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13. ABSTRACT (Maximum 200 Words) Insulin receptor substrate 1 and 2 are downstream signaling molecules for growth factors and adhesion receptors that are implicated in breast cancer. In this report, we dissect the individual roles of IRS-1 and IRS-2 in the promotion and progression of breast carcinoma. We show that IRS-1 and IRS-2 play distinct roles in the malignant progression of breast carcinoma. IRS-1 and IRS-2 are both required for cell proliferation, with IRS-2 being a better mediator of cell proliferation. We also show that IRS-2 but not IRS-1 is required for cell survival in the absence of exogenous growth factors and in invasion. In fact, IRS-1 function might reduce the efficacy of IRS-2 in these processes. Altogether, our findings suggest that IRS-1 is involved in the early stages of breast cancer establishment and that IRS-2 is required for its maintenance and progression to malignancy.				
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Introduction

Understanding the signaling mechanisms that lead to cancer onset and progression is essential for providing better tools for inhibiting these processes. I have begun to investigate the insulin receptor substrate (IRS) family of proteins that I believe are key mediators of signaling pathways that lead to breast cancer. The IRS proteins are docking proteins that do not contain intrinsic kinase activity but transmit signals downstream of several growth factor and cytokine receptors including the insulin and insulin-like growth factor I (IGF-I) receptors (1). Importantly, some of these receptors have been implicated in breast cancer. For example, increased expression of the IGF-IR in breast carcinoma cells has been reported and this increased expression is associated with a poor prognosis for breast cancer patients (2). Increased levels of IGF-I and IGF-II, ligands for the IGF-IR, have also been reported in breast cancer, and overexpression of these growth factors in transgenic mice leads to mammary tumorigenesis (3,4). To further emphasize the importance of the IRS proteins in breast cancer, we recently found that these docking proteins are phosphorylated and recruit phosphatidylinositol-3 kinase (PI3K) upon ligation of the $\alpha 6\beta 4$ integrin in breast carcinoma cells (5).

There is growing evidence to support an important function for $\alpha 6\beta 4$ in breast cancer. For example, like the IGF-IR, increased expression of the $\alpha 6$ and the $\beta 4$ subunits correlates with a poor prognosis in breast cancer patients (6,7). Furthermore, previous studies by Shaw and colleagues (8) have shown that expression of the $\alpha 6\beta 4$ integrin increased breast carcinoma cell invasion and this effect was due to increased activation of PI3K. Our recent findings that the IRS proteins are involved in the $\alpha 6\beta 4$ -mediated activation of PI3K supports further the importance of these docking proteins to breast carcinoma progression.

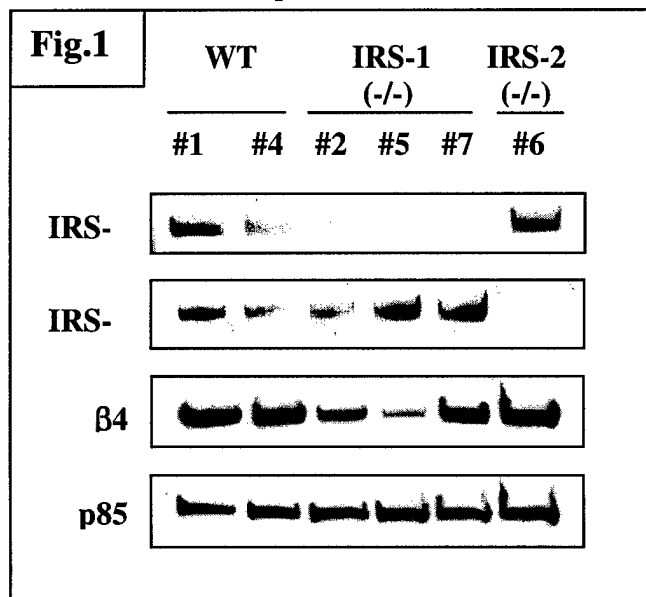
During the last year, 5/15/2002-5/14/2003, I have been able to elucidate a functional difference between IRS1 and IRS2 in breast carcinoma progression and further identify the signaling pathway involved in mediating their functions. IRS-1 and IRS-2 null cell lines (IRS1(-/-) and IRS2(-/-) cells, respectively) that have been isolated in our lab from polyoma middle T- derived mammary tumors from IRS-1 and IRS-2 knockout mice were compared to wild type tumor cells in their ability to proliferate, survive in serum-free medium and invade *in vitro*. IRS-1 deletion caused a decrease in the ability of the cells to proliferate while enhancing their ability to invade, and had no effect on cell survival. Deletion of IRS-2 on the other hand, further reduced cell proliferation and inhibited their invasion as well as induced apoptosis in serum-free medium. Taken together, these results indicate that both IRS-1 and IRS-2 must be functional for cell proliferation. However, only IRS-2 is important for breast carcinoma cell survival and invasion.

