2011-05-11

Insulin Receptor Substrate-2 (IRS-2): A Novel Hypoxia-Responsive Gene in Breast Cancer: A Dissertation

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INSULIN RECEPTOR SUBSTRATE-2 (IRS-2): A NOVEL HYPOXIA-RESPONSIVE GENE IN BREAST CANCER

A Dissertation Presented

By

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Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

MAY 11, 2011

CANCER BIOLOGY

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Signature page

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May, 11 2011

Dedication

I'd like to dedicate this work to my mom, Galina Mardilovich, who was diagnosed with metastatic breast cancer at the age of 45 and passed away at 51, two weeks before my qualifying exam. She taught me determination and stamina that I needed so much during school. Her strength, courage, endless optimism and incredible love of life will always be my inspiration.

Acknowledgements

I'd like to express my heartfelt gratitude to my mentor, Dr. Leslie Shaw for the guidance and support she has provided me throughout my entire Ph.D. training process. She has given me a complete freedom to move where the science takes me and guided me patiently through my project. She has been a constant source of not only help and advice, but also encouragement and optimism that helped me through my work. The phrase "the glass is half full" will always remind me of her. I wouldn't be a strong and confident scientist without her encouragement and unlimited help and support.

I thankfully acknowledge the contributions of my Thesis Research Advisory Committee members, Dr. Arthur Mercurio (chair), Dr. Brian Lewis, Dr. Stephen Lyle, Dr. Merav Socolovsky, Cancer Biology Department, UMassMed for their guidance though my project. Dr. Andrew Kung, Harvard Medical School, my external examiner also has my thankfulness for his invaluable suggestions and comments.

I am especially grateful to my current and previous lab members, Maura Byrne, Dr. Shannon Pankratz, Jenny Janusis, Xiaoqing Yang, Justine Landis, Jen Clark, Rasika Rohatgi, and Dr. Anuradha Seshadri for their outstanding scientific advise, kind support, and a warm, friendly environment, filled with lots of candy and laughter. And a special thanks to Yumiko Fine for invaluable moral support through my toughest days and excellent Flamenco lessons, and Dushani Palliyaguru, my undergrad student for achieving so much and making me a proud mentor. It has been a real pleasure to work with you all.

I'd like to thank my amazing friends from UMass, Minakshi Guha, Jenny Xie, Dimitra Efthymiadis, Ermelinda Porpiglia, Dushani Palliyaguru, Swarna Mehrotra, and Ling Lin, who have been with me through the whole experience, for their unconditional support, understanding of the graduate student hardships, and also lunches, shopping and endless coffee breaks – everything that made it a fun and joyful time. This experience wouldn't have been the same without you! Also, my friends Julia and Alex Krasheninin, Natasha and Eugene Sharov, who have become my second family, for believing in me, supporting me, and for making this place my second home.

And finally, I am eternally grateful to my family, my parents, Ivan and Galina Mardilovich and my sister, Anastasia, for their tremendous moral support and unconditional love that has given me the strength to always move forward. I wouldn't have been here without you!

Abstract

Breast cancer is the most common malignancy among women in the U.S. While many successful treatments exist for primary breast cancer, very few are available for patients with metastatic disease. The purpose of this study was to understand the role of Insulin Receptor Subtrate-2 (IRS-2) in breast cancer metastasis. IRS-2 belongs to the IRS family of cytoplasmic adaptor proteins that mediate signaling from cell surface receptors, many of which have been implicated in cancer. Although the IRS proteins are highly homologous in structure and have some complementary functions, growing evidence supports that the IRS proteins have unique roles in cancer. IRS-1 has been shown to promote tumor cell proliferation, while IRS-2 has been positively associated with cancer cell invasion, glycolysis and tumor metastasis. In the current work, we identified IRS-2 as a novel hypoxia-responsive gene in breast carcinoma cells. In contrast, IRS-1 expression does not increase in response to hypoxia, supporting the notion of their non-overlapping functions. Hypoxia promotes the adaptation and resistance of cancer cells to chemo- and radiation therapy, and also promotes tumor cell survival, invasion and metastasis by selecting for aggressive tumor cells that can survive under stressful low oxygen conditions. We have shown that IRS-2 upregulation in response to hypoxia promotes Akt signaling and tumor cell viability and invasion. We identified a cell context-dependent role for Hypoxia Inducible Factor (HIF) in the regulation of IRS-2 expression in hypoxia, with HIF-2 playing a more dominant role than HIF-1. We also demonstrate that binding of Snail, a regulator of the EMT, to the IRS-2 promoter keeps the chromatin in an open conformation

that is permissive for HIF-dependent transcription of IRS-2 in hypoxia. IRS-2 is not upregulated by hypoxia in well-differentiated epithelial-like carcinoma cells that do not express Snail, implicating IRS-2 gene expression as part of the EMT programming. In summary, we have identified an endogenous mechanism by which cancer cells can shift the balance of IRS-1 and IRS-2 to favor IRS-2 expression and function, which promotes survival, invasion, and ultimately metastasis. Understanding the mechanism of IRS-2 regulation by hypoxia may reveal new therapeutic targets for metastatic breast cancer.

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CHAPTER I: Introduction

Breast cancer is the most common and the second most deadly malignancy among women in the US [1]. Many successful treatments exist for localized breast cancer. Almost 98 percent of women diagnosed with localized breast cancer have a five-year survival rate. However, only 27 percent of patients diagnosed with metastatic disease reach a 5-year survival [1]. Therefore it is crucial to focus our research on understanding the mechanisms of metastasis in order to develop more effective treatments for aggressive types of breast cancer. Our lab has been focusing on understanding the mechanism of breast cancer metastasis, and particularly the role of the Insulin Receptor Substrate (IRS) proteins in this process using *in vivo* and *in vitro* models. The purpose of this work was to identify novel roles for IRS-2 in breast cancer metastasis.

Insulin Receptor Substrate – 2 (IRS-2)

IRS-2 belongs to the Insulin Receptor Substrate (IRS) family of cytoplasmic adaptor proteins that includes four members: IRS-1, IRS-2, IRS-3, and IRS-4. The IRS proteins were initially identified as adapter proteins for the insulin receptor (IR) and have been extensively studied in the context of insulin signaling [2]. Later they were found to also function downstream of the insulin-like growth factor-1 receptor (IGF-1R) [3]. In addition, a few studies have identified the IRS proteins to mediate signaling downstream of other receptors, including several cytokine receptors, Growth Hormone (GH), Vascular Endothelial Growth Factor (VEGF, KDR), and EGF receptor, as well as αVß3 and α6ß4 integrins [4-7]. IRS-1 and IRS-2 are ubiquitously expressed in all tissues in the body and are the most common contributors to regulation of insulin-dependent proliferation, growth

and metabolism [2]. Expression of IRS-4 is restricted to brain, kidney, thymus, and liver and IRS-3 is expressed only in rodents [8, 9].

IRS-1 was the first IRS family member to be characterized as an endogenous substrate of the insulin receptor kinase. It was discovered in an anti-phosphotyrosine immunoblot screening of IR substrates in hepatoma cells after insulin stimulation [10]. IRS-2 was identified as an alternative insulin receptor substrate in *Irs-1*$^{-/-}$ mice [11]. It was originally cloned from myeloid progenitor cells and named 4PS. Sequence analysis revealed significant levels of homology with the IRS-1 protein and later IRS-2 was found to be expressed in many cell types and tissues [12]. IRS-2 was also shown to be phosphorylated in response to insulin stimulation and recruit Phosphoinositide 3-OH kinase (PI3K) and Grb2 in liver and muscle cells to support insulin signaling of *Irs-1*$^{-/-}$ mice [11].

All IRS proteins share some homology in structure, with about 75% homology in the N-termini, and about 30% homology in the C-termini (Figure1.1). They contain plekstrin homology (PH) and phosphotyrosine binding (PTB) domains in their N-termini and multiple tyrosine phosphorylation sites in their C-termini. Upon activation of upstream receptors, IRS proteins are recruited to the receptor at the membrane via the PH domain, which contributes to protein-protein and protein-phospholipid interactions [13-17]. The PH domain is followed by the PTB domain, which interacts with the NPXY motifs in the activated receptors [16, 17]. IRS-2 also contains a unique motif, the kinase regulatory loop-binding (KRLB) domain in its N-terminus. The KRLB domain interacts with the tyrosine kinase domain of the insulin receptor and is thought to function to limit tyrosine phosphorylation of IRS-2 [16, 18]. Tyrosine 628 (Tyr628) within the IRS-2 KRLB domain

is phosphorylated by the insulin receptor, but the turnover of the phosphorylation is slow and has an inhibitory effect on the receptor kinase activity [19].

IRS proteins do not have intrinsic kinase activity. They act as scaffold proteins to recruit downstream effectors to the receptors thus determining the outcome of the IRS-mediated activation of signaling pathways [20]. Upon binding to the upstream receptor, the IRS proteins are phosphorylated on multiple tyrosine residues in their C-termini, generating phosphotyrosine-binding sites that recruit downstream effectors, including the p85 subunit of PI3K, the tyrosine phosphatase SHP2, Grb-2, Fyn, c-Crk, CrkII and Nck (Fig.1.1) [2, 12, 21-27]. The majority of studies performed to understand IRS protein interactions with receptors and downstream effectors have been performed on IRS-1 alone, making the assumption that IRS-1 and IRS-2 behavior is identical based on their homology. Therefore additional studies are needed to characterize IRS-2 specific interactions. A recent study identified additional potential interacting proteins for IRS-1 and IRS-2 by utilizing phosphorylated versus non-phosphorylated bait peptides to analyze all potential phosphotyrosine dependent interaction sites in IRS-1 and IRS-2 [28]. However, these interactions have not yet been confirmed *in vivo* in response to physiological stimuli. Binding of the effector proteins to the tyrosine-phosphorylated IRS protein initiates activation of the downstream signaling cascades resulting in changes in cell metabolic responses, gene expression, translation, survival, and migration (Fig.1.2).

The IRS proteins are homologous in structure and have some complementary functions. However, knockout mouse models provide evidence that they exhibit unique functions in metabolic regulation. *Irs-1*$^{-/-}$ mice are born small and never develop diabetes due to an increase in insulin production, implicating Irs-1 in somatic growth regulation

[29]. Mice deficient for Irs-1 develop insulin resistance but do not progress to diabetes because they maintain normal pancreatic beta-cell numbers [29]. *Irs-2*$^{-/-}$ animals have a normal body size, but small brain and exhibit about 50% decrease in neuronal proliferation [30, 31]. Unlike *Irs-1*$^{-/-}$ mice, *Irs-2*$^{-/-}$ mice develop type 2 diabetes due to peripheral insulin resistance and beta-cell failure [30]. *Irs-2*$^{-/-}$ females are also infertile, which together with evidence from insulin-signaling in *Drosophila* and *C. elegans*, supports a conserved mechanism for integrating reproduction and metabolism [32]. *Irs-4*$^{-/-}$ mice are phenotypically normal, with only mild growth, reproductive and insulin sensitivity defects [33]. Recent evidence suggests that IRS proteins also play unique roles in cancer.

IRS-1 and IRS-2 in cancer

Many of the pathways activated downstream of IRS-signaling have been implicated in tumorigenesis and cancer progression. Therefore, expression and function of IRS-1 and IRS-2 has been analyzed in several cancers, but most extensively in breast cancer. IRS-1 expression is increased in patients with estrogen receptor (ER)-positive breast cancer and is associated with worse disease-free survival prognosis [34]. Another group found a positive correlation between IRS-1 expression and Ki-67 staining in ER-positive tumors and a negative correlation in ER-negative tumors [35]. These combined findings suggest that IRS-1-mediated signaling in breast tumors may contribute to tumor growth. Similar to breast cancer, IRS-1 also seems to have a positive role in ovarian tumor cell growth. Treatment of ovarian carcinoma cells with all-trans retinoic acid (ATRA), which causes growth arrest, decreases IRS-1 levels [36]. The role of IRS-1 in cancer cell proliferation is

also supported by *in vitro* studies. Overexpression of IRS-1 in T47D breast carcinoma cells that do not express either IRS-1 or IRS-2 results in increased cell proliferation [37]. Interestingly, overexpression of IRS-2 in the same cells increases cell motility [37]. Similarly, IGF-1-dependent phosphorylation of IRS-1 promoted cell growth and IRS-2 promoted cell motility in malignant pleural mesothelioma cell lines [38]. In addition, IGF-1 has been shown to predominantly induce IRS-2 phosphorylation rather than IRS-1 phosphorylation in human breast cancer cells selected for metastatic behavior *in vivo* [39]. Introduction of an IRS-2 antisense mRNA into these metastatic cells results in decreased IGF1-induced cell motility and anchorage-independent growth [39]. Therefore while IRS-1 has been linked with regulation of tumor cell proliferation, IRS-2 has been connected with tumor cell motility and invasion.

In some cancer types, IRS-1 expression has been reported to correlate negatively with tumor progression. Specifically, IRS-1 expression was found to be high in well-differentiated breast tumors and decrease in poorly differentiated tumors [40]. It was found to negatively correlate with tumor grade and lymph node involvement in ductal carcinoma [41]. Similar to breast cancer, IRS-1 expression is lost in 43% of human samples of stage I non-small cell lung cancer (NSCLC). However the loss of IRS-1 did not correlate with disease-free survival, indicating that it was an early stage event in NSCLC [42]. Based on the different roles of IRS-1 and IRS-2 in cancer progression, these results suggest that in certain cancer types, the switch from IRS-1 to IRS-2 signaling becomes a determining factor in tumor progression, rather than simply the levels of expression of either one. Therefore careful analysis of expression of both IRS-1 and IRS-2 is necessary to create a full picture of the role each IRS protein in tumor progression.

In vivo mouse models also support that IRS-1 and IRS-2 play unique roles in tumor development and progression. Work from our own lab using the Polyoma Middle T (PyV-MT) mouse model of mammary tumor progression, where the PyV-MT oncogene is expressed under control of the mouse mammary tumor virus (MMTV) promoter, restricting it to the mammary gland epithelium, has revealed that IRS-1 and IRS-2 have independent non-overlapping roles in breast cancer. *PyV-MT:Irs-2*$^{-/-}$ tumors are significantly impaired in their ability to metastasize, whereas *PyV-MT:Irs-1*$^{-/-}$ tumors that have increased levels of Irs-2 expression and tyrosine-phosphorylation, have a higher rate of metastasis than their wild-type counterparts [43, 44]. Another group using the *PTEN*$^{+/-}$ mouse model of prostate and endometrial cancer has confirmed our *in vivo* results. Specifically, deletion of Irs-2 in this model has no impact on tumor initiation, but significantly suppresses tumor invasion [45]. Therefore, a positive regulation of invasion may be one of the mechanisms by which IRS-2 promotes tumor progression and metastasis.

Another possible mechanism by which IRS-2 can promote tumor progression is by positively regulating glycolysis. The switch to glycolysis from aerobic phosphorylation in tumor cells has been previously shown to correlate with more metastatic potential [46]. Our lab has shown that IRS-2 regulates the localization of glucose transporter-1 (GLUT-1) to the cell surface to promote glycolysis [47]. PyV-MT:Irs-2$^{-/-}$ tumor cell lines have significantly lower rates of aerobic glycolysis than PyV-MT wild-type and Irs-1$^{-/-}$ cell lines. Expression of exogenous Irs-2 in Irs-2$^{-/-}$ cells restores glycolysis to the wild-type cell levels. Importantly, suppression of Glut-1 expression decreases Irs-2-dependent invasion in PyV-MT mouse mammary tumor cells. Therefore, IRS-2 may contribute to breast cancer metastasis by positive regulation of aerobic glycolysis.

IRS-2 has also been shown to be important for tumor cell survival, which may also contribute to its role in promoting tumor progression. *PyV-MT:Irs-2*$^{-/-}$ mammary tumors exhibit significantly more apoptosis than wild-type or *Irs-1*$^{-/-}$ tumors [48]. Moreover, PyV-MT:Irs-2$^{-/-}$ tumor cell lines are significantly more sensitive to serum starvation-induced cell death than wild-type or Irs-1$^{-/-}$ cells [48]. Down-regulation of IRS-2 expression in human hepatoma Hep3B cells that express high levels of this protein has also been shown to increase apoptosis levels in these cells [49].

Transgenic Irs-1 and Irs-2 overexpression mouse models provide evidence that both IRS-1 and IRS-2 have oncogenic potential. Overexpression of either Irs-1 or Irs-2 protein alone in the mammary gland is sufficient to drive tumor formation in mice. However, no differences in tumor metastasis were observed in this study [50]. This finding contradicts the previously accepted notion of different roles of IRS-1 and IRS-2 in breast cancer progression. However, it is possible that when overexpressed, IRS-1 and -2 can gain functions that are absent at normal physiological expression levels.

Little is known about the function of the IRS proteins in other types of cancer. Only a few studies have analyzed expression levels of the IRS proteins in different types of tumor samples in correlation with tumor progression and outcomes. In general, IRS protein levels have been found to be elevated in tumor samples, compared to normal tissue. For example, one study found expression of IRS-1, -2, and -4 elevated at mRNA and protein levels in 80% of analyzed human hepatocellular carcinoma samples [51]. IRS-1 protein levels were also found to be increased in medulloblastoma, pancreatic and ovarian carcinoma [36, 52, 53]. A study of 54 primary prostate tissue samples found a trend towards increased IRS-1 expression in malignant biopsies compared to normal tissue [54].

Increased IRS-1 levels in medulloblastoma were associated with high levels activation of the downstream pathway, Erk-1, Erk-2, and Akt [53]. To date, the only study to examine IRS-4 expression in human cancer reported increased expression of IRS-4 in hepatocellular carcinoma [55].

Overall IRS-2 expression has also been found to be elevated in several types of cancer. IRS-2 expression was found increased in two different murine models of hepatocellular carcinoma. Its expression increased with tumor progression, with lower levels in preneoplastic lesions and higher levels in tumoral nodules [56]. IRS-2 expression was also elevated in human hepatocellular carcinoma specimens and in human hepatoma cell lines at both protein and mRNA levels [56]. Human pancreatic cancer cell lines also show elevated IRS-2 mRNA and protein levels. Treatment of pancreatic carcinoma cell lines with IGF-1 has a mitogenic effect associated with IRS-2 phosphorylation and association with PI3K. Inhibition of PI3K attenuates the effect of IGF-1, suggesting that IRS-2 may contribute to mitogenic signaling via the PI3K pathway in pancreatic cancer [57]. The function of the IRS protein in cell signaling depends on the cell type. It seems that the role of IRS-1 and IRS-2 is not uniform in all types of cancer, however the trend exists that both proteins my lead to tumor growth and progression through different mechanisms.

One caveat to all of the expression studies, however, is that the IRS proteins can be phosphorylated on serine residues through negative feedback loops, which inhibits their function [58]. Therefore, expression of the IRS proteins alone may not reflect the functional status of these adaptor proteins. We have previously observed a positive correlation between increased Irs-1 serine phosphorylation and metastasis in our PyV-MT

mouse mammary tumor model [59]. Additional studies are needed to establish the expression and function of the IRS proteins in human cancer and to determine if their relative expression levels have prognostic or predictive value.

Regulation of IRS-2 expression

Since the IRS proteins are phosphorylated in order to perform their signaling functions, their activity is heavily regulated at post-translational levels. Prolonged stimulation with IGF-1 or insulin and sustained activation of downstream pathways leads to activation of the negative feedback loop mediated by mTOR [60-65]. P70S6-kinase phosphorylates the IRS proteins on serine/threonine (Ser/Thr) residues leading to decreased IRS tyrosine phosphorylation, ubiquitination and subsequent proteasomal degradation [58, 66-68]. Phosphorylation on Ser/Thr residues in close vicinity to the PTB domain may also prevent IRS protein binding to upstream receptors [62, 69]. Similarly, phosphorylation of Ser residues near the PI3K-binding motif can inhibit binding of the p85 subunit of PI3K to the IRS proteins [70]. The majority of the studies examining the effect of negative feedback loops on IRS activity and phosphorylation were performed using IRS-1. In contrast, little is known on the specific effect of the negative regulation of IRS-2. Therefore, the unique functions of IRS-1 and IRS-2 in tumor progression may be due to different sensitivity to negative feedback regulation resulting in different strength and duration of the downstream signaling pathways [71-73].

There is very little information about the regulation of IRS-2 expression at the transcriptional level in cancer. As mentioned above, *Irs-1*$^{-/-}$ tumors have increased Irs-2

expression and phosphorylation. The fact that IRS-2 expression increases to compensate for the decrease in IRS-1 levels, altering downstream signaling, suggests that the outcome of IRS signaling lies in the balance between IRS-1 and IRS-2 expression levels. Shifting the balance towards higher expression of IRS-2, which promotes tumor invasion and metastasis, may be a crucial step in promoting tumor progression. Therefore it is important to understand the regulation of IRS-2 expression. Several studies have revealed that despite similar structure and some promoter homology, IRS-1 and IRS-2 gene expression is regulated by independent mechanisms and in response to different stimuli [74, 75]. Since IRS proteins were first discovered as insulin signaling intermediates, their expression was best studied in the context of insulin signaling in normal cells and in diabetes.

In normal hepatocytes, insulin downregulates IRS-2 expression through the PI3K/Akt pathway by regulating binding of the Forkhead box (FOXO) transcription factors to the insulin response element (IRE) sequences in the IRS-2 promoter to maintain signaling balance [76, 77]. Therefore, the negative regulation of IRS signaling occurs at both post-translational and transcriptional levels, in order to effectively prevent over-activation of the pathway. On the other hand, glucose has been shown to positively regulate IRS-2 expression, but not IRS-1 expression in pancreatic beta-cells. Glucose treatment not only induces IRS-2 transcription but also IRS-2 phosphorylation, therefore increasing its signaling efficiency [78]. Thus, IRS-2 expression is effectively regulated through positive and negative mechanisms with a quick response to changes in glucose and insulin homeostasis.

Adjacent to the IRE site in the IRS-2 promoter, an E-box element has been shown to be involved in the regulation of IRS-2 expression by Transcription Factor E3 (TFE3) and

Transcription Factor EB (TFEB) in normal liver cells in mice [79]. E-boxes, often found in promoters of genes involved in metabolism, are consensus *cis*-elements for the basic helix-loop-helix (bHLH) family of transcription factors, including Microphthalmia family of transcription factor, which includes TFE3 and TFEB. The same group identified a positive role for FOXO in regulating IRS-2 transcription, indicating a dual role for FOXO transcription factors in mediating insulin signaling. To counteract the positive action of FOXO and TFE3/B at the IRS-2 promoter, Sterol Regulatory Element-Binding Protein (SREBP) factors have been shown to negatively regulate IRS-2 transcription by competing with FOXO and TFE3 factors for binding to the E-box/IRE region in the IRS-2 promoter in mouse hepatocytes *in vivo* [80].

IRS-2 gene expression is also tightly regulated in cancer cells through multiple independent mechanisms. Hormone regulation of gene expression often occurs in cancer cells. IRS-1 and IRS-2 expression are modulated in response to different hormonal stimulation. IRS-1 has been found to be an oestrogen-responsive gene [81]. In contrast to IRS-1 expression, IRS-2 transcription can be directly regulated by progesterone in progesterone receptor (PR)-positive breast cancer cells [81, 82]. Both progesterone and IGF-1 signaling pathways play a crucial role in breast cancer progression. Progestin stimulation before IGF-1 treatment of breast cancer cells results in increased IRS-2 levels and phosphorylation and binding to downstream proteins such as PI3K and Grb-2 leading to enhanced IRS-2-dependent signaling [83].

Other growth factors have also been found to affect IRS-1 and IRS-2 expression. Both fibroblast growth factor (FGF) and epidermal growth factor (EGF) can modulate IRS-1 expression in the MCF7 breast cancer cell line [84]. EGF has also been found to induce

IRS-2 expression in SUM159 breast carcinoma cells via activation of JNK signaling and increased cFos expression [85]. However, this effect is not common to all growth factors. Platelet-derived growth factor (PDGF) cannot change IRS expression, indicating that certain growth factors specifically regulate IRS-1 or IRS-2 expression [84].

