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<b>13. SUPPLEMENTARY NOTES</b>					
<b>14. ABSTRACT</b> The insulin-like growth factor (IGF) system has been shown to play role in breast cancer tumorigenesis and metastasis. Insulin receptor substrate (IRS) adaptor proteins are recruited to the IGF-1 receptor (IGF-1R) in response to IGF-I to activate downstream MAPK and PI3K signaling. Data from our lab suggests that different isoforms (IRS-1 and IRS-2) exhibit a selective propensity for one of these signaling pathways to drive cellular behavior, where IRS-1 drives proliferation through Grb2/MAPK activation and IRS-2 stimulates motility through PI3K/Akt induction. Our findings suggest that overlapping and distinct sets of genes are driven by IRS-1 and IRS-2 following IGF-1 exposure. While both isoforms regulate expression, early (4h) IRS-2 genes linked to motility and late IRS-1 (24h) genes linked to proliferation. IGF-I-induced upregulation in the transforming growth factor beta 2 (TGFβ2) gene suggested that IRS isoforms link the IGF and TGFβ pathways. Comparative analysis and hierarchical clustering of our IRS-driven microarrays with published data sets uncovered numerous commonly regulated genes. Moreover, certain subsets were linked to poor patient survival. Our data suggest that IGF stimulation of breast cancer cells results in distinct profiles of gene expression that are dependent on IRS adaptor protein expression and these signatures may reveal poor prognosis.					
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## **Introduction:**

The insulin-like growth factor (IGF) pathway plays an integral role during breast tumor establishment, propagation and therapeutic resistance. Recruitment of insulin receptor substrate (IRS) adaptor proteins to the type 1 IGF receptor (IGF-1R) following IGF-I ligand stimulation dictates initiation and subsequent activation of multiple oncogenic kinases. Activation of the Grb2/MAPK and PI3K/Akt pathways are prime examples of this phenomenon and are key downstream IRS response elements known to drive the expression of multiple gene targets as global expression patterns in cancer cells.

Expression of distinct IRS isoforms in T47D-YA cells (T47D-CO strain that is IRS null) has revealed that IRS-1 links IGF-1R to proliferation and growth via the MAPK pathway, while IRS-2 drives a motile phenotype via the PI3K pathway in breast cancer cells (1). However, the degree and manner in which IRS proteins mediate global gene expression has yet to be definitively determined (2,3). This study seeks to identify IRS-driven regulatory gene elements and networks in to better define the impact of IGF-I on transcriptional profiles. Therefore, we hypothesize that IRS-1 mediated proliferation and IRS-2 induced migration is dependent upon Grb2/MAPK and PI3K/Akt activation and that IRS species differentially regulate patterns of global gene expression.

## **Body:**

**Specific Aim 1: Identify how Grb2 and p85 interaction with IRS influences MAPK and PI3K associated signaling and biology.**

*1.1) Do Grb2 and p85 interact differentially in the IRS-1 and IRS-2 T47D-YA clones?*

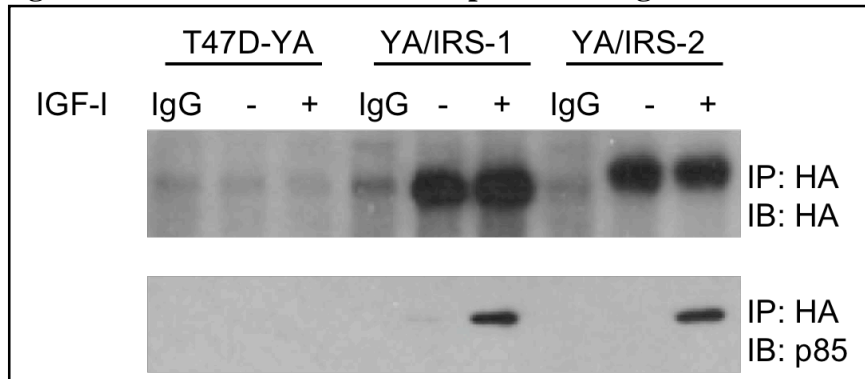
*1.2) Will mutation of the proposed Grb2 and p85 binding site of IRS alter association?*

*1.3) How will biological behavior be affected in vitro by the loss of Grb2 and p85 binding to IRS?*

We have determined that both IRS-1 and IRS-2 rapidly recruit p85 in response to IGF-I ligand stimulation in our T47D-YA/IRS-1 and 2 clones (Figure 1). Determining the role of Grb2 and IRS-1/2 binding has proven unsuccessful.

