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14. ABSTRACT In this project, we have analyzed the role of the IGF signaling system in prostate cancer initiation and progression. Our original hypothesis stated that, since the IGF system is an important factor in tumorigenesis and prostate cancer, that genes and proteins that were differentially expressed in cells that expressed different levels of the IGF-I receptor that mediates the intracellular effects of the IGFs may themselves constitute potential diagnostic factors or therapeutic targets. During the period of this award, we have analyzed differential gene expression and protein secretion in genetically engineered metastatic and non-metastatic prostate cancer cells expressing different levels of the IGF-IR. These studies were in direct support of the original statement of work. In addition, we have: 1) defined the transcriptional regulation of the IGF-I gene; 2) reported the regulation of IGF signaling by saw palmetto; 3) described the interaction between the IGF-IR and the androgen receptor; 4) identified a novel product of the Her2 gene that regulates IGF action; and 5) reviewed the role of IGF action in prostate cancer. These findings are represented by the peer-reviewed publications summarized in this final report and provided as appendices.					
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Introduction

This project was aimed at elucidating the role of insulin-like growth factor (IGF) action, specifically that of the cell-surface IGF-I receptor, in prostate cancer initiation, progression, and metastasis. The statement of work entailed a combined geneomic and proteomic analysis of IGF-regulated genes and secreted proteins that were associated with metastasis. Specifically, since variation in the levels of the IGF-I receptor regulates in vitro metastasis in prostate cancer cells, the effect of IGF-I receptor expression levels in non-metastatic and metastatic prostate cancer cells was examined using microarray gene profiling and surface-enhanced laser-desorption/ionization-time-of flight (SELDI-TOF) mass spectrometry. These data and ongoing extensions of these studies are described below.

In addition to the studies directly mandated by the statement of work, we also completed a number of related studies and efforts under during the project period that acknowledge their support by this award. These additional studies comprised analyses of the transcriptional regulation of IGF-I receptor gene expression, regulation of IGF signaling by dietary factors utilized as therapies, the relationship between IGF-IR and androgen receptor action, and the description of a novel mechanism of coordinate regulation of IGF and EGF signaling in cancer. These various studies are summarized below, and the pertinent manuscripts are included as appendices.

Body

Results in support of original statement of work.

The original statement of work for this project comprised the following tasks:

Task 1: Completion of microarray analysis, months 1-6 (Specific aim 1).

Task 2: Completion of preliminary SELDI-TOF analyses of conditioned media from M12-LISN and M12-LNL6 cultures, months 1-12 (Specific aim 2).

Task 3: Generation of probes for Northern and/or RPA analysis and verification of differential gene expression in M12-LISN and M12-LNL6 cells, months 6-12 (Specific aim 1).

Task 4: In-gel purification and proteolysis or on-chip proteolysis of differentially secreted proteins and tandem mass spec analysis, months 13-24 (Specific aim 2).

Task 5: Generation of total RNA from laser-microdissected samples of benign prostate epithelium, prostate adenocarcinoma and metastatic lesions, months 13-18 (Specific aim 3).

Task 6: Design and synthesis of Taqman probes for selected IGF-I receptor target genes and the IGF-IR and validation of probe effectiveness in M12-LISN and M12-LNL6 cells, months 13-18 (Specific aim 3).

Task 7: Tandem mass spec microsequencing of peptides from proteins not identified in Task 4 and design of probes for use in Task 8, months 19-30 (specific aim 2).

Task 8: Analysis of differential gene expression in prostate samples using real-time quantitative RT-PCR, months 24-36 (Specific aim 3).

The sections immediately following detail the progress made in addressing each of these tasks.

Task 1. Completion of microarray analysis of genes differentially expressed in LISN and LNL6 cells that express different levels of IGF-I receptor and which are, respectively, non-metastatic and metastatic in nude mouse xenografts. We have completed this analysis using three independent RNA preparations from each cell line grown in defined medium with 5% FBS and have analyzed each sample using triplicate arrays that each contain >12,000 sequence-verified, non-redundant human cDNA clones. Data were analyzed by accepted means of normalization, statistical verification and false-discovery rate analyses. These data demonstrate that there are specific genes that are constantly differentially expressed in LISN and LNL6 cells.

Table 1. Genes up-regulated in metastatic (LISN) cells

<u>Accession #</u>	<u>Gene Name</u>	<u>Fold change</u>
AA401457	hypothetical protein	41
AA188416	ubiquitin-conjugating enzyme E2-binding protein	28
AA488418	A kinase anchor protein 2 (AKAP-KL)	21
R93875	nucleosome assembly protein-like 1	14
AA701860	folliculin	9
AA733061	nucleoporin p54	9
AA448468	caspase 8, apoptosis-related cysteine protease	8
AA454639	F-box only protein 9	8
AA423792	EST-similar to human IL-17 receptor	6
AA039929	degenerative spermatocyte	6
AA428341	methyl-CpG binding domain protein 2	4
AA487148	TATA box binding protein-associated factor	4
AA452130	protein phosphatase 2	4
W47667	MAP kinase 8	2.5

Table 2. Genes down-regulated in metastatic (LISN) cells

<u>Accession #</u>	<u>Gene Name</u>	<u>Fold change</u>
AA129552	neuroblastoma Ras oncogene	-4.5
AA129552	forkhead box M1	-4
AA443982	protein phosphatase 1	-4
AA456882	integrin cytoplasmic domain-associated protein	-3.5
AA056148	reticulin 3	-3.5
AA134570	RAB23 protein	-3
H69334	pirin	-3
AA421977	DR1-associated protein 1	-3

Task 2. SELDI-TOF analysis of conditioned media from M12-LISN and M12-LNL6 cells. Cells were grown in defined media supplemented with 5% FBS under either standard tissue culture conditions or in a 3-dimensional high-aspect rotating wall vessel system that more closely mimics in vivo conditions. Samples were prepared one of two ways: 1) conditioned media was used without precipitation or fractionation; or 2) conditioned media was precipitated in cold acetone at -20°C and washed in 95% ethanol. Pellets were dissolved in 6M urea and concentrated in a Centrplus YM-50 to remove serum albumin. The flow-through was precipitated again as above and resuspended in 500 mM Tris-HCl, pH 7.4. Ciphergen NP20 (normal phase) and WCX-2 (weak cation-exchange) arrays were used in SELDI analyses. 10 µl of conditioned media was applied overnight at 4°C in a humidity chamber. Spots were washed 5X with binding buffer and 2X with water. EAM was applied

while spots were still moist. Chips were analyzed at 220, 250 and 270 laser intensity on a Ciphergen PBS-II system.

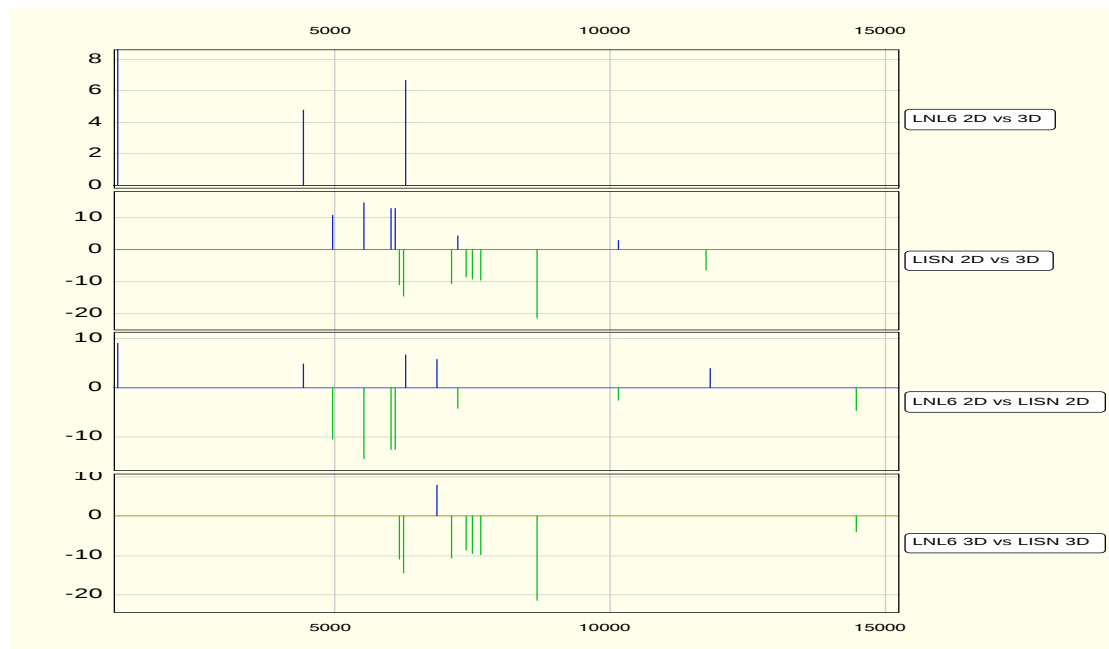


Figure 1. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on NP-20 chips. 3-15 kDa range is shown.

SELDI-TOF profiling revealed a discrete set of proteins in the 4-25 kDa range that were differentially present in the CM of the LNL6 and LISN lines grown under 2-dimensional versus 3-dimensional conditions, as well as proteins that were differentially present in CM of LNL6 and LISN cells under either culture condition. Some of these proteins were differentially expressed between the different lines under both conditions. Thus, changes in IGF-I receptor expression of the degree characteristic of metastatic versus non-metastatic prostate cancer cells are associated with alterations in the secreted protein profile.

