 transfected RKO cells.

Flow cytometric analysis was also conducted for the SAS cell line, with similar results. The untransfected cells were negative for red fluorescence (Figure 8), while the mRuby2, mRuby2-OGDH2, and mRuby2-mutant OGDH2 transfectants showed definite positivity (Figure 9).

FACS: SAS Parent

Figure 8 Flow cytometric analysis of red fluorescence in untransfected SAS cells.

FACS: SAS Transfectants

Figure 9 Flow cytometric analysis of ruby red fluorescence in mRuby2, mRuby2-OGDH2, and mRuby2-mutant OGDH2 in transfected SAS cells.

Flow cytometric analysis of the RCC4 cell line was conducted to confirm successful transfection. Due to lower transfection efficiency in this cell line, the success of the transfection was more difficult to establish through microscopy alone. Flow cytometry, however, revealed a population of unambiguously red fluorescent transfectants (Figure 10). Further flow cytometric analysis and sorting of this cell line will be necessary before the construct is used for investigative purposes.

Figure 10 Flow cytometric analysis of red fluorescence in RCC4 VHL cells.

Hypoxic response of wild type and stable mutant proteins

In order to determine the stability of the fusion protein and endogenous OGDH2 in hypoxia, RKO cells were treated with hypoxia and analyzed by immunoblot. Figure 11 shows hypoxic destruction of the wild type mRuby2-OGDH2 and endogenous OGDH2 proteins, when probed with antibodies targeting alpha-KGDH (Figures 11) and xpress epitopes (Figure 12).

Figure 11 Western blot results showing endogenous OGDH2 protein and wild type and mutant fusion proteins in transfected RKO cells (alpha-KGDH antibody).

Figure 12 Western blot showing wild type and stable mutant fusion proteins in transfected RKO cells (xpress antibody).

The stable mutant fusion protein exhibited resistance to hypoxic degradation (Figures 11 and 12). Surprisingly, both fusion and endogenous OGDH2 proteins appeared to show enhanced expression following 0.5 mM DMOG treatment.

Chapter 4: Discussion

Reporter gene constructs are widely used to study cellular physiology in a variety of contexts, including hypoxia. They are generally constructed by attaching a gene encoding an easily quantifiable protein to an upstream promoter of interest (Doran, Kulkarni-Datar, Cool, & Brown, 2011). Commonly used reporters include beta galactosidase, fluorescent proteins, chloramphenicol acetyl transferase, and Luciferase (Cecic et al., 2007; Doran et al., 2011).

In hypoxic applications, reporter genes offer a number of advantages and potential challenges. In contrast to many widely used indicators of oxygenation status, such as NITP, EF5, and pimonidazole, which require that the tissue be fixed prior to analysis, reporter gene constructs can be used to gauge oxygenation levels in live cells in both in vivo and in vitro models (Vordermark, Shibata, & Brown, 2001).

Fluorescent reporters hold particular appeal, since they enable microscopic visualization of cellular localization and co-localization patterns in various physiologic and treatment conditions. This was particularly advantageous in our case, given the putative mitochondrial localization of the OGDH2 protein. The visible fluorescence conferred by the reporter gene allowed us to view a distinctive pattern of localization associated with our fusion protein. This pattern was strongly suggestive of mitochondrial localization, and co-localization of the fusion protein with a mitochondrial protein is planned to provide confirmation.

Fluorescent reporters could also be used to reveal patterns of interaction between OGDH2 and other proteins and complexes. The E1 subunit of the alpha-ketoglutarate complex, of which OGDH2 represents one splice variant, typically complexes with E2 and E3 subunits. It remains to be established whether all splice variants function as part of the same complex. A fluorescent reporter may also shed light on aspects of OGDH2 destruction. Presumably, this occurs in the cytoplasm, where the proteasome is found, however it is unclear as to how mitochondrial OGDH2 might be removed for destruction. Changes in the distribution pattern of the fluorescent protein in response to manipulation of key proteins or experimental conditions may offer clues as to how this occurs. Additionally, fluorescent reporters allow for the generation of striking and informative images and for relatively easy, quantitative sorting of cells based on the presence of the protein of interest.

A potential limitation of the use of fluorescent reporters in hypoxia is the requirement of molecular oxygen for chromophore development. Formation of the chromophore has been described as a three-step process, beginning with protein folding, followed by cyclization of the chromophore tripeptide, and finishing with the oxidation of the cyclized chromophore, which is the slowest step (Reid & Flynn, 1997). It follows that, in low oxygen conditions, the degree of fluorescence may not reliably reflect the quantity of the fusion protein that is produced (Cecic et al., 2007). Both green and red fluorescent proteins have been shown to exhibit reduced reporter activity in low oxygen conditions despite unchanged levels of the reporter protein (Cecic et al., 2007; Coralli, Cemazar, Kanthou, Tozer, & Dachs, 2001; Vordermark et al., 2001). Other types of

reporters, such as luciferase and thymidine kinase similarly require molecular oxygen for fully reliable reporter activity (Cecic et al., 2007).