While our early reports suggested that both IRS-1 and IRS-2 recruit Grb2 (IRS-1 associates to Grb2 with a greater propensity), we have since revealed that in our model system and this interaction carries with it a significant degree of non-specificity. Inclusion of an IgG control during an HA-tagged IRS immunoprecipitation (IP)/Grb2 immunoblot (IB) or the reverse (IP:Grb2, IB: HA-tagged IRS) repeatedly resulted in the presence of Grb2 protein in the control lane. More specifically, an increase in the IGF-I lane was similar to levels of Grb2 in the IgG control lane. To our dismay, this phenomenon persisted for several months, regardless of cell condition, reagents and antibodies. Therefore, we now seek to answer the questions pertaining to Specific Aim 1 via a different experimental approach.

**Figure 1. IRS-1 and IRS-2 recruit p85 following IGF-I**



*Cells were exposed to IGF-I for 10 minutes and protein lysates collected. HA-tagged IRS proteins were immunoprecipitated with an anti-HA or non-specific IgG antibody and immunoblotted for either HA or p85.*

While physical association is important to understanding the role of IRS and downstream signaling activation, we recognize that any result obtained from mutated IRS species will be cumbersome to relate directly *in vivo* and clinically. Therefore, we are now employing the highly selective MAPK and PI3K inhibitors U0126 and LY294002 in order to block IRS-induced activation of the two signaling pathways. Following drug inhibition and IGF-I ligand stimulation, cells will be collected and mRNA species analyzed by RT-qPCR for IRS species and relevant genes. We anticipate that alterations in gene regulation are more telling and truly reflective of how early IRS/Grb2/p85 interactions shape a cellular response. More importantly, this approach will circumvent the limitations of the T47D-YA/IRS clonal cell line model. This strategy allows feasible examination of a multitude of normal and cancerous breast cell lines (e.g. MCF-10A, MCF-7, MDA-MB-231, F11, etc.) more reflective of clinical disease.

We will use the aforementioned approach to answer the third sum aim (1.3), whereby in response to selective inhibitors and IGF-I stimulation cellular proliferation and motility will be measured. These experiments are currently ongoing and we anticipate completion of Specific Aim 1 in the immediate future.

**Specific Aim 2: Dissect differences between IRS-1 and IRS-2 expression by gene array analysis and establish isoform-specific biomarkers and genetic signatures.**

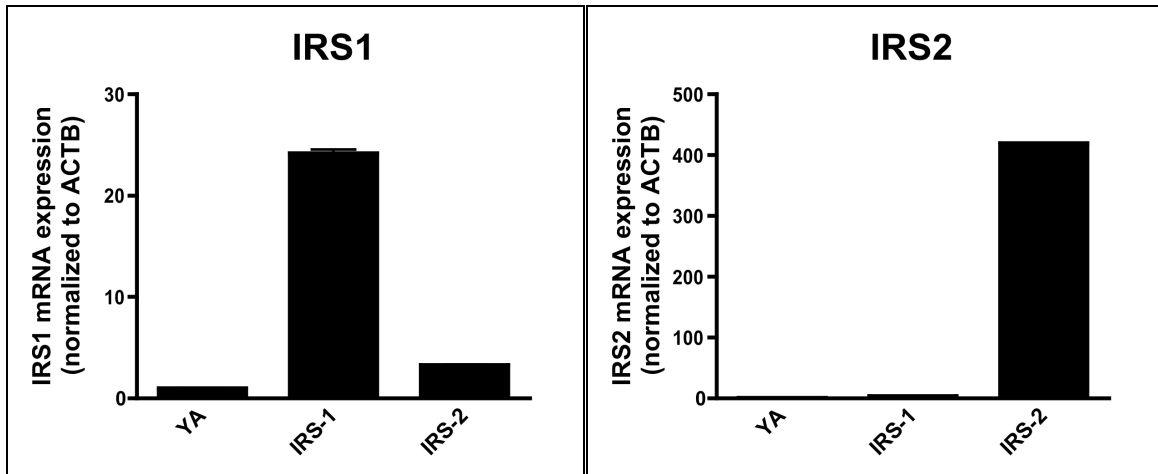
**2.1) How is global gene expression influenced by differential expression of IRS proteins?**

