

# Hypoxia Regulates Insulin Receptor Substrate-2 Expression to Promote Breast Carcinoma Cell Survival and Invasion

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## 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 show 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 hypoxia-inducible factor-dependent manner in hypoxic environments. IRS-2 is active to mediate insulin-like growth factor I-dependent signals in hypoxia, and enhanced activation of Akt in hypoxia is dependent on 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.** [Cancer Res 2009;69(23):8894–901]

## 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-I (IGF-I) and insulin receptors, several cytokine receptors, prolactin, growth hormone and vascular endothelial growth factor (VEGF) receptors, and members of the integrin receptor family (1). The IRS proteins act as scaffold proteins to recruit signaling molecules to the receptors to regulate intracellular signaling cascades (2). 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 (1, 3). 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 (4, 5). Similarly, *Irs-2* expression is elevated in tumors that arise in *PTEN*<sup>+/-</sup> mice, and deletion of *Irs-2* suppresses tumor growth and progression to invasive disease (6).

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 their wild-type (WT) counterparts (5). IGF-I predominantly induces IRS-2 phosphorylation in MDA-MB-231 human breast carcinoma cells selected for metastatic behavior *in vivo* (7). Introduction of an IRS-2 antisense mRNA into these metastatic cells results in decreased IGF-I-induced cell motility and anchorage-independent growth (7). Similarly, expression of IRS-2 in T47D breast carcinoma cells results in increased cell motility in response to IGF-I stimulation (8). 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 (9). 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 toward invasive and metastatic disease (10, 11).

Rapidly growing tumors develop areas of low oxygen tension, or hypoxia, when their growth outpaces the development of new blood vessels (12). 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 (13). In addition, hypoxia upregulates signaling pathways that facilitate invasion and survival (14, 15). Therefore, exposure of tumor cells to hypoxia creates a selection for cells with a more aggressive, invasive behavior (16, 17). To conserve energy in hypoxic conditions, overall gene expression is suppressed, and primarily, genes that are essential for low-oxygen/nutrient adaptation are expressed (18). Specifically, genes that are upregulated in response to hypoxia are involved in angiogenesis, DNA damage responses, glycolysis, and survival (15). Upregulation of these genes in response to hypoxia ultimately leads to increased metastatic potential (15).

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.

## Materials and Methods

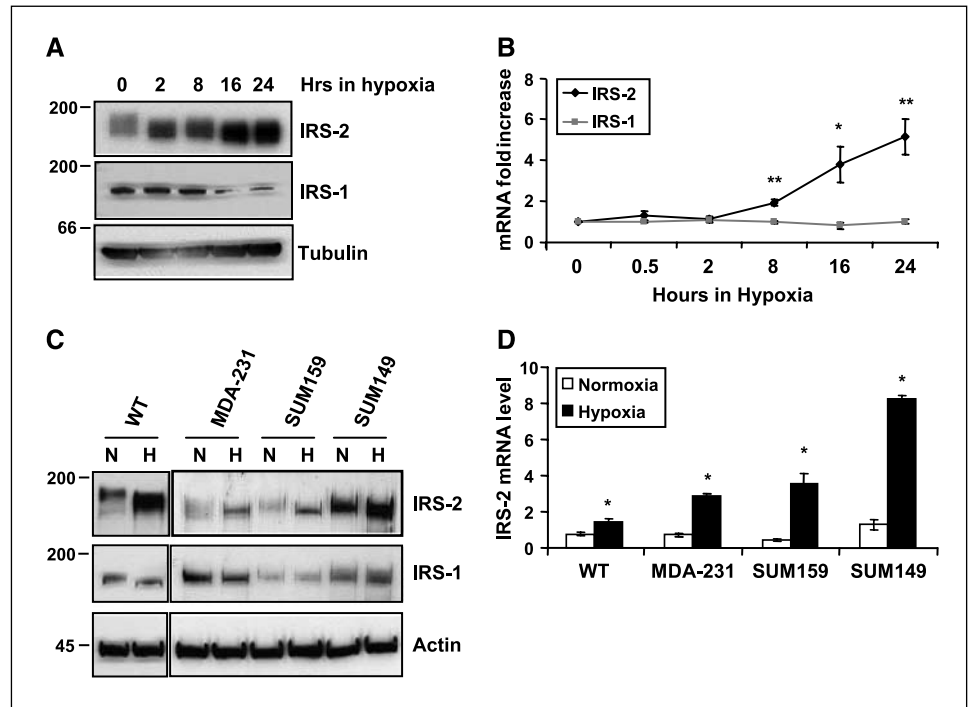
**Cell lines and hypoxia treatment.** Mouse mammary tumor cell lines were isolated from PyV-MT-derived *WT* and *Irs-2*<sup>-/-</sup> tumors as described previously (5). MDA-MB-231 human breast carcinoma cells were obtained from American Type Culture Collection. SUM159 and SUM149 human breast carcinoma cells were a gift from Dr. A. Mercurio (University of Massachusetts Medical School, Worcester, MA).