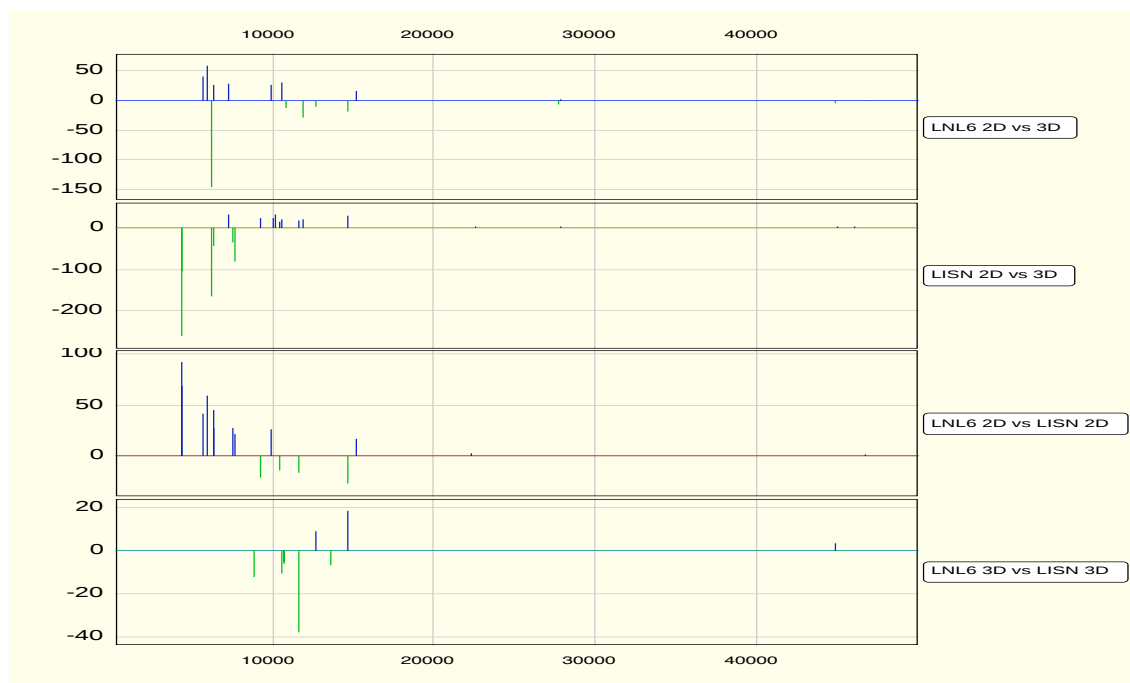


Figure 2. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on NP-20 chips. 5-50-kDa range is shown.

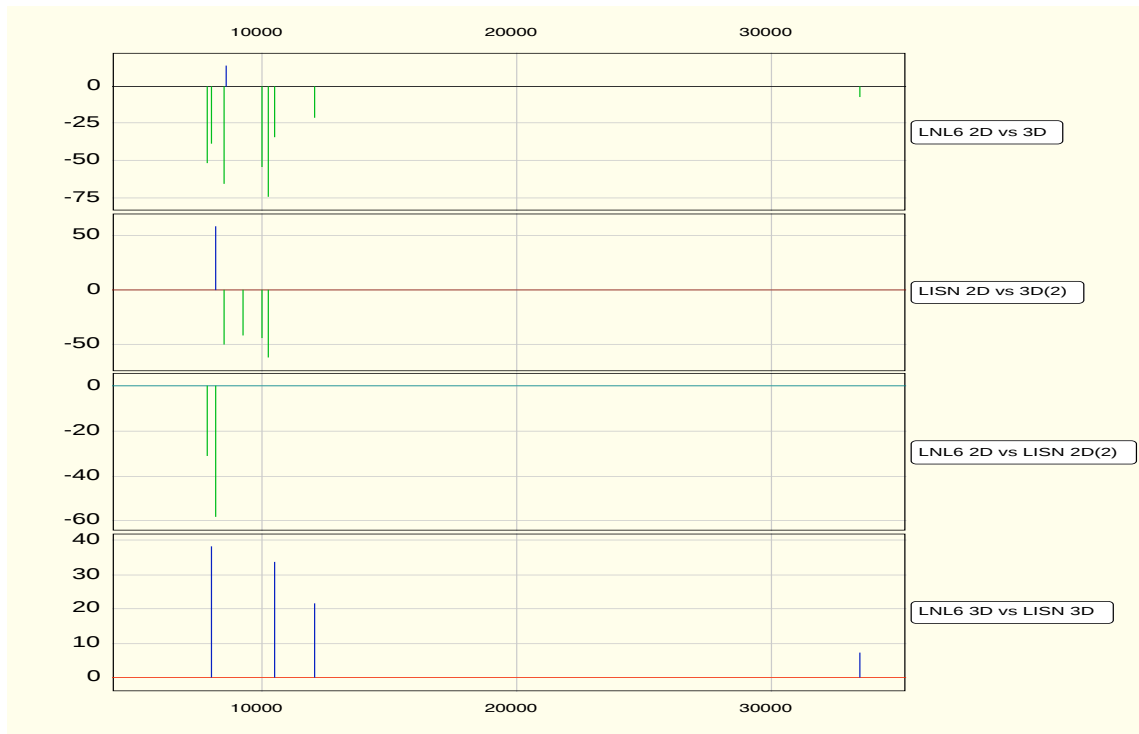


Figure 3. Difference spectra of LISN and LNL6 cells grown in 2-D and 3-D culture analyzed on WCX2 chips. 3-15-kDa range is shown.

The SELDI data shown above was presented at the 86th Annual Meeting of the Endocrine Society in New Orleans, LA, June, 2004. (Denley, A., Carroll, J.M., Nagalla, S.R., and Roberts, C.T., Jr., Proteomic analysis of IGF-regulated proteins in prostate cancer cells.)

Task 3. Generation of probes for Northern and/or RPA analysis and verification of differential gene expression in M12-LISN and M12-LNL6 cells. The differential expression of a number of the most highly regulated genes in Tables 1 and 2 have now been validated by quantitative RT-PCR.

Current status of work outlined in original statement of work.

During the last two years of this project, we participated in the design of a novel array comprised of ~2100 human genes predicted to encode secretory proteins. Proprietary gene analysis software was employed to scan the human genome project database to select genes predicted to encode secreted gene products based on algorithms designed to assess the presence of signal sequences and the presence of likely cleavage sites proximal to hydrophobic transmembrane segments. This approach selects for genes whose only likely product is secreted, as well as other categories of genes that could potentially encode both cell-surface and secreted products (e.g., growth hormone and cytokine receptor genes that produce both transmembrane and “shed” isoforms), since the latter proteins may constitute viable biomarkers. This analysis produced ~2100 genes out of the ~32,000 analyzed. These have been employed to screen normal prostate tissue and a number of cases of prostate cancer. The table below lists the genes that were 1) specifically expressed in prostate vs. other tissues examined and 2) significantly up-regulated in prostate cancer vs. normal prostate tissue. This study represents an alternative approach to tasks 5 and 6.

Table 3. Secretory genes found to be up-regulated in CaP vs. normal prostate tissue.

Gene ID	Fold Change	Gene Symbol	Gene Name
AA040387	4.09789	XPNPEP2	X-prolyl aminopeptidase (aminopeptidase P) 2, membrane-bound
AA459208	3.45208	FVT1	Follicular lymphoma variant translocation 1
W42723	3.22154	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
T72220	3.21218	RBP4	Retinol binding protein 4, plasma
AA487582	3.19130	EXT1	Exostoses (multiple) 1
AA609955	2.84302	HYPE	Huntingtin interacting protein E
AA451904	2.68464	WFDC2	WAP four-disulfide core domain 2
AA779457	2.62008	BMP5	Bone morphogenetic protein 5
AA905669	2.41953	PZP	Pregnancy-zone protein
AI217172	2.41513	VIP	Vasoactive intestinal peptide
AI139437	2.38875	KLK7	Kallikrein 7 (chymotryptic, stratum corneum)
AA133920	2.27653	PRL	Prolactin
AA677287	2.25938	FGL1	Fibrinogen-like 1
AI214881	2.19955	TG	Thyroglobulin
AA416552	2.18287	CXCL16	Chemokine (C-X-C motif) ligand 16
R80217	2.16503	PTGS2	Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
AA775447	2.05771	A2M	Alpha-2-macroglobulin
AA775880	1.99022	CCL27	Chemokine (C-C motif) ligand 27
AA424513	1.96548	MMP27	Matrix metalloproteinase 27
AA284668	1.95824	PLAU	Plasminogen activator, urokinase
AA455222	1.95735	PLAUR	Plasminogen activator, urokinase receptor
AA436410	1.92975	BCAT2	Branched chain aminotransferase 2, mitochondrial
W16715	1.91515	NELL1	NEL-like 1 (chicken)
AA669222	1.89851	MMP19	Matrix metalloproteinase 19
T67549	1.89485	PLGL	Plasminogen-like
AA969504	1.87357	IFNG	Interferon, gamma
AA609262	1.85627	OSTM1	Osteopetrosis associated transmembrane protein 1
R10973	1.80254	PAPPA	Pregnancy-associated plasma protein A, pappalysin 1
H68848	1.79419	APOH	Apolipoprotein H (beta-2-glycoprotein I)
AA868278	1.75041	CRISP2	Cysteine-rich secretory protein 2
AA780059	1.74633	C5	Complement component 5
AA425227	1.74200	MMP9	Matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)
AA628410	1.72676	SERPINA4	Serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antitrypsin), anti
AA125872	1.70895	ANGPT2	Angiopoietin 2
AA670200	1.67577	PCOLCE	Procollagen C-endopeptidase enhancer
AA633747	1.66421	COL6A2	Collagen, type VI, alpha 2
AA478724	1.65102	IGFBP6	Insulin-like growth factor binding protein 6
AA459401	1.64685	KLK10	Kallikrein 10
W37306	1.63898	PTH	Parathyroid hormone
R89067	1.62187	KNG1	Kininogen 1
T54298	1.61975	ANGPTL4	Angiopoietin-like 4
AA446899	1.60458	MAN1B1	Mannosidase, alpha, class 1B, member 1
AA284278	1.58574	ADAMTSL1	ADAMTS-like 1
AA479058	1.57887	THPO	Thrombopoietin (myeloproliferative leukemia virus oncogene ligand, megakaryocyte growth and development factor)
AA478481	1.55640	COL12A1	Collagen, type XII, alpha 1

AI200114	1.54085	GZMB	Granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)
AI017010	1.51806	OSTM1	Osteopetrosis associated transmembrane protein 1
R51912	1.51176	SST	Somatostatin
N66396	1.51004	COL11A2	Collagen, type XI, alpha 2
AA630120	1.50867	VEGFB	Vascular endothelial growth factor B
AA401397	1.50742	KLK13	Kallikrein 13
AI369841	1.50082	SNX26	Sorting nexin 26

We are now performing a similar analysis of RNAs isolated from LISN and LNL6 cells grown under 2-D and 3-D culture conditions. In parallel, we are performing differential 2-dimensional gel electrophoretic analyses of conditioned media from these cell lines grown under 2-D and 3-D conditions to identify differentially abundant spots and to obtain protein identification by MALDI-TOF and qTOF analyses. These studies will comprise successful completion of the remaining tasks of the original statement of work.