**2.2) Do patterns of global gene expression correlate with human breast tumor databases?**

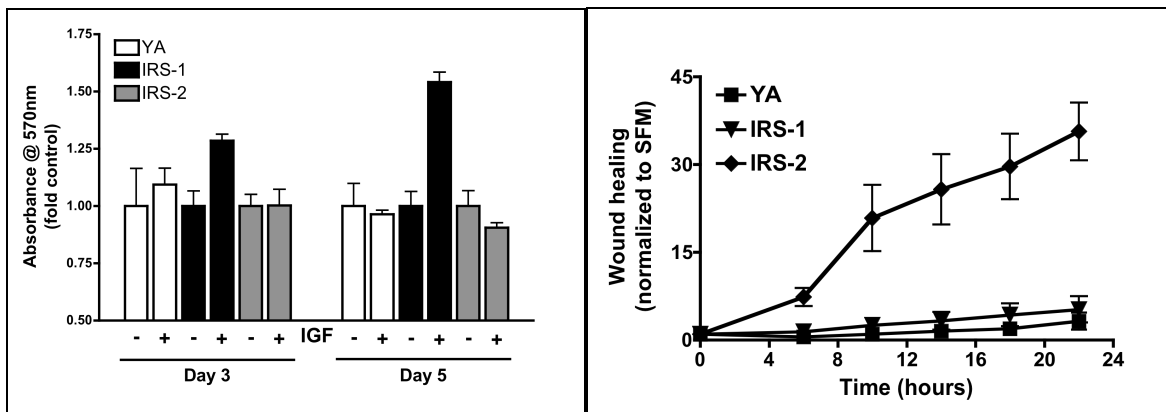
Our initial goal, aimed at dissecting differences between IRS-1 and IRS-2 expression by gene array analysis in order to establish isoform-specific biomarkers and genetic signatures, was predicated on stable cell lines generated previously (1). Prior to submitting mRNA for microarray analysis we discovered that the signaling phenotype in the IRS-2 expressing T47D-YA cells did not coincide with earlier characterization. As a result, we regenerated clones from the T47D-YA/IRS-2 cell line and selected two new clones demonstrating IRS-2-specific

signaling (PI3K activation) and cellular behavior (motility) (Figure 2). We observed increased proliferation in IRS-1 cells alone and enhanced motility in IRS-2 cells as determined by MTT monolayer growth assay and scratch wound analysis.

**Figure 2. IRS-1/2 expression and behavioral characterization**



**Figure 2A.** RT-qPCR results depicting mRNA expression of the following cell lines: T47D-YA (“YA”), T47D-YA/IRS-1 (clone #10 and #20 combined “IRS-1”), and T47D-YA/IRS-2 (subclone #1 and #6 combined “IRS-2”).



**Figure 2B.** Assessment of proliferation (Left, MTT assay) and motility (Right, scratch wound assay) following IGF-I treatment in T47D-YA, IRS-1 and IRS-2 cell lines.

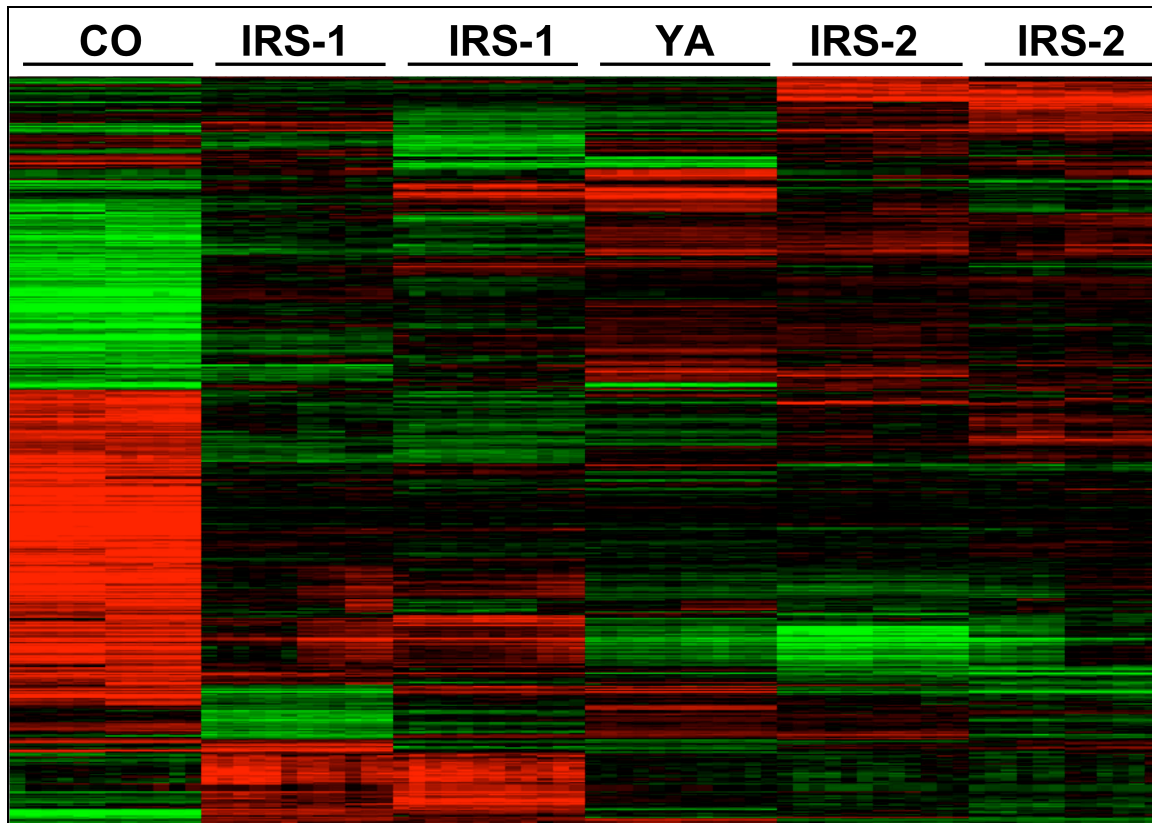
Following confirmation of the clones we proceeded to microarray analysis. As proposed, cells were plated at a density of  $3 \times 10^6$  in 150mm dishes, allowed to equilibrate overnight, and the media was replaced by SFM (no fibronectin) for 24h prior to stimulation. At time = 0 cells were treated with SFM (+ fibronectin) alone or IGF-I and RNA collected using the Qiagen - RNeasy Mini Kit or 5 Prime - PerfectPure RNA Tissue Kit according to the manufacturer instructions (Fisher Scientific) at 4h and 24h. RNA quantity was determined by 260:280 assay and quality