Some tumor suppressors and oncogenes have been shown to regulate expression of the IRS proteins. Expression of the tumor suppressor BRCA1 negatively regulates IRS-1 expression through epigenetic changes of histones [86]. Steroid Receptor Coactivator (SRC)-3 (also known as Amplified in breast cancer 1 or AIB1) can induce IRS-1 and IRS-2 expression. AIB1 is an oncogene often overexpressed in human tumors and known to induce growth of hormone-insensitive cancer cells. AIB1 has been shown to directly affect IRS-1 transcription by cooperating with the activator protein-1 (AP-1) transcription factor [87, 88].

IRS-2 expression is also the target of PTEN signaling [89]. PTEN is a potent tumor suppressor that antagonizes PI3K signaling by dephosphorylating PtdIns-3,4,5-P3. Both overexpression of PTEN and inhibitors of PI3K signaling not only increase IRS-2 levels but also increase its association with the p85 regulatory subunit of PI3K subunit, in MDA-MB-468 human breast carcinoma cells, therefore allowing more intense activation of PI3K signaling [90]. As a negative regulator of IRS-2 expression, PTEN sustains a baseline of activation of signaling pathways [89].

Recently, IRS-2 expression has also been shown to be regulated by micro RNAs (miR). Ectopic expression of tumor suppressor micro RNA-153 (miR-153) results in decreased Akt activation due to decreased IRS-2 expression in glioblastome cells [91]. Induction of miR-153 expression at endogenous levels by treatment with chromatin-

modifying drugs also results in suppression of IRS-2, Bcl-2, and Mcl-1 expression. Growing evidence suggests a complex regulation of IRS-2 expression at both transcriptional and post-translational levels to mediate IRS-2-dependent signaling in normal and malignant cells. Combined effects of external stimuli and cell signaling determines the outcome of overall IRS-2 expression.

Hypoxia and tumor progression

Rapidly growing tumors often exhibit areas of low oxygen content, or hypoxia. Oxygen concentration in normal tissues can vary from 20 percent atmospheric oxygen in the lungs to about 5 percent oxygen in other organs [92]. The concentration of oxygen in tumors has been found to be overall lower than in adjacent tissues. It decreases gradually from the outer edge towards the center of the tumor, where it can be as low as 0.3-0.5 per cent [92]. However, the distribution of oxygen in the tumor often depends on tumor vascularization and on the proximity of major blood vessels to the tumor [92]. Hypoxic, or oxygen-deprived, areas often appear in tumors that outgrow the rate of blood vessel formation, and is often associated with necrosis due to oxygen and nutrient deficiency. Most solid tumors greater than $1mm^3$ in size exhibit a regional hypoxic environment due to slow growth of blood vessels [93]. Normal cells do not survive under such conditions, but cancer cells often develop resistance to hypoxia, leading to increased invasiveness, survival and metastatic potential [94, 95].

The above changes occur as a result of altered gene expression in response to decreased oxygen concentration in the tumor microenvironment. Overall gene expression

is shut down in response to hypoxia through mTOR downregulation to sustain cell viability and only expression of genes that are important for adaptation and survival in hypoxia is upregulated [96, 97]. Genes that are increased by hypoxia regulate survival, DNA damage responses, invasion, metabolic changes and tumor angiogenesis [97-100]. Among survival genes that are upregulated by hypoxia are Bcl2, PDK-1 and -3, and also the heat-shock family member HSP70, which also has been implicated in tumor cell growth [101-104]. An essential group of genes that are upregulated by hypoxia are metabolic pathway genes. These include glucose transporter-1 (GLUT-1), aldolase A (ALDOA), phosphoglycerate kinase 1 (PGK1), and lactate dehydrogenase A (LDHA). Increased expression of these genes leads to cell adaptation to low oxygen conditions thorough shifting cellular metabolism from oxidative phosphorylation to oxygen-independent anaerobic glycolysis for ATP production [97-100, 105, 106]. This process results in the increased production of lactic acid, a byproduct of glycolysis, which in turn alters the pH homeostasis of the tumor microenvironment. Many carcinomas have an increased concentration of lactic acid, which is also associated with increased risk of metastasis [107]. Low pH levels in the tumor can indirectly increase the metastasis rate by promoting degradation of the basal membrane and adjacent connective tissue to facilitate tumor cell dissemination. A highly acidic tumor microenvironment also creates an additional selective pressure for tumor cell survival, creating a new population of highly aggressive cells that are resistant to low oxygen and nutrient conditions, and low pH environment. Tumor hypoxia can also directly induce tumor cell invasion and motility. It has been shown to induce tumor metastasis by increasing tumor cell motility in both MDA-MB-231 and MCF-7 human breast carcinoma cell lines [108-110].

Prolonged hypoxia-induced oxidative stress also results in tumor cell resistance to radiation and chemo therapy [111, 112]. Hypoxia has been shown to upregulate not only anti-apoptotic genes but also drug transporter proteins such as MDR1 that lowers the concentrations of cytotoxic drugs in the cells [113]. Upregulation of pyruvate dehydrogenase kinase-3 (PDK-3) by hypoxia has also been shown to increase drug resistance in Hela cells [101].

Another crucial effect of hypoxia on tumor progression is promoting epithelial to mesenchymal transition (EMT) in cancer cells. EMT is a process by which epithelial cells lose their polarity and are converted to a mesenchymal phenotype. It is considered a critical event in morphogenetic changes during embryonic development, wound healing, and cancer metastasis. Cells undergoing EMT have a less differentiated morphology, lose expression of epithelial markers (E-cadherin, cytokeratin) and express mesenchymal markers (vimentin), and become more motile. The presence of cells with these features within a tumor is often associated with increased metastasis, drug resistance and poor prognosis [114, 115].

Hypoxia has been shown to induce EMT directly within 72 hours in several epithelial-like cancer cell lines, including HepG2 hepatoma, PANC-1 pancreatic carcinoma, HT-29 colon cancer, and MCF-7 breast carcinoma cell lines [116]. The early EMT switch in hypoxia was found to be dependent on increased levels of reactive oxygen species [116]. Tumor hypoxia has been shown to promote EMT through several mechanisms. For example, the E-cadherin transcriptional repressor TWIST is positively regulated by hypoxia inducible factor-1 (HIF-1) transcription factor [115]. Notch has been shown to positively regulate expression and stability of the transcription factor Snail,

another EMT regulator, in hypoxia [117]. HIF-1 has also been shown to directly regulate Snail expression in mouse cells [118]. ERbeta has also been shown to maintain epithelial phenotype in prostate cancer via inhibition of HIF-1-dependent VEGF expression, therefore inhibiting VEGF-A receptor neuropilin-1 signaling leading to exclusion of Snail from the nucleus [119].

The importance of the tumor microenvironment, and hypoxia in particular, for tumor metastasis is highlighted in several studies that investigated the effect of anti-angiogenic treatment on tumor progression. Short-term treatment with single anti-angiogenic agent was shown to have a profound effect on tumor growth and size. However, the tumor shrinkage caused by the treatment resulted in severe hypoxia inside the tumor due to a disrupted vasculature. This led to acquired resistance of tumor cells to hypoxia and subsequent tumor outgrowth. Most importantly, treated tumors showed a significantly higher rate of local invasion, distant metastasis, and decreased overall survival than tumors in untreated animals [120, 121]. Two independent groups have obtained identical results with different anti-angiogenesis treatments in different mouse models of pancreatic cancer, glioblastoma and melanoma tumor progression. Therefore it is crucial to understand the effect of hypoxia on carcinoma cell behavior and regulation of hypoxic gene expression.

Hypoxic regulation of gene expression

The expression of the majority of hypoxia-responsive genes is regulated by a family of transcription factors, the Hypoxia Inducible Factors (HIF). The HIF transcription factors

are heterodimers consisting of two basic helix-loop-helix (bHLH) Per/Arnt/Sim (PAS) proteins, HIF-alpha and HIF-beta. The beta subunit (aryl hydrocarbon receptor nuclear translocator (ARNT)) is expressed constitutively and is independent of the oxygen concentration. The alpha subunit levels are regulated in an oxygen-dependent manner. The alpha subunit exists in three isoforms, HIF-1alpha, HIF-2alpha (EPAS1), and HIF-3alpha (IPAS). HIF-1 and HIF-2 share similar structure and are well characterized. HIF-3alpha exists as multiple splice variants, some of which can inhibit HIF-1alpha and HIF-2alpha activity in a dominant-negative manner [122]. HIF-3alpha lacks a transactivation domain and acts as a repressor of HIF-1alpha activity by interfering with its ability to bind to HIF-1beta [122].

Both HIF-1alpha and HIF-2alpha have two transactivation (TAD) domains, one at the N-terminus (NTAD) and one at the C-terminus (CTAD) [123]. Under high oxygen concentrations, HIF-1alpha and HIF-2alpha are constitutively expressed but rapidly degraded by an oxygen-sensing mechanism. HIF-alpha becomes hydroxylated at one or both of two highly conserved prolyl residues located near NTAD by the members of the prolyl hydroxylase domain (PHD) family of proteins [124-126]. This hydroxylation event creates a binding site for the von Hippel-Lindau (VHL) tumor suppressor protein, which is a component of an ubiquitin ligase complex [124, 127, 128]. HIF-alpha is then ubiquitinated and targeted for proteasomal degradation [129, 130]. The activity of PHD proteins depends entirely on oxygen concentration. At decreased oxygen levels, HIF-alpha can no longer be hydroxylated. It dimerizes with the beta subunit, translocates to the nucleus, and binds to the promoters of genes that contain a hypoxia responsive enhancer element (HRE), with the consensus sequence 5’-(A/G)CGTG-3’ [131].

Factor-inhibiting HIF (FIH) can also inhibit HIF-1alpha activity in an oxygen-dependent manner. It hydroxylates asparaginyl residues in the HIF-1alpha CTAD, preventing recruitment of coactivators p300 and CBP [132, 133]. Unlike PHD proteins, FIH remains active at lower oxygen concentrations and therefore can inhibit HIF transactivation activity under more moderate hypoxic conditions, when PHD proteins are inactive. Other mechanisms can also regulate HIF-alpha stability. For example, inhibition of HSP90 function can promote HIF-1alpha degradation in a VHL-independent manner. Histone deacetylase inhibitors can also promote HIF-1alpha degradation in a VHL-independent manner, which involves increased interaction between HIF-1alpha and HSP70, after the disruption of HSP70/HSP90 signaling mediated by HDAC-6 activity [134, 135].

HIF-1 and HIF-2 share regulation of expression of many hypoxia-responsive genes, but also have unique target genes [136]. For example, HIF-1 has been shown to be the major regulator of expression of glycolytic enzymes [136]. HIF-2 on the other hand has been shown to be a dominant regulator of EPO expression [137, 138]. Their activity also differs under long- and short-term hypoxia. Most of the early hypoxia-responsive genes are regulated either by HIF-1 alone or are a common HIF-1 and HIF-2 target. At later time points, HIF-1 expression and expression of its specific target genes have been shown to decrease, while HIF-2 activity increases [139]. The differences in transcriptional activity between HIF-1 and HIF-2 can also be due to different sensitivity for FIH activity. HIF-2alpha is relatively resistant to FIH1-mediated inhibition, compared to HIF-1alpha [140]. The specificity of HIF-1 and HIF-2 in regulating transcription of unique target genes also may result from unique co-activators for each HIF and specific transcription factors that HIF interacts with in regulating gene expression.

The transcriptional activity of HIF largely depends on its co-activators, recruitment of which is mediated by the TAD domains. The most common co-activator of HIF is p300/ Creb-binding protein (CBP). Its main function is to stabilize the transcription initiation complex and to recruit histone acetyl-transferase enzymes, such as the redox factor (Ref-1) [141]. Ref-1 has some histone acetyl-transferase activity and has been shown to regulate formation of the hypoxia-inducible transcriptional complex on the VEGF promoter in rat endothelial cells [142]. Several co-activators have been found to be specific for HIF-2alpha. For example, transcription factor Ets1 has been shown to interact exclusively with HIF-2alpha in regulating VEGF receptor 2 (Flk-1) transcription [143]. Another Ets family transcription factor, Elk-1, cooperates with HIF-2alpha to activate expression of CITED-2 in hypoxia in MCF-7 human breast carcinoma cell line [144].

A large number of hypoxia-responsive genes can also be upregulated in a HIF-independent manner. Multiple transcription factors have altered activity through changes in phosphorylation, localization, expression, or dimerization partners in hypoxia. For example, AP1 is upregulated by hypoxia through increased expression of cFos and cJun [145, 146]. VHL has also been implicated in regulating gene expression in hypoxia independent from HIF [147, 148].

Often both mechanisms, HIF-dependent and –independent, are combined to achieve a higher and more prolonged expression of hypoxia-responsive genes. Many of the genes regulated by hypoxia contain enhancer elements for multiple hypoxia-regulated factors. For example, VEGF, a well-known common HIF-1 and HIF-2 target gene, has also been shown to be regulated by Ras in a HIF-independent manner in hypoxia in colon cancer [149]. The role of Ras in hypoxic expression of VEGF was identified when a VEGF

promoter construct with a mutated HRE was found to still be upregulated by hypoxia. Overexpression of K-ras had a synergistic effect with hypoxia on VEGF expression. This shows that VEGF expression is regulated by both HIF-dependent and HIF-independent mechanisms. In another example of hypoxic regulation of gene expression by multiple transcription factors, hypoxic endothelin-1 expression in vascular endothelial cells requires AP-1, GATA-2, and NF-1 for HIF-1 binding and subsequent recruitment of p300/CBP to the hypoxia-responsive complex at the promoter [150]. In addition, transcription factors can regulate the expression and activity of each other in hypoxia. For example, Forkhead Transcription Factors (FOXO) modulate HIF expression and activity in a cooperative regulation of hypoxic gene expression [151, 152]. AP-1 coactivator Jab1 (Jun activation domain-binding protein-1) can interact with HIF-1alpha and increase its stability [153]. Therefore, all hypoxia-responsive transcription factors work in a coordinated manner to tightly regulate gene expression in response to environmental stimuli.

Rationale for the project

Hypoxia is known to be a potent effector of tumor progression. It increases tumor cell metastatic potential by altering cellular metabolism from oxidative phosphorylation to anaerobic glycolysis. Moreover, cyclic hypoxia results in a permanent switch to glycolysis even in the presence of oxygen, or aerobic glycolysis, a common feature of highly aggressive cancer cells. Hypoxia has also been shown to induce cell invasion, survival, and drug-resistance through altered gene expression, and promote cell motility through EMT induction. Since IRS-2 has been implicated in several of the cellular processes altered by

hypoxia, including cell motility, invasion, survival, glycolysis, and metastasis, we sought to determine if IRS-2 was a hypoxia-responsive gene and if its upregulation contributed to the effect of hypoxia on tumor cells.

Based on the positive role of IRS-2 in tumor progression and metastasis, IRS-2 could be a valid target for designing new therapies. However, since the IRS-2 protein does not have enzymatic activity, it cannot be used as a treatment target. Therefore, understanding the mechanism of regulation of its transcription could uncover new avenues for targeting IRS-2 expression in cancer cells.

Figures

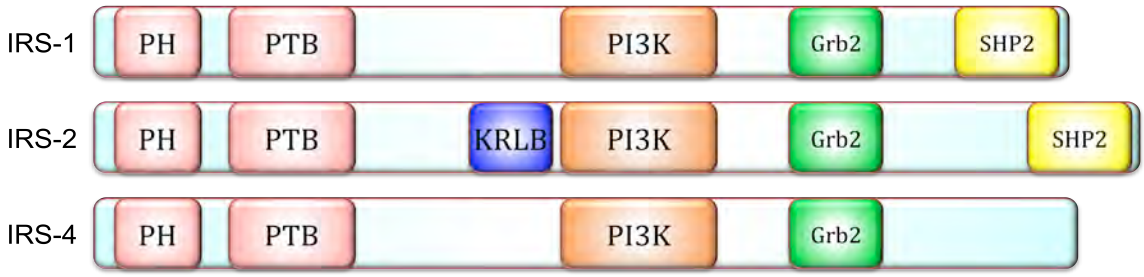

Figure 1.1. Schematic of the IRS protein family. Interaction domains of the IRS proteins are indicated. PH (pink), pleckstrin homology domain; PTB (purple), phosphotyrosine binding domain; KRLB (blue), kinase regulatory loop binding domain; PI3K (orange), region containing multiple PI3K binding motifs; Grb-2 (green), Grb-2 binding site; SHP-2 (yellow), SHP-2 binding site.

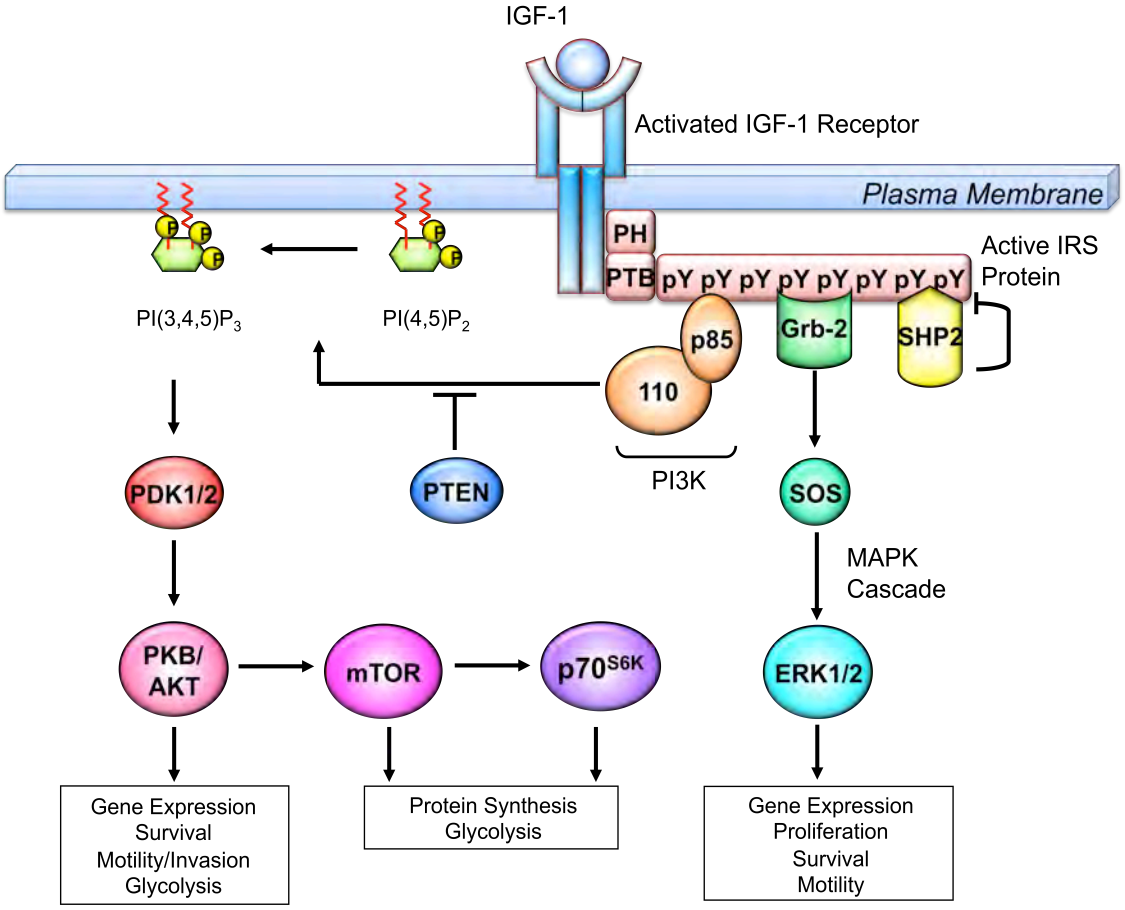

Figure 1.2. Signaling via the IRS proteins. The IRS proteins are recruited to activated cell surface receptors via PH/PTB domains in their N-termini. Once bound, they are phosphorylated on tyrosine residues in their C-termini. The phosphorylation of tyrosine residues (pY) creates docking sites for the recruitment of downstream signaling effectors. Subsequently, downstream signaling pathways are activated that can regulate gene expression, protein synthesis, glycolysis, cell proliferation, survival and motility/invasion.

CHAPTER II: Hypoxia regulates Insulin Receptor Substrate-2 expression to promote breast carcinoma cell survival and invasion

Parts of this chapter represent work published as:

Hypoxia regulates Insulin Receptor Substrate-2 expression to promote breast carcinoma cell survival and invasion.

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Cancer Res. 2009 Dec 1; 69(23): 8894-901.

Abstract

Insulin Receptor Substrate-2 (IRS-2) belongs to the IRS family of adaptor proteins that function as signaling intermediates for growth factor, cytokine and integrin receptors, many of which have been implicated in cancer. Although the IRS proteins share significant homology, distinct functions have been attributed to each family member in both normal and tumor cells. In cancer, IRS-2 is positively associated with aggressive tumor behavior. In the current study, we demonstrate that IRS-2 expression, but not IRS-1 expression, is positively regulated by hypoxia, which selects for tumor cells with increased metastatic potential. We identify IRS-2 as a novel hypoxia responsive gene and establish that IRS-2 gene transcription increases in a HIF-dependent manner in hypoxic environments. IRS-2 is active to mediate IGF-1-dependent signals in hypoxia, and enhanced activation of Akt in hypoxia is dependent upon IRS-2 expression. Functionally, the elevated expression of IRS-2 facilitates breast carcinoma cell survival and invasion in hypoxia. Collectively, our results reveal a novel mechanism by which IRS-2 contributes to the aggressive behavior of hypoxic tumor cells.

Introduction

Insulin Receptor Substrate-2 (IRS-2) belongs to the IRS family of cytoplasmic adaptor proteins that function as signaling intermediates for activated cell surface receptors. The IRS proteins are immediate downstream effectors of the insulin-like growth factor-1 (IGF-1) and insulin receptors, several cytokine receptors, prolactin, growth hormone (GH) and vascular endothelial growth factor (VEGF) receptors, and members of the integrin receptor family [154]. The IRS proteins act as scaffold proteins to recruit signaling molecules to the receptors to regulate intracellular signaling cascades [155]. Although IRS-1 and IRS-2 share significant homology and both have been implicated in tumorigenesis, distinct functions for these adaptor proteins in cancer progression have been identified [50, 156]. In this regard, IRS-2 is positively associated with aggressive tumor behavior. In MMTV-PyV-MT mice, mammary tumor metastasis is significantly diminished in the absence of Irs-2, and Irs-2 activation is enhanced in Irs-1-deficient tumors that are highly metastatic [43, 59]. Similarly, Irs-2 expression is elevated in tumors that arise in PTEN-/+ mice, and deletion of Irs-2 suppresses tumor growth and progression to invasive disease [45].

Mechanistic experiments aimed at understanding how IRS-2 contributes to tumor progression have revealed a role for this adaptor protein in regulating cell invasion and survival. PyV-MT-derived mammary tumor cells that lack Irs-2 expression are less invasive and more sensitive to apoptosis induced by serum deprivation than are their wild type counterparts [48]. IGF-1 predominantly induces IRS-2 phosphorylation in MDA-MB-231 human breast carcinoma cells selected for metastatic behavior *in vivo* [157].

Introduction of an IRS-2 antisense mRNA into these metastatic cells results in decreased IGF-1-induced cell motility and anchorage-independent growth [157]. Similarly, expression of IRS-2 in T47D breast carcinoma cells results in increased cell motility in response to IGF-1 stimulation [158]. One mechanism by which IRS-2 promotes mammary tumor cell invasion is through the regulation of GLUT-1 localization to the cell surface to increase glucose uptake and enhance aerobic glycolysis [159]. Tumor cells depend more upon glycolysis than oxidative phosphorylation to generate ATP and studies have shown that it provides tumor cells with a selective advantage in their ability to progress towards invasive and metastatic disease [160, 161].

