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Abstract

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Comments

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Hypoxia-inducible Factor Regulates αvβ3 Integrin Cell Surface Expression

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Hypoxia-inducible factor (HIF)-deficient placentas exhibit a number of defects, including changes in cell fate adoption, lack of fetal angiogenesis, hypocellularity, and poor invasion into maternal tissue. HIF is a heterodimeric transcription factor consisting of α and β aryl hydrocarbon receptor nuclear translocator or ARNT) subunits. We used undifferentiated trophoblast stem (TS) cells to characterize HIF-dependent adhesion, migration, and invasion. *Arnt*$^{-/-}$ and *Hif*α$^{-/-}$ TS cells exhibit reduced adhesion and migration toward vitronectin compared with wild-type cells. Furthermore, this defect is associated with decreased cell surface expression of integrin αvβ3 and significantly decreased expression of this integrin in focal adhesions. Because of the importance of adhesion and migration in tumor progression (in addition to placental development), we examined the affect of culturing B16F0 melanoma cells in 1.5% oxygen (O_2). Culturing B16F0 melanoma cells at 1.5% O_2 resulted in increased αvβ3 integrin surface expression and increased adhesion to and migration toward vitronectin. Together, these data suggest that HIF and O_2 tension influence placental invasion and tumor migration by increasing cell surface expression of αvβ3 integrin.

INTRODUCTION

Oxygen (O_2) deprivation or "hypoxia" acting through hypoxia-inducible factors (HIFs) influences cell adhesion and migration in a number of biological contexts. For example, HIF regulates progenitor cell adhesion and migration in ischemic tissue by inducing expression of SDF-1 and promotes tumor invasion through the tyrosine kinase receptor MET (Pennacchietti *et al*., 2003; Ceradini *et al*., 2004). *Hif1*α$^{-/-}$ macrophages exhibit poor migration and Matrigel invasion (Cramer *et al*., 2003), and directed migration of renal cell carcinoma cells is regulated by HIF activation of the chemokine receptor CXCR4 (Staller *et al*., 2003). HIF activity also is clearly important during placentation (Kozak *et al*., 1997; Adelman *et al*., 2000): placentas from embryos lacking HIF exhibit a variety of defects, including poor invasion of the maternal decidua (Adelman *et al*., 2000; Cowden Dahl, Mack, Compernolle, Adelman, Carmeliet, and Simon, unpublished data). Furthermore, tumor hypoxia is often clinically correlated with increased metastatic potential (Vaupel *et al*., 2001). The integrin adhesion receptors promote placental invasion, macrophage adhesion and migration, and metastatic progression of neoplasms (Zhou *et al*., 1997b; Ding *et al*., 1999; Jin and Varner, 2004), and hypoxia has been shown to increase mRNA levels of multiple integrins (β3, β2, and α5) (Walton *et al*., 2000; Iwaki *et al*., 2004; Koike *et al*., 2004; Kong *et al*., 2004). Therefore, HIF regulates cell motility in a variety of developmental and pathological situations.

The transcriptional response to hypoxia is mediated by the dimeric basic-helix-loop-helix-PAS (bHLH-PAS) transcription factor HIF. HIF is composed of an α subunit (HIF1α or HIF2α) and β subunit (aryl hydrocarbon receptor nuclear translocator; ARNT) and activates transcription by binding to hypoxic response elements (HREs) scattered throughout the genome (Gu *et al*., 2000). HIFα is labile under normoxic conditions, but stabilized by hypoxia, enabling dimerization with ARNT to induce genes such as angiogenic factors, glycolytic enzymes, glucose transporters, and erythropoietin (Wang and Semenza, 1993; Firth *et al*., 1994; Liu *et al*., 1995; Forsythe *et al*., 1996; Semenza *et al*., 1996; Ema *et al*., 1997; Okino *et al*., 1998). *Arnt*$^{-/-}$ mice die by embryonic day (E) 10.5 with defects in angiogenesis, cardiogenesis, hematopoiesis, and placentation (Kozak *et al*., 1997; Maltepe *et al*., 1997; Adelman *et al*., 1999, 2000; Abbott and Buckalew, 2000). *Hif1*α$^{-/-}$ embryos also die by E10.5, exhibiting angiogenic and cardiac defects (Iyer *et al*., 1998; Ryan *et al*., 1998; Compernolle *et al*., 2003). Mutations in *Hif2*α also result in embryonic lethality, with *Hif2*α$^{-/-}$ animals exhibiting defects in cardiac function, angiogenesis, and lung maturation (Tian *et al*., 1998; Peng *et al*., 2000; Compernolle *et al*., 2002). Therefore, HIF activity is essential for viability and cardiovascular development during embryogenesis.

Arnt$^{-/-}$ placentas are avascular, hypocellular, and exhibit aberrant cell fate adoption in addition to poor invasion. To study mechanisms underlying HIF regulation of multiple aspects of placentation, we used undifferentiated trophoblast stem (TS) cells from wild-type, *Arnt*$^{-/-}$, *Hif1*α$^{+/-}$*Hif2*α$^{+/-}$, and *Hif1*α$^{-/-}$*Hif2*α$^{-/-}$ embryos. TS cells are nontransformed, immortal precursor stem cells derived from blastocysts capable of differentiating into multiple placental

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lineages (e.g., trophoblast giant cells, spongiotrophoblasts, and syncytiotrophoblasts).

Because HIF-deficient placentas exhibit poor maternal invasion, we determined whether HIF regulates trophoblast invasion through integrin-mediated adhesion to the extracellular matrix (ECM). We show here that undifferentiated *Arnt*$^{-/-}$ and *Hif1α*$^{-/-}$*Hif2α*$^{-/-}$ TS cells exhibit reduced αvβ3 integrin-mediated adhesion and migration due to decreased cell surface expression of this integrin. These studies explain in part the poorly invasive phenotype observed in *Arnt*$^{-/-}$ and *Hif1α*$^{-/-}$*Hif2α*$^{-/-}$ placentas in vivo. Moreover, because HIF regulates αvβ3 integrin-mediated adhesion, we reasoned that hypoxia also may up-regulate αvβ3 integrin expression in tumors, leading to increased metastasis. Melanoma metastases contain hypoxic regions, and metastatic progression is associated with increased αvβ3 integrin expression (Gehlsen *et al*., 1992; Nip *et al*., 1992; Lartigau *et al*., 1997; Felding-Habermann *et al*., 2002). Poorly and highly metastatic melanoma cells (B16F0 and B16F10, respectively) were used to study hypoxic regulation of adhesion pathways that may be relevant to tumor metastasis. We show that hypoxia induces αvβ3 integrin cell surface expression in poorly metastatic B16F0 melanoma cells, resulting in increased adhesion and migration.

MATERIALS AND METHODS

Antibodies

The Novus HIF1α antibody (clone H1α67) was used for HIF1α electrophoretic mobility shift assay (EMSA) "supershift." PE-RMV-7 from BD Biosciences PharMingen (San Diego, CA) was used for fluorescence-activated cell sorting (FACS) of αv integrin, and the Chemicon International (Temecula, CA) polyclonal αv integrin antibody was used for immunoprecipitation and Western blot assays. For β3 integrin, 2C9.G2 subclone from BD Biosciences PharMingen was used for blocking function, FACS, immunoprecipitation, immunoelectron microscopy (immuno-EM), and immunofluorescence. For β1 integrin, MAB1997 was used for immunoprecipitation. Additionally, the Chemicon International β3 integrin and β5 integrin antibodies were used in Western blot assays. For FACS analysis, BD Biosciences PharMingen's Ha2/5 was used for β1 integrin, the Santa Cruz Biotechnology (Santa Cruz, CA) (H-96) for β5 integrin, or BD Biosciences PharMingen's GoH3 for α6 integrin. For immunofluorescence, 58K (Abcam, Cambridge, MA), anti-calreticulin (StressGen Biotechnologies, San Diego, CA), phospho-Y397FAK (BD Transduction Laboratories, Lexington, KY), and anti-mouse-Alexa-fluor 488, anti-rabbit-Alexa-fluor 488, and streptavidin Alexa-fluor 568 from Molecular Probes (Eugene, OR) were used.

EMSAs

EMSAs were performed in binding buffer consisting of 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 1 mM $MgCl_2$, 5 mM dithiothreitol, 1 mM EDTA, and 5% glycerol to which 0.1 mg/ml bovine serum albumin (BSA), 5 μg of nuclear extract, and 10^5 cpm of probe were added per 20 μl of reaction. The binding site sequence of the probe (E24F) is 5′-GCCCTACGTGCTGCCTCGCATGGC-3′. Cold cAMP response element-binding protein (CREB) competitor oligo (100× excess) containing the CREB binding site from the somatostatin promoter was added: 5′-GATCGCCTCCTTGGCTGACGTCAGAGAGCTAG-3′.