The challenges posed by the oxygen requirement do not preclude the use of fluorescent reporters in all hypoxic applications. Vordermark and colleagues demonstrated that, the fluorescence of green fluorescent protein (GFP) in a human fibrosarcoma cell line was impaired only at near-anoxic levels (below 0.02% O₂) and this impairment was readily alleviated by reoxygenation (Vordermark et al., 2001). Hypoxic treatment of cells in the present study was conducted at an oxygen level of 1%, which was well above the level at which impairment of fluorescence would be anticipated, based on the findings of Vordermark and colleagues. These findings may not accurately predict the utility of our construct, however, as our ruby fluorescent reporter may interact differently with molecular oxygen. The role of oxygen in chromophore formation has not been thoroughly investigated for the mRuby2 reporter in human cell lines. The utility of the closely related mCherry reporter has been demonstrated in hypoxic bacteria, in which no reduction in fluorescence per unit OD was observed with oxygen depletion by *Mycobacterium tuberculosis* (Carroll et al., 2010). Others investigating the effects of hypoxia on a monomeric red fluorescent reporter, however, found a significant reduction in reporter activity levels relative to reporter protein at oxygen levels as high as 2% (Cecic et al., 2007).

The requirement of molecular oxygen for chromophore formation can generally be managed in hypoxic applications by allowing a period of reoxygenation prior to analysis. Some authors have recommended a 3 hour reoxygenation period for full

chromophore maturation (Koshikawa, Takenaga, Tagawa, & Sakiyama, 2000).

Vordermark and colleagues found that fluorescence increased steadily post-hypoxic treatment, with a maximum at 4 hours; however their data also showed reoxygenation to be unnecessary at oxygen levels over 0.02% (Vordermark et al., 2001). In the present work, strong fluorescence was observed with FACS analysis carried out as soon as possible following hypoxic treatment. Due to the need to trypsinize, centrifuge, and resuspend the hypoxia-treated cells prior to FACS analysis, some reoxygenation is both unavoidable and difficult to control precisely with the methods used here.

While the period of reoxygenation to which our cells were subjected appears, based on our preliminary data, to be sufficient for chromophore development, a greater concern may be the recovery of OGDH2 protein levels during this period. The time frame of OGDH2 recovery has not been precisely determined; however, Western blot data showed that it returns to approximately normal levels within an hour following return to normoxic conditions (Figures 10 and 11). The challenge, then, is to accomplish FACS analysis at a time point which allows enough reoxygenation for reliable reporter activity without permitting substantial restoration of OGDH2 levels. Further investigation of chromophore maturation and OGDH2 recovery, with precise timepoints will establish optimal timing of post-treatment analyses of OGDH2 and fusion protein levels.

The use of the RCC4 renal cell carcinoma cell line offers a solution to potential problems posed by the oxygen requirement for chromophore development and by post-hypoxia recovery of OGDH2 protein levels. Renal cell carcinomas feature a mutation which results in loss of function of the VHL complex. The complex recognizes and

ubiquitinates HIF1 alpha following its hydroxylation by prolyl hydroxylase 3 in normoxic conditions. With loss of VHL function, HIF1 becomes constitutively active. Comparison of the RCC4 wild type cell line to its counterpart with restored VHL function permits the study of HIF1 activation without the need for hypoxia. This is ideal in our case, since it obviates the need for reoxygenation for chromophore maturation and allows for sustained HIF1 activation until the time of FACS analysis.

When a reporter construct is to be used in hypoxic applications, the choice of promoter is especially important. Some promoters, including the cytomegalovirus immediate/early promoter (CMV IE) used in our study, contain binding sites for proteins that are activated in hypoxic stress, such as CREB, AP1, SP1, and NF-KB (Doran et al., 2011). Consequently, hypoxic induction or inhibition of the promoter that is independent of HIF1 activation is a potential problem. Our hypoxia stable mutant serves as a useful control in this instance, and immunoblotting showed little change in the quantity of the mutant protein in normoxic and hypoxic conditions. Preliminary data also show that the wild-type fusion protein responds to hypoxia in a manner that is similar to its endogenous counterpart. While there is potential for confounding due to hypoxic induction or inhibition of the promoter, this does not appear to influence the transcriptional activation of our construct to an appreciable degree. mRNA analysis would provide further confirmation that any observed effects are post-translational.

Further evaluation of our wild type and mutant OGDH2 reporter constructs is necessary to ensure that the behavior of the fusion proteins accurately reflects that of the endogenous counterparts. Following validation of each cell line, they will be used to

assess OGDH2 function and localization patterns in normoxia and hypoxia. Next steps also include the construction of an shRNA library of genes that are essential to OGDH2 destruction.

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