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Insulin promotes growth in breast cancer cells through the type I IGF receptor in insulin receptor deficient cells

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Abstract

Breast cancer is the most common cancer in women. The upregulation of insulin-like growth factor (IGF) system observed in certain types of breast cancers was linked to growth, metastasis, and survival resulting in multiple strategies designed to target the type I IGF receptor (IGF-1R) in breast cancer. These attempts failed to prove beneficial and it has been suggested that insulin receptor (IR) could also play an important role in breast cancer biology. To better understand the IR's role in breast cancer cells, the receptor was deleted from MCF-7L cells using CRISPR technology, and fluorescence-assisted cell sorting was used to obtain clone 35 (CL35). It was found that CL35 activated signaling pathways upon insulin stimulation despite the absence of IR expression. We hypothesized that CL35 used a surrogate receptor for sustained growth and development. IGF-1R was able to activate insulin signaling and growth in CL35. Thus, insulin may play a central role in regulating breast cancer growth due to its ability to activate all the receptors of the IGF family. These findings argue that dual targeting of IR and IGF-IR may be required to inhibit breast cancer growth.

Keywords

cell-signaling; breast cancer; IGF system; type I IGF receptor; insulin receptor; CRISPR

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Introduction:

Breast cancer is a major health concern in women. It is estimated that breast cancer contributes to approximately 30 percent of newly diagnosed cancer cases in females and is the second cause of women's cancer mortality after lung cancer [1]. The prognosis of early-stage breast cancer is favorable while survival rates worsen if breast cancer cells metastasize [2]. While adjuvant therapies in early-stage breast cancer reduce the risk of distant recurrence, there still is a need to identify new breast cancer targets to improve outcomes.

Cancer cells continually proliferate, this sustained growth can be mediated by growth factor receptor tyrosine kinases (RTKs) [3-5]. RTKs activate downstream signaling pathways associated with normal cell proliferation, motility, and survival. In cancer, these same RTKs mediated pathways contribute to malignant growth, survival, and metastasis and drive therapeutic resistance [6, 7]. These cellular processes that RTKs regulate make them desirable targets for the treatment of breast cancer.

The type I IGF receptor (IGF-1R) and insulin receptor (IR) are RTKs belonging to the insulin-like growth factor (IGF) signaling system. It has been demonstrated that the IGF system plays a role in the growth and development of the mammary gland [8-10]. Various studies linked the IGF system to play a role in different sub-categories of breast cancer [11]. IGF-IR was considered a viable target to treat breast cancer as suggested by pre-clinical studies [12, 13]. The studies showed that blockade of IGF-IR signaling reduced breast cancer growth, development, and metastasis.

Given these preclinical findings, there were significant efforts to create IGF-IR targeted drugs for breast cancer and other cancers including tyrosine kinase inhibitors and monoclonal antibodies [14, 15]. While these drugs were efficacious in inhibiting breast cancer growth *in vitro* and preclinical model systems, these results were not seen in clinical trials and essentially all anti-IGF-1R drug development has been abandoned [16-20]. The close homology between IGF-IR and IR and metabolic disruption of the anti-IGF-1R strategies resulting in hyperinsulinemia and hyperglycemia [21] implicate a potential role for IR. Further, expression levels of IGF-1R was not used as an inclusion criteria for any of the clinical trials [17].

IR is recognized for its contribution to maintaining glucose homeostasis of the body. IR has two splice variants i.e. IR-A and IR-B. While IR-A lacks exon 11 which shortens the extracellular α subunit by 12 amino acids compared to IR-B. Functionally IR-A is extensively expressed in fetal tissues that require growth and development. The IR-A: IR-B ratio was skewed towards IR-A in thyroid cancer, lung, breast, and colorectal cancer. On the other hand, the function of IR-B is linked with metabolic homeostasis and is expressed in the liver, fat, and muscle[22-24]. IR-A is reported to have higher affinity for IGF-2[25], a ligand abundant in adult human serum.

In research conducted by Fagan et al, it was observed that when breast cancer cells acquire tamoxifen resistance, they lose expression of IGF-IR but signaled through insulin receptors [26]. The cells that were resistant to endocrine therapy were more sensitive to IR inhibition

than the treatment naïve cells [27]. It is reported that the expression of IR is upregulated in breast cancer [28] and high levels of IR conferred poor prognosis [29]. Depleting the gene expression of IR using shRNA technique showed a reduction in oncogenic growth and metastasis [30].

Although the clinical trial was unsuccessful in showing the efficacy of cixutumumab an IGF-IR targeted monoclonal antibody, it was noted that these patients had over-expression of total IR compared to IGF-1R. The survival analysis suggested that patients with the highest quartile of IR expression had a very poor prognosis. Thus, indicating that IR could be essential for growth and survival of endocrine-resistant breast cancer [18]. Thus, it is possible that IR is also a viable target for treatment of breast cancer especially in endocrine resistant cells.

While previous studies have reported the effects of IR signaling inhibition by reducing IR expression using shRNA or by blocking the receptor signaling using inhibitors. The long-term consequences of complete deletion of IR in cells sensitive to both insulin and IGF signaling has not been studied. Here CRISPR Cas9 technology was used to delete IR from MCF-7L cells. We obtained a clone that lacked IR expression (CL 35) by cell sorting from a knockout pool using fluorescence-assisted cell sorting (FACS). While these cells lacked IR, insulin was still able to signal and stimulate growth via IGF-1R.