Additional research efforts supported by this award.

Review of the field-published in Genes, Chromosomes, and Cancer 36: 113-120 (2003)

The IGF1 Receptor Gene: A Molecular Target for Disrupted Transcription Factors. Haim Werner and Charles T. Roberts, Jr.

The biological actions of the insulin-like growth factors, IGF-I and IGF-II, are mediated by their activation of the IGF-I receptor (IGF-IR), a transmembrane heterotetramer linked to the ras-raf-MAPK and PI3K-PKB/Akt signal transduction cascades. The IGF-IR displays potent mitogenic, antiapoptotic, and transforming activities, and is a prerequisite for oncogenic transformation. A number of transcription factors have been identified that control the expression of this gene and therefore determine, to a significant extent, the proliferative status of the cell. The purpose of this review is to summarize data showing that, under normal physiological conditions, expression of the IGF-IR is under inhibitory control by a family of negative growth regulators or tumor suppressors. Cells with a reduced number of cell-surface receptors are unable to progress through the cell cycle and remain in a postmitotic state. Loss-of-function mutation of tumor suppressors in certain cancers results in transcriptional derepression of the IGF-IR gene, with ensuing increases in the levels of IGF-IR and increased proliferative capacity. Understanding the molecular mechanisms responsible for transcriptional regulation of the IGF-IR gene will prove important in designing novel therapies aimed at targeting the IGF axis.

Study #1-published in Endocrinology 145: 3205-3214 (2004)

Saw Palmetto Extract Suppresses Insulin-Like Growth Factor-I Signaling and Induces Stress-Activated Protein Kinase/c-Jun N-Terminal Kinase Phosphorylation in Human Prostate Epithelial Cells. Teri I. Wadsworth, Julie M. Carroll, Rebecca A. Mallinson, Charles T. Roberts, Jr., and Charles E. Roselli

A common alternative therapy for benign prostatic hyperplasia (BPH) is the extract from the fruit of saw palmetto (SPE). BPH is caused by nonmalignant growth of epithelial and stromal elements of the prostate. IGF action is important for prostate growth and development, and changes in the IGF system have been documented in BPH tissues. The main signaling pathways activated by the binding of IGF-I to the IGF-I receptor (IGF-IR) are the ERK arm of the MAPK cascade and the phosphoinositol-3-kinase

(PI3K)/protein kinase B (PKB/Akt) cascade. We tested the hypothesis that SPE suppresses growth and induces apoptosis in the P69 prostate epithelial cell line by inhibiting IGF-I signaling. Treatment with 150 µg/ml SPE for 24 h decreased IGF-I-induced proliferation of P69 cells and induced cleavage of the enzyme poly(ADP-ribose)polymerase (PARP), an index of apoptosis. Treatment of serum-starved P69 cells with 150 µg/ml SPE for 6 h reduced IGF-I-induced phosphorylation of Akt (assessed by Western blot) and Akt activity (assessed by an Akt kinase assay). Western blot analysis showed that SPE reduced IGF-I-induced phosphorylation of the adapter protein insulin receptor substrate-1 and decreased downstream effects of Akt activation, including increased cyclin D1 levels and phosphorylation of glycogen synthase kinase-3 and p70s6k. There was no effect on IGF-I-induced phosphorylation of MAPK, IGF-IR, or Shc. Treatment of starved cells with SPE alone induced phosphorylation the proapoptotic protein JNK. SPE treatment may relieve symptoms of BPH, in part, by inhibiting specific components of the IGF-I signaling pathway and inducing JNK activation, thus mediating antiproliferative and proapoptotic effects on prostate epithelia.

Study #2-published in Prostate 61: 276-290 (2004)

Androgen Receptor (AR) Expression in AR-Negative Prostate Cancer Cells Results in Differential Effects of DHT and IGF-I on Proliferation and AR Activity Between Localized and Metastatic Tumors. Stephen R. Plymate, Marie K. Tennant, Stephen H. Culp, Lillie Woodke, Marco Marcelli, Ilsa Colman, Peter S. Nelson, Julie M. Carroll, Charles T. Roberts Jr., and Joy L. Ware

Two features of the progression from organ-confined to metastatic prostate cancer are dysregulation of the androgen receptor (AR) and a decrease in insulin-like growth factor-type-I receptor (IGF-IR) expression. The purpose of this study was to determine the effect of changes in IGF-IR expression on AR activity. M12 human prostate cells were stably transfected with an AR expression construct to produce the M12-AR parental (PAR) cell line. PAR cells were implanted orthotopically into nude mice and M12-AR primary (PRI) cell lines were derived from intraprostatic tumors and metastatic cell lines (MET) were derived from PRI tumors that had metastasized to diaphragm or lung. Tumor formation in the prostate by PAR cells was decreased significantly compared to M12 controls. PAR, PRI, and MET cells expressed equivalent amounts of AR protein; however, IGF-IR expression was increased significantly in PAR and PRI cells. IGF-IR expression decreased in MET lines to the levels seen in M12 control cells. IGF-I significantly enhanced dihydrotestosterone (DHT)-stimulated, but not basal, AR transcriptional activity in PRI cells. In MET cells, IGF-I significantly suppressed DHT-stimulated transcriptional activity. In MET cells in which the IGF-IR was re-expressed from a retroviral vector, the effects of DHT and IGF-I on AR activity were similar to those seen in PRI cells. This study demonstrates that the changes in IGF-IR expression exhibited by this model of metastatic progression cause significant alterations in AR signaling and suggest that this interaction may be an important aspect of the changes seen in AR function in disease progression in vivo.

Study #3-published in Cancer Research 65: 1849-57 (2005)

Androgens Up-regulate the Insulin-like Growth Factor-I Receptor in Prostate Cancer Cells. Giuseppe Pandini, Rossana Mineo, Francesco Frasca, Charles T. Roberts, Jr., Marco Marcelli, Riccardo Vigneri, and Antonino Belfiore

In this study, we show that androgens up-regulate insulin-like growth factor-I receptor (IGF-IR) expression and sensitize prostate cancer cells to the biological effects of IGF-I. Both dihydrotestosterone and the synthetic androgen R1881 induced an ~6-fold increase in IGF-IR

expression in androgen receptor (AR)–positive prostate cancer cells LNCaP. In accordance with IGF-IR up-regulation, treatment with the nonmetabolizable androgen R1881 sensitized LNCaP cells to the mitogenic and motogenic effects of IGF-I, whereas an IGF-IR blocking antibody effectively inhibited these effects. By contrast, these androgens did not affect IGF-IR expression in AR-negative prostate cancer cells PC-3. Reintroduction of AR into PC-3 cells by stable transfection restored the androgen effect on IGF-IR up-regulation. R1881-induced IGF-IR up-regulation was partially inhibited by the AR antagonist Casodex (bicalutamide). Two other AR antagonists, cyproterone acetate and OH-flutamide, were much less effective. Androgen-induced IGF-IR up-regulation was not dependent on AR genomic activity, because two AR mutants, AR-C619Y and AR-C574R, devoid of DNA binding activity and transcriptional activity were still able to elicit IGF-IR up-regulation in HEK293 kidney cells in response to androgens. Moreover, androgen-induced IGF-IR up-regulation involves the activation of the Src-Erk pathway, because it was inhibited by both the Src inhibitor PP2 and the MEK-1 inhibitor PD98059. The present observations strongly suggest that AR activation may stimulate prostate cancer progression through the altered IGF-IR expression and IGF action. Anti-androgen therapy may be only partially effective, or almost ineffective, in blocking important biological effects of androgens, such as activation of the IGF system.

Study #4-under revision for the Journal of Biological Chemistry (2006)

Modulation of Insulin-like Growth Factor Signaling by Herstatin, an Alternatively Spliced Her-2 (erbB-2) Gene Product. Lara S. Shamieh, Julie M. Carroll, Elaine Hart, Gail M. Clinton, and Charles T. Roberts, Jr.

