GENERATION OF ENGINEERED SMALL PROTEIN SCAFFOLDS AND INSULIN RECEPTOR TARGETING IN HUMAN BREAST CANCER

A THESIS

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF THE UNIVERSITY OF MINNESOTA

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Douglas Yee, Advisor

April 2017

ACKNOWLEDGEMENTS & DEDICATION

First and foremost, I wish to express my gratitude to my advisor, Dr. Douglas Yee for allowing me to join the lab and financially supporting me throughout my entire Ph.D. training at the University of Minnesota. It has been my pleasure to be part of the lab, working on a translational project, which I believe may one day make a difference in the general science and oncology fields. After I joined the lab, I had the opportunity to collaborate on a ligand engineering project with Dr. Benjamin Hackel from the Department of Chemical Engineering. I had not thought of myself as an engineer or developer of a protein/binder/drug. This was certainly not the easiest project I had imagined, but the experience was very fascinating and rewarding. Having gone through the transition from a person with little protein engineering and molecular biology background to actually identifying promising and translational drugs, I am extremely grateful to many people, especially Dr. Yee and Dr. Hackel for their tremendous support and patient. Their inspiration and mentorship to my scientific endeavors have been immeasurable. The projects presented some challenges and delays at the beginning, but Dr. Yee and Dr. Hackel were willing to take time from their busy schedules to provide guidance and advice on technical hurdles and scientific inquiries. I am honored to have this rare opportunity to work with a great physician and world-renowned scientists.

I would also like to thank my current and former thesis committees, Dr. Jill Siegfried, Dr. Deepali Sachdev, Dr. Hiroshi Hiasa and Dr. Sundaram Ramakrishnan for spending their valuable time reviewing my proposal and thesis,

and participating in my annual committee meetings and final defense. Your suggestions and feedback on my research and career are greatly appreciated.

Moreover, I am incredibly thankful to work with many talented, helpful and respectful scientists and students. To all past and present Yee lab and Hackel lab members, I could not ask for a better working and learning environment. You have made these five years an enjoyable and meaningful experience. Your assistance, weather it is a piece of technical advice or a friendly criticism, is valued. Further, I would like to extend my appreciation to a few friends that listened to my complains when experiments were not as expected, and that explored the city and tasted culinary delights with me. You have made the Minnesota cold so much more tolerable. Yes, it is you, I thank you!

Last but not least, this thesis is dedicated to my friends and family who have supported and encouraged me throughout the process. I deeply appreciate the help in any forms: mentally, emotionally, intellectually or monetarily. I am doubtful that I would have achieved this milestone as a scientist and person without these wonderful people supporting me. It means a lot to me, I thank you!

ABSTRACT

The insulin-like growth factor (IGF) system is a well-studied growth regulatory pathway implicated in breast cancer tumorigenicity and drug resistance. The pivotal members of IGF system, the type I insulin-like growth factor (IGF1R) and insulin receptor (InsR) are homologous receptors necessary for signal transduction by their cognate ligands insulin, insulin-like growth factor-I and –II. A number of drugs, including monoclonal antibodies (mAbs) directed against IGF1R, small molecule tyrosine kinase inhibitors (TKIs) and IGF ligand neutralizing antibodies have been developed and tested in clinical trials. Early trials suggested benefits in delaying tumor progression, unfortunately, none of the anti-IGF1R mAbs has, thus far, shown significant benefits in phase III clinical trials. Although preclinical studies of anti-IGF1R mAbs showed promising results using endocrine-sensitive models, these antibodies were evaluated in breast cancer patients with endocrine-resistant tumors. When our group generated endocrine-resistant breast cancer models, we showed that IGF1R expression was lacking and anti-IGF1R mAb treatment was inefficacious in treating these endocrine-resistant cells. Inaccurate recapitulation of human diseases explains the disappointing outcomes of the trials. This finding also suggests that IGF1R inhibition is not an appropriate treatment in treating breast cancer patients whom are resistant to endocrine treatments.

Unlike IGF1R, InsR expression is not affected in the endocrine-resistant breast cancer model. Several studies have shown a shift in gene signatures from IGF- to insulin-mediated growth and differentiation in the absence of IGF1R. Our group has shown an increase of insulin sensitivity in breast cancer cells when

IGF1R is downregulated and insulin/InsR alone is sufficient to drive metastasis and tumor growth *in vivo*. However, InsR has been intentionally avoided as a potential target in cancer therapy due to its major function in glucose homeostasis. There are, currently no InsR-specific inhibitor or molecular diagnostics available. This reason has become the motivation for my work.

The first section of the work examines the roles of InsR and efficacy of InsR inhibition in endocrine-resistant breast cancer. The model we used was a tamoxifen resistant (TamR) cells derived from estrogen receptor positive breast cancer cell lines. These TamR cells did not simply lose IGF1R expression and function, but also gained sensitivity to insulin stimulation compared to their parental cells. We used three different targeting mechanisms to disrupt the functions of InsR: 1.) InsR short hairpin RNA (shIR) to knock down endogenous InsR expression, 2.) a small InsR-blocking peptide, S961 and 3.) an InsR down-regulator mAb (clone 83-7). These methods showed consistent results that suppression of InsR function in TamR cells successfully blocked insulin-mediated signaling, monolayer proliferation, cell cycle progression and anchorage-independent growth. This strategy, however was not effective in the parental cells, which were sensitive to endocrine treatments, likely because of the presence of IGF1R/InsR hybrid receptors. Down-regulation of IGF1R with monoclonal antibody in conjunction with shIR or S961 was more effective in blocking IGF- and insulin-mediated signaling and growth in the parental cells compared with single-receptor targeting alone. Our finding showed TamR cells were stimulated by InsR and were not sensitive to IGF1R inhibition, whereas in

tamoxifen-sensitive parental cells, the presence of both receptors, especially hybrid receptors, allowed cross-reactivity of ligand-mediated activation and growth. Surprisingly, the synergistic inhibitory effects were not achievable with anti-IGF1R and anti-InsR mAbs in the parental cells. When this combination treatment was tested in triple-negative breast cancer cells, an additive inhibition of ligand-mediated signaling was observed, especially in the samples treated with IGF-II, suggesting that they may potentially benefit from the combinational IGF1R/InsR therapy. Although the signaling result of IGF1R/InsR blocking antibodies seems promising in triple-negative breast cancer, no functional assay has yet been done to prove their efficacy.

The second section of my dissertation focuses on the generation and characterization of InsR-evolved small protein scaffolds based on the T7 phage Gene 2 protein (Gp2). Gp2 is one of the potential small protein scaffolds for ligand engineering and has the capable for mutation to generate new binding functions. Our long-term goal is to create effective InsR inhibitors and/or diagnostic tools. Using yeast surface display and directed evolution, we identified three Gp2 variants, known as Gp2 #1, #5 and #10 with low nanomolar binding affinity to cell-surface InsR with weak cross-reactivity to IGF1R of Gp2#1. These Gp2 variants inhibited insulin-mediated monolayer proliferation in both endocrine-sensitive and -resistant breast cancer, but did not down-regulate InsR expression. Gp2 #5 and Gp2#10 disrupted InsR function by inhibiting ligand-induced receptor activation. In contrast, Gp2#1 did not block InsR phosphorylation. Notably, Gp2#1 binding was enhanced by pre-treatment of cells

with insulin suggesting a unique receptor-ligand binding mode. These Gp2 variants are the first non-immunoglobulin protein scaffolds to target insulin receptor and present compelling opportunity for modulation of InsR signaling.

Taken together, targeting both IGF1R and InsR is optimal in endocrine-sensitive, endocrine-resistant breast cancer and potentially triple-negative breast cancer to fully suppress the IGF system. Since double inhibition using mAbs might not be an ideal approach in endocrine-sensitive breast cancer, future studies using one of the Gp2 variants as alternative options, either as a single or in combination with other agents may provide some insights with regards to IGF targeting in breast cancer.

TABLE OF CONTENTS

LISTS OF FIGURES

Chapter 1.

Chapter 2.

ABBREVIATIONS

AI – Aromatase inhibitor

CDK4/6 – cyclin-dependent kinases 4 and 6

DBD – DNA binding domain

DMBA – 7,12-dimethylbenz(a)anthracene

ER – estrogen receptor

ERE – estrogen response element

FACS – fluorescence activated cell sorting

GAPDH – glyceraldehyde 3-phosphate dehydrogenase

GH – growth hormone

Gp2 – T7 phage gene 2 protein

HER2 – human epidermal growth factor receptor 2

IGF-I – insulin-like growth factor I

IGF-II – insulin-like growth factor II

IGFBP – insulin-like growth factor binding protein

IGF1R – type I insulin-like growth factor receptor

IGF2R – type II insulin-like growth factor receptor

InsR – insulin receptor

IP – immunoprecipitation

IRR – insulin-receptor-related receptor

IRS-1 – insulin receptor substrate 1

IRS-2 – insulin receptor substrate 2

LBD – ligand binding domain

mAb – monoclonal antibody

MAPK – mitogen activated protein kinase

mTOR – mammalian target of rapamycin

MTT – thiazolyl blue tetrazolium bromide

PARP – poly ADP-ribose polymerase

PET – positron emission tomography

PI3K – phosphatidylinositol-3 kinase

PR – progesterone receptor

PVDF – polyvinylidene difluoride

qRT-PCR – quantitative real-time polymerase cycle reaction

S6K – ribosomal protein S6 kinase

SDS-PAGE – sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SERM – selective estrogen receptor modulator

SERD – selective estrogen down-regulator

TamR – tamoxifen resistant

TKI – tyrosine kinase inhibitor

TNBC – triple negative breast cancer

Chapter 1

Introduction

Statistical facts and therapeutic options in breast cancer

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death among women in the United States. According to American Cancer Society, an estimated 61,000 cases of *in situ*, more than 249,000 invasive new cases and 40,000 deaths are expected in 2016. About one in eight women will develop breast cancer at some point in their life time. Although the incidence is prevalent, the survival rate for breast cancer is one of the highest among the solid cancers. Death rate from breast cancer has declined by 38% since around the 1990s, with larger decreases in women younger than 50 years old due to improvements in early detection and treatments. The chance that a woman will die from breast cancer is 3% [1]. The majority of the breast cancer deaths was due to recurrence and metastasis. Statistics from National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) database show that breast cancer found localized has a five-year relative survival rate of 98.8%. As it spreads to regional lymph nodes, the five-year survival rate drops to 85.2% and further decreases to 26.3% when the cancer metastasizes to distant parts of the body [2].

The standard treatments for breast cancer include at least one of the following: mastectomy/lumpectomy, radiation therapy, chemotherapy, endocrine therapy and/or targeted therapy, depending on the stages, subtypes and molecular status of the tumors. Surgery and radiation therapy are both effective treatments in removing and destroying breast tumors. Chemotherapy is one of the oldest methods that has been used to treat cancer by killing highly dividing

cells. This traditional method has been proven effective, however, cytotoxicity is inevitable due to its indiscriminating killing effects on normal healthy cells. Moreover, as these neoplastic cells are not completely killed or suppressed, this residual population enables regrowth of tumors that no longer respond to a wide variety of drugs and ultimately ensue resistance to cancer drugs [3]. Thus, identifying new therapies, either alone or in combination with traditional therapies, to treat breast cancer is crucial.

Endocrine and targeted therapies are relatively newer therapies as they target specific characteristics in cancer cells, in hope to control tumor growth with reduced potential cytotoxic effects. Often, cancer cells display evaded programmed cell death, enhanced growth signals and proliferative properties via elevated or dysregulated hormonal or growth factor pathways. Targeting these pathways may be an alternative approach to the traditional methods that have not been very successful. One good example of targeted therapies in breast cancer is trastuzumab, a monoclonal antibody directed against the human epidermal growth factor receptor-2 (HER2). Targeting growth factor receptors have be proved to be effective. However, since HER2 is only expressed in a subpopulation of breast cancer, other tumor types lacking HER2 expression do not benefit from this therapy. Thus, there is a need to explore other targets and growth factor pathways.

The IGF system

The insulin-like growth factor (IGF) family, which consists of multiple receptors, extracellular ligands (IGF-I, IGF-II, insulin) and binding proteins

(IGFBPs 1-6), belongs to the receptor tyrosine kinase superfamily and has been implicated in tumorigenesis and malignant phenotypes. There are at least five different types of receptors in the system, including the type I IGF receptor (IGF1R), insulin receptor (InsR), IGF1R/InsR hybrid receptor, insulin-receptor-related receptor (IRR) and the type II IGF receptor (IGF2R).

Unlike sother receptor members of the IGF family, IGF1R and InsR have been extensively studied for decades. They are dimeric receptors and share 45-65% similarity in ligand-binding domains and 84% in the catalytic domains [4, 5]. Each monomer is synthesized as a heavily glycosylated single chain proreceptor and proteolytically cleaved to yield α and β subunits with molecular weights of around 135 kDa and 90 kDa, respectively [6]. The α subunit contains extracellular ligand binding sites; while the transmembrane β subunit contains the intracellular tyrosine kinase domain. Two αβ subunits are then covalently linked by disulfide bonds to form either a holo-receptor, which is composed of two identical monomers, or a heterodimer, which is made of a single chain of InsR and a single chain of IGF1R. The heterodimer is also known as IGF1R/InsR hybrid receptor. InsR exists in two isoforms: Isoform A and B due to alternative splicing of exon 11. As a result, InsR-A, lacking exon 11 is 12 amino acids shorter than InsR-B. InsR-B tends to be a metabolic receptor and is mainly expressed in adult muscle, liver and fat; whereas InsR-A is predominantly expressed during fetal development and in cancers.

IRR was first discovered due to its high homology to family members [7]. However, IRR is an orphan receptor as no endogenous ligand has been

identified despite efforts including the whole genome search for substances with high sequence similarity to insulin or IGFs [7-9]. It is mainly expressed in the kidney and has only been described as an alkali sensor and regulator in the body [10]. On the other hand, IGF2R, which is also known as cation-independent mannose-6-phosphate receptor lacks tyrosine kinase activity and signal transduction capability [11]. Studies have shown its important functions in multiple cellular pathways and that loss of IGF2R is associated with progression of tumorigenesis. One of its roles is facilitating the activation of latent TGF-β by inducing G_1 arrest in cancer cells [12-14]. IGF2R also acts as a sequester for many extracellular ligands such as IGF-II [15, 16]. When IGF2R expression is low, IGF-II bioavailability increases and therefore promotes IGF-II signaling via IGF1R. Thus, the field has also referred IGF2R as a IGF-II “sink” and may be a potential tumor suppressor. Due to its multiple functions in cellular processes, IGF2R’s role in breast tumor is not fully understood.

Three known ligands IGF-I, IGF-II and insulin bind to their receptors with different binding affinity and specificity. At physiological concentrations, IGF-I binds solely to IGF1R; IGF-II binds to IGF1R and InsR-A; while insulin binds to InsR with higher affinity for InsR-A. However, in certain circumstances in such that IGF-I is at much higher concentration than the preferential ligand insulin, IGF-I can bind to InsR. Similar situation can also apply to insulin and IGF1R. The affinities of IGF1R and InsR for their ligands are as following: InsR, insulin >> IGF-II > IGF-I; IGF1R, IGF-I > IGF-II >> insulin; IGF2R, IGF-II > IGF-I (insulin does not bind). Upon ligand binding, the receptors undergo autophosphorylation

within the tyrosine kinase domain and subsequently initiate signal transduction pathways. A number of adaptor proteins, such as insulin receptor substrates (IRS-1, IRS-2), Grb2 and Shc are recruited to the membrane. These events then trigger various downstream signaling cascades including mitogen activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3K). These pathways regulate a wide array of transcription factors and gene expressions, ultimately resulting in protein synthesis, cell growth, proliferation, metabolism and survival [17, 18].

. IGF-I and IGF-II are single-chain polypeptides of about 7.5 kDa and are highly homologous, sharing 70% sequence similarity and 50% homology to proinsulin [19]. Despite their high homology, these ligands have very different origins. IGF-I and IGF-II are mainly produced in hepatocytes under the control of growth hormone (with much lesser degree for IGF-II) and circulated in the blood at 20-80 nM concentrations. Under normal condition, hypothalamus releases growth-hormone-releasing hormone and somatostatin to regulate the production of growth hormone (GH) in pituitary. Once GH is released, it binds to GH receptor in the liver, which in turn stimulates the production of IGF-I in an endocrine fashion. Circulating IGF-I acts on the negative feedback loop of GH/IGF-I axis and maintains their physiological serum concentrations [20]. In addition, IGF-I and IGF-II can also expressed in target tissues such as kidney, skeletal muscle, spleen, brain and heart, suggesting that they can also act through autocrine and paracrine manners [21-23]. IGF-I plays critical roles in skeletal muscled physiology and the change in IGF-I level at different stages of

life is particularly responsible for the pubertal growth spurt. IGF-I level is low at birth; it starts to increment in the childhood and peaks during puberty, reaching around 2-3 folds more than adult level [24, 25].

In contrast, IGF-II level does not change with age. It is preferentially expressed in early embryonic and fetal development. It may substitute for IGF-I when IGF-I level is low, however not much is known about IGF-II's physiological functions in adulthood. Most studies have implicated its roles in early stages of carcinogenesis and a variety of tumors growth; some evidence has shown that IGF-II local expression contributes to metabolic syndrome, abnormalities in cardiac architecture (enlarged left ventricle, bradycardia) and atherosclerotic lesions [26-28].