Materials and Methods

Cells and reagents

MCF-7L was kindly provided by C. Kent Osborne (Baylor College of Medicine, Houston, TX) and maintained in improved MEM Richter's modification medium (zinc option) supplemented with 5% FBS, and 11.25 nM insulin. MCF-7L karyotyping and gene expression profiling have shown that these cells are consistent with the originally described MCF-7 cell line (data not shown). MCF-7L insulin receptor knock out CL 35cells were maintained in the same media as parental cell line MCF-7L. MCF-7L TamR cells [26] were maintained in phenol-red free IMEM (zinc option) supplemented with 11.25 nM insulin, 5% charcoal/dextran-treated FBS and 100 nM 4-OH tamoxifen. All cell lines were confirmed to be mycoplasma negative and STR (short-tandem repeat) profiling is performed annually on these cells (data not shown). All cells were grown at 37°C in a humidified atmosphere containing 5% CO_2. All growth media were supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin and purchased from Gibco®. Trace elements B 1000x solution was from Corning. Transferrin human 50mg/ml was purchased from SIGMA.

Estradiol (E2) and tamoxifen (Tam) were from Sigma. IGF-I, IGF-II and insulin were purchased from Gemini and Eli Lily, respectively. S961 peptide was generously provided by Novo Nordisk, Denmark. OSI-906 was purchased from Abcam. IRS1, IGF-IR, pIGF-IR/pIR, pAKT, AKT, pErk1,2, Erk1,2 antibodies were from Cell Signaling Technology, Inc. (Danvers, MA). Insulin receptor antibody for immunoprecipitation and immunoblotting was from Santa Cruz. Insulin receptor antibody clone 83-7 (hybridoma) was kindly provided by Ken Siddle from University of Cambridge, UK. Anti-phospho-Tyr antibody (PY20) is a

product of BD Biosciences (San Jose, CA, 1:5,000), which was used for detection of IRS phosphorylation.

CRISPR Knock out Insulin Receptor in MCF-7L cells

The CRISPR was performed using Synthego® gene knockout kit. The single guide RNA (sgRNA) sequence designed by the company is **UCUUGUAGUGUGUCCCGGCA.** The sgRNA was mixed with the cas9 to form ribonucleoprotein (RNP) complexes, which was then delivered into the cells using reverse lipofection. The optimized sgRNA to cas9 ratio (5:1) was obtained by trial and error method recommended by the company to obtain satisfactory level of editing efficiency.

Analyzing CRISPR editing results

CRISPR editing efficiency and type of edits were obtained by using Synthego's ICE (Inference of CRISPR Edits) tool, which uses Sanger sequencing data to produce quantitative analysis of CRISPR editing. Primers used for Sanger sequencing were designed in our lab using Primer3 and created against the gene code for human insulin receptor. Forward primer is **CGCGTTACTCAATCCCCTGT** and reverse primer is **CCCGGAAGAGCAGCAAGTAA**. After lipofection, genomic DNA was isolated from the cells using QIAamp DNA Mini Kit (Catalog# 51304). The polymerase chain reaction (PCR) was then carried out using AmpliTaq Gold™ 360 Master Mix. The resulting cDNA was then analyzed on 1% agarose gel to confirm the PCR product size (around 483 bp range). The PCR product was purified by using QIAquick PCR Purification Kit (Catalog# 28104). The purified sample along with the forward primer was sent for Sanger sequencing at the University of Minnesota's genomic center. Sanger sequence files were then loaded into ICE software for CRISPR editing analysis.

Selection of insulin receptor knock out clones

CRISPR edited mixed population cells with the highest editing efficiency were used for flow cytometry analysis and Florescent Assisted Cell Sorting (FACS) to obtain single cell clone. Briefly, cells were detached using trypsin, washed with PBS and labeled with an insulin receptor antibody 83-7 at concentration of 1 μg/ml and incubated for one hour at 4°C. After wash with cold PBS, cells were incubated with Alexa Fluor 647 conjugated anti-Mouse IgG (H+L) secondary Antibody, (Invitrogen Catalog# A-21235) for one hour at 4°C in a dark room. Fluorescence was analyzed on BD FACSCanto flow cytometer and quantified using FlowJo software. Sorting of cells was done using BD FACSAria II. Insulin receptor negative cells were obtained in a single cell format in 96 well plate for continued culture and Immunoblotting analysis to confirm negative expression of insulin receptor clones.

Immunoblotting analysis

Cells were plated at a density of 8×10^5 cells in 60 mm diameter dishes and incubated overnight in complete medium. Cells were washed twice with PBS and starved in serum-free medium (SFM) for 24 hours. Cells were then treated with ligands and/or IGFIR/IR inhibitors at concentration and time indicated in the figures. After washed with ice-cold PBS and lysed with TNESV buffer of 50 mM Tris-Cl (pH 7.4), 1% Nonidet P-40, 2 mM EDTA

(pH 8.0), 100 mM NaCl, 10 mM sodium orthovanadate, and complete protease inhibitor cocktail (Roche Diagnostics), the lysates were centrifuged at 12,000 g for 30 minutes at 4°C. Protein concentrations were measured using a bicinchoninic acid protein assay reagent kit (Pierce). Whole cell lysates (50 μg) were boiled in 5X Laemmli loading buffer, separated by 8% SDS-PAGE, transferred to PVDF membrane and immunoblotted for primary antibodies overnight at 4°C and secondary antibody for one hour in room temperature.