Northern Blot Analysis

Undifferentiated TS and embryonic stem (ES) cells were cultured for 16 h at 20% or 1.5% O_2 and then washed with Dulbecco's phosphate-buffered saline (DPBS). RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Fifteen micrograms of total RNA was electrophoresed in 1% agarose containing formaldehyde. The RNAs were transferred to Hybond N^+ membranes (Amersham Biosciences, Piscataway, NJ). Full-length HIF1α and β-tubulin cDNAs were labeled as probes.

Cell Culture

Arnt$^{+/+}$ and *Arnt*$^{-/-}$ TS were described previously (Adelman *et al*., 2000) and derived by standard protocols (Tanaka *et al*., 1998). *Hif1α*$^{+/-}$*Hif2α*$^{+/-}$, and *Hif1α*$^{-/-}$*Hif2α*$^{-/-}$ TS cells are described by Cowden Dahl, Mack, Compernolle, Adelman, Carmeliet, and Simon (unpublished data). Briefly, TS cells were maintained in an undifferentiated state on mouse embryo fibroblasts (MEFs) in the presence of FGF4 and heparin. For all experiments, TS cells were cultured in the absence of MEFs but supplemented with MEF-conditioned media containing FGF4 and heparin. TS cells were plated on glass coverslips for immunofluorescence assays. B16F0 and B16F10 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in DMEM supplemented with 10% serum, HEPES, penicillin, streptomycin, and L-glutamine. B16 melanoma cells were cultured either under standard culture conditions (20% O_2) or at 1.5% O_2 (in an IG750 3 gas incubator; Jouan, Winchester, VA) for 24 h. CO_2 is maintained at 5% for all experiments.

Immunoprecipitation of Biotin-labeled Cell Surface Proteins

TS or melanoma cells were cultured to 75% confluence, placed on ice, and washed with ice cold DPBS with calcium and magnesium. Cells were then incubated in 1 mg/ml NHS-LC-Biotin (Pierce Chemical, Rockford, IL) for 90 min on ice and washed three times with 50 mM glycine in DPBS. Cell lysates were prepared in NP-40 buffer, pH 7.5 (0.1 M Tris-Base, 1% NP-40, 10 μg/ml Pefabloc, 1 mM E-64, 5 μg/ml leupeptin, 1 μg/ml pepstatin, 0.02 mg/ml aproptinin, and 0.5M EDTA). Debris was pelleted, and supernates were transferred to new tubes. Lysates were precleared overnight with protein A/G beads (Pierce Chemical). Bicinchoninic acid assays were performed on precleared lysates to determine protein concentration. Immunoprecipitations were performed on 250 μg of protein by using protein A/G beads and anti-αv integrin (Chemicon International), anti-β3 integrin (BD Biosciences PharMingen) or anti-β1 integrin (Chemicon International). After immunoprecipitation, beads were washed thrice with MDB buffer (50 mM Tris, pH 7.5, 0.1% SDS, 0.05% NP-40, and 0.3 M NaCl) and thrice with Tris-wash buffer (10 mM Tris, pH 7.5). Lysates were then resuspended in nonreducing sample buffer and run on an 8% SDS-polyacrylamide gel.

FACS Analysis

Cell surface expression of integrins was determined by FACS analysis. Cells were trypsinized and washed in DPBS and blocked with anti-CD16/32 for 15 min at room temperature. After washing in 1% BSA in DPBS, cells were incubated with primary antibody for 30 min on ice, washed with 1% BSA/DPBS, pelleted, and resuspended in isotonic buffer. Propidium iodide was used to exclude dead cells. FACS data were analyzed using CellQuest software, and error bars represent ±SE. Student's *t* tests were performed to determine statistical significance.

Adhesion, Migration, and Invasion Assays

Adhesion assays were performed on CytoMatrix cell adhesion strips containing fibronectin (FN), vitronectin (VN), collagen IV, and laminin (LN) according to manufacturer's instructions (Chemicon International). The concentration of ECM proteins and BSA for the adhesion and migration assays is not provided by Chemicon International. Briefly, strips were rehydrated with DPBS. Cells were prepared in a single cell suspension in serum-free media, and 1×10^5 cells were added to each well of the adhesion strips. Strips were incubated for 1 h at 37°C and washed twice with DPBS, stained with 0.2% crystal violet in 10% ethanol (EtOH), washed again, and solubilized in 50% 0.1 M NaH_2PO_4 in 50% EtOH. Absorbance was read at 560 nm on a microplate reader. Quantitative cell migration assays (QCM-FN and QCM-VN; Chemicon International) were performed on feeder-free TS cells according to manufacturer's instructions. Briefly, 2.5×10^5cells were placed in each well of FN-, VN-, or BSA-coated Boyden chambers. Cells were allowed to migrate for 16 h, and chambers were stained with crystal violet. Dye was then solubilized and read on a microplate reader. Absorbance was normalized to migration toward BSA. Invasion was measured on BD BioCoat Matrigel (BD Biosciences, San Jose, CA) invasion chambers according to manufacturer's instructions. Briefly, inserts were rehydrated with RPMI 1640 medium. Cells (4×10^5) were added to chambers and incubated for 22 h. Chambers were then cleaned and stained with crystal violet. Solubilized dye was measured on a microplate reader.

Western Blots

Cells were washed in DPBS and lysed in NP-40 buffer (described above). Lysates were cleared by centrifugation, and equal amounts of protein were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose, and the membrane was blocked with 3% BSA in Tris-buffered saline/Tween 20 and incubated with antibodies before development with ECL.

Immunoelectron Microscopy

Briefly, samples were rinsed in buffer and fixed in 2% paraformaldehyde + 0.2% glutaraldehyde in DPBS for 30 min and permeablized with 0.1% saponin. Fixative was removed, cells washed, and residual aldehyde was inactivated by treatment with 0.1% NaBH for 10 min. Cells were incubated with 1% ovalbumin + 5% normal horse or donkey serum and 0.2% cold water fish skin gelatin for 60 min at room temperature to prevent nonspecific binding. Primary antibody incubation was performed overnight at a dilution of 1:100

cells washed and treated with ultrasmall gold secondary overnight. After washing with DPBS cells were fixed in 1% glutaraldehyde in DPBS for 15 min. Cells were washed with DPBS and then with deionized water before enhancing with silver stain. After silver staining cells were osmicated with 1% osmium and dehydrated in graded alcohol and infiltration aided by propylene oxide and embedded in Epon. Ultrathin sections were mounted on Formvar-coated slotted copper grids and imaged in JEOL JEM 1010 aided by AMT HR-12 software and Hamamatsu Charge-coupled device camera.

Fluorescence Confocal Microscopy

TS cells were plated on uncoated coverslips and then fixed for 10 min at room temperature in –20°C acetone, washed in DPBS, permeabilized with DPBS/0.2%Triton X-100, and blocked in 0.2% BSA in DPBS-Tween 20. Cells were then incubated with primary and conjugated-secondary antibodies at room temperature for 1 h each. Coverslips were mounted on slides. Samples were viewed by confocal microscopy. Images were acquired for both red and green lasers and merged in Photoshop.

Statistical Analysis

Each experiment was performed at least three times. Error bars represent ± SE. Student's *t* tests were performed to determine statistical significance for the following assays: adhesion, migration, invasion, FACS, and immuno-EM quantitation.

RESULTS

TS Cells Exhibit High Levels of HIF Activity When Cultured in 20% O_2

$Arnt^{-/-}$ TS cells exhibit a number of phenotypes reminiscent of defects observed in $Arnt^{-/-}$ placentas, including aberrant cell fate adoption upon differentiation and poor adhesion to ECM (see below). Because differentiated $Arnt^{-/-}$ TS cells exhibit defects in cell fate adoption, we chose to confine the experiments exclusively to *undifferentiated* TS cells. We determined that the degree of differentiation in both wild-type and $Arnt^{-/-}$ TS cells was very low based on trophoblast lineage-specific gene expression, morphology, and DNA content (our unpublished data). Interestingly, defects in $Arnt^{-/-}$ TS cells are detected under both normoxic and hypoxic conditions. To demonstrate that the $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cell phenotypes observed under normoxic conditions are mediated by the HIF pathway, we assessed the degree of HIF activity in normoxic and hypoxic TS cells. First, we examined mRNA expression of HIF1α and HIF2α in TS cells. For most experiments, we used two independent $Arnt^{+/+}$ (0 and 2) and $Arnt^{-/-}$ (4 and 10) TS cell lines. In Figure 1A, both $Arnt^{+/+}$ (0 and 2) and $Arnt^{-/-}$ (4 and 10) TS cell lines *were* examined for gene expression at 20% and 1.5% O_2. Because tubulin expression is regulated by O_2 tension, the 28S RNA band serves as an important second loading control. Even though the abundance of HIF1α mRNA somewhat varied between TS cell lines, HIF1α mRNA was present at two- to threefold higher levels under both normoxia (20% O_2) and hypoxia (1.5% O_2) in all TS cell lines compared with wild-type ES cells (Figure 1A). Although HIF2α is present in ES cells (our unpublished data), HIF2α RNA was not detected in undifferentiated TS cells, indicating that all TS HIF activity is therefore due to HIF1α/ARNT dimers. We then performed an EMSA to detect HIF DNA binding to an HRE residing in the erythropoietin (EPO) 3′ enhancer. Low levels of HIF binding activity were observed in normoxic ES cells, which increased upon exposure to 1.5% O_2 (Figure 1B, lanes 1 and 2). Importantly, antibody against HIF1α completely shifted the hypoxic DNA binding complex, indicating that the inducible complex contains HIF (Figure 1B, lane 3). In comparison with ES cells, HIF DNA binding activity was detected in TS cells at relatively high levels at 20% O_2; DNA binding activity was further enhanced by hypoxia and supershifted with α-HIF1α antibody (Figure 1B, lanes 4–6). These data suggested that HIF target genes might be regulated by HIF in TS cells even under normoxic conditions. As expected, no HIF DNA binding activity was present in $Arnt^{-/-}$ TS cells (Figure 1B, lanes 7–9). Furthermore, we observed decreased expression of the HIF target genes vascular endothelial growth factor (VEGF), ERO1-Lα, phosphofructose kinase (PFK), and Glut-1 (our unpublished data) in normoxic TS cells. Therefore, we concluded that HIF is partially active under normoxic conditions in $Arnt^{+/+}$ TS cells and conducted all subsequent experiments at 20% O_2 to study a homogenous stem cell population.