Rapidly growing tumors develop areas of low oxygen tension, or hypoxia, when their growth outpaces the development of new blood vessels [162]. Tumor cells that can develop a metabolic self-sufficiency through anaerobic glycolysis can survive in stressful environments that lack oxygen and other essential nutrients for energy production [163]. In addition, hypoxia upregulates signaling pathways that facilitate invasion and survival [164]. Therefore, exposure of tumor cells to hypoxia creates a selection for cells with a more aggressive, invasive behavior [165]. To conserve energy in hypoxic conditions, overall gene expression is suppressed and primarily genes that are essential for low-oxygen/nutrient adaptation are expressed [166]. Specifically, genes that are upregulated in response to hypoxia are involved in angiogenesis, DNA damage responses, glycolysis and survival [167]. Upregulation of these genes in response to hypoxia ultimately leads to increased metastatic potential [167].

Given that IRS-2 has been implicated in promoting both tumor cell survival and invasion, and regulating tumor cell glycolysis, we sought to determine if IRS-2 expression

is regulated by hypoxia, and if this adaptor protein contributes to breast carcinoma cell behavior in hypoxic microenvironments. In this study, we report that IRS-2 expression is increased upon exposure to hypoxia in breast carcinoma cells. The elevated expression of IRS-2 in response to hypoxia facilitates breast carcinoma cell survival and invasion.

Results

IRS-2 expression increases in response to hypoxia.

To determine if IRS-2 expression is regulated by hypoxia, MDA-MB-231 breast carcinoma cells were incubated for increasing periods of time in hypoxic (0.5% oxygen) conditions. When compared with the level of IRS-2 expression in cells maintained in normoxia (0 hours), both IRS-2 protein and mRNA expression increased significantly in response to hypoxia (Fig.2.1A and B). The induction of IRS-2 mRNA occurred within 8 hours of exposure to hypoxia, with maximal levels observed after 16-24 hours. IRS-1 mRNA expression did not increase in response to hypoxia over the same time course (Fig.2.1B). However, IRS-1 protein expression decreased after 16 hrs in hypoxia, a result that is consistent with a previous report that IRS-1 can be degraded through a caspase-mediated cleavage in response to hypoxia [168].

To determine if upregulation of IRS-2 expression is a common response of breast carcinoma cells to hypoxic conditions, IRS-2 expression was evaluated in additional cell lines. As was observed for MDA-MB-231 cells, IRS-2 mRNA and protein expression increased after 16 hrs in hypoxia in SUM159 and SUM149 human breast carcinoma cells, and also in a mouse mammary tumor cell line (PyV-MT:*WT*) (Fig.2.1C and D) [48]. IRS-1 protein and mRNA levels either remained unchanged (human) or decreased (mouse) in response to hypoxia in these additional cell lines (Fig.2.1C and E).

IRS-2 is competent for signaling in hypoxic environments.

IRS-2 is phosphorylated on multiple tyrosine and serine residues in response to different stimuli, which results in a mobility shift on SDS-PAGE gels [155]. We observed that IRS-2 mobility increases after exposure to hypoxia for more than 12 hours, which could reflect a decrease in tyrosine phosphorylation and a corresponding decrease in IRS-2 signaling. To determine if IRS-2 retains functional activity in hypoxia, we examined the signaling potential of IRS-2 in hypoxic cells. IRS-2 tyrosine phosphorylation levels and binding to the regulatory subunit of PI3K (p85) were compared in SUM159 cells that were incubated for 15 hrs in normoxia or hypoxia in the presence or absence of IGF-1 (50 ng/ml), or stimulated with IGF-1 for 15 minutes at the end of the 15-hour incubation. In normoxia, IRS-2 tyrosyl-phosphorylation increased in response to both acute and long-term IGF-1 stimulation, and PI3K recruitment increased in parallel (Fig.2.2A). In hypoxia, IRS-2 phosphorylation levels and association with PI3K were elevated 2.7-fold in the absence of exogenous IGF-1 stimulation when compared with cells maintained in normoxia, and both tyrosine phosphorylation and PI3K interactions were increased further in response to acute IGF-1 stimulation (15 min) (Fig.2.2A). Long-term IGF-1 stimulation under hypoxic conditions resulted in decreased IRS-2 expression, tyrosine-phosphorylation and association with p85 (Fig.2.2A), most likely due to the activation of a negative feedback mechanism in response to prolonged activation of this pathway [169]. Therefore, IRS-2 is functionally active and capable of signaling in hypoxic cells.

To investigate further how IRS-2 contributes to the hypoxic tumor response, activation of intracellular signaling pathways in PyV-MT:*WT* and PyV-MT:*Irs-2*$^{-/-}$ mammary tumor cell lines were examined in either normoxia or hypoxia. MAPK activation

was induced by hypoxia in both cell lines and there was a modest increase in activation in the presence of IGF-1 (Fig.2.2B). Activation of Akt was increased 16-fold in WT cells in response to hypoxia, and activation was enhanced further in response to IGF-1 stimulation (Fig.2.2B). However, Akt activation did not increase in hypoxia in the absence of IRS-2 (Fig.2.2B).

IRS-2 promotes tumor cell viability in hypoxia.

Genes that allow cells to adapt to low oxygen conditions are upregulated in hypoxia [170]. To determine if enhanced IRS-2 expression contributes to cell survival in hypoxia, PyV-MT:*WT* and PyV-MT:*Irs-2*$^{-/-}$ mammary tumor cells were incubated in hypoxia or normoxia for 24 hours in complete culture medium. PyV-MT:*Irs-2*$^{-/-}$ cells were significantly more sensitive to hypoxia-induced apoptosis than PyV-MT:*WT* cells (Fig.2.3A). To confirm a role for IRS-2 in the survival of mammary tumor cells in hypoxic environments, IRS-2 expression was suppressed by shRNA in PyV-MT:*WT* cells (Fig.2.3B). Cells expressing IRS-2-specific shRNA were more apoptotic in hypoxia when compared with cells expressing vector alone or parental PyV-MT:*WT* cells (Fig.2.3C). A similar impact of IRS-2 on survival in hypoxia was observed for the human SUM159 cell line. Cells expressing two independent IRS-2 shRNA targeting sequences that showed the best decrease in IRS-2 expression (Fig.2.3D) exhibited a significant increase in hypoxia-induced apoptosis when compared with parental or GFP shRNA expressing cells (Fig.2.3E).

IRS-2 promotes tumor cell invasion in hypoxia.

Stable shRNA-mediated knockdown of IRS-2 in MDA-MB-231 cells (Fig.2.3F) did not sensitize these cells to hypoxia-induced apoptosis (Fig.2.3G). MDA-MB-231 cells are likely resistant to apoptosis in response to IRS-2 suppression because they express mutated Ras, which can directly activate survival signaling pathways, bypassing the need for IRS-2 [171]. However, suppression of IRS-2 expression impaired significantly the ability of this highly metastatic cell line to invade in hypoxic conditions. Hypoxic cells with decreased IRS-2 levels were over 50% less invasive than parental or control GFP shRNA expressing cells (Fig.2.4A). A similar 40 % decrease in invasion in hypoxia was observed in SUM159 cells expressing IRS-2 snRNA (Fig.2.4B). However, since SUM159 cells are also apoptotic under the same conditions, we evaluated whether the decrease in invasion was due to increased apoptosis. Repeating the experiment in the presence of the DEVD apoptosis inhibitor showed that suppression of invasion was the result of apoptosis and IRS-2 was not required for the invasion of SUM159 cells in hypoxia (Fig.2.4C). Taken together with the differential impact of IRS-2 on the survival of breast carcinoma cells, our data support that IRS-2 promotes either breast carcinoma survival or invasion in hypoxia in a cell type-dependent manner, which is likely to reflect the heterogeneity of signaling pathway activity in tumor cells.

Hypoxia regulates IRS-2 transcription.

To examine the mechanism by which IRS-2 expression is enhanced by hypoxia, we compared IRS-2 protein and mRNA stability under normoxic and hypoxic conditions in

murine PyV-MT:*WT* and human MDA-MB-231 cells. Cells were incubated for 16 hours in hypoxia and then treated with either cycloheximide (CH) or Actinomycin D (ActD) for additional time periods to inhibit protein translation or mRNA transcription, respectively. IRS-1 and IRS-2 protein expression decreased in both cell lines after addition of CH in both normoxic and hypoxic conditions (Fig.2.5A). Densitometric analysis revealed that IRS-2 protein is slightly less stable in hypoxia when compared with normoxia (Fig.2.5B). The decreased gel mobility observed for both IRS-1 and IRS-2 after treatment with CH is most likely due to increased ubiquitination, as it has been previously shown that the IRS proteins can be degraded through ubiquitin-dependent proteasomal degradation [169, 172]. IRS-2 mRNA stability was similar in hypoxia and normoxia for both murine PyV-MT:WT and human MDA-MB-231 cell lines (Fig.2.5C).

Next, we sought to determine if IRS-2 mRNA expression is regulated by hypoxia at the level of gene transcription. MDA-MB-231 cells were pretreated with ActD for 30 minutes prior to hypoxic exposure to block *de novo* gene transcription. Cells were incubated for 16 hours in hypoxia in the continued presence of ActD or vehicle (DMSO), or left untreated. ActD pre-incubation inhibited the upregulation of IRS-2 expression in response to hypoxia (Fig.2.6A). To investigate further the hypoxic regulation of IRS-2 transcription, MDA-MB-231 cells were transiently transfected with a pGL3-luciferase plasmid containing 2.3 Kb or 1Kb of the human IRS-2 promoter [173]. A 2.5 fold induction of luciferase activity was observed in hypoxia for the IRS-2 promoter, which mimics the fold change in endogenous IRS-2 mRNA expression in MDA-MB-231 cells (Fig.2.6B).

A modest upregulation of IRS-2 protein in the absence of an mRNA increase was sometimes observed in response to acute hypoxia (0.5-2 hours) (Fig.2.1A). To test if this increase was regulated by translation or protein stability, MDA-MB-231 cells were treated with cycloheximide (CH) before exposure to hypoxia for 0.5, 2, or 4 hours. Untreated cells showed a small upregulation of IRS-2 protein at 0.5 hours in hypoxia. This effect was enhanced in the cells pre-treated with CH, indicating that the upregulation of IRS-2 protein expression in response to acute hypoxia does not require translation and is likely the result of a transient increase in protein stability (Fig.2.6C).

HIF-1 and HIF-2 are required for the regulation of IRS-2 transcription in response to hypoxia.

The HIF family of transcription factors are the major regulators of hypoxia-induced transcription [131, 174, 175]. To determine if HIF-1 or HIF-2 play a role in regulating IRS-2 expression in response to hypoxia, an siRNA targeting approach was used to transiently suppress HIF-1α or HIF-2α expression (Fig.2.7A). Knockdown of HIF-1α or HIF-2α alone did not significantly inhibit IRS-2 protein or mRNA expression in hypoxia. However, simultaneous knockdown of both HIF-1α and HIF-2α prevented IRS-2 protein and mRNA upregulation in response to hypoxia (Fig.2.7B).

Discussion

In this study, we identify IRS-2 as a hypoxia-responsive gene that contributes to breast carcinoma cell survival and invasion in hypoxic environments. Exposure of breast carcinoma cells to hypoxia increases IRS-2 expression, but not IRS-1 expression, at the level of gene transcription. IRS-2 is phosphorylated on tyrosine residues and recruits PI3K in response to IGF-1 stimulation in hypoxia, indicating that IRS-2 is functionally active to mediate signaling in low oxygen conditions. In this regard, activation of Akt in response to hypoxia is dependent upon IRS-2 expression. Functionally, IRS-2 can protect cells from apoptosis and promote invasion in hypoxic environments. Collectively, our results provide a novel mechanism by which IRS-2 contributes to the aggressive behavior and metastasis of hypoxic tumor cells.

IRS-1 and IRS-2 expression are differentially regulated by hypoxia in breast carcinoma cells, a finding that adds to a growing body of evidence that these homologous adaptor proteins are not functionally redundant. IGF-1-dependent signaling through IRS-1 or IRS-2 in human breast carcinoma cells stimulates proliferation or migration/invasion, respectively [158]. IRS-2, but not IRS-1, has been implicated in metabolic regulation in tumor cells, through the regulation of glycolysis [159]. *In vivo*, mammary tumors that lack Irs-2 expression are significantly impaired in their ability to metastasize, and Irs-1 cannot compensate for this function [48]. In fact, in the absence of Irs-1 expression, Irs-2 expression and signaling increase in cell lines in culture and in tumors, and metastasis is enhanced [176]. Taken together, these findings infer that the balance of IRS-1 and IRS-2 expression can significantly impact tumor cell function and progression. Shifting the IRS

balance in favor of IRS-2 would promote metabolic independence, invasive ability and survival, factors that contribute to the metastatic potential of a tumor. The fact that hypoxia concurrently suppresses IRS-1 expression while upregulating IRS-2 expression reveals a novel endogenous mechanism by which this balance is altered to favor tumor progression.

The regulation of IRS-2 gene expression by hypoxia implicates IRS-2 in the adaptation of tumor cells to hypoxia and reveals a novel mechanism by which hypoxic cells acquire a more aggressive behavior after exposure to low oxygen conditions. Hypoxia occurs in areas of tumors that are poorly vascularized, which results in decreased oxygen delivery to the tumor cells [177]. Overall, gene expression is suppressed in hypoxia as a mechanism to conserve energy in this stresssful microenvironment, which is often lacking in nutrient availability as well [167]. In general, the genes that are expressed in hypoxic environments are essential for tumor cells to survive in, and ultimately adapt to, low oxygen conditions. For example, genes that regulate anaerobic glycolysis are coordinately expressed in hypoxia to facilitate energy production when oxidative phosphorylation is inhibited by insufficient oxygenation [131]. Genes such as VEGF are also upregulated to increase angiogenesis and restore normoxic conditions [178]. Chronic exposure to hypoxia creates a selection for cells with a tolerance for hypoxia and these cells become more invasive and metastatic [165]. The impact of this selective pressure is highlighted by recent studies revealing that anti-angiogenic therapy alone may provide only short term benefit for many cancer patients because the disruption of blood vessels leads to increased hypoxia, and patients will go on to develop metastatic disease [120, 121]. These studies underscore the importance of understanding how tumor cells maintain their viability in hypoxia. Our current findings that IRS-2 contributes to breast carcinoma cell survival in

hypoxia, along with our previous demonstrations that IRS-2 regulates aerobic glycolysis and positively contributes to mammary tumor metastasis, identify this adaptor protein as a key mediator of signals that influence tumor cell responses to hypoxia.

Our data reveal that one mechanism by which IRS-2 contributes to the hypoxic tumor response is by sustaining activation of Akt in hypoxia. In our previous *in vivo* studies, Akt signaling was upregulated in PyV-MT:*Irs-1*$^{-/-}$ tumors that have enhanced Irs-2 expression and association with PI3K, providing evidence that our *in vitro* findings linking IRS-2 with Akt activation are recapitulated in tumors [176]. A number of studies have implicated Akt signaling in positively regulating tumor cell survival in hypoxia and several mechanisms for its action have been proposed [179]. Akt negatively regulates the function of pro-apoptotic downstream effectors including the FOXO transcription factors and the pro-apoptotic protein Bad [180]. IRS-2 regulates FOXO function through Akt in mouse embryo fibroblasts, and this regulatory pathway has been proposed to control nutrient homeostasis [181]. Viability and growth are also influenced by the Akt-dependent regulation of genes that control energy production through the switch from oxidative phosphorylation to anaerobic glycolysis for ATP generation [182]. Akt signaling can also enhance the expression of HIF-1α to amplify the expression of HIF target genes [183, 184]. In this regard, SUM149 cells, which lack PTEN and have elevated Akt activity, exhibited the greatest induction of IRS-2 expression in hypoxia [185]. As mentioned previously, tumors that arise in PTEN+/- mice also have elevated IRS-2 expression [45]. These findings raise the possibility that in tumor cells with PI3K pathway mutations, hypoxia provides a second “positive hit” by upregulating IRS-2 expression to counterbalance

negative feedback regulation of IRS-2, and by doing so enhancing downstream PI3K signaling to promote tumor progression.

Hypoxic regulation of IRS-2 expression requires the function of either HIF-1 or HIF-2. The HIFs are major regulators of hypoxia-responsive gene transcription and each factor consists of two subunits, HIF-α and HIF-β/ARNT [186]. HIF-1α and HIF-2α subunits are highly homologous, and both contain basic helix-loop-helix (bHLH), Per/ARNT/Sim (PAS), and oxygen-dependent degradation (ODD) domains [186]. In low oxygen conditions, the HIF-α subunit is stabilized and it interacts with HIF-β to form the active HIF transcription factor, which binds to HRE sequences in target genes [187]. HIF-1 and HIF-2 can regulate both unique and common target genes [136, 188]. Suppression of both HIF-1α and HIF-2α was required to prevent hypoxia-dependent upregulation, indicating that IRS-2 is a common target gene for HIF-1 and HIF-2. However, given the lack of a canonical HRE in the hypoxia responsive region of the promoter that we identified and the delayed timing of the increase in IRS-2 expression in response to hypoxia, additional factors are likely to contribute to the regulation of IRS-2 expression. A number of transcription factors have been identified that stimulate gene expression in response to hypoxia, and these factors either act in cooperation with HIF, or are regulated by HIF, to alter hypoxic gene expression [189]. Importantly, some of these transcription factors have been previously implicated in the regulation of IRS-2 gene expression including AP-1, the forkhead transcription factors FOXO1 and FOXO3a, and CREB [80, 85].

The delayed timing of IRS-2 expression and the fact that a functional HRE site is not required for hypoxia-dependent regulation indicates that IRS-2 may be an indirect HIF target. We hypothesize that HIF regulates the expression of an intermediate transcription factor that binds to the IRS-2 promoter directly. A number of transcription factors have been identified that regulate gene expression in response to hypoxia, and these factors either act independently or in cooperation with HIF to alter hypoxic gene expression [170]. Many of these factors are targets of HIFs, and their expression increases in a HIF-dependent manner. Importantly, some of these transcription factors have been previously implicated in the regulation of IRS-2 gene expression. AP-1 is required for the EGF-induced regulation of IRS-2 expression [85]. AP-1 can be regulated by hypoxia in a cell type-specific manner and it modulates target gene expression in hypoxia by cooperating with and altering the activity of other transcription factors, including HIF-1 [170, 190]. Members of the forkhead transcription family, FOXO1 and FOXO3a, positively regulate IRS-2 expression in primary hepatocytes through an insulin response element (IRE) sequence in the promoter [80]. Expression of the FOXO3a transcription factor is upregulated by hypoxia in a HIF-1-dependent manner in fibroblasts and in MCF-7 breast carcinoma cells [151]. This upregulation leads to activation of a feedback loop in which FOXO3a in turn induces transcription of CITED2, a negative regulator of HIF-1 function to inhibit expression of HIF-1 pro-apoptotic target genes [151]. In mouse islet β-cells, IRS-2 expression is positively regulated by c-AMP-mediated activation of CREB [191]. CREB phosphorylation and activation can be regulated by hypoxia [192]. In addition, CBP/p300, a CREB co-activator, forms a DNA-bound complex with HIF-1 to regulate hypoxia-responsive gene expression, including EPO and VEGF [141]. Moreover, several

studies have shown that the CRE-binding transcription factors ATF-1 and CREB can bind to the canonical HRE sequence, while HIF-1 can also bind to the CRE element to activate transcription of target genes [193, 194].

In summary, we have established a novel mechanism by which hypoxia selects for aggressive tumor behavior and promotes metastatic disease. The identification of IRS-2 as a hypoxia-responsive gene that regulates signaling pathways important for tumor cell survival and invasion in hypoxic environments opens a new avenue for investigation into how this pathway could be manipulated for therapeutic benefit.

Materials and Methods

Cell lines and hypoxia treatment. Mouse mammary tumor cell lines were isolated from PyV-MT-derived wild-type (*WT*) and *Irs-2*$^{-/-}$ tumors as described previously [48]. MDA-MB-231 human breast carcinoma cells were obtained from ATCC. SUM159 and SUM149 human breast carcinoma cells were a gift from Dr. A. Mercurio (UMass Medical School).

For hypoxia exposure, cells were maintained at a constant gas mixture of 0.5% oxygen, 94.5% nitrogen and 5% carbon dioxide in an InVivo$_2$ Hypoxia Workstation (Ruskinn Technology Ltd) for periods of time indicated in each Figure Legend.

Cycloheximide and Actinomycin-D treatments. Cells were incubated in normoxia or hypoxia for 16 hours and then cycloheximide (20ug/ml) (Sigma) or Actinomycin-D (10uM) (Sigma) were added to the cell culture medium for 1, 3, or 6 hours of incubation. Cells were also pretreated with Actinomycin-D (10uM) or DMSO for 30 minutes in normoxia before being transferred to hypoxia.

Luciferase assays. The human 1 Kb IRS-2 promoter-luciferase expression plasmid was a gift from Dr. J. Goldstein (UT Southwestern Medical Center) [195]. The human 2.3 Kb IRS-2 promoter luciferase plasmid was a gift from Dr. A. Lee (Baylor College of Medicine) [85, 173].

Cells were plated in triplicate wells of a 24-well plate and co-transfected with the promoter-luciferase plasmids (0.25 ug) and a pRL-CMV renilla luciferase plasmid (0.25 ug) (Promega). After an overnight incubation, duplicate plates were incubated either in

hypoxia or normoxia for 24 hours. Cells were assayed for firefly and renilla luciferase activity using the Dual-Glo Luciferase Assay System (Promega).

RNAi-mediated suppression of gene expression. HIF1α and HIF2α smart pool siRNAs (Dharmacon) were transfected using Oligofectamine reagent (Invitrogen). Lentiviral vectors containing murine Irs-2 small hairpin RNAs (shRNA) were a gift from Dr. B. Lewis (UMass Medical School). Lentiviral vectors containing human IRS-2 and GFP small hairpin RNAs were obtained from Open Biosystems (Huntsville, AL).

RNA extraction and real-time quantitative PCR (RQ-PCR). mRNA was extracted from cells using the RNeasy kit (Qiagen), treated with DNaseI (Invitrogen), and converted to cDNA using SuperscriptII® Reverse Transcriptase (Invitrogen). Gene expression was quantified using Syber Green RT-PCR master mix reagents (Applied Biosystems). The Delta Ct method was used to quantify the relative expression of each gene. IRS expression was normalized to either murine GAPDH or human Actin. Primers used in RQ-PCR: mouse Irs-1 (forward: 5'-ctggacatcacagcagaatga-3' and reverse: 5'-ggtaaacaaaactgtaacggatgc-3'), mouse Irs-2 (forward: 5'-cactggagctttgccctct-3' and reverse: 5'-gctggtagcgcttcactctt-3'), mouse GAPDH (forward: 5'-ggagaaacctgccaagtatga-3' and reverse: 5'-accaggaaatgagcttgaca-3'), human IRS-1 (forward: 5'-caactggacatcacagcagaa-3' and reverse 5'-actgaaatggatgcatcgtacc-3'), human IRS-2 (forward: 5'-ccaccatcgtgaaagagtga-3' and reverse: 5'-cagagtccacagatgtttccaa-3'), human Actin (forward: 5'-tgagcgcggctacagctt-3' and reverse: 5'-tccttaatgtcacgcacgattt-3').

Immunoprecipitation and immunoblotting. IRS-2 immunoprecipitations and immunoblots were performed as described previously [48] using the following antibodies: IRS-2 (immunoblot, EMD Biosciences, Inc.; immunoprecipitation, Bethyl Labs); phosphotyrosine (PY99; Santa Cruz Biotechnology); p85 (gift from Dr. A. Toker, Harvard Medical School); IRS-1 (Bethyl Labs); tubulin (Sigma). All other antibodies were purchased from Cell Signaling Technology, Inc. Band intensities were quantified by densitometry using LabWorks Analysis Software (UVP, Inc.).

Apoptosis assays. Cells were plated at equal densities and allowed to adhere to plates in normoxia for 24 hours before being transferred to hypoxia for an additional 24 hours. For apoptosis assays with SUM159 cells, the culture medium was replaced with serum-free RPMI 1640 (GIBCO®) supplemented with 1g/L glucose prior to their transfer to hypoxia. Cells were analyzed using the Annexin V-PE Apoptosis Detection Kit (BD Pharmingen™).

Invasion assays. Cells were incubated in hypoxia for 16 hours prior to the invasion assay and then maintained in the hypoxia chamber during assay preparation. Invasion assays were performed as described previously [159].