Immunoprecipitation

500μg protein cell lysate was used for immunoprecipitation with 1:50 IGFIR or IR antibodies in 0.5 ml TNSEV buffer for 2 h at 4°C. Then, 25 μl proteinA/G plus agarose beads was added with gentle rock at 4°C overnight. Beads were washed five times and pulldown proteins were used for immunoblotting with pIGFIR/IR antibodies.

Monolayer cell growth

Cells in serum-containing medium were seeded as triplicate sets into 24-well plates with 10,000 cells per well. Next day cells were switched to serum-free medium (SFM Improved MEM phenol red free zinc option, 10 mM HEPES, Trace Element 1:1000, 2μg/ml Fibronectin, Transferrin 1:25000) or base media (Modified IMEM phenol red free) overnight. Then cells were treated with different ligands for 3 to 5 days. Live cell number was quantified at day 3 and 5 after treatment by the MTT assay. In brief, 60 μl of 5 μg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution in SFM was added to each well. After incubation for 2 h at 37°C, wells were aspirated, and formazan crystals were lysed with 500μl of solubilization solution (95% DMSO+5% Improved Minimal Essential Medium). Absorbance was measured with a plate reader at 570 nm by using a 670 nm differential filter.

Results:

Development of CL 35 by CRISPR and analysis using Inference of CRISPR edits (ICE).

Using the CRISPR gene editing tool with a gRNA targeting exon 2 i.e., in the early coding region of the IR gene within exon 2 (Figure 1A). An IR knockout pool from MCF-7L cells was obtained (Figure S1A-C). To further screen for cells with deletion of IR, FACS was performed to visualize two sets of the population (Figure S1D i.e., one that was IR positive and another that was IR negative. We then selected the negative population, performed index sorting in a 96 well-plate, and obtained CL 35. Sequencing analysis of CL 35 shows the changes in sequencing post-gRNA cut-site region in comparison to the control sequence (data not shown). The indel distribution graph showed the presence of a single homogenous population of CL 35 with a 4 base-pair deletion (data not shown). To validate that CL 35 is an IR null cell, expression was evaluated by flow cytometry using an IR monoclonal antibody [31]. Flow cytometry analysis demonstrated that CL 35 lacked the expression of IR compared to wild-type MCF-7L cells (figure 1B).

Residual insulin signaling activation upon stimulation in CL 35.

To further examine protein expression, we evaluated IR expression by immunoblot. Figure 2 shows that IR was not detected in CL 35 consistent with expression levels detected by

flow cytometry (Figure 1B). As expected, 5nm IGF-I initiated phosphorylation of IGF1R/IR in both the parental cells and CL 35. However, 10nM insulin also resulted in downstream signaling in parental and CL 35 cells as shown by tyrosine phosphorylation (PY20), p-Akt, and p-Erk1,2. We have previously shown that the band detected by PY20 blotting is IRS-1/IRS2 in MCF-7 cells [32]. Thus, cells lacking IR expression (CL 35) still showed insulin-stimulated downstream signaling. These results were not expected as the absence of IR in CL 35 should make cells unresponsive to insulin. To further explore the ability of insulin to initiate signaling in CL 35, we exposed cells to both IGF-I and insulin over a range of concentrations.

Insulin and IGF-I both activated signaling through the IGF-I receptor in IR deleted cells.

Previous research has suggested that insulin can bind to IGF-IR although with a 100-fold lower affinity in comparison to IR [33]. Additionally stimulating endothelial cells with as high as 10nM of insulin activated IGF-IR tyrosine phosphorylation [34]. Since IGF-I, insulin, IR, and IGF-IR belong to the same family of ligands and receptors we stimulated wild-type MCF-7L and CL 35 cells with varying concentrations of IGF-I or insulin. CL 35 was more sensitive to IGF-I stimulation in comparison to MCF-7L with signaling initiated at concentrations as low as 0.5nM IGF-I (Figure 3A). We found that CL 35 was less sensitive to insulin stimulation for signaling activation (Figure 3B) compared to the wild-type cells. Concentrations as low as 1nM resulted in phosphorylation of IRS-1 in wild-type cells as detected by PY20 blotting, while a 10-fold higher concentration was necessary to show similar signaling in CL 35. This data suggests that at higher concentrations insulin may activate IGF-IR a Receptor highly homologous to IR [35].

To evaluate IGF-1R function in CL 35 cells, we stimulated cells with ligands and used receptor specific inhibitors to evaluate the contribution of the receptor. S961 is a small peptide that disrupts insulin binding to IR, S961 was able to block insulin-mediated signaling in MCF-7L selected for resistance to tamoxifen (MCF-7LTamR) which lack IGF-IR [27]. However, S961 did not inhibit 10nM insulin signaling in either wild-type MCF-7L or CL 35 (Figure 4A) suggesting that IGF-1R could mediate the effects of insulin. We next used OSI-906 which is an IGF-IR and IR dual tyrosine kinase inhibitor (Figure 4B) [36]. We observed that in the presence of OSI-906 insulin-mediated signaling events were blocked in MCF-7L and CL 35 which suggests that insulin can only signal through the IGF family of receptors.