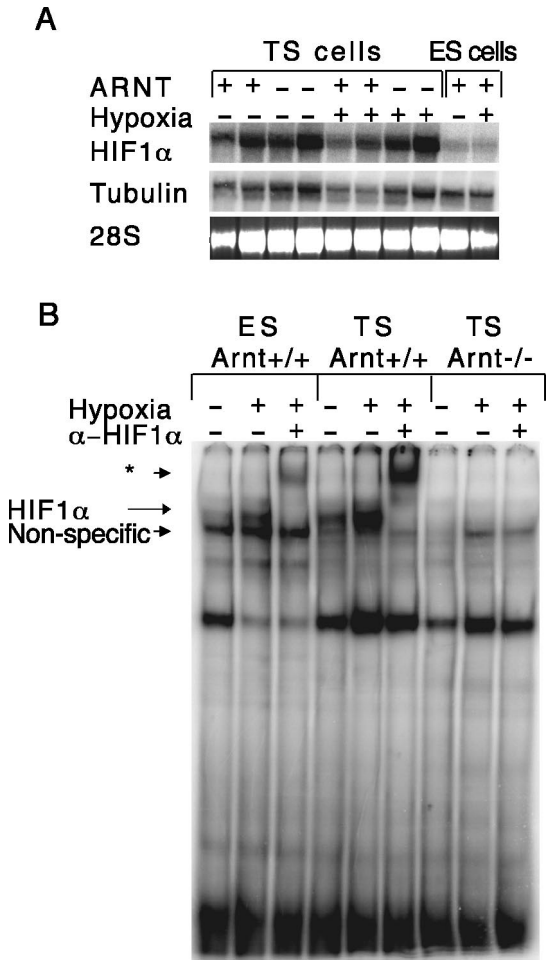

Figure 1. TS cells exhibit high levels of HIF activity when cultured in 20% O_2. (A) Northern blot analysis for HIF1α mRNA levels in two independent $Arnt^{+/+}$ TS cell lines, two independent $Arnt^{-/-}$ TS cell lines, and wild-type ES cells. Tubulin and 28S RNA serve as loading controls. As expected, HIF1α mRNA levels are not increased by culture at 1.5% O_2 in either TS or ES cells. Each of these experiments was performed three times. (B) EMSA analysis showing HIF DNA complex formation. $Arnt^{+/+}$ ES cells demonstrate low levels of the HIF complex under normoxic conditions, but complex formation is enhanced under hypoxia. In addition to the hypoxia-inducible DNA complex, ES cells harbor a constitutive nonspecific complex as indicated. $Arnt^{+/+}$ TS cells exhibit HIF complex formation under normoxia and hypoxia, but no complex forms in the absence of ARNT. HIF complexes are supershifted with an anti-HIF1α antibody, as indicated by the asterisk.

$Arnt^{-/-}$ TS Cells Exhibit Poor Migration and Adhesion

Because $Arnt^{-/-}$ trophoblasts exhibit poor invasion into maternal tissue, we investigated whether HIF activity plays a

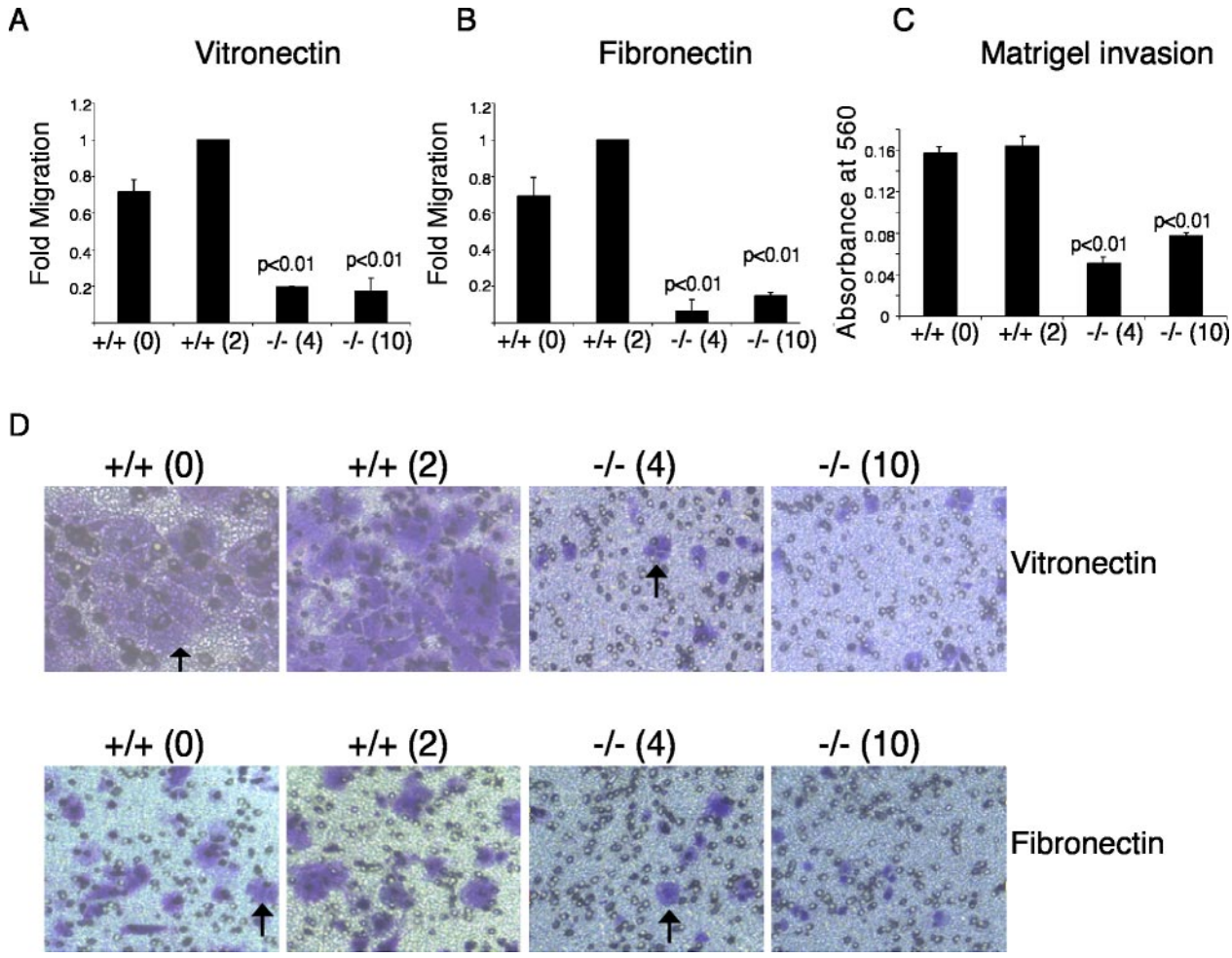

Figure 2. $Arnt^{-/-}$ TS cells exhibit impaired migration toward vitronectin and fibronectin and reduced invasion into Matrigel. (A) Boyden chamber assays for migration toward vitronectin. (B) Boyden chamber assays for migration toward fibronectin. (C) Invasion of Matrigel. (D) Photographs of migration toward vitronectin (top) and migration toward fibronectin (bottom). Original magnifications, 100×. Arrows indicate stained cells that have migrated through Boyden chamber pores. Each experiment was performed three times in duplicate. Error bars represent ± SEM. Student's *t* tests were used for statistical significance.

role in undifferentiated TS cell migration. Migration toward the ECM proteins vitronectin and fibronectin was analyzed using a Boyden chamber assay, and migration was normalized to the wild-type control +/+(2) TS cells for each experiment. Migration toward vitronectin was decreased by 5- and 5.9-fold, respectively, in two independent $Arnt^{-/-}$ TS cell lines compared with wild-type cells ($p < 0.01$ and $p < 0.01$) (Figure 2A). Additionally, migration toward fibronectin was decreased by 16.7- and 6.8-fold in $Arnt^{-/-}$ TS cell lines (Figure 2B). Photographs of the Boyden chambers showing decreased migration toward vitronectin (top) and fibronectin (bottom) are presented in Figure 2D. We also measured the ability of TS cells to invade basement membrane (Matrigel), which contains collagens, laminin, proteoglycans, matrix degrading enzymes and their inhibitors, and growth factors. Both $Arnt^{+/+}$ cell lines exhibited roughly equivalent invasion. In contrast, $Arnt^{-/-}$ TS cell lines displayed decreased invasion (3.2- and 2.1-fold, respectively) into Matrigel compared with the control +/+(2) cells ($p < 0.01$) (Figure 2C). We concluded that trophoblasts lacking HIF activity exhibit poor migration and invasion.