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**Figure 1.** Hypoxia induces IRS-2 expression. MDA-MB-231 cells were incubated in normoxia (0 h) 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 real-time quantitative PCR (RQ-PCR). Results were plotted as the fold change in mRNA levels compared with normoxic levels. Points, mean of three independent experiments; bars, 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 h. 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**). Columns, mean of three independent experiments; bars, SEM. \*,  $P \leq 0.008$  relative to normoxic levels.



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<sub>2</sub> Hypoxia Workstation (Ruskin 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 h and then cycloheximide (20  $\mu$ g/mL; Sigma) or Actinomycin-D (10  $\mu$ mol/L; Sigma) were added to the cell culture medium for 1, 3, or 6 h of incubation. Cells were also pretreated with Actinomycin-D (10  $\mu$ mol/L) or DMSO for 30 min 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 (University of Texas Southwestern Medical Center, Dallas, TX; ref. 19). The human 2.3 Kb IRS-2 promoter luciferase plasmid was a gift from Dr. A. Lee (Baylor College of Medicine, Houston, TX; refs. 20, 21). IRS-2 promoter plasmids [0.6 Kb (–732 to –116) and 0.3 Kb (–428 to –116)] were generated by serial SmaI digestion of the 1 Kb IRS-2 promoter.

Cells were plated in triplicate wells of a 24-well plate and cotransfected with the promoter-luciferase plasmids (0.5  $\mu$ g) and a pRL-CMV renilla luciferase plasmid (0.1  $\mu$ g; Promega). After an overnight incubation, duplicate plates were incubated either in hypoxia or normoxia for 24 h. Cells were assayed for firefly and renilla luciferase activity using the Dual-Glo Luciferase Assay System (Promega).

**RNAi-mediated suppression of gene expression.** Hypoxia-inducible factor (HIF)-1 $\alpha$  and HIF-2 $\alpha$  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 (University of Massachusetts Medical School, Worcester, MA). Lentiviral vectors containing human IRS-2 and green fluorescent protein (GFP) shRNAs were obtained from Open Biosystems.

**RNA extraction and real-time quantitative PCR.** Messenger RNA 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 reverse transcription-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 glyceraldehyde-3-phosphate dehydrogenase or human Actin.

**Immunoprecipitation and immunoblotting.** IRS-2 immunoprecipitations and immunoblots were performed as described previously (5) 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, Boston, MA), IRS-1 (Bethyl Labs), and 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 h before being transferred to hypoxia for an additional 24 h. For apoptosis assays with SUM159 cells, the culture medium was replaced with serum-free RPMI 1640 (Life Technologies) supplemented with 1 g/L glucose before 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 h before the invasion assay and then maintained in the hypoxia chamber during assay preparation. Invasion assays were performed as described previously (9).

**Statistics.** All data are represented as an average  $\pm$  SEM. All statistical analyses were performed using the unpaired Student's *t* test.

## 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 hour), both IRS-2 protein and mRNA expression increased significantly in response to hypoxia (Fig. 1A and B). The induction of IRS-2 mRNA occurred within 8 hours of exposure to hypoxia, with maximal levels observed after 16 to 24 hours. IRS-1 mRNA expression did not increase in response to hypoxia over the same time course (Fig. 1B). However, IRS-1 protein expression decreased after 16 hours 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 (22). A modest upregulation of IRS-2