Mature insulin, which consists of two polypeptides chains A and B linked by disulfide bonds has a molecular weight of about 5.8 kDa. It is first produced by pancreatic β-cells as a pre-proinsulin and is processed into proinsulin in rough endoplasmic reticulum and Golgi, respectively. Its activation is preceded by the cleavage of C-peptide. Unlike IGFs, insulin production, excretion and activity are not influenced by GH [24, 29]. Insulin mainly affects metabolism of hepatocytes, myocytes and adipose tissue cells. However, a number of studies including our group have shown that insulin's effect is not limited to glucose homeostasis. Insulin can also drive mitogenicity.

Six IGF binding proteins (IGFBPs) have been identified, sharing conserved protein structure and high binding affinity for IGF-I and IGF-II. Thus, they are characterized as modulators of IGF bioavailability, signaling and activity

[30, 31]. However, their functions remain controversial. They are six distinct proteins with each having evolved with exclusive functions and properties [32]. IGFBP-3 is the most abundant IGFBP in the circulation. It binds to about 90% of the IGFs and has an affinity of about one order magnitude higher than that of IGF1R [33]. Many studies have examined the relationship between circulating IGFBP-3 level and cancer risk or prognosis, no consistent evidence has been found [34-38]. Although IGFBP-3 may act as a IGF-I suppressor by diminishing their bioavailability to IGF1R, some discussion indicated that IGFBP-3 stabilizes IGF-I in the binary or tertiary complex and facilitates IGF transport through body compartment [39]. In contrast, IGFBP-1 and IGFBP-2 mainly involve in diet and metabolic balance [40, 41]. While IGFBP-3 is positively regulated by GH, both IGFBP-1 and IGFBP-2 are suppressed by GH and further decreased by insulin secretion. Patients with chronic hyperinsulinemia or obesity are associated with lower IGFBP-1 and IGFBP-2 [41]. Studies suggested that the inversely relationship between IGFBP-1/IGFBP-2 and insulin in a hyperinsulinemic condition increases free-IGF and favors IGF activity. However, circulating IGFBP-1 and IGFBP-2 alone are not associated with cancer risks [42, 43]. On the other hand, IGFBPs 4-6 do not seem to play critical roles in regulating endocrine IGFs. They have been shown to exhibit IGF-independent actions and may be regulated in a cell-specific manner. At present, there is no clinical indication for measuring these binding proteins [44].

ER system and hormonal therapy in breast cancer

Estrogen receptor (ER) belongs to the nuclear receptor superfamily and functions as a transcription factor for a number of target genes [45]. Binding to its ligand – estrogen, which is a natural steroid hormone – allows ER to regulate various physiological processes in a broad range of target tissue in human body, including breast, genital tract, cardiovascular, bone and brain [46].

There are two forms of ER: ERα and ERβ, which are encoded by genes *ESR1* (chromosome 6) and *ESR2* (chromosome 14), respectively [47]. Both ERα and ERβ are composed of 6 domains termed A to F from N- to C-terminus. Domain AB, which is also known as activation function (AF-1) is a ligand-independent region that involves transcriptional activation and protein-protein interaction with other transcription factors. Domain C is the DNA-binding domain (DBD), containing two zinc finger motifs; while the domain D is the hinge, serving as the rotational site for conformational alteration of the receptor. Domain E is the ligand-binding domain (LBD) that allows homo- or hetero- dimerization upon ligand binding. This LBD also harbors activation function 2 (AF-2), where its transcriptional activation function including conformational change of the receptor is dependent on ligand binding. Both AF-1 and AF-2 are the important domains that influence transcriptional activity of the ERs. They recruit a range of co-regulatory protein complexes to assist transcriptional activation of target-gene expression [48, 49]. Domain F is still poorly understood. ERα and ERβ display high conservation, approximately 97% in the DBD but considerably less conservation, approximately 59% in the LBD (Figure 1.1). This suggests that they

interact with similar estrogen response element (ERE) sequences but may have different ligand-binding characteristics [49].

After many years of extensive research on estrogen and ER, it has become clear about the contribution of ER in breast cancer development. Estrogen was never suspected as a carcinogen until about decades ago, when it was discovered as a key growth factor for driving breast carcinoma and contributed to the majority of the breast cancer cases. (Estrogen was added into the carcinogen list in 2002 by National Institute of Environmental Health Science.) ER has become the major prognostic and therapeutic determinant in the breast cancer clinic. About 75% of the breast cancer patients are estrogen receptor positive (ER+). This is usually referred to high ERα expression in the breast tumors. ERα is only expressed in small percentage (7-10%) in normal breast tissues, in contrast to ERβ expression that is relatively high (80-85%) [50-52]. Studies demonstrated during tumor progression, ERα expression level rose, while ERβ expression level decreased [53]. Overexpression of recombinant ERβ inhibited ERα-induced transcription [54, 55], proliferation [56, 57], migration [58] and sensitized cells to tamoxifen [59], indicating that ERβ may serve as a tumor suppressor. However, some studies have shown ERβ expression was associated with high proliferation (Ki67 expression) and high tumor grade [53, 60]. The interplay between ERα and ERβ has become more complicated and the definitive role of ERβ in breast carcinogenesis is still elusive.

Blocking ER pathway has proven beneficial in neoadjuvant, adjuvant and metastatic settings [61-63]. There are three major classes of anti-estrogen

agents with different targeting mechanisms of action. 1.) selective estrogen receptor modulators (SERMs), 2.) estrogen receptor down regulators (SERDs), and 3.) aromatase inhibitors (AIs). SERMs such as tamoxifen prevent the binding of estrogen to ERs by blocking the recruitment of co-activators to AF-2 but not AF-1, thus blocking the activation of nuclear ERs. Since AF-1 remains open, partial estrogen agonist activity in skeletal, cardiovascular, central nervous system and gastrointestinal tract is expected [64]. Unlike SERMs, SERDs such as fulvestrant, is a pure antiestrogen with no agonist activity. Binding of fulvestrant to ERs impairs receptor dimerization, blocks nuclear localization of the receptor, blocks the transcriptional activation of AF-1 and AF-2 and subsequently promotes ER degradation. The ability of SERDs to reduce cellular ER level suggests its better efficacy compared to that of tamoxifen in postmenopausal women with breast cancer [65]. On the other hand, AIs such as letrozole, anastrozole and exemestane do not interact with ERs, but rather, interfere the conversion of androgen into estrogen. Moreover, they also lack estrogen agonist activity, providing a rational for the development of AIs as an alternative therapy to tamoxifen [66].

Crosstalk between ER and IGF systems

Estrogen functions through two distinct signaling: ligand-dependent genomic and ligand-independent non-genomic pathways. In the genomic pathway, estrogen diffuses into the cell and bind ER, which is located in the nucleus. Once activated, ERs dimerize and bind to specific response elements known as estrogen response elements (EREs) in the promoter of target genes.

Estrogen binding, in this case induces a conformational change in the receptors, allowing the release of co-repressors and recruitment of co-activators such as AIB1 and ARC1 to the appropriate sites. Direct binding of activated ER to ERE is referred as the classical genomic pathway. However, ER regulated gene expressions do not solely rely on this classical mechanism. Around one third of the genes in humans that are regulated by ERs do not contain ERE-like sequences. ER can also indirectly associate with promoters of target genes through protein-protein interactions with other DNA-binding transcription factors such as AP-1, SP-1 and NF-kB, in an ERE-independent manner [67-69]. In this non-classical genomic pathway, ER, acting as a co-activator regulates the transcription of target genes that lack ERE but have a binding element for its tethered transcription proteins.

In addition to the genomic pathway, estrogen can also mediate signal transduction pathway through plasma membrane. Evidence has shown the presence of ERs at the plasma membrane and they are responsible for extranuclear, rapid and non-genomic actions [70-72]. Non-genomic actions are frequently associated with the activation of various protein-kinase cascades such as adenylate cyclase, phospholipase C, MAPK/ERK and PI3K/AKT pathways [73]. Thereby, membrane ERs potentially associate with many growth factor receptors such as IGF1R, EGFR and contribute to a variety of cell proliferation, differentiation and metabolic functions [67, 74].

The cross-talk between ERα and IGF pathway is well-established and occurs bidirectional. Estrogen influences the IGF system at multiple levels by

sensitizing the IGF system. Estrogen increases the expression of both IGF1R and IRS-1 in breast cancer cells, resulting in enhanced IGF signaling and activation of downstream pathways such as ERK and AKT [75]. Total and phosphorylated IRS-1 is upregulated with estrogen in MCF-7L xenografts. Removal of estrogen halted tumor growth and decreased IRS-1 expression [75]. Estrogen increases IGF-II secretion and affects breast cancer in an autocrine manner [76]. Furthermore, estrogen decreases IGFBP-3 expression, an IGF ligand sequester; while a pure antiestrogen, ICI-182780 increases IGFBP-3 level in breast cancer cells [77]. Reciprocally, IGF-I activates ER-mediated signaling in a ligand-independent manner [78-80], particularly through the phosphorylation of ER at both Serine 118 and Serine 167 residues [81, 82]. IGF-I induces ERα expression, increases phosphorylation of co-activators and other regulatory proteins of ERα activity [79, 80, 83] and potentiates ERα transcriptional activity by increasing the expression of ERα target genes, e.g. progesterone receptor [84]. When recombinant IGFBP-1 was used as an inhibitor for IGF-I action, it abolished IGF-I-dependent ERα activity and growth in breast cancer cells [85]. Reduced serum IGF-I was also observed in patients treated with tamoxifen [86, 87].

Estrogen was originally believed to exert its proliferative effects through direct activation of gene expression (via genomic action). Evidence has suggested many of these estrogen-responsive genes are key components of growth factor pathways (including the IGF pathway), which in turn, not only contribute to the already existing proliferation matters, but can also directly

reactivate the ER pathway. The synergistic relationship of ER and growth factors intensifies the growth-promoting environment in breast cancer and also adds another level of complexity to the treatments of breast cancer, suggesting that multiple level of inhibitions may be necessary.

Targeting IGF system

In addition to the crosstalk of IGF1R with ER pathway, IGF system itself has also been implicated in different types of malignancies, including breast cancer [88-91]. Activation of IGF1R stimulates cell proliferation [92, 93], enhances survival [93, 94], regulates cell metabolism [95] and promotes metastasis [94, 96]. Collective evidence has shown that blocking IGF system inhibits growth and metastasis *in vitro* and *in vivo* [97, 98]. Multiple strategies targeting IGF system have been developed and tested in the clinical trials as shown below.

Neutralization of IGF-I and IGF-II ligands

MEDI-573 (MedImmune) is a dual-specific neutralizing antibody against IGF-I and IGF-II ligands. Several pre-clinical studies have shown efficacy in blocking IGF-driven cell proliferation *in vitro* [99], tumor growth [100, 101] and angiogenesis [99] in sarcoma xenografts *in vivo*. Combination of MEDI-573 with either mTOR inhibitor (rapamycin and AZD2014) [101] or anti-IGF1R (MAB391) [99] were more effective in blocking tumor growth. In phase I dose-escalation study, MEDI-573 was well tolerated with no notable perturbation in metabolic homeostasis and preliminary anti-tumor activity has also been observed (NCT00816361 and NCT01340040). A phase II clinical trial of MEDI-573 testing

in patients with hormone-sensitive metastatic breast cancer is underway (NCT01446159).

BI-836845 (Boehringer Ingelheim) is another IGF-I/IGF-II neutralizing antibody. Most of the studies are still in phase I trials testing patients with various solid tumors. One of them is a phase I/II study of BI-836845 in combination with exemestane (aromatase inhibitor) and everolimus (mTOR inhibitor) testing in ER+/HER2- advanced breast cancer patients (NCT02123823).

Targeting downstream IGF/insulin receptors

AKT/PI3K/mTOR is one of the main downstream networks of receptor activation. Around 40% of ER+, 23% HER-positive and 8% basal-like breast tumors present *PIK3CA* mutations [102]. *TP53* mutation, however is much more prevalent in basal-like (84%) and HER2-positive (75%) compared to luminal (22%) breast tumors. *AKT1* and *PTEN* mutations have also been reported, but they are relative less frequent in breast cancer [103]. Few AKT inhibitors have made their way to clinical evaluation. Ipatasertib (GDC-0068, Genentech), an ATP-competitive AKT inhibitor demonstrates safety profile and disease control in a subgroup of patients in a phase I study testing patients with solid tumors [104]. AZD-5363 (AstraZeneca) and Afuresertib (GSK2110183, Novartis) are being evaluated in phase II trials, including an ongoing study testing patients with invasive ER+ breast cancer (NCT02077569).

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase located downstream of PI3K/AKT signaling cascade. It has appeared to be a favorable alternative target for cases with *PIK3CA* mutations since a

downstream blockage is able to fully suppress the aberrant PI3K/AKT activation due to the mutation. This is less likely with an upstream inhibition. However, some caution is warranted in using mTOR inhibitors. mTOR in conjunction with ribosomal protein S6 kinase (S6K), which is one of mTOR downstream molecules, mediate negative feedback of PI3K/AKT action by inhibiting IRS-1 function. The phosphorylation of IRS-1 at multiple residues, including serine 636/696 by mTOR and serine 270/307/636/1001 by S6K promotes IRS-1 degradation [105] and subsequently AKT inhibition. This negative feedback loop of mTOR is disrupted with mTOR suppression. A combination treatment of mTOR inhibitor with PI3K or IRS-1 inhibitors may be an option. Dual-specific PI3K/mTOR inhibitor, such as NVP-BEZ235 are in preclinical and clinical studies although some toxicities have been reported. Currently, there are two mTOR inhibitors approved for use in treating cancer in the United States: everolimus (Novartis) and temsirolimus (Pfizer). Ridaforolimus (Merck) has recently been dispproved.by the FDA. Everolimus combined with an AI improved progression-free survival in patient with ER+ advanced breast cancer who have progressed on nonsteroidal AI in the phase III trials and this regime has incorporated into clinical practice [106, 107].

Cyclin-dependent kinases 4 and 6 (CDK4/6) are protein kinases in cell cycle regulating DNA replication and cell division. The cell cycle is divided into phases: G_0 (quiescence), G_1 (pre-DNA synthesis), S (DNA synthesis), G_2 (pre-division), and M (cell division). The progression from G_1 to S is a critical checkpoint in protecting the cell from abnormal replication, and that process is

tightly regulated by CDK4/6 and cyclin D, the expression of which is controlled by IGF/insulin [108]. Palbociclib (Pfizer), a CDK4/6 inhibitor is approved in 2015 for use in treating postmenopausal women with metastatic breast cancer who are resistant to endocrine treatments [109]. A phase III clinical trial of Palbociclib has shown efficacy in combination with fulvestrant in women with ER+/HER- advanced metastatic breast cancer [110].

Another major downstream network of IGF/insulin receptor activation is Ras/Raf/MEK/ERK pathway. Alterations of this pathway, including mutations and upregulation of MAPKs have been well documented in many cancers. Genes *MAP3K1* and *MAP2K4* are one of the highest mutated genes in breast cancer after *PIK3CA* and *TP53* [103], resulting in upregulation or constitutive activation of MAPK pathway. This is particularly observed in the basal-like/triple-negative breast cancer cells. U0126 is an example of selective inhibitor of MEK1/2 that lacks pharmaceutic properties for clinical evaluation. However, it is an invaluable research tool for dissecting the MEK/ERK pathway. The status of additional approaches and targets of this signaling pathway that are under preclinical and clinical evaluations are described in this article [111].

Other potential downstream interventions include S6K inhibitor, H89 [112] and IRS-1 inhibitor, NT157 [113]. They did not receive as much attention compared to other targets, however few investigators, including our group have shown some interesting outcomes with these inhibitors.

Blockage of receptor activation

Past years, IGF1R has been the central focus of IGF interference. Many humanized monoclonal antibodies have been designed and developed to bind specifically to IGF1R, and not InsR. This class of drugs interferes the binding of ligands to the receptors by triggering receptor endocytosis and subsequently its degradation in the endosome [114]. Few examples of these antibodies are Figitumumab (Pfizer), Dalotuzumab (Merck), cixutumumab (Imclone) and Ganitumab (Amgen) [17]. They have been evaluated in clinical trials, either as single agents or in combination with other antitumor drugs in various cancers. Unfortunately, these trials have shown no benefit in the tested patients in late-phase clinical trials. Substantial toxicities, including elevated GH and circulating IGF-I levels, hyperglycemia and hyperinsulinemia have been reported [115-117]. The anti-IGF1R monoclonal antibodies disrupt the negative feedback control of endocrine IGF-I as the brain fails to detect IGF-I levels. This results in an increase in serum GH and IGF-I production by liver. The elevated GH also induces insulin resistance, probably due to increased lipolysis and free fatty acid production from the liver, thus resulting in elevated insulin production by pancreas [20]. The enhanced levels of GH, IGF-I and insulin from anti-IGF1R monoclonal antibodies might activate InsR unwantedly. Since anti-IGF1R monoclonal antibodies do not block InsR, this may be a concern. InsR could certainly drive survival and compensatory signaling and blunt the therapeutic effects of anti-IGF1R monoclonal antibodies.