ECM is composed of a number of proteins that serve as ligands for integrins, including fibrous proteins (e.g., collagen), linker proteins (e.g., fibronectin, laminin, and vitronectin), and glycosaminoglycans. We reasoned that the decreased migration exhibited by $Arnt^{-/-}$ TS cells might be due to a defect in ECM adhesion. TS cells were plated on vitronectin-, fibronectin-, collagen IV-, or laminin-coated plates for 1 h to measure binding to different substrates. Binding to vitronectin was decreased by approximately fivefold in both $Arnt^{-/-}$ TS cell lines ($p < 0.01$) (Figure 3A), whereas adhesion to fibronectin was decreased by ~1.8-fold ($p < 0.001$) (Figure 3B). Because integrin $\alpha v\beta 3$ is the prototypic receptor for vitronectin and has affinity for fibronectin, we determined whether the ECM adhesion defects were due to a decrease in $\alpha v\beta 3$ activity. We treated $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells with a $\beta 3$ integrin-blocking antibody and then performed adhesion assays. Adhesion to vitronectin was greatly decreased in $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells by blocking $\beta 3$ integrin activity ($p < 0.01$); however, adhesion to fibronectin was not (Figure 3, C and D). This suggested that inhibition of $\beta 3$ activity is sufficient to decrease adhe-

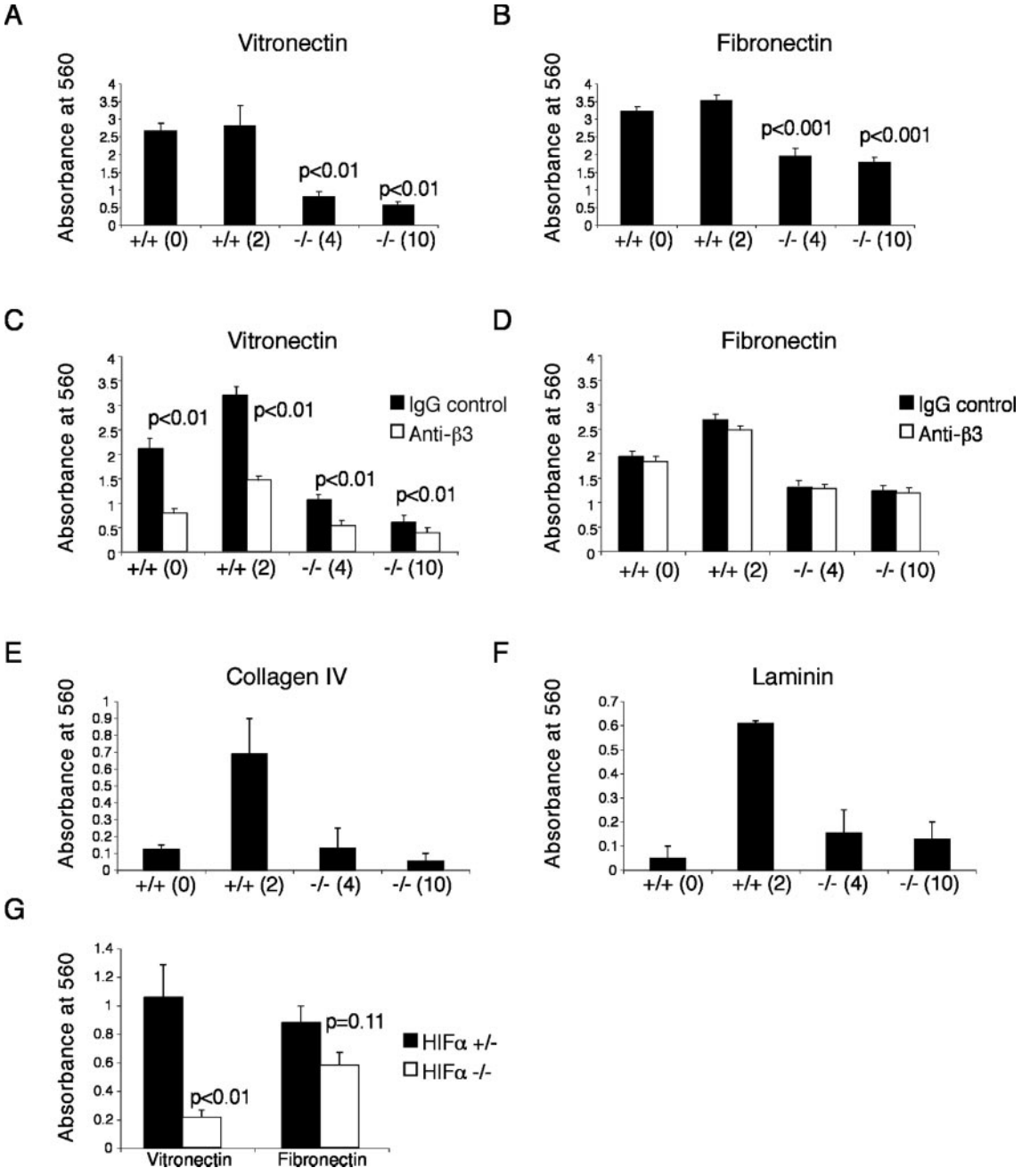

Figure 3. $Arnt^{-/-}$ TS cells adhere poorly to both vitronectin and fibronectin. $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells were plated on strips coated with vitronectin (A) or fibronectin (B) and stained with crystal violet. Solubilized crystal violet incorporated by adherent cells was measured on a plate reader at 560 nm. Two independent wild-type and mutant lines were used. Treatment of TS cells with a blocking antibody for β3 integrin reduces vitronectin (C) but not fibronectin (D). $Arnt^{-/-}$ TS cells adhere to collagen IV (E) and laminin (F) as well as $Arnt^{+/+}$ TS line 0. $Hif\alpha^{-/-}$ TS cells demonstrate a substantial reduction in adhesion to vitronectin ($p < 0.01$) and are somewhat reduced in adhesion to fibronectin coated-strips compared with $Hif\alpha^{+/-}$ TS cells (G). Each experiment was performed three times in triplicate. Error bars represent ± SEM. Student's *t* tests were used for statistical significance.

sion to vitronectin, but blocking β3 activity alone does not inhibit adhesion to fibronectin. Adhesion to collagen IV and laminin was equivalent between $Arnt^{-/-}$ cell lines and one of the wild-type cell lines +/+(0) (Figure 3, E and F). The second wild-type cell line [+/+(2)] exhibited more adhesion to laminin and collagen IV than the other lines, indicating that this effect was not genotype specific. To demonstrate that the decrease in adhesion to vitronectin and fibronectin was due to inactivation of the HIF pathway, ECM adhesion for TS cells lacking both *Hif1α* and *Hif2α* (designated $Hif\alpha^{-/-}$) was examined. $Hif\alpha^{-/-}$ TS cells exhibited a fivefold decrease in adhesion to vitronectin compared with $Hif1a^{+/-}$ $Hif2a^{+/-}$ ($Hif\alpha^{+/-}$) TS cells ($p < 0.01$) (Figure 3G). Adhesion to fibronectin was also slightly decreased in $Hif\alpha^{-/-}$ TS cells (1.5-fold), but this change was not significant. Importantly, adhesion of $Hif\alpha^{+/-}$ TS cells to laminin was equivalent to