To confirm that insulin signaled through IGF-IR a neutralizing humanized and specific IGF-IR monoclonal antibody was used with the two ligands [37]. huEM164 blocked insulin-mediated signaling in CL 35 consistent with insulin activation of IGF-IR in the absence of IR (Figure 4C). In contrast, insulin signaling in the parental MCF-7L cells was not inhibited by huEM164 due their expression of IR. immunoprecipitation was used to further determine if insulin could stimulate IGF-1R in CL 35 cells, (Figure 4D). Total cell lysates were immunoprecipitated with an anti-IRβ and immunoblotted with p-Irβ/p-IGF-Irβ and no phosphorylated protein was detected in any of the treatment groups of CL 35 (lower panels). In contrast, immunoprecipitation with an anti-IGF-IR antibody followed by p-Irβ/p-IGF-Irβ immunoblotting showed insulin stimulated receptor phosphorylation in both parental and

CL 35 cells (upper panels). Thus, signaling observed in insulin stimulated CL 35 occurred through IGF-IR.

IGF-IR signaling drives the growth of CL 35 cells

To understand the implications of signaling events on the proliferation of MCF-7L and CL 35, monolayer cell growth experiments were used to examine ligand stimulated proliferation in serum free media. CL 35 were more sensitive to IGF-I stimulation at each concentration we evaluated compared to parental cells (Figure 5A) which could be due to a lack of hybrid IGF-1R/IR receptors in CL35. These data were consistent with the enhanced IGF-I stimulated IGF1R signaling in CL 35 (Figure 3A). Insulin stimulated growth in both cells although the parental cells were slightly more sensitive than CL35 which is most notable at the 0.3nM concentration of insulin (Figure 5B). Stimulating CL 35 with ten times this low concentration (3nM) of insulin stimulated growth consistent with the activation of p-Erk 1,2 observed in insulin stimulated CL 35 cells (Figure 3B).

Serum-free media formulations contain both transferrin and fibronectin. To determine if insulin-mediated proliferation was affected by either of these proteins, cell proliferation assays were also performed in the absence of these proteins. In the protein free medium (based medium), response to insulin was robust in the MCF-7L parental cells and the addition of fibronectin and transferrin did not alter this response. CL35 cells were minimally stimulated by insulin in the protein free (base med) and the fibronectin (FN) containing media, but full responses were seen when transferrin (TF) was added (Figure 5C). These findings suggest that transferrin and IGF-1R signaling interact and iron has been shown to stimulate estrogen receptor positive cells [38]. While the mechanism for this interaction is uncertain, these data show that insulin acting through IGF-1R enhanced cell growth in specific experimental conditions and show that other signaling pathways impinge on cell growth via IR.

Discussion:

To further study the role of IR in breast cancer cell biology, we generated an IR null cell line clone 35 from MCF-7L parental cells through Cas9/CRISPR targeting of exon 2. Despite the absence of IR, insulin-mediated signaling events were still observed which was unexpected as IR is thought to mediate the physiologic effects of insulin in normal and malignant tissues.

Insulin is reported to bind to IGF-IR although, at significantly lower affinity. Since IGF-1R and IR share homology and belong to the same receptor family, we hypothesized that insulin may potentially signal through IGF-IR. To investigate this MCF-7L and CL 35 were stimulated with increasing concentrations of insulin or IGF-I. Enhanced IGF-I sensitivity of CL 35 may be due to the elimination of hybrid IGF-I/IR receptors when the IR gene is deleted thereby increasing holo-IGF-IR receptors which are high affinity binders of IGF-I. During selection of CL 35, there were some signaling differences observed such as a greater baseline activation of Erk 1,2 and Akt in comparison to MCF-7L. As both of these signaling pathways are essential for cell survival, it seems possible that constitutive activation of these downstream effects were necessary to select the IR-deficient clone. Furthermore, the

immunoblot analysis revealed that Erk 1,2 was preferentially activated over Akt during 3nM insulin stimulation of CL 35 consistent with the growth effects observed in the MTT assay . These differences in signaling pathways between MCF-7L and CL 35 may be due to clonal variation that occurred when selecting Cl35, but it is possible that they reveal important differences between IR and IGF-IR function in breast cancer.

CL 35 requires higher amounts of insulin to activate signaling in comparison to MCF-7L cells is indicative of a possible insulin-IGF-IR signaling axis. A non-selective tyrosine kinase inhibitor (OSI-906) was able to abrogate insulin mediated signaling in CL 35 suggesting that the effect was mediated through one of the two receptors. HuEM164, an IGF-IR-specific monoclonal antibody, was able to effectively inhibit signaling events caused by insulin stimulation of CL 35 and we concluded that insulin activated signaling through IGF-1R. The results obtained from the use of specific inhibitors on CL 35 in addition to the co-immunoprecipitation experiment further supported a role for insulin stimulation of IGF-IR in IR deficient cells.