The reasons why we did not see the effects of monoclonal antibodies on the endocrine systems in the preclinical animal models is due to the substantial

difference between human and murine endocrine systems [118]. Mice have low circulating levels of IGF-II postnatally; while humans have high levels of IGF-II [119]. Besides, these monoclonal antibodies are developed specifically against human IGF1R and thus have minimal effects on murine IGF1R. Although blocking the IGF1R with monoclonal antibodies has shown tumor inhibition in mice, mouse model does not fully recapitulate a human system, and thus it is not a perfect model for studying the effects of endocrine disruptors like IGF1R inhibitors. Another explanation for the failure of the anti-IGF1R monoclonal antibody trials could be the lack of molecular biomarkers to predict the benefits from anti-IGF1R treatments [118]. An exploratory analysis of serum biomarkers suggested that elevated levels of IGFs and IGFBP may potentially be predictive markers for anti-IGF1R treatment, however later trials could not confirm this observation and found no correlation between levels of IGF-I, IGFBP-2 and IGFBP-3 with the treatment of anti-IGF1R monoclonal antibody. IGF1R mRNA expression, cell surface expression, copy number and mutation status were not associated with tumor responsiveness to IGF1R inhibition in osteosarcoma [120]. Perhaps, other biomarkers outside of serum IGF-I and IGF1R expression levels need to be exploited.

Other than antibodies, IGF1R activation can also be blocked by tyrosine kinase inhibitors (TKIs), which are small molecules competing for the ATP binding site in the catalytic domain of the β subunit of the receptors. Most of the drugs of this class originally aimed to target IGF1R's ATP binding site. Due to high homology of the catalytic domains between IGF1R and InsR, these TKIs

lack IGF1R selectivity and exhibit some inhibitory effects on InsR. BMS-754807 (Bristol-Myers Squibb) and linsitinib (OSI-906, OSI Pharmaceuticals) are two examples of TKIs, targeting both IGF1R and InsR. They have shown efficacy in blocking tumor growth *in vitro* and *in vivo.* Combination studies with either chemotherapy, hormonal or targeted agents have shown synergistic benefits [121-123]. Phase I/II studies of OSI-906 showed safety profile, but failed to show anti-tumor activity in unselected relapsed non-small-cell lung cancer patients [124, 125]. So far, no result of the clinical trials testing BMS-754807 has been released.

Roles of InsR in breast cancer

While InsR is often thought to associate with blood sugar management and diabetic condition, it is also a key growth regulatory factor that can stimulate mitogenic phenotypes similarly to IGF1R. Insulin has been long considered essential for the growth and development of rodent mammary [126]. Studies looking into the roles of insulin and InsR in cancer have been conducted as early as in the 1970s. Insulin administration influenced mammary tumor growth induced by a carcinogen, 7,12-dimethylbenz(a)anthracene (DMBA) [127] and the mammary tumor regression was observed when the rats were given alloxan diabetes to destroy tumor pancreatic β-cells, resulting in insulin deficiency [128]. In a non-obese mouse model of type II diabetes, insulin accelerates breast cancer development and progression [129]. Furthermore, our lab has shown that down-regulation of InsR in breast cancer cells and xenografts caused reduced proliferation, angiogenesis, angiogenesis and lymphangiogenesis [130].

In addition to the data from animal studies, elevated InsR levels are detected on human breast, colon, kidney, lung, ovary and thyroid carcinomas [131]. In breast cancer specifically, approximately 80% of the samples have an IR content 2-20 fold higher than normal breast tissues [132]. Human disease states such as type II diabetes and obesity, which are often accompanied by hyperinsulinemia, are associated with an increased risk for many cancer types, including breast cancer. Breast cancer patients with high insulin levels are much more likely to suffer from metastasis, disease recurrence and reduced survival. The effects of hyperinsulinemia in human are thought to be cancer-promoting due to elevated circulating level of insulin, chronic exposure of insulin on peripheral tissues and reduced insulin sensitivity of the pancreas to regulate glucose levels, which may lead to hyperglycemia and low grade inflammation [133]. Higher glucose consumption by cancer cells, in contrast to nonmalignant cells, has been well observed and recorded. Most cancer cells have altered metabolism and predominantly produce energy through the fermentation of glucose to lactate regardless of oxygen availability (Warburg effect) [134]. Therefore, hyperinsulinemia can reinforce the permissive role of sufficient energy substrate availability by cancer cells and is recognized as an underlying cause of cancer.

Cumulative evidence of clinical and epidemiological studies suggests that insulin and InsR involve in the formation and maintenance of malignant phenotypes. The levels and phosphorylation of InsR are associated with poor survival in breast cancer [135, 136]. Overexpression of InsR also predicts a poor

survival among patients with non-small-cell lung cancer [137]. Although a study suggested differently that high InsR expression is an independent predictor of a favorable clinical outcomes in early stage breast cancer, the IGF-II levels were significantly higher in patients with low tumor InsR expression, suggesting that ligand levels may also affect the clinical outcomes independent of the receptor levels [138]. They also mentioned the lack of sensitive quantitative assessment in the study that might not pick up the effects of down-regulation of InsR. No doubt, the InsR role in cancer is established and may be an important implication considering that the clinical trials of IGF1R blocking antibodies are not efficient in controlling tumor growth in the tested patients. Targeting both IGF1R and InsR may be a better choice for cancer therapy. However, the biological response of insulin on cancer cell proliferation and invasiveness cannot be predicted solely based on the insulin content [139]. The prognostic value of InsR inhibition in the clinical setting of human malignancies needs to be defined.

Rationale and purpose of this study

Generation of endocrine resistant breast cancer models demonstrated the lack of IGF1R expression and inefficacy of IGF1R inhibition. InsR, which is a closely related receptor of IGF1R remains intact and functional in these models. The purpose of the study is to understand how InsR influences the development of endocrine resistance. The work is to determine the role of InsR in endocrine resistant breast cancer model, to evaluate the efficacy of InsR inhibition in human breast cancer, and to develop and characterize small molecular agents of InsR for therapeutic purposes.

Hypothesis

InsR serves as a compensatory pathway to IGF1F and InsR inhibition is necessary to overcome acquired resistance breast cancer to endocrine therapy.

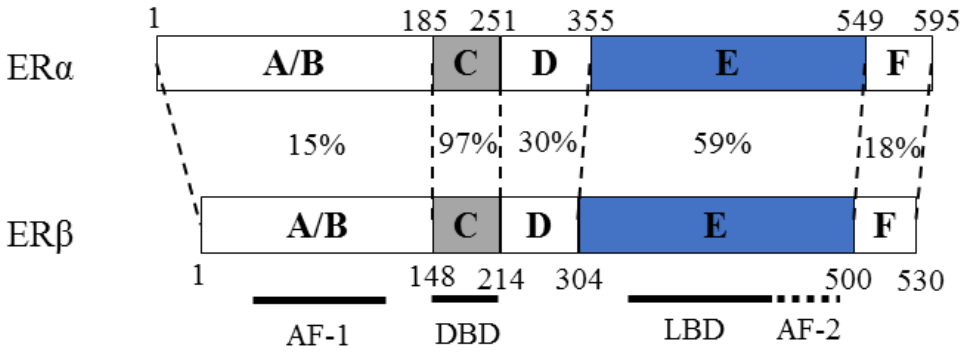


Figure 1.1. Comparison of primary structure between ERα and ERβ. They are composed of 6 domains labelled from A to F from N- to C-terminus. Degree of homology of each domain between the two ER isoforms is represented as percentage.

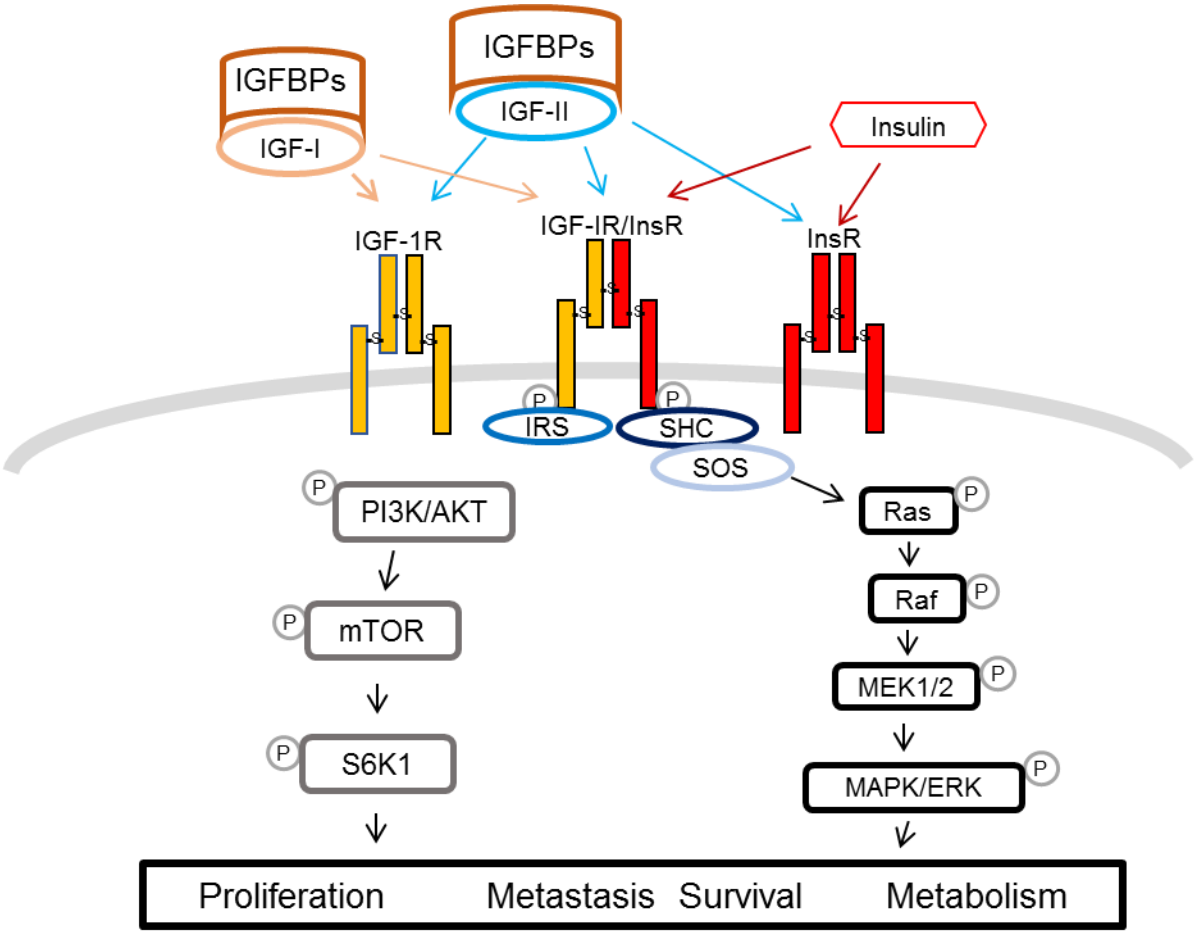


Figure 1.2. Schematic diagram of IGF family. IGF-I and IGF-II and insulin are natural ligands of transmembrane tetrameric receptors: IGF1R, InsR and hybrid receptors. The bioavailability of IGF-I and IGF-II are regulated by IGFBPs. Upon ligand binding, activated and phosphorylated receptors recruit a number of adaptor proteins and activate two major pathways: PI3K/AK and MAPK/ERK pathways. Uncontrolled activation of these receptors in cancer result in aberrant proliferative phenotypes.

Chapter 2

Targeting insulin receptor in human breast cancer, particularly in endocrine-resistant breast cancer

Note: Majority of the results were published in

Chan JY, LaPara K, Yee D. Disruption of insulin receptor function inhibits proliferation in endocrine-resistant breast cancer cells. Oncogene. 2016; 35(32):4235-4243.

Introduction

Approximately 75% of the breast cancer cases express estrogen receptor-α, representing the most prevalent breast cancer subtype [140]. Patients with ER-positive breast cancer can be treated by inhibiting ER function. This strategy has been successful in early stage and advanced breast cancer [61, 62], but a significant proportion of patients never responded to ER inhibition (*de novo* or primary resistance) or have progression after a prolonged period of therapy (acquired or secondary resistance) [141, 142]. Endocrine resistance still poses a key clinical problem. Recently, targeting of mTORC1 and CDK4/6 have been used to treat ER-positive tumors [143, 144], but there is still a need for additional strategies, aiming to delay or ideally overcome resistance to endocrine therapy.

This system has been implicated in cancer development as well as crosstalk with ER, suggesting that it may contribute to the regulation of ER-positive breast cancer [145, 146]. IGF1R is an estrogen regulated gene and enhances ER transcriptional activity, suggesting co-targeting of ER and IGF1R might be clinical useful [84, 147]. A number of anti-IGF inhibitors including anti-IGF1R mAbs, TKIs and ligand neutralizing antibodies were developed primarily to target IGF1R and IGF ligands while leaving InsR unperturbed [118]. Despite the hope that anti-IGF1R targeted therapies would provide clinical benefit in endocrine resistant breast cancer, we showed that TamR cells lacked IGF1R expression [148]. This finding was validated in women with breast cancers – recurrent endocrine treated tumors showed lower level of IGF1R compared to the pre-treated tumors [149, 150]. Thus, it would be unlikely for anti-IGF1R mAbs to have clinical activity in endocrine resistant cells. These observations likely

explain why the results of phase III clinical trials of anti-IGF1R mAbs tested in endocrine resistant population have been negative [151].

Unlike IGF1R, InsR is not an estrogen regulated gene and its level remained intact in TamR cells. InsR is closely related to IGF1R, sharing 84% similarity within catalytic domain, 45-65% in ligand-binding domain and more than 50% in the overall amino acid sequence [6]. The highly homologous InsR activates almost identical downstream signaling cascades in a ligand-dependent fashion. On the loss of IGF1R function, osteoblasts shifted from IGF- to insulin-mediated growth and differentiation [152]. Down-regulation of IGF1R in breast cancer increased sensitivity to insulin [153]. In addition, a patient tumor developed an increased InsR gene copy number while being treated with, and eventually becoming resistant to endocrine therapy [154]. Although InsR expression in cancer has been documented for several decades [130-132, 155], InsR inhibition has been intentionally avoided because of concern over disrupting glucose homeostasis.

InsR inhibitors have been developed as dual IGF1R/InsR tyrosine kinase inhibitors: BMS-754807 and OSI-906. These two drugs have completed several clinical trials, including a phase II study against ER+ breast cancer resistant to aromatase inhibitors. The trial has completed but the results have not been disclosed (*NCT01225172*). Early clinical evidence suggests that TKIs are safer than originally anticipated. Although hyperglycemia was evident in patients treated with OSI-906, encouraging disease control was observed in patients [156, 157].

In this study, we determined that InsR signaling serves as a bypass pathway and compensates for the loss of IGF1R in TamR breast cancer cells. We suppressed InsR functions using three different mechanisms in TamR versus parental breast cancer cells. Our data showed that InsR inhibition alone blocked signaling and cell proliferation in TamR cells but not in the parental cells. When anti-IGF1R mAb was given in conjunction with S961 or shIR, a complete suppression of insulin-stimulated growth in endocrine-sensitive parental breast cancer cells was observed. An additive down-regulation of receptor levels and a synergistic blockage of ligand-mediated signaling using combination treatments of anti-IGF1R and anti-InsR mAbs were also observed in a triple negative breast cancer cells, suggesting the involvement of hybrid receptors in the mediation of IGF/insulin in breast cancer cells. Thus, dual inhibition of IGF1R and InsR is necessary for optimal suppression of this signaling system.

Results

TamR cells were more sensitive to insulin compared to their parental cells

MCF-7L and T47D are ER-positive human breast cancer cell lines and are estrogen sensitive and can be inhibited by SERMs such as tamoxifen. We previously generated TamR cells from MCF-7L and T47D and showed reduced IGF1R expression levels and a lack of efficacy of anti-IGF1R monoclonal antibodies in TamR cells. However, AEW541, a dual TKI that targets both IGF1R and InsR was able to inhibit insulin- and IGF-stimulated signaling and growth [148].

To better understand the role of insulin/InsR in TamR cells, we treated MCF-7L, T47D and their TamR cells with increasing concentrations of insulin for 15 minutes. As measured by AKT, P70S6K and MAPK phosphorylation, insulin signaling stimulated phosphorylation at lower levels of insulin in TamR cells compared to MCF-7L or T47D parental cells (Figure 2.1). Monolayer growth and soft agar assays showed greater proliferation (Figure 2.2) and colony formation (Figure 2.3) at lower concentrations of insulin in TamR cells compared to their parental cells.

Genetic knockdown of InsR reduced insulin-regulated signaling and growth in TamR cells, but not in the parental cells.