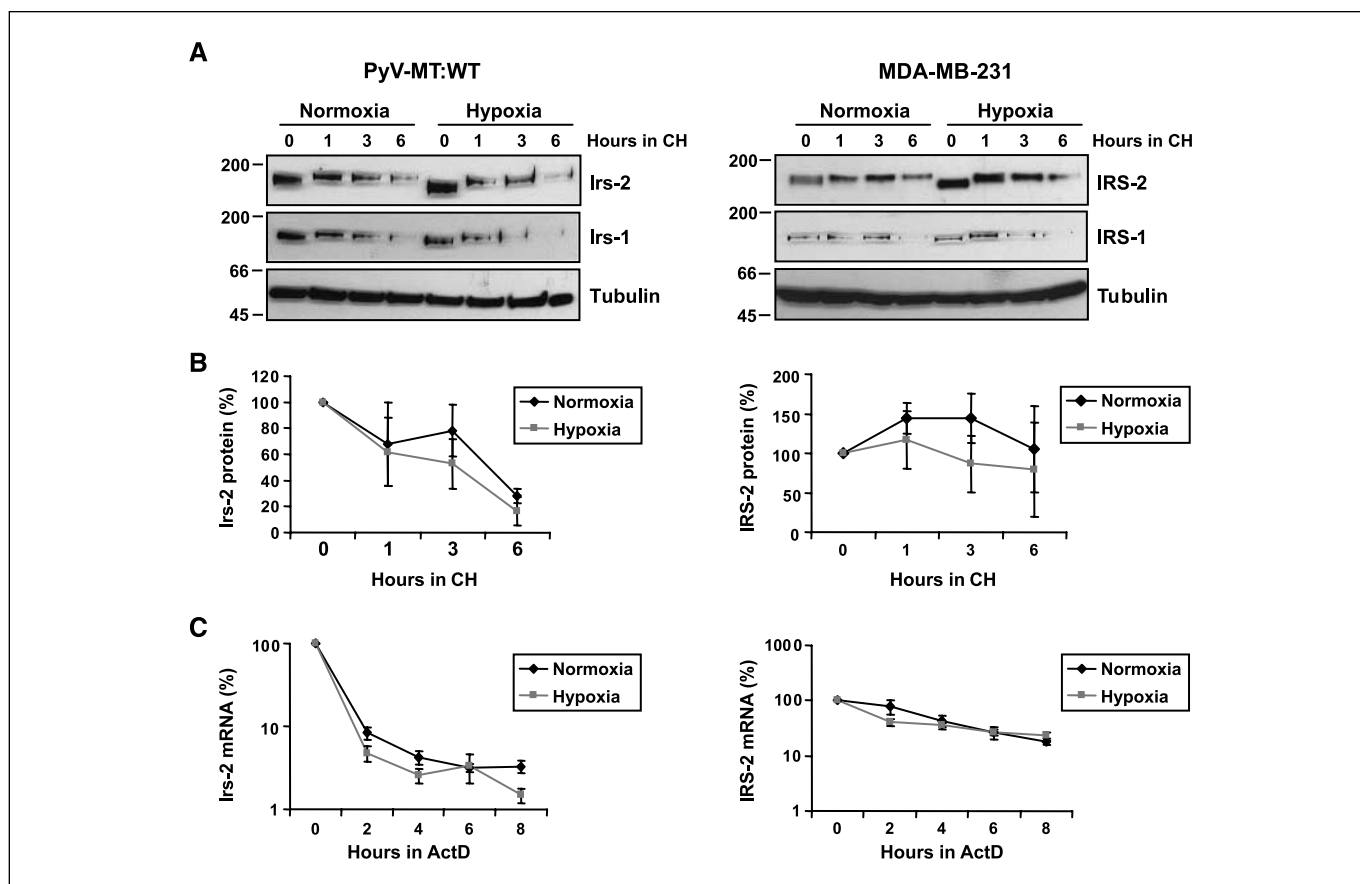
protein in the absence of an mRNA increase was sometimes observed in response to acute hypoxia (2 hours; Fig. 1A). This increase is likely due to increased IRS-2 protein stability because pretreatment of cells with cycloheximide did not prevent this early IRS-2 protein increase (data not shown).

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 hours in hypoxia in SUM159 and SUM149 human breast carcinoma cells, and also in a mouse mammary tumor cell line (PyV-MT:WT; Fig. 1C and D; ref. 5). IRS-1 protein and mRNA levels either remained unchanged (human) or decreased (mouse) in response to hypoxia in these additional cell lines (data not shown; Fig. 1C).