IGF-1R activation also had implication in cell growth. In the absence of IR, IGF-IR sustained insulin-mediated signaling and growth under certain physiologic conditions. Taken together the results indicate that targeting IGF-IR and IR should be targeted in breast cancers that rely on the IGF system for their growth. Further, these preclinical data suggest that neutralizing the IGF ligands alone may be insufficient to block signaling through IGF-1R as suggested by tne negative results obtained from the breast cancer trials which tested the IGF-ligand neutralizing antibody xentuzumab [39]. However, targeting both receptors, especially IR will create challenges in maintaining metabolic homeostasis in the body.

The results indicate, CL 35 cells lacking IR are able to harness insulin for growth via IGF-IR which suggests that both receptors from the IGF family i.e. IGF-IR and IR need to be targeted in therapeutic settings. Therefore combining inhibitors selective to IR-A and IGF-IR would be preferred to prevent compensatory mechanisms from sustaining growth of breast cancer cells dependent on IGF family. These data highlight a functional difference in the IR splice variants which may have implications for cancer biology. Thus specific targeting the IR-A isoform over the IR-B isoform would help address the challenges of targeting cancer cell insulin effects while avoiding the IR requirements for normal tissues.

In conclusion, dual targeting of IR and IGF-IR may be required to block signaling through this family of receptors and ligands as cells lacking IR still respond to insulin. Challenges in developing IR-A specific drugs need to be overcome in order to translate these findings into the clinic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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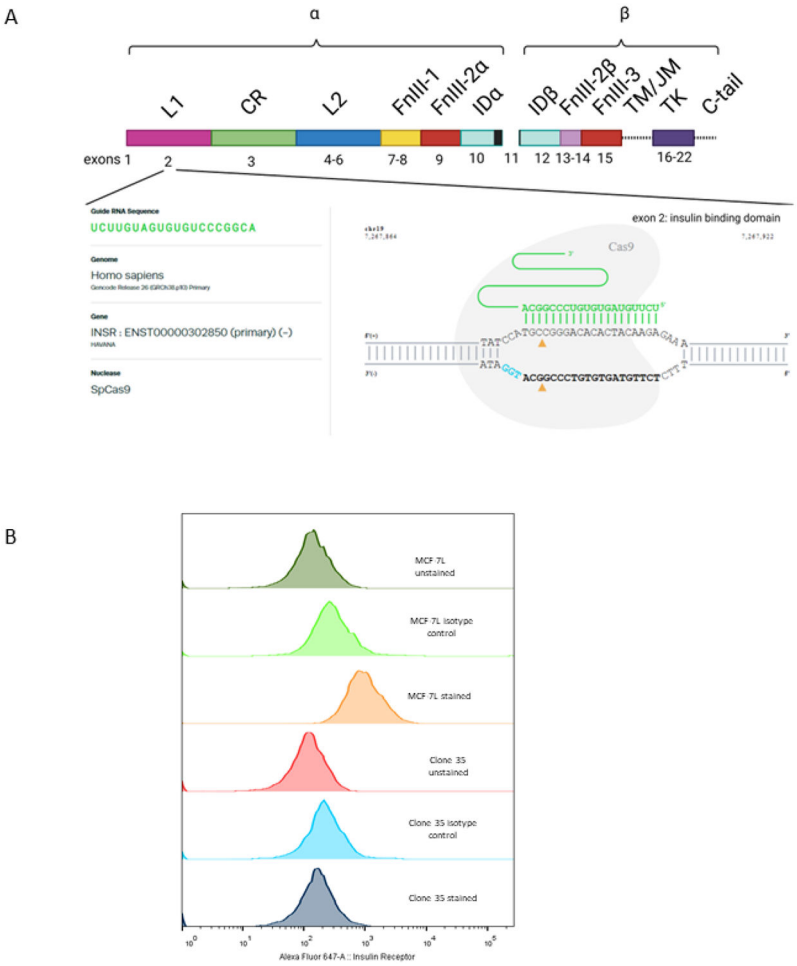


Figure 1: CL 35 obtained from MCF-7L cells lacks the expression of IR.

A) A schematic representation of the gRNA binding to the target site in the genome i.e. exon 2 to delete IR expression (Synthego CRISPR Design, Knockout Guide Design. 2019. v1.3. Synthego; 3/17/2023.

B) Flow cytometry analysis of wild-type MCF-7L and CL 35 using histogram plot along with their corresponding mean florescence intensities. Cells were unstained or stained with 2ug/μl of 83-7 insulin receptor monoclonal antibody or an isotype control antibody for 1 hour followed by a secondary incubation of AF-647 for 1 hour.