Stable InsR knockdown cell lines were generated in MCF-7L, T47D and their TamR cells using lentiviral shRNA. IGF1R level was unaffected in both MCF-7L and T47D parental cells. Compared to control shRNA, shIR#6 was a more efficient knockdown construct than shIR#2 as down-regulations of InsR protein and mRNA levels were greater in cells transduced with shIR#6, validated by immunoblotting (Figure 2.4) and qRT-PCR (Figure 2.5). A reduction of insulin-mediated signaling was measured by IGF1R/InsR, AKT, MAPK, P70S6K and IRS (pY-20) phosphorylation after InsR knockdown in TamR cells especially in shIR#6 TamR cells. Surprisingly, this was not the case in parental cells even though the InsR level was significantly down-regulated (Figure 2.4).

To further examine if InsR knockdown affected biological functions, cells were studied by cell cycle analyses, monolayer growth, and anchorage-independent growth assays. Down-regulation of InsR abolished insulin-mediated

proliferation (Figure 2.6), cell cycle progression (Figure 2.7) and anchorage-independent growth (Figure 2.8) in MCF-7L and T47D TamR cells, but not in MCF-7L or T47D parental cells. Re-introduction of recombinant human InsR (hIR) in shIR#2 and shIR#6 transduced MCF-7L TamR cells rescued insulin-mediated signaling (Figure 2.9) and cell proliferation (Figure 2.10).

In contrast, InsR overexpression in both MCF-7L and TamR cells showed a mild up-regulation of InsR protein level and also insulin-mediated phosphorylation of IRS (pY-20), AKT, P70S6K and MAPK (Figure 2.11A). However, only MCF-7L TamR cells showed a significant increase in cell proliferation (Figure 2.11B).

S961 inhibited insulin-regulated PI3K/MAPK signaling and growth in TamR cells, but not in parental cells.

S961, a small peptide was synthesized and shown to be a competitive antagonist with a slightly higher affinity to InsR than insulin and partial agonist effects at lower concentrations [158]. S961 has very low affinity for IGF1R. To examine the effect of S961 in endocrine resistance cells, cells were pre-treated with increasing concentrations of S961 before exposing cells to either IGF-I or insulin. S961 did not inhibit IGF-I or insulin-regulated signaling in MCF-7L and T47D parental cells even at high concentration as shown by IGF1R/InsR, AKT and MAPK phosphorylation (Figure 2.12A and 2.13A). Similarly, S961 did not inhibit insulin or IGF-I stimulated cell cycle progression (Figure 2.14A and 2.15A) and anchorage-independent growth in parental cells (Figure 2.16A and 2.17A). Even though agonist effects of S961 have been reported, we did not observe

S961-induced signaling or cell proliferation in these breast cancer cells (Figure 2.21 and 2.22).

In contrast, S961 blocked insulin-regulated signaling even at concentrations as low as 1 nM concentration in TamR cells (Figure 2.12B and 2.13B). Similar sensitivity was reflected in anchorage-independent growth assay (Figure 2.16B and 2.17B) and cell cycle analysis (Figure 2.14B and 2.15B), where 1 nM concentration of S961 fully diminished insulin-stimulated colony formation growth and S-phase induction, respectively in MCF-7L TamR cells. A higher S961 concentration was needed to fully block insulin-stimulated growth in T47D TamR cells. Since TamR cells lack IGF1R, they do not respond to IGF-I stimulation.

83-7 mAb down-regulates InsR, thus inhibiting insulin-stimulated signaling and growth in TamR cells.

Monoclonal antibody clone 83-7 (83-7 mAb) binds alpha-subunit of InsR allosterically without interfering insulin binding [159] and is specific for InsR binding although its functional roles in cells are not well studied. The antibody has been reported to stimulate lipogenesis, inhibit lipolysis, and activate receptor kinase by cross-linking receptor molecules [160].

To explore the effect of 83-7 mAb in cancer cells, we pre-treated 83-7 mAb overnight before treating with IGF-I, IGF-II, or insulin in MCF-7L, T47D and TamR cells. As shown in Figure 2.18, 83-7 mAb did not induce receptor phosphorylation. In contrast, 83-7 mAb down-regulated InsR and effectively blocked IGF-II and insulin-stimulated IGF1R/InsR, IRS, AKT and MAPK

phosphorylation (Figure 2.18). To study the biological effects of InsR, anchorage independent growth assays showed that 83-7 mAb effectively inhibited insulin-stimulated colony formation (Figure 2.19). Although 83-7mAb caused some InsR down-regulation in the parental cells, there was little to minimal inhibitory effect of 83-7 mAb on MCF-7L and T47D parental cells in terms of IGF-I, IGF-II or even insulin-stimulated signaling and anchorage-independent growth (Figure 2.18 and 2.19).

Inhibition of InsR was not effective in parental breast cancer cells due to the presence of IGF1R/InsR hybrid receptors.

To further explore why InsR inhibition was not effective in MCF-7L and T47D parental cells, we used immunoprecipitation and immunoblotting to examine the ability of these cells to express IGF1R/InsR hybrid receptors. Co-immunoprecipitation studies showed that IGF1R/InsR hybrid receptors are present in MCF-7L and T47D parental cells but not in TamR cells because of their downregulation of IGF1R (Figure 2.20). As previously shown, S961 was not effective in parental cells. To inhibit IGF1R and hybrid receptors, we used HuEM164 (also known as AVE1642), an anti-IGF1R mAb shown to specifically bind IGF1R and result in its down-regulation [161]. In the parental cells, insulin signaling was more completely extinguished by the use of both IGF1R mAb and S961 (Figure 2.21 and 2.22).

Similarly, when shIR transduced parental cells was treated with HuEM164, a synergistic inhibitory effect was achieved, as measured by IGF1R/InsR, IRS, AKT and MAPK phosphorylation. Unlike S961, down-regulation of InsR by

HuEM164 however was able to only partially block insulin-mediated signaling in parental cells (Figure 2.23 and 2.24). The most complete inhibition of ligand signaling was achieved by the combination of shIR#6 and HuEM164.

83-7 and HuEM164 mAbs provided no synergistic benefits to ER+ breast cancer

Since mAbs are commonly used to treat cancer in the clinic, we were curious if 83-7 would be a better choice for combination treatments. MCF-7L cells were treated with 83-7 and/or HuEM164 mAbs overnight prior to western blot analysis. Although the expression levels of IGF1R and InsR were significantly reduced in MCF-7L treated with HuEM164 and 83-7 mAbs, compared to individual treatment (Figure 2.25), IGF-II-mediated signaling was not fully suppressed., HuEM164 alone was sufficient to block IGF-I- and insulin-mediated signaling in MCF-7L, which is consistent to the previously shown results. To further evaluate the combinational benefits in a functional context, MTT monolayer proliferation assay and cell cycle analysis were performed. Interestingly, not only was 83-7 mAb not able to provide a combined inhibitory effect in conjunction with HuEM164, 83-7 mAb interfered with the function of HuEM164 in blocking IGF-I-driven monolayer proliferation (Figure 2.26) and cell cycle progression (Figure 2.27).

Combination treatments of 83-7 and HuEM164 fully suppressed ligand-mediated signaling in triple negative breast cancer cells

MDA-MB-231 and MDA-MB-435 are triple-negative breast cancer (TNBC) cell lines. They both express moderate amounts of IGF1R and InsR and respond

to IGF-I, IGF-II and insulin stimulations. Unlike MCF-7L, 83-7 mAb alone inhibited insulin-mediated signaling in MDA-MB-231. The combination treatments of 83-7 and HuEM164 mAbs synergistically blocked IGF-II-regulated signaling, which was not achieved with single agent inhibition (Figure 2.28). The western blot data of MDA-MB-435 was not clear because of the high basal level of p-AKT (Figure 2.29). However, down-regulation of receptor levels by 83-7 and HuEM164 mAbs were additive. LCC6, which is a derivative of MDA-MB-435, can be an alternative TNBC model. Moreover, constitutively activation of MAPK was noticeable in these cell lines.

Discussion

While the insulin/InsR signaling system is responsible for glucose homeostasis; it is also a cellular growth factor. Metabolic syndrome associated with obesity and type II diabetes, both states of relative insulin resistance resulting in hyperinsulinemia are associated with cancer risk [162]. Breast cancer patients who have these conditions are more likely to suffer metastatic disease, disease recurrence, and mortality [163, 164]. When a number of anti-IGF1R mAbs trials failed to show benefits in cancer patients, one mechanistic explanation is the presence of InsR acting as a compensatory pathway to IGF1R inhibition and IGF1R loss [118]. Our endocrine resistant model showed greater sensitivity towards insulin when IGF1R expression level is lacking (Figure 2.1, 2.2 and 2.3). Other studies have also shown similar pattern in prostate cancer and pancreatic neuroendocrine tumors *in vivo*, where InsR induces mitogenic

activities and compensates for IGF1R inhibition or resistance to anti-IGF1R [165, 166].

InsR exists in two isoforms: InsR-A and InsR-B due to alternative splicing of exon 11, differing by 12 amino acids. Previous studies have shown that InsR-B tends to be a metabolic receptor expressed in adult muscle, liver, and fat. InsR-B binds only insulin at physiological concentrations. In contrast, InsR-A, a predominant isoform during fetal development is commonly expressed in cancer and binds with high affinity to insulin and IGF-II [167, 168]. Up-regulation of InsR-A has been reported in breast, ovarian, lung, colon cell lines and/or human tumors and is thought to mediate tumorigenesis and survivor in response to insulin and IGF-II [169-173]. However, our endocrine-resistant model did not show an increase in InsR-A/InsR-B ratio of mRNA level compared to parental cells (data not shown). To date, antibodies have not been developed that can distinguish between levels of InsR isoforms, thus the exact protein expression of isoforms is uncertain in cells. In tumors, the data regarding the role of InsR-A are derived from mRNA levels detected by PCR. Additional study is needed to determine if InsR-B has an important role in cancer biology.

In this study, we blocked InsR function by three different techniques: 1.) genetic knockdown of InsR using lentiviral shRNA, 2.) competitively blocked of insulin binding to its receptor by S961 and 3.) down-regulation of InsR without affecting insulin binding by a mAb. These different techniques showed consistent results, where inhibiting InsR was effective in the inhibition of insulin-regulated signaling and growth in TamR breast cancer cells, but not in parental cells.

These data show that insulin signaling is important in endocrine resistant cells, but less relevant to parental MCF-7L and T47D cells. The presence of IGF1R or hybrid receptors and little of holo-InsR make insulin only a weak mitogen in parental cells (Figure 2.20). However, in TamR cells, where there is little IGF1R, InsR becomes the predominant receptor driving insulin- (and IGF-II) stimulated growth. Thus, InsR is an important target in TamR cells. Moreover, IGF1R/InsR hybrid receptors allows cancer cells to expand their ligand binding capacity. Insulin can still signal through the other available IGF1R heterodimer upon InsR inhibition; likewise, IGF-I may signal through InsR in a hybrid confirmation suggesting single target inhibition of IGF1R or InsR is not sufficient to suppress hybrid receptor signaling.

Unfortunately, there is no reliable method to quantify the level of hybrid receptors in cells or patient tumors making it difficult to predict response to antibody-based therapy on only examining levels of receptor expression. However, the use of broader range of receptor biochemical inhibition such a TKI may provide a better therapeutic advantage.

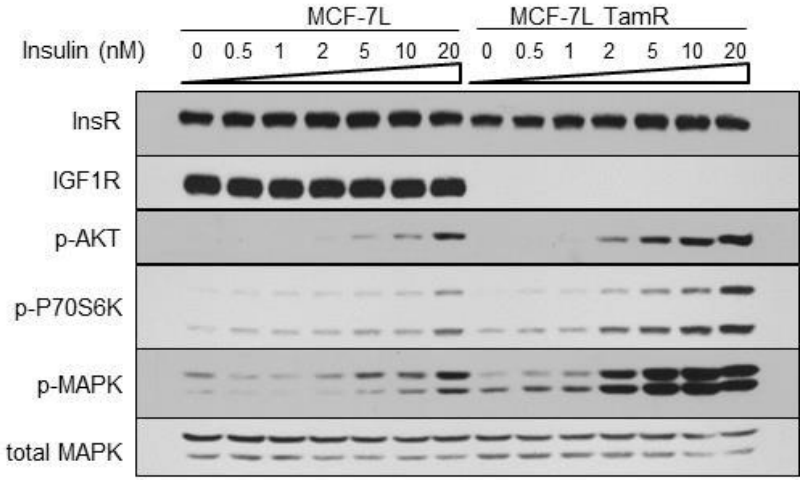
TNBCs are the most aggressive form of breast cancer. They remain non-targetable due to their vast heterogeneity and lack of the three markers (ER-, PR- and HER2-). There is no targeted therapy approved for TNBC. This type of tumors is usually treated with conventional chemotherapy and radiation therapy. In our study, the signaling result of IGF1R/InsR blocking antibodies did seem promising in MDA-MB-231 (Figure 2.28), even though we have yet to show functional data to prove their efficacy. Our lab has shown that down-regulation of

InsR using shRNA reduced tumor growth and metastasis of LCC6 in mice [130], suggesting that TNBC may benefit from InsR/IGF1R inhibition. Similar functions of insulin receptor have been described in an ER-negative model of mouse breast cancer [174]. In this system, InsR suppression was necessary to inhibit murine breast cancers in both normal and hyper-insulinemic hosts.

The major concern about targeting InsR is the resulting disruption of glucose homeostasis in normal tissues. Hyperglycemia can be managed by metformin, a commonly used drug for type 2 diabetes that reduces hepatic gluconeogenesis, circulating insulin level, and stimulates glucose uptake in muscle independent of insulin [175, 176]. However, the ability of metformin to directly affect cancer cell biology outside of modulating serum insulin levels is not understood. While there are preclinical data suggesting that metformin has little effect in models of non-diabetic rodent models [177], the clinical benefits of metformin in non-diabetic women with breast cancer awaits reporting of an adjuvant clinical trial where women were assigned to receive metformin or placebo for five years after surgical therapy for breast cancer (NCT01101438).

In summary, direct targeting of InsR would be a preferable strategy. Here, we show that monoclonal antibodies and a competitive peptide inhibitor have active against InsR, but there could have significant metabolic effects *in vivo*. As noted the two isoforms of InsR provide a theoretical strategy to only inhibit the cancer associated function of InsR signaling. If InsR-A specific agents could be developed, then this would not perturb the metabolic functions of InsR-B in normal tissues.

A


MCF-7L
MCF-7L TamR
Insulin (nM)
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0 0.5 1 2 5 10 20
InsR
IGF1R
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p-P70S6K
p-MAPK
total MAPK

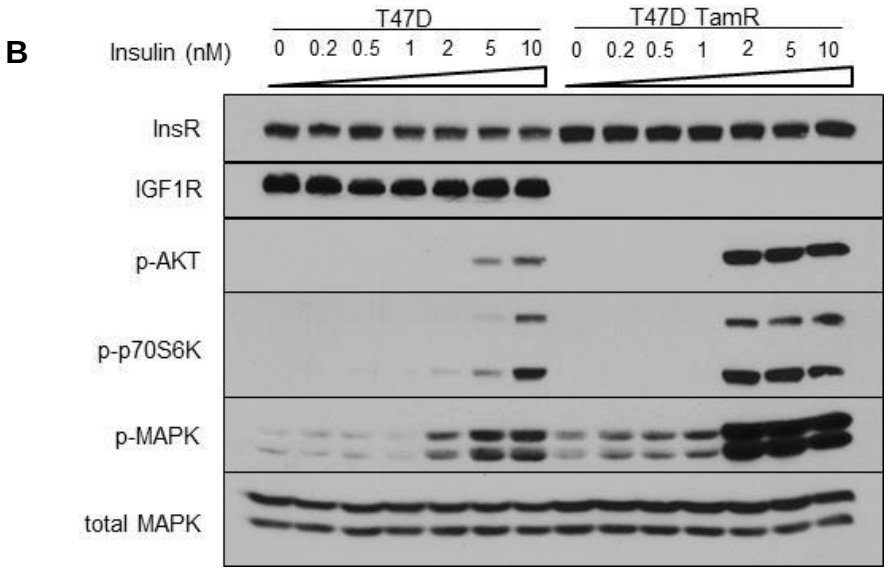
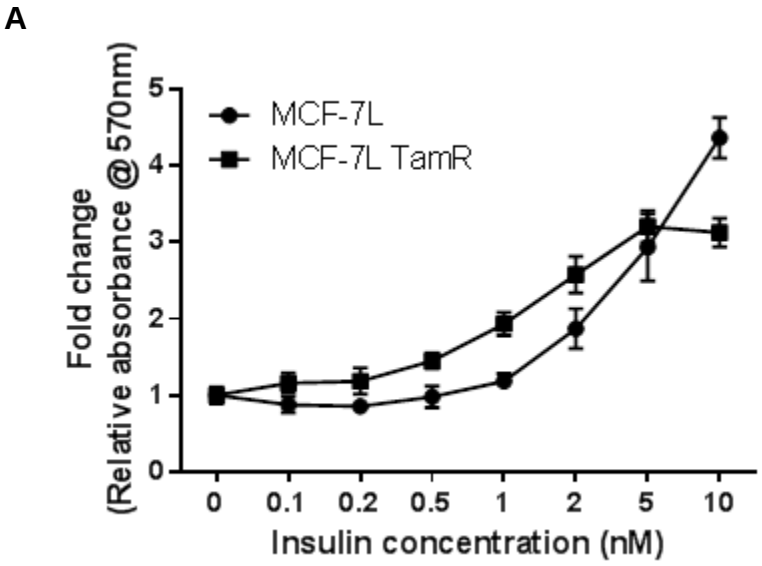


Figure 2.1. Insulin-mediated PI3K and MAPK activation was stronger in TamR cells compared to their parental counterparts.
(A) MCF-7Ls and **(B)** T47Ds were plated, serum starved for 24 hours and treated with increasing concentrations of insulin for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected to the indicated immunoblotting analyses.