Statistics. All data are represented as an average +/- standard error. All statistical analyses were performed using the unpaired Student's *t*-test.

Figures

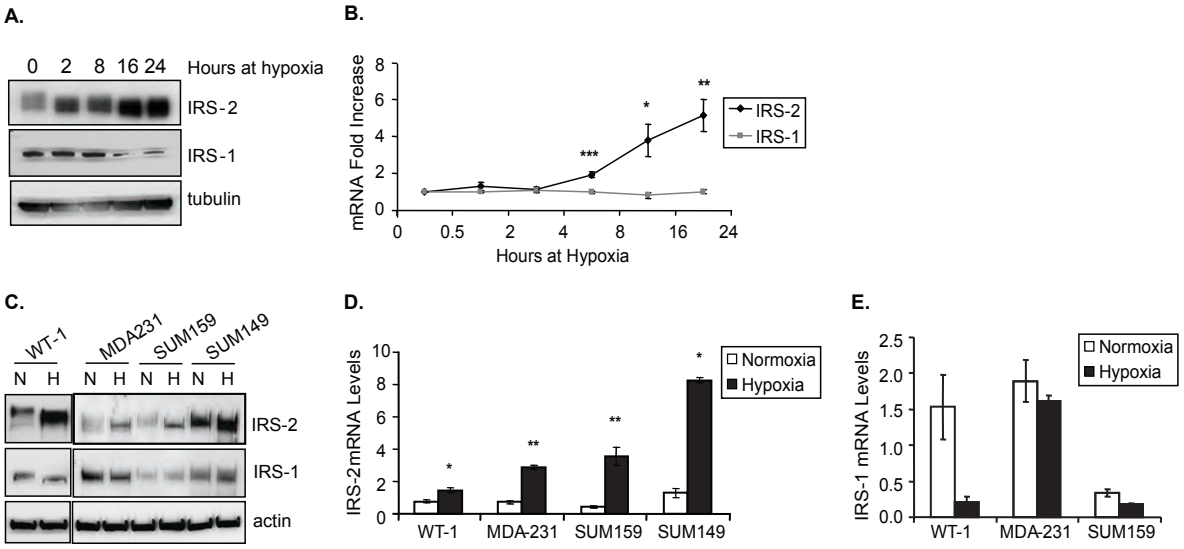

Figure 2.1. Hypoxia induces IRS-2 expression. MDA-MB-231 cells were incubated in normoxia (0 hrs) or exposed to hypoxia for the time periods indicated. A) Cell extracts were immunoblotted with antibodies that recognize IRS-2, IRS-1 or Tubulin. B) IRS-1 and IRS-2 mRNA levels were determined by RQ-PCR. Results were plotted as the fold change in mRNA levels compared with normoxic levels. The data shown represent the mean of three independent experiments (+/-SEM). *, $p < 0.04$ and **, $p < 0.004$ relative to normoxic levels. C and D) PyV-MT:*WT* murine mammary tumor cells and MDA-MB-231, SUM159, and SUM149 human breast carcinoma cells were incubated either in normoxia or hypoxia for 16 hours. Cell extracts were immunoblotted with antibodies that recognize IRS-2, IRS-1 or Actin (C). IRS-2 mRNA levels were determined by RQ-PCR (D). The data shown represent the mean of three independent experiments (+/-SEM). *, $p \leq 0.008$ relative to normoxic levels. E) PyV-MT:*WT* murine mammary tumor cells, MDA-MB-231 and SUM159 human breast carcinoma cells were incubated either in normoxia or hypoxia for 16 hours. IRS-1 mRNA levels were determined by RQ-PCR. The data shown represent the mean of three independent experiments (+/-SEM). *, $p \leq 0.02$ relative to normoxic levels.

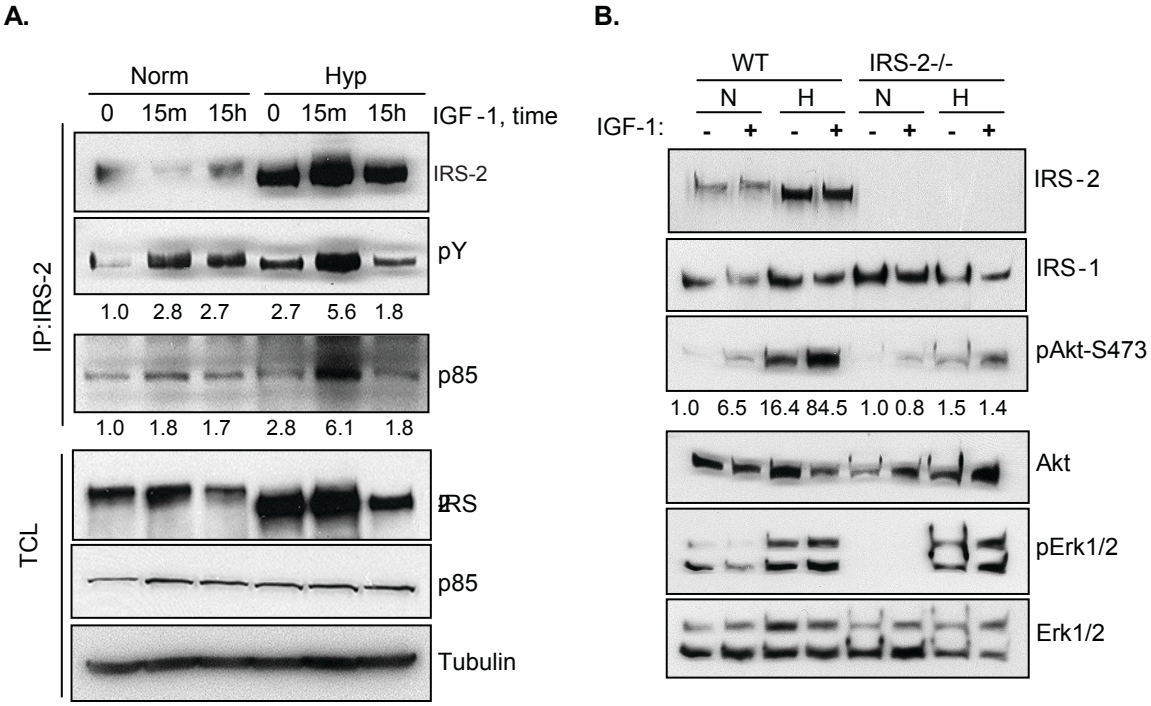

Figure 2.2. IRS-2 signaling in hypoxia. A) SUM159 cells were incubated in normoxia or hypoxia for 15 hrs in the presence or absence of IGF-1, or stimulated with IGF-1 for the final 15 minutes of the incubation. Cell extracts were immunoprecipitated with IRS-2-specific antibodies and immunoblotted with antibodies that recognize phosphotyrosine (pTyr) or the p85 regulatory subunit of PI3K. The pTyr blot was stripped and reprobed with IRS-2 antibodies. The densitometric values shown represent the fold change in IRS-2 phosphorylation and p85-association relative to unstimulated (0 hr) normoxic conditions. Cell extracts were also immunoblotted for total IRS-2, p85, and tubulin levels. TCL, total cell lysates. B) PyV-MT:*WT* cells were incubated in normoxia or hypoxia for 24 hrs in the presence or absence of IGF-1. Cell extracts were immunoblotted with antibodies that recognize IRS-2, IRS-1, phospho-Akt-S473, total Akt, phospho-Erk1/2 and total Erk1/2. Phospho-Akt levels were normalized to total Akt and the numbers represent phospho-Akt levels relative to unstimulated normoxic phospho-Akt levels.

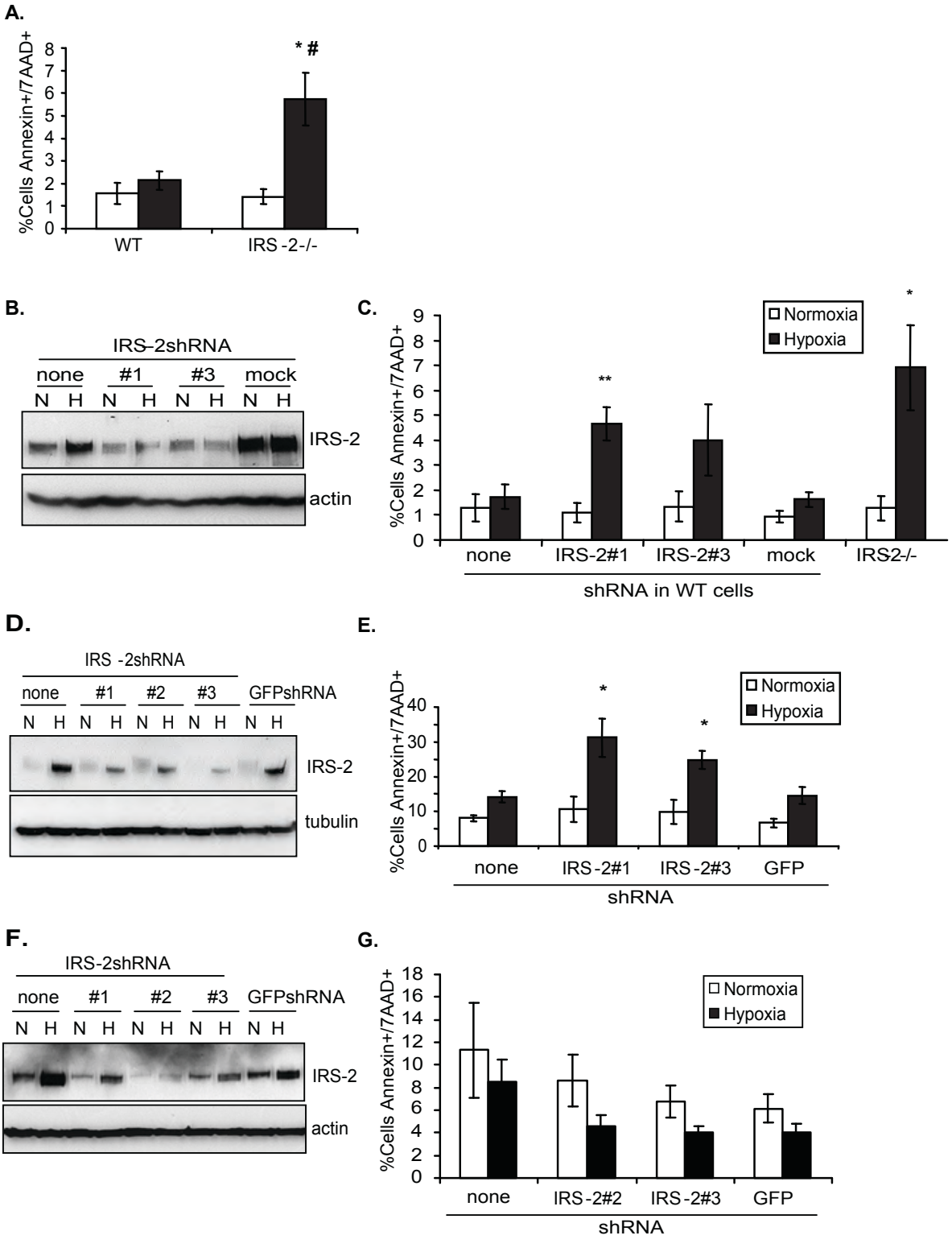
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Figure 2.3. IRS-2 promotes cell survival in hypoxia. A) PyV-MT:*WT* and PyV-MT:*Irs-2*$^{-/-}$ cells were incubated in normoxia or hypoxia for 24 hrs in complete growth medium. Apoptosis was determined by Annexin V-PE and 7-AAD staining. *, $p < 0.02$ relative to PyV-MT:*WT* apoptosis in hypoxia; #, $p < 0.007$ relative to PyV-MT:*Irs*2$^{-/-}$ apoptosis in normoxia. B and C) PyV-MT:*WT* subclones stably expressing either an Irs-2-targeted shRNA or a pLKO vector alone (PyV-MT:*WT*/mock) were incubated in normoxia or hypoxia for 24 hrs. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or Actin (B). Apoptosis was determined by Annexin V-PE and 7-AAD staining (C). *, $p < 0.04$ relative to PyV-MT:*WT*/mock apoptosis in hypoxia. D and E) SUM159 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 hrs in serum-free RPMI media supplemented with 1 g/L glucose. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or Tubulin (D). Apoptosis was determined by Annexin V-PE and 7-AAD staining (E). *, $p < 0.05$ relative to SUM159/GFPshRNA apoptosis in hypoxia. F and G) MDA-MB-231 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 hrs in serum-free RPMI media supplemented with 1 g/L glucose. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or Actin (F). Apoptosis was determined by Annexin V-PE and 7-AAD staining. Data shown represent the mean of 3 independent experiments (+/-SEM) (G).

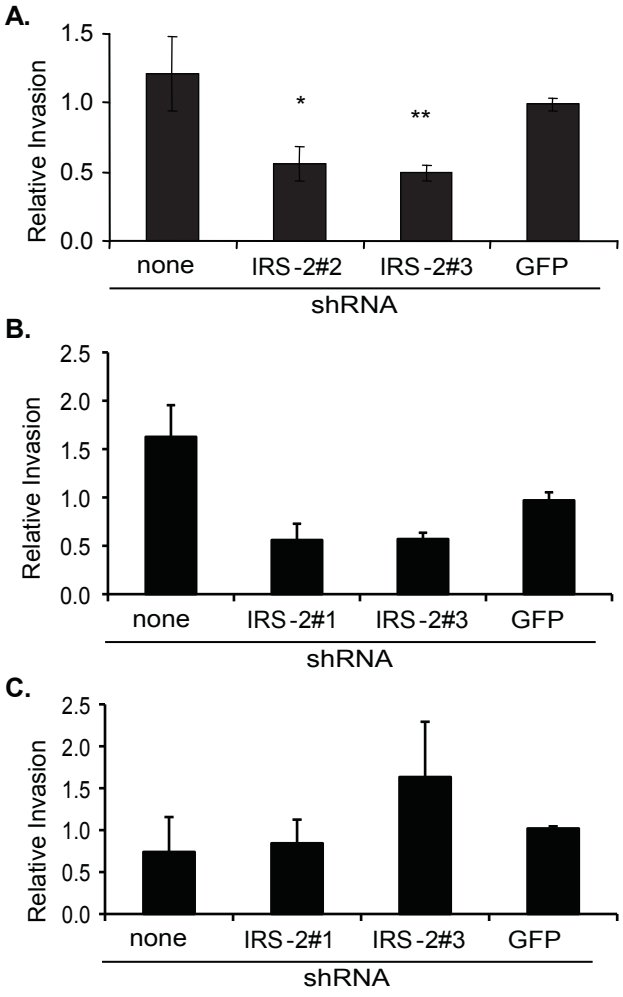

Figure 2.4. IRS-2 promotes invasion in hypoxia. A) MDA-MB-231 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 hrs in serum-free RPMI media supplemented with 1 g/L glucose. Cells were assayed for their invasive potential using a Matrigel Transwell assay. The data were plotted as relative numbers compared with invasion levels of GFPshRNA-expressing cells. The data shown represent the mean (+/-SEM) of 5 independent experiments performed in duplicate. *, $p < 0.01$ and **, $p = 0.0002$ relative to MDA-MB-231/GFPshRNA invasion. B) SUM159 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 hrs in serum-free RPMI media supplemented with 1 g/L glucose. Cells were assayed for their invasive potential using a Matrigel Transwell assay. The data were plotted as relative numbers compared with invasion levels of GFPshRNA-expressing cells. The data shown represent the mean (+/-SEM) of 5 independent experiments performed in duplicate. C) the experiment in B) was repeated in the presence of DEVD apoptosis inhibitor.

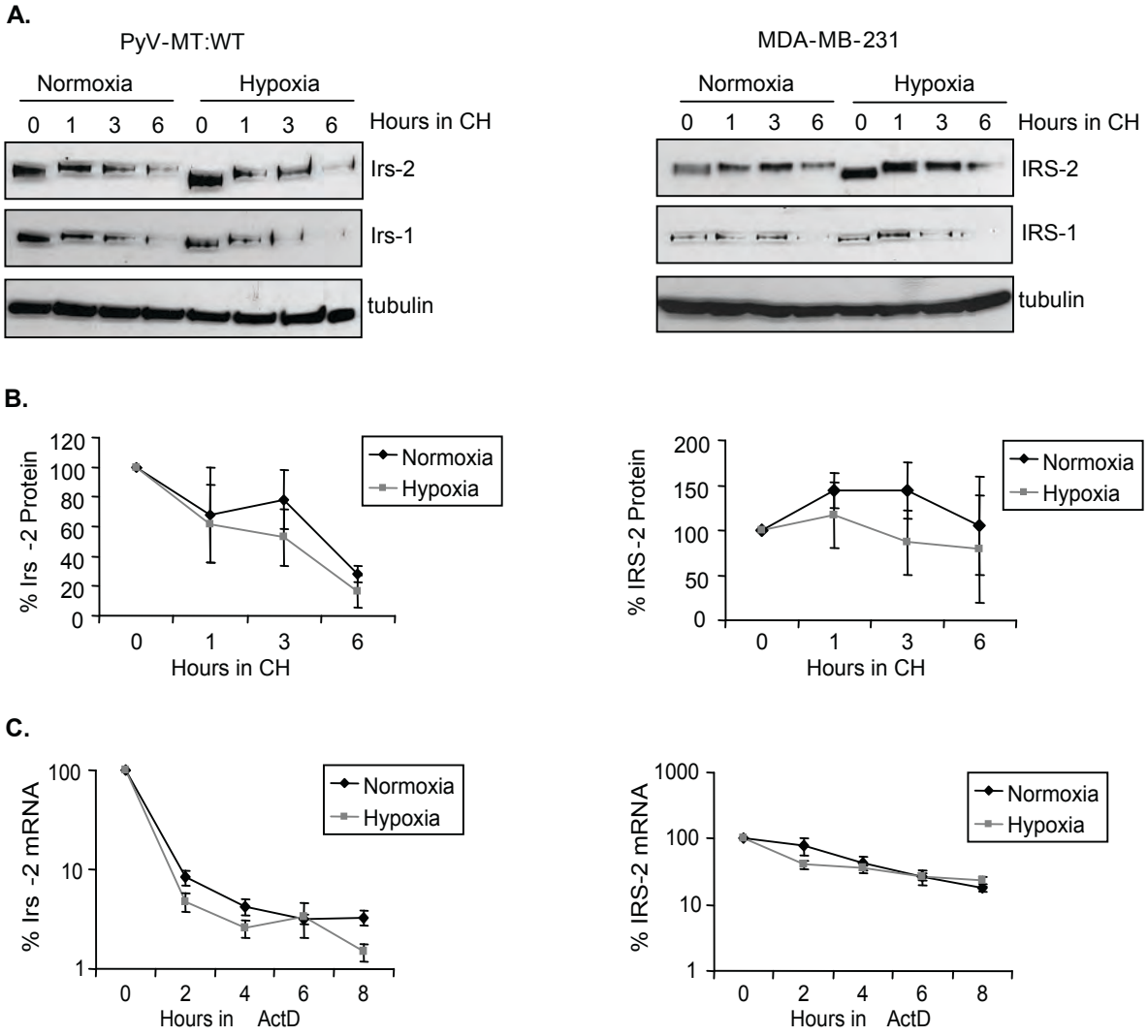

Figure 2.5. IRS-2 protein and mRNA stability are not increased by hypoxia. PyV-MT:*WT* (left) and MDA-MB-231 (right) cells were incubated in either normoxia or hypoxia for 16 hrs (0 hrs) before the addition of cycloheximide (A and B) or Actinomycin D (C) for the time periods indicated. A) Cell extracts were immunoblotted with antibodies that recognize IRS-2, IRS-1 or Tubulin. B) IRS-2 expression levels in (A) were quantified by densitometry. The data shown represent the mean of three independent experiments (+/- SEM). C) IRS-2 mRNA levels were quantified by RQ-PCR and the change in mRNA levels (%) relative to no treatment (0 hrs) was determined. The data shown represent the mean of three independent experiments (+/- SEM).

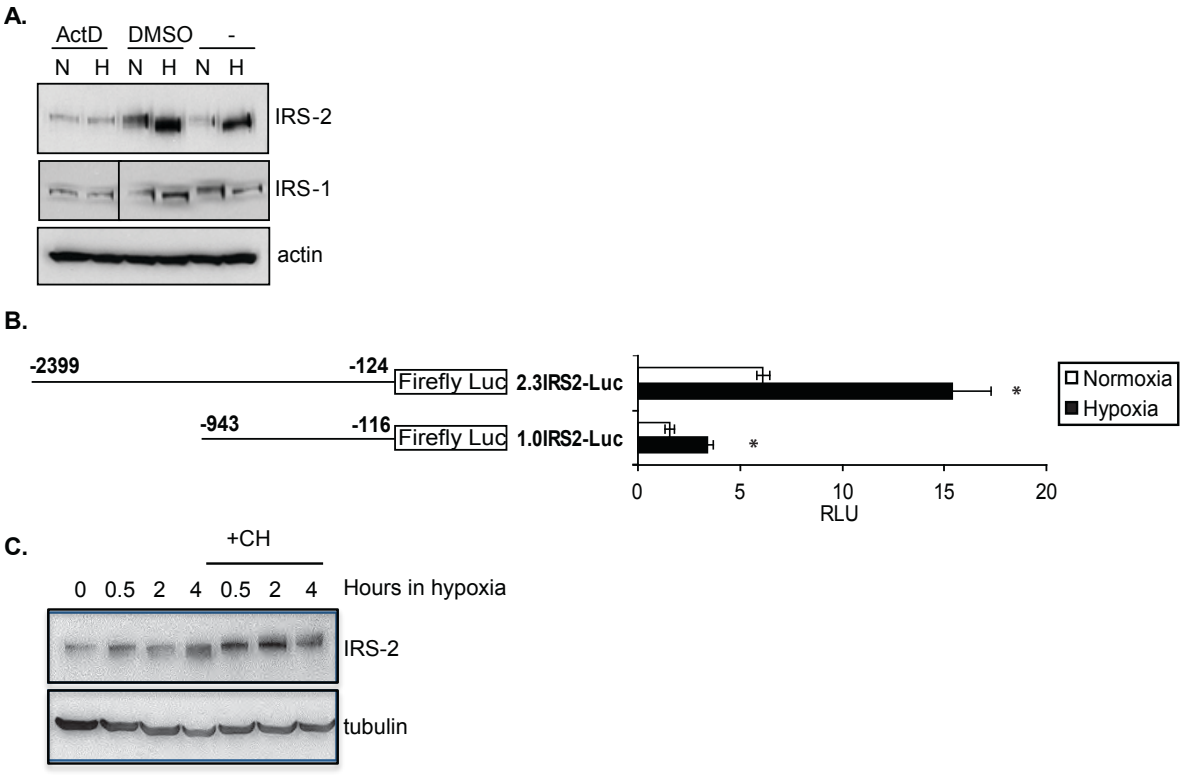

Figure 2.6. Hypoxia regulates IRS-2 transcription. A) MDA-MB-231 cells were pre-treated with ActD or DMSO prior to incubation in hypoxia for 16-hours. Cell extracts were immunoblotted with antibodies that recognize IRS-2, IRS-1 or Actin. The vertical line in the IRS-1 blot denotes different exposures. (B) MDA-MB-231 cells were transfected with the indicated promoter-luciferase constructs and assayed for luciferase activity after incubation in normoxia or hypoxia for 24 hrs. The relative luciferase values are shown and they represent the mean of four independent experiments (+/- SEM). *, $p \leq 0.003$ and **, $p < 0.0002$ relative to normoxic luciferase values. C) MDA-MB-231 cells were incubated in hypoxia with or without Cycloheximide for the indicated periods of time. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or tubulin.