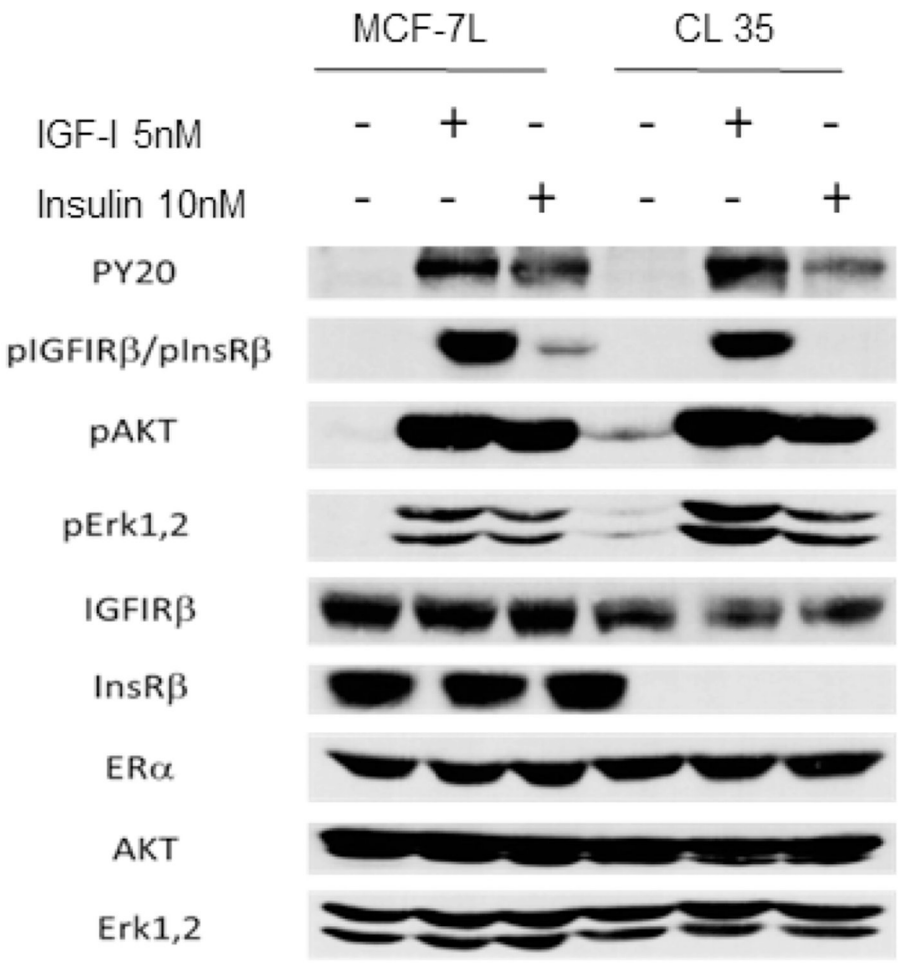


Figure 2: Insulin activated signaling in IR negative CL 35 cells.

Whole cell lysates from MCF-7L and CL 35 treated with either insulin (Ins) 10nM or insulin like growth factor-1 (I) 5nM for 10 minutes and were analyzed by immunoblot for the expression of insulin receptor (IR), phosphorylated insulin receptorβ/insulin like growth factor-1 receptor beta (p-IRβ/ IGF-1Rβ), tyrosine phosphorylation (PY20), phosphorylated protein kinase B (p-Akt), p-Erk1,2 with Akt and Erk1,2 serving as the loading controls.

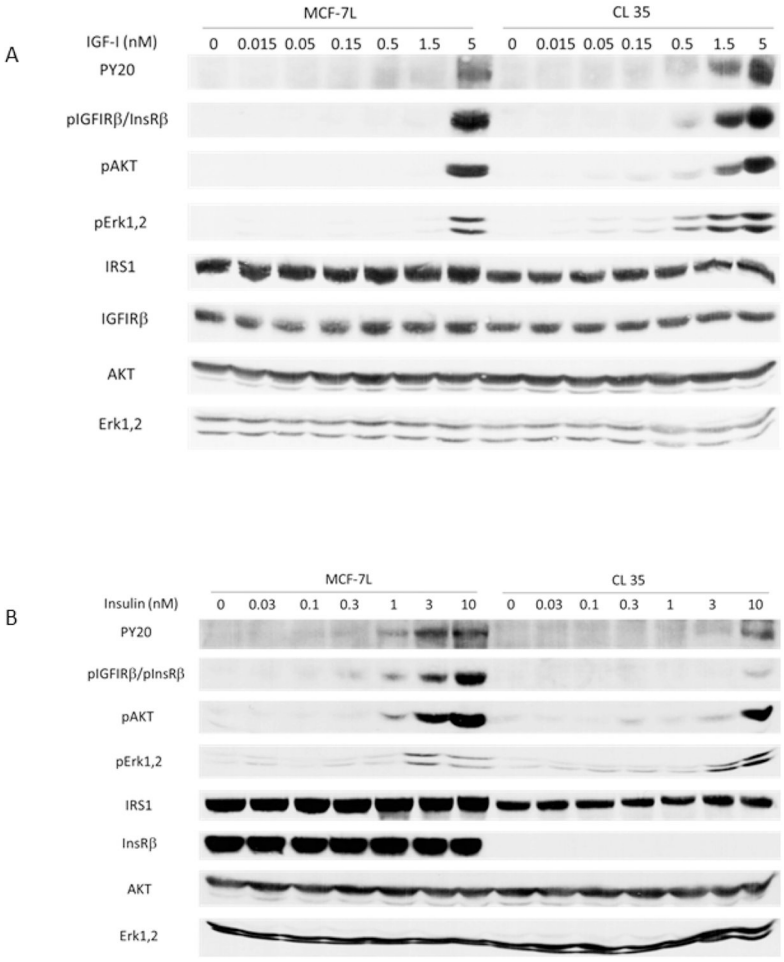
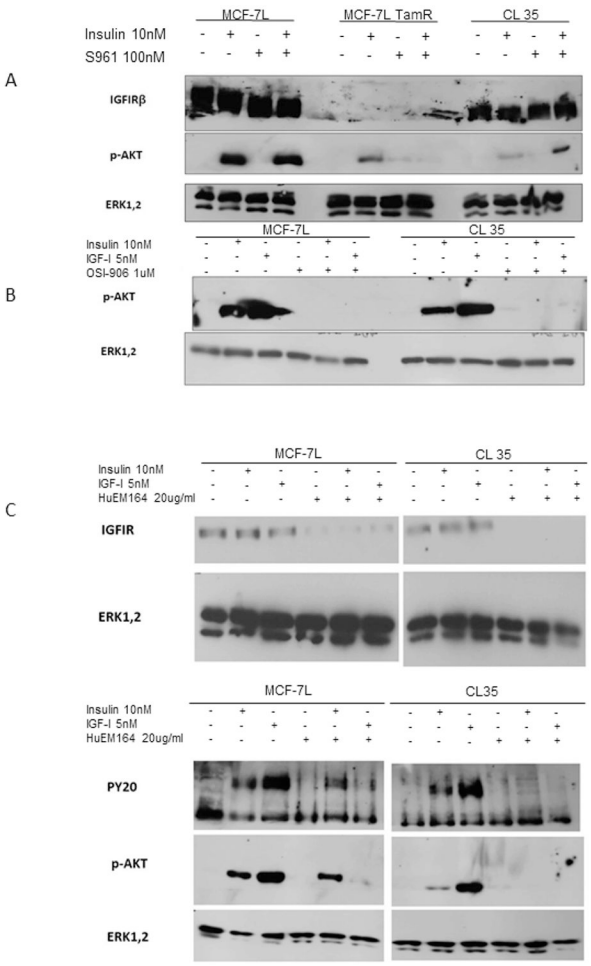