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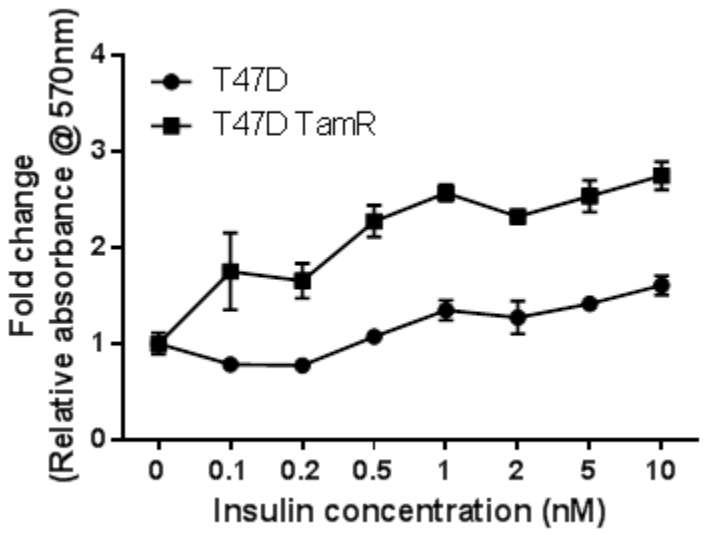
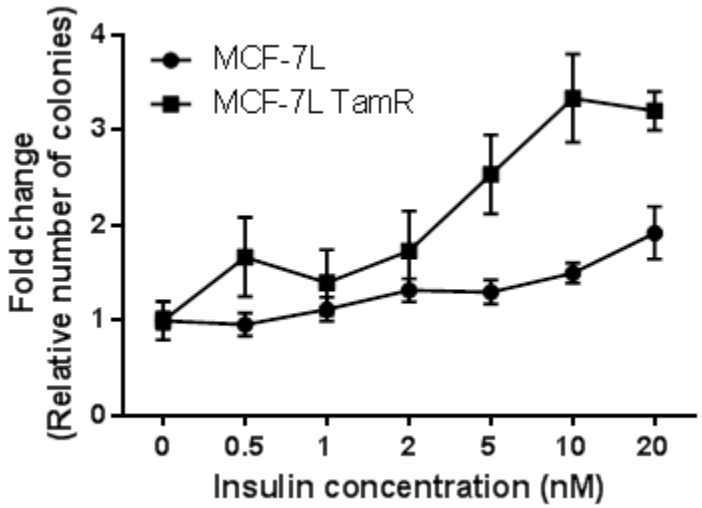


Figure 2.2. Insulin treatment stimulated greater monolayer proliferation in TamR cells compared to their parental counterparts.
Cell monolayer growth of **(A)** MCF-7Ls and **(B)** T47Ds were measured using MTT proliferation assay. Cells were serum starved for 24 hours and then treated with increasing concentrations of insulin. Readings were taken 5 days later. The results were normalized to untreated group. Values were normalized to untreated group and were presented as fold change (mean ± standard deviation, n=3).

A



B

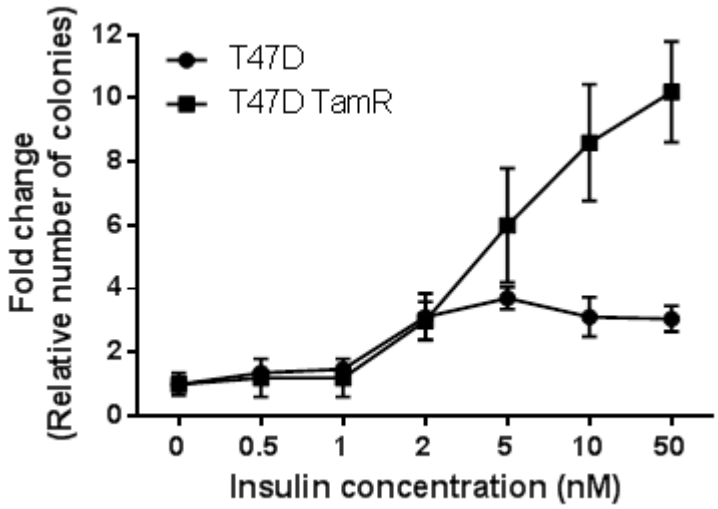
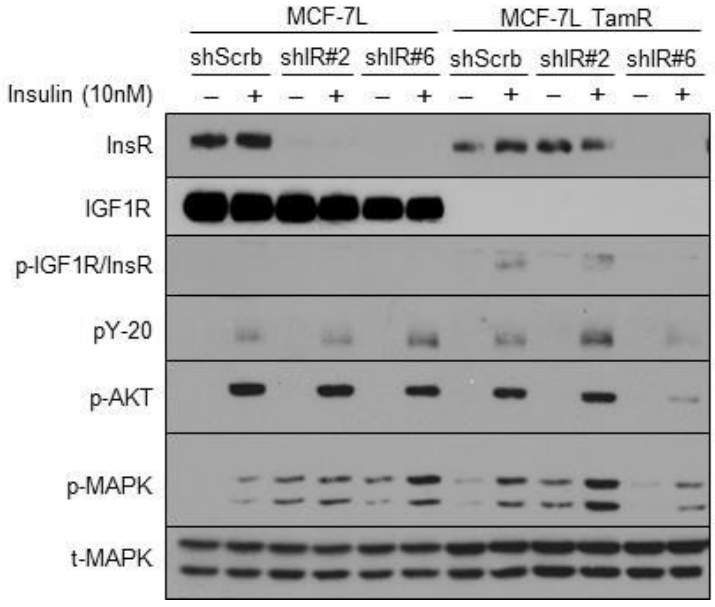


Figure 2.3. Insulin stimulated more colony formation in TamR cells compared to their parental counterparts

Anchorage-independent growth assay was carried out on **(A)** MCF-7Ls and **(B)** T47Ds. Colonies formed were counted 14 days and 20 days later, respectively. Values were normalized to untreated group and were presented as fold change (mean ± standard deviation, n=3).

A



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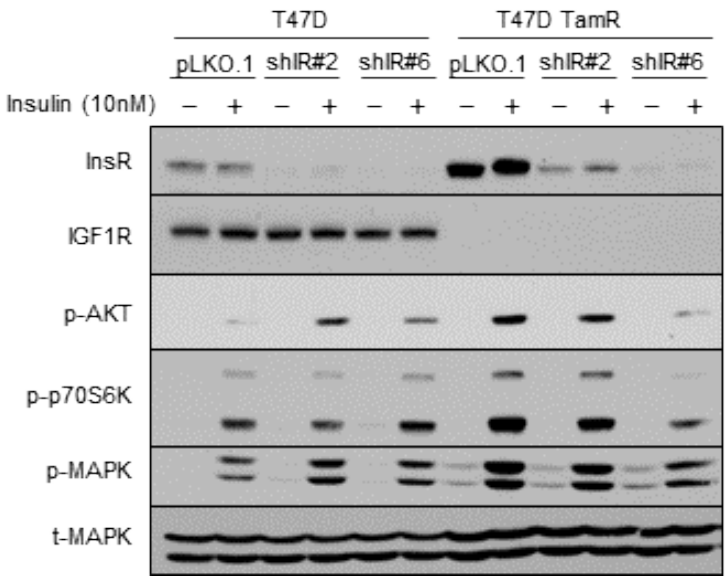


Figure 2.4. Knockdown of InsR using short hairpin RNA reduced insulin-regulated signaling in TamR cells, but not in their parental cells.
(A) MCF-7Ls and **(B)** T47Ds were InsR knock-downed with lentiviral shRNA (shIR#2, shIR#6) or plasmid control (shSrcb or pLKO.1). InsR protein levels were determined using Western blot analyses. Cells were plated, serum started for 24 hours and treated with or without 10nM insulin for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

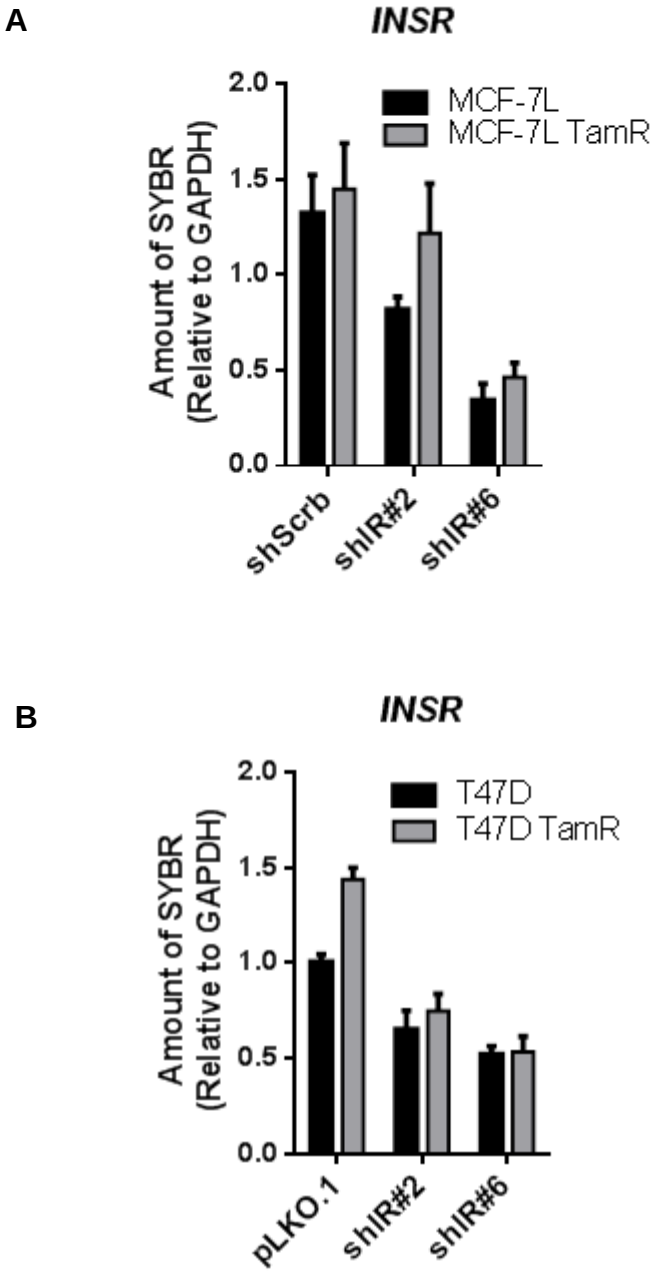


Figure 2.5. Measurement of mRNA level of InsR after knockdown using short hairpin RNA.

InsR mRNA expression levels of **(A)** MCF-7Ls and **(B)** T47Ds were determined using qRT-PCR analysis, total RNA was collected from cells in full media. Data was normalized to housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. Results represent mean ± standard deviation of triplicates from three independent experiments.

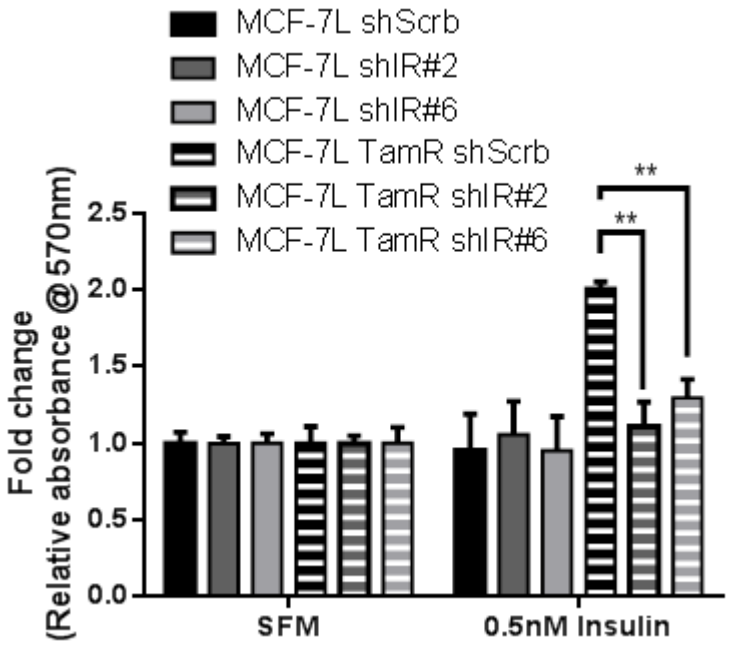


Figure 2.6. Knockdown of InsR using short hairpin RNA abolished insulin-driven monolayer proliferation in TamR cells.

Cell monolayer growth was measured using MTT assay. Transduced MCF-7L cells were serum starved for 24 hours and treated with or without insulin for 5 days. Values were normalized to untreated group and were represented in fold change (mean ± standard deviation, n=3). Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated versus treated groups and shIR versus control groups. **, $p<0.01$.

Cell lines	Insulin (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
MCF-7L shScrb	0	47.8 ± 7.8	27.4 ± 1.2	9.28 ± 3.8
	1	49.4 ± 4.1	29.0 ± 1.9	14.6 ± 2.9
	10	45.5 ± 3.0	35.1 ± 1.5	11.9 ± 5.1
MCF-7L shIR#2	0	51.2 ± 6.6	27.8 ± 2.8	9.9 ± 5.7
	1	53.9 ± 3.7	27.2 ± 2.6	12.4 ± 1.6
	10	49.4 ± 7.3	35.7 ± 5.1	6.71 ± 4.0
MCF-7L shIR#6	0	49.6 ± 8.2	30.9 ± 9.7	11.6 ± 3.8
	1	52.1 ± 7.3	28.2 ± 2.0	11.7 ± 3.2
	10	44.4 ± 5.5	38.1 ± 4.1	7.7 ± 7.0
MCF-7L TamR shScrb	0	55.3 ± 1.9	24.5 ± 2.4	14.4 ± 1.1
	1	39.0 ± 15.3	34.2 ± 1.4	11.9 ± 4.6
	10	41.4 ± 1.7	35.6 ± 2.7	13.8 ± 4.6
MCF-7L TamR shIR#2	0	39.8 ± 1.6	17.8 ± 1.4	26.2 ± 3.8
	1	39.8 ± 3.5	16.3 ± 1.6***	28.5 ± 6.6
	10	38.0 ± 3.5	22.1 ± 3.1***	22.0 ± 5.3
MCF-7L TamR shIR#6	0	45.7 ± 3.0	20.3 ± 4.3	19.2 ± 3.8
	1	43.7 ± 5.9	19.7 ± 2.7***	21.2 ± 2.2
	10	44.6 ± 5.6	20.7 ± 0.1***	19.7 ± 5.8

Cell lines	Insulin (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
T47D pLKO.1	0	53.0 ± 5.0	24.8 ± 2.5	11.3 ± 5.4
	1	53.8 ± 6.8	25.6 ± 1.4	10.7 ± 6.1
	10	41.9 ± 8.2	33.3 ± 2.2	13.1 ± 0.8
T47D shIR#2	0	50.5 ± 2.4	26.9 ± 0.7	11.0 ± 1.4
	1	52.4 ± 8.6	25.0 ± 3.7	12.1 ± 0.8
	10	40.4 ± 8.7	33.8 ± 5.5	13.4 ± 1.2
T47D shIR#6	0	52.2 ± 8.1	27.1 ± 6.2	7.5 ± 1.3
	1	49.2 ± 5.9	28.1 ± 6.0	10.9 ± 0.5
	10	35.9 ± 8.1	33.6 ± 1.3	10.8 ± 7.0
T47D TamR pLKO.1	0	60.3 ± 6.5	21.0 ± 5.1	13.0 ± 1.3
	1	55.2 ± 4.2	27.9 ± 6.4	10.7 ± 3.9
	10	42.0 ± 2.8	36.8 ± 1.6	10.7 ± 2.6
T47D TamR shIR#2	0	63.9 ± 3.6	20.3 ± 1.7	8.9 ± 0.2
	1	52.5 ± 8.6	19.8 ± 2.0^{ns}	17.7 ± 5.8
	10	49.0 ± 7.1	25.8 ± 1.0*	14.1 ± 3.4
T47D TamR shIR#6	0	54.8 ± 9.9	19.5 ± 0.7	12.8 ± 2.6
	1	54.7 ± 8.5	18.6 ± 1.4*	15.3 ± 4.9
	10	48.6 ± 5.0	22.8 ± 1.9***	14.8 ± 1.5

Mean ± SD (n = 3) ns, not significant; *, p<0.05; ***, p<0.001
shIRs versus control of relative treatment groups

Figure 2.7 knockdown of InsR affected the distribution of cell cycle phases in TamR cells, but not in the parental cells.

Cells were plated, serum starved for 24 hours and treated with indicated insulin concentrations overnight. Cell cycle analysis was performed using flow cytometry.