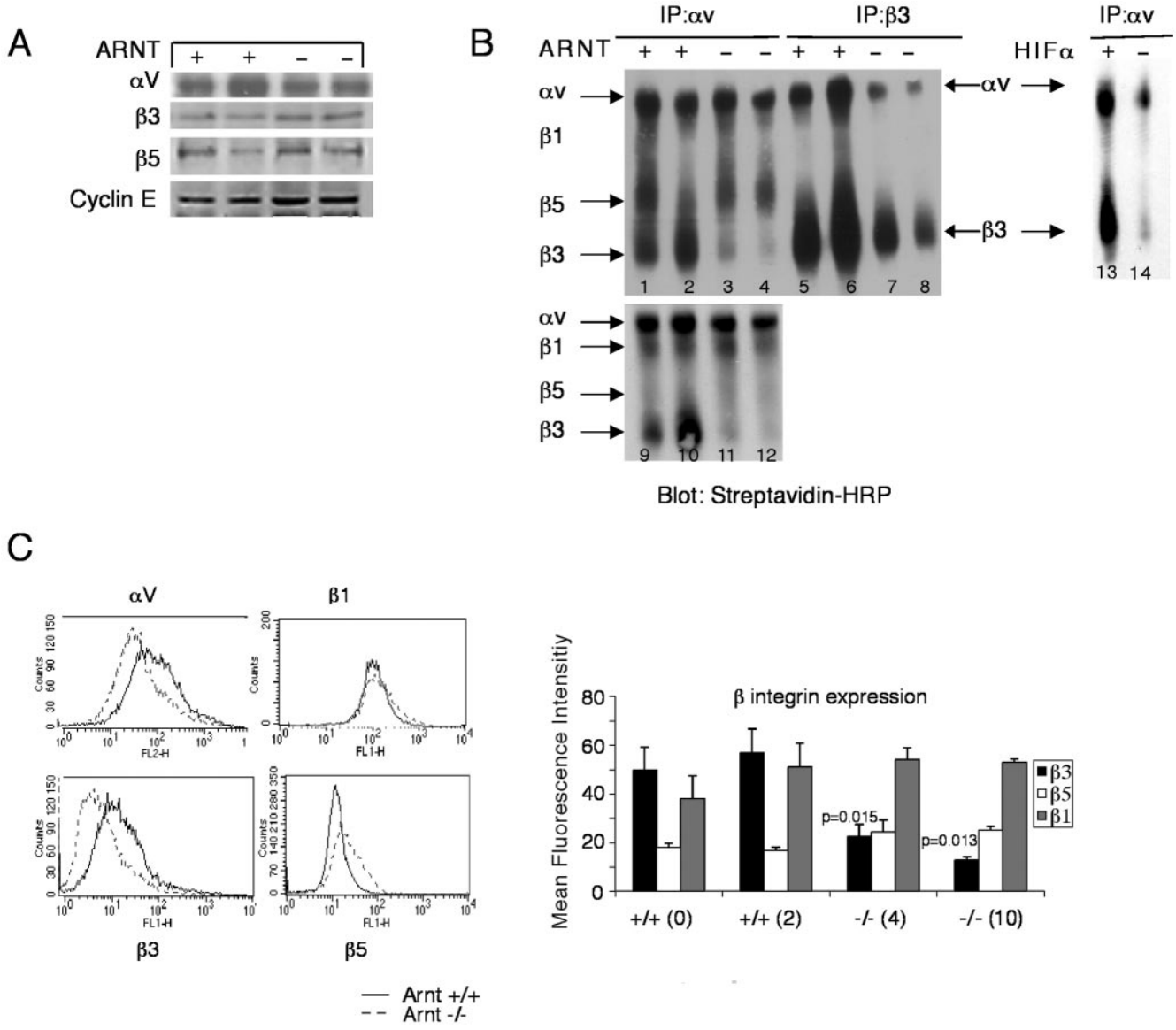

Figure 4. TS cells lacking *Arnt* exhibit reduced cell surface αvβ3 integrin. (A) Immunoblots showed that integrins αv, β3, and β5 are expressed equivalently in wild-type and mutant TS whole cell lysates. Cyclin E is used as a loading control. (B) TS cells were surface labeled with biotin, and lysates were immunoprecipitated with antibodies for αv integrin or β3 integrin and separated by nonreducing SDS-PAGE, which was subsequently blotted with streptavidin-HRP. Positions of the individual integrins are indicated. Lanes 1, 2, 5, 6, 9, and 10 are $Arnt^{+/+}$ cell lysates; lanes 3, 4, 7, 8, 11, and 12 are $Arnt^{-/-}$ cell lysates; lane 13 is $Hif\alpha^{+/-}$ cell lysate, whereas lane 14 is $Hif\alpha^{-/-}$ cell lysate. (C) $Arnt^{+/+}$ and $Arnt^{-/-}$ cells were analyzed by FACS for surface expression of integrins αv, β1, β3, and β5. Representative histograms are shown as well as bar graphs for the mean fluorescence intensity of at least three independent experiments for each integrin. Error bars represent ± SEM. Student's *t* tests were performed to determine statistical significance. Of note, only one $Arnt^{-/-}$ TS line exhibited reduced surface integrin αv expression.

$Hif\alpha^{-/-}$ TS cells (our unpublished data). We concluded that $Arnt^{-/-}$ and $Hif\alpha^{-/-}$ TS cells exhibit striking defects in adhesion to vitronectin and fibronectin.

In the Absence of ARNT, αvβ3 Integrin Does Not Localize to the Cell Surface

Because the adhesion and migration defects were specific for the ECM proteins vitronectin and fibronectin, we evaluated a role for HIF in the regulation of adhesion receptors. As stated above, integrin αvβ3 is a receptor for both vitronectin and fibronectin. Furthermore, αvβ3 integrin activity has been shown to be important in cytotrophoblast invasion in the human placenta (Zhou *et al*., 1997a). First, we determined whether HIF regulates expression of αvβ3 integrin. We performed Western blots on TS cell extracts to examine protein levels for αv, β3, and β5 integrins. We found comparable levels of each of these integrins in whole cell lysates prepared from $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells (Figure 4A). Cyclin E was used as a loading control. These data suggest that the αv and β3 integrins are not regulated at the level of transcription or translation and therefore are not likely to be direct HIF target genes in TS cells.

Next, we examined the cell surface expression of αvβ3 integrin on $Arnt^{+/+}$, $Arnt^{-/-}$, $Hif\alpha^{+/-}$, and $Hif\alpha^{-/-}$ TS cells.

TS cell surfaces were biotinylated, and whole cell lysates were immunoprecipitated with antibodies to either αv integrin or β3 integrin. Immunoprecipitations (IPs) were fractionated by nonreducing SDS-PAGE, blotted, and probed with streptavidin-horseradish peroxidase (HRP). The top left panel of Figure 4B presents an experiment where IPs were washed under high salt conditions (0.6 M NaCl), causing β1 integrin to dissociate from αv integrin. The bottom panel represents IPs washed less stringently (0.3 M NaCl), and the position of β1 integrin is indicated. *Arnt*$^{+/+}$ TS cells lysates immunoprecipitated with antibody to αv integrin exhibited expression of αvβ1, αvβ5, and αvβ3 integrin dimers on their cell surface (Figure 4B, lanes 1, 2, 9, and 10). Additionally, when *Arnt*$^{+/+}$ extracts were immunoprecipitated with β3 integrin antibody, substantial αvβ3 integrin was detected on the cell surface (Figure 4B, lanes 5 and 6). In contrast, *Arnt*$^{-/-}$ TS cells showed dramatically decreased αvβ3 integrin on the cell surface when lysates were immunoprecipitated with antibody against αv integrin (lanes 3, 4, 11, and 12) or β3 integrin (lanes 7 and 8). The top right panel of Figure 4B depicts *Hifα*$^{+/-}$ and *Hifα*$^{-/-}$ TS cells that have been biotinylated and immunoprecipitated with integrin αv. *Hifα*$^{-/-}$ TS cells exhibit reduced surface αvβ3 integrin compared with *Hifα*$^{+/-}$ TS cells (Figure 4B, lanes 13 and 14). Importantly, *Arnt*$^{-/-}$ TS cells expressed comparable levels of αvβ1 integrin and αvβ5 integrin on their cell surface compared with *Arnt*$^{+/+}$ TS cells. We also used FACS analysis to determine the extent of cell surface expression for integrins αv, β1, β3, and β5. Only one of the *Arnt*$^{-/-}$ TS cell lines expressed less αv integrin on the cell surface than wild-type cells, so this is not a genotype-specific phenomenon. Instead, we found that β3 integrin cell surface expression was decreased in both *Arnt*$^{-/-}$ TS cell lines (Figure 4C) whereas integrins β1 and β5 were expressed at wild-type levels. In conclusion, HIF regulation of integrin αvβ3 cell surface expression seems to mediate adhesion to vitronectin and fibronectin.