Body

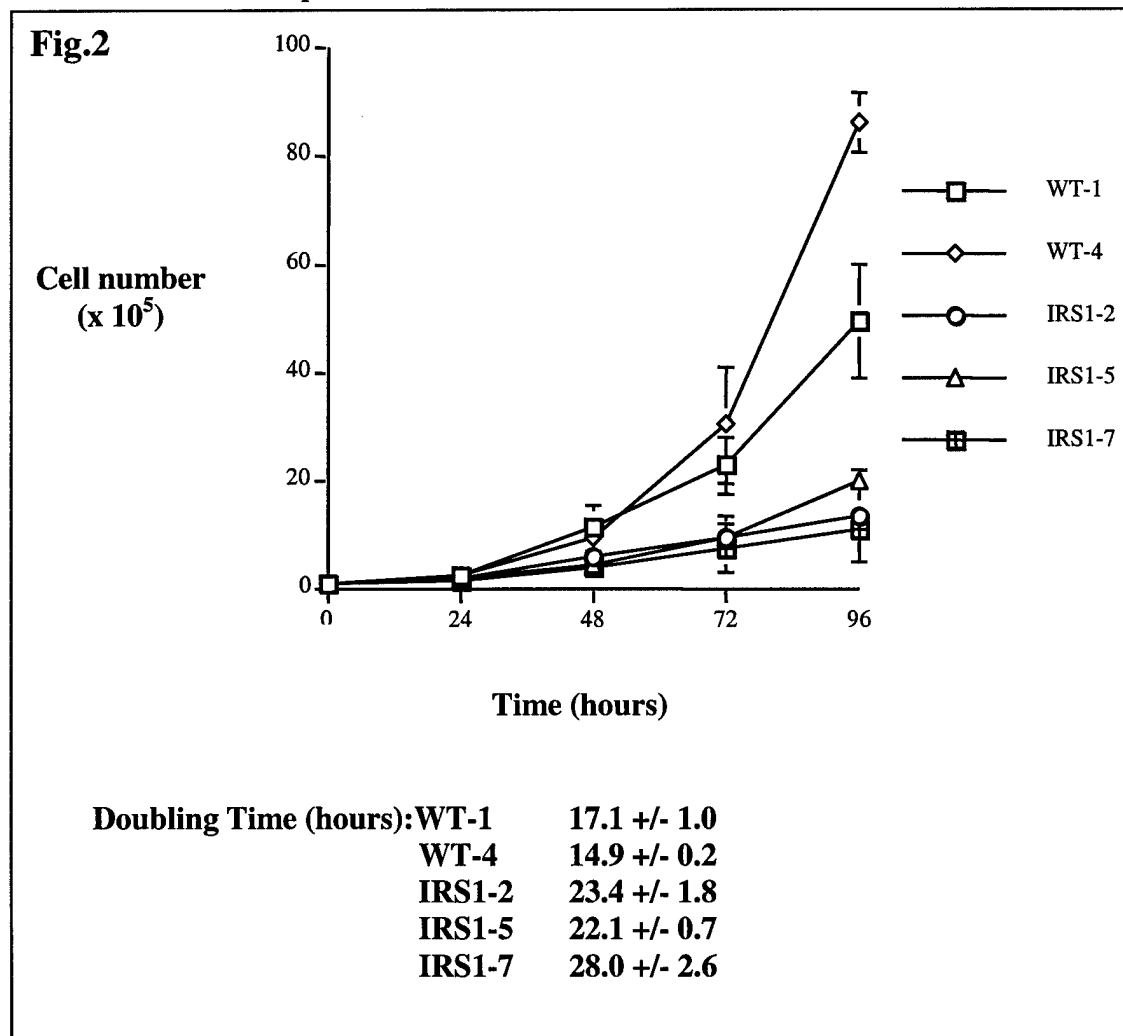
Given the importance of the IRS family of proteins as downstream signaling molecules for the IGF1 receptor and $\alpha 6\beta 4$ integrin, two known markers for breast carcinoma progression, I have begun to investigate their role in mediating these receptors functions. The IRS family includes IRS-1, IRS-2, IRS-3 and IRS-4, which all share structural homology and contain some common and some unique downstream effector binding sites (9,10). IRS-1 and IRS-2, but not IRS-3 and IRS-4, are expressed in the mammary glands (M. White, personal communication) and there is some direct evidence