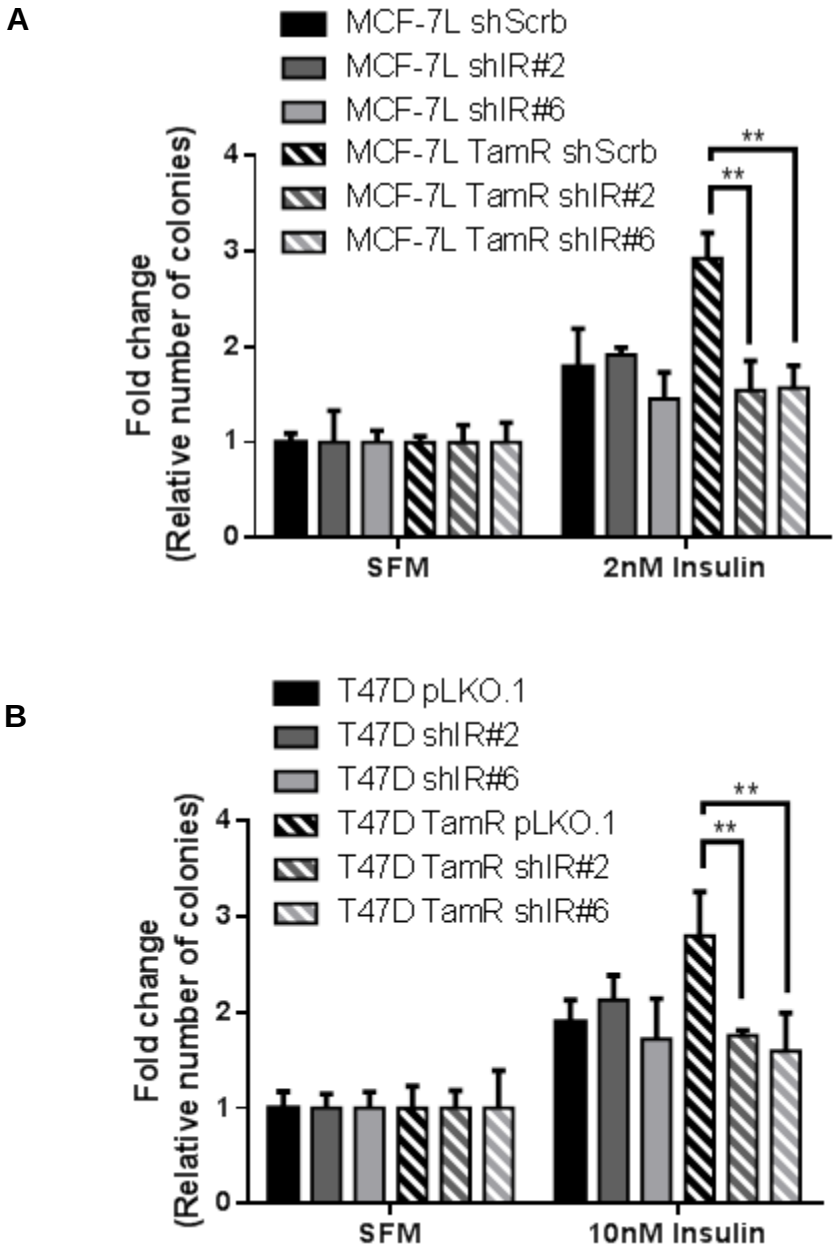


Figure 2.8. Knockdown of InsR using short hairpin RNA abolished anchorage-independent growth in TamR cells.

Anchorage-independent growth of lentiviral transduced **(A)** MCF-7Ls and **(B)** T47Ds was measured after 19 days and 25 days, respectively using soft agar assay. Values were normalized to untreated group and were represented in fold change (mean ± standard deviation, n=3). Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated versus treated groups and shIR versus control groups. **, $p<0.01$.

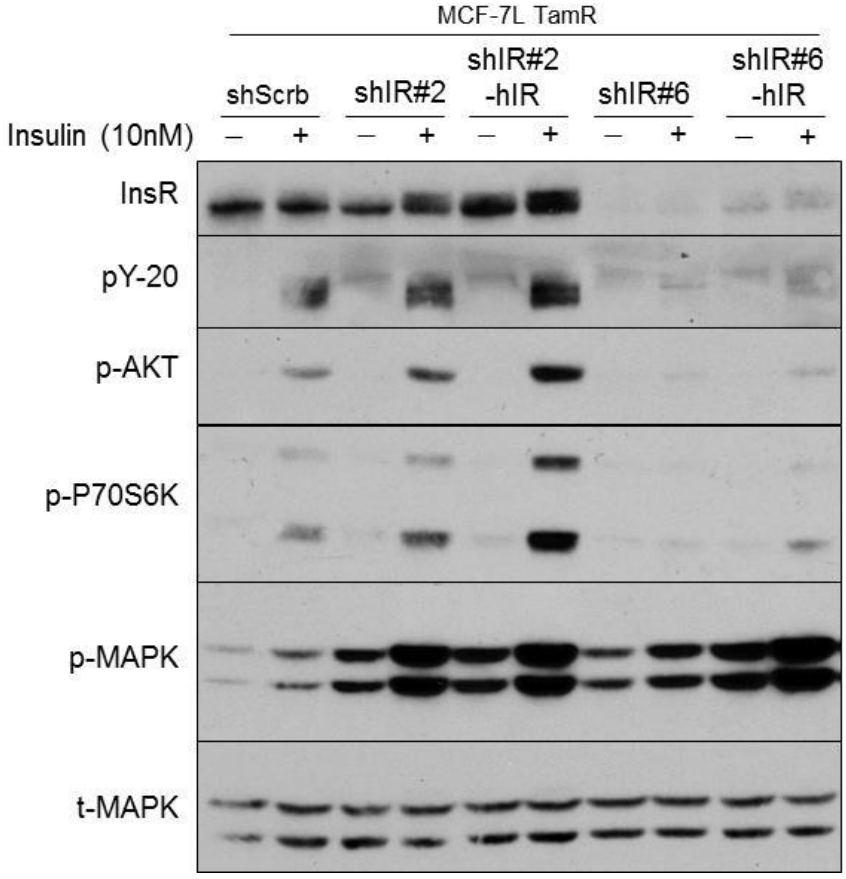


Figure 2.9. Expression of hIR-GFP cDNA mildly restored InsR expression and signaling.

Lentiviral transduced MCF-7L TamR cells were transiently transfected with hIR-GFP cDNA and selected under 500 µg/mL G418 for at least a week. Cells were serum starved for 24 hours and treated with 10nM insulin for 15 minutes before collecting for SDS-PAGE and immunoblotting analyses.

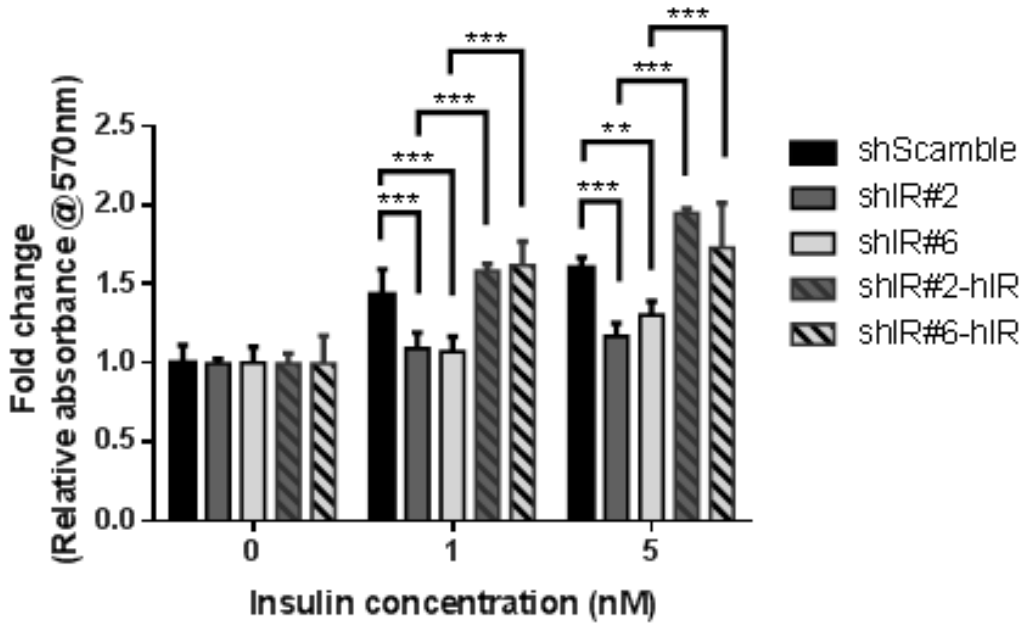


Figure 2.10, Expression of hIR-GFP cDNA reversed the knockdown effect of shIR in MCF-7L TamR cells.

Cell monolayer growth was carried out using MTT assay, where the cells were serum starved for 24 hours and treated without and with insulin for 5 days before the reading was taken. Graphs represent mean ± standard deviation of triplicates. Two-way ANOVA with Bonferroni comparison was performed. **, $p<0.01$; ***, $p<0.001$.

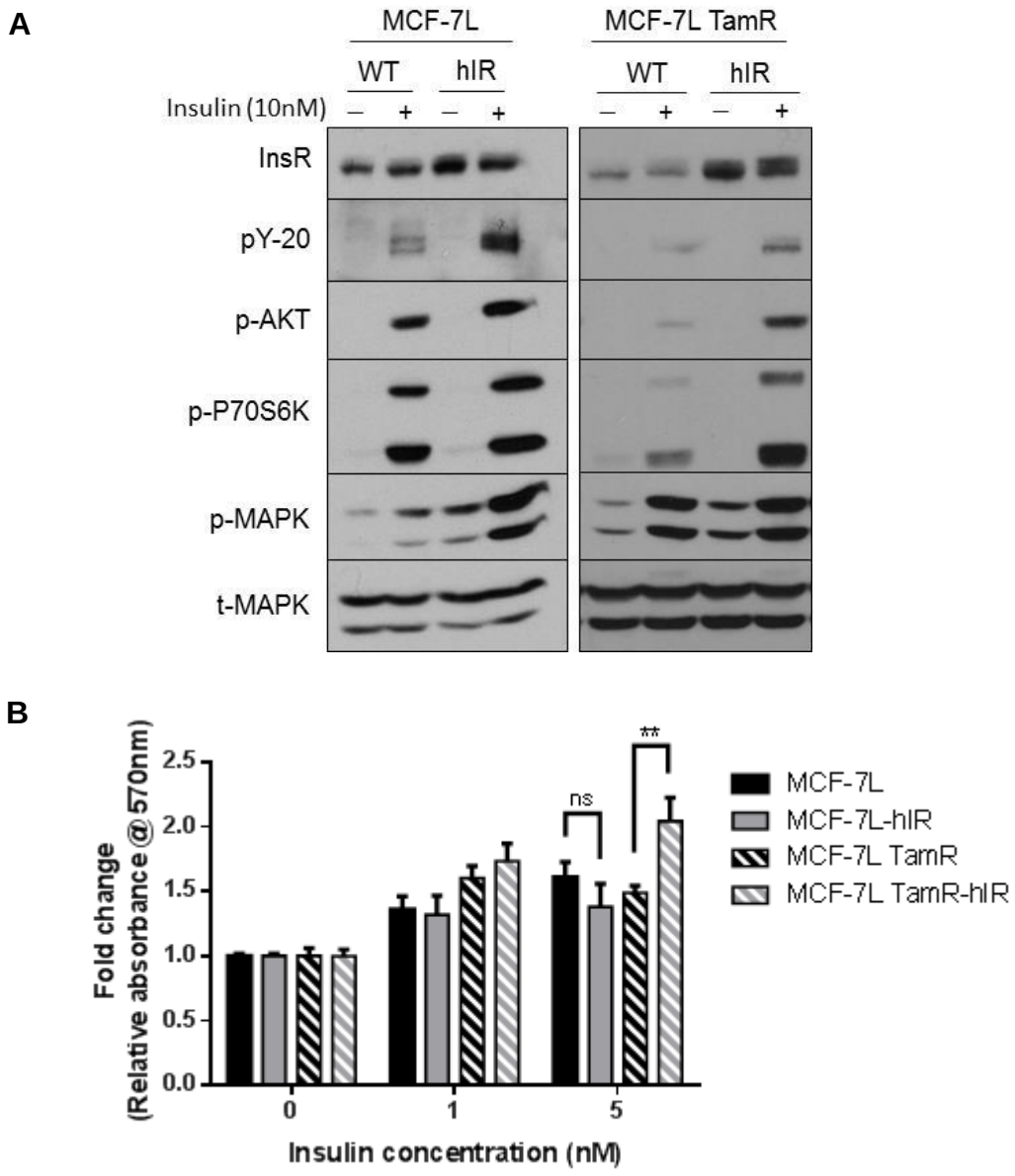


Figure 2.11. Exogenous expression of human *InsR* gene facilitated insulin-regulated signaling and cell growth in TamR but not in the parental cells. MCF-7L and TamR were transiently transfected with hIR-GFP under 500 µg/mL G418 for at least a week. **(A)** Cells were serum starved for 24 hours and treated with or without 10nM insulin for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses. **(B)** Cell monolayer growth was measured using MTT assay. Cells were serum starved for 24 hours and treated with indicated insulin concentrations for 6 days before the reading was taken. Graphs represent mean ± standard deviation of triplicates. Two-way ANOVA with Bonferroni comparison was performed. ns = not significant; **, $p<0.01$.

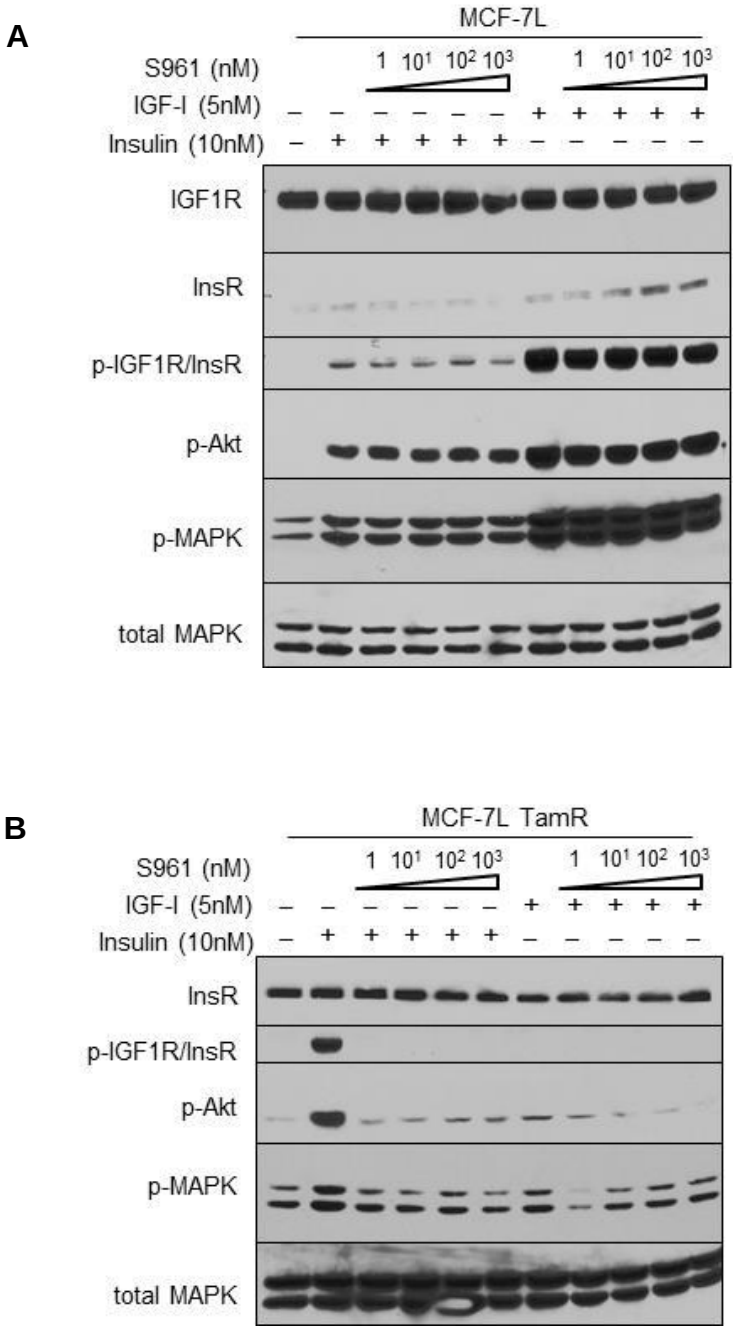


Figure 2.12. S961 inhibited insulin-mediated PI3K/MAPK signaling in MCF-7L TamR, but not in the parental MCF-7L cells.

(A) MCF-7L and **(B)** MCF-7L TamR cells were serum starved overnight and pre-treated with varying concentrations of S961 for 30 minutes before treating the cells with either 10nM insulin or 5nM IGF-I for 10 minutes. Whole cell lysates were separated by SDS-PAGE and immunoblotted for indicated antibodies.