via the Agilent Bioanalyzer 2100 to ensure RNA banding conservation. Isolated RNA samples were then submitted to the University of Minnesota Biomedical Genomics Center - Microarray Facility for biotin labeling, synthesis and hybridization to the Affymetrix U1330 Plus 2.0 array.

All arrays were normalized using GC-RMA process embedded in GeneData Refiner and further normalized to corresponding untreated states to isolate IGF-I response independently of basal differences between cell lines. T-tests were performed between groups using GeneData Expressionist with P-values < 0.05 and a minimum average fold-change of 1.5 was set as a cutoff. Hierarchical clustering was carried out on log base 2 transformed data generated using Gene Cluster 3.0. Data was visualized and images generated using Java TreeView.

Upon comparison of basal differences between cell lines (correction for changes induced by IGF-I treatment and over time) we determined that IRS expression alone altered basal gene expression (Figure 3). Supervised clustering revealed over 700 transcripts were differentially expressed at the basal level.

**Figure 3. IRS expression alters basal gene expression**

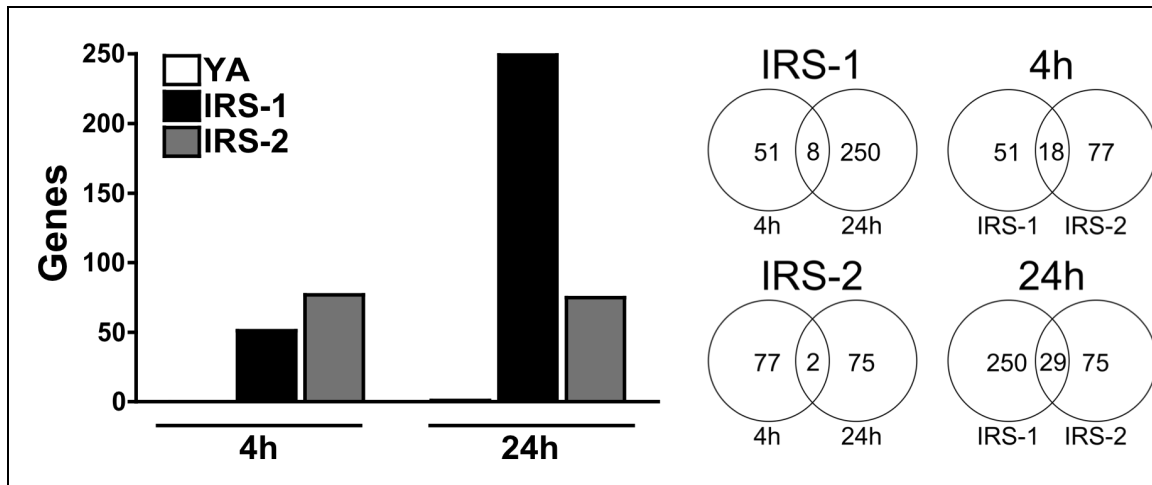


*Gene cluster analysis where the y-axis represents transcripts and the x-axis represents all treatment and time-point conditions (e.g. CO columns from left to right = 4h SFM, 4h IGF, 24h SFM, 24h IGF with each group in triplicate for a total of 6 cell lines and 72 total points).*

In addition to basal gene changes, we observed a number of distinct and overlapping IGF induced genes when we compared IGF-I to SFM conditions at both the early and late time points (Figure 4). We unexpectedly observed that while IGF-I induced gene expression changes in the

parental T47D-CO cell (IRS-1/2 expressing), absence of IRS an IRS-null status (T47D-YA) resulted in a loss of IGF-induced gene expression (only 1 gene was altered at the late time point). Expression of IRS-1 and IRS-2 resulted in a rescue of IGF-I gene induction and a substantial increase in gene expression. When the IRS-1 and IRS-2 clones were compared to one another alone, we discovered an early set of motile genes regulated in the IRS-2 clones and proliferative set of genes modulated late in the IRS-1 clones. These data support an early gene induction by IRS-2 and late by IRS-1 that may link each isoform to their exclusive IGF-I-regulated cell behavior.

