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Suppression of Insulin Receptor Substrate 1 (IRS-1) Promotes Mammary Tumor Metastasis▽

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The insulin receptor substrate (IRS) proteins are cytoplasmic adaptors that organize signaling complexes downstream of activated cell surface receptors. Here, we show that IRS-1 and IRS-2, despite significant homology, play critical yet distinct functions in breast cancer, and we identify specific signaling pathways that are influenced by IRS-1 using the polyoma virus middle-T (PyV-MT) transgenic mouse model of mammary carcinoma and Irs-1 null (*Irs1*$^{-/-}$) mice. The absence of Irs-1 expression enhanced metastatic spread significantly without a significant effect on primary tumor growth. Orthotopic transplant studies revealed that the increased metastatic potential of *Irs1*-deficient tumor cells is cell autonomous. Mammary tumors that developed in PyV-MT::*Irs1*$^{-/-}$ mice exhibited elevated Irs-2 function and enhanced phosphatidylinositol 3-kinase/Akt/mTor activity, suggesting that one mechanism by which Irs-1 impedes metastasis is to suppress Irs-2-dependent signaling. In support of this mechanism, reduction of Irs-2 expression in *Irs1*$^{-/-}$ tumor cells restored mTor signaling to wild-type levels. PyV-MT::*Irs1*$^{-/-}$ tumors also exhibited a significant increase in vascular endothelial growth factor expression and microvessel density, which could facilitate their dissemination. The significance of our findings for human breast cancer is heightened by our observation that Irs-1 is inactivated in wild-type, metastatic mammary tumors by serine phosphorylation. Collectively, our findings reveal that inactivation of IRS-1 enhances breast cancer metastasis and support the novel hypothesis that IRS-1 has metastasis suppressor functions for breast cancer.

The insulin receptor substrate (IRS) proteins are cytoplasmic docking proteins that function as essential signaling intermediates downstream of activated cell surface receptors, including the insulin, insulin-like growth factor 1 (IGF-1), prolactin, growth hormone (GH), and vascular endothelial growth factor (VEGF) receptors, members of the integrin receptor family, and select cytokine receptors (38, 68, 70, 78, 81, 85). The IRS proteins are recruited to receptors through pleckstrin homology and phosphotyrosine-binding domains in their N termini and mediate their functions by organizing signaling complexes at sites of receptor activation (81). Upon binding they are phosphorylated on tyrosine residues in their C termini, creating multiple phosphotyrosine binding motifs that recruit downstream effectors, including phosphatidylinositol 3-kinase (PI3K), Grb-2, Fyn, and Shp-2 to initiate intracellular signaling cascades (81). The IRS proteins were originally identified as substrates of the insulin receptor, and they have been predominantly studied for their role in metabolic signaling (81). Although the IRS proteins are highly homologous, there is evidence for unique functions for each of the four IRS family members. Irs-1 null mice are born small and remain runted throughout life, and these mice develop insulin resistance. However, Irs-1 null mice do not develop diabetes, because they maintain normal numbers of pancreatic beta cells (3). In contrast, Irs-2 null mice are a normal size at birth but exhibit brain defects and develop early-onset diabetes due to a combination of peripheral insulin resistance and beta-cell failure (66, 83, 84). Irs-3 null mice are phenotypically normal, and Irs-4 null mice have mild reproductive and insulin sensitivity defects (16, 42).

IRS-1 and IRS-2 are the IRS family members that are expressed in normal mammary epithelial tissue, both in humans and in mice, and in breast cancer (37, 52). To date, more is known regarding the role of IRS-2 in breast cancer than that of IRS-1. The importance of IRS-2 for breast cancer progression was first suggested by the fact that the IGF-1 receptor no longer promotes mitogenesis but it is still required for metastasis in estrogen receptor-negative (ER−) breast carcinoma cell lines which lack or have decreased IRS-1 expression but retain IRS-2 expression (15). In these cell lines, which signal predominantly through IRS-2, IGF-1 stimulates breast carcinoma cell motility (6, 14, 33, 86). IRS-2 is also an essential intermediate in the activation of PI3K and promotion of breast carcinoma invasion by the α6β4-integrin receptor (70). The most compelling data that support a critical role for IRS-2 in breast cancer were provided by our study that examined the impact of Irs-2 deficiency on the polyoma virus middle-T (PyV-MT) mouse model of mammary tumor progression (52). We found that Irs-2-deficient mice did not exhibit differences in mammary tumor onset or growth compared to mice with a wild-type (WT) genetic background. However, they did exhibit

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significantly reduced rates of metastasis (52). Mammary tumor cells derived from the PyV-MT::*Irs2*$^{-/-}$ mice also exhibited attenuated invasion and cell survival. These findings not only highlighted the importance of IRS-2 for breast cancer progression, but they also revealed that IRS-1 is not sufficient to promote metastasis.

The inability of Irs-1 to compensate for the loss of Irs-2 in mammary tumor metastasis in vivo supports the hypothesis that IRS-1 functions in breast cancer in a manner distinct from that of IRS-2. However, the existing data on IRS-1, which were derived largely from studies on human breast carcinoma cell lines, are conflicting and have not provided definitive insight into the contribution of IRS-1 to breast cancer. For example, suppression of IRS-1 expression in ER+ breast carcinoma cell lines inhibits IGF-1-dependent proliferation and survival, suggesting that IRS-1 plays a positive role in breast cancer (9, 53). However, ER− breast carcinoma cell lines, which express low levels of IRS-1, are more invasive and metastatic than ER+ cells (32, 70). In the current study, we examined rigorously the role of Irs-1 in mammary tumorigenesis and progression and investigated the specific pathways that are influenced by Irs-1 function. Our data reveal that loss of Irs-1 expression stimulates metastatic spread significantly. The significance of our findings for human breast cancer is heightened considerably by our novel observation that Irs-1 is selectively inactivated in metastatic mammary tumors.

MATERIALS AND METHODS

Mice. Female C57Bl/6, *Irs1*$^{+/-}$ mice were generated previously and were bred for 10 generations into the FVB genetic background (3). Female FVB *Irs1*$^{+/-}$ mice were crossed with male FVB mice that were transgenic (+/−) for the PyV-MT antigen under the control of the mouse mammary tumor virus promoter (Jackson Labs) to generate PyV-MT$^{+/-}$::*Irs1*$^{+/-}$ male mice. Female *Irs1*$^{+/-}$ mice were bred to male PyV-MT$^{+/-}$::*Irs1*$^{+/-}$ mice, and female offspring from this cross that were PyV-MT$^{+/-}$ and were wild type for the Irs-1 allele (PyV-MT) or homozygous null for the Irs-1 allele (PyV-MT::*Irs1*$^{-/-}$) were saved for further analysis. Genotyping was performed by PCR using oligonucleotides specific for the Irs-1 gene (5′-GCCAGGCACCAGCATCTTCG-3′ and 5′-TGG CCGCTCCCGAATTCAAT-3′), the neomycin gene (5′-GCTACCCGTGATA TTGCTGAAGAG-3′), and as described previously for the PyV-MT transgene (24). PyV-MT::*Irs2*$^{-/-}$ female mice were generated as described previously (52).

Beginning at 15 days of age, the mice were palpated every 5 days to detect the onset of mammary tumor development. At 75, 80, or 85 days, the tumors were dissected and measured using calipers. The total tumor volume was determined using the following formula: volume = $(4/3)(\pi)(1/2 \times \text{smaller diameter})^2(1/2 \times \text{larger diameter})$. Portions of the tumors and the lungs were either snap frozen or fixed in 10% buffered formalin. Blood was also collected at the time of sacrifice (100 μl) and spun at 1,000 rpm for 10 min at 4°C to obtain serum.

To examine the ability of Irs-1-deficient PyV-MT mammary tumor cells to grow and metastasize in a wild-type-Irs genetic background, PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumor cells (2×10^6) were resuspended in Matrigel (50 μl) and injected into the number 3 and number 8 thoracic mammary glands of female nu/nu mice. At an equivalent tumor burden [tumor size determined using the following formula: volume = width^2 × (length/2)], the tumors and lungs were dissected and portions of each were either snap-frozen or fixed in 10% buffered formalin (50).

IHC. Formalin-fixed tissue was embedded in paraffin and sections were stained either with hemotoxylin and eosin (H&E) or with antibodies for histology and immunohistochemistry (IHC) analysis. For IHC, tissue sections were deparaffinized and rehydrated before endogenous peroxidase activity was quenched in 3% H_2O_2 for 5 min. For detection of estrogen receptor α (ERα), tissue sections were incubated in citrate buffer, pH 6, at 95°C in a steamer for 35 min, followed by incubation overnight at 4°C with primary antibodies. To detect endothelial cells, tissue sections were digested with *N-p*-tosyl-L-phenylalanyl chloromethyl ketone-trypsin (0.05%) for 28 min before overnight incubation at 4°C with rat anti-mouse CD31 (2.5 μg/ml; BD Biosciences). The primary antibodies were amplified with biotinylated anti-rat (CD31) or anti-rabbit (ERα) immunoglobulin G (Vector Laboratories) and detected using the Vectastain Elite ABC reagent (Vector Laboratories) with 3,3′-diaminobenzidine as the substrate. CD31 staining was quantitated using NIH Image 1.61 software. To detect apoptotic cells, tissue sections were stained using the ApopTag Plus peroxidase in situ apoptosis detection kit according to the manufacturer's instructions (Chemicon, Temecula, CA).