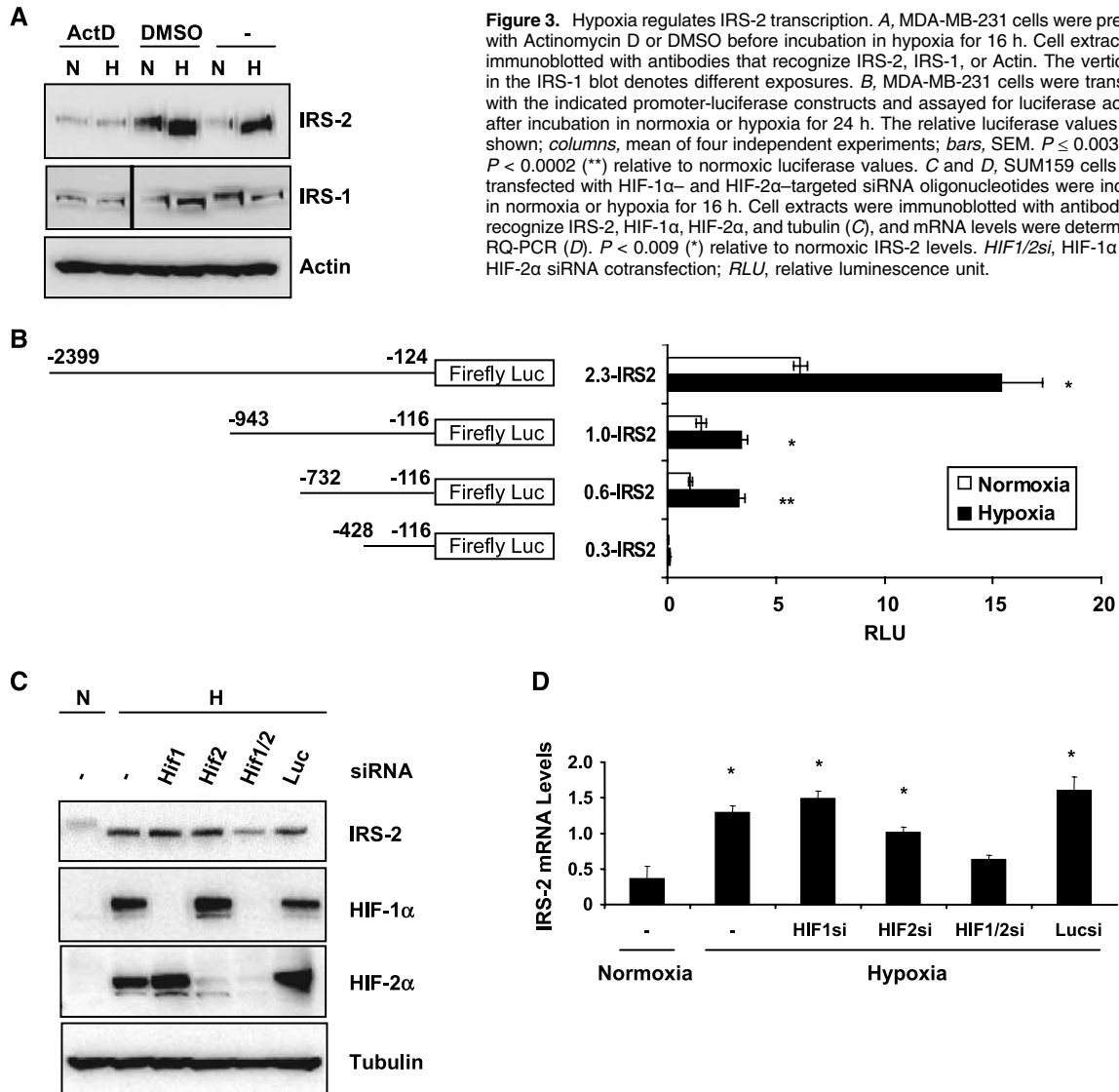
**Hypoxia regulates IRS-2 transcription.** To examine the mechanism by which IRS-2 expression is enhanced by hypoxia, we compared IRS-2 protein and mRNA stability under normoxic and hypoxic conditions in murine PyV-MT:WT and human MDA-MB-231 cells. Cells were incubated for 16 hours in hypoxia and then treated with either cycloheximide or Actinomycin D 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 cycloheximide in both normoxic and hypoxic conditions (Fig. 2A). Densitometric analysis revealed that

IRS-2 protein is slightly less stable in hypoxia when compared with normoxia (Fig. 2B). The decreased gel mobility observed for both IRS-1 and IRS-2 after treatment with cycloheximide 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 (23, 24). IRS-2 mRNA stability was similar in hypoxia and normoxia for both murine PyV-MT:WT and human MDA-MB-231 cell lines (Fig. 2C).

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 Actinomycin D for 30 minutes before hypoxic exposure to block *de novo* gene transcription. Cells were incubated for 16 hours in hypoxia in the continued presence of Actinomycin D or vehicle (DMSO), or left untreated. Actinomycin D preincubation inhibited the upregulation of IRS-2 expression in response to hypoxia (Fig. 3A). 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 of the human IRS-2 promoter (20). 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. 3B). To identify a hypoxia-responsive region of the IRS-2 promoter, pGL3 plasmids containing progressive deletions of the IRS-2 promoter were evaluated for luciferase activity in hypoxia. A decrease in normoxic promoter activity was



**Figure 2.** 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 h (0 h) before the addition of cycloheximide (CH; A and B) or Actinomycin D (ActD; 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. Points, mean of three independent experiments; bars, SEM. C, IRS-2 mRNA levels were quantified by RQ-PCR and the change in mRNA levels (%) relative to no treatment (0 h) was determined. Points, mean of three independent experiments; bars, SEM.