for the involvement of these IRS homologs in breast cancer. In primary human breast cancers, high IRS-1 expression correlates with an increased likelihood of tumor recurrence and a decreased survival rate (11). In addition, IRS-1 expression is regulated by the estrogen receptor and the level of IRS-1 expression is decreased in response to anti-estrogens such as tamoxifen and ICI 182,780 (12). This regulation of IRS-1 expression has been hypothesized to be a mechanism by which these anti-estrogens decrease breast carcinoma growth. IRS-2 is not regulated by estrogen and anti-estrogen drugs in this fashion (11). However, expression of IRS-2 but not IRS-1, correlates with an invasive, metastatic phenotype in breast carcinoma cells (13). These data suggest that despite their overall similarity in structure and some shared downstream effectors, IRS-1 and IRS-2 may regulate distinct signaling pathways that differentially influence breast carcinoma promotion and progression. This hypothesis is supported further by the fact that distinct IRS-1 and IRS-2 subcellular localization, phosphorylation patterns, and effector recruitment have been observed in other cell types (13). Also, IRS-1 and IRS-2 knock out mice exhibit different phenotypes with regard to growth and glucose metabolism (1). The overall goal of my research is to establish the contribution of IRS-1 and IRS-2 to $\alpha 6\beta 4$ -dependent signaling and breast cancer. Such research will provide us with better tools for diagnosing and predicting breast cancer behavior and lead to identifying potentially new therapeutic targets.

To elucidate any functional differences between IRS1 and IRS2 in breast carcinoma cells, I have utilized IRS-1 and IRS-2 null cell lines that have been isolated in our lab from polyoma middle T (PyV-MT) - derived mammary tumors from IRS-1 and IRS-2 knockout mice. Subclones from each knockout tumor cell line were compared to subclones isolated from wild type (WT) tumor cell lines in three essential functions that promote the progression of breast carcinoma, namely proliferation, survival and invasion *in vitro*. Figure 1 shows the expression profile of relevant proteins in the different subclones used in the different experiments.



Earlier studies have shown that IRS-1 is required for IGF1-induced cell proliferation (12,14,15). To test the effect of knocking out IRS-1 or IRS-2 on cell proliferation, cells from each subclone were plated at equal numbers in 10%FBS/DMEM media, in 5 x60mm dishes. At 24h time intervals, attached cells were trypsinized, stained with 4% trypan blue and counted under the microscope. Cell number on each day for each subclone was plotted. As indicated in Figure2, IRS-1 (-/-) cells show a dramatic decrease in proliferation rate compared to WT. In culture IRS-2 (-/-) cells seem to be growth inhibited and preliminary results (not shown) indicate that these cells also proliferate at a low rate compared to WT cells. These results indicate that the both IRS-1 and IRS-2 are necessary for breast carcinoma cell proliferation. Western blot analysis (not shown) revealed that under normal growth conditions, phosphorylated mitogen activated protein kinase (MAPK) is reduced in IRS-2 (-/-) subclones compared to WT and IRS-1 (-/-) subclones. However, no difference in phosphorylated AKT, a downstream signaling molecule of PI3K was noticed. This observation indicates that IRS-2 promotes cell proliferation through a MAPK-dependent pathway, and PI3K/AKT signaling pathway is not involved in this process. Further analysis, by use of PI-3K and MAPK inhibitors, is required to confirm this conclusion.



To test the role of IRS-1 and IRS-2 in cell survival, PyV-MT subclones were plated at 7.5×10^5 cells/100mm dish in 10%FBS/DMEM. After 2.5h, when the cells have attached, cells were washed with phosphate buffered saline (PBS) twice then maintained in 10%FBS or 0.1%BSA/DMEM for 24h, at which time cells are ~ 50% confluent. Attached and floating cells were collected, washed with PBS, then stained with propidium iodide (PI) and analyzed by FACS. The average of the results of four experiments is shown in Figure3a.