ELISAs. VEGF-A protein was measured using the murine VEGF Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems) according to the manufacturer's instructions.

Real-time quantitative PCR (RQ-PCR). Total RNA was isolated from lung tissue and tumors using the RNeasy extraction kit (QIAGEN). Quantitative analysis of gene expression was obtained using the ABI 7300 sequence detection system and TaqMan One-Step RT-PCR master mix reagents (Applied Biosystems). PyV-MT mRNA was amplified using the following primers and probe: forward primer, 5′-AGCCCGATGACAGCATATCC-3′; reverse primer, 5′-GG TCTTGGTCGCTTTCTGGA-3′; Taqman probe, 5′-CGGACCCCCCCAGAA CTCCTGT-3′. The murine VEGF-A primer pairs and probe were obtained from Applied Biosystems. The transcript quantity in each RNA sample was normalized to that of glyceraldehyde-3-phosphate dehydrogenase using the TaqMan Pre-Developed assay reagent for rodents (Applied Biosystems).

Immunoprecipitation and immunoblotting. Frozen tumors were homogenized at 4°C in T-PER tissue protein extraction reagent (Pierce Biotechnology, Inc., Rockford, IL), containing 1 mM sodium orthovanadate, and protease inhibitors (Complete Mini; Roche Applied Science, Indianapolis, IN). Tissue culture cells were solubilized at 4°C for 10 min in a 20 mM Tris buffer, pH 7.4, containing 0.14 M NaCl, 1% NP-40, 10% glycerol, 1 mM sodium orthovanadate, and protease inhibitors. Nuclei were removed by centrifugation at 12,000 × *g* for 10 min. Aliquots of tissue or cell extracts containing equivalent amounts of protein were incubated for 3 h at 4°C with antibodies and protein A Sepharose (Amersham Biosciences) with constant agitation. The beads were washed three times in extraction buffer. Laemmli sample buffer was added to the samples, which were then incubated at 95°C for 4 min. Immune complexes, as well as aliquots of cell and tumor extracts containing equivalent amounts of total protein, were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters.

For immunoblotting, the filters were blocked for 1 h with a 50 mM Tris buffer, pH 7.5, containing 0.15 M NaCl, 0.05% Tween 20 (TBST), and 5% (wt/vol) dry milk. The filters were incubated overnight at 4°C in the same buffer containing primary antibodies. After three 10-min washes in TBST, the filters were incubated for 1 h in blocking buffer containing horseradish peroxidase-conjugated secondary antibodies. After three 10-min washes in TBST, proteins were detected by enhanced chemiluminescence (Pierce). For RC-20 phosphotyrosine immunoblots, the filters were blocked for 1 h with a 10 mM Tris buffer, pH 7.5, containing 0.5 M NaCl, 0.1% Tween 20 (RC-20 buffer), and 2% (wt/vol) Carnation dry milk. The filters were washed briefly in RC-20 buffer and then incubated overnight at 4°C in RC-20 buffer containing 3% (wt/vol) and a 1:500 dilution of the RC-20 antibody. After a wash, the filters were incubated for 1 h in blocking buffer containing horseradish peroxidase-conjugated streptavidin and the proteins were detected by enhanced chemiluminescence.

The following antibodies were used for immunoprecipitation or immunoblotting: IRS-1 (Bethyl Labs); IRS-2 (immunoblot, Calbiochem, EMD Biosciences, Inc., Darmstadt, Germany; immunoprecipitation, Bethyl Labs); phosphotyrosine (RC-20; BD Biosciences, San Diego, CA; and 4G10; Upstate Biotechnology, Charlottesville, VA); p85 (a generous gift from Alex Toker); β4-integrin subunit (a generous gift from Arthur Mercurio); ERα, IGF-1 receptor (IGF-1R) β-subunit, and PP2A (Y307) catalytic subunit (Santa Cruz Biotechnology, Santa Cruz, CA); PP2A catalytic subunit (BD Biosciences); PTEN (Chemicon, Temecula, CA); and cyclin D1 (Biosource, Camarillo, CA). All other antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Cell lines, siRNA, and transfections. PyV-MT mammary tumor cell lines were established as described previously (52). Small interfering RNA (siRNA) oligonucleotides for Irs-1 and Irs-2 targeting were as follows: NNUAACCGGUUU CGAAAGAGA (siIrs1 no. 1), NNAGCCGGUCCUCUCUUACUA (siIrs1 no. 2), NNGGCAGGACCUUCCCAGUAA (siIrs2 no. 1), and NNCUACACGCC UAUCGCUAGA (siIrs2 no. 2). The Irs-1 siRNA oligonucleotides were combined equally into one pool and transfected into PyV-MT tumor cells in OPTI-MEM (Invitrogen) using Oligofectamine (Invitrogen) according to the manufacturer's recommendations. The final concentration for siRNA transfection was 400 nM. The Irs-2 siRNA oligonucleotides were transfected individually into PyV-MT::*Irs1*$^{-/-}$ tumor cells at a final concentration of 200 nM. Twenty-four hours after transfection, the cells were incubated in Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin for an additional 24 h.

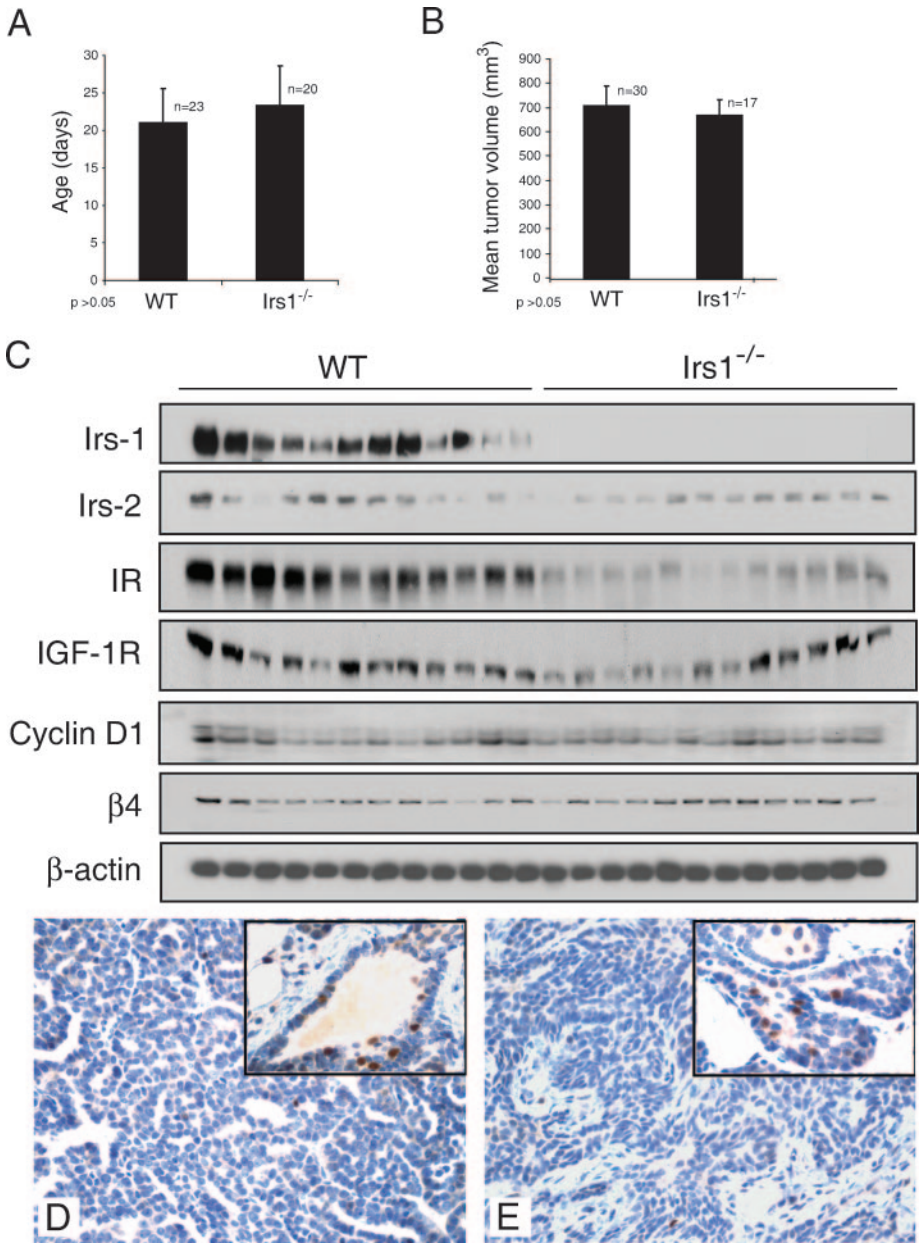

FIG. 1. Analysis of Irs-1 involvement in PyV-MT mammary tumor development. (A) Female PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice were monitored for the age at which mammary tumors were first palpable. The mean age in days for palpable tumors (± SEM) ($P > 0.05$) is shown. The number of mice analyzed for each genotype is indicated. (B) Female PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice were analyzed for their total tumor burden at 80 days of age. The mean tumor volume for each genotype (± SEM) ($P > 0.05$) is shown. The number of mice analyzed is indicated. (C) Biochemical analysis of representative PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumors. Aliquots of mammary tumor extracts that contained equivalent amounts of total protein were immunoblotted with antibodies specific for Irs-1, Irs-2, insulin receptor (IR) β-subunit, IGF-1R β-subunit, cyclin D1, β4, or β-actin. WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice. (D and E) Sections from PyV-MT (D) and PyV-MT::*Irs1*$^{-/-}$ (E) tumors were stained with antibodies specific for ERα. Inset, normal glands showing positive ERα staining.