Figure 3: CL 35 gains sensitivity to IGF-I in comparison to wild-type MCF-7L cells

A) Whole cell lysate obtained from MCF-7L and CL 35 treated with increasing concentrations of IGF-1 (0nM-5nM) for 10 minutes and then immunoblotted for the expression of insulin like growth factor receptor beta (IGFIRβ), phosphorylated insulin receptorβ/ insulin like growth factor-1 receptor beta (p-IRβ/ IGF-1Rβ), tyrosine phosphorylation (PY20), phosphorylated protein kinase B (p-Akt), p-Erk1,2 with Akt serving as the loading control.

B) Whole cell lysate obtained from MCF-7L and CL 35 treated with increasing concentrations of insulin (0-10nM) for 10 minutes and then immunoblotted for the expression of insulin receptor beta (InsRβ), phosphorylated insulin receptorβ/ insulin like growth factor-1 receptor beta (p-IRβ/IGF-1Rβ), tyrosine phosphorylation (PY20), phosphorylated protein kinase B (p-Akt), p-Erk1,2 with Akt serving as the loading control.



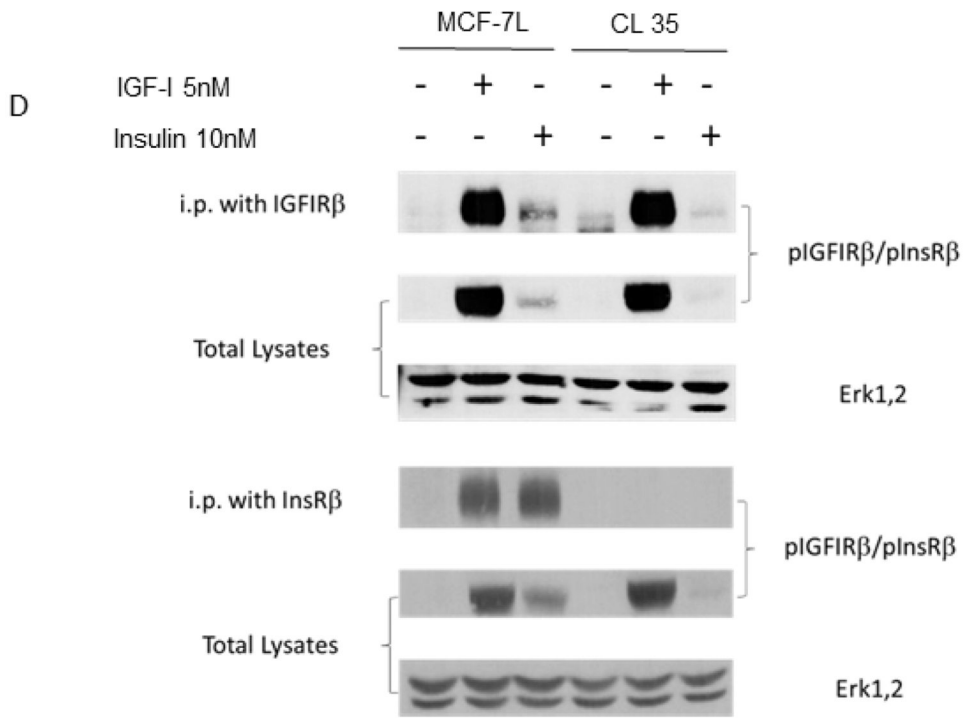


Figure 4: Insulin signaled through IGF-IR in IR negative CL 35 cells.