**Figure 4. IRS was required to induce global gene expression in an IGF-I and time-dependent manner**



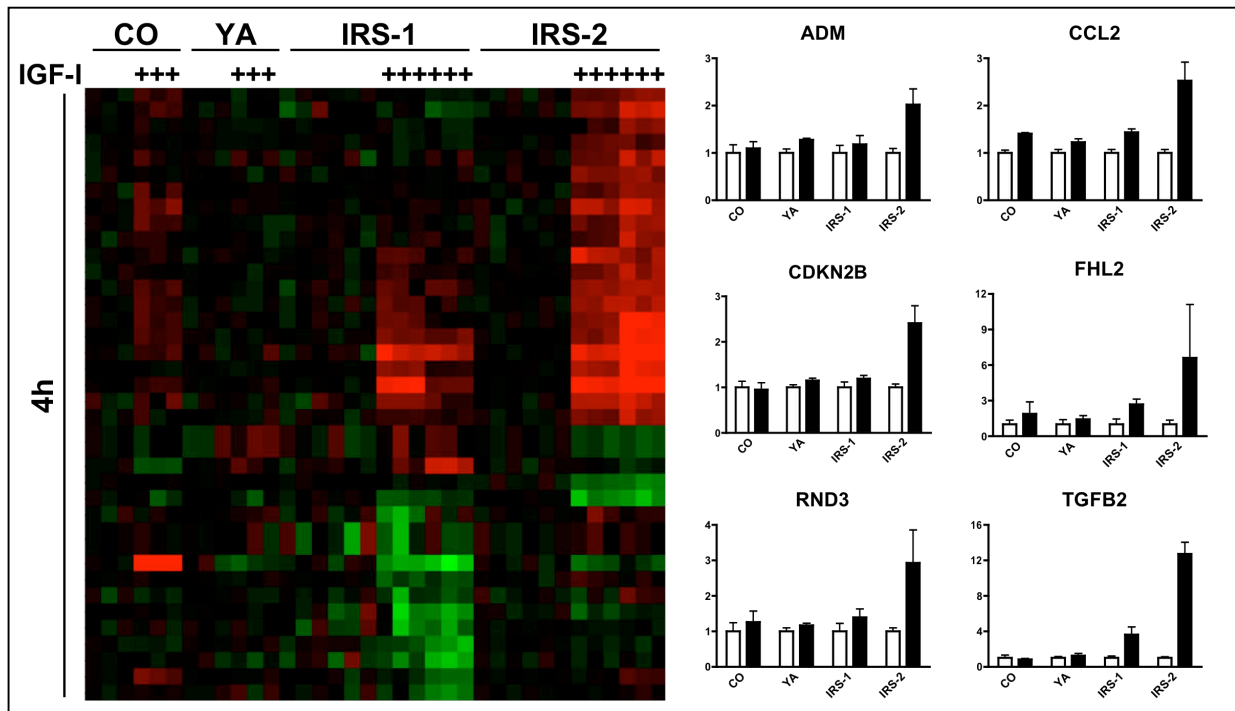
*Left- Bar graph at represents the number of IGF-regulated genes in comparison to SFM (IRS clones grouped) at 4 and 24 hours. Right- Venn diagrams represent a comparison of genes between the IRS-1 and IRS-2 cells that were distinct or overlapping at the 4 or 24 hour time points.*

We have validated a number of genes from the microarray analysis by RT-qPCR analysis. Early findings indicate a number of genes from the array are reproducible and specific to IRS-1 and/or IRS-2 (Figure 5).

The heat map depicted is a comparison of IGF-I-treated IRS-1 vs. IRS-2 clones and reaffirm that a number of early IRS-2 and late IRS-1 genes. In addition, we present here six early genes (ADM, CCL2, CDKN2B, FHL2, RND3, TGFB2) from the IRS-2 cells that have been independently shown to correlate with cell motility, metastasis and breast cancer.