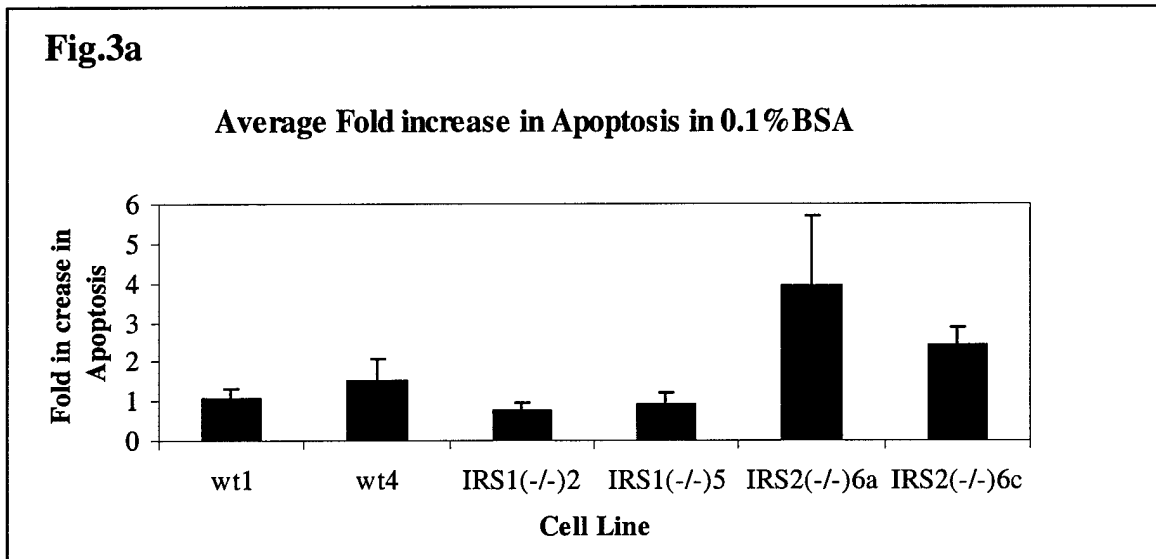


Figure 3a demonstrates that under serum-free conditions, IRS-2 (-/-) cells exhibited 3-5 fold increase in apoptosis compared to cells maintained in serum-containing media. WT clones were also affected by serum-starvation to a lesser extent (1.5 fold increase in apoptosis). However, there was no effect on IRS-1 (-/-) cell survival. These results indicate that IRS-2, but not IRS-1 function, is important for cell survival. Serum-starvation for 48h enhanced the apoptosis rate of WT and IRS-2 (-/-) cells but had no effect on IRS-1 (-/-) cells. Previous studies have shown that IRS-1 is important for IGF1-mediated cell survival in breast cancer cells and other cell types (16,17). IRS-2 on the other hand, was shown to be unimportant for this process. This report shows for the first time that IRS-2 is the main player in promoting breast carcinoma cell survival under serum-free (stress) conditions.

To further investigate the downstream signaling pathways involved in cell survival, WT and IRS-2 (-/-) cells were plated as above and maintained in 10%FBS/DMEM containing 10uM PI3K inhibitor LY294002, 10uM MAPK inhibitor PD98059 or equal volume of DMSO as a control. IRS-1 (-/-) cells were plated in duplicates and maintained in 10%FBS or 0.1%BSA/DMEM media containing the inhibitors. Cells were analyzed 24h later as mentioned above. The results of 3-6 assays are represented in Figures 3b and 3c.

Figure 3b shows that LY294002 treatment of IRS-2 (-/-) cells increased their apoptosis rate compared to the control cells, however there was no effect on WT cell survival. PD98059 treatment had no effect on cell survival for any of the cell types. Extended treatment of the cells with PD98059 did not result in apoptosis (data not shown). Extended LY294002 treatment of IRS-2 (-/-) cells further enhanced their apoptosis rate while it had no effect on WT cell survival (data not shown).