The luciferase control siRNA was obtained from Dharmacon Research, Inc. (Lafayette, CO).

Statistics. For the densitometric analyses of immunoblots, the results were compared using the Mann-Whitney U test, and the values are presented as means ± standard errors (SEM) for each group. Fisher's exact test and Pearson chi-square test were employed to test the significance of the incidence of lung metastasis. The apoptotic rates were compared using Student's *t* test. All statistical analyses were carried out with SPSS 11.5 statistical software, and a *P* value of 0.05 was considered statistically significant. Corresponding significance levels are indicated in the figures.

RESULTS

Irs-1 expression is not required for PyV-MT-dependent mammary tumor initiation and tumor growth. The PyV-MT oncogene transforms cells by activating signaling molecules that include PI3K, Shc, and Src family kinases (25, 79). The molecular changes that occur during progression of mammary tumors that develop in response to PyV-MT have been de-

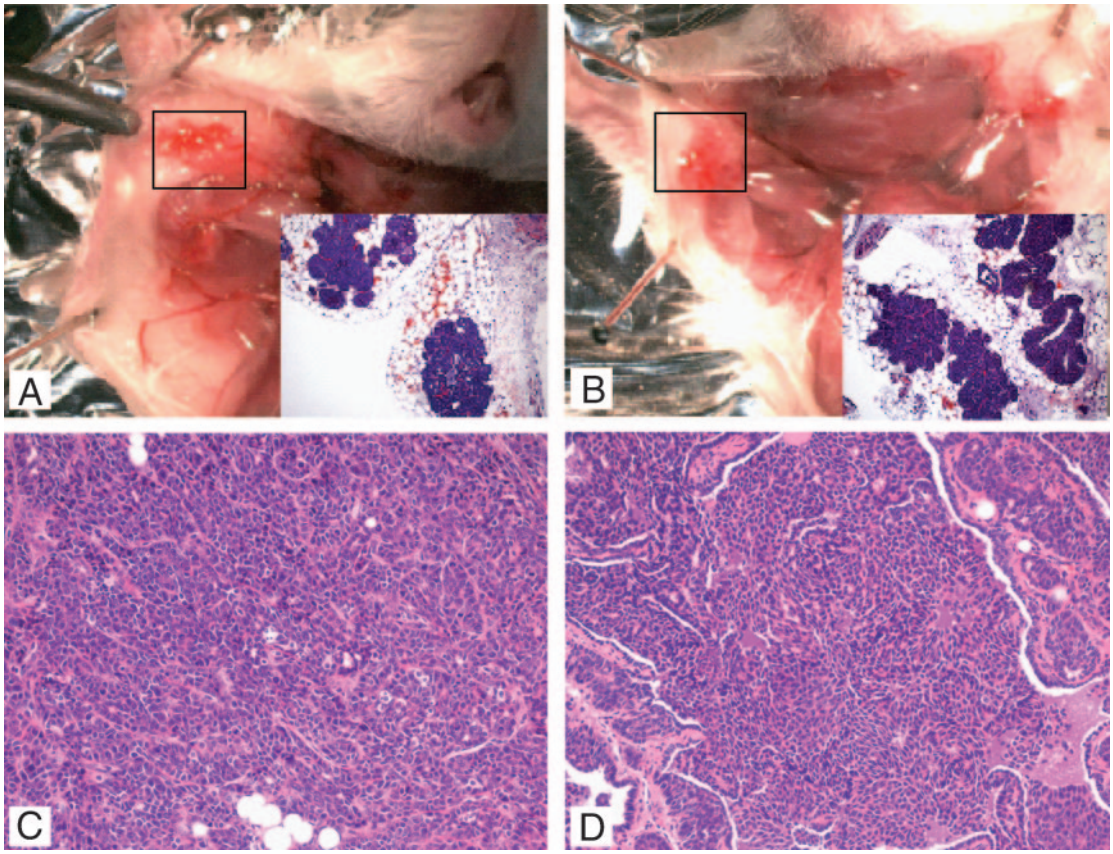

FIG. 2. Histological characteristics of PyV-MT (A and C) and PyV-MT::*Irs1*$^{-/-}$ (B and D) mammary tumors. (A and B) PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice at 24 and 25 days, respectively, the time for onset of palpable tumors. Inset, H&E-stained sections from the same mice (magnification, ×100). (C and D) Solid, nodular, high-grade tumors that lack glandular structure (magnification, ×200).

scribed, and these tumors mimic key features of human breast cancer (40). We have previously shown that Irs-1 and Irs-2 are expressed in PyV-MT-derived mammary tumors and that Irs-2 expression is not required for PyV-MT-dependent mammary tumor initiation or primary tumor growth (52). To assess if Irs-1 is required for PyV-MT-dependent tumorigenesis, female PyV-MT$^{+/-}$ transgenic mice or transgenic mice that were null for the Irs-1 allele (PyV-MT::*Irs1*$^{-/-}$) were analyzed for mammary tumor development. Mammary gland development is normal in the absence of Irs-1 (37). Tumors were first detected at similar ages in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice (Fig. 1A and 2A and B). The total tumor burdens did not differ significantly between these mice at 80 days of age (Fig. 1B). Taken together with our previous findings with PyV-MT::*Irs2*$^{-/-}$mice, our results demonstrate that expression of either Irs-1 or Irs-2 is sufficient for PyV-MT-dependent mammary tumor initiation and growth and suggest that Irs-1 and Irs-2 are either functionally redundant or do not contribute to early stages of tumor development (52).

Characterization of Irs-1 null tumors. To determine if a loss of Irs-1 expression influences the progression of PyV-MT-derived mammary tumors from hyperplasia to invasive carcinoma, we assessed specific markers that increase or decrease during progression to late-stage disease in this model (40). ERα is expressed in normal mammary epithelial cells, and its expression diminishes during tumor progression (11, 40, 71); cyclin D1 expression increases during the progression from hyperplastic lesions to late-stage carcinomas, which reflects the increased mitotic indices of these poorly differentiated tumors (40, 73). Both PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors were negative for ERα expression (Fig. 1D and E). Moreover, the absence of Irs-1 did not alter the expression of cyclin D1 in the mammary tumors (52) (Fig. 1C). These results support the conclusion that Irs-1 is not required for progression to malignancy, since overall tumor development was not delayed in the absence of this docking protein. We evaluated the expression and activation of the IGF-1 and insulin receptors in the tumors because they are upstream regulators of the IRS proteins and have been implicated in breast cancer. Whereas the IGF-1R was expressed at equivalent levels in the WT and *Irs1*$^{-/-}$ tumors, insulin receptor expression was reduced in the absence of Irs-1 (Fig. 1C). However, we did not detect activation of these receptors in either of the tumor genotypes, suggesting that other upstream regulators of IRS function are responsible for activating the IRS proteins in PyV-MT-derived tumors (data not shown) (38, 68, 70, 78, 81, 85). We also assessed the expression of the β4 integrin subunit as a marker of the epithelial content of the tumors and found equivalent expression levels, supporting that the overall tumor/stromal ratios were not altered by the loss of Irs-1 (48) (Fig. 1C). Total Irs-2 expression did not increase significantly in PyV-MT::*Irs1*$^{-/-}$ tumors to compensate for the absence of Irs-1 (Fig.

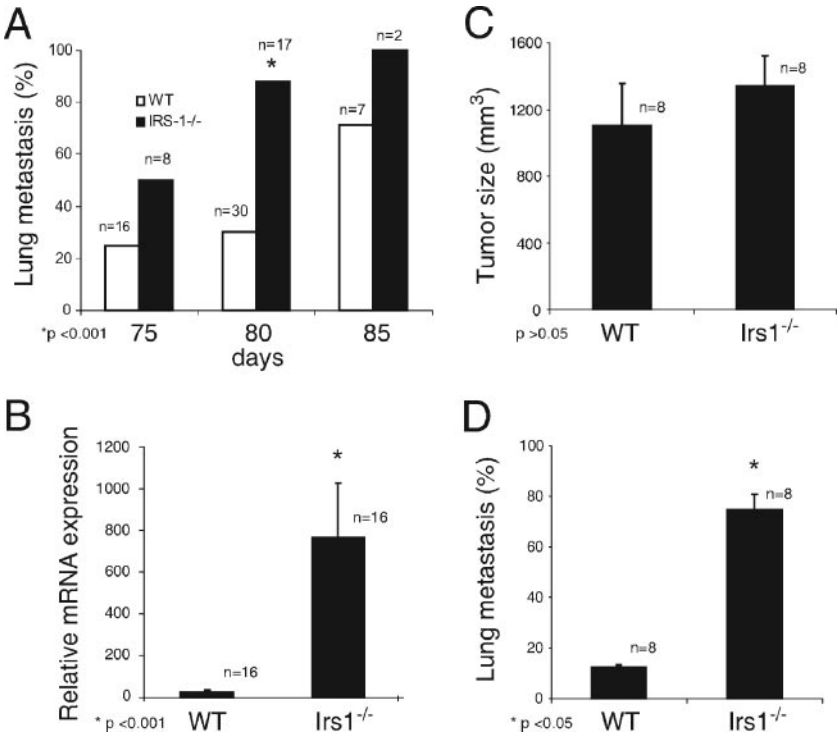

FIG. 3. Analysis of Irs-1 involvement in PyV-MT mammary tumor metastasis. (A) Lungs from female PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice were sectioned and screened microscopically for the presence of metastatic lesions. Five representative sections from each lung were analyzed. The percentage of mice that scored positively for metastatic lesions at each time point (75, 80, and 85 days) is shown (for WT versus *Irs1*$^{-/-}$ results at 80 days, $P < 0.001$). (B) PyV-MT mRNA was amplified and quantified from the lungs of 80-day-old PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mice by RQ-PCR. The data shown are the mean mRNA expression levels (± SEM) from 16 mice of each genotype. ($P < 0.001$.) (C) Mean tumor burden (± SEM) of PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors grown orthotopically in the mammary fat pads of female nu/nu mice. The number of mice analyzed is indicated. ($P > 0.05$.) (D) Lungs from mice with PyV-MT and PyV-MT::*Irs1*$^{-/-}$ orthotopic tumors were sectioned and screened microscopically for the presence of metastatic lesions. Five representative H&E sections from each lung were analyzed. The percentage of the mice that scored positively for metastatic lesions is shown. ($P < 0.05$.) WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice.