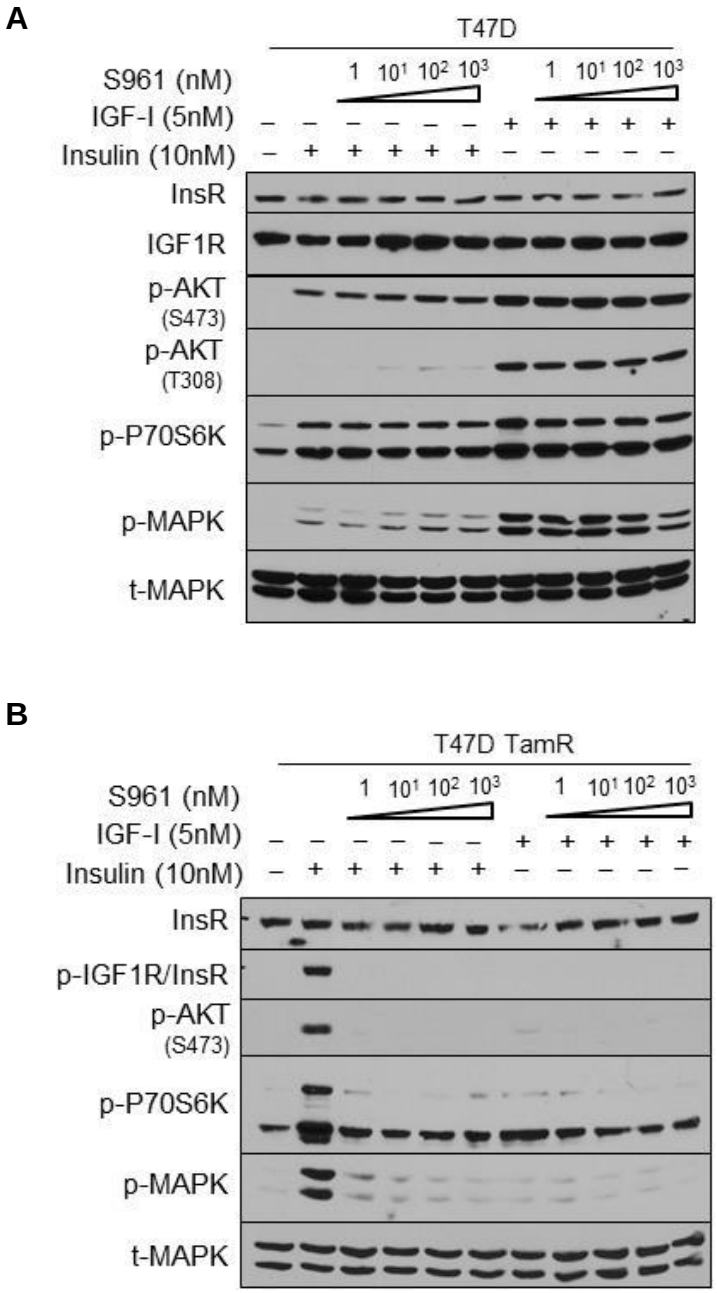


Figure 2.13. S961 inhibited insulin-mediated PI3K/MAPK signaling in T47D TamR, but not in the parental T47D cells.

(A) T47D and **(B)** T47D cells were serum starved and pre-treated with increasing concentrations of S961 for 30 minutes before treating the cells with either 10nM insulin or 5nM IGF-I for 10 minutes. Whole cell lysates were separated by SDS-PAGE and immunoblotted for indicated antibodies.

A

MCF-7L

treatment	S961 (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
SFM	0	64.9	20.4	14.7
Insulin	0	32.7	45.0	22.3
	1	34.9	44.4	20.7
	10	39.5	43.0	17.5
	100	38.4	41.8	19.8
IGF-I	0	34.7	48.2	17.1
	1	34.2	40.1	25.7
	10	33.8	47.2	19.1
	100	34.7	46.2	19.2

B

MCF-7L TamR

treatment	S961 (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
SFM	0	66.0	19.1	15.0
Insulin	0	30.9	32.7	36.4
	1	60.1	24.3	15.6
	10	64.6	19.7	15.7
	100	64.3	17.1	19.4
IGF-I	0	59.9	26.0	14.1
	1	59.2	20.1	20.7
	10	66.9	20.2	12.9
	100	63.0	20.4	16.7

Figure 2.14. S961 inhibited S phase induction stimulated by insulin in MCF-7L TamR, but not in the parental MCF-7L cells.
(A) MCF-7L and **(B)** MCF-7LTamR cells were plated, serum starved for 8 hours before treating with varying concentrations of S961 and either 10nM insulin or 5nM IGF-I overnight. Cell cycle analysis was performed using flow cytometry and analyzed using FlowJo.

A

T47D

treatment	S961 (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
SFM	0	50.5	28.4	11.5
IGF-I	0	23.3	50.9	10.6
	1	34.4	42.6	8.3
	10	30.5	45.6	5.2
	100	42.8	34.8	9.9
Insulin	0	30.5	43.5	14.7
	1	34.2	45.1	9.2
	10	34.4	44.2	13.5
	100	31.7	42.2	12.9

B

T47D TamR

treatment	S961 (nM)	Phases (%)		
		G_0/G_1	S	G_2/M
SFM	0	61.9	17.4	11.9
IGF-I	0	50.8	22	17.9
	1	53.7	20.4	17.5
	10	52.2	23.9	10.9
	100	54.0	22.5	13.4
Insulin	0	40.1	37.3	10.3
	1	46.9	30.5	16.9
	10	54.7	22.1	15.0
	100	55.4	19.8	16.2

Figure 2.15. S961 inhibited S phase induction stimulated by insulin in T47D TamR, but not in the parental T47D cells.

(A) T47D and **(B)** T47D TamR cells were plated, serum starved for 24 hours before treating with increasing concentrations of S961 in conjunction with either 10nM insulin or 5nM IGF-I overnight. Cell cycle analysis was performed using flow cytometry.

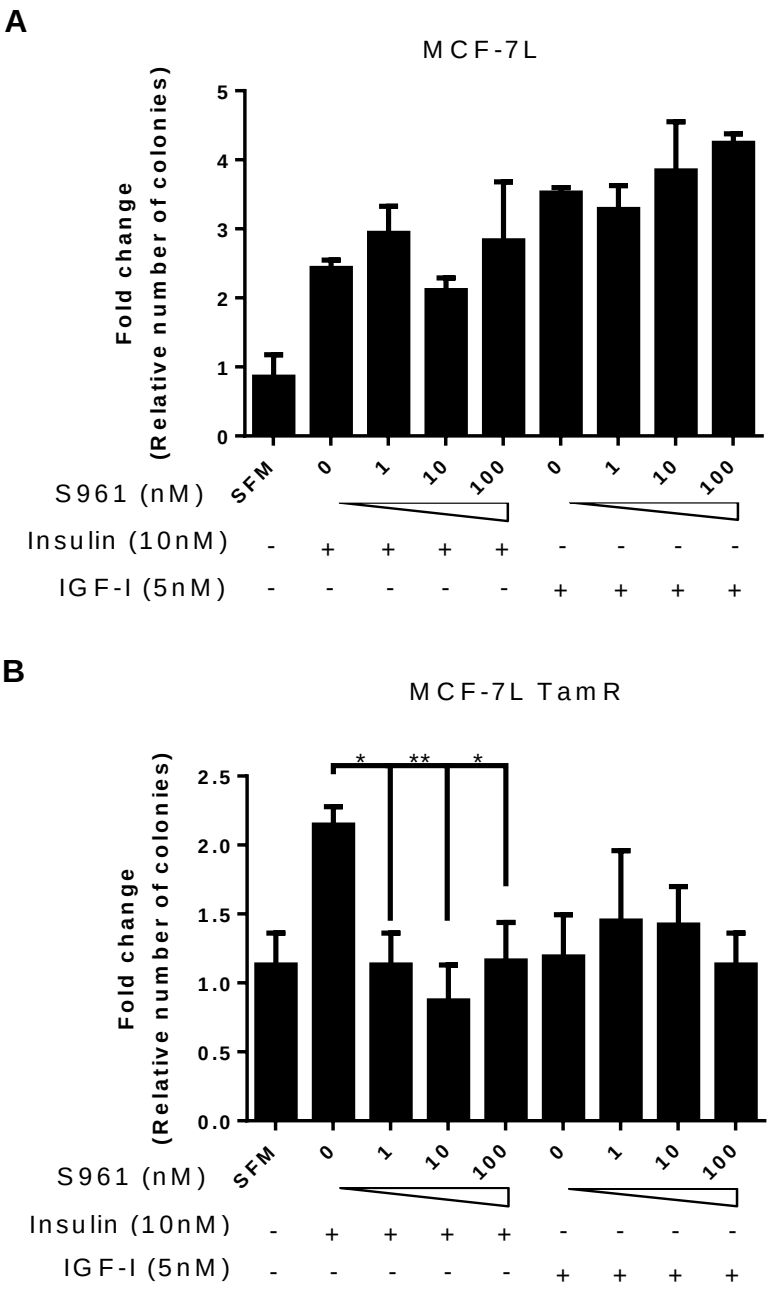


Figure 2.16. S961 inhibited insulin-driven colony formation in MCF-7L TamR, but not in the parental MCF-7L cells.

Anchorage-independent growth assay was carried out on **(A)** MCF-7L and **(B)** MCF-7L TamR cells treated with varying concentrations of S961 and either 10nM insulin or 5nM IGF-I. Treatments were spiked in after 7 days. Colonies formed were counted 14 days later. Two-way ANOVA with Bonferroni comparison was performed to compare between treated and untreated group. *, $p<0.05$; **, $p<0.01$.

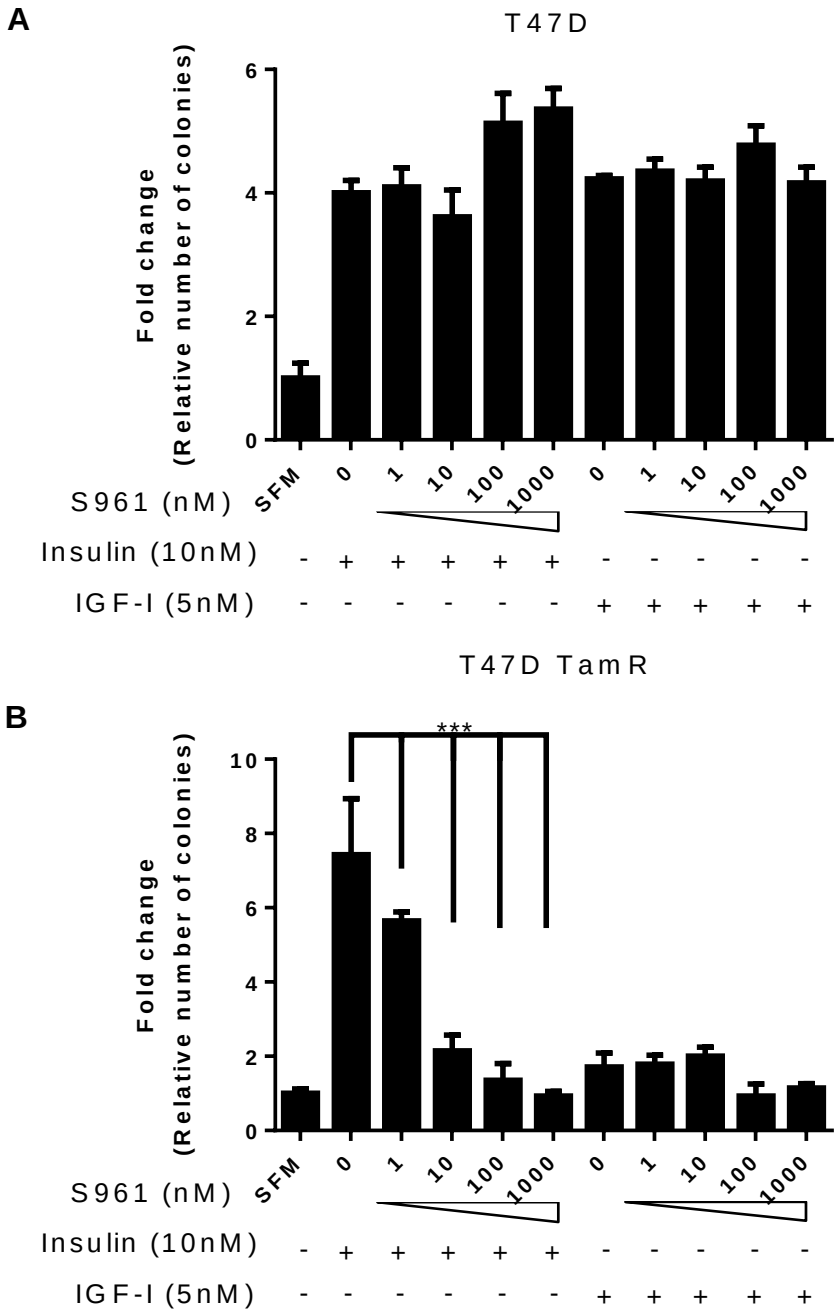


Figure 2.17. S961 inhibited anchorage-independent growth in T47D TamR, but not in the parental T47D cells.

Soft agar assay was carried out on **(A)** T47D and **(B)** T47D TamR cells treated with increasing concentrations of S961 and either 10nM insulin or 5nM IGF-I. Treatments were spiked in after 7 days. Colonies formed were counted 15 days after plated. Two-way ANOVA with Bonferroni comparison was performed to compared between treated and untreated group. ***, $p<0.001$.

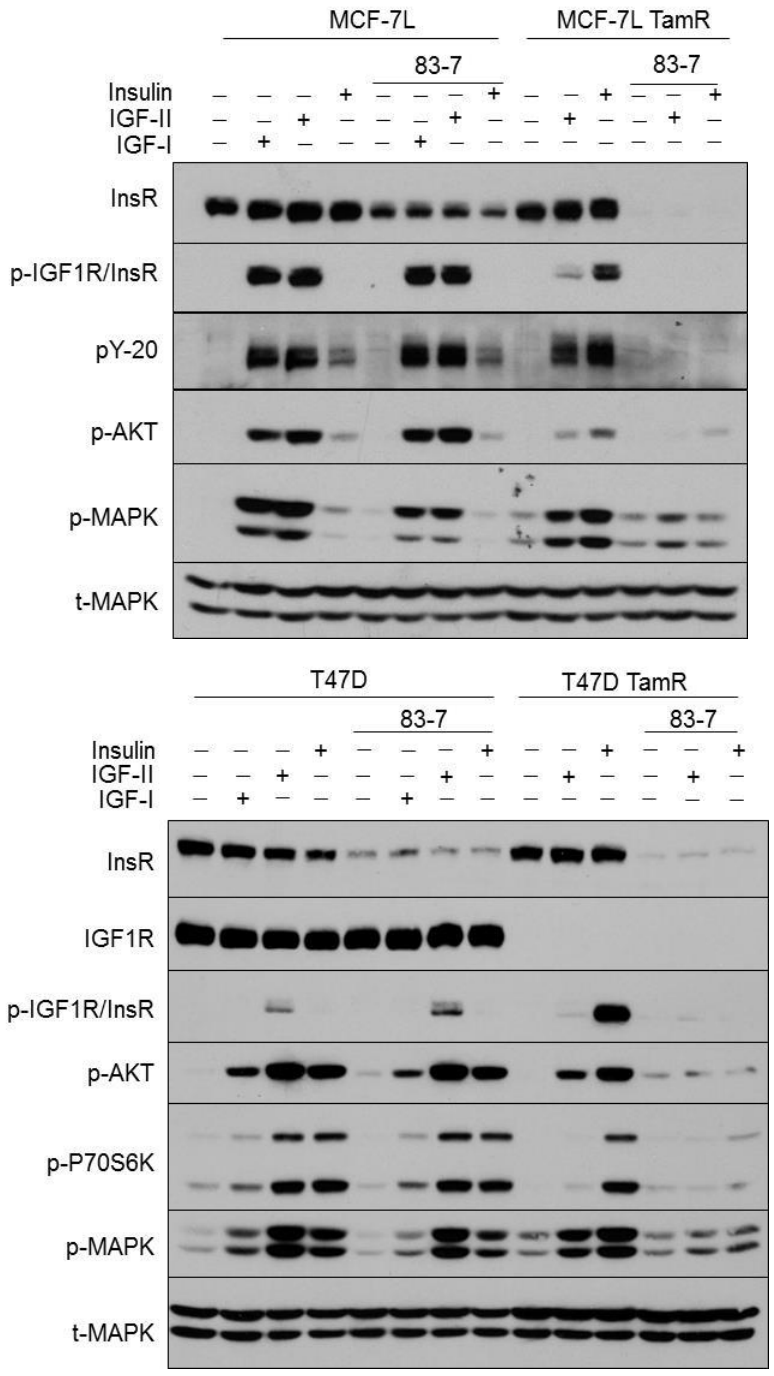


Figure 2.18 Down-regulation of InsR by anti-InsR monoclonal antibody clone 83-7 inhibited insulin-stimulated signaling in TamR cells.
MCF-7L and T47D cells were serum starved and pre-treated with 2 µg/mL of 83-7 overnight before treating with either 5nM IGF-I, 10nM IGF-II or 10nM insulin for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