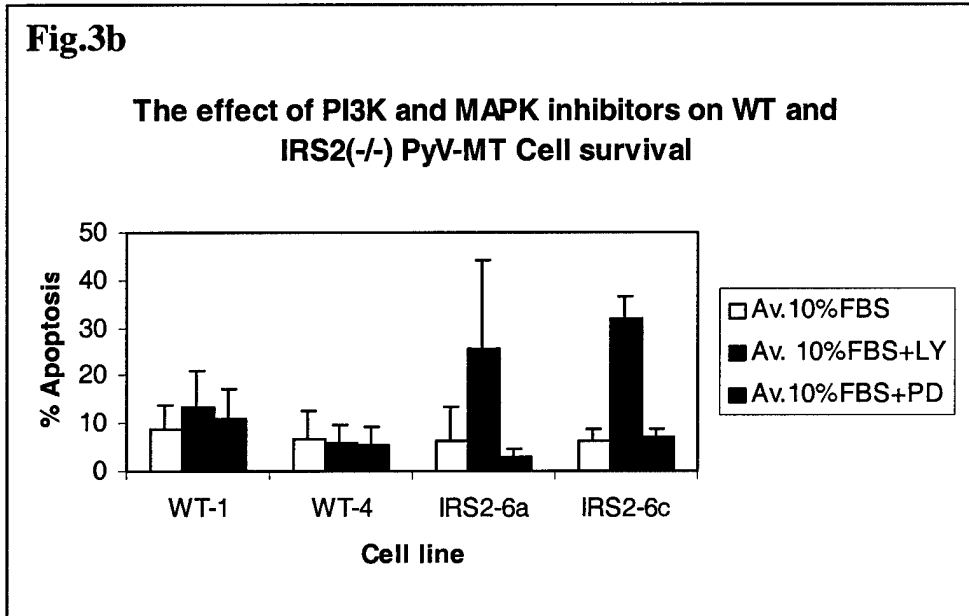
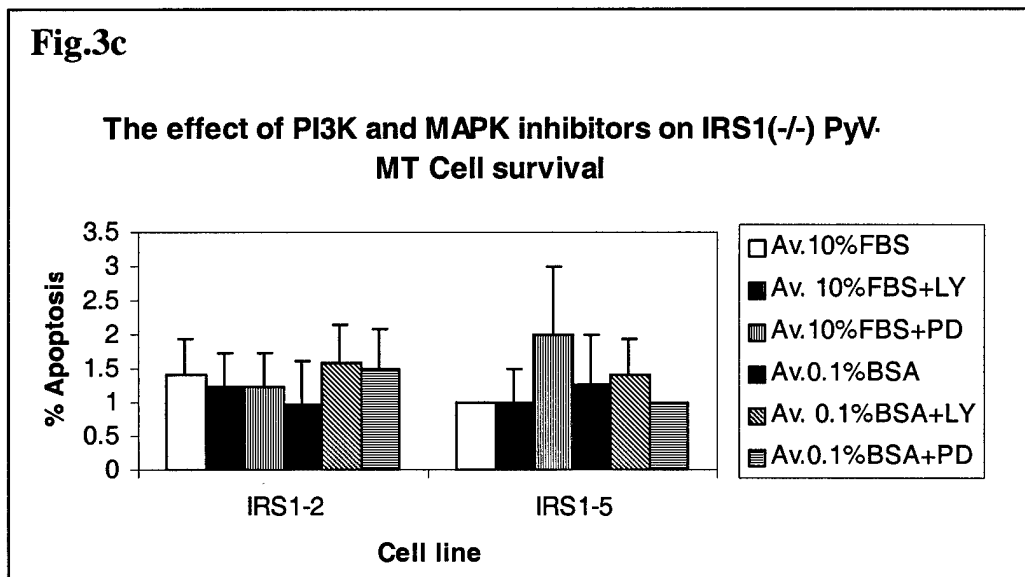
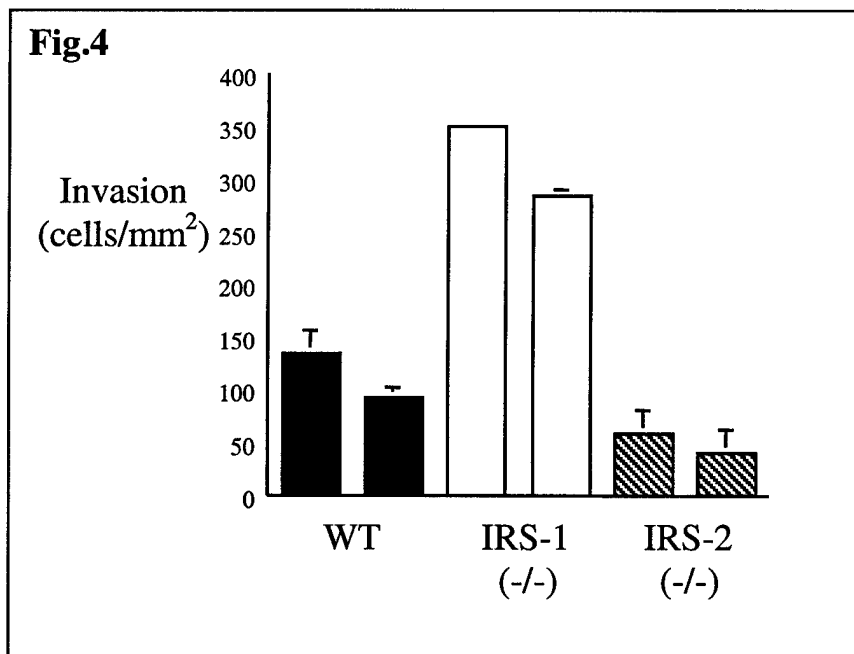