1C). Finally, PyV-MT expression was equivalent in the PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors (data not shown).

Analysis of the PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors at the earliest time of palpable detection revealed that the tumors arose in a multifocal manner in both genetic backgrounds (Fig. 2A and B). High-grade tumors developed in both groups of mice, with areas of solid tumor nests lacking significant glandular structure and containing large, pleiotropic nuclei with little cytoplasm (Fig. 2C and D). Invasion of tumor cells into the surrounding stromal tissue was observed more frequently in the PyV-MT::*Irs1*$^{-/-}$ tumors than in the tumors from PyV-MT mice (data not shown). These in vivo results are consistent with our previously published findings demonstrating that *Irs1*$^{-/-}$ mammary tumor cells are more invasive in vitro in Matrigel invasion assays than wild-type mammary tumor cells, and they support that loss of Irs-1 promotes invasive potential (52). The histology and behavior of the PyV-MT::*Irs1*$^{-/-}$ tumors contrasts with the more well-differentiated histology that we observed in the absence of Irs-2, suggesting that Irs-1 may play a suppressive role in later stages of tumor progression (52).

Loss of Irs-1 promotes mammary tumor metastasis in a cell-autonomous manner. PyV-MT mammary tumors are characterized by a high incidence of lung metastasis (24). To test our hypothesis that Irs-1 regulates mammary tumor metastasis, we microscopically screened five independent H&E-stained sections of the lungs from each of the PyV-MT or PyV-MT::*Irs1*$^{-/-}$ mice. An increased incidence of lung metastasis was observed for the PyV-MT::*Irs1*$^{-/-}$ mice compared with results for the PyV-MT mice at all time points examined (75, 80, and 85 days). We expanded the number of mice at the 80-day time point, and the difference in metastasis was found to be statistically significant (WT versus *IRS-1*$^{-/-}$, $P < 0.001$; Fig. 3A). To quantify the differences in metastasis, PyV-MT mRNA expression in the lungs of the 80-day mice was measured using real-time PCR. PyV-MT is not expressed in the lungs of normal mice, and the amount of PyV-MT mRNA is a measure of tumor burden in this tissue (52). A significantly higher level of PyV-MT mRNA expression was measured in the lungs from PyV-MT::*Irs1*$^{-/-}$ mice than in the lungs from PyV-MT mice ($P < 0.001$; Fig. 3B). These results confirm that Irs-1 deficiency increases mammary tumor metastasis.

The ability of tumors to grow and metastasize is influenced by stromal cells and other factors in the tumor microenvironment, as well as systemic conditions (82). Many stromal cells, including fibroblasts, endothelial cells, and immune cells, express IRS-1, and the absence of IRS-1 protein in these diverse cell types could influence tumor metastasis. To determine if the impact of Irs-1 deficiency on metastasis is an intrinsic property of the tumor cells in the PyV-MT::*Irs1*$^{-/-}$ mice, the

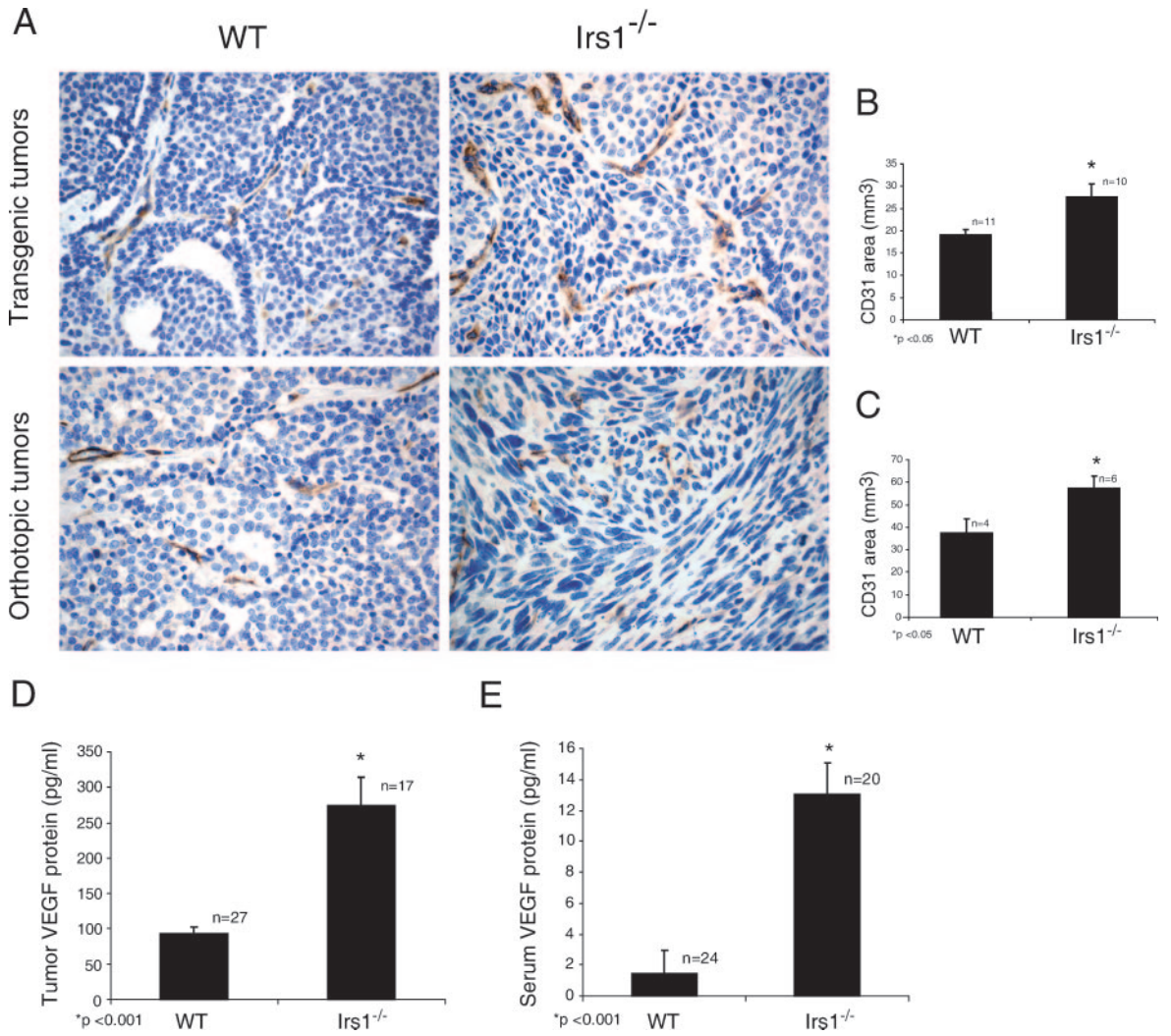

FIG. 4. Analysis of tumor angiogenesis in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumors. (A) PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors from transgenic and nu/nu mice were stained with antibodies specific for CD31 to detect tumor angiogenesis. (B and C) Quantification of angiogenesis in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic (B) or orthotopic (C) mammary tumors. The results shown are the mean area of CD31 staining (± SEM). The number of mice analyzed is indicated. Magnification, ×400. (D and E) Quantification of VEGF-A protein levels in the tumors (D) or serum (E) of PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic mice. The data shown are the mean concentrations of VEGF-A (± SEM). WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice.

ability of *Irs1*$^{-/-}$ mammary tumor cells to develop tumors and metastasize in an *Irs1*$^{+/+}$ genetic background was examined. When PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumor cells were injected into the mammary fat pad of female nu/nu mice, 100% of the mice developed tumors. In contrast to the equivalent growth rates observed with the transgenic null mice, PyV-MT::*Irs1*$^{-/-}$ tumors grew faster than PyV-MT tumors when grown orthotopically, suggesting that loss of Irs-1 in the stromal microenvironment may negatively influence tumor growth. An increased incidence of lung metastasis was detected for the mice with PyV-MT::*Irs1*$^{-/-}$ tumors compared with results for mice with PyV-MT tumors of an equivalent size (Fig. 3C and D). These results demonstrate that the increased metastasis observed in the transgenic PyV-MT::*Irs-1*$^{-/-}$ mice did not result from systemic or stromal alterations but was due to an absence of Irs-1 expression in the mammary tumor cells. Similar to the tumors from the knockout mice, both PyV-MT and PyV-MT::*Irs1*$^{-/-}$ orthotopic tumors were solid in overall appearance, and the *Irs1*$^{-/-}$ tumors had a greater frequency of stromal invasion (Fig. 4). Taken together, our results suggest that IRS-1 may have metastasis suppressor functions in breast cancer.