**Figure 5. Early IRS-2 genes link to breast cancer metastasis**

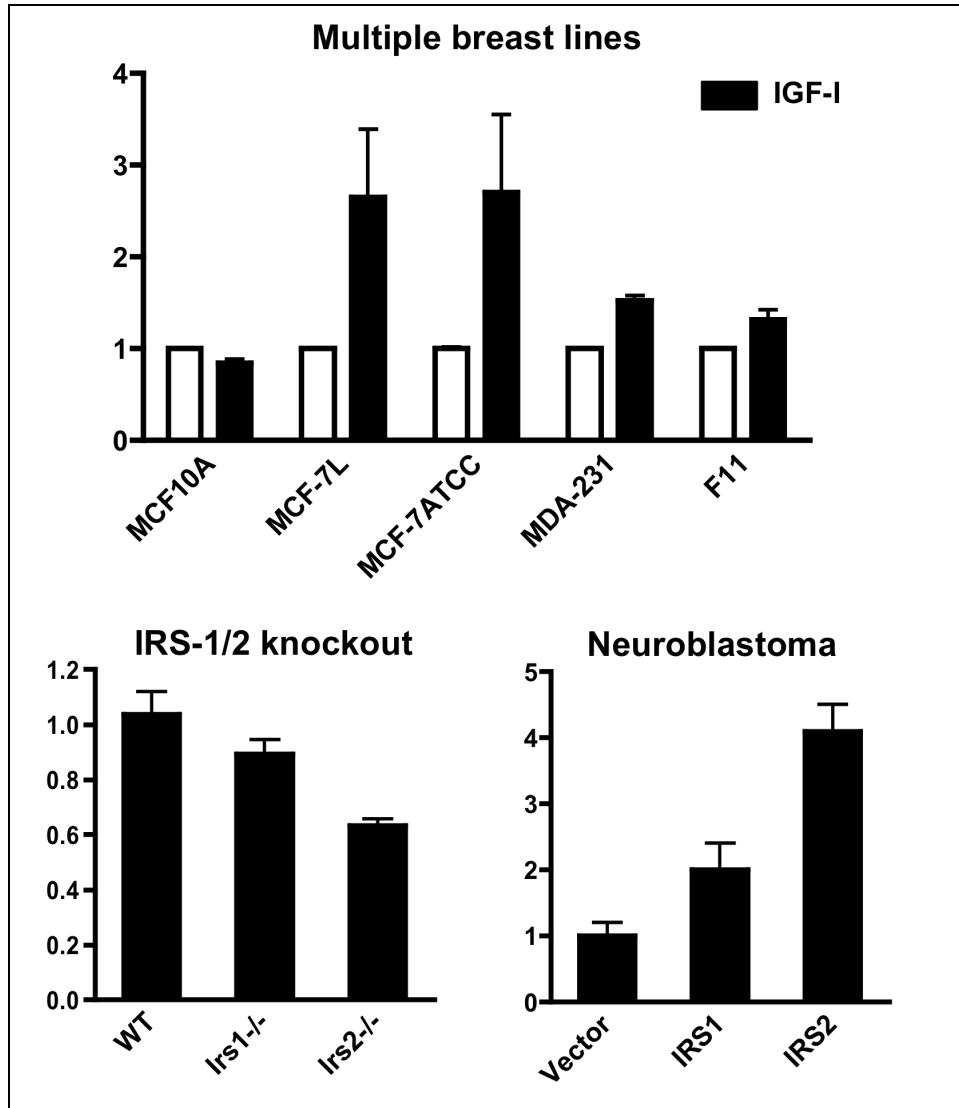


*Left- Heat map is a comparison of IGF-treated IRS-1 clones vs. treated IRS-2 clones at the early 4 hour time point. Right- RT-qPCR of six confirmed IRS-2 genes linked to motility. Open bars indicate SFM conditions and black bars indicate IGF-treated.*

A number of reports have demonstrated that transforming growth factor beta (TGF $\beta$ ) pathway regulates multiple facets of breast tumor biology, including metastasis. Interestingly, a 10-fold upregulation of TGF $\beta$ 2 gene expression by IGF-I was observed in the IRS-2 clones. In addition, IGF-I induced TGF $\beta$ 2 expression in IRS-1 clones, albeit to a lesser yet significant degree. This suggested a potential link between the IGF pathway via IRS expression and TGF $\beta$ 2 regulation.

As a result, we looked to both additional breast cancer lines and a non-breast cancer model of malignancy. The following cell lines express both IRS-1 and IRS-2 and are behaviorally regulated (proliferation and/or motility) by IGF-I stimulation: MCF-7, MCF-7 ATCC, MDA-231, F11 (MDA-231 BO). IGF-I induced TGF $\beta$ 2 expression in all four lines (Figure 6). IGF-I did not induce TGF $\beta$ 2 expression in the immortalized, non-transformed human mammary epithelial cell line, MCF-10A, and served as a negative control. Additionally, we confirmed that IRS expression linked to TGF $\beta$ 2 expression via confirmation in mammary tumor RNA obtained from an IRS-knockout mouse model (2) and an IRS-overexpression SH-EP neuroblastoma model (4). Specifically and relatively, loss of IRS-2 resulted in the lowest TGF $\beta$ 2 expression and IRS-2 overexpression resulted in the highest TGF $\beta$ 2 expression.