**Figure 3.** Hypoxia regulates IRS-2 transcription. **A**, MDA-MB-231 cells were pretreated with Actinomycin D or DMSO before incubation in hypoxia for 16 h. 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 h. The relative luciferase values are shown; *columns*, mean of four independent experiments; *bars*, SEM.  $P \leq 0.003$  (\*) and  $P < 0.0002$  (\*\*) relative to normoxic luciferase values. **C** and **D**, SUM159 cells transfected with HIF-1 $\alpha$ - and HIF-2 $\alpha$ -targeted siRNA oligonucleotides were incubated in normoxia or hypoxia for 16 h. Cell extracts were immunoblotted with antibodies that recognize IRS-2, HIF-1 $\alpha$ , HIF-2 $\alpha$ , and tubulin (**C**), and mRNA levels were determined by RQ-PCR (**D**).  $P < 0.009$  (\*) relative to normoxic IRS-2 levels. *HIF1/2si*, HIF-1 $\alpha$  and HIF-2 $\alpha$  siRNA cotransfection; *RLU*, relative luminescence unit.

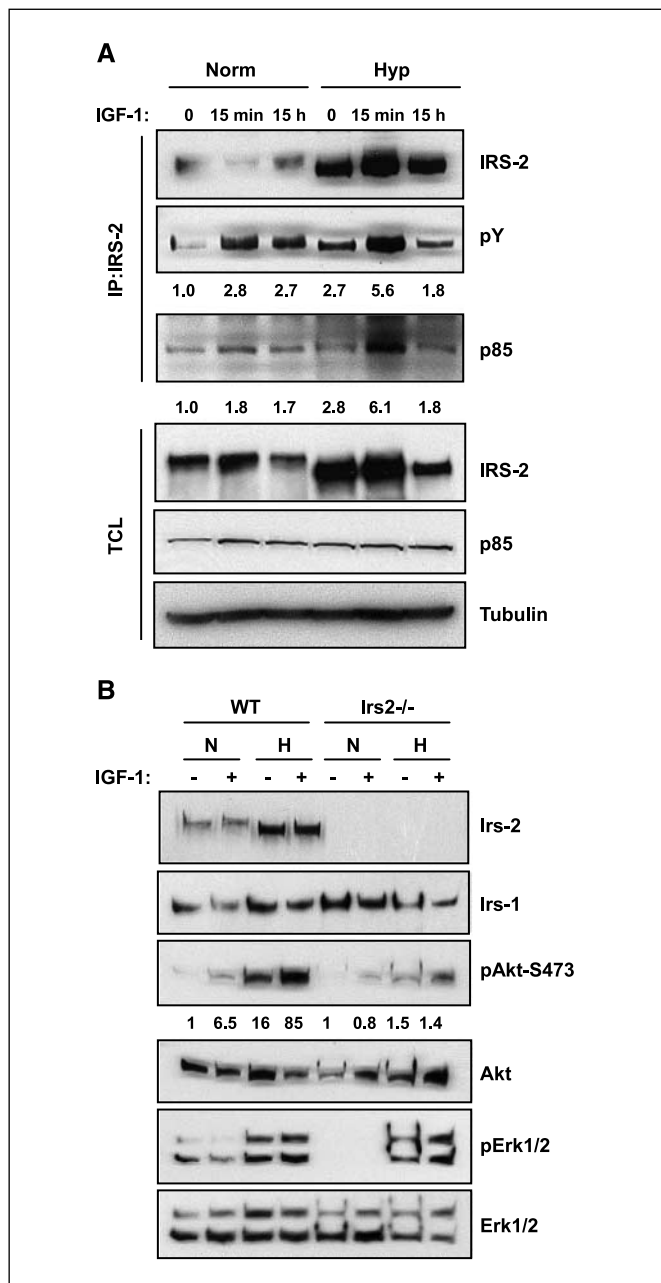
observed upon progressive deletion of the IRS-2 promoter, with a total loss of activity between -732 and -428 of the promoter. An insulin response element that is important for the regulation of basal IRS-2 expression has been identified previously in this region (19). However, the IRS-2 promoters that retain luciferase activity in normoxia (1 and 0.6 Kb) also exhibited a 2.5-fold upregulation in activity in response to hypoxia (Fig. 3B). Taken together, our data establish that transcription is required for hypoxic regulation of IRS-2 expression and identify a hypoxia-responsive region between -732 and -428 of the IRS-2 promoter.