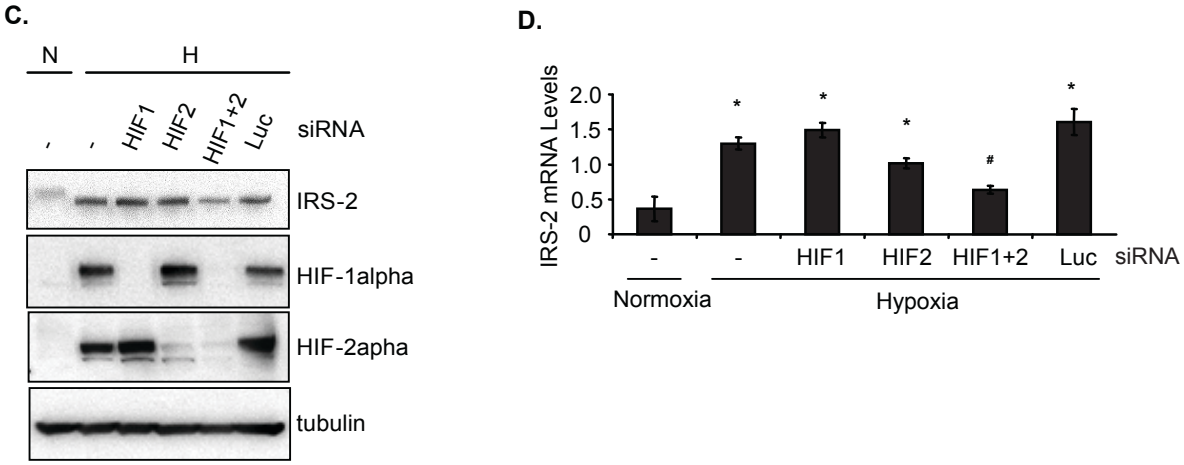

Figure 2.7. HIF is required for hypoxic IRS-2 expression. A and B) SUM159 cells transfected with HIF-1α and HIF-2α-targeted siRNA oligonucleotides were incubated in normoxia or hypoxia for 16 hrs. Cell extracts were immunoblotted with antibodies that recognize IRS-2, HIF-1α, HIF-2α and tubulin (A) and mRNA levels were determined by RQ-PCR (B). *, $p < 0.009$ relative to normoxic IRS-2 levels; HIF1/2si, HIF-1α and HIF-2α siRNA co-transfection.

CHAPTER III: Hypoxia Inducible Factors (HIFs) cooperate with Snail in the regulation of IRS-2 expression in hypoxia

Abstract

Insulin Receptor Substrate-2, an adaptor protein for multiple receptors, including the insulin and IGF-1 receptors, has been implicated in the regulation of cellular processes leading to cancer progression, invasion, and metastasis. IRS-2 expression varies between different cell types and often correlates with a more aggressive and invasive phenotype. Regulation of IRS-2 expression has been shown to change in response to several stimuli in breast cancer but remains not well understood. Here we report that mesenchymal-like breast cancer cell lines that exhibit invasive and metastatic behavior have the ability to upregulate IRS-2 expression in hypoxia. We have found that the constitutive presence of Snail at the IRS-2 promoter in these cells is required for its upregulation. We show that while both HIF-1 and HIF-2 can bind to the proximal IRS-2 promoter, HIF-2 plays a dominant role in this regulation. Together our data implicate EMT in the ability of breast cancer cells to upregualte IRS-2 expression in hypoxia.

Introduction

Insulin Receptor Substrate-2 (IRS-2) is a member of the IRS family of cytoplasmic adaptor proteins. Originally identified as signaling effectors of the insulin receptor, the IRS proteins also function as signaling intermediates for the closely related Insulin Like Growth Factor-1 receptor (IGF-1R), as well as some cytokine and integrin receptors [196]. The IRS proteins are recruited to receptors through plextrin homology (PH) and phosphotyrosine binding (PTB) domains in their N-termini and they are phosphorylated on tyrosine residues in their C-termini, creating multiple binding motifs that recruit downstream effectors to regulate intracellular signaling cascades that modify cell behavior [155]. Although the IRS proteins share considerable homology, many studies have shown that they can regulate distinct cellular functions in response to common stimuli, indicating that they have unique signaling capabilities. IRS-2 has been implicated in many aspects of tumor biology that are considered important for cancer progression. Specifically, IRS-2 promotes breast cancer cell invasion and migration, enhances tumor cell survival and positively regulates tumor cell glycolysis, all of which are features of metastatic cells [43, 157, 159]. *In vivo* studies support the importance of IRS-2 for metastasis as PyV-MT-derived mammary tumors are significantly impaired in their ability to metastasize in the absence of IRS-2, and are more metastatic in the absence of IRS-1, when IRS-2 expression and signaling are enhanced [43, 59, 156].

Despite the growing evidence for the importance of IRS-2 for tumor progression, relatively little is known regarding how its gene expression is regulated in tumors. IRS-2 is a progesterone-responsive gene and it is directly regulated by the progesterone receptor

(PR) [197]. However, many tumors that are PR-negative maintain expression of IRS-2, indicating that this is not an essential pathway for its expression. In normal hepatocytes and some breast cancer cells, IRS-2 expression increases in response to inhibition of PI3K signaling by chemical inhibitors or by overexpression of PTEN [89]. The mechanism of this induction of expression is likely through enhanced FOXO nuclear localization and direct binding to the IRS-2 promoter. EGF-mediated signaling can also positively regulate IRS-2 expression in breast carcinoma cells through JNK activation and direct AP1 interaction with the IRS-2 promoter [85]. Recently, we reported that *IRS-2* is a hypoxia-responsive gene and that the upregulation of IRS-2 expression in response to hypoxia requires the hypoxia inducible factors HIF-1 and HIF2 [198].

Oxygen concentration in the tumor microenvironment fluctuates during tumor development. When tumors outgrow the pace of blood vessel formation, areas with a poor blood supply are created within the tumor, resulting in low nutrient and oxygen concentration (hypoxia) [92]. Prolonged exposure to hypoxic conditions, and also cycles of normoxia/hypoxia, can result in adaptation of tumor cells to these stressful conditions. This leads to the selection of cells that are resistant to low oxygen, nutrient, and pH environment, and therefore are highly invasive and metastatic [95]. In this regard, induction of IRS-2 in hypoxic environments stimulates Akt signaling and promotes both breast carcinoma cell survival and invasion [198]. Understanding hypoxic regulation of gene expression is crucial for uncovering the mechanisms underlying the metastatic potential of tumor cells. In this report, we investigated the mechanism of IRS-2 transcriptional regulation in response to hypoxia. We have identified a hypoxia-responsive region in the proximal IRS-2 promoter and have established a cell context-dependent

cooperative mechanism involving HIF-1/HIF-2 and Snail in the hypoxic regulation of IRS-2 expression.

Results

Identification of a hypoxia-responsive region in the human IRS-2 promoter.

As the first step in determining how IRS-2 expression is regulated by hypoxia, a series of deletion constructs were generated using the 1Kb human IRS-2 promoter-luciferase construct that we originally used to demonstrate that IRS-2 is a hypoxia-responsive gene [173, 195, 198]. MDA-MB-231 cells were transiently transfected for 24 hrs with each of the deletion constructs, exposed to 0.5% oxygen (hypoxia) or normoxic cell culture conditions for an additional 24 hours, and then assayed for luciferase activity. Deletion of the 1Kb promoter (-943 to -116, relative to translation start site) to 0.6Kb (-731 to -116) decreased basal promoter activity in normoxia, but a 2.5 fold increase in activity was maintained in response to hypoxia (Fig.3.1A). Further deletion of the region between -731 to -505 (0.4Kb promoter) resulted in a significant reduction of both normoxic and hypoxic IRS-2 promoter activity to background levels (Fig.3.1A), indicating that this deleted region is crucial for promoter activity. To test further the activity of this region of the promoter, a construct containing only -731 to -493 of the IRS-2 promoter (0.2Kb promoter) upstream of luciferase was generated. The luciferase activity of this minimal promoter construct was equivalent to that of the 2.3Kb IRS-2 promoter in both normoxia and hypoxia. Moreover, deletion of this region from the 1Kb promoter (Δ0.2Kb promoter) resulted in ablation of both normoxic and hypoxic activity, confirming that this region is required for basal activity of the IRS-2 promoter and can be upregulated by hypoxia.

Since Δ0.2 promoter activity was at or near background levels, its responsiveness to hypoxia could not be tested. To analyze its ability to be upregulated by hypoxia, a fusion

construct of Δ0.2 IRS-2 and the SV40 promoter was created. The 0.2Kb-IRS2 and Δ0.2Kb-IRS-2 sequences were placed upstream of the SV40 promoter in the basic pGL3 vector and luciferase activity stimulated by the fusion promoters was analyzed in both normoxic and hypoxic conditions. We observed a minimal upregulation in SV40 promoter activity in response to hypoxia. Therefore, to make sure that only IRS-2 promoter responsiveness to hypoxia is compared, all Luciferase results were normalized to the SV40 values at either normoxic or hypoxic conditions. The 0.2Kb-IRS2/SV40 chimeric promoter showed a two-fold upregulation in activity in response to hypoxia, whereas only a 1.4 fold increase in hypoxia was observed for the Δ0.2Kb-IRS2/SV40 chimeric promoter (Fig.3.1B). Taken together with the deletion analysis data, our results demonstrate that the region of the IRS-2 promoter between -731 to -493 contains regulatory elements that are required for increased gene expression in response to hypoxia.

Role of HRE elements in human IRS-2 promoter activity.

We have previously reported that either HIF-1 or HIF-2 are required for hypoxic upregulation of IRS-2 mRNA expression [198]. To investigate further the mechanism by which IRS-2 expression is positively regulated by hypoxia, we analyzed the IRS-2 promoter for the presence of consensus HIF regulatory element (HRE) sites (5'-nA/GCGTGn-3') [187]. There is one consensus HRE contained within the 1Kb IRS-2 promoter at position -123 to -118 (Fig.3.1A). However, this HRE is not present within the hypoxia-responsive 2.3Kb IRS-2 promoter, which terminates at -124, nor within the 0.2Kb-IRS2 minimal hypoxia-responsive region, suggesting that it is not necessary for the

hypoxic upregulation of IRS-2 promoter activity. To confirm this, the HRE was mutated in the 0.6Kb IRS-2 promoter construct and luciferase activity was measured under both normoxic and hypoxic conditions. Although the HRE-mutant promoter exhibited reduced basal luciferase activity in normoxia, it retained hypoxia-induced upregulation, confirming that this HRE is not required for hypoxic regulation of IRS-2 (Fig.3.1C). Next, we searched for HRE sequences outside of the proximal 2.3 Kb IRS-2 promoter and found an additional HRE in the coding region of the human IRS-2 gene (+88 to +94, relative to translation start site). To test the contribution of this HRE to hypoxic regulation of IRS-2, a construct was generated that included this additional HRE (-731 to +222). Inclusion of this region significantly inhibited IRS-2 promoter activity to background levels in both normoxia and hypoxia. We conclude from these data that the HRE at position +88 does not play a role in the regulation of IRS-2 expression by hypoxia, and that the region from -116 to +222 may contain negative regulatory elements (Fig.3.1A).

Analysis of the minimal hypoxia-responsive region in the IRS-2 promoter.

We analyzed the 0.2Kb hypoxia-responsive region of the human IRS-2 promoter to identify potential transcription factor binding sites that could play a role in promoting increased IRS-2 expression in hypoxia. Within this region, we identified an E-box (5'-CACATG-3'), an Insulin Response Element (IRE, 5'-TGTTTTG-3'), which has been previously identified to be regulated by FOXO in normal liver cells *in vivo* [80], and an AP1 binding site (Fig.3.1C). To determine the potential role of these sites in the response of the IRS-2 promoter to hypoxia, each site was mutated individually in the context of the

0.6Kb promoter and the luciferase activity of each mutant promoter was assayed in MDA-MB-231 cells after exposure to normoxia or hypoxia. Mutation of the AP1 site decreased normoxic activity, but the fold-increase in activity in hypoxia was maintained (Fig.3.1C). Mutation of the IRE had no effect on promoter activity in either normoxic or hypoxic conditions. In contrast, mutation of the E-box significantly decreased basal promoter activity, but mutant promoter activity was still upregulated by hypoxia over 3 fold (Fig.3.1C). Similarly, mutation of the E-box in the context of the larger 2.3Kb IRS-2 promoter resulted in a significant decrease in luciferase activity in normoxia but did not affect its ability to be upregulated by hypoxia, confirming the importance of this E-Box for basal IRS-2 promoter activity (Fig.3.1D).

The E-Box in the IRS-2 promoter overlaps with an imperfect HRE sequence (5'-ACATG-3'). To investigate the possibility that HIF binding to either this site or the consensus HRE at -123 is sufficient for hypoxic induction, combined mutations in the E-box and HRE were generated in the 0.6Kb promoter. Combined E-box/HRE mutations marginally decreased the fold upregulation of 0.6 IRS-2 promoter activity by hypoxia, but this promoter was still upregulated 2 fold by hypoxia (Fig.3.1C). Mutation of the IRE, which is adjacent to the E-box, in combination with the E-Box mutation reduced the fold-induction of promoter activity by hypoxia over that observed for the E-box mutant alone, suggesting that this region in the promoter may contribute to the regulation of IRS-2 expression by hypoxia (Fig.3.1C). A cooperation between these two sites has been previously reported for the regulation of IRS-2 expression by insulin in hepatocytes [79].

HIFs cooperate with Snail to regulate IRS-2 expression in hypoxia.

To further our investigation of the mechanism by which IRS-2 expression is regulated by hypoxia, we next focused on potential factors that are known to bind to consensus E-box sequences. The cell lines that we have demonstrated to upregulate IRS-2 expression in hypoxia (MDA-MB-231, SUM-159, and SUM149) have a mesenchymal-like, invasive phenotype [198]. IRS-2 has been previously implicated in contributing to the epithelial-mesenchymal transition (EMT) [85, 199]. In light of these findings, we hypothesized that factors associated with regulating the mesenchymal phenotype could be involved in the regulation of IRS-2 expression. For this reason, we investigated the possibility that Snail, a factor that binds to E-box sequences and regulates the EMT could play a role in regulating IRS-2 expression in response to hypoxia. Snail expression was transiently suppressed in SUM159 and MDA-MB-231 cells by siRNA transfection before exposure of the cells to hypoxia. Suppression of Snail resulted in only a small reduction of IRS-2 mRNA and protein expression in hypoxia (Fig.3.2A and C). In our previous experiments to investigate HIF involvement in IRS-2 regulation, we observed that suppression of HIF-1 or HIF-2 alone resulted in only a small reduction in hypoxic IRS-2 expression in SUM-159 cells, but that suppression of HIF-1 and HIF-2 together decreased hypoxic IRS-2 expression to almost normoxic levels [198]. To determine if Snail cooperates with either HIF-1 or HIF2 to regulate IRS-2 expression, combined knockdowns of Snail with HIF-1alpha or HIF-2alpha were performed and the effect on IRS-2 mRNA and protein expression was evaluated in normoxia and hypoxia. Simultaneous knockdown of Snail and either HIF-1alpha or HIF-2alpha inhibited the hypoxic induction of IRS-2 mRNA by ~50% in SUM-159 cells (Figs.3.2C and D). In MDA-MB-231 cells, co-

suppression of Snail and HIF-1alpha did not reduce IRS-2 mRNA expression, whereas knockdown of Snail and HIF-2alpha together completely inhibited the upregulation of IRS-2 mRNA expression in response to hypoxia (Figs.3.2A and B). Taken together, these results support that Snail can cooperate with both HIF-1 and HIF-2 to regulate IRS-2 expression, but that the relative contribution of HIF-1 and HIF-2 is cell context dependent.

Role of HIF-1 and HIF-2 in IRS-2 expression.

To investigate the differences we observed in the contribution of HIF-1 and HIF-2 to regulating hypoxic expression of IRS-2 in the two independent cell lines we evaluated their expression in these cells. Comparison of HIF-1alpha and HIF-2alpha expression after 24hrs in hypoxia revealed that the two cell lines each express both HIFs, however, SUM159 cells express predominantly HIF-1alpha, while MDA-MB-231 cells express predominantly HIF-2alpha (Fig.3.2E). In a time-course analysis, HIF-1alpha expression was induced and sustained in SUM159 cells at the later hypoxia time-points when IRS-2 expression is increased, while HIF-1alpha expression peaked between 2-8 hrs of hypoxia and was significantly diminished at the later time points in MDA-MB-231 cells (Fig.3.2F). HIF-2alpha expression was induced at later time points in both cell lines. SUM159 cells also express markedly higher levels of Snail than MDA-MB-231 cells, which provides a possible explanation for the less efficient inhibition of hypoxic IRS-2 expression in the combined knockdown experiments (Fig.3.2E).

To examine further the relative roles of HIF-2 and Snail in hypoxic IRS-2 expression, we evaluated if overexpression of either factor alone could increase IRS-2

expression in normoxia. Transient overexpression of HIF-2alpha markedly increased IRS-2 expression, when expressed alone or in combination with Snail. However, Snail overexpression alone had no effect on IRS-2 protein levels (Fig.3.3A and B).

To investigate if Snail, HIF-1 and HIF-2 directly regulate IRS-2 expression, the binding of each factor to the IRS-2 promoter was evaluated under normoxic and hypoxic conditions by chromatin immunoprecipitation (ChIP) analysis, using primers that amplify the region of the IRS-2 promoter surrounding the E-box. For these experiments we used SUM-159 cells, which express all three factors at the 24 hr hypoxic time-point. Snail was present at the IRS-2 promoter in normoxia and the level of binding did not increase in hypoxia (Fig.3.3C). HIF-1 and HIF-2 were both present at the IRS-2 promoter in normoxic conditions, and their binding increased in response to hypoxia, as did the binding of PolII (Fig.3.3D). These results support our hypothesis that both HIF-1 and HIF-2 can regulate IRS-2 expression in hypoxia, but that they likely do so in a cell context-dependent manner, dependent upon the expression levels of each factor. These results also explain our previously published observation that suppression of both HIF-1 and HIF-2 was required to inhibit the hypoxic upregulation of IRS-2 expression in SUM159 cells [198].

Role of Snail-driven EMT in hypoxic upregulation of IRS-2 expression.

Our data demonstrate that the binding of Snail to the IRS-2 promoter is not increased by hypoxia, and the overexpression of Snail alone does not increase IRS-2 expression. HIF has been previously shown to selectively regulate expression of genes that are actively transcribed in normoxia [200]. Therefore, we hypothesized that the

constitutive presence of Snail at the IRS-2 promoter in mesenchymal-like cells may facilitate hypoxia-dependent regulation of IRS-2 expression by maintaining the promoter in a "permissive," open state and allowing HIF-dependent transcription in hypoxia. To test this hypothesis, IRS-2 expression levels were compared in invasive mesenchymal-like cells (MDA-MB-231 and SUM159) and well-differentiated non-invasive epithelial-like cells (MCF7 and T47D) in normoxia and hypoxia. MCF-7 and T47D cells have significantly lower basal expression levels of IRS-2 than MDA-MB-231 and SUM-159 cells in normoxia. Moreover, these well differentiated cells fail to increase IRS-2 expression in response to hypoxia (Fig.3.4A and B). MCF-7 and T47D cells express both HIF-1 and HIF-2 and have comparative levels of Snail to MDA-MB-231. However, these cells have significantly lower levels of nuclear Snail than MDA-MB-231 and SUM159 cell lines in hypoxia (Fig.3.4B). To analyze if Snail or HIF-2 binds directly to the IRS-2 promoter in epithelial-like cell lines, ChIP experiments were performed with MCF7 cells. Consistent with low levels of IRS-2 expression, we observed very weak PolII binding, compared to SUM159 cells, and a faint presence of Snail at normoxia that disappeared under hypoxic conditions. As expected, Snail was also not bound to the E-cadherin promoter, indicating that Snail is not active in MCF7 cells. No HIF-1 or HIF-2 binding to the IRS-2 promoter was observed in MCF7 cells in normoxia or hypoxia, supporting our hypothesis that Snail binding to the IRS-2 promoter may be required for HIF-dependent transcription of IRS-2. As controls for HIF-1 and HIF-2 activity in MCF7 cells, we confirmed binding of each factor to the promoters of their target genes, PGK-1 and EPO, respectively (Fig.3.4C).

In contrast to MDA-MB-231 and SUM159 cells, overexpression of HIF-2 in MCF7 cells did not increase IRS-2 expression (Fig.3.5A), indicating the importance of Snail for

HIF-dependent expression of IRS-2. To confirm a role for Snail in the regulation of hypoxic IRS-2 expression, Snail was overexpressed in MCF-7 for 48 hours before exposure of cells to hypoxia or normoxia for an additional 24 hours. A mild upregulation in IRS-2 mRNA expression was observed in response to hypoxia, which was not observed in vector alone transfected cells (Fig.3.5B). To ensure that the exogenous expression of Snail was localized to the nucleus, nuclear fractionation was performed with MCF7 cells overexpressing Snail. Overexpressed Snail was localized to the nucleus and was not retained in the cytoplasm, (Fig.3.5C).

To test the role of endogenously upregulated Snail in regulation of IRS-2 expression, MCF-7 cells were treated with TGF-β in charcoal-stripped serum (CSS) containing media for 6 days to induce EMT before exposing the cells to hypoxia. CSS-containing medium was used to remove estrogen from the media in order to promote the EMT process. MCF7 cells have been previously shown to acquire EMT-like features when they lose estrogen-dependence [201]. Cells treated with CSS+TGF-beta showed a decrease in normoxic IRS-2 mRNA levels, but were able to upregulate expression in hypoxia, unlike cells maintained in normal growth medium supplemented with FBS. The level of hypoxic IRS-2 mRNA in TGF-beta-treated cells was higher than hypoxic IRS-2 mRNA levels in untreated cells that showed a significant decrease in IRS-2 expression in hypoxia (Fig.3.5D).

To demonstrate that binding of Snail to the IRS-2 promoter is important for HIF-binding, direct binding of Snail and HIF-2 to the IRS-2 promoter in normoxia and hypoxia in MCF7 cells after TGF-beta treatment was analyzed by ChIP. In contrast to untreated

MCF7 cells (Fig.3.4C), an increased binding of Pol II and Snail to the IRS-2 promoter in hypoxia was observed, which was also associated with HIF-2 binding (Fig.3.5E).

To test if constitutive binding of Snail to the IRS-2 promoter maintains chromatin in an opened conformation, we analyzed histone 3 methylation on lysine 4 status (H3K4) of the IRS-2 promoter in normoxia and hypoxia in untreated MCF-7 cells or cells that have been treated with TGF-beta. We found that overall H3K4 status did not change with the treatment in normoxia, but treated cells showed increased H3K4 levels under hypoxic conditions (Fig3.5F).

IRS-2 expression associates with Snail expression in metastatic mammary tumors *in vivo*.

To confirm our *in vitro* data on the role of HIF and Snail in IRS-2 expression, IRS-2, HIF-1, HIF-2, and Snail expression were analyzed in PyV-MT wild-type mammary tumors. We found a strong correlation of increased IRS-2 expression and tumor metastasis. HIF-1 expression fluctuated in the panel of mouse tumors and did not correlate with either IRS-2 expression or tumor metastasis. HIF-2 levels were slightly elevated in two out of four metastatic tumors but did not correlate with IRS-2 expression (Fig.3.6). Expression of Snail was increased in metastatic tumors. We observed a trend in the correlation between Snail and IRS-2 expression. These results support our *in vitro* findings that while dependent on cell context, either HIF-1 or HIF-2 can regulate IRS-2 expression. Additional mechanisms are also required to permit HIF-dependent transcription of IRS-2 in hypoxia, and this may be mediated by Snail.

Discussion

In this report, we identify a region of the proximal IRS-2 promoter that is necessary and sufficient for the hypoxic regulation of IRS-2 expression. An E-box located in this minimal hypoxia-responsive region is required for the basal activity of the IRS-2 promoter. Both E-box and adjacent IRE sites are important for hypoxic upregulation of IRS-2 promoter activity. We demonstrate that HIF-1 and HIF-2 can bind directly to the region of the IRS-2 promoter containing the E-box in SUM159 breast carcinoma cells. However, our data also support that the specificity of HIF function in regulating IRS-2 expression is determined by the relative cellular expression levels of each factor, and that HIF-2 may play a more dominant role than HIF-1 in this regulation. We have also established cell context-dependent conditions that determine the ability of IRS-2 expression to be regulated by hypoxia. Specifically, upregulation of IRS-2 by hypoxia is observed only in breast carcinoma cell lines that have endogenous basal expression levels of IRS-2, which occurs predominantly in highly invasive cells with a mesenchymal-like phenotype. The IRS-2 promoter is permissive for responding to hypoxia-dependent factors in these cells, but not in non-invasive, epithelial-like cell lines. One factor that we identify to be important for the hypoxic increase in IRS-2 transcription is the E-box-binding transcription factor Snail, a positive regulator of the EMT. Taken together, our data suggest that the EMT is permissive for regulation of IRS-2 expression by hypoxia.