Herstatin, a product of alternative splicing of the HER-2 gene, consists of subdomains I and II of the ectodomain of the HER-2 receptor tyrosine kinase, followed by a 79-amino acid C-terminal domain encoded by intron 8. Previous studies have shown that herstatin binds to the ectodomain of multiple members of the EGF receptor (EGFR) family, and that binding to EGFR and HER-2 blocks receptor dimerization and ligand activation. Herstatin was recently found to also bind to the IGF-I receptor (IGF-IR), which exhibits signaling crosstalk and contains regions of high homology with the EGFR family (1). We, therefore, investigated the impact of herstatin expression on IGF-I signaling and proliferation in parental and herstatin-transfected MCF-7 breast cancer cells. IGF-IR levels, as well as IGF-I-mediated IGF-IR tyrosine phosphorylation, were reduced several-fold in two different clones of herstatin-expressing cells. Down-regulation did not appear to be caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF-7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not affect IGF-IR levels. Examination of the impact of herstatin on IGF-I-specific signaling revealed strong inhibition of tyrosine phosphorylation of IRS-1, while IRS-2 activation was enhanced. Although IGF-IR tyrosine phosphorylation was strongly reduced, herstatin expression did not inhibit, but stimulated, IGF-I-mediated ERK activation, while IGF-I activation of the PI3K-Akt/PKB pathway was inhibited. Altered IGF-IR signaling culminated in loss of IGF-I-mediated cell growth and survival in herstatin-expressing clonal cell lines. These studies demonstrate that herstatin profoundly modulates IGF-I-stimulated signaling and proliferation in MCF-7 breast cancer cells, either through direct interaction with the IGF-IR or indirectly, by modulating cross-talk with the EGFR family.

Key Research Accomplishments

- o Determination of genes differentially regulated by IGF-I receptor levels in metastatic and non-metastatic prostate cancer cells (tasks 1 and 3).

- o Identification of specific molecular weight species that are differentially secreted by metastatic and non-metastatic prostate cancer cells under 2-D and 3-D culture conditions (task 2).
- o Review of the state of research in IGF-I receptor action in tumorigenesis.
- o Demonstration of effectiveness of saw palmetto in modulating IGF-I receptor signaling in prostate cancer cells.
- o Description of functional interactions between IGF-I receptor and androgen receptor signaling in prostate cancer cells and in tumor xenograft models.
- o Characterization of regulation of IGF-I receptor gene expression by androgen receptor action in prostate cancer cells.
- o Discovery of inhibition of IGF signaling by a novel product of the her-2/neu/erbB2 gene.

Reportable Outcomes

- o Review article summarizing state of the field in IGF-I receptor role in tumorigenesis. Werner, H., and Roberts, C.T., Jr. The IGF-I receptor gene: a molecular target for disrupted transcription factors. *Genes Chromosomes Can.* 36:113-120 (2003).
- o Abstract describing SELDI-TOF analyses of LISN and LNL6 cells in 2-D and 3-D culture presented at 86th Annual Meeting of the Endocrine Society in New Orleans, LA, June, 2004. Denley, A., Carroll, J.M., Nagalla, S.R., and Roberts, C.T., Jr., Proteomic analysis of IGF-regulated proteins in prostate cancer cells.
- o Research article describing effect of saw palmetto extract on IGF signaling in P69 prostate cell line parent of LISN-LNL6 system accepted for publication. Wadsworth, T.L., Carroll, J.M., Roberts C.T., Jr., and Roselli, C.E. Saw palmetto extract suppresses IGF-I signaling and induces SAPK/JNK phosphorylation in human prostate epithelial cells. *Endocrinology* 145: 3205-3214 (2004).
- o Research article describing interactions between IGF and androgen receptor signaling in LISN and LNL6 cells and derivatives. Plymate, S.R., Tennant, M.K., Culp, S.H., Woodke, L., Marcelli, M., Colman, I., Nelson, P.S., Carroll, J.M., Roberts, C.T., Jr., and Ware, J.L. Androgen receptor (AR) expression in AR-negative prostate cancer cells results in differential effects of DHT and IGF on proliferation and AR activity between localized and metastatic tumors. *Prostate* 61: 276-290 (2004).
- o Idea Development Award application on androgen receptor regulation of IGF-I receptor expression funded by USAMRMC, CDRMP, PCRP, 12/04.
- o Research article describing AR regulation of IGF-IR gene expression. Pandini, G., Mineo, R., Frasca, F., Roberts, C.T., Jr., Marcelli, M., Vigneri, R., and Belfiore, A. Androgens up-regulate the IGF-I receptor in prostate cancer cells. *Cancer Res.* 65: 1849-1857 (2005).
- o Research article describing control of IGF action by herstatin. Shamieh, L., Carroll, J.M., Hart, E., Clinton, G.M., and Roberts, C.T., Jr. Modulation of IGF signaling by herstatin. *J. Biol. Chem.*, in press (2006).

Conclusions

As outlined in the introduction section above, we have made significant progress in a number of areas of prostate cancer research, both directly related to the original statement of work and in closely related areas. These studies have elucidated clinically relevant aspects of the IGF signaling system and its control by, and interaction with, other signaling and regulatory systems that are themselves implicated in prostate cancer.

References

None.

Appendices

Pre-print of study # 4 is attached

**MODULATION OF INSULIN-LIKE GROWTH FACTOR SIGNALING BY HERSTATIN,
AN ALTERNATIVELY SPLICED HER-2 (erbB-2) GENE PRODUCT**

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Running title: Herstatin regulation of IGF signaling

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ABSTRACT

Herstatin, a product of alternative splicing of the HER-2 gene, consists of subdomains I (L1) and II (S1) of the HER-2 receptor tyrosine kinase, followed by a 79-amino acid C-terminal domain encoded by intron 8. We have previously shown that herstatin binds to multiple members of the EGF receptor (EGFR/erbB/HER) family, and that binding to EGFR and HER-2 blocks dimerization and ligand activation. Herstatin also binds to the IGF-I receptor (IGF-IR), which exhibits signaling crosstalk, and contains regions of high homology with, the EGFR family (1). In this study, we have extended these latter findings by investigating the effects of herstatin on IGF system expression and action in MCF-7 breast cancer cells. IGF-IR expression and IGF-stimulated IGF-IR tyrosine phosphorylation were significantly reduced in two different clones of herstatin-expressing cells. These effects were not caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF-7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not affect IGF-IR levels. Herstatin also inhibited the expression and IGF-induced tyrosine phosphorylation of IRS-1, while IRS-2 expression and activation was not affected. Although IGF-IR and IRS-1 tyrosine phosphorylation was strongly reduced, herstatin did not inhibit, but stimulated, IGF-I-mediated ERK activation, while IGF-I activation of the PI3K-Akt/PKB pathway was modestly inhibited. Altered IGF-IR signaling culminated in loss of IGF-I-mediated cell growth and survival in herstatin-expressing cells. These studies demonstrate that herstatin profoundly modulates IGF-I-stimulated signaling and proliferation in MCF-7 breast cancer cells through direct interaction with the IGF-IR and/or by modulating crosstalk with the EGFR family.

INTRODUCTION

Receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) and the insulin-like growth factor-I receptor (IGF-IR) families, play key signaling roles in fundamental cellular processes. The EGFR family, which includes the EGFR (HER-1/erbB1), HER 2/neu/erbB2, HER-3/erbB3, and HER 4/erbB4, has been shown to mediate key cellular processes such as growth and differentiation (2-4). The IGF-IR family, which includes the IGF-IR, the insulin receptor, and the insulin receptor-related receptor, has also been shown to participate in an overlapping array of biological processes (5-11). While the expression and biological effects of these receptor families are essential for normal growth and development, aberrant expression leads to a variety of human cancers (12-15).

The four members of the EGFR family each contain an extracellular ligand-binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (16-18). Eleven ligands, each containing an EGF core domain, bind with high affinity to these receptors, except HER-2, causing the formation of receptor homo- or heterodimers. This dimerization results in receptor activation and autophosphorylation of specific C-terminal tyrosine residues (4,17,19-22), which enables the subsequent recruitment and tyrosine phosphorylation of SH2-domain-containing signaling molecules, leading to the initiation of two major intracellular signaling pathways, the (generally) anti-apoptotic PI3K/Akt/PKB and mitogenic ERK cascades (12,23,24).

The IGF-IR, in contrast to other RTKs, consists of a pre-formed, disulfide-linked, heterotetramer (25,26). Ligand binding to the extracellular α subunits leads to a conformational change in the transmembrane β subunits and autophosphorylation of tyrosine residues in the catalytic domain. The subsequent phosphorylation of additional tyrosines, particularly in the juxtamembrane domain of the β subunit, provides docking sites for PTB and SH2-domain-containing scaffolding/adaptor proteins, including the insulin receptor substrates IRS-1 and IRS-2. These adaptor proteins then activate signaling pathways such as the PI3K and ERK cascades that are also activated by the EGFR family (27).

Both the EGFR and IGF-IR families are major regulators of cell growth and survival, and dysregulation of either receptor family can lead to uncontrolled growth and tumorigenesis. Recent evidence suggests that there is crosstalk between these RTKs, which may allow coordinated control of cellular responses in normal and tumor cells

(reviewed in (1)). Sustained activation of a mitogenic ERK signal by the EGFR is dependent on a functional IGF-IR (28). Recently, the converse was also shown to be true, in that activation of ERK by the IGF-IR requires a functional EGFR (5,29,30). Additionally, it has been shown in several cell types that IGF-I stimulation of the IGF-IR leads to activation of the EGFR and, coordinately, the ERK pathway, through proteolytic activation and autocrine release of HB-EGF (30-32). IGF-I-induced coordinate activation of ERK through EGFR and IGF-IR is in contrast to IGF-I-induced activation of Akt, which is unaffected by EGFR-specific inhibitors (30,32). These data suggest that crosstalk between the EGFR and the IGF-IR controls activation of the ERK signaling pathway, but not the PI3K-Akt/PKB pathway. In addition to coordination of signal transduction, Ahmed et al. have recently reported that the EGFR co-immunoprecipitates with the IGF-IR in mammary epithelial cells, and that phosphorylation of the complexed EGFR is enhanced by treatment with IGF-I (29). Another recent study has described an association between IGF-IR and HER-2 (X).