**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 (25–27). 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-1 $\alpha$  or HIF-2 $\alpha$  expression (Fig. 3C). Knockdown of HIF-1 $\alpha$  or HIF-2 $\alpha$  alone did not significantly inhibit IRS-2 protein or mRNA expression in hypoxia.

However, simultaneous knockdown of both HIF-1 $\alpha$  and HIF-2 $\alpha$  prevented IRS-2 protein and mRNA upregulation in response to hypoxia (Fig. 3D).

**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 (2). We observed that IRS-2 mobility increases after exposure to hypoxia for >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 phosphoinositide 3-kinase (PI3K; p85) were compared in SUM159 cells that were incubated for 15 hours in normoxia or hypoxia in the presence or absence of IGF-I (50 ng/mL), or stimulated with IGF-I 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-I stimulation, and

PI3K recruitment increased in parallel (Fig. 4A). In hypoxia, IRS-2 phosphorylation levels and association with PI3K were elevated 2.7-fold in the absence of exogenous IGF-I stimulation when compared with cells maintained in normoxia, and both tyrosine phos-



**Figure 4.** IRS-2 signaling in hypoxia. *A*, SUM159 cells were incubated in normoxia or hypoxia for 15 h in the presence or absence of IGF-I, or stimulated with IGF-I for the final 15 min of the incubation. Cell extracts were immunoprecipitated with IRS-2-specific antibodies and immunoblotted with antibodies that recognize phosphotyrosine (pY) or the p85 regulatory subunit of PI3K. The phosphotyrosine 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 h) 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 h in the presence or absence of IGF-I. 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.

phorylation and PI3K interactions were increased further in response to acute IGF-I stimulation (15 minutes; Fig. 4A). Long-term IGF-I stimulation under hypoxic conditions resulted in decreased IRS-2 expression, tyrosine-phosphorylation, and association with p85 (Fig. 4A), most likely due to the activation of a negative feedback mechanism in response to prolonged activation of this pathway (23). Therefore, IRS-2 is functionally active and capable of signaling in hypoxic cells.