Loss of Irs-1 expression enhances tumor angiogenesis by increasing VEGF protein expression. One hallmark of metastatic tumors is their ability to stimulate robust tumor angiogenesis (26). To determine if the enhanced metastasis we observed in the absence of Irs-1 corresponded to a stimulation of angiogenesis, we assessed the microvascular density in the PyV-MT::*Irs1*$^{-/-}$ and PyV-MT tumors by staining for the endothelial cell-specific marker CD31 (67). Tumor vascular densities were significantly higher in PyV-MT::*Irs1*$^{-/-}$ tumors than in PyV-MT tumors, whether established in PyV-MT transgenic mice or as orthotopic tumors in nu/nu mice ($P < 0.05$ for both transgenic and orthotopic tumors) (Fig. 4A to C).

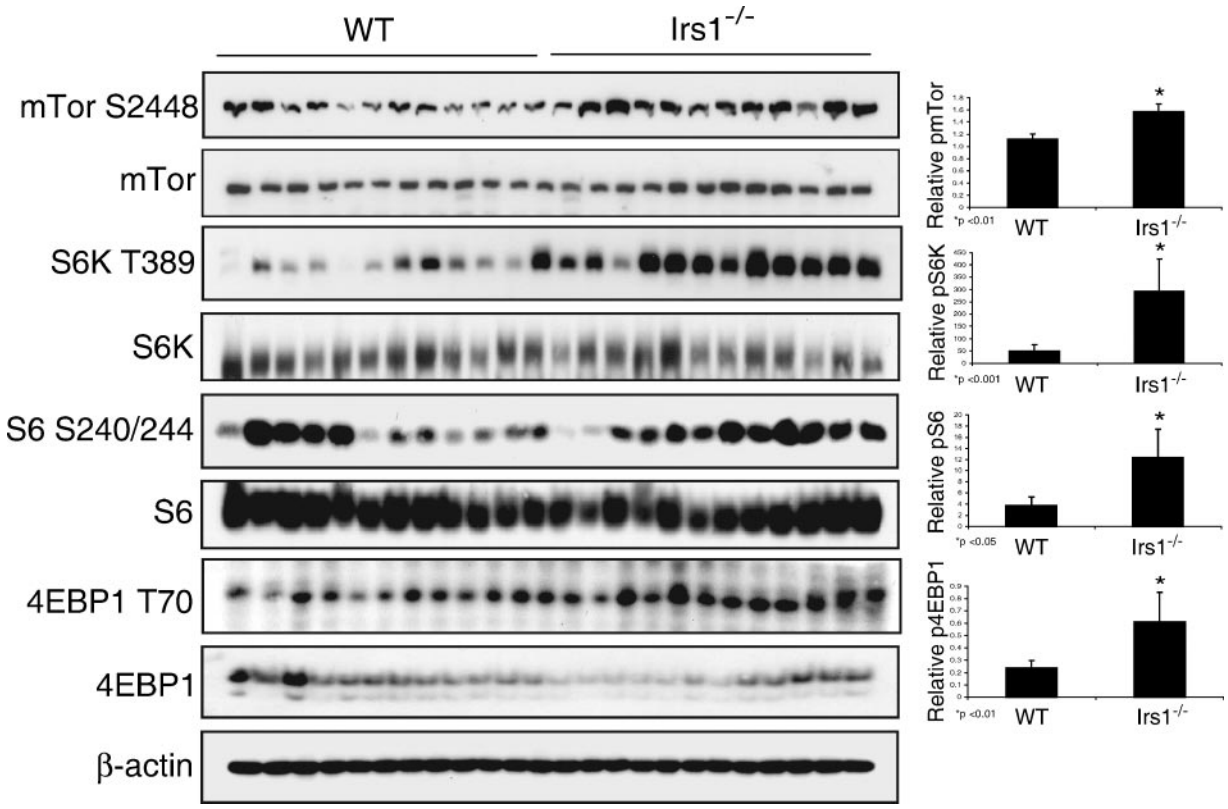

FIG. 5. Evaluation of mTor activation and signaling in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic mammary tumors. Aliquots of tumor extracts from PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumors containing equivalent amounts of total protein were immunoblotted with antibodies specific for phosphoserine-2448 of mTor (mTor S2448), total mTor, phosphothreonine-389 of S6 kinase (S6K T389), total S6 kinase, phosphoserine-240/244 of S6 (S6 S240/244), total S6, phosphothreonine-70 of 4EBP1 (4EBP1 T70), total 4EBP1, or β-actin. For densitometry, $n = 12$ for each genotype. The relative levels of phosphorylation are shown in the graphs to the right of each immunoblot pair. WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice.

To address the mechanism by which angiogenesis was enhanced in Irs-1-deficient tumors, VEGF-A protein levels were measured in tumors from PyV-MT or PyV-MT::*Irs1*$^{-/-}$ mice by ELISA. VEGF-A expression was significantly higher in PyV-MT::*Irs1*$^{-/-}$ tumors than in PyV-MT tumors ($P < 0.001$) (Fig. 4D). VEGF-A protein levels were also significantly elevated in the serum of the PyV-MT::*Irs1*$^{-/-}$ tumor-bearing mice ($P < 0.001$) (Fig. 4E). VEGF expression can be regulated by both transcriptional and posttranscriptional mechanisms (43, 45). To determine whether the increased protein levels in the PyV-MT::*Irs1*$^{-/-}$ mice resulted from enhanced mRNA transcription, RQ-PCR was performed using primers that recognize all isoforms of VEGF-A. The level of VEGF-A mRNA in the PyV-MT::*Irs1*$^{-/-}$ tumors did not differ significantly from the level of VEGF-A mRNA in the PyV-MT tumors, indicating that VEGF-A expression is increased at the posttranscriptional level in the absence of Irs-1 (data not shown).

Activation of mTor signaling in Irs-1$^{-/-}$ mammary tumors. The fact that VEGF-A expression is increased in PyV-MT::*Irs1*$^{-/-}$ tumors in the absence of an increase in mRNA expression led us to hypothesize that VEGF expression may be controlled through an increase in protein translation. To investigate the mechanism by which VEGF translation could be regulated, we examined the activation of mTor, a central regulator of the translational machinery (28). To do so, we evaluated the phosphorylation of mTor on serine-2448 (S2448) in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumor extracts, because phosphorylation at this site is indicative of mTor activation (10, 30). As shown in Fig. 5, phosphorylation of mTor S2448 was significantly increased in PyV-MT::*Irs1*$^{-/-}$ tumors compared with results for PyV-MT tumors. To examine further if the mTor pathway was activated at higher levels in the absence of Irs-1, we assessed the activation status of two major downstream effectors of mTor, p70-S6 kinase (S6K) and 4EBP1, both of which are involved in regulating protein synthesis. S6K is phosphorylated on threonine-389 by mTor, and this phosphorylation event promotes full activation of the kinase to phosphorylate the ribosomal protein S6 (56). 4EBP1 is phosphorylated on multiple residues, of which threonine-70 is mTor dependent (20). Phosphorylation of 4EBP1 promotes its dissociation from the translation initiation factor eIF4E, thereby promoting cap-dependent translation (21). Both S6K and 4EBP1 were phosphorylated at significantly higher levels in the PyV-MT::*Irs1*$^{-/-}$ tumors than in the PyV-MT tumors, confirming that mTor-dependent signaling is elevated in Irs-1-deficient tumors (Fig. 5). In addition, S6 phosphorylation was increased significantly in the PyV-MT::*Irs1*$^{-/-}$ tumors, providing evidence for enhanced S6K activation (Fig. 5).

Cell survival is increased in Irs-1-deficient mammary tumors. In addition to its important role as a translational