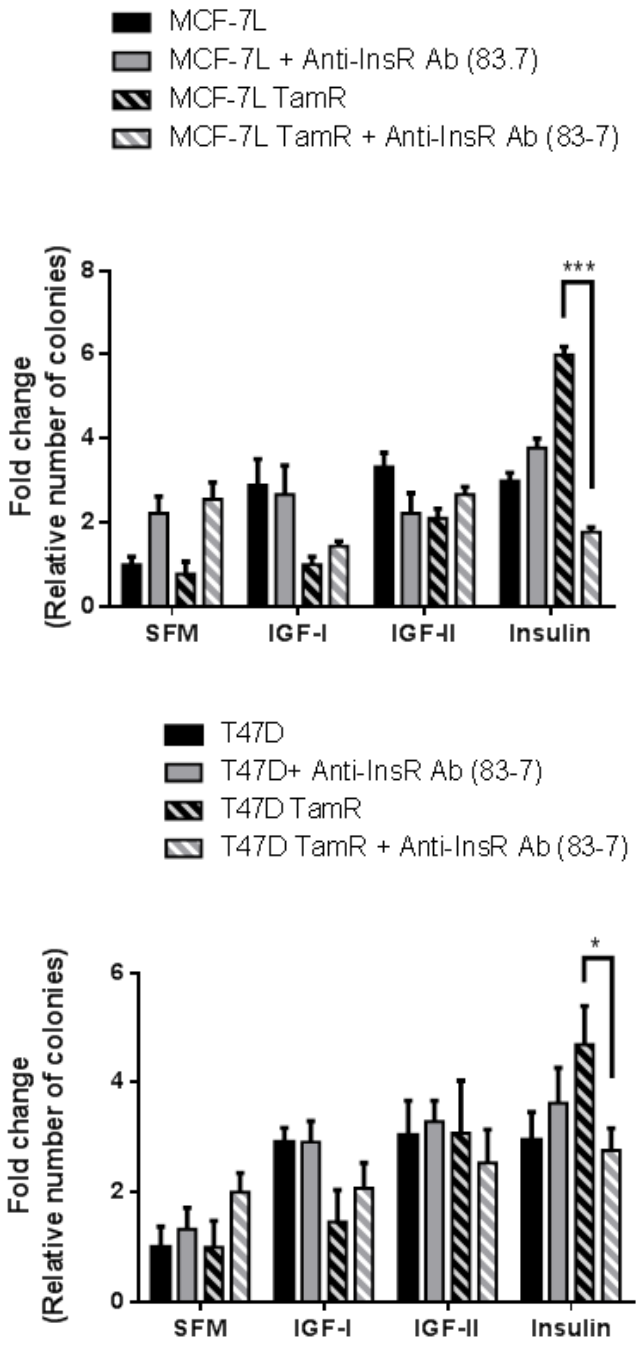


Figure 2.19. Monoclonal antibody clone 83-7 against InsR inhibited anchorage-independent growth in TamR cells.

Anchorage-independent growth assay was carried out on MCF-7Ls and T47Ds treated with 2 µg/mL of 83-7 without or with either 10nM IGF-I or 10nM insulin. Colonies formed were counted 14 days for MCF-7Ls and 21 days for T47D cells after first plated. Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated vs. treated groups. *, $p<0.05$; ***, $p<0.001$.

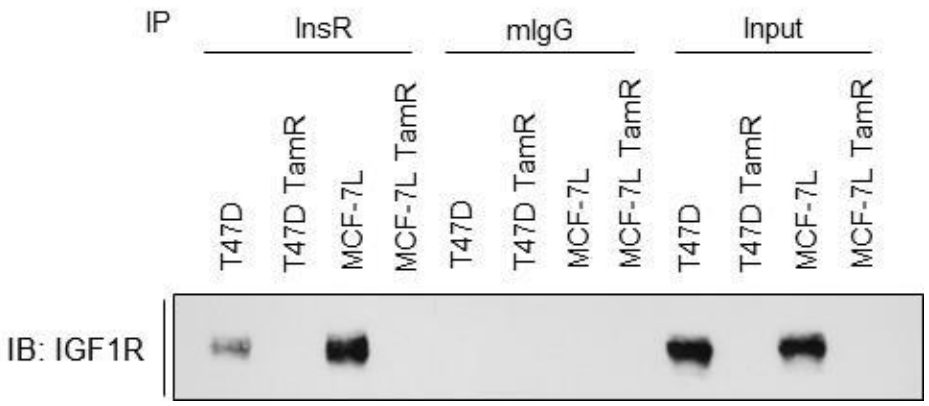


Figure 2.20. IGF1R/InsR hybrid receptors were present in MCF-7L and T47D parental cells.

The presence of IGF1R/InsR hybrid receptors was identified via immunoprecipitation (IP). Whole cell lysates were collected in full media and immunoprecipitated with either anti-InsR antibody or mouse IgG overnight. IP was then resolved with SDS-PAGE and subjected for IGF1R immunoblotting.

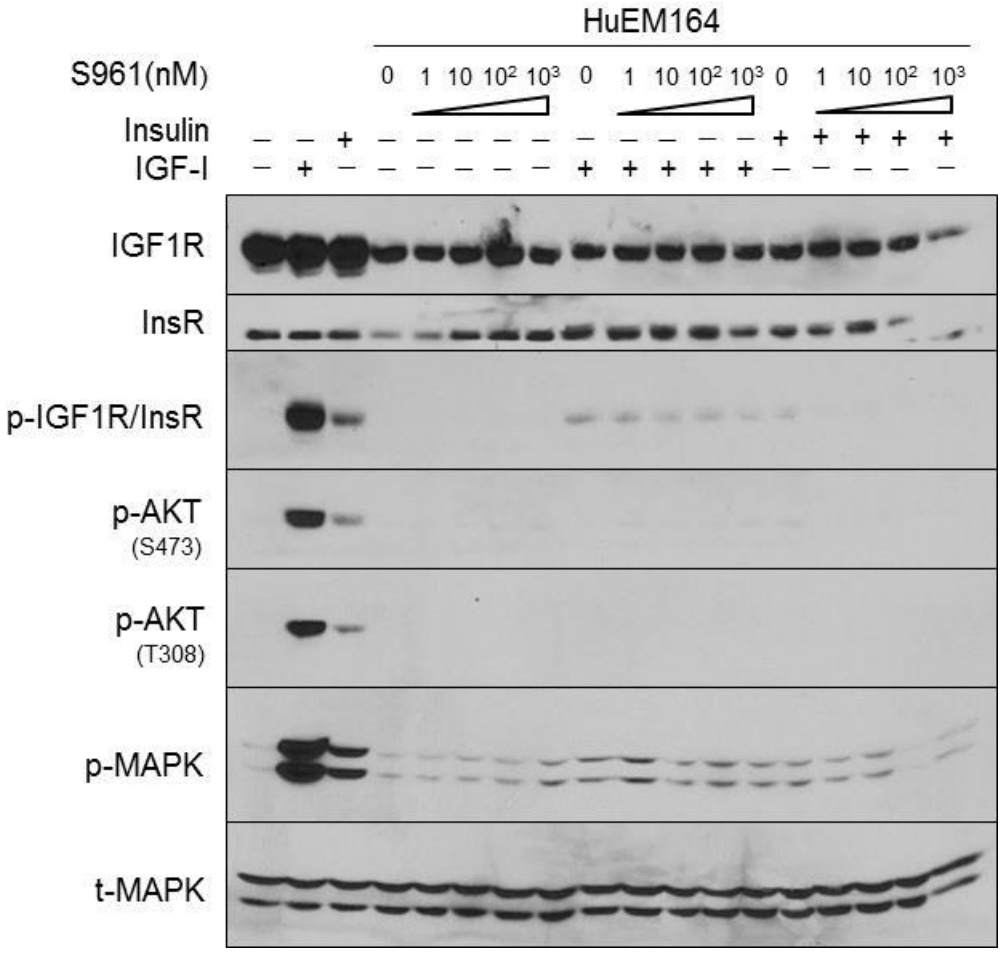


Figure 2.21. HuEM164 was required to fully suppress IGF-I- and insulin-stimulated signaling in MCF-7L parental cells, which was not achieved with S961 alone.

MCF-7L parental cells were serum starved overnight, pre-treated with 20 µg/mL HuEM164 for 3 hours and varying concentrations of S961 for 30 minutes before exposing to either 10nM insulin or 5nM IGF-I for 10 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for immunoblotting analyses.

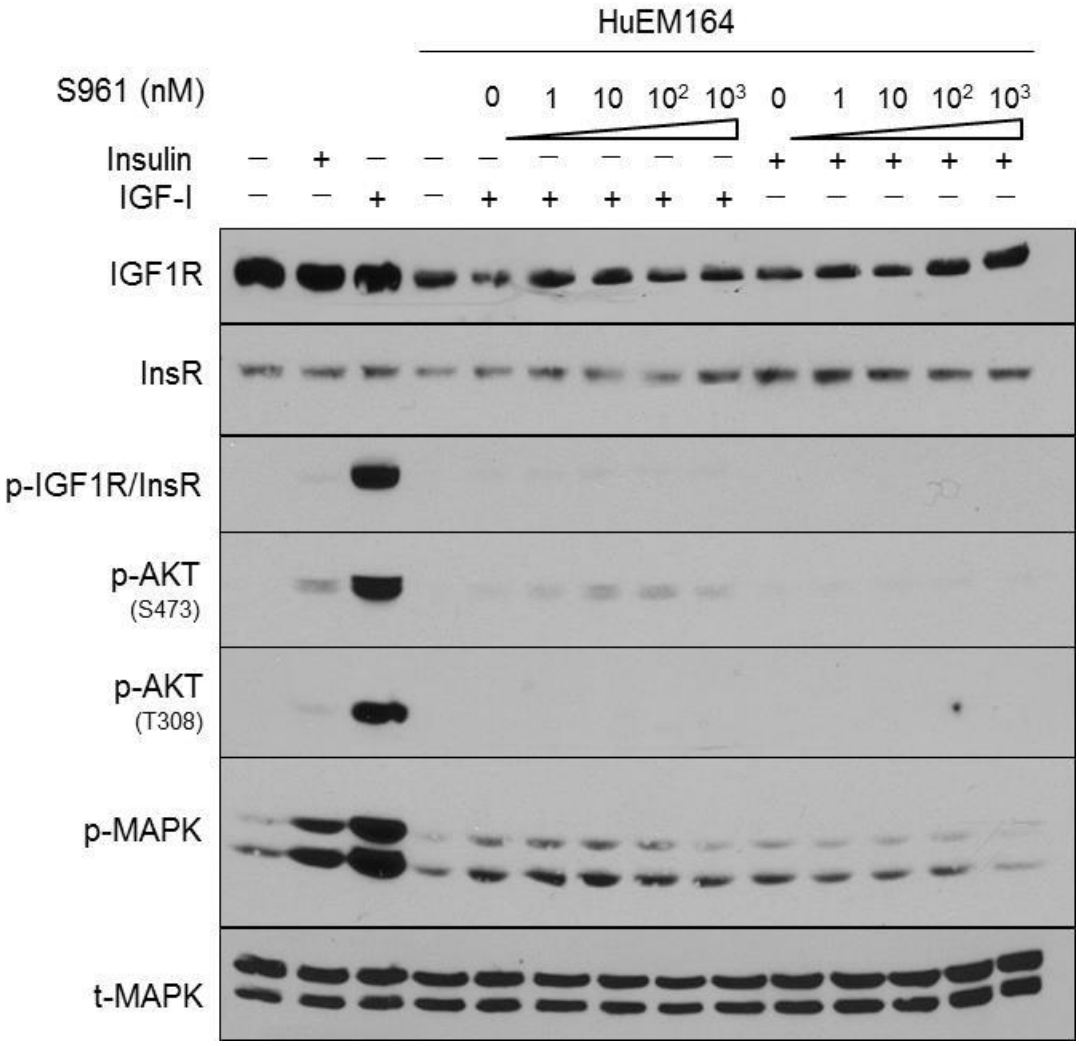


Figure 2.22. Combination treatment of S961 and HuEM164 effectively blocked insulin-mediated signaling in T47D parental cells.
T47D parental cells were serum starved overnight, pre-treated with 20 µg/mL HuEM164 for 3 hours and varying concentration of S961 for 30 minutes before treating with either 10nM insulin or 5nM IGF-I for 10 minutes. Whole cell lysates were collected and separated by SDS-PAGE and subjected for immunoblotting analyses.

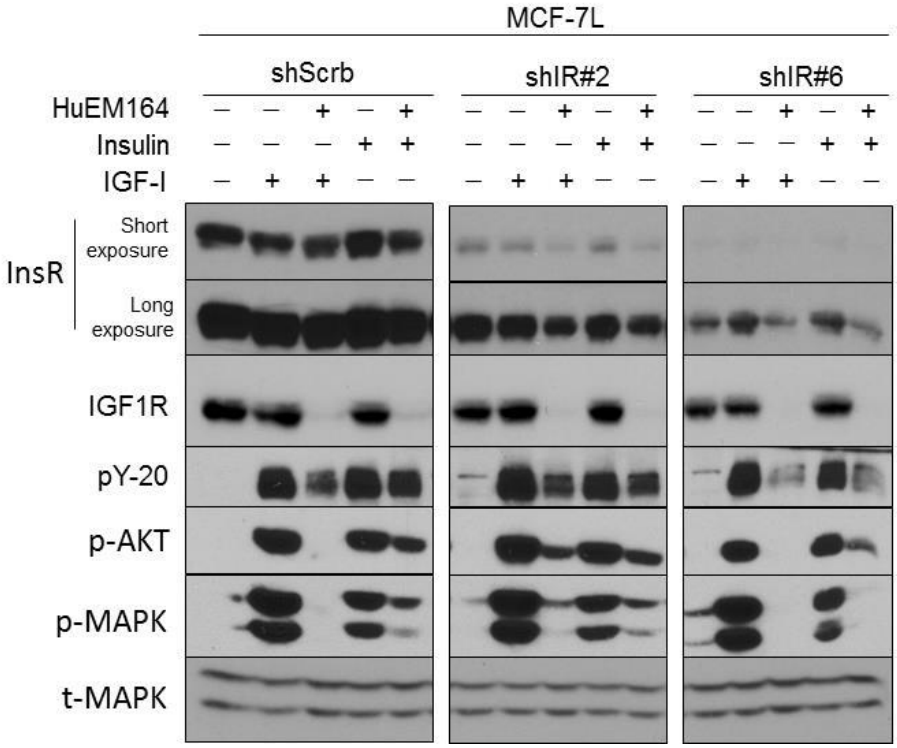


Figure 2.23. HuEM164 partially down-regulated InsR and blocked insulin-stimulated signaling in MCF-7L parental cells.
Lentiviral transduced MCF-7L cells were serum starved overnight and pre-treated with 20 µg/mL HuEM164 overnight before exposing to either 10nM insulin or 5nM IGF-I for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

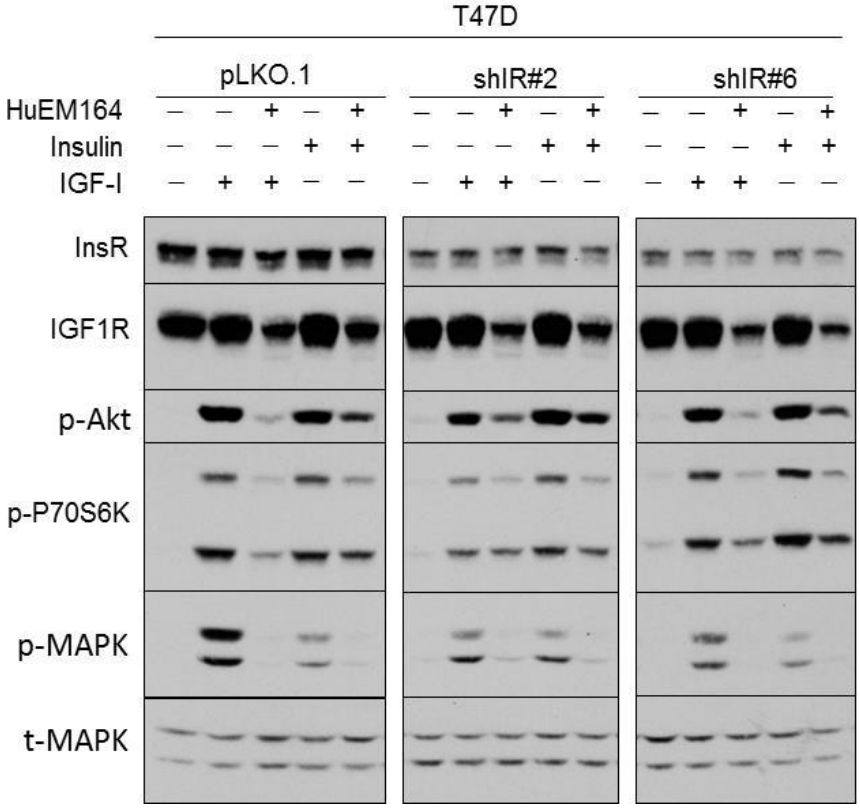


Figure 2.24. IGF1R down-regulation is necessary to effectively block insulin-regulated signaling in T47D parental cells.
Lentiviral transduced T47D parental cells were serum starved for 24 hours and pre-treated with 20 µg/mL HuEM164 for 1 hour before treating with 5nM IGF-I or 10nM insulin for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