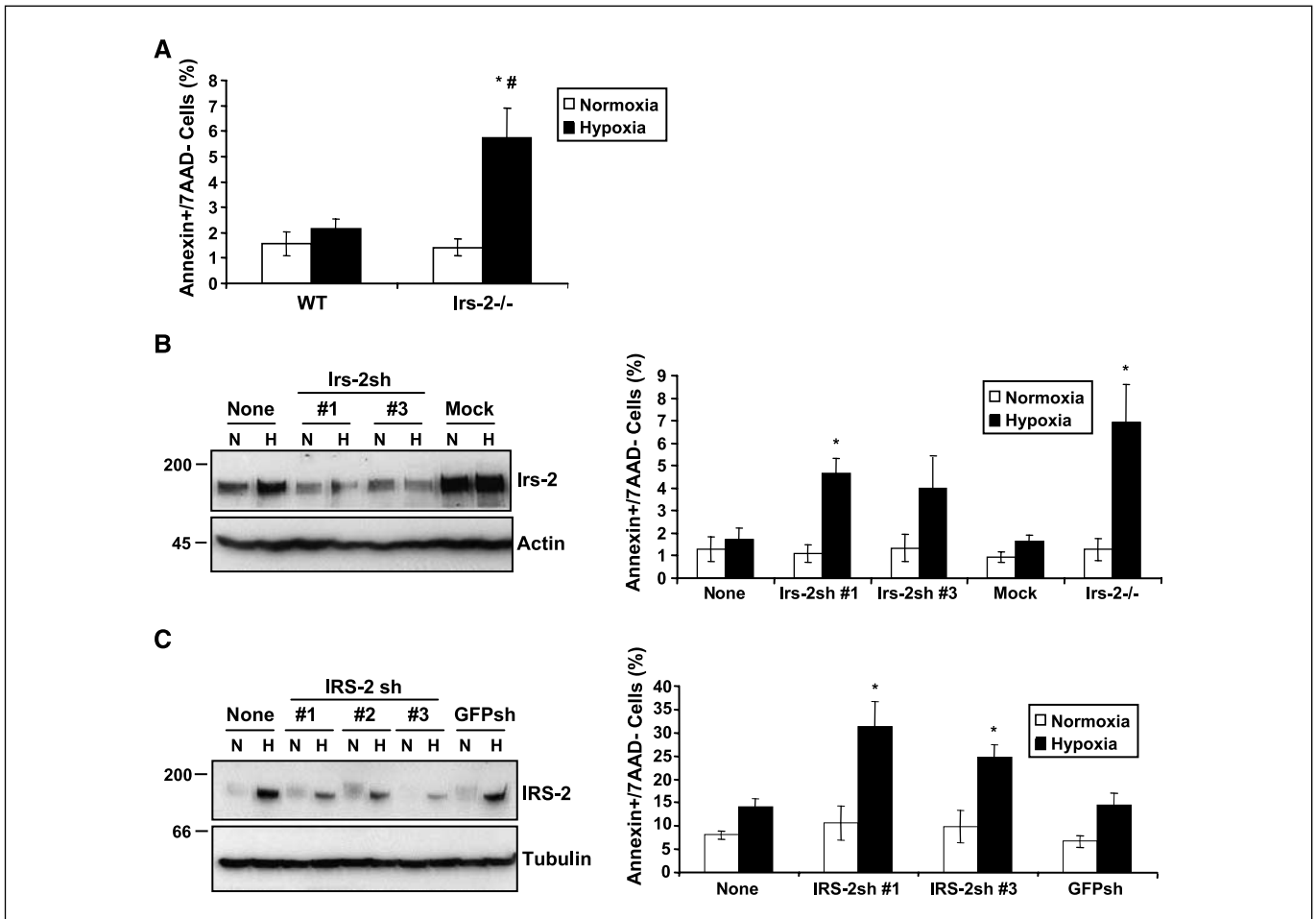
To investigate further how IRS-2 contributes to the hypoxic tumor response, activation of intracellular signaling pathways in PyV-MT:WT and PyV-MT:*Irs-2*<sup>-/-</sup> mammary tumor cell lines were examined in either normoxia or hypoxia. Mitogen-activated protein kinase activation was induced by hypoxia in both cell lines and there was a modest increase in activation in the presence of IGF-I (Fig. 4B). Activation of Akt was increased 16-fold in WT cells in response to hypoxia, and activation was enhanced further in response to IGF-I stimulation (Fig. 4B). However, Akt activation did not increase in hypoxia in the absence of IRS-2 (Fig. 4B).

**IRS-2 promotes tumor cell viability in hypoxia.** Genes that allow cells to adapt to low oxygen conditions are upregulated in hypoxia (15, 18). To determine if enhanced IRS-2 expression contributes to cell survival in hypoxia, PyV-MT:WT and PyV-MT:*Irs-2*<sup>-/-</sup> mammary tumor cells were incubated in hypoxia or normoxia for 24 hours in complete culture medium. PyV-MT:*Irs-2*<sup>-/-</sup> cells were significantly more sensitive to hypoxia-induced apoptosis than PyV-MT:WT cells (Fig. 5A). 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. 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. 5B). 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 exhibited a significant increase in hypoxia-induced apoptosis when compared with parental or GFP shRNA-expressing cells (Fig. 5C).

**IRS-2 promotes tumor cell invasion in hypoxia.** Stable shRNA-mediated knockdown of IRS-2 in MDA-MB-231 cells did not sensitize these cells to hypoxia-induced apoptosis (data not shown). 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 (28). 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. 6). In contrast, suppression of IRS-2 did not inhibit the invasion of PyV-MT:WT and SUM159 cells in hypoxia (data not shown). 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.

## 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. Either HIF-1 or HIF-2 is required for this



**Figure 5.** IRS-2 promotes cell survival in hypoxia. *A*, PyV-MT:WT and PyV-MT:Irs-2<sup>-/-</sup> cells were incubated in normoxia or hypoxia for 24 h 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<sup>-/-</sup> apoptosis in normoxia. *B*, 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 h. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or Actin (*left*). Apoptosis was determined by Annexin V-PE and 7-AAD staining (*right*). \*,  $P < 0.04$  relative to PyV-MT:WT/mock apoptosis in hypoxia. *C*, SUM159 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 h in serum-free RPMI supplemented with 1 g/L glucose. Cell extracts were immunoblotted with antibodies that recognize IRS-2 or tubulin (*left*). Apoptosis was determined by Annexin V-PE and 7-AAD staining (*right*). \*,  $P < 0.05$  relative to SUM159/GFPshRNA apoptosis in hypoxia.