A) Immunoblot of whole cell lysates obtained from MCF-7L, MCF-7L TamR and CL 35 treated with insulin 10nM for 5 minutes, IGF-I 5nM for 5 minutes and S961 100nM for 24 hours and immunoblotted for IGF-1Rβ, p-Akt(S473) with Erk 1,2 acting as loading control.

B) Immunoblot of whole cell lysates obtained from MCF-7L and CL 35 treated with insulin 10nM for 5 minutes, IGF-I 5nM for 5 minutes and OSI-906 1uM for 4 hours. Lysates were analyzed for p-Akt and Erk1,2 served as loading control.

C) Immunoblot of whole cell lysates obtained from MCF-7L and CL 35 treated with insulin 10nM for 5 minutes, IGF-I 5nM for 5 minutes and pre-treated with HuEM164 20ug/ml for 4 hours. Lysates were analyzed for IGF-IRβ, PY20 (p-IRS), p-Akt, with Erk1, representing as loading control.

D) Whole cell lysates were collected from MCF-7L, and CL 35 cells stimulated with insulin 10nM for 5 minutes, IGF-I 5nM for 5 minutes and immunoprecipitated (IP) with either IGF-IRβ or IRβ. IP was then resolved with SDS-PAGE and subjected to immunoblotting for pIGF-Irβ/pIRβ and Erk1,2 functioning as loading control

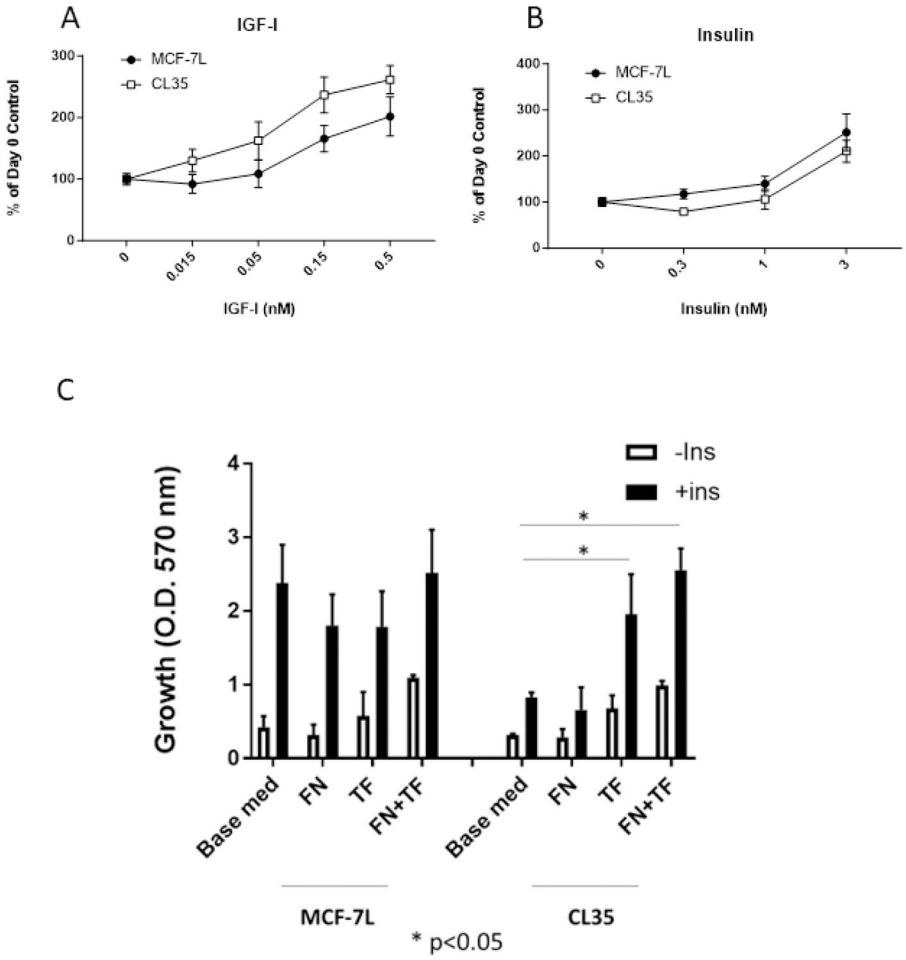


Figure 5: Insulin stimulated growth in Cl 35 in the presence of transferrin

A) MCF-7L and clone 35 cells stimulated with increasing amounts of IGF-I and cell numbers were estimated after 5 days using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Growth conditions were performed in serum-free media including addition of transferrin and fibronectin.

B) Similar growth evaluation was performed with increasing concentrations of insulin under the same growth conditions.

C) MCF-7L or CL 35 cells were stimulated with or without insulin in based medium (Base med), fibronectin (FN), transferrin (TF) or both (FN+TF). Growth was estimated by MTT assay after 5 days. In CL 35 cells, insulin stimulated growth was increased in TF containing media compared to non-TF (Base med) containing media ($p<0.05$).