Figure 3c shows that neither LY294002 or PD98059 had an effect on IRS-1 (-/-) cell survival in the presence or absence of serum. Again, extended treatment of the cells with the inhibitors did not cause apoptosis.



The above results indicate that IRS-2, but not IRS-1, is important for breast carcinoma cell survival. The presence of IRS-1 may actually render the cells more susceptible to apoptosis, possibly by competing with IRS-2 for downstream signaling molecules that are necessary for IRS-2 mediated cell survival. The results also indicate that while IRS-1 appears to utilize a PI3K-dependent pathway to promote cell survival under normal growth conditions, IRS-2 operates through a PI3K and MAPK-independent pathway to promote cell survival in the presence or absence of exogenous survival factors.

The role of IRS-1 and IRS-2 in cell invasion was also assessed. Work in our lab has shown that IRS-2 phosphorylation and subsequent activation of PI3K is necessary for β 4-mediated cell invasion (5). IRS-2 is also involved in IGF-1-induced cell migration (18). To analyze the role of IRS-1 and IRS-2 in invasion, PyV-MT cells were plated in triplicates at 5×10^4 cells/well in the top chamber of matrigel-coated transwells. NIH3T3 conditioned media was put in the lower chamber of the transwells and the cells were allowed to invade for 4h, 37°C . The top of the transwells were then swabbed with cotton swabs to remove the cells that did not invade through the membrane. Cells on the bottom of the membrane were fixed in methanol then stained with crystal violet and counted. Figure 4 shows the results of a representative assay. Deletion of IRS-1 caused a 2-2.5 fold increase in invasion compared to WT cells, while deletion of IRS-2 caused ~2 fold decrease in invasion compared to WT cells. These results indicate that IRS-2, but not IRS-1, is required for cell invasion. Again, the presence of IRS-1 actually inhibits cell invasion, possibly by competing with IRS-2 for downstream signaling molecules that are necessary for IRS-2 mediated cell invasion.



Key research accomplishments

The results of the studies on the role of IRS-1 and IRS-2 in breast carcinoma progression can be summarized as follows:

1. Both IRS-1 and IRS-2 are necessary for cell proliferation.
2. IRS-2, but not IRS-1, is required for cell survival in the absence of exogenous survival factors.
3. IRS-2, but not IRS-1, is important for cell invasion.
4. IRS-1 appears to be involved in the initial steps of breast carcinoma formation, while IRS-2 function is required for later stages of breast carcinoma progression.

Reportable outcomes

Awwad, R.A., M.A. Byrne, and L.M. Shaw. IRS-1 and IRS-2 function differently to promote the malignant progression of breast carcinoma. Manuscript in preparation.

Conclusions

Previous studies in our laboratory have shown that IRS-1 and IRS-2 are both activated down stream of $\alpha 6\beta 4$ and that IRS-2 is required for $\alpha 6\beta 4$ - mediated cell invasion. In this report the specific roles of IRS-1 and IRS-2 in breast carcinoma are further investigated. Despite their structural homology and shared downstream signaling molecules, IRS-1 and IRS-2 function in different processes that are necessary for breast carcinoma promotion and progression. It appears that IRS-1 is important for the initial steps in establishing breast tumors, but IRS-2 contributes to later stages in the progression to malignancy. These findings are further supported by what others have reported with respect to loss of IRS-1 expression in advanced breast cancer (19,20). The outcome of this project highlights the importance of the IRS proteins as prognostic markers for breast carcinoma progression. Because of their distinct functions they can be used as targets for the development of proper diagnostic and therapeutic agents for breast cancer.

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