Because of the important role of the EGFR family in malignant growth, extensive effort has been directed toward the development and characterization of specific inhibitors. Effective tumor inhibition has been achieved clinically with inhibitors that antagonize the EGFR and HER-2 (33,34). Several studies suggest that redundant signaling through the IGF-IR maintains the activation of pathways necessary for survival in the presence of EGFR family inhibitors. *In vitro*, IGF-IR signaling in MCF-7/HER-2 and SKBR-3 breast carcinoma cells protects against inhibition by Herceptin, an anti-Her-2 monoclonal antibody (35). The inhibitory effects of AG1478, an EGFR inhibitor, can also be overcome in glioblastoma multiforme cells by overexpression and increased signaling through the IGF-IR (36). Most recently, it has been shown in breast and prostate cancer cell lines that acquired resistance to Iressa, a small-molecule EGFR inhibitor, occurs through increased IGF-IR activation and signaling (37,38).

Recent efforts have also been directed at targeting the IGF-IR family. Inhibition of tumor growth with two IGF-IR small-molecule inhibitors has been documented with solid tumor xenografts and leukemic malignancies (39,40). Specific anti-IGF-IR antibodies have been recently developed that have shown efficacy in inhibition of IGF-stimulated proliferation and tumorigenesis (41-43). Additionally, *in vitro* combinatorial therapy, using Herceptin to block HER-2, and a dominant-negative form of the IGF-IR in breast carcinoma cells, revealed synergy between the two treatments and led to increased growth inhibition (44). Recently, a bivalent monoclonal antibody to the EGFR

and IGF-IR has been described (45,46). Use of this di-antibody resulted in increased growth inhibition compared to that achieved with either anti-EGFR or anti-IGF-IR antibodies alone (45).

In this study, we investigated the impact of a cellular pan-EGFR family inhibitor, herstatin, on IGF-I signaling. Herstatin, the product of alternative splicing of the HER-2 gene transcript, consists of the N-terminal portion of the HER-2 RTK, followed by a novel 79-amino acid C-terminal domain (47). Herstatin is unique in that it binds with nM affinity to all members of the EGFR family (48), and its binding to EGFR and HER-2 blocks receptor activation (47,49-51). We have recently demonstrated that herstatin also binds with lower affinity to the IGF-IR compared to the EGFR ($K_d \approx 150$ nM vs 15 nM) (48), presumably to a site in the ectodomain that has homology with the EGFR (52). We, therefore, determined the effects of herstatin on IGF-I signaling system expression and signaling in MCF-7 mammary carcinoma cell lines. Our data demonstrate that herstatin action represents a novel mechanism of cross-regulation of the EGFR and IGF-IR families.

MATERIALS AND METHODS

Cell culture

MCF-7 breast carcinoma cells were obtained from the American Type Culture Collection and maintained at 37°C/5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and gentamicin (0.25 µg/ml). Media and supplements were purchased from Gibco BRL-Life Technologies (Grand Island, NY). Herstatin-expressing MCF-7 clones (MCF-7/Hst cells), previously characterized (50), were maintained under the same conditions as parental MCF-7 cells in media supplemented with 0.5 mg/ml G418 sulfate.

Antibodies

All primary antibodies were used at a 1:1000 dilution unless otherwise indicated. Polyclonal antibodies [IGF-IR and IRS-1 (N-terminus)] and monoclonal antibody PY20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal ERK 1/2 and polyclonal pERK 1/2, Akt/PKB, and IRS-1 antibodies were purchased from Cell Signaling Technologies (Boston, MA). Monoclonal herstatin and polyclonal IRS-2

antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal pAkt/PKB antibody was purchased from BioSource International (Hopkinton, MA).

Western immunoblotting and immunoprecipitation

Cells were grown to ~80% confluency, serum-starved overnight in DMEM, and treated with 14 nM EGF (SOURCE???) or 5 nM IGF-I (GroPep, Australia) for the times indicated. For Western blots, cells were washed twice with ice-cold PBS and lysed in SDS sample buffer (53) without reducing agent and boiled for 5 min. After clarification by centrifugation at 13,000 rpm for 5 min., supernatant was collected and protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad; Hercules, CA). Dithiothreitol (100 mM) and bromophenol blue (0.1% (w/v)) were then added and samples were boiled again for 5 min. Twenty-mg aliquots of protein were analyzed by 10% SDS-PAGE and electrotransferred onto nitrocellulose (Amersham Pharmacia Biotech; Piscataway, NJ). Blots were probed with a phospho-specific antibody, stripped in 5x stripping buffer (53) and reprobed with the respective pan antibody. For immunoprecipitation, cells were washed twice with ice-cold PBS, lysed in NP-40 buffer [1% NP-40, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.2% SDS], containing protease inhibitors (Roche Diagnostics; Indianapolis, IN), 1 mM NaVO₄, and 1 mg/ml pepstatin. Lysates were cleared and protein concentration was determined as above. For IGF-IR, 1 mg of whole-cell lysate protein was immunoprecipitated with 10 µg of anti-IGF-IR antibody and incubated overnight at 4°C while rocking. For IRS-1 and IRS-2, 500 µg of whole-cell lysate protein was incubated overnight with 5 or 10 µg antibody, respectively. 100 µl of protein A-agarose bead slurry (Amersham Pharmacia Biotech) was added for 2 hours rocking at 4°C. Three washes were performed, and the pellet was boiled in 2x SDS sample buffer (53). The beads were spun down and the supernatant loaded onto a 10% (IGF-IR) or 7% (IRS-1/2) SDS-PAGE and immunoblotted as above. Blots were probed with PY20, stripped, and reprobed with their respective antibodies. Binding of primary antibodies was detected by enhanced chemiluminescence (Amersham), and film exposures were quantified using a scanning densitometer (Bio-Rad).

Cellular growth and survival

For determination of overall growth/survival, cells (4×10^4) were plated in quadruplicate in 24-well plates, incubated in serum-free DMEM for 24 hours, and treated

with either 5 nM IGF-I (GroPep; Adelaide, Australia) or an equivalent volume of vehicle (10 mM HCl). At the indicated time-points, cell monolayers were washed with PBS and incubated for 30 minutes at 37°C with 30 µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium) inner salt Aqueous One Solution (Promega; Madison, WI) dissolved in 270 ml PBS] per well. Absorbance readings were obtained at 490 nm in a Bio-Tek plate reader.

DNA synthesis was measured by incorporation of BrdU...

Apoptosis was assessed...

EGFR inhibitor studies

Control MCF-7 cells were serum-starved overnight and treated with the EGFR kinase AG1478 (Sigma) or vehicle (DMSO) for 5 min. prior to the addition of 14 nM EGF or 5 nM IGF-I. After 5 min. of growth factor treatment, cell lysates were prepared and analyzed for ERK and Akt/PKB activation as described above.

RESULTS

Effect of herstatin on the expression of IGF signaling molecules

The studies described below demonstrate the effects of herstatin expression on IGF-I signaling. In preparation for those studies, we first examined the effect of herstatin expression on basal levels of the signaling molecules comprising the IGF signaling system. The expression of herstatin in MCF-7 cells resulted in the down-regulation of several components of the IGF signaling system (Fig. 1). Both IGF-IR and IRS-1 protein levels were decreased 5-fold, while IRS-2 protein levels were modestly up-regulated. There was no apparent difference in the levels of total ERK; however, there was a shift from a preponderance of ERK1 to ERK2, as well as an increase in the apparent size of ERK1. Akt/PKB levels were modestly affected, with an average 2-fold decrease seen in herstatin-expressing cells.

Effect of herstatin on IGF-IR activation

To evaluate the effect of herstatin on activation of the IGF-IR by IGF-I, we examined tyrosine phosphorylation of IGF-IR immunoprecipitated from IGF-I-treated MCF-7 and MCF-7/Hst cells. In MCF-7 cells, IGF-I robustly stimulated IGF-IR tyrosine phosphorylation, which represents the initial autophosphorylation stage of IGF-IR

activation. In MCF-7/Hst cells, however, there was only a small increase in IGF-IR phosphorylation, which corresponds to an approximately 8-fold reduction in activation (Fig. 2). This decreased activation reflects, in part, the decrease in IGF-IR expression consistently seen in herstatin-expressing cells (see Fig. 1), as well as diminished tyrosine phosphorylation (Fig. 2). Reduced IGF-IR expression and activation by IGF-I (and IGF-II) were also observed in a second clonal line of herstatin-expressing cells (data not shown).

IGF-I activation of IRS-1 and IRS-2

To further investigate the effects of herstatin expression on IGF-I-mediated signaling, we examined the activation of IRS-1 and IRS-2, signaling molecules immediately downstream of the IGF-IR. IGF-I-induced phosphorylation of IRS-1 was severely reduced in MCF-7/Hst cells compared to control cells (Fig. 3A & B). This decreased tyrosine phosphorylation of IRS-1 was a result of both decreased expression of IRS-1 (~5-fold; see Figure 1), as well as an apparent 6-fold decrease in the efficiency of IRS-1 immunoprecipitation in herstatin-expressing cells. This reduction in the amount of IRS-1 immunoprecipitated from herstatin-expressing cells was also seen with a second, N-terminally directed IRS-1 antibody (data not shown). Together, the combined effects of decreased IRS-1 expression and immunoprecipitation efficiency resulted in an ~30-fold difference in the amount of IRS-1 in immunoprecipitates from control and herstatin-expressing cells. This was similar to the difference in tyrosine-phosphorylated IRS-1; therefore, the decrease in IRS-1 protein immunoprecipitated from herstatin-expressing cells was equivalent to the decrease in IRS-1-associated phosphotyrosine. Thus, the relative activation of IRS-1 was similar in control and herstatin-expressing cells. In contrast, the levels of activated (tyrosine-phosphorylated) IRS-2 were slightly increased in herstatin-expressing cells, which was proportional to the slight increase in IRS-2 protein seen in herstatin-expressing cells (Fig. 3 C & D & Fig. 1).