Our observation that IRS-2 expression is increased by hypoxia only in poorly differentiated, invasive breast carcinoma cells adds to the growing evidence that IRS-2 is associated with more aggressive tumor behavior and provides a mechanistic explanation for

how expression is regulated in metastatic cells. Using a PyV-MT mouse model of tumor progression, we previously observed that mammary tumor metastasis is significantly reduced in the absence of Irs-2 expression, and increased in *Irs-1*$^{-/-}$ tumors that show enhanced levels of Irs-2 expression and function. Additionally, we now show that IRS-2 expression is enhanced in metastatic primary tumors from *PyV-MT:WT* mice when compared with non-metastatic primary tumors. Importantly, Irs-1 cannot compensate for the loss of Irs-2 for metastasis, and its expression is negatively regulated by hypoxia. Therefore, hypoxia can shift the IRS signaling balance in favor of IRS-2 to promote a more aggressive tumor cell phenotype. Experiments using cells derived from PyV-MT-derived tumors demonstrated that Irs-2 positively regulates survival and invasion, two important properties of metastatic tumor cells [43, 59]. The mouse studies correlate well with earlier reports showing increased IRS-2 expression and activation levels in human breast carcinoma cells after selection for metastatic behavior *in vivo* [157]. Specifically, IGF-1 stimulated an increase in IRS-2 phosphorylation and tumor cell migration in MDA-MB-435 and MDA-MB-231 cells that were isolated from metastatic sites when compared with their parental cell lines. Many studies have demonstrated that hypoxia stimulates tumor cell migration and invasion *in vitro* and that it selects for greater metastatic potential *in vivo*. The ability of hypoxia to selectively upregulate IRS-2 expression would provide those tumor cells that are in a permissive state a metastatic advantage by enhancing Akt-mediated signaling and increasing their potential to survive and invade.

The cell context-dependent regulation of IRS-2 by hypoxia that is revealed in our current study has been demonstrated previously for HIF-dependent gene regulation. The HIF consensus binding sequence (5’-(A/G)CGTG-3’) is found widely throughout the

genome but only several hundred genes have been validated to be direct HIF targets [200]. Therefore, there must be additional factors that determine if a gene will respond to HIF regulation. Comparison of ChIP-chip profiles with gene expression profiles has revealed a preferential hypoxia-dependent binding of HIF-1 to genes that were already transcriptionally active in normoxia [200]. This finding provides an explanation for why the subset of genes that are induced by hypoxia can vary significantly in different cells, since basal gene expression can also vary markedly. IRS-2 is actively transcribed and expressed in poorly-differentiated breast carcinoma cells under normal culture conditions and its expression is increased in response to hypoxia. Both HIF-1 and HIF-2 bind directly to the proximal IRS-2 promoter in hypoxic conditions and promote gene transcription. Although basal, normoxic IRS-2 expression can be detected in more well-differentiated breast carcinoma cell lines, the level of expression is significantly lower than that observed for more mesenchymal-like cells. ChIP analysis confirmed that neither HIF-1 nor HIF-2 bound to the IRS-2 promoter in hypoxia, indicating that this basal level of expression is insufficient to support hypoxic induction of HIF binding.

Although both HIF-1 and HIF-2 can bind to the proximal IRS-2 promoter and contribute to the regulation of IRS-2 gene transcription, our data support that HIF-2 is likely to play a more dominant role than HIF-1 in this regulation. This HIF-2 preference is supported by the report that IRS-2 is one the most highly expressed genes that is associated with HIF-2 activity in VHL-deficient renal cell carcinoma [202]. HIF-1 and HIF-2 bind to the same consensus HRE sequence and can regulate the expression of many common target genes. There is also evidence to support that these factors can regulate unique target genes. For example, HIF-1 is the major regulator of expression of glycolytic enzymes and HIF-2

has been shown to be a dominant regulator of EPO expression [136-138]. One explanation for the differences in activity of the two factors is the different timing of their stabilization upon exposure of cells to hypoxia. Generally, HIF-1 activity is strongly associated with gene expression at early, acute hypoxic time periods and HIF-2 activity increases in response to prolonged, chronic oxygen deprivation [139]. In breast carcinoma cells, IRS-2 expression increases at later hypoxia time-points, when HIF-2 activity is greater than that of HIF-1. HIF-1 may be able to regulate IRS-2 expression if its expression is sustained at longer time points, as observed in SUM-159 cells. However, the fact that IRS-2 mRNA expression does not increase at earlier time points in MDA-MB-231 cells, when HIF-1 peaks in expression, suggests that co-regulatory factors must be upregulated by prolonged hypoxia to induce IRS-2 expression. The specificity of HIF-1 and HIF-2 in regulating transcription of unique target genes has been shown to result from unique co-activators for each HIF. The most common co-activator of HIF is p300/Creb-binding protein (CBP). Its main function is to stabilize transcription initiation complex and to recruit histone acetyl-transferase enzymes, such as the redox factor (Ref-1) [141, 142]. Several co-activators have been found to be specific for HIF-2. For example, transcription factor Ets1 has been shown to interact exclusively with HIF-2alpha in regulating VEGF receptor 2 (Flk-1) transcription [143]. Another Ets family transcription factor, Elk-1, cooperates with HIF-2 to activate expression of CITED-2 in hypoxia in MCF-7 human breast carcinoma cell line [144]. Binding sites for some of the above factors can be found in the IRS-2 promoter, including Ets, suggesting a potential role for an interaction among multiple factors in regulating hypoxic IRS-2 expression.

Tumor cells that express higher levels of IRS-2 under normal (i.e. normoxic) cell culture conditions and upregulate expression in hypoxia have mesenchymal-like morphology and are likely to have undergone an EMT [85, 198]. Snail is one of several transcription factors that are upregulated by stimuli that promote an EMT and its major function is to repress genes that promote an epithelial phenotype such as E-Cadherin. However, increasing evidence shows that in a different context, Snail can play the role of a transcriptional activator. For example, Snail positively regulates expression of Wnt-target genes through an interaction with beta-Catenin [203]. Snail has also been shown to associate with early growth response gene 1 (EGR-1) and stimulatory protein 1 (SP-1) transcription factors to positively regulate TPA-induced transcription of p15(INK4b) in HepG2 cells [204]. Therefore, the outcome of Snail function, as a repressor or an activator, depends on factors that Snail cooperates with to mediate transcription. Our data support that Snail cooperates with the HIFs, although whether this is through a direct interaction or an indirect cooperation still needs to be determined. There is previous evidence that Snail can cooperate with HIFs when bound to E-box sequences. Specifically, the Snail homolog in C. Elegans positively regulates transcription by sharing target genes with members of the basic helix-loop-helix family of transcription factors, which includes HIF-1 and HIF-2 [205]. We hypothesize that Snail binding to the IRS-2 promoter at the E-box creates a permissive chromatin environment for the binding of HIFs. Furthermore, other factors that promote EMT and recognize E-boxes, such as Slug and Twist, may also contribute to IRS-2 expression in cells in which they are expressed and functional. The inability of the Snail knockdown alone to inhibit IRS-2 expression in hypoxia could be explained by an incomplete suppression of expression, or by compensation by these other factors.

Additionally, non-EMT factors that regulate IRS-2 expression through the E-box, such as the TFE family in hepatocytes, may also be permissive for the hypoxic regulation of IRS-2.

Snail activity and hypoxia have both been previously demonstrated to be associated with EMT and also with tumor metastasis. EMT occurs during both normal (in development) and pathological (in cancer) processes when normal epithelial cells or well-differentiated tumor cells, respectively, are induced by extracellular stimuli to adopt a more migratory and invasive phenotype. This allows tumor cells to survive and spread to secondary sites in the body. Hypoxia is one such environmental stimulus that induces changes in gene expression that facilitate the transition to a mesenchymal status. The requirement for Snail in the hypoxic regulation of IRS-2 expression links these factors with aggressive tumor behavior. The fact that overexpression of Snail in MCF7 cells resulted in an increase in hypoxic IRS-2 expression suggests that IRS-2 regulation may be a part of the EMT programming of gene expression. In this regard, the IRS proteins have been implicated previously in the EMT process. Specifically, IRS-1 expression and phosphorylation is inversely correlated with the ability of TGF-beta to stimulate an EMT in A549 lung carcinoma cells [199]. IRS-1 expression and tyrosine phosphorylation is decreased in response to TGF-beta, and overexpression of IRS-1 can inhibit the TGF-beta-induction of EMT both at the level of morphology and gene expression. With regard to IRS-2 and EMT, IRS-2 expression is upregulated by EGF in a JNK-dependent manner in SUM159 cells and it is required for the enhanced EMT-associated migration that occurs in response to this stimulation [85]. Consistent with the inverse roles of IRS-1 and IRS-2 in EMT, hypoxia suppresses IRS-1 expression at the same time it induces IRS-2 expression,

shifting the balance of IRS signaling to favor IRS-2 and promote tumor cell invasion and metastasis [198, 206].

In summary, we have elucidated a novel context-dependent cooperative mechanism for the regulation of IRS-2 expression that involves the hypoxia-induced factors HIF-1 and HIF-2 and the EMT regulator Snail. Given its important role downstream of multiple receptors that are active in cancer cells, understanding how IRS-2 expression is regulated may lead to a more broad understanding of the role of IRS-2 in cancer and a more extensive use of IRS-2 expression as a marker for tumor progression.

Materials and Methods

Cell lines, hypoxia, and TGFbeta treatment. MDA-MB-231, MCF-7, and T47D human breast carcinoma cells were obtained from ATCC. SUM159 human breast carcinoma cells were a gift from Dr. A. Mercurio (UMass Medical School).

For hypoxia exposure, cells were maintained at a constant gas mixture of 0.5% oxygen, 94.5% nitrogen and 5% carbon dioxide in an $InVivo_2$ Hypoxia Workstation (Ruskinn Technology Ltd) for periods of time indicated in each Figure Legend.

For TGFbeta treatment, MCF7 cells were treated with 5ng/ml TGFbeta combined with 5% charcoal-stripped serum containing media for 6 days, prior to exposure to hypoxia as above.

Generation of IRS-2 promoter serial-deletion Luciferase constructs: The human 1Kb IRS-2 promoter-luciferase expression plasmid was a gift from Dr. J. Goldstein (UT Southwestern Medical Center) [195]. The human 2.3Kb IRS-2 promoter luciferase plasmid was a gift from Dr. A. Lee (Baylor College of Medicine) [85, 173]. All other constructs were generated by serial digestion of the 1Kb IRS-2 promoter construct with SmaI restriction enzyme.

Site-directed mutagenesis. The HRE, IRE, E-box, and AP1 binding sites in IRS-2 promoter were mutated using the QuickChange® XL Site-Directed Mutagenesis Kit (Stratagene), following the manufacturer's protocol. The primers used for mutagenesis were: HRE mutant, Forward, 5'-

CCTGGGCCCGAGGGACGaaaGATGGTACCGTACGC-3’; and Reverse, 5’-GCGTACGGTACCATCtttCGTCCCTCGGGCCCAGG-3’; E-box mutant, Forward, 3’-GCCTGCGTAACGCCGAGTaAaAaGTTGTTTTGCTCTTCTTAG-5’, and Reverse, 3’-CTAAGAAGAGCAAAACAACtTtTtACTCGGCGTTACGCAGGC-5’; AP1 mutant, Forward, 3’- CACTCGGTGCGCGATGTGTTAaaaACTGTGCGGCGG-5; and Reverse. 3’- CCGCCGCACAGTtttTAACACATCGCGCACCGAGTG-5’; primers for IRE mutation were designed according to [80] to eliminate FOXO binding: Forward, 3’-GTAACGCCGAGTCACATGTTaTTTTaCTCTTCTTAGTTCAGTCAC-5’, Reverse, 3’-GTGACTGAACTAAGAAGAGtAAAAtAACATGTGACTCGGCGTTAC-5’. Double E-box/IRE mutant was created by addition of the IRE mutation to the E-box mutant promoter using the following primers: Forward, 3’-GCGTAACGCCGAGTAAAAAGTTaTTTTaCTCTTCTTAGTTCAGTC-5’, and Reverse, 3’- GACTGAACTAAGAAGAGtAAAAtAACTTTTTACTCGGCGTTACGC-5’.

Luciferase assays. Cells were plated in triplicate in 24-well plates and co-transfected with the promoter-luciferase plasmids (0.4 ug) and a pRL-CMV renilla luciferase plasmid (0.1 ug) (Promega). After an overnight incubation, duplicate plates were incubated either in hypoxia or normoxia for 24 hours. Cells were assayed for firefly and renilla luciferase activity using the Dual-Glo Luciferase Assay System (Promega). All results were normalized to pGL3 vector alone activity at normoxia or hypoxia.

RNAi-mediated suppression of gene expression. HIF-1alpha, HIF-2alpha, and Snai1 smart pool siRNAs (Dharmacon) were transfected using Oligofectamine reagent (Invitrogen) following manufacturer's instructions.

Overexpression of cDNA. Snail and HIF-2 cDNA constructs (provided by Dr. Mercurio, UMASS, and Dr. McKnight, UT Southwestern, respectively) were transfected using Lipofectamine 2000 reagent (Ivitrogen) following manufacturer's instructions.

RNA extraction and real-time quantitative PCR (RQ-PCR). mRNA was extracted from cells using the RNeasy kit (Qiagen), treated with DNaseI (Invitrogen), and converted to cDNA using SuperscriptII® Reverse Transcriptase (Invitrogen). Gene expression was quantified using Syber Green RT-PCR master mix reagents (Applied Biosystems). The Delta Ct method was used to quantify the relative expression of each gene. IRS expression was normalized to either murine GAPDH or human Actin. Primers used in RQ-PCR: human IRS-2 (forward: 5'-CCACCATCGTGAAAGAGTGA-3' and reverse: 5'-CAGAGTCCACAGATGTTTCCAA-3'), human Actin (forward: 5'-TGAGCGCGGCTACAGCTT-3' and reverse: 5'-TCCTTAATGTCACGCACGATTT-3').

Immunoblotting. All immunoblots were performed as described previously [48] using the following antibodies: IRS-2 (EMD Biosciences, Inc.); HIF-1alpha (Novus); HIF-2alpha (Santa Cruz), tubulin (Sigma); hnRNP (Santa Cruz); Snail (Cell Signaling Technology, Inc.).

Nuclear and Cytoplasmic Cellular Fractionation: Nuclear fractionation was performed using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) according to manufacturer's instrutions.

Chromatin-immunoprecipitation. ChIP was performed using a previously published protocol [207]. Briefly, cells (10 million per IP) were fixed with 1 % formaldehyde in culture media for 10 min at room temperature. The reaction was quenched with the addition of 125 mM glycine for an additional 5 minutes. Cells were washed with cold PBS, scraped and collected by centrifugation at 2500 rpm for 10 minutes. Cells were lysed in lysis buffer (150 mM NaCl, 50mM Tris-HCl, pH7.5, 5mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 0.5% Nonidet P-40) supplemented with complete protease inhibitors (Roche). Nuclei were collected by centrifugation at 10,000 rpm for 1 minute, then washed once with lysis buffer without protease inhibitors and resuspended in ChIP buffer (150 mM NaCl, 50mM Tris-HCl, pH 7.5, 1mM EDTA, 1% Triton X-100, 0.1% Nonidet P-40). The nuclei were sonicated using Sonicator3000 (Msonix, Inc.) with a microtip at power level 3 for 5 pulses (for SUM-159 cells), or 8 pulses (for MDA-MB-231 and MCF7 cells) of 15 seconds followed by 30 seconds rest on ice, to reduce the chromatin fragments to an average 100 – 1000 bp. After sonication, lysates were cleared by centrifugation at 12000 rpm for 10 minutes at 4C. Soluble chromatin was pre-cleared with non-specific IgG (Santa Cruz) and Protein A-Sepharose beads. The chromatin was diluted at a 1:3 ratio with ChIP buffer and incubated with 2 or 4 ug antibody or non-specific IgG at 4^{o}C overnight. Immune complexes were collected by incubation with Protein A-Sepharose that had been pre-incubated with BSA and ssDNA for 2 hours at 4^{o}C. Beads were washed

twice with wash buffer I (50mM Tris, 1M NaCl, 1mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, pH7.5), twice with wash buffer II (10mM Tris, 0.25 M NaCl, 1mM EDTA, 0.5% Nonidet P-40, 0.5% Na deoxycholate, pH7.5), and twice with TE (10mM Tris-HCl, 1mM EDTA, pH8.0). Beads were resuspended in buffer C (50mM Tris-HCl, pH7.5, 10Mm EDTA, 1% SDS) and shaken at 14000 rpm at 65^{o}C for 10 minutes. Subsequently, TE and proteinase K (Roche) were added and incubated at 42^{o}C for 4-8 hours. NaCl was added to a final concentration of 0.5M and the samples were de-crosslinked at 65^{o}C overnight. DNA was extracted using the phenol-chloroform method.

The following antibodies were used for ChIP assays: PolII (Active Motif), Snail (Santa Cruz), HIF-2 (Santa Cruz), HIF-1 (Novus Biologicals), H3K4 (Millipore), IgG (Santa Cruz). The PolII antibody was used at a concentration of 2 ug per IP. All other antibodies were used at a concentration of 4 ug per IP.

IRS-2, EPO, PGK1, and E-cadherin promoters were amplified by semi-quantitative PCR and the PCR products were resolved on a 1.5% agarose gel. The following primers were used in PCR reactions: human IRS-2 promoter, forward 5'-TGCTCTTCTTAGTTCAGTCACTCGG-3', reverse 5’-TGTTGCTGCTGCTGCTGCC -3’, negative control human IRS-2 intron region, forward 5’-CTAGGCTTGATGCCCACTTC-3’, reverse 5’-GCTCCACCTTCAAACTGCTC-3’. The human PGK1, EPO, and E-cadherin promoters were amplified using previously published primer sequences [138, 200, 208]

Statistics. All data are represented as an average +/- standard error. All statistical analyses were performed using the unpaired Student’s *t*-test.

Figures

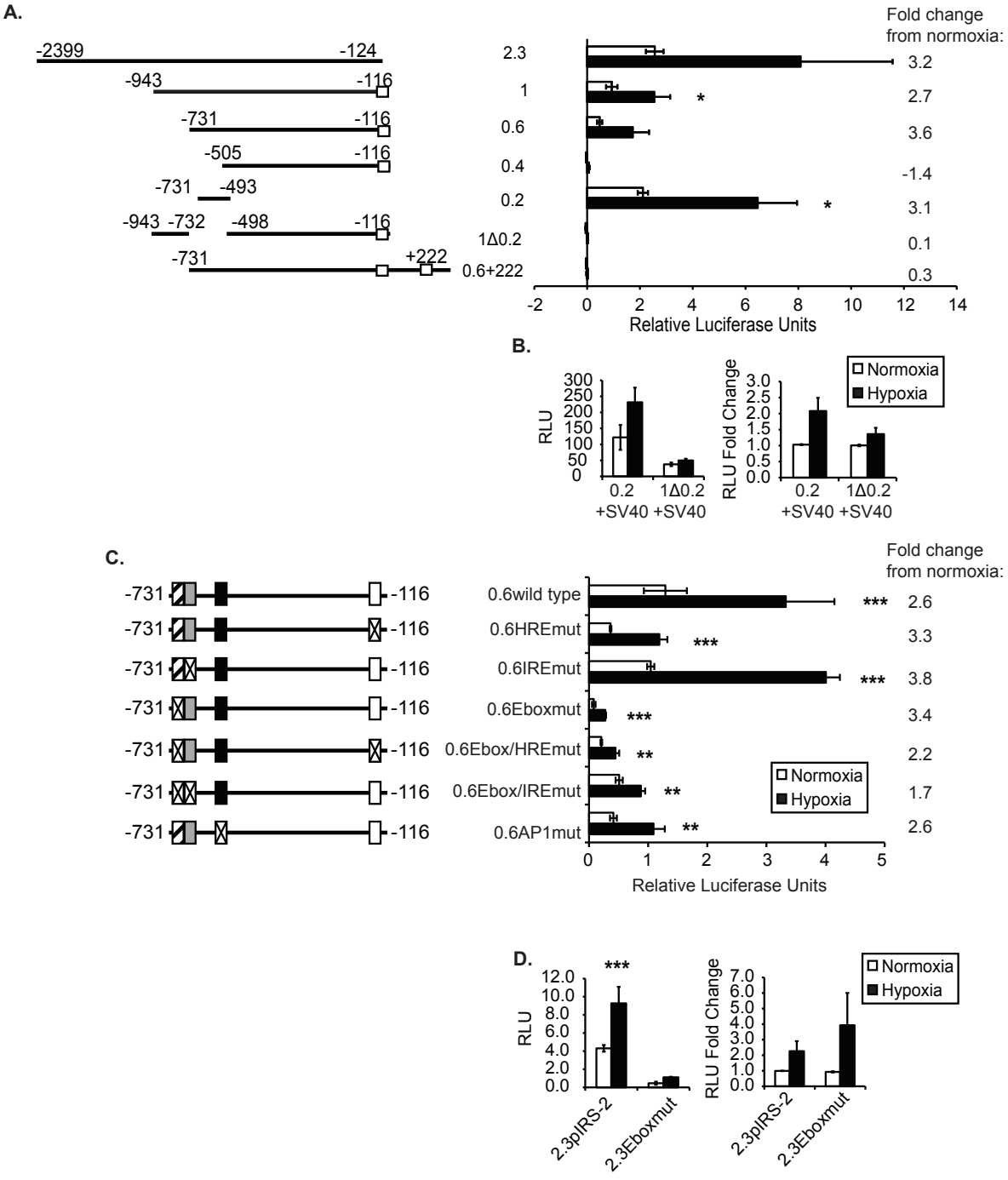
A.
-2399
-124
-943
-116
-731
-116
-505
-116
-731
-493
-943
-732
-498
-116
-731
+222
2.3
1
0.6
0.4
0.2
1Δ0.2
0.6+222
Fold change from normoxia:
3.2
2.7
3.6
-1.4
3.1
0.1
0.3
Relative Luciferase Units
B.
RLU
RLU Fold Change
Normoxia
Hypoxia
0.2 +SV40
1Δ0.2 +SV40
C.
0.6wild type
0.6HREmut
0.6IREmut
0.6Eboxmut
0.6Ebox/HREmut
0.6Ebox/IREmut
0.6AP1mut
Fold change from normoxia:
2.6
3.3
3.8
3.4
2.2
1.7
2.6
Relative Luciferase Units
D.
RLU
RLU Fold Change
2.3pIRS-2
2.3Eboxmut

Figure 3.1. Identification and characterization of hypoxia-responsive region in the human IRS-2 promoter. A) MDA-MB-231 cells were transfected with the human IRS-2 promoter-Luciferase constructs for 24 hours, followed by exposure to hypoxia (0.5% oxygen) or maintained at normoxia (regular cell culture incubator) for an additional 24 hours. The cells were lysed and relative Luciferase expression values were analyzed. The schematic on the left represents the different promoter constructs; the white box shows location of an HRE. The data shown represents at least three independent experiments +/- SEM; *, $p<0.045$. B) The minimal hypoxia-responsive region of the human IRS-2 promoter (0.2) or the 1Kb IRS-2 promoter lacking this region (1Δ0.2) was placed upstream of the SV40 promoter in in pGL3 Luciferase construct and activity of each chimeric promoter was analyzed in normoxia and hypoxia. The data shown represents at least three independent experiments +/- SEM; **, $p\leq0.02$. C) Luciferase activity of mutant 0.6Kb human IRS-2 promoter constructs was analyzed as above. Striped rectangle shows the location of the E-box, grey rectangle – IRE, black rectangle – AP1 binding site, white rectangle – HRE, crossed rectangle – mutated site. The data shown represents at least three independent experiments +/- SEM; **, $p\leq0.03$, ***, $p\leq0.005$. D) Luciferase activity of wild type or E-box-mutant 2.3Kb human IRS-2 promoter analyzed in normoxia or hypoxia as above. The data shown represents at least three independent experiments +/- SEM; ***, $p\leq0.007$.