Because β3 integrin seemed to be translated normally in *Arnt*$^{-/-}$ TS cells, we examined cellular localization of β3 integrin by performing immunofluorescence confocal microscopy analysis. TS cells grow as epithelial sheets, so the micrographs shown consist of undifferentiated cells within a sheet and not individual cells. We first determined whether β3 integrin localized to the Golgi apparatus by analyzing colocalization between β3 integrin and 58K, a microtubule-binding Golgi protein (Bashour and Bloom, 1998). *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ TS cells were initially stained for β3 integrin (in red) and 58K (in green) by using fluorescently conjugated antibodies (Figure 5A). The yellow color present in the merged image of both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ TS cells indicates colocalization between β3 integrin and 58K, suggesting that β3 integrin is present in the Golgi (Figure 5A). We subsequently assessed colocalization between β3 integrin (red) and the ER protein calreticulin (green). β3 integrin was detected in the ER in both *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ TS cells (Figure 5B). When integrins are activated, they cluster at focal adhesions to mediate attachment to the ECM (Giancotti and Ruoslahti, 1999). Focal adhesions are composed of structural proteins, cytoskeletal proteins, and signaling molecules. On integrin clustering, focal adhesion kinase (FAK) becomes phosphorylated at tyrosine 397 (Rodriguez-Fernandez, 1999). To determine whether β3 was present in TS cell focal adhesions, colocalization between β3 integrin and phosphorylated FAK was examined. Although integrin β3 was localized with phosphorylated FAK in wild-type cells, very little β3 integrin was associated with focal adhesions in *Arnt*$^{-/-}$ TS cells (Figure 5C). In agreement with the decreased β3 integrin observed by FACS and immunoprecipitation assays, β3 integrin transits through the Golgi and ER in *Arnt*$^{-/-}$ TS cells but is not transported to focal adhesions at the cell surface.

To further analyze β3 subcellular localization, we performed immuno-EM for β3 integrin. Immuno-EM is an unbiased approach used to examine the association of a particular protein with ultrastructural features or subcellular compartments. In *Arnt*$^{+/+}$ TS cells, β3 integrin-specific gold particles were localized near the cell surface and in small intracellular clusters (Figure 5E). In direct contrast, β3 integrin was never present on the cell surface in *Arnt*$^{-/-}$ cells. Instead, there were large clusters of intracellular β3-specific gold particles, indicating that β3 integrin was localized in the cell interior (Figure 5F). These gold particle aggregates seemed to be in membrane-bound compartments, which may be some type of vesicle. To quantitate these findings, we counted small gold particle clusters (1 gold particle) or large gold particle clusters (≥3 gold particles) for at least nine electron micrographs for *Arnt*$^{+/+}$ and *Arnt*$^{-/-}$ TS cells. *Arnt*$^{-/-}$ TS cells had significantly more large clusters of gold particles ($p = 0.048$) and fewer small clusters of gold particles (Figure 5D) than wild-type cells. Therefore, β3 integrin seems to be accumulating in a post-Golgi, membrane-bound compartment in *Arnt*$^{-/-}$ TS cells. Accumulation in this compartment either prevents transport of β3 integrin to the cell surface or β3 integrin accrues in this location because it is unable to be transported properly to the surface. These results provide further evidence that β3 integrin is intracellular but does not reach the cell surface in TS cells deficient for HIF.

Hypoxia Regulates αvβ3 Integrin-mediated Adhesion and Migration in Melanoma Cells

Integrin expression and activation is important in both developmental processes and pathological conditions. It has been reported that hypoxia is associated with radiation resistance in tumors and predicts poor patient outcome (Vaupel *et al.*, 2001). Additionally, αvβ3 integrin expression correlates with metastasis or an increased invasive phenotype in a number of tumor cell types such as melanoma, ovarian carcinoma, hepatocellular carcinoma, and breast cancer (Carreiras *et al.*, 1996; Wong *et al.*, 1998; Nejjari *et al.*, 2002; Jin and Varner, 2004). We examined the possible regulation of αvβ3 integrin by hypoxia in melanoma cells. We assayed both highly metastatic B16F10 murine melanoma cells and poorly metastatic B16F0 cells for cell surface expression of αvβ3 integrin. Based on FACS analysis, B16F10 cells exhibited 50% more αv integrin ($p < 0.001$) and 25% more β3 integrin ($p = 0.01$) on the cell surface than B16F0 cells (Figure 6, A and B). Unlike the TS cells, culturing cells at 1.5% O_2 for 24 h induced αvβ3 integrin protein expression in B16F0 and F16F10 cells approximately twofold (our unpublished data). Of note, the highly metastatic B16F10 cells were more adherent to vitronectin ($p < 0.01$) and laminin ($p = 0.025$) than the poorly metastatic B16F0 cells (Figure 6C). To further investigate the role of hypoxia in tumor progression, we examined changes in adhesion properties of melanoma cells in response to hypoxic culture conditions. We subjected B16F0 cells to 24 h of 1.5% O_2 and measured adhesion to fibronectin, vitronectin, and laminin. Hypoxia increased B16F0 adhesion to fibronectin and vitronectin by 1.6-fold ($p = 0.017$) and 1.7-fold ($p = 0.001$), respectively, compared with culture in 20% O_2 (Figure 7A). In contrast, adhesion to laminin was unaltered by hypoxic exposure (Figure 7A).

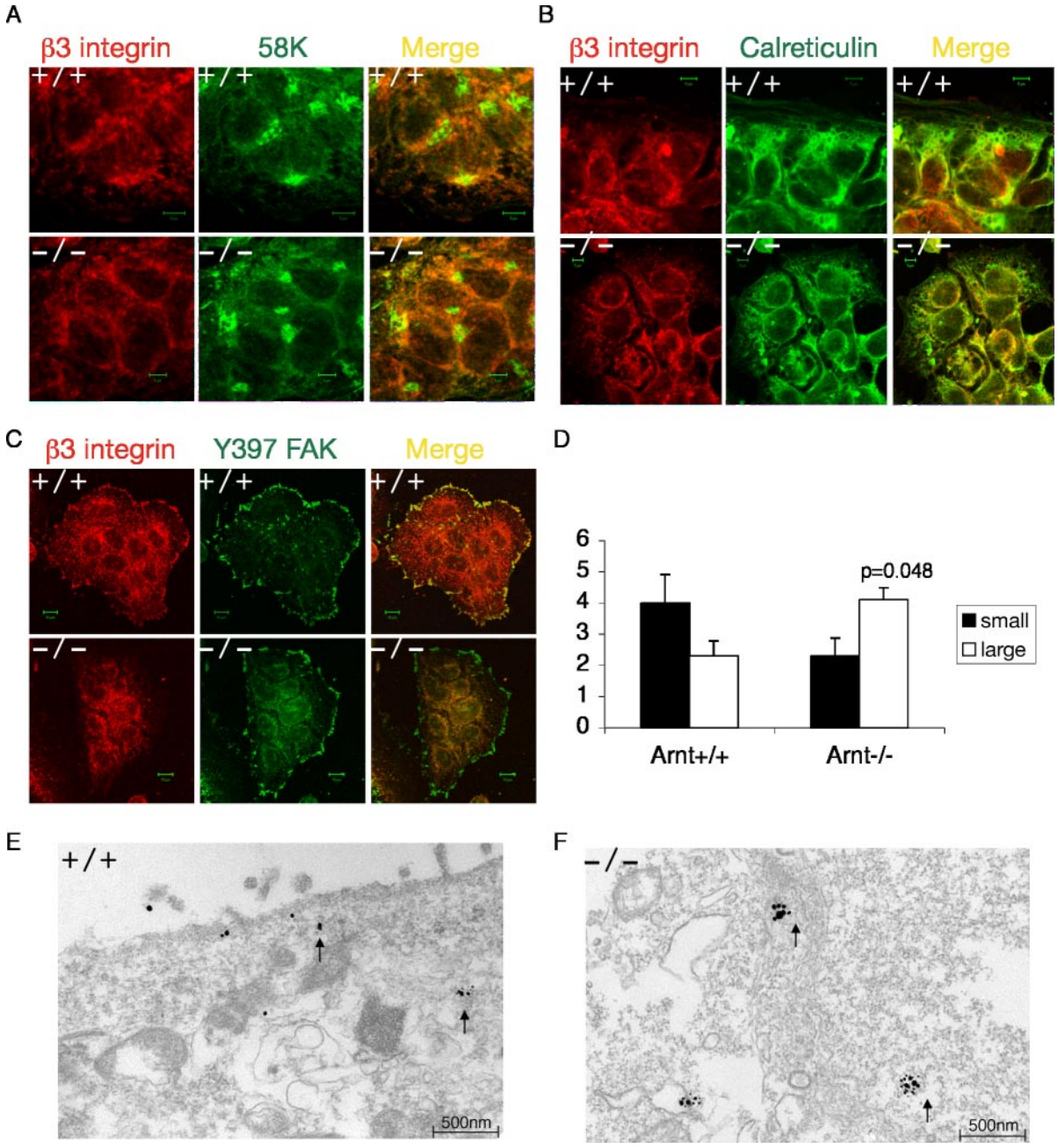

Figure 5. Subcellular localization of integrin β3 in TS cells. Confocal immunofluorescence microscopy was performed on $Arnt^{+/+}$ and $Arnt^{-/-}$ TS cells showing colocalization of β3 integrin with the Golgi marker 58K (A), calreticulin in the ER (B), and phosphorylated FAK Y397 (C). Integrin β3 is in red and the other markers are in green. The yellow image is a red/green merge to show colocalization. Intracellular small and large gold particle clusters for β3 integrin in wild-type and $Arnt^{-/-}$ TS cells from immunoelectron micrographs was quantitated (D). Immunoelectron micrographs for β3 integrin in $Arnt^{+/+}$ (E) and $Arnt^{-/-}$ TS cells (F). Bar, 500 nm. Error bars represent ± SEM. Student's *t* tests were performed to determine statistical significance.