IGF-I activation of ERK and PKB

Herstatin has been shown to differentially affect ERK pathway activation. Specifically, herstatin had no effect on EGF-stimulated ERK activation in 3T3 cells over-expressing EGFR, but did inhibit heregulin activation of ERK in MCF-7 cells (49, FARIDA ref, 54?). Herstatin expression did not inhibit overall activation of the ERK signaling pathway in IGF-I-treated MCF-7 cells. ERK phosphorylation was rapid and transient, with a

maximal response at 5 minutes in parental cells. In herstatin-expressing cells, the timing of the maximal response was the same, but the amplitude of total ERK activation, indicated by enhanced phospho-ERK, was enhanced several-fold (Fig. 4). Interestingly, we observed a specific stimulation of ERK2, while there was no change in the activation of ERK1. Furthermore, we consistently observed an increase in the apparent size of ERK1. This may correspond to the appearance of an ERK1 splice variant, or a post-translational modification (55-57). In contrast, IGF-I activation of the PI3K pathway, as assessed by the overall level of Akt/PKB phosphorylation, was reduced by 2-fold in MCF-7/Hst cells (Fig. 5). This effect is similar to the previously reported inhibition of EGF and heregulin-stimulated Akt/PKB activation in 3T3 and MCF-7 cells (49, 50, 54). Thus, herstatin expression did not reduce, but enhanced, ERK2 signaling, but attenuated the anti-apoptotic Akt/PKB signaling cascade. Similar effects, i.e., enhanced ERK2 activation and decreased Akt/PKB activation, were also seen in a second, independent herstatin-expressing MCF-7 clone (data not shown).

Herstatin reduces IGF-I-stimulated growth and survival in MCF-7 cells

Previous studies have shown that stable expression of herstatin in MCF-7 cells blocked heregulin-stimulated proliferation (50). The inhibition of IGF-IR signaling observed in herstatin-expressing cells suggested that herstatin may also interfere with IGF-I-mediated growth and survival. To further investigate the effect of herstatin on IGF-I action, we examined the IGF-I-induced growth of parental MCF-7 cells and two clones stably transfected with herstatin, MCF-7/Hst#1 and MCF-7/Hst#2. Parental MCF-7 cells grew in response to IGF-I, whereas cell viability decreased in the absence of growth factor. Both of the MCF-7/Hst clones, however, failed to exhibit IGF-I-stimulated growth (Fig. 6).

Herstatin blocks EGF signaling

Previous studies have demonstrated that the EGFR is involved in IGF-I signaling (1, 5, 29-32). Therefore, the observed effects on IGF-I signaling may have been an indirect effect of herstatin-mediated inhibition of the EGFR. To determine whether EGF-stimulated signaling was attenuated by herstatin, we compared the ability of EGF to activate the ERK and PI3K-Akt/PKB cascades in control and herstatin-expressing MCF-7 cells. As shown in Fig. 7, EGF treatment of control cells elicited robust ERK and Akt/PKB phosphorylation, which was severely reduced in cells expressing herstatin.

These data demonstrate that herstatin blocks both heregulin and EGF-stimulated signaling in MCF-7 cells.

Effect of EGFR inhibition on IGF-IR expression

Herstatin expression had a striking effect on the levels of the IGF-IR. To determine if the observed effects of herstatin on IGF-IR levels were an indirect result of decreased EGFR action, we investigated whether specific inhibition of EGFR mimicked the effects of herstatin. Treatment with the EGFR inhibitor, AG1478, prevented EGF-stimulated activation of ERK (data not shown). However, neither short-term nor long-term treatment with AG1478 resulted in the down-regulation of IGF-IR levels that was seen in herstatin-expressing cells (Fig. 8).

DISCUSSION

An understanding of the effects of herstatin, an autoinhibitor of the EGFR family, on IGF-I signaling is critical to defining the overall mode of action of herstatin and to further clarify the mechanisms that link the actions of these two important RTK families. We have previously shown that herstatin blocks heregulin signaling and proliferation in MCF-7 cells (50). This study shows that EGF signaling is also blocked in these cells. To further assess the interplay between herstatin and the IGF-IR, initially suggested by binding of herstatin at nM concentrations to the ectodomain of the IGF-IR (47), we examined IGF-I signaling and proliferation in MCF-7 breast carcinoma cells in which signaling through the EGFR family is disabled.

We found a striking effect of herstatin expression on several aspects of IGF-I signaling. Foremost, herstatin expression resulted in down-regulation of IGF-IR expression and an 8-fold decrease in IGF-I-induced IGF-IR tyrosine phosphorylation (Fig 1 and 2). Herstatin expression also resulted in a striking decrease in IRS-1 activation, which is immediately downstream of the IGF-IR in the IGF-I signaling pathway (Fig 3). Most importantly, this altered signaling culminated in a loss of IGF-I-mediated survival of herstatin-expressing MCF-7 cells (Fig. 6).

In contrast to the blockade of EGF and heregulin-induced ERK activation, IGF-I stimulation of ERK was not inhibited, even though IGF-IR levels were reduced several fold (Fig. 4). Therefore, the extent of IGF-IR activation did not parallel the effects on the downstream ERK signaling cascade. Thus, the low levels of activated IGF-IR appeared

to be sufficient to fully activate ERK signaling. Although ERK1 activation was unaffected, we observed a shift in the size of ERK1 in herstatin-expressing cells. We speculate that this size shift may be due to alternative splicing of the ERK1 gene, and may represent the ERK1b splice variant, which is 2.6 kDa larger than ERK1 (55-57). ERK1b has an altered ability to interact with MEK1 and may, therefore, result in a differential signaling profile (56). Interestingly, in herstatin-expressing cells, we also observed a preferential activation of ERK2 relative to ERK1 (Fig 3). Recent studies have implicated activation of ERK2, but not ERK1, in apoptosis (58-61). Therefore, the preferential activation of ERK2 in herstatin-expressing cells may contribute to the loss of IGF-I-mediated survival demonstrated in Fig. 6.

The effects of herstatin expression on the signaling factors immediately downstream of the IGF-IR, IRS-1 and IRS-2, were complex and distinct. Herstatin reduced both IRS-1 expression and immunoprecipitation efficiency, with a concomitant decrease in IGF-I-stimulated tyrosine phosphorylation (Fig 1 and Figure 3 A & B). The mechanisms responsible for the two former effects are unclear. With respect to the differential immunoprecipitation of IRS-1 in control vs herstatin-expressing cells, it is possible that herstatin alters the subcellular localization or association pattern of IRS-1, such that the availability of IRS-1 to interact with multiple antibodies is attenuated. One possibility is that nuclear translocation of IRS-1, which has been observed in multiple cell types, including MCF-7 cells, is affected by herstatin expression (62). In contrast, herstatin expression did not significantly affect expression or activation of IRS-2. The differential enhancement of IGF-I-stimulated IRS-1 and IRS-2 activation by herstatin may reflect the fact that feedback mechanisms, such as patterns of inhibitory serine phosphorylation, differ between IRS-1 and IRS-2 (63). Interestingly, previous studies have shown that IRS-1, but not IRS-2, is important in IGF-I-mediated inhibition of apoptosis, an effect that may underlie the inhibitory effects of herstatin on cell viability seen in the current study (64). Combinatorial effects of herstatin expression that include decreased expression and activation of the IGF-IR and its immediate downstream signaling molecule, IRS-1, reduction in activation of Akt, and an increase in activation of ERK2, may all contribute to the retarded growth of herstatin-expressing MCF-7 cells (Fig. 5).

There are several potential mechanisms through which herstatin may modulate IGF-IR signal transduction and, thereby, IGF-I action. First, herstatin may directly bind to intracellular IGF-IR in the secretory pathway; alternatively, secreted herstatin may

interact at the cell surface, since we have previously determined that it binds to the ectodomain of the IGF-IR with nanomolar affinity (48). However, since herstatin binds to all EGFR family members, and with higher affinity than to IGF-IR, the impact of herstatin on IGF-I signaling may be indirect and needs to be further investigated in cells that do not express the EGFR family.

A second possibility is that the modulation of IGF-I signaling is a secondary effect due to blockade of EGFR family signaling. Ample evidence exists for an IGF-I-stimulated autocrine loop that results in the release of heparin-binding EGF (HB-EGF) and, consequently, in the activation of the EGFR (32). To examine whether the effect of herstatin on down-regulation of the IGF-IR occurs via the EGFR, we blocked EGFR activation (using the EGFR-specific kinase inhibitor, AG1478) in parental MCF-7 cells. While the inhibitor fully blocked EGF-induced ERK activation (data not shown), it failed to mimic herstatin-mediated down-regulation of the IGF-IR (Fig. 8). However, we cannot rule out the possibility that longer-term effects of herstatin expression are involved, or that modulation of the other members of the EGFR family indirectly affects IGF-I signaling.

A third possibility is that herstatin may modulate the formation of hetero-oligomers between the IGF-I and EGF receptors. Recent evidence suggests that the EGFR is present in IGF-IR immunoprecipitates, suggesting the interesting possibility that herstatin may disrupt EGFR/IGF-IR hetero-oligomers (29). Regardless of whether this mechanism entails a direct or indirect effects of herstatin on the IGF-IR, the results presented here demonstrate a profound modulation of IGF-I signaling by an alternative product of the HER-2 gene.

The roles of both the EGFR and IGF-IR families in neoplastic growth and malignancies have been well documented. Over-expression and autocrine stimulation of both receptor families and their ligands has been implicated in a variety of carcinomas (65-69). Acquired resistance to Iressa, an EGFR inhibitor, in breast and prostate cancer cells is mediated by activation and signaling of the IGF-IR (37,38). Furthermore, IGF-IR signaling has been shown to protect HER-2-overexpressing breast carcinoma cells from the inhibitory effects of Herceptin, an anti-HER-2 monoclonal antibody (35). Thus, therapeutic strategies that are directed at both of these signaling systems would be expected to have significant advantages over those that target a single growth factor pathway. Our data suggest that herstatin is an inhibitor that may block proliferative signals from two distinct families of RTKs.