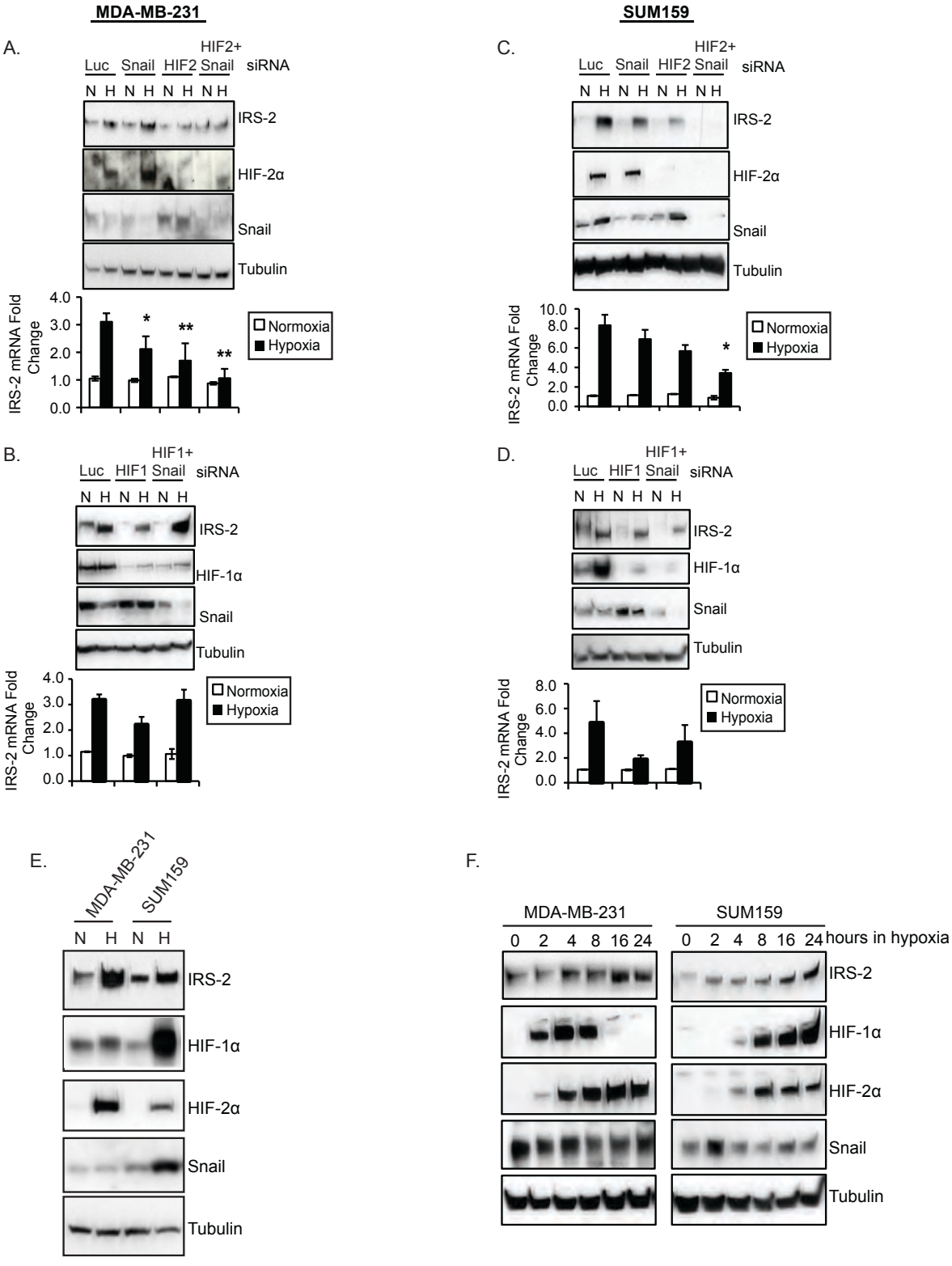
MDA-MB-231
SUM159
A.
B.
C.
D.
E.
F.
HIF2+
HIF1+
Luc
Snail
HIF2
HIF1
siRNA
N H N H N H N H
IRS-2
HIF-2α
HIF-1α
Snail
Tubulin
IRS-2 mRNA Fold Change
Normoxia
Hypoxia
hours in hypoxia
0 2 4 8 16 24

Figure 3.2. HIF-2 cooperates with Snail to regulate the hypoxic IRS-2 expression. A, B) MDA-MB-231 cells were transiently transfected with indicated siRNAs 24 hours prior to exposure to 0.5% oxygen (hypoxia) or normoxia (regular cell culture incubator) for an additional 24 hours. IRS-2 mRNA levels were analyzed by real-time PCR (left). IRS-2 protein levels were analyzed by immunoblot (right). C, D) SUM159 cells were transiently transfected with the indicated siRNAs and IRS-2 mRNA (left) and protein (right) levels were analyzed as above. All IRS-2 mRNA levels were plotted as fold change relative to the corresponding normoxic value. Each graph shows results of three independent experiments +/- SEM. *, $p<0.05$, **, $p<0.009$, relative to Luciferase siRNA control hypoxia sample. E) MDA-MB-231 and SUM159 cells were plated 24 hours prior to exposure to hypoxia (H) or normoxia (N) for an additional 24 hours. Transcription factor expression was analyzed by immunoblot. F) MDA-MB-231 and SUM159 cells were plated 24 hours prior to exposure to hypoxia for the indicated times. Total protein levels were analyzed by immunoblot.

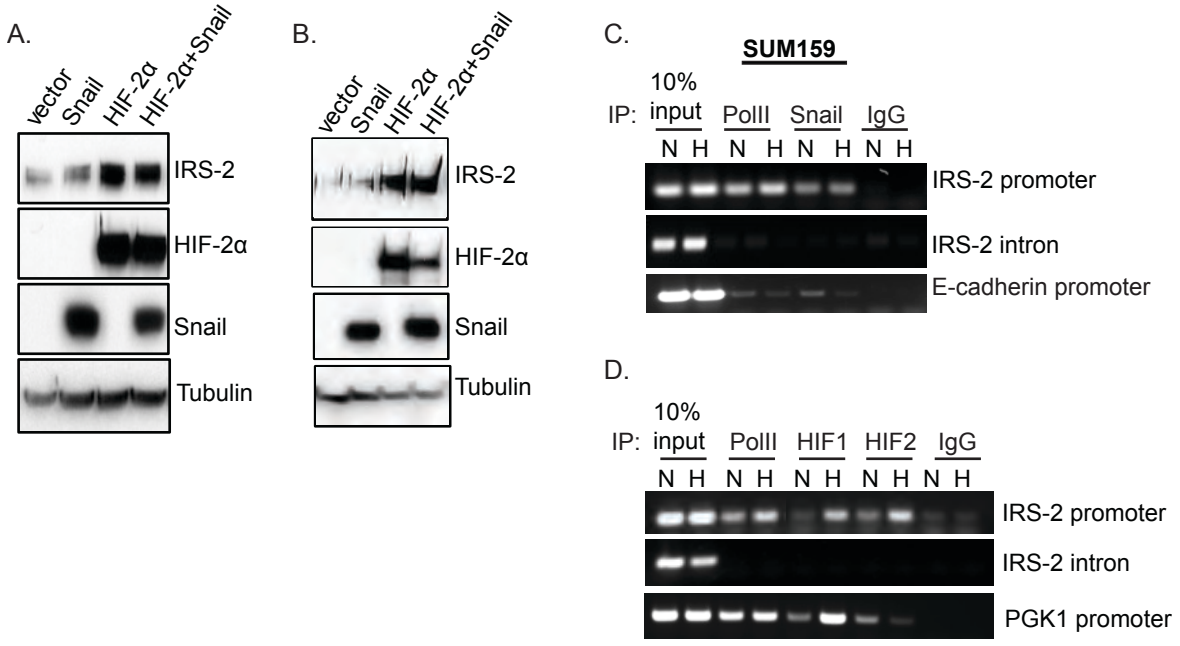

Figure 3.3. Snail and HIF bind directly to the IRS-2 promoter in hypoxia. A,B) HIF-2 and Snail were overexpressed in MDA-MB-231 (A) and SUM159 (B) cells for 48 hours. Cells were lysed and changes in IRS-2, HIF-2, and Snail protein levels were analyzed by Western blot. C) SUM159 cells were exposed to normoxia (N) or 0.5% oxygen (H) for 24 hours and binding of Snail to the IRS-2 promoter was analyzed by ChIP. IRS-2 intron sequence was used as a negative control, the E-cadherin promoter was used as a Snail-binding control. D) SUM159 cells were exposed to normoxia (N) or 0.5% oxygen (H) for 24 hours and binding of HIF-1 and HIF-2 to the IRS-2 promoter was analyzed by ChIP. IRS-2 intron sequence was used as a negative control, the PGK-1 promoter was used as a HIF-binding positive control.

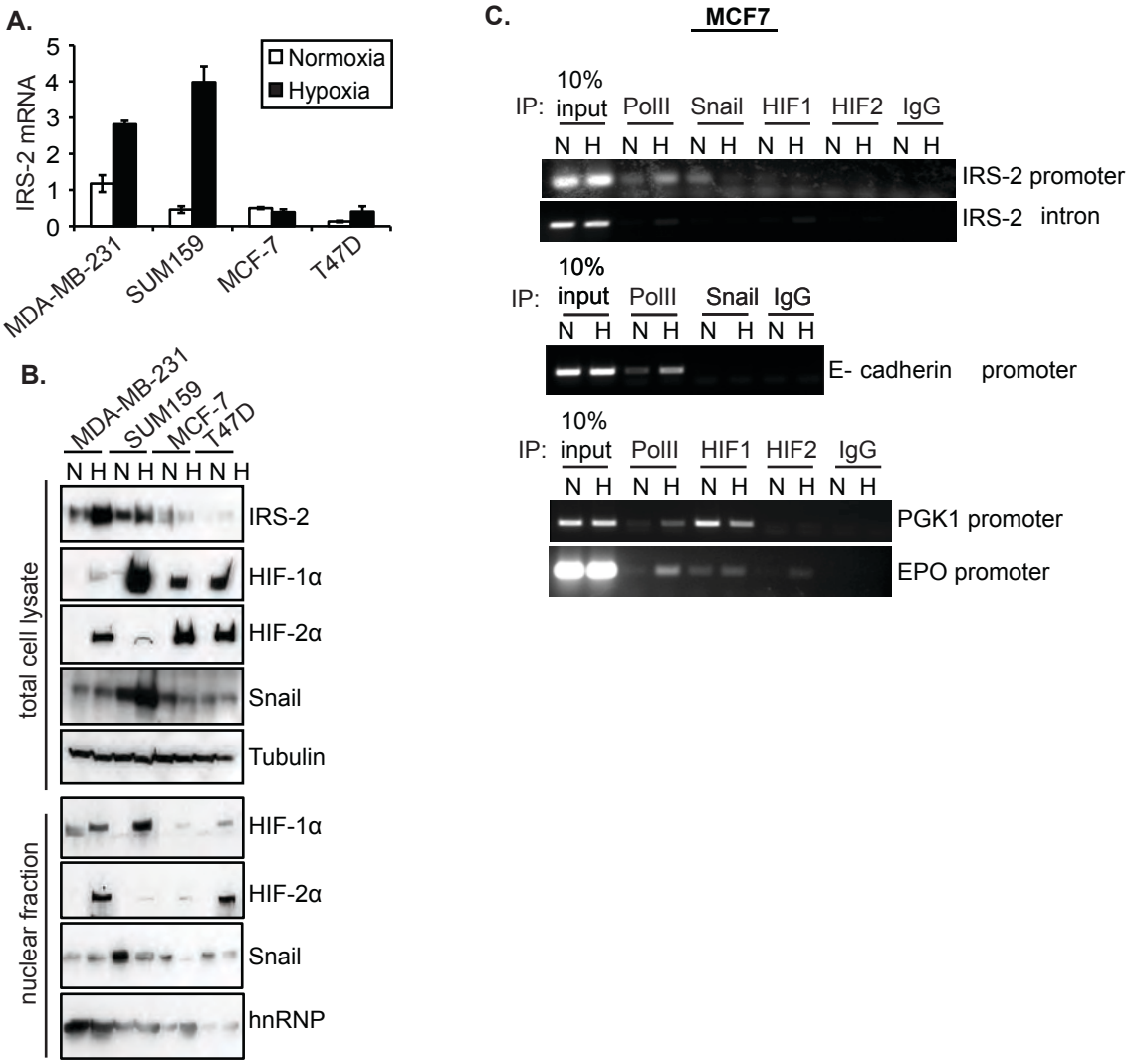

Figure 3.4. Hypoxic IRS-2 expression in epithelial-like breast carcinoma cell lines. A) The indicated cell lines were exposed to normoxia (N) or 0.5% oxygen (H) for 24 hours. IRS-2 mRNA levels were analyzed by real-time PCR. B) The indicated cell lines were exposed to normoxia (N) or hypoxia (H) as above and total protein levels of HIF-1, HIF-2, and Snail were analyzed by immunoblot (top). The same cells were subjected to nuclear fractionation and nuclear levels of HIF-1, -2, and Snail protein levels were analyzed by immunoblot (bottom). C) MCF7 cells were exposed to normoxia (N) or hypoxia (H) for 24 hours and binding of the indicated transcription factors to the IRS-2 promoter was analyzed by ChIP. IRS-2 intron sequence was used as a negative control; the E-cadherin promoter was used as a Snail-binding control; the PGK-1 promoter was used as a HIF-1 binding control; the EPO promoter was used as a HIF-2 binding control.

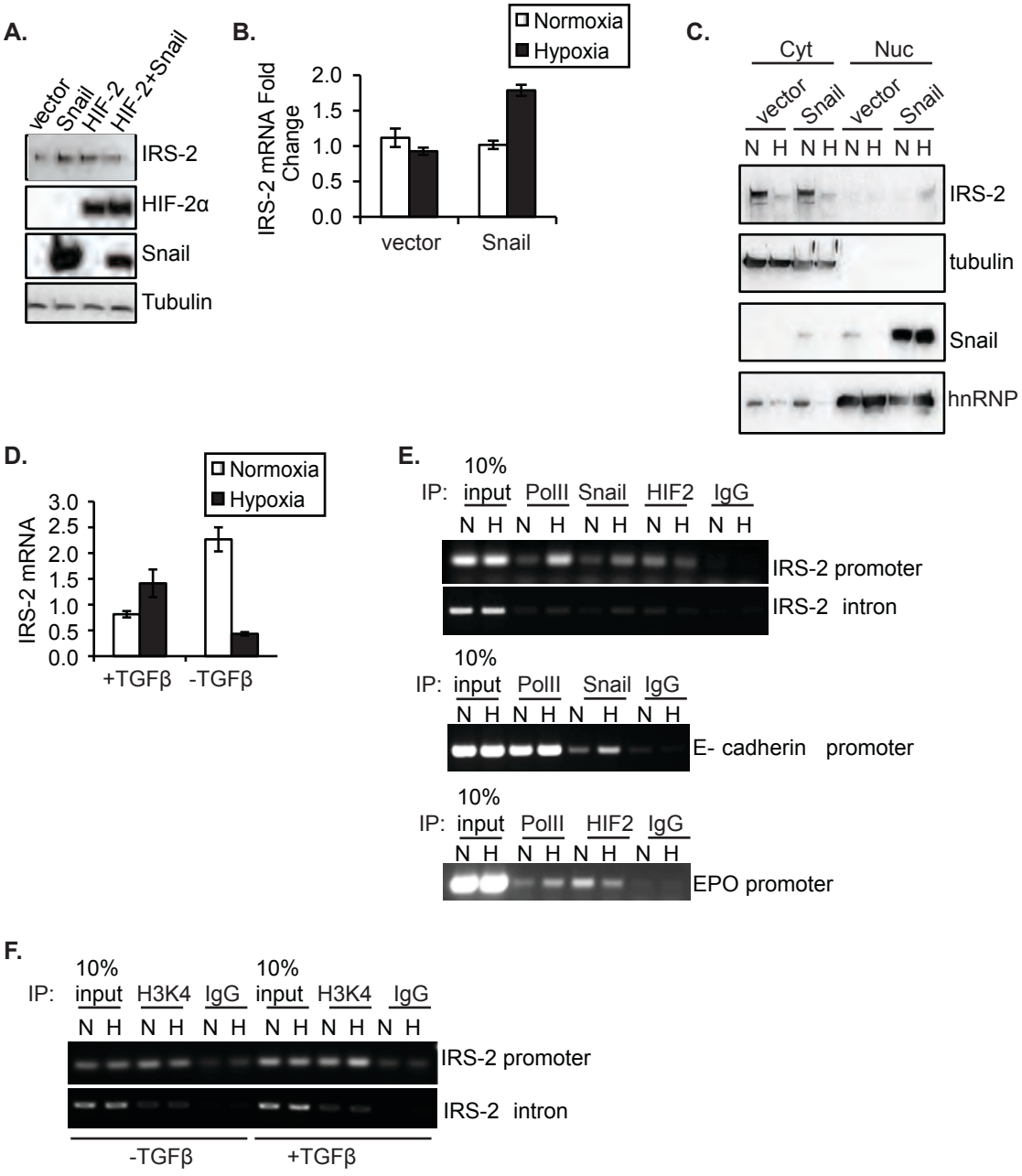

Figure 3.5. Role of Snail-induced EMT in hypoxic IRS-2 transcription. A) HIF-2 and Snail were overexpressed in MCF7 cells for 48 hours. Total IRS-2, Snail, and HIF-2 protein levels were analyzed by immunoblot. B) Snail was overexpressed in MCF7 cells as above before exposure to normoxia or hypoxia for an additional 24 hours. IRS-2 mRNA levels were analyzed by real-time PCR. C) Snail was overexpressed in MCF7 cells before exposure to normoxia (N) or hypoxia (H) as above. Cells were subjected to cytoplasmic (Cyt)/ Nuclear (Nuc) fractionation and levels of exogenous Snail protein were analyzed by immunoblot. D) MCF7 cells were treated with 5ng/ml TGF-beta in charcoal-stripped serum (CSS) containing medium or left untreated in regular FBS-containing growth

medium for 6 days. The cells were exposed to hypoxia or maintained in normoxia for an additional 24 hours. IRS-2 mRNA levels were detected by real-time PCR. E) Binding of Snail and HIF-2 to the IRS-2 promoter in the MCF7 cells treated with TGF-beta was analyzed by ChIP. IRS-2 intron sequence was used as a negative control; the E-cadherin promoter was used as a Snail-binding control; the EPO promoter was used as a HIF-2 binding control. F) MCF7 cells treated with TGF-beta as above were used in the ChIP assay to determine changes in histone 3 methylation on lysine 4 (H3K4).

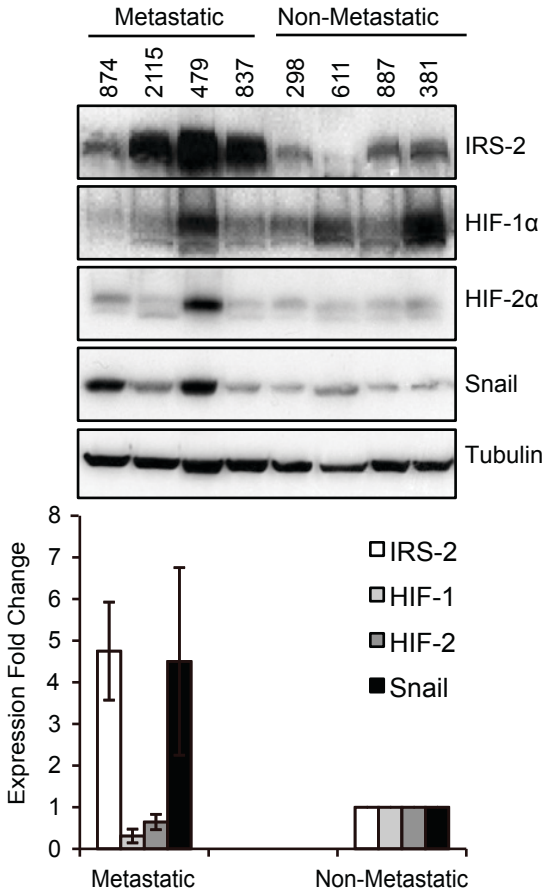

Figure 3.6. IRS-2 correlates with Snail expression in PyV-MT mouse mammary tumors. Total levels of the indicated proteins were analyzed by immunoblot in *PyV-MT:WT* mouse mammary tumor lysates. The graph gelow shows densitometry results. All values were normalized to loading control and plotted as expression fold change from non-metastatic tumors.

CHAPTER IV: Discussion

IRS-2 is an adaptor protein for insulin and IGF-1 receptors. Its expression and function have been heavily investigated and its role in cancer has been emphasized. This study was designed to investigate the expression and function of IRS-2 in breast cancer. Since hypoxia promotes tumor cell survival, invasion, and glycolysis leading to tumor progression and enhanced metastasis and IRS-2 has been implicated in all of the above processes, we sought to investigate a possible regulation of IRS-2 expression and function by hypoxia in breast cancer. We observed a strong correlation between IRS-2 expression and hypoxia in multiple mouse and human breast cancer cell lines, while IRS-1 expression was decreased. We showed that IRS-2 was fully functional under hypoxic conditions and promoted Akt activation even in the absence of external stimuli. Increased IRS-2 expression in hypoxia also promoted cell survival and invasion in a cell type-dependent manner. We also analyzed the mechanism of IRS-2 upregulation in hypoxia and found that protein and mRNA stability were not affected by hypoxia and transcription was required for its upregulation. Detailed analysis of the human IRS-2 promoter revealed a hypoxia responsive region (from -731 to -493) in the human IRS-2 promoter. We showed that binding of Snail to this region was a necessary condition for HIF-dependent transcription of IRS-2 in hypoxia.

Our finding of differential regulation of IRS-1 and IRS-2 expression by hypoxia supports the notion that these two proteins have independent functions in breast cancer and shows that their expression is regulated by unique mechanisms. The expression of IRS-1 and -2 has been previously shown to be regulated by different mechanisms. For example, IRS-1 is an estrogen-responsive gene, while IRS-2 expression has been shown to be regulated by progesterone [81, 197, 209]. Our finding that hypoxia not only promotes IRS-

2 upregulation but also simultaneously decreases IRS-1 levels indicates that the shift to the preferential signaling through IRS-2 may be one of the crucial steps in tumor cell progression to a more aggressive phenotype and hypoxia may be one of the determining factors of this shift.

One of the mechanisms by which IRS-2 can preferentially promote tumor metastasis is by supporting tumor cell survival. Here we show that hypoxic upregulation of IRS-2 promotes Akt activation, which has been previously shown to promote survival through different mechanisms [210-212]. For example, Akt can protect cells from apoptosis and promote survival by phosphorylation of the Bcl-2 pro-apoptotic protein family member BAD [213]. IRS-2 overexpression has been shown to promote resistance to glucose-induced caspase-3 activation though Akt dependent BAD phosphorylation in neuroblastoma [214]. Akt also negatively regulates the function of pro-apoptotic downstream effectors including the FOXO transcription factors [180]. IRS-2 has been shown to regulate FOXO activity through Akt in mouse embryo fibroblasts [181]. Our findings of a role for IRS-2 in Akt activation in hypoxia correlates with the previously published observation from our lab of increased Akt and mTor activation in *PyV-MT:Irs1*$^{-/-}$ mammary tumors that signal through Irs-2 [176]. A more recent study has also shown that suppression of IRS-2 expression by miRNA-153 in glioblastoma cells increases apoptosis levels [91].