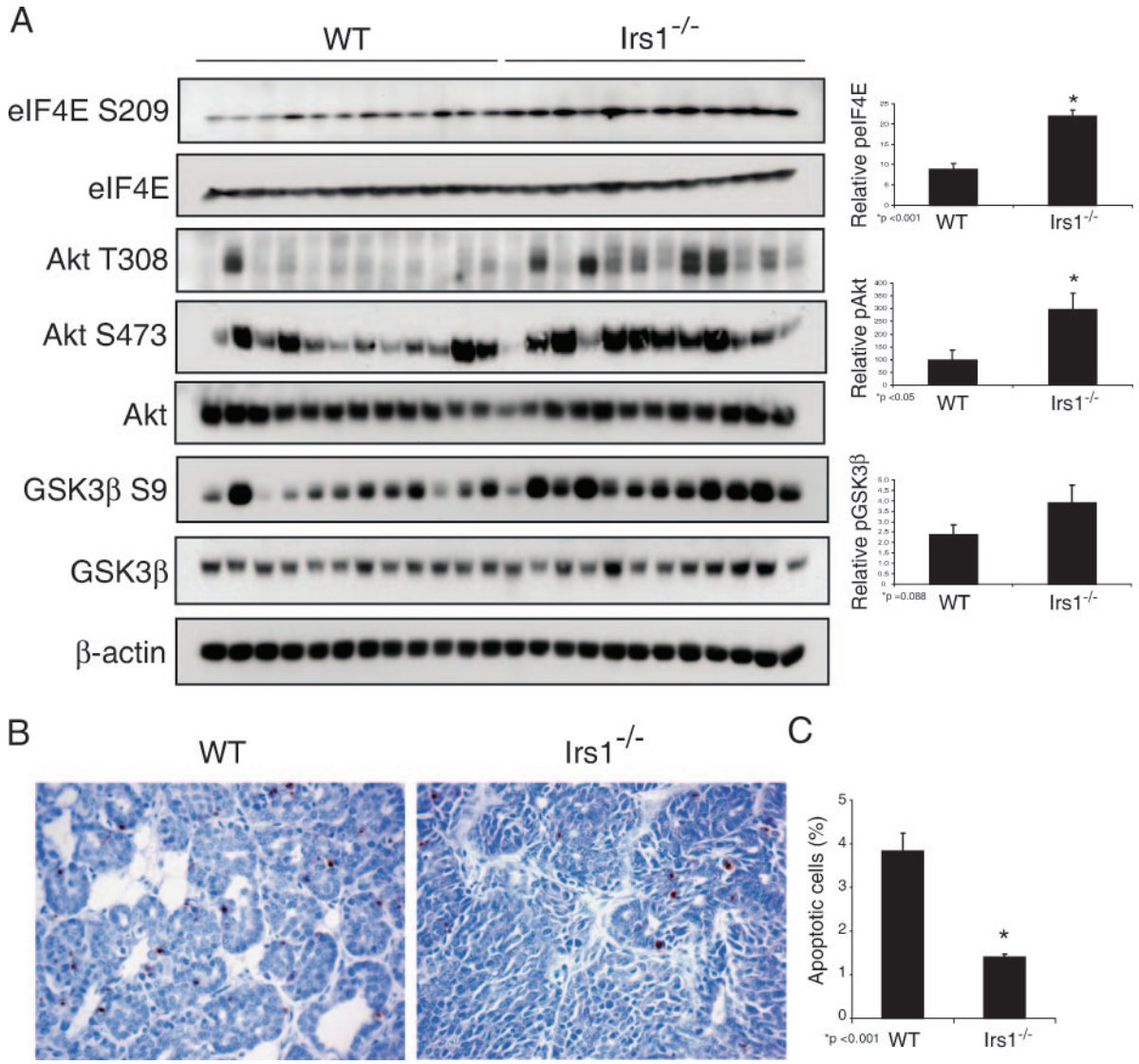

FIG. 6. Absence of Irs-1 increases tumor cell survival. (A) Aliquots of tumor extracts from PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic mammary tumors containing equivalent amounts of total protein were immunoblotted with antibodies specific for phosphoserine-209 of eIF4E (eIF4E S209), total eIF4E, phosphothreonine-308 of Akt (Akt T308), phosphoserine-473 of Akt (Akt S473), total Akt, phosphoserine-9 of GSK-3β (GSK3β S9), total GSK-3β, or β-actin. For densitometry, $n = 12$ for each genotype except for GSK-3β ($n = 11$). The relative levels of phosphorylation are shown in the graphs to the right of each immunoblot pair. (B and C) Terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling analysis of PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic mammary tumors. (B) Representative images from PyV-MT and PyV-MT::*Irs1*$^{-/-}$ mammary tumors stained with Apoptag reagent are shown (magnification, ×400). (C) The percentages of ApopTag-positive nuclei in the tumor sections were determined using the following formula: number of ApopTag-positive nuclei/total nuclei ($P < 0.01$). $n = 20$ fields (4 random fields from 5 tumors) for each group (PyV-MT or PyV-MT::*Irs1*$^{-/-}$ mice). WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice.

regulator, eIF4E has also been implicated in oncogenesis through its contribution to survival signaling (72, 80). To determine if eIF4E activity was increased in PyV-MT::*Irs1*$^{-/-}$ tumors, we evaluated the phosphorylation of eIF4E on serine-209. As shown in Fig. 6A, eIF4E phosphorylation was significantly elevated in PyV-MT::*Irs1*$^{-/-}$ tumors compared with results for PyV-MT tumors. We also assessed the status of Akt signaling in the tumors, because Akt is a central regulator of survival signaling pathways and it is commonly activated in many types of cancer (41, 44, 72, 77, 80). Moreover, Akt is an upstream regulator of mTor function (31, 47). Akt activity is regulated by phosphorylation on two key residues, serine-473 and threonine-308 (2, 63). Both residues were phosphorylated at higher levels in PyV-MT::*Irs1*$^{-/-}$ tumors than in PyV-MT tumors (Fig. 6A). In addition, phosphorylation of GSK-3β on serine-9, an Akt-dependent site, was also significantly increased, confirming that Akt activity was enhanced in Irs-1-deficient tumors (Fig. 6A) (13).

To evaluate directly if cell survival was increased in PyV-MT::*Irs1*$^{-/-}$ tumors, apoptosis was assessed in PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumor sections by ApopTag staining. Although apoptotic cells were present in tumors from both genetic backgrounds, the percentage of apoptotic cells was markedly decreased in the PyV-MT::*Irs1*$^{-/-}$ tumors ($P <$