The data obtained in this study were obtained with MCF-7 cells and were based on two independent herstatin-expressing clones in comparison to control cells. Although MCF-7 cells are a valuable and established model for the study of cellular regulatory mechanisms relevant to breast cancer, it will be desirable to extend these results to other cell types. Constitutive expression of herstatin is, however, toxic to most other cells that we have analyzed; thus, further studies will be facilitated by exploiting conditional, regulated expression models that we are currently developing.

Current receptor-directed therapeutics are typically targeted at a single receptor or receptor family, which may explain, in part, their limited clinical efficacy. Recently, a hetero-bi-functional monoclonal antibody that targets both the EGFR and IGF-IR was found to block both EGF and IGF-I-induced activation of Akt/PKB and ERK, resulting in strong inhibition of xenograft growth (45,46). We suggest that herstatin may have significant promise as a novel anti-cancer agent, since it acts as a multi-functional inhibitor that suppresses signaling from both the EGFR and IGF-IR families.

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FIGURE LEGENDS

Figure 1. Effect of herstatin expression on the expression levels of IGF signaling proteins. Sub-confluent MCF-7 and MCF-7/Hst cells were extracted and signaling protein levels were assessed by Western blot.

Figure 2. Herstatin modulation of IGF-I activation of the IGF-IR. MCF-7 and MCF-7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipitated with an IGF-IR antibody and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IGF-IR expression and tyrosine phosphorylation using anti-IGF-IR and PY20 anti-phosphotyrosine antibodies, respectively. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot of IGF-IR immunoprecipitated from IGF-I-treated MCF-7 and MCF-7/Hst cells. **(B)** A graphical representation of two independent experiments of IGF-I-induced activation of the IGF-I receptor.

Figure 3. Effect of herstatin on IGF-I activation of IRS-1 and IRS-2. MCF-7 and MCF-7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute time course, and harvested in NP-40 lysis buffer. 1 mg of cell lysate was immunoprecipitated with IRS-1 (A & B) or IRS-2 (C & D) antibodies and protein A-agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IRS expression and tyrosine phosphorylation. Western blots were scanned and quantified by densitometry. **(A)** Representative IRS-1 immunoprecipitation and analysis with antiphosphotyrosine PY20 antibody. Both light and dark exposures of the IRS-1 immunoprecipitation are shown. **(B)** Graphical representation of 3 separate experiments. **(C)** Representative IRS-2 immunoprecipitation and analysis with anti-phosphotyrosine PY20 antibody. **(D)** Graphical representation of 3 separate experiments.

Figure 4. Effect of herstatin on IGF-I activation of ERK. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5 nM IGF-I at 37°C over a 60-minute time course. Cell lysates (50 µg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with ERK and phospho-ERK antibodies. **(A)** Representative Western

blot showing IGF-I-induced ERK activation in MCF-7 and MCF-7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 5. Effect of herstatin on IGF-I activation of Akt/PKB. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5nM IGF-I at 37°C over a 60- minute time course. Cell lysates (50 µg) were separated on a 10% SDS-PAGE gel and then analyzed by Western blotting with Akt and phospho-Akt antibodies. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot showing IGF-I-induced Akt/PKB activation in MCF-7 and MCF-7/Hst cells. **(B)** Graphical representation of 3 separate experiments.

Figure 6. Effect of herstatin on IGF-I-stimulated cell proliferation. Parental MCF-7 breast carcinoma cells and **(A)** low and **(B)** high herstatin-expressing clones were serum-starved for 24 hours and then treated with 5 nM IGF-I or vehicle. Growth was determined by an MTS assay as described in Materials and Methods and was assessed at the indicated days.

Figure 7. Effect of herstatin on EGF-stimulated signaling. MCF-7 and MCF-7/Hst cells were serum-starved and treated with 5 nM EGF at 37°C for the times indicated. Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and ERK and Akt activation were analyzed by Western blotting as described in the legends to Figures 3 and 4. Western blots were scanned and quantified by densitometry. **(A)** Effect of herstatin expression on EGF-induced ERK activation. **(B)** Effect of herstatin expression on EGF-induced Akt/PKB activation.

Figure 8. Effect of AG1478 on IGF-IR expression. MCF-7 cells were treated with AG1478 for the times indicated. Cells were lysed and lysates were run on a 10% SDS-PAGE gel and analyzed by Western blot.

Figure 2.

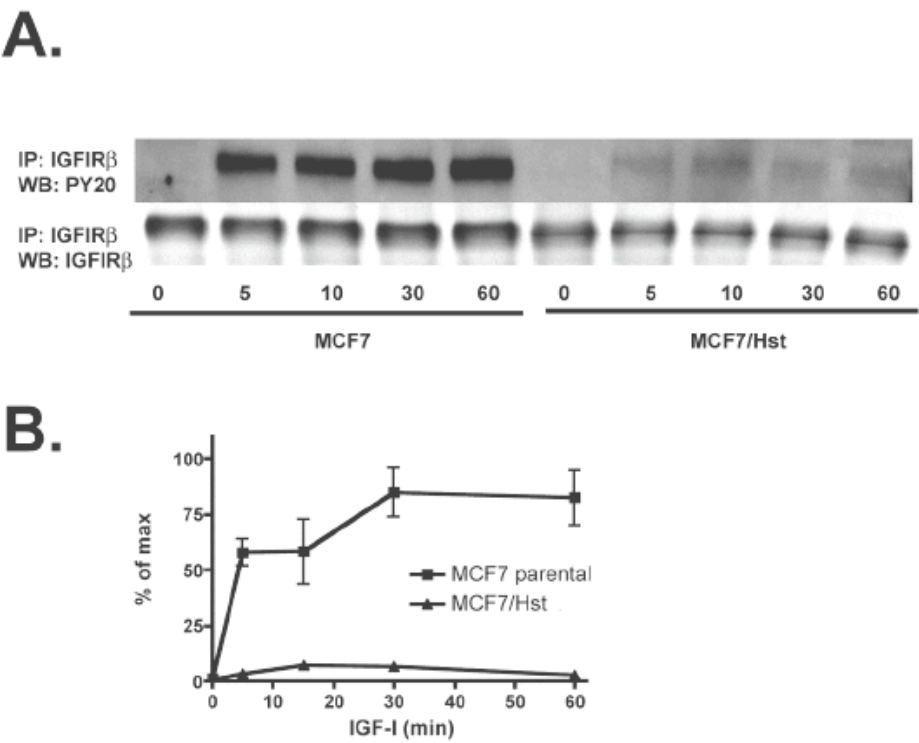


Figure 3.

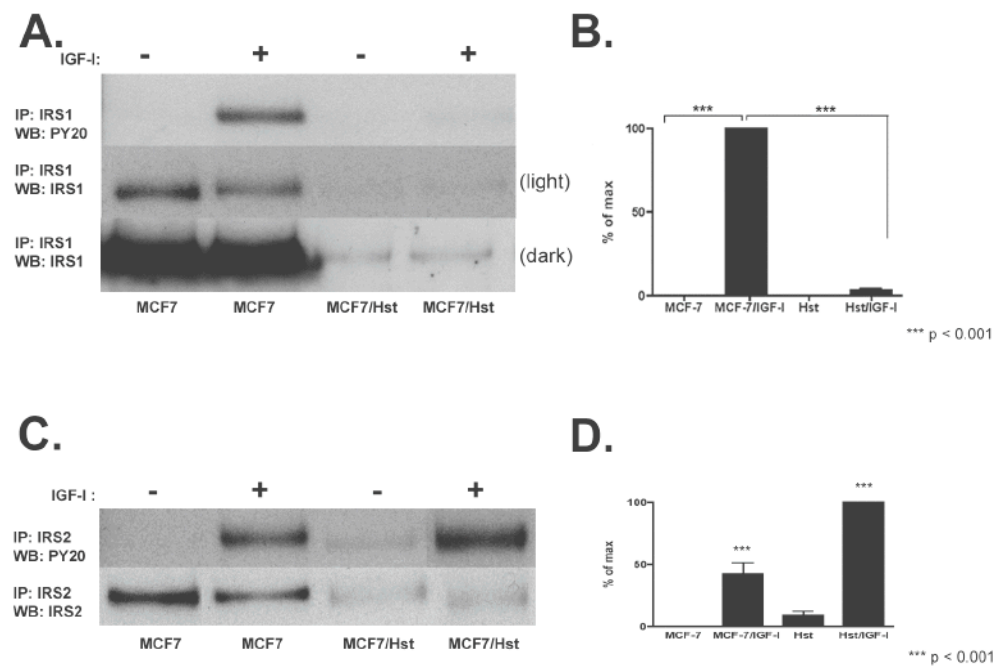


Figure 4.