We also measured B16F0 cell migration toward vitronectin. Treatment of B16F0 cells with 1.5% O_2 resulted in increased vitronectin-associated migration ($p = 0.045$) (Figure 7B). Assays for invasion of B16F0 and B16F10 cells into Matrigel were performed; unfortunately, both cell lines failed to perform in these experiments When a blocking antibody for β3 integrin was added to B16F0 cells, adhesion to vitronectin under both normoxia and hypoxia was decreased ($p < 0.01$) (Figure 7C), suggesting that adhesion to vitronectin is mediated by β3 integrin. In contrast to B16F0 cells, exposure of B16F10 cells to 1.5% O_2 for 24 h did not significantly increase adhesion to vitronectin (our unpublished data). We then determined the effect of hypoxia on αvβ3 integrin cell surface expression. B16F0 cells were cultured at 20% O_2 and 1.5% O_2 for 24 h, and cells were harvested, stained with specific antibodies, and analyzed by FACS. B16F0 cells exhibited increased αv integrin ($p < 0.001$) and β3 integrin ($p < 0.01$) cell surface expression under hypoxia (Figure 7D). However, cell surface expression of α6 and β1 integrins did not increase after hypoxic exposure (Figure 7D).

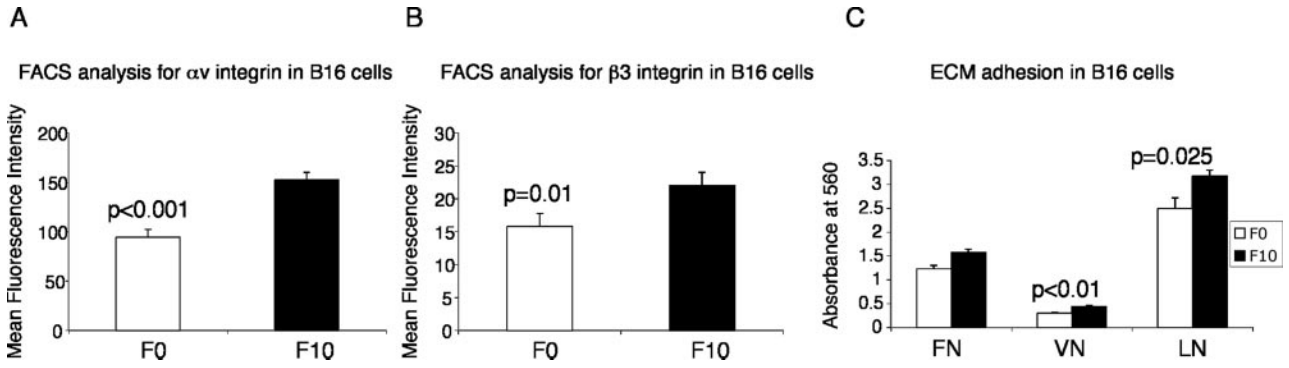

Figure 6. Poorly metastatic B16F0 melanoma cells express less cell surface αvβ3 integrin than highly metastatic B16F10 cells. (A) Mean fluorescence intensity for αv integrin on B16F0 and B16F10 cells based on FACS analysis. (B) Mean fluorescence intensity for β3 integrin on B16F0 and B16F10 cells. (C) B16F0 and B16F10 cells were plated on strips coated with FN, VN, or LN and stained with crystal violet. Solubilized crystal violet incorporated by adherent cells was measured on a plate reader at 560 nm. Error bars represent ± SEM. Student's *t* tests were performed to determine statistical significance.

B16F0 cell surfaces also were labeled with biotin to detect membrane-bound proteins. Immunoprecipitation with antibodies to αv integrin confirmed that B16F0 cells exhibited increased αvβ3 integrin cell surface expression under hypoxia, whereas immunoprecipitation with an antibody for β1 integrin showed no change in the abundance of integrin β1 on the cell surface after hypoxic treatment (Figure 7E). In conclusion, hypoxia specifically induces αvβ3 integrin expression in the poorly metastatic B16F0 melanoma cells, resulting in increased adhesion and migration. However, the mechanism of hypoxic induction is slightly different from that of TS cells because hypoxia increases αvβ3 total cellular protein in B16F0 melanoma cells.

DISCUSSION

Data presented here demonstrate that HIF signaling is important for both adhesion of undifferentiated TS cells to vitronectin and migration properties of these cells, via control of subcellular localization of αvβ3 integrin. We also show that hypoxia promotes tumor cell migration through regulation of αvβ3 integrin-mediated adhesion. Therefore, the HIF signaling pathway and O_2 availability are important in cell motility and adhesion in a number of biological contexts.

HIF Regulates Adhesion and Migration

Placental development initially occurs in a natural O_2 gradient, and O_2 sensing via HIF activity in the placenta is critical for trophoblast cell fate adoption and trophoblast invasion (Adelman *et al*., 2000; Cowden Dahl, Mack, Compernolle, Adelman, Carmeliet, and Simon, unpublished data). Trophoblast adhesion and invasion are critical for embryo implantation into the maternal tissue and subsequent placentation. Therefore, we examined the ability of HIF to regulate trophoblast adhesion and migration. Because embryos lacking ARNT or the HIFα subunits exhibit shallow placental invasion, we used TS cells derived from $Arnt^{-/-}$ blastocysts to assess whether the invasion defect in vivo is autonomous to trophoblasts. Normoxic undifferentiated TS cells were analyzed because they represent the most homogenous cell population. TS cells exhibit unusually high levels of HIF activity at 20% O_2 conditions, thereby allowing us to study HIF-dependent processes under normal O_2 tensions. TS cells deficient in the ARNT subunit of HIF migrated and adhered poorly to vitronectin and fibronectin, suggesting HIF regulates these events. The specificity of the adhesion defect suggests that HIF specifically mediates adhesion through the αvβ3 integrin pair and not through other integrins.

Other groups have examined hypoxic regulation of αvβ3 integrin in endothelial cells. Hypoxia transcriptionally stimulates integrin αv and β3 mRNA levels in human umbilical vein endothelial cells (HUVECs) (Walton *et al*., 2000) and integrin β2 in human monocytes (Kong *et al*., 2004). However, we did not observe changes in integrin αvβ3 protein levels in hypoxic TS cell or HUVECs (Figure 4C; out unpublished data). Treatment of bovine retinal endothelial cells with exogenous VEGF induces αv and β3 integrin expression; however, VEGF treatment does not alter expression of either αv integrin or β3 integrin in TS cells (Suzuma *et al*., 1998; our unpublished data). In $Arnt^{-/-}$ TS cells, β3 integrin is transcribed, translated, and transits through the Golgi and ER, but it fails to cluster at focal adhesions on the cell surface. Therefore, the regulation of αvβ3 integrin in TS cells seems to be distinct from that in endothelial cells.

As the trophectoderm of the blastocyst differentiates into trophoblasts, cell polarity, motility, and adhesion are altered (Sutherland, 2003). Integrins α5, α6, αv, αIIb, β1, and β3 are expressed on the surface of blastocysts (Sutherland *et al*., 1993; Schultz *et al*., 1997). Antibodies against integrins α5, αv, β1, and β3 block adhesion of blastocysts to fibronectin, an early event in trophoblast differentiation; therefore, αvβ3 integrin-mediated adhesion may be important in trophoblast differentiation and function (Schultz and Armant, 1995). After the embryo implants into the uterus, integrin localization changes. Contact with uterine ECM induces protein trafficking of the laminin receptors α5β1 integrin and α7β1 integrin from the basal surface to the apical surface of the trophectoderm (Schultz *et al*., 1997; Klaffky *et al*., 2001; Sutherland, 2003). Trafficking of integrins in trophoblasts seems to be an important regulatory step in trophoblast differentiation.