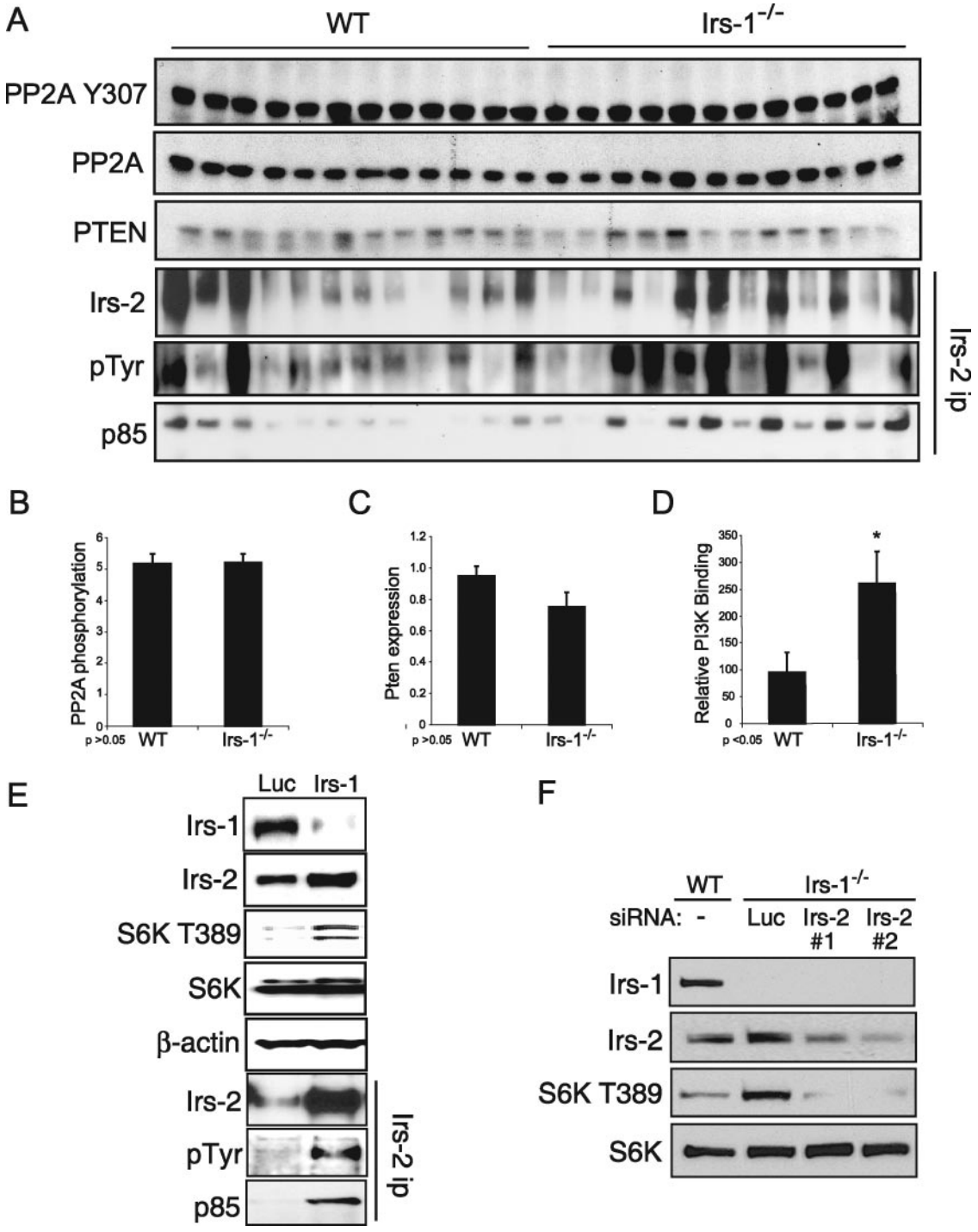

FIG. 7. Irs-2-dependent signaling in the absence of Irs-1. (A) Aliquots of tumor extracts from PyV-MT and PyV-MT::*Irs1*$^{-/-}$ transgenic mammary tumors containing equivalent amounts of total protein were immunoblotted with antibodies specific for phosphotyrosine-307 of the catalytic subunit of PP2A (PP2A Y307), total catalytic subunit of PP2A, or PTEN. Aliquots of tumor extracts were also immunoprecipitated with an antiserum specific for IRS-2, and the immune complexes were immunoblotted with phosphotyrosine-specific antibodies (RC-20). The pTyr immunoblots were subsequently stripped and reprobed with Irs-2-specific and p85-specific polyclonal antisera. (B) Densitometric analysis of relative PP2A phosphotyrosine levels ($n = 12$ for each genotype; $P > 0.05$). (C) Densitometric analysis of relative PTEN expression ($n = 12$ for each genotype; $P > 0.05$). (D) Densitometric analysis of p85 binding to Irs-2. ($n = 11$ for each genotype; $P < 0.05$). WT, PyV-MT mice; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ mice (E) PyV-MT tumor cells were transfected with either a control luciferase siRNA (Luc) or Irs-1-specific siRNAs (Irs-1). Aliquots containing equivalent amounts of total protein were immunoblotted with antibodies specific for Irs-1, Irs-2, phosphosthreonine-389 of S6 kinase (S6K T389), total S6K, or β-actin (upper panels). Aliquots of the cell extracts containing equivalent amounts of total protein were immunoprecipitated with an Irs-2-specific antiserum and immunoblotted with phosphotyrosine-specific antibodies (4G10). The immunoblots were subsequently stripped and reprobed with Irs-2- and p85-specific antibodies (lower panels). (F) PyV-MT::*Irs1*$^{-/-}$ tumor cells were transfected with either a control luciferase siRNA (Luc) or two different Irs-2-specific siRNAs (Irs-2 #1 and Irs-2 #2). Aliquots containing equivalent amounts of total protein were immunoblotted with antibodies specific for Irs-2, Irs-1, phosphothreonine-389 of S6 kinase (S6K T389), or total S6K. WT, PyV-MT cells; *Irs1*$^{-/-}$, PyV-MT::*Irs1*$^{-/-}$ cells.

0.001) (Fig. 6B and C). Taken together, our results provide evidence that Irs-1 deficiency triggers the activation of tumor cell survival signaling pathways that may facilitate tumor cell metastasis.

Irs-2 activation in the absence of Irs-1. IRS-1 contains multiple PI3K binding sites and can participate in the activation of Akt and mTor in many model systems (51, 81). However, the activation of these signaling pathways was increased when Irs-1 expression was abolished, which raises the question as to how Akt and mTor activation are enhanced in the absence of Irs-1. Reduced expression of PTEN, a lipid phosphatase that negatively regulates PI3K signaling, or decreased activation of PP2A, a serine-threonine phosphatase that negatively regulates both Akt and S6K, would enhance Akt/mTor signaling (49, 55). Mutation of PTEN has been reported to compensate for the absence of IRS-1 in prostate carcinoma cells (59). However, PTEN expression and PP2A activation were not significantly different in the PyV-MT and PyV-MT::*Irs1*$^{-/-}$ tumors (Fig. 7A to C). In contrast to the increased metastasis that we observed in PyV-MT::*Irs1*$^{-/-}$ mammary tumors, we have previously demonstrated that Irs-2-deficient mammary tumors have a reduced metastatic potential (52). We hypothesized that Akt and mTor signaling could be elevated in Irs-1-deficient tumors through increased Irs-2 function. In support of this mechanism, Irs-2 was more heavily tyrosine phosphorylated in the PyV-MT::*Irs1*$^{-/-}$ tumors than in the PyV-MT tumors (Fig. 7A). Importantly, the amount of p85 regulatory subunit of PI3K that coimmunoprecipitated with Irs-2 was significantly higher in PyV-MT::*Irs1*$^{-/-}$ tumors, indicating that Irs-2-dependent PI3K activation was increased ($P < 0.05$) (Fig. 7A and D).

To investigate further the connection between Irs-1 and Irs-2 expression and to determine if Irs-1 directly influences Irs-2 expression and function, we transiently knocked down Irs-1 expression in PyV-MT mammary tumor cells using siRNA oligonucleotides. Irs-2 expression increased in tumor cells when Irs-1 expression was suppressed (Fig. 7E). Irs-2 tyrosine phosphorylation and association with PI3K and activation of the downstream effector S6K also increased when Irs-1 expression was downregulated, as we had observed with the PyV-MT::*Irs1*$^{-/-}$ mammary tumors. To investigate the contribution of Irs-2 to the enhanced activation of Akt/mTor signaling pathways in the absence of Irs-1, we suppressed Irs-2 expression transiently in *Irs1*$^{-/-}$ tumor cells. When Irs-2 expression was knocked down to wild-type levels in the PyV-MT::*Irs1*$^{-/-}$ tumor cells, S6K activation was also restored to the level observed in wild-type cells (Fig. 7F). These in vitro results support that Irs-2 compensates for the loss of Irs-1 and in doing so enhances the activation of signaling pathways that promote tumor metastasis.

Irs-1 is inactivated in metastatic tumors. The results from our PyV-MT tumor studies demonstrate that mammary tumor cells have a greater metastatic potential in the absence of Irs-1 expression. To investigate the physiological relevance of our findings for human breast cancer metastasis, we assessed whether Irs-1 expression or function was suppressed in metastatic mammary tumors. Irs-1 expression was maintained in PyV-MT metastatic tumors at levels equivalent to or higher than those in nonmetastatic (PyV-MT::*Irs2*$^{-/-}$) tumors. IRS-1 function can be negatively regulated by serine phosphorylation, which prevents tyrosine phosphorylation and blocks the interaction of IRS-1 with downstream effectors, effectively inhibiting downstream signaling (22). To investigate if Irs-1 function is inactivated in metastatic tumors, we examined the phosphorylation status of several serine residues in Irs-1. Irs-1 was consistently phosphorylated on all of the serine residues that we examined in the metastatic tumors (Fig. 8). In contrast, serine phosphorylation of Irs-1 in the nonmetastatic tumors was sporadic, and none of the serine residues was consistently phosphorylated in the tumors we examined. To determine the effect of serine phosphorylation on Irs-1 function in the metastatic tumors, Irs-1 tyrosine phosphorylation was assessed. The level of Irs-1 tyrosine phosphorylation in the metastatic tumors was markedly lower than that observed for Irs-1 in the nonmetastatic tumors, demonstrating that Irs-1 function was suppressed in metastatic tumors (Fig. 8).

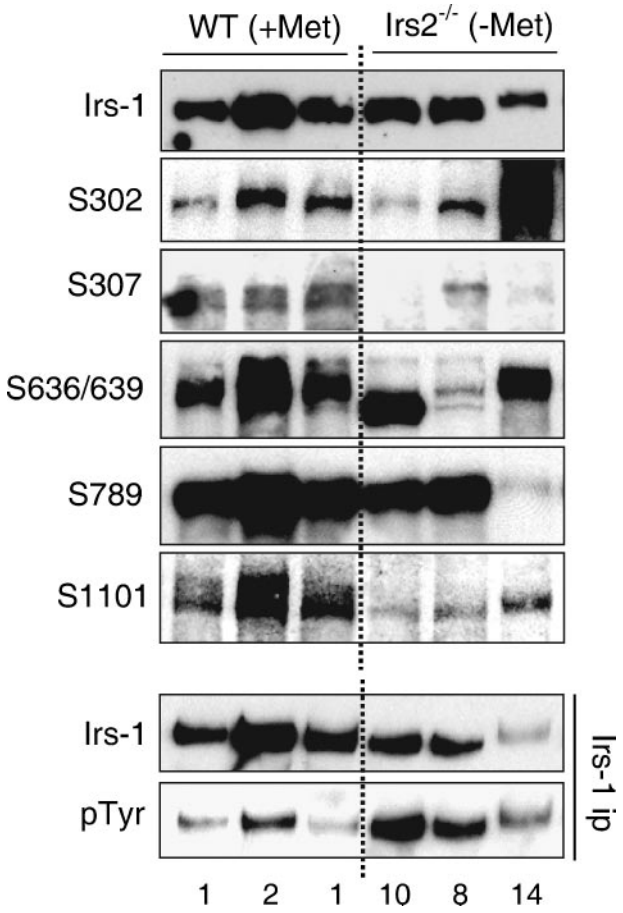

FIG. 8. Irs-1 is inactivated in metastatic PyV-MT mammary tumors. Aliquots of tumor extracts from PyV-MT and PyV-MT::*Irs2*$^{-/-}$ mammary tumors containing equivalent amounts of total protein were immunoblotted with antibodies specific for total Irs-1 or phosphoserine Irs-1 (S302, S307, S636/S639, S789, and S1101) (upper panels). Aliquots of tumor extracts from WT and *Irs2*$^{-/-}$ transgenic mammary tumors containing equivalent amounts of total protein were immunoprecipitated with an Irs-1-specific antiserum and then immunoblotted with phosphotyrosine-specific antibodies. The immunoblots were subsequently stripped and reprobed with Irs-1-specific antibodies (lower panels). The numbers below indicate the relative levels of Irs-1 tyrosine phosphorylation in each tumor. WT, PyV-MT mice; *Irs2*$^{-/-}$, PyV-MT::*Irs2*$^{-/-}$ mice.

DISCUSSION

In this study, we demonstrate that mammary tumor metastasis is enhanced in the absence of Irs-1. Specifically, mammary tumor latency and growth were not altered in Irs-1-deficient mouse mammary tumor virus–PyV-MT transgenic mice compared with those for wild-type littermates, but lung metastasis increased significantly in the absence of Irs-1 expression. The increased metastatic potential of Irs-1-deficient tumor cells was maintained when these cells were grown orthotopically in the mammary fat pad of nu/nu mice, supporting a cell-autonomous function for Irs-1 in mammary tumor metastasis. Activation of Irs-2 was enhanced in Irs-1-deficient mammary tumors, a novel finding that contributes to a growing body of evidence that Irs-2 is a positive regulator of tumor progression. Irs-1-deficient tumors are characterized by enhanced activation of the Akt and mTor signaling pathways, as well as by increased VEGF expression and microvascular density. Importantly, we demonstrate that Irs-1 function is suppressed in wild-type metastatic mammary tumors. This result validates our findings from the Irs-1-deficient mice and supports the novel hypothesis that IRS-1 has metastasis suppressor functions for breast cancer.