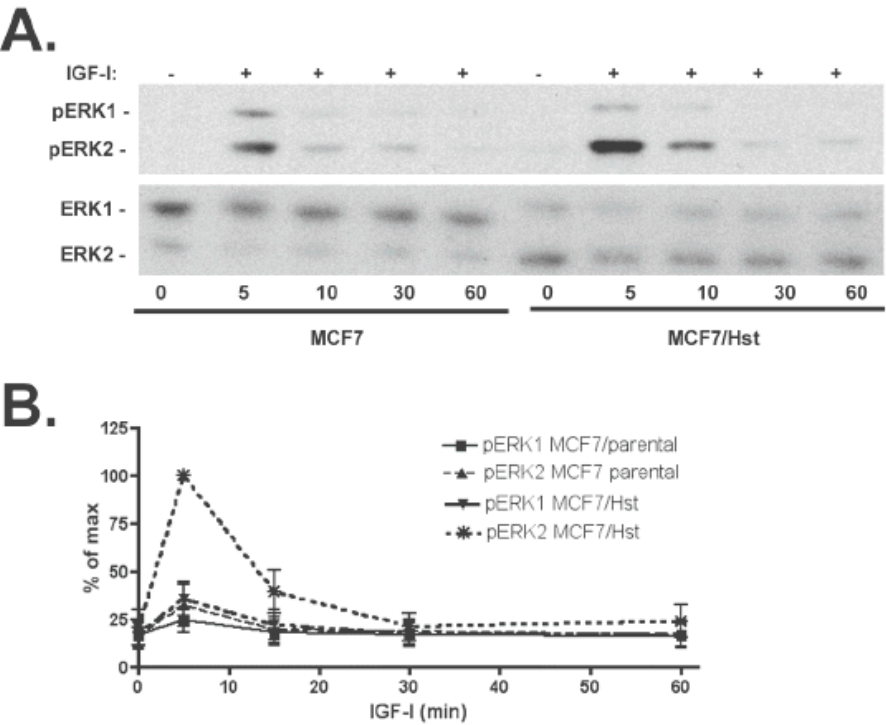


Figure 5.

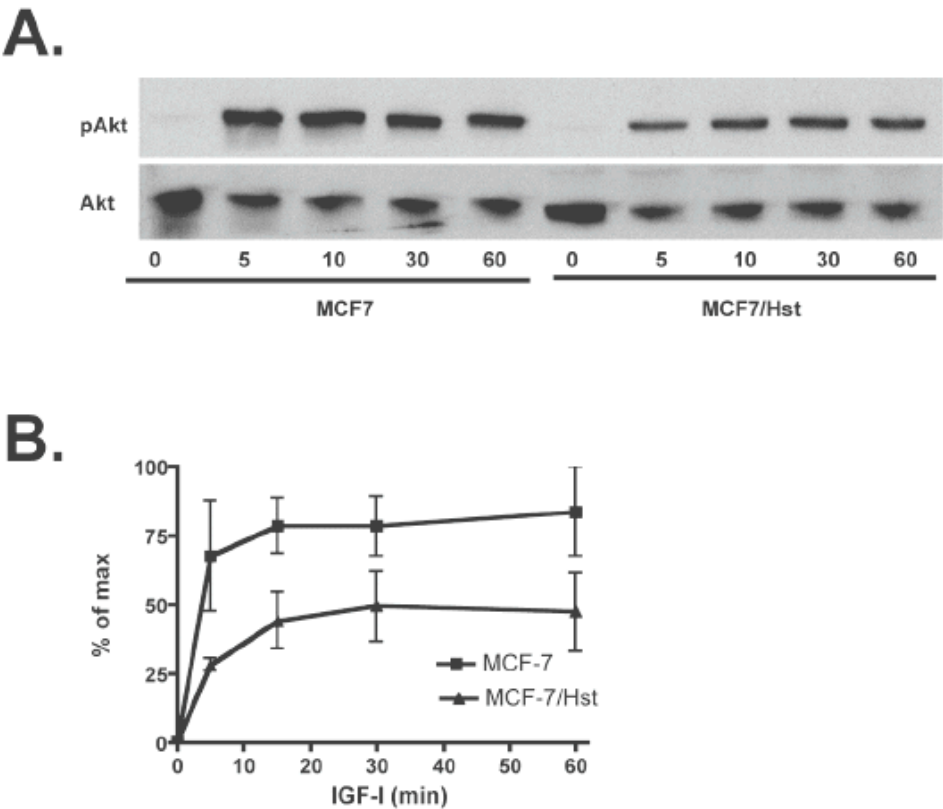
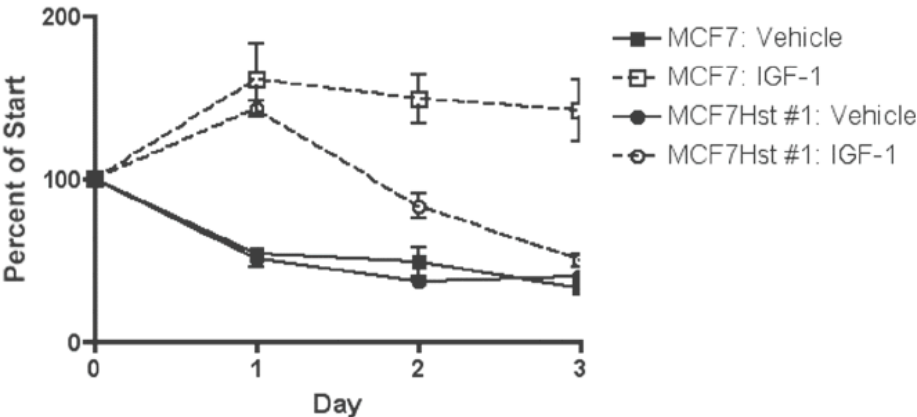


Figure 6.

A.



B.

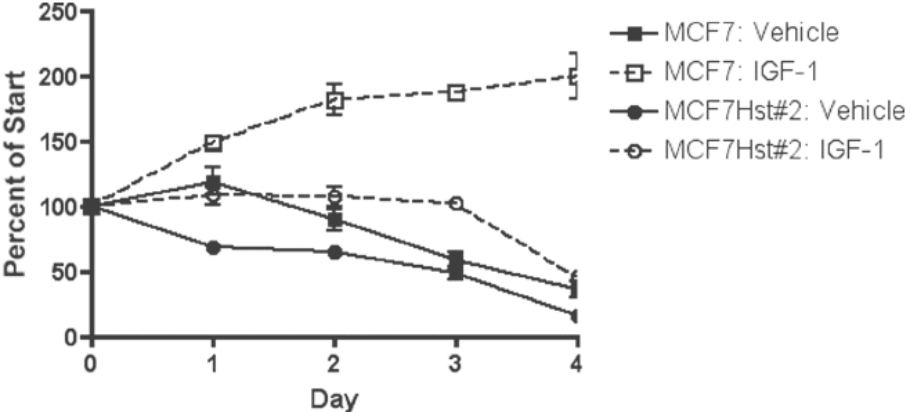


Figure 7.

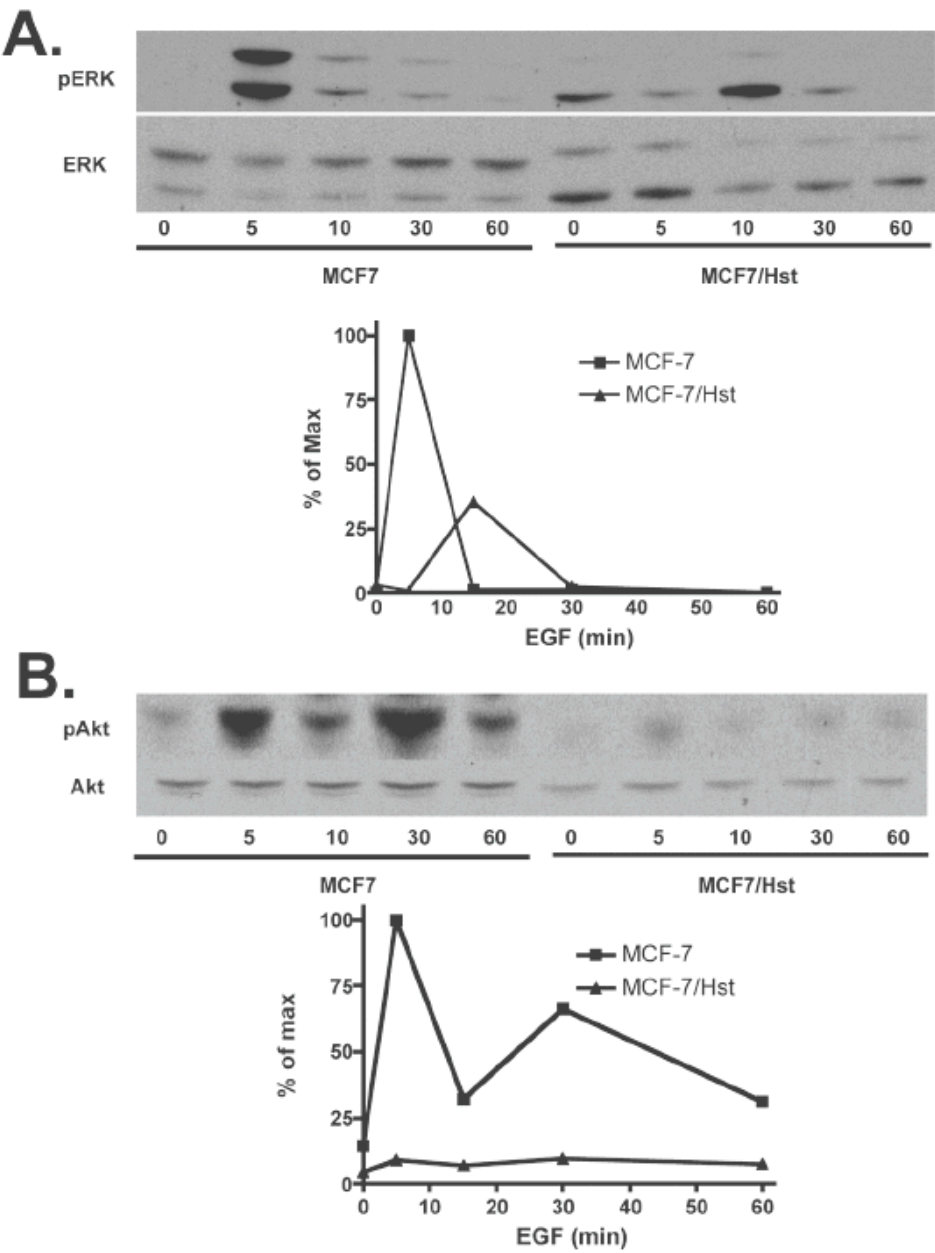


Figure 8.