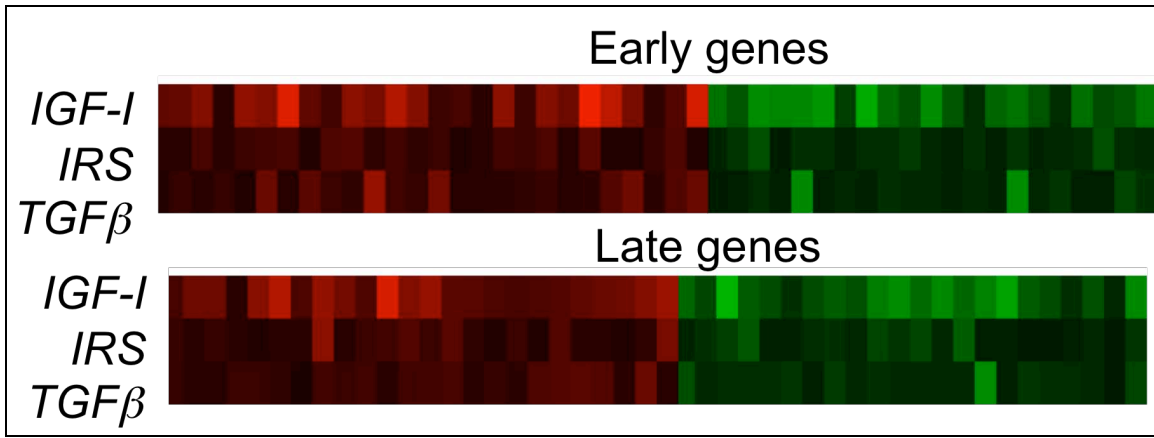
**Figure 6. TGF $\beta$ 2 expression was regulated in an IGF-I and IRS-1/2-dependent fashion in multiple cancer models**



*TGF $\beta$ 2 expression was assessed by quantitative RT-PCR in multiple models. Top - multiple breast cancer lines (IGF-I response measured at 4h). Bottom left - IRS-knockout mouse model (Shaw). Bottom right - IRS-overexpressing SH-EP neuroblastoma cells (Feldman). All data was normalized to the GUSB housekeeper gene.*

We then compared our arrays with published IGF-I (MCF-7) and TGF $\beta$ -derived (MCF10A, MDA-231, HaCaT, HPL1) microarrays (5, 6) and found 75 genes that were regulated in common between these signatures (Figure 7). Further analysis revealed a large degree of overlap in terms of both direction (upregulation vs downregulation) and magnitude (fold-induction).

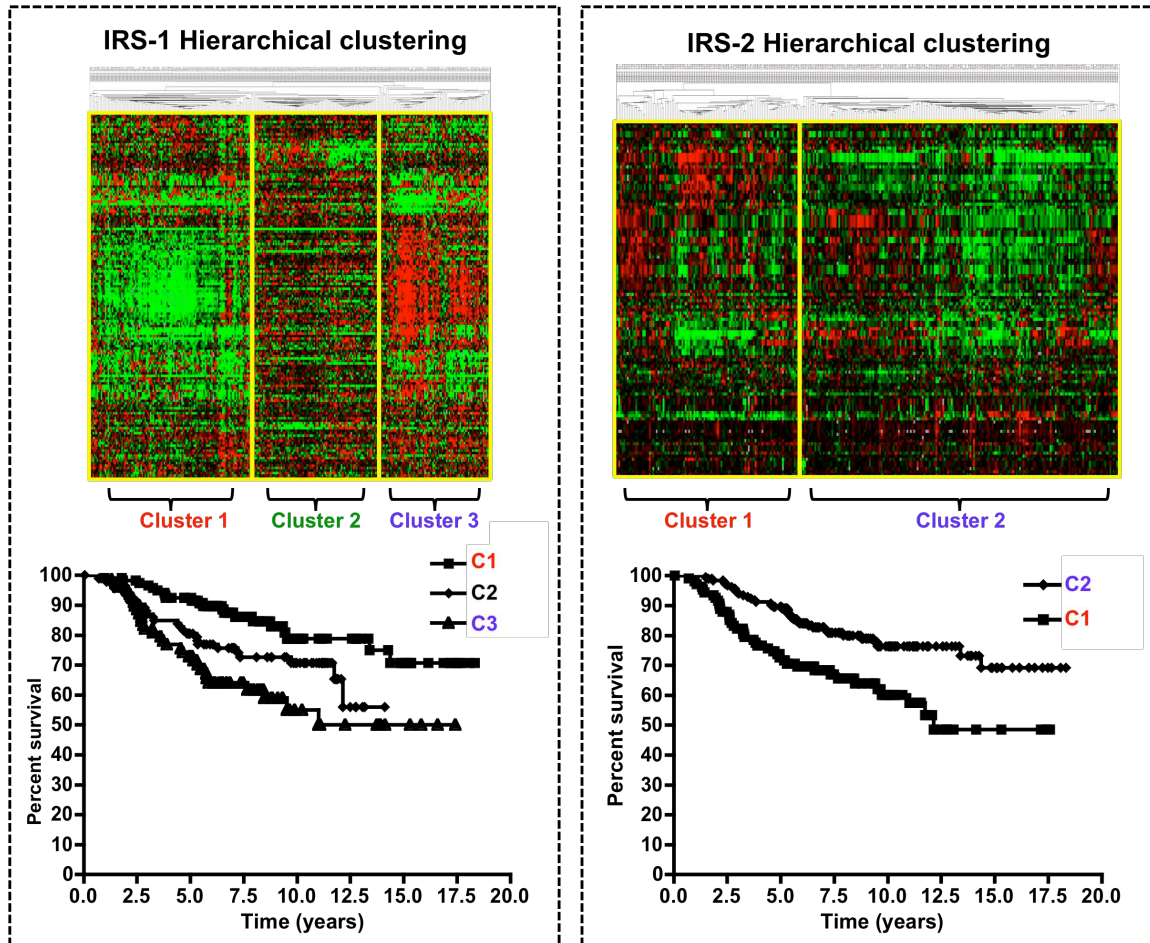
**Figure 7. IGF-I and TGF $\beta$  triggered overlapping patterns of gene expression in various breast cancer lines**