The role of IRS-2 in survival under hypoxic conditions varied between cell lines. We found that SUM159 cells but not MDA-MB-231 cells depended on IRS-2 for survival in hypoxia. MDA-MB-231 survival in hypoxia was not affected by IRS-2 knockdown likely because these cells express mutated Ras, which can directly activate survival

signaling pathways bypassing the need for IRS-2 [171]. On the other hand, IRS-2 knockdown decreased invasion in MDA-MB-231 but not in SUM159 cells in hypoxia. These results indicate that the IRS-2 role in cell behavior in hypoxia depends on the cell context and the pathways that cells rely on for invasion or survival.

Hypoxia is known to upregulate expression of glycolytic genes resulting in a cellular metabolic switch. IRS-2-dependent activation of Akt in hypoxia may further promote this process. Akt has been shown to promote viability and growth through regulation of genes that control the metabolic switch from oxidative phosphorylation to anaerobic glycolysis [182]. Moreover, our lab has previously shown a positive role for IRS-2 in aerobic glycolysis [159]. Therefore, sustained Akt activation downstream of IRS-2 signaling may be one mechanism by which IRS-2 promotes glycolysis under hypoxic conditions.

In the current work, we focused on understanding the mechanism of regulation of IRS-2 expression under hypoxic conditions. We identified cell context-dependent roles for HIF-1 and -2 in the regulation of hypoxic IRS-2 expression. We established that in SUM159 cells, that showed high HIF-1 levels at prolonged hypoxia, both HIF-1 and -2 could contribute to IRS-2 upregulation in hypoxia. While in MDA-MB-231 cells, that showed low HIF-1 and high HIF-2 levels at prolonged hypoxia, when IRS-2 was upregulated, only HIF-2 was required for hypoxic IRS-2 upregulation. These results suggest that the role of HIF-1 in IRS-2 expression depends on cell context, while HIF-2 is the dominant regulator of IRS-2 expression. In line with our findings, another group has identified IRS-2 as one of the main HIF-2 target genes in a microarray screen of gene expression changes after treatment of cells with a HIF-2-specific inhibitor [202]. HIF-1

and -2 are known to have both common and unique target genes. Activation of expression of unique target genes can depend on the timing of stabilization of each factor. HIF-1 has been shown to regulate expression of the early-response genes in hypoxia and HIF-2 has been shown to have higher activity at prolonged hypoxia. The finding that IRS-2 is only upregulated after prolonged hypoxia correlates with the role of HIF-2 in its expression. However, SUM159 cells show HIF-1 stabilization after prolonged hypoxia, which in part can explain a role for HIF-1 in IRS-2 expression in these cells.

Another possible explanation for a different role of each HIF in transcriptional activation of IRS-2 is potential differences in co-activating factors. Several co-activators have been found to be specific for HIF-1 and HIF-2. For example, transcription factor Ets1 has been shown to interact exclusively with HIF-2alpha in regulating VEGF receptor 2 (Flk-1) transcription [143]. Another Ets family transcription factor, Elk-1, cooperates with HIF-2alpha to activate expression of CITED-2 in hypoxia in MCF-7 human breast carcinoma cell line [144]. On the other hand, cooperation of hepatocyte nuclear factor 4 (HNF-4), a liver- and kidney-specific transcription factor, with HIF-1, is essential for EPO transcription in hepatocytes [215, 216]. Therefore, differences in total expression or activity of specific co-factors between SUM159 and MDA-MB-231 cell lines can determine the specific cell context-dependent role of HIF-1 and HIF-2 in hypoxic IRS-2 expression.

HIF-1 and HIF-2 regulate transcription by binding to the HRE element (5'-(A/G)CGTG-3') in the promoter of target genes. However, several studies suggest that HIF can also bind to a non-consensus HRE [217]. The E-box (5'-CACATG-3') in the IRS-2 promoter resembles a non-consensus HRE site. Our ChIP data indicate that HIF-2 and

HIF-1 (in SUM159 cells only) could potentially bind to the E-box element in the IRS-2 promoter. However, due to the limitations of the ChIP assay, the sonicated fragments of analyzed DNA range from 100 to 1000 bp, it is not possible to conclude that the HIFs bind directly to the E-box. They could bind to any region of the IRS-2 promoter within 1Kb from the E-box. Further investigation is required to show the binding specificity of HIF to the E-box. Luciferase assays with HIF overexpression or EMSA can be used to identify the regions of the IRS-2 promoter that are regulated by the HIF. Similarly, the IRS-2 promoter with a mutated E-box element can be used in this assay to determine if HIF binds to and regulates IRS-2 expression through this site. Two additional HRE sequences (5'-RCGTG) in the reverse direction (5'-CACGR) can be found at the 5'-end of the 2.3Kb promoter. These sites however are not present in the 1Kb, 0.6, or 0.2Kb IRS-2 promoters that are responsive to hypoxia. This suggests that HIF may possibly bind to these regions through indirect interactions mediated by other transcription factors. Alternatively, the activity of these promoters could be regulated in a HIF-independent manner. For example, AP1 is upregulated by hypoxia through increased expression of cFos and cJun and does not require HIF [145, 146]. The tumor suppressor VHL has also been implicated in regulating gene expression in hypoxia independent from HIF [147, 148]. Luciferase assays and EMSA will not be informative, however, in the case of promoter regulation by HIF mediated by chromatin changes or through interactions over long range genomic intervals. HIF has been shown to preferentially act through the long-distance binding on the promoters of target genes [221]. Binding of HIF to the IRS-2 promoter does not eliminate this possibility. Chromosome conformation capture (3C) analysis will be necessary to investigate if HIF associates with the IRS-2 promoter over long range genomic intervals.

We have identified a positive role for Snail in hypoxic regulation of IRS-2 expression. Snail is mostly known for its function as a transcriptional repressor of E-cadherin expression. However, more recent evidence has emerged that Snail can play a positive role in regulating transcription. For example, it has also been shown to positively regulate expression of Wnt-target genes through interaction with beta-Catenin and expression of p15(INK4b) by cooperating with early growth response gene 1 (EGR-1) and stimulatory protein 1 (SP-1) [203, 204]. Snail has also been shown to activate transcription of target genes in C. Elegans *in vivo* by sharing E-box elements in the promoters of the target gene with bHLH transcription factors, which includes HIF [205]. Therefore, the outcome of Snail activity, repressor or activator, depends on the transcription factors and co-factors that Snail cooperates with to mediate transcription.

We found that Snail overexpression could not induce IRS-2 transcription, indicating that Snail activity alone was not sufficient for IRS-2 expression. However constitutive binding of Snail to the IRS-2 promoter was required for HIF-dependent IRS-2 transcription in mesenchymal-like breast carcinoma cell lines. This suggests that Snail may keep the chromatin in an open conformation, therefore permitting the HIF-dependent transcription of IRS-2 in hypoxia. Consistent with this, HIF transcription factors have been previously shown to have specificity in regulating expression of genes with active promoters [200]. Snail has been previously shown to alter chromatin conformation through direct interaction with histone-modifying enzymes. In its repressive role, Snail has been shown to bind histone demethylase LSD1 and to recruit protein arginine methyltransferase 5 (PRMT5) to the target E-cadherin promoter [218-220].

Our data suggest that binding of Snail to the IRS-2 promoter does not induce transcription directly, but leads to chromatin modification. We hypothesize that this leads to an open chromatin conformation that is permissive for HIF-dependent transcription. Further analysis is necessary to investigate our hypothesis. In order to show that chromatin modification is necessary for increased expression of IRS-2 in response to hypoxia MCF7 cells can be treated with histone deacetylase (HDAC) and histone methyltransferase inhibitors before exposure to hypoxia. According to our hypothesis, this treatment would lead to an open chromatin conformation that should allow increased expression of IRS-2 in MCF7 cells in hypoxia. Similarly, treatment of MDA-MB-231 cells with acetyltransferase and histone demethylase inhibitors should prevent the increase in IRS-2 expression.

Evidence of direct interaction of Snail with chromatin modifying enzymes and changes in this interaction under hypoxic conditions is also necessary. This can be done by co-immunoprecipitation and co-ChIP experiments to show not only changes in protein association but also their recruitment directly to the IRS-2 promoter *in vivo* in hypoxia. Additionally, we need to show that Snail binding leads to histone modification. Overexpression of Snail in MCF7 cells (at exogenous or endogenous levels induced by TGF-beta treatment) followed by treatment with histone-methyltransferase inhibitors should prevent the Snail-mediated increase in IRS-2 expression in hypoxia.

Apart from acting on chromatin, Snail could recruit HIF to the IRS-2 promoter in hypoxia through cooperation with hypoxia-regulated co-factors. No evidence exists for an interaction of Snail with HIF-1 or HIF-2 either directly or in a protein complex. Co-immunoprecipitation experiments are needed to answer the question whether Snail interacts

with HIF. Alternatively, Snail could be responsible for mediating a long-distance regulation of IRS-2 expression by HIF in hypoxia.

An alternative explanation for our ChIP and double knock-down data is that Snail and HIF can represent two independent mechanisms of regulation of IRS-2 expression in hypoxia. Snail may bring other transcription factors to the IRS-2 promoter in hypoxia in HIF-independent manner. Moreover, Snail can possibly play an indirect role in hypoxic IRS-2 expression by regulating expression of one or more factors that directly regulate IRS-2 expression in hypoxia.

The importance of the IRE site in combination with the E-box for hypoxic upregulation of IRS-2 promoter activity suggests a possible role for FOXO. However we did not observe an increase in FOXO nuclear localization when cells were exposed to hypoxia (Appendix 1E). Moreover, since the IRE mutation alone had no effect on hypoxia responsiveness of the IRS-2 promoter, the double mutation most likely affects binding of other factors than FOXO. A wide screening of binding proteins is necessary to determine the range of transcription factors that can bind to the E-box/IRE region and how their binding is affected by the mutations. A biotin-labeled oligo with the wild-type and mutant E-box-IRE region can be used in a pull down assay with lysates from normoxic and hypoxic cells. It is also possible that the double mutation can create a site favourable for binding of a hypoxic repressor.

The finding that E-box mutation alone significantly decreases basal promoter activity in the Luciferase assays suggests that other E-box binding factors may directly regulate IRS-2 promoter activity and basal IRS-2 expression. This opens a possibility that several independent mechanisms are involved in hypoxic IRS-2 expression. If Snail does

not play a role in regulation of IRS-2 expression by hypoxia, other transcription factors may be responsible for modulating IRS-2 expression.

We have previously identified an Ets-1, a specific HIF-2 co-activator, binding site in the 0.4Kb region of the human IRS-2 promoter (Appendix 1A). This region has no basal activity because it lacks the crucial E-box element, but close proximity of the Ets-binding site may increase HIF-2 specificity for the IRS-2 promoter. Therefore we tested the possible role of Ets-1 in hypoxic IRS-2 expression. Single siRNA-mediated knockdown of Ets-1, however, had no effect on hypoxic IRS-2 expression (Appendix 1B). We have not performed a double Ets-1 knockdown with HIF-2 or Snail to identify a possible cooperation of these factors. It would be interesting to test this possibility. If Ets-1 cooperation with HIF-2 is required for hypoxic IRS-2 upregulation, double knockdown of Ets-1 and Snail should have the same effect on hypoxic IRS-2 expression as HIF-2 and Snail combined knockdown. Similarly, we tested whether NFkappaB played a role in hypoxic IRS-2 expression. We identified five consecutive NFkappaB binding sites in the same region of the IRS-2 promoter, but the knockdown of the p65 subunit of NFkappaB alone had no effect on IRS-2 expression in hypoxia (Appendix 1A,D). Since the 0.2Kb IRS-2 promoter construct that lacks the 0.4Kb region with the Ets-1 and NFkappaB binding sites maintains relatively high activity in hypoxia, the 0.4Kb region is not required for IRS-2 promoter activity. However, the factors that bind to this region may enhance IRS-2 transcription. We have also identified a strong cooperative role of TFEB and HIF-2 factors in the regulation of hypoxic IRS-2 transcription (Appendix 1C). However, due to the lack of a commercially available specific TFEB antibodies, we were not able to pursue this question further. The TFEB transcription factor belongs to the Microphthalmia family of

basicHLH transcription factors. It has been shown to mediate IRS-2 expression through an E-box interaction in mouse liver cells in response to insulin signaling [79]. TFEB has never been previously implicated in hypoxic gene expression and it would be interesting to understand how its activity is regulated and how it might contribute to IRS-2 expression in hypoxia. In the future, specific TFEB andibodies can be made to investigate if TFEB directly binds to the IRS-2 promoter in breast cancer cells and if its binding changes and and affects HIF binding in hypoxia.

Specificity protein 1 (Sp1) housekeeping transcription factor has also been previously implicated in the regulation of IRS-2 expression in neuroblastoma and hepatoma cells [223, 224]. Sp1 cooperates with different factors in the same region of the IRS-2 promoter that we identified in a cell type-dependent manner. Sp1 competes with ZBP89 factor to positively regulate IRS-2 expression in response to serum starvation in neuroblastoma cells [223]. Binding of the ZBP89 factor has been shown to inhibit IRS-2 promoter activity and its binding is increased by PI3K signaling [223]. In the HepG2 hepatoma cell line, Sp1 cooperates with nuclear factor 1 (NF1) in the positive regulation of IRS-2 expression in an ERK-dependent manner in response to multiple stimuli, including oxidative stress and interleukin 1 beta (IL1B) stimulation [224]. As a known house-keeping transcription factor, and a positive regulator of IRS-2 transcription, Sp1 may play a role similar to Snail, maintaining the chromatin in an open conformation at the IRS-2 promoter to permit for efficient HIF-dependent transcription in hypoxia. Its role in hypoxic IRS-2 expression can be investigated in the future using a similar approach we used for Snail.

While the majority of this study was designed to understand the mechanism of regulation of hypoxic IRS-2 expression in human cells, we originally identified IRS-2 as a hypoxia-responsive gene in mouse cells. While the overall sequence and structure of the mouse IRS-2 promoter differs from the human promoter, the hypoxia-responsive region that we identified in the human IRS-2 promoter has a high level of homology with the mouse promoter. Moreover, the E-box element and adjacent IRE and SREBP binding sites are conserved among species, suggesting a similar role for these factors in IRS-2 expression in mouse and human cells [79]. The mouse IRS-2 promoter also contains additional consensus HRE elements that are absent from the human promoter, suggesting a more direct role of HIF in hypoxic IRS-2 expression in mouse cells. Cloning of the mouse IRS-2 promoter would be necessary to answer these questions. Correlation of IRS-2 expression with HIF-2 but not HIF-1 levels in mouse PyV-MT wild-type mammary tumors also suggests a dominant role of HIF-2 in IRS-2 expression. However, the fact that some metastatic tumors have high levels of IRS-2, but low levels of both HIF-1 and HIF-2, suggests factors other than hypoxia such as growth factors (EGF and FGF), may contribute to increased IRS-2 expression *in vivo*. [84, 85].

We observed that despite uniform expression of HIF-1 and HIF-2 among different breast carcinoma cell lines, only mesenchymal-like cells could upregulate IRS-2 expression in hypoxia. IRS proteins have been previously implicated in EMT. IRS-1 expression and phosphorylation has been shown to have an inverse correlation with EMT in the A549 lung cancer cell line [199]. A recent study has also shown an inverse correlation between E-cadherin and IRS-1 expression in a subset of non-small cell lung cancer that have undergone EMT [225]. Consistent with these findings, hypoxia, known to induce EMT,

also results in decreased IRS-1 levels alongside with upregulation of IRS-2 expression [198, 206]. The requirement of Snail for hypoxic IRS-2 expression in breast carcinoma cell lines *in vitro* and a strong correlation between Snail and IRS-2 expression in PyV-MT tumors *in vivo*, suggest that increased IRS-2 expression in tumor cells in response to hypoxia may be part of the EMT programming. In this case, exposure of tumor cells to hypoxia and other changes in the tumor microenvironment that lead to EMT, leads to "activation" of the IRS-2 promoter via Snail, therefore increasing overall IRS-2 expression, and allowing cells to upregulate expression in response to hypoxia. This upregulation may lead to enhanced activation of the Akt signaling axis, resulting in increased survival and invasiveness of the cells under hypoxic condition. Moreover, cells with increased IRS-2 expression may be able to permanently switch to glycolysis, even under aerobic condition, once they leave the hypoxic tumor environment, therefore passing through selection for more aggressive subpopulation of cells, ultimately increasing tumor metastasis.

This work was focused on IRS-2 expression and function in breast cancer. In regards to other types of cancer, there is little information on the role of IRS-2 in tumor progression. Most studies are focused on the overall expression levels of IRS proteins that have been found to be elevated in the majority of tumors compared to normal tissue. IRS-2 has been found to be overexpressed in both murine and human hepatocellular carcinoma, as well as hepatoma cell lines [51, 56]. Irs-2 has been shown to be important for tumor invasion in $PTEN^{+/-}$ mouse model of prostate and endometrial tumor progression [45]. Similar to our findings, IRS-2 seems to be important for tumor progression in other cancers, but a thorough analysis of its expression and function in different backgrounds is

necessary to fully understand the role of this protein in cancer progression and the possibility to use it as a prognostic and treatment marker.

Conclusions:

The role of the IRS proteins in cancer development and progression has recently gained a wide attention. In breast cancer, where these proteins have been studied the most, IRS-2 has been shown to be associated with cancer cell invasion, survival, and tumor metastasis. Our work provides strong evidence for a direct involvement of IRS-2 in tumor progression by promoting invasion and survival thorough sustained Akt signaling in hypoxia. Although the importance of overall IRS-2 expression levels in cancer has been generally accepted, there is still little known about the mechanisms of the regulation of its expression. In this work, we focused on the regulation of IRS-2 expression under hypoxic conditions and identified a mechanism that allows aggressive cell lines to induce IRS-2 expression in response to hypoxia. Since IRS-2 does not have any intrinsic kinase activity, it is more difficult to target for therapy. However, detailed understanding of the regulation of IRS-2 expression may provide a possibility to design treatment to target its expression. For example, specific HIF-2 inhibitors have been investigated as potential chemotherapy agents [202]. Moreover, its strong association with metastatic markers (such as hypoxia and EMT) makes it a potential marker for tumor progression and possible treatment outcome.

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APPENDIX

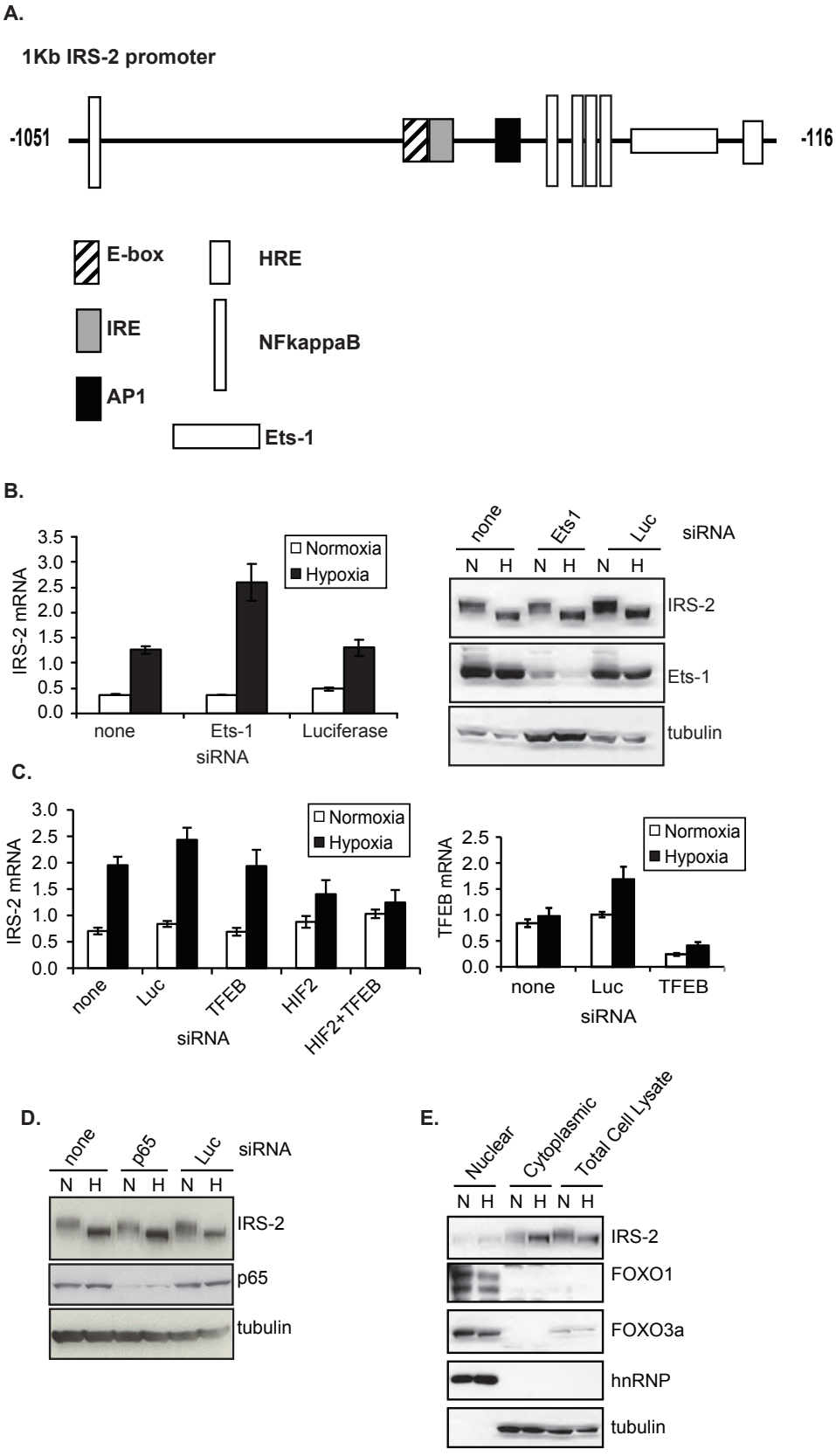
A.
1Kb IRS-2 promoter
-1051
-116
E-box
HRE
IRE
NFkappaB
AP1
Ets-1
B.
IRS-2 mRNA
Normoxia
Hypoxia
none
Ets-1
Luciferase
siRNA
none
Ets1
Luc
siRNA
N H N H N H
IRS-2
Ets-1
tubulin
C.
IRS-2 mRNA
Normoxia
Hypoxia
none
Luc
TFEB
HIF2
HIF2+TFEB
siRNA
TFEB mRNA
Normoxia
Hypoxia
none
Luc
TFEB
siRNA
D.
none
p65
Luc
siRNA
N H N H N H
IRS-2
p65
tubulin
E.
Nuclear
Cytoplasmic
Total Cell Lysate
N H N H N H
IRS-2
FOXO1
FOXO3a
hnRNP
tubulin

Figure 1. Analysis of the role of additional transcription factors in regulation of IRS-2 expression in hypoxia. A) 1Kb promoter schematic. Transcription factor binding sites are indicated as follows: striped square – E-box, grey square – IRE, black square – AP1 binding site, white square – HRE, vertical rectangle – NfkappaB binding site, horizontal rectangle – Ets-1 binding site. B) MDA-MB-231 cells were transfected with Ets-1 or Luciferase (Luc) siRNA and exposed to hypoxia (H) or normoxia (N) for 24 hours. Total mRNA was extracted and IRS-2 mRNA levels were analyzed by real-time PCR (left). IRS-2, Ets-1, and tubulin were analyzed by immunoblot (right). C) MDA-MB-231 cells were transfected with the indicated siRNA (Luc = Luciferase) and exposed to normoxia or hypoxia for 24 hours. Total mRNA was extracted and changes in IRS-2 (left) and TFEB (right) mRNA levels were analyzed by real-time PCR. D) MDA-MB-231 cells were transfected with p65 NFkappaB subunit or Luciferase (Luc) siRNA and exposed to hypoxia (H) or normoxia (N) for 24 hours. IRS-2, p65, and tubulin were analyzed by immunoblot. E) MDA-MB-231 cells were incubated in normoxia (N) or hypoxia (H) for 24 hours. IRS-2, FOXO1, FOXO3a, tubulin, and hnRNP protein levels in nuclear and cytosolic fractions, and total cell lysate were analyzed by Western blot.