Placental development is coordinated with invasion into maternal decidua. In preeclampsia, cytotrophoblast invasion is greatly compromised. Preeclampsia is a pregnancy-associated disease that affects 4–5% of all pregnancies and can be fatal (VanWijk *et al*., 2000). Hypoxia may affect placental invasion in vivo because preeclamptic placentas are hypoxic and exhibit reduced invasion into maternal deciduae (Van-

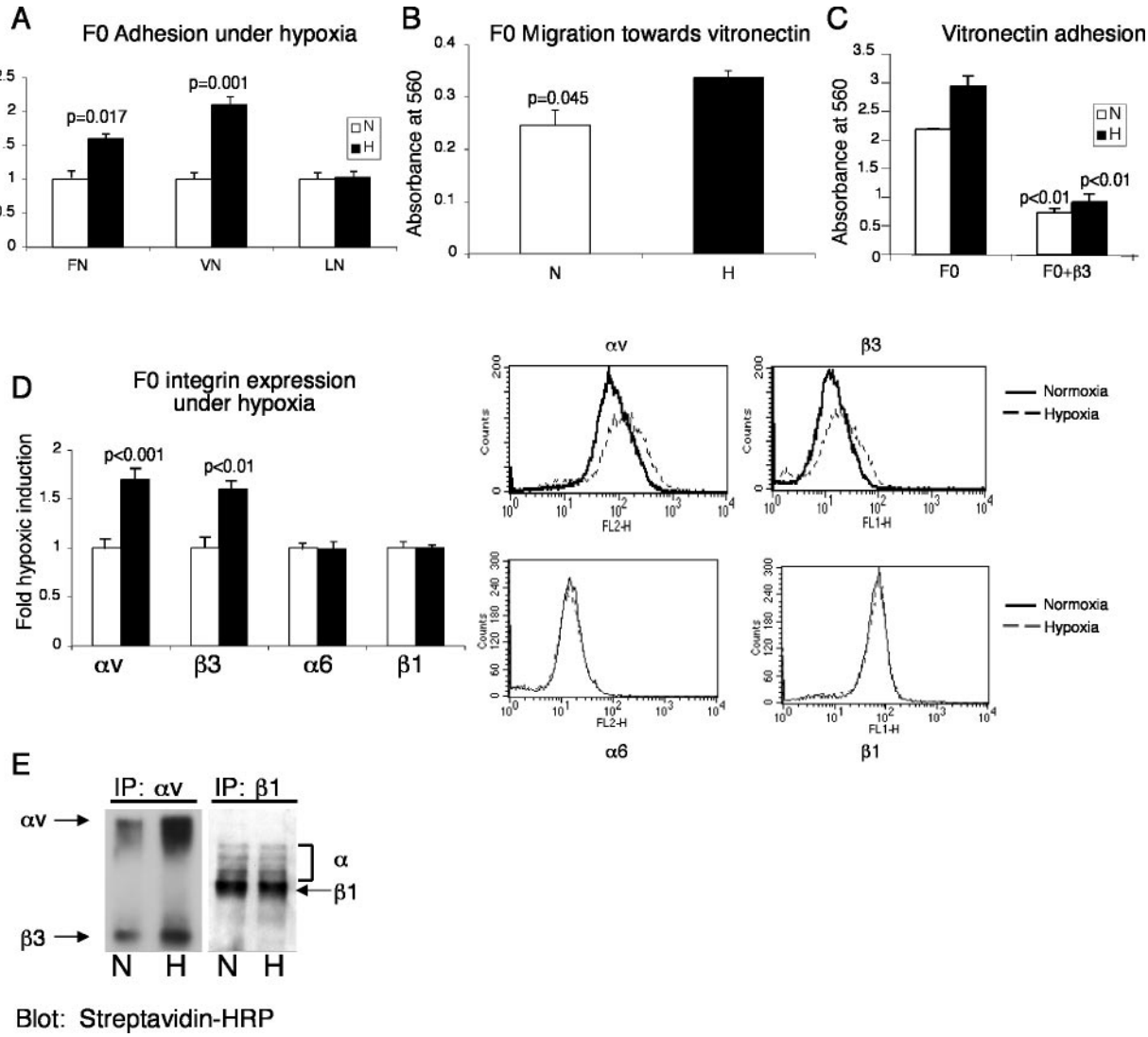

Figure 7. Adhesion to vitronectin and migration toward vitronectin is increased in B16F0 melanoma cells cultured in 1.5% O_2 versus 20% O_2, via increased cell surface expression of $\alpha v\beta 3$. (A) An increase in adhesion to FN and VN is observed in hypoxic B16F0 cells. Adhesion to LN is not changed by hypoxia. (B) Migration toward vitronectin is also enhanced by hypoxia in F0 cells. (C) Adhesion to vitronectin under normoxia and hypoxia is mediated by the $\beta 3$ integrin because a blocking antibody for $\beta 3$ integrin diminishes adhesion to vitronectin. (D) B16F0 cells exhibit increased $\alpha v\beta 3$ integrin surface expression when treated with 1.5%O_2 for 24 h. Surface levels of $\alpha 6$ and $\beta 1$ integrins are unaltered by hypoxia. FACS analysis for integrins αv, $\beta 3$, $\alpha 6$, and $\beta 1$ was performed on cells cultured in either 20% O_2 (solid line) or 1.5%O_2 (dotted line). (E) B16F0 cells were surface labeled with biotin and immunoprecipitated for αv integrin or $\beta 1$ integrin. IPs were separated by nonreducing SDS-PAGE and blotted with streptavidin-HRP. Positions of αv integrin, $\beta 3$ integrin, and $\beta 1$ integrin are indicated. Error bars represent ± SEM. Student's *t* tests were performed to determine statistical significance.

Wijk *et al*., 2000; Goldman-Wohl and Yagel, 2002). Preeclamptic placentas express low levels of $\beta 3$ integrins throughout the placenta compared with normal placentas (Zhou *et al*., 1997a), suggesting that this integrin may be important in proper placental progression. Furthermore, $\alpha v\beta 3$ integrin and $\alpha IIb\beta 3$ integrin regulate migration and trophoblast outgrowth on fibronectin (Rout *et al*., 2004). Interestingly, $\beta 3$ integrin is expressed throughout the murine placenta. However, the subcellular localization of $\beta 3$ integrin is different for distinct trophoblast subtypes (Bowen and Hunt, 1999). $\beta 3$ integrin is localized to the membrane in labyrinthine trophoblasts, whereas it is diffuse and cytoplasmic in spongiotrophoblasts (Bowen and Hunt, 1999), suggesting that $\beta 3$ integrin localization and expression may be a developmentally regulated event. $\beta 3$ integrin is not essential for placentation because integrin $\beta 3^{-/-}$ mice are viable; however, $\beta 3^{-/-}$ mice exhibit reduced fecundity. $\beta 3$ integrin function is likely important for normal pregnancy, because the viability of integrin $\beta 3^{-/-}$ embryos is reduced due to placental defects (Hodivala-Dilke *et al*., 1999). Of note, 80% of αv integrin-null embryos die at midgestation with placental defects such as a reduced labyrinthine layer, fewer intermingled fetal and maternal blood vessels, and aberrant spongiotrophoblast and giant cell layers (Bader *et al*., 1998). We have shown that HIF activity is critical to proper establishment of each placental layer (Cowden Dahl, Mack, Compernolle, Adelman, Carmeliet, and Simon, unpublished data). HIF may regulate trophoblast differentiation and invasion, in part, through regulation of $\alpha v\beta 3$ integrin. Importantly, TS cells differentiated for 4 d also fail to express high

levels of cell surface αvβ3 integrin (our unpublished data), suggesting that αvβ3 integrin is not regulated properly during differentiation either. These results strongly suggest that misexpression of αvβ3 may occur in HIF-deficient placentas. Integrin-mediated defects are not as severe as those noted for HIF-deficient placentas, likely due to misexpression of additional target genes in HIF mutants. In summary, HIF regulation of genes critical to placentation in addition to the timing of αvβ3 integrin surface expression contributes to placental invasion of maternal tissue.

HIF is critical to trophoblast adhesion and migration and is also important in these processes in a variety of disease states. For example, hypoxia and oxidative stress are major factors in inflammation, cardiovascular disease, and cancer (Paul *et al*., 2004; Peyssonaux and Johnson, 2004). HIF is critical to inflammatory responses because myeloid cells lacking HIF1α are defective in migration and invasion (Cramer *et al*., 2003). Similar to our findings in TS cells, αvβ3 integrin may be important in the normal oxygen-regulated inflammatory responses of macrophages. For example, macrophage differentiation is marked by increased expression of αvβ3 integrin (Lafrenie *et al*., 2002).

Hypoxia Promotes Tumor Cell Motility

As tumors expand, they can outgrow their vascular supply, resulting in regions of the tumor deficient in O_2 and nutrients. Induction of genes in this hypoxic environment is important for further tumor progression. For example, VEGF induction in melanomas enhances both tumor angiogenesis and metastasis (Rofstad and Danielsen, 1999). In a variety of human cancer, tumor hypoxia is associated with increased metastatic potential. Although many mechanisms are involved in this process, changes in integrin activity may contribute to metastatic progression. We propose that one way tumor cells become more motile is by hypoxic exposure, because hypoxic exposure enhances αvβ3 integrin surface expression and adhesion to vitronectin in poorly metastatic B16F0 cells. Additionally, modulation of αvβ3 integrin expression could have consequences for tumor angiogenesis, which is linked to αvβ3 integrin. To this end, integrin inhibitors are currently in clinical trials as cancer therapeutics.

In summary, we demonstrate that HIF activity is a critical regulator of undifferentiated TS cell adhesion and migration. Functional inactivation of HIF results in decreased αvβ3 integrin cell surface expression. αvβ3 integrin is not appropriately transported to the cell surface of $Arnt^{-/-}$ TS cells, which adhere poorly to αvβ3 integrin ligands such as vitronectin and fibronectin. Furthermore, hypoxia regulates adhesion and migration of mouse melanoma cells through the regulation of αvβ3 integrin expression at the plasma membrane. These data illustrate that HIF activity controls adhesion, migration, and invasion through regulation of a key integrin in both development and cancer.

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