*Published MCF-7 IGF-I (Creighton) and TGF $\beta$ -derived (Padua) microarrays were compared to our T47D arrays. A set of commonly regulated genes (IGF-I and TGF $\beta$ -induced) was derived based on a fold-cutoff (1.2) and p-value (0.05) and cluster analysis performed. Figures depict both early and late gene induction overlap.*

To explore the clinical relevance of the signatures we developed, we examined the NKI 295 dataset that was used to establish the 70-gene profile of prognosis (7) via hierarchical clustering analysis (Figure 8). Strikingly, we discovered that patient survival was heavily influenced by the degree to which tumor expression correlated to the conserved IRS signatures. A high degree of correlation resulted in the poorest disease free survival and an inverse correlation resulted in an improved disease free survival. These data indicate prognostic and possible predictive value of the IRS signatures in breast cancer patients.

**Figure 8. IRS-1 and IRS-2 signatures derived *in vitro* conveyed poor prognosis in human tumors**



*Hierarchical clustering analysis was performed comparing the IRS-1 (left) and IRS-2 (right) gene signatures derived from the T47D-YA/IRS clones in the NKI-295 human tumor set. Patient survival from each cluster (IRS-1 clustered into 3 groups and IRS-2 clustered in 2 groups) was determined and Kaplan Meier curves plotted.*

### **Key Research Accomplishments**

- IRS proteins were required to confer ligand response as the presence of a functional IGF-IR alone in T47D-YA cells was not sufficient to induce gene expression following IGF-I exposure.
- IRS expression altered basal gene expression in an isoform-specific manner as cluster analysis revealed distinct genetic and genomic clonal redundancy with partial overlap.
- IGF-I-induced early IRS-2 and late IRS-1-driven gene expression in breast cancer cells.

- Early IRS-2 and late IRS-1 regulated genes exhibited distinct isoform signatures.
- A subset of IRS-1 and IRS-2 linked genes further confirm their role in tumor cell growth, proliferation, adhesion and motility.
- Multiple models of cancer confirmed IRS and IGF-I-driven TGF $\beta$ 2 expression.
- IRS-driven gene signatures exhibited high homology to published IGF-I and TGF $\beta$  gene expression patterns.
- IRS-1 and IRS-2 signatures derived *in vitro* conveyed poor prognosis in human tumors.

### **Reportable Outcomes**

#### Presentations

Becker MA, Byron S, Sarver A, Yee D. IRS isoforms differentially mediate global gene expression patterns in T47D breast cancer cells. Gordon Research Conference: Insulin-Like Growth Factors In Physiology & Disease, Ventura, CA, March 2009. *Poster Presentation*

Becker MA, Byron S, Sarver A, Yee D. IRS isoforms differentially mediate global gene expression patterns in T47D breast cancer cells. AACR Annual Meeting, Denver, CO, April 2009. *Poster Presentation*

Becker MA, Ibrahim YH, Byron SA, Sarver AL, Silverstein KS, Lee AV, Yee D. IGF-induced gene profiles are dependent on IRS expression and convey prognostic value in human breast cancer. San Antonio Breast Cancer Symposium, San Antonio, TX, December 2009. *Poster Presentation*

### **Conclusion**

IRS proteins were required for IGF-I-induced gene expression and distinct patterns of regulation indicate early motile IRS-2 and late proliferative IRS-1 signatures as potential breast cancer biomarkers. These data suggest that IGF stimulation of breast cancer cells results in distinct profiles of gene expression that are dependent on IRS adaptor protein expression. In addition, some of the “IRS-regulated” genes are shared in common with other gene signatures of poor prognosis. With the use of anti-IGF therapies in breast cancer, attention should focus on the use of these profiles as prognostic and predictive biomarkers. Current analyses to further validate these IRS-1 and IRS-2 signatures to human breast tumors are ongoing.

### **Significance**

This study emphasizes the need for IRS-specific signatures as biomarkers in breast cancer downstream of the IGF-IR. Identification of IRS-driven gene targets and expression patterns

may provide novel biomarkers and correlate clinically with current prognostic and predictive indicators for breast cancer.

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