hypoxia-dependent increase in IRS-2 expression. IRS-2 is phosphorylated on tyrosine residues and recruits PI3K in response to IGF-I 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 on 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-I –dependent signaling through IRS-1 or IRS-2 in human breast carcinoma cells stimulates proliferation or migration/invasion, respectively (8). IRS-2, but not IRS-1, has been implicated in metabolic regulation in tumor cells, through the regulation of glycolysis (9). *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 (5). In fact, in the absence of Irs-1 expression, Irs-2 expression and signaling in-

crease in cell lines in culture and in tumors, and metastasis is enhanced (4). 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 (16). Overall, gene expression is suppressed in hypoxia as a mechanism to conserve energy in this stressful microenvironment, which is often lacking in nutrient availability as well (15). 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 (15, 25). Genes such as VEGF are also upregulated to increase angiogenesis and restore normoxic conditions (29). Chronic exposure to hypoxia creates a selection for cells with a tolerance for hypoxia, and these cells become more invasive and metastatic (16, 17). The impact of this selective pressure is highlighted by recent studies revealing that antiangiogenic 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 (30, 31). 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*<sup>-/-</sup> 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 (4). 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 (32). Akt negatively regulates the function of proapoptotic down-

stream effectors including the FOXO transcription factors and the proapoptotic protein Bad (33). IRS-2 regulates FOXO function through Akt in mouse embryo fibroblasts, and this regulatory pathway has been proposed to control nutrient homeostasis (34). 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 (35). Akt signaling can also enhance the expression of HIF-1 $\alpha$  to amplify the expression of HIF target genes (36, 37). In this regard, SUM149 cells, which lack PTEN and have elevated Akt activity, exhibited the greatest induction of IRS-2 expression in hypoxia (38). As mentioned previously, tumors that arise in PTEN<sup>+/-</sup> mice also have elevated IRS-2 expression (6). 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- $\alpha$  and HIF- $\beta$ /ARNT (39). HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits are highly homologous, and both contain basic helix-loop-helix, Per/ARNT/Sim, and oxygen-dependent degradation domains (39). In low oxygen conditions, the HIF- $\alpha$  subunit is stabilized and it interacts with HIF- $\beta$  to form the active HIF transcription factor, which binds to HRE sequences in target genes (40). HIF-1 and HIF-2 can regulate both unique and common target genes (41, 42). Suppression of both HIF-1 $\alpha$  and HIF-2 $\alpha$  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 that have been identified 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 (43). Importantly, some of these transcription factors have been previously implicated in the regulation of IRS-2 gene expression including activator protein-1 (AP-1), the forkhead transcription factors FOXO1 and FOXO3a, and cAMP-responsive element binding protein (21, 44, 45).

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.

## Disclosure of Potential Conflicts of Interest

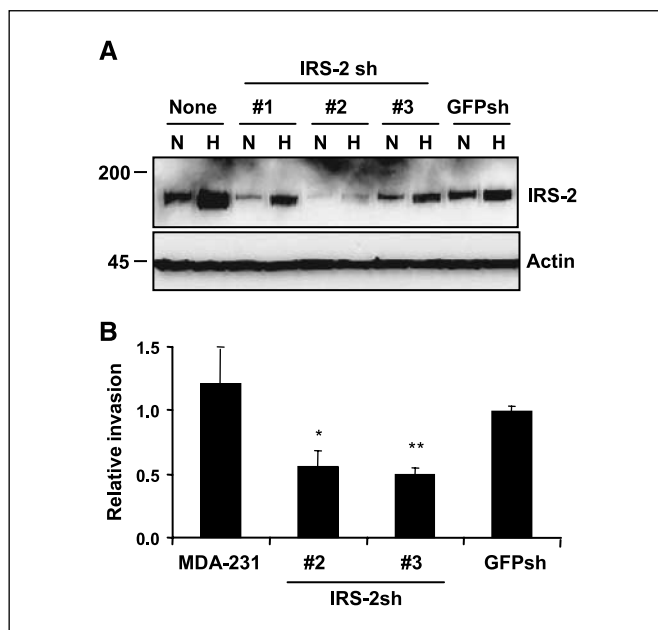
No potential conflicts of interest were disclosed.

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**Figure 6.** IRS-2 promotes invasion in hypoxia. MDA-MB-231 cells stably expressing IRS-2-targeted shRNAs or a GFP-targeted shRNA were incubated in either normoxia or hypoxia for 24 h. *A*, cell extracts were immunoblotted with antibodies that recognize IRS-2 or Actin. *B*, 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. *Columns*, mean of five independent experiments performed in duplicate; *bars*, SEM. \*,  $P < 0.01$  and \*\*,  $P = 0.0002$  relative to MDA-MB-231/GFPshRNA invasion.

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