A novel and unexpected finding from our study is that Irs-2 activity increased in Irs-1-deficient mammary tumors. This activation of Irs-2, as indicated by increased tyrosine phosphorylation and association with PI3K, was coincident with an enhanced activation of Akt and mTor. Knock-down of Irs-2 restored the activation of these signaling molecules to wild-type levels, supporting that Irs-2 is responsible for their enhanced activation in the absence of Irs-1. Our observed increase in Irs-2 function was not anticipated, because activation of mTor signaling has been shown to inhibit IRS expression and function (18, 69). Negative feedback regulation of the IRS proteins was first demonstrated in insulin-dependent signaling, and this feedback pathway is essential for regulating insulin sensitivity and glucose homeostasis (1, 39, 74). Serine phosphorylation of the IRS proteins by multiple kinases, including the mTor-dependent p70-S6 kinase, can interfere with their function by targeting these docking proteins for inactivation and/or proteasomal degradation (22). Inactivation occurs either through inhibition of recruitment to upstream regulatory receptors or through disruption of interactions with downstream effectors (22). In tumors that arise from mutations in pathways that impede mTor signaling, such as the TSC1 or TSC2 genes, p70-S6 kinase is constitutively active (27, 69). These tumors grow quite large but rarely progress to invasive carcinoma, because both IRS-1 and IRS-2 are degraded in response to S6 kinase-mediated phosphorylation (46, 69). In contrast to the benign nature of the TSC1/2 mutant tumors, we observed that Irs-1-deficient tumors, which have increased mTor signaling, progress more rapidly to metastasis. We hypothesize that the reason for this discrepancy is that Irs-2 is resistant to negative feedback regulation, which leads to enhanced function in the Irs-1-deficient tumors.

The fact that Irs-2 activation is enhanced in Irs-1-deficient tumors that are more metastatic substantiates our previous finding that Irs-2 is a positive regulator of mammary tumor progression. Specifically, we demonstrated that mammary tumor metastasis was significantly diminished in the absence of Irs-2 (52). One mechanism by which IRS-2 is likely to contribute to tumor metastasis is through its ability to regulate cell motility. Irs-2-deficient mammary tumor cells are less invasive, and Irs-1-deficient tumor cells, which signal exclusively through Irs-2, are more invasive, both in vitro and in vivo, than their wild-type counterparts (52). IGF-1 promotes cell motility and invasion in breast carcinoma cell lines that express IRS-2 but not in cell lines that express only IRS-1 (14, 33, 86). Similar influences of the IRS proteins on motility have been found in other types of tumor cell lines. Specifically, IRS-2-dependent signaling promotes cell motility and invasion in neuroblastoma and mesothelioma cells, while expression of IRS-1 in LNCaP prostate carcinoma cells decreases motility (29, 34, 59). In addition to being more motile, mammary tumor cells that express only Irs-2 are also resistant to stress-induced apoptosis. Therefore, a second mechanism by which IRS-2-dependent signaling contributes to metastasis is by regulating survival signaling pathways (52). In fact, enhanced IRS-2 signaling could explain the discrepancy between our data showing that IRS-1 deficiency promotes tumor cell survival and previous studies reporting that "knock-down" of IRS-1 expression in MCF-7 breast carcinoma cells increases apoptosis. MCF-7 cells express predominantly IRS-1, and when IRS-1 is suppressed in these cells, IRS-2 is not present to compensate for the loss (9, 53). However, in PyV-MT mammary tumor cells, like ER− human breast carcinoma cells, Irs-2 is expressed and its function is enhanced, which leads to an increased activation of PI3K, a major upstream regulator of signaling pathways that regulate both cell survival and invasion. Given that both IRS-1 and IRS-2 can recruit and activate PI3K, other factors must influence the outcomes of PI3K signaling downstream of these two adaptor proteins, or IRS-1 would be capable of promoting metastasis to the same extent as IRS-2. Distinct intracellular compartmentalization of IRS-1 and IRS-2 can impact the functional outcome of signals transmitted through these adaptor proteins (17). Furthermore, differential sensitivities of IRS-1 and IRS-2 to negative feedback regulation could alter the longevity and intensity of signals initiated through each protein, modifying cellular responses to PI3K activation and impacting tumor metastasis.

Our conclusion that IRS-1 impedes metastasis, which was obtained using Irs-1-deficient mice, implies that mechanisms exist in tumors to suppress IRS expression or function to promote progression. Indeed, a major observation from our study is that Irs-1 is expressed in metastatic wild-type mammary tumors but that it is phosphorylated on serine residues and inactivated. This finding raises the novel hypothesis that IRS-1 functions as a metastasis suppressor in breast cancer. Taken together with our previous work showing that IRS-2 promotes metastasis, it appears that the IRS proteins are prime candidates for prognostic markers for breast cancer progression. Interestingly, however, neither IRS-1 nor IRS-2 has been identified as a marker of tumor progression or metastasis by microarray analysis, supporting our conclusion that changes in IRS function occur primarily at the level of protein expression or activation and not through changes in gene expression. To date, IRS-2 protein expression in human breast tumors has not been evaluated, and only a few studies have directly assessed IRS-1 protein expression. One study reported strong down-regulation of IRS-1 in grade 3, poorly differentiated breast

tumors, confirming our prediction for a positive correlation between low IRS-1 and poor prognosis (64). However, separate studies have reported that high IRS-1 expression is predictive of a greater incidence of recurrence and a decreased patient survival rate (36, 60). A caveat to these last reports that is highlighted by our own data is that overall expression of IRS-1 or IRS-2 may not reflect the activation status of these docking proteins, and IRS-1 may be expressed, but not active, in these tumors with a poor prognosis. In future studies, it will be important to identify specific serine or tyrosine phosphorylation events that can be used to assess functional activity of these proteins in tumors to evaluate the prognosis more accurately.

Our molecular analysis of Irs-1-deficient tumors revealed that they express significantly more VEGF-A than do wild-type tumors and that VEGF-A is increased in the serum of Irs-deficient tumor-bearing mice. These tumors also exhibited a significant increase in microvessel density, which is consistent with increased VEGF expression. The possibility that increased VEGF-A expression and microvessel density in response to loss of Irs-1 promote progression is supported by numerous studies on human breast tumors that have correlated these parameters with disease progression and negative outcome, as well as by recent transgenic studies in which overexpression of VEGF-A in the mammary gland resulted in increased tumor metastasis in mice (12, 54, 65, 75). VEGF contributes to metastasis in a paracrine manner by promoting tumor angiogenesis and also acts in an autocrine manner to support the survival of metastatic tumor cells outside of their normal microenvironment, where exogenous stimuli are absent and intrinsic survival mechanisms are required (4, 5, 65). Given our finding that mTor activation is enhanced in Irs-$1^{-/-}$ tumors and the fact that mTor is a key regulator of protein translation, it is feasible that mTor-dependent VEGF translation is stimulated in the absence of Irs-1. In fact, mTor-dependent regulation of VEGF-A translation was recently reported for tumors that develop in response to overexpression of the Her2/Neu oncogene (35). mTor is emerging as an essential regulator of tumorigenesis and progression in many types of cancer, including breast cancer, and the notion that protein translation can be a regulatory checkpoint for tumor progression is gaining support (23, 57). Indeed, many oncogenes, growth regulatory genes, and survival genes that are associated with cancer are regulated at the level of translation initiation (45, 61). Thus, it is likely that in addition to VEGF-A, other proteins that play important roles in promoting tumor metastasis are up-regulated in a translation-dependent manner in response to increased mTor activity when IRS-1 is inactivated in tumors.

The relevancy of our studies for human breast cancer is highlighted by the fact that mouse mammary tumors that develop in response to the PyV-MT oncogene in an Irs-1-deficient background are phenotypically similar to aggressive, advanced human breast tumors that are associated with poor patient prognosis. Elevated levels of VEGF-A in tumors and sera correlate with a poor prognosis for breast cancer patients (12, 19, 62). Akt activation is positively associated with disease progression and occurs early, at the ductal carcinoma in situ stage, and persists throughout tumor development (7). mTor and its downstream effector p70-S6 kinase are both expressed and activated at high levels in invasive human breast carcinomas, and both of these signaling molecules are associated with an increased risk for disease recurrence (7, 35, 76, 87). Expression of the translation initiator eIF4E is also upregulated in human cancers, including breast, and it has been linked to metastasis (8, 58). In the absence of Irs-1, mouse mammary tumors exhibit increased activity of all of these signaling molecules, confirming that at the molecular level, loss of Irs-1 closely mimics the progression of human breast cancer to metastatic disease.

In summary, we have established that suppression of Irs-1 promotes mammary tumor metastasis, and we provide evidence that Irs-1 is inactivated in metastatic tumors. Taken together with our previous study demonstrating that loss of Irs-2 impedes mammary tumor metastasis, we now have strong evidence that IRS-1 and IRS-2 play distinct roles in breast cancer metastasis. Our novel findings support the possibility that IRS activity, rather than expression, may be a valuable predictive indicator of metastasis in human breast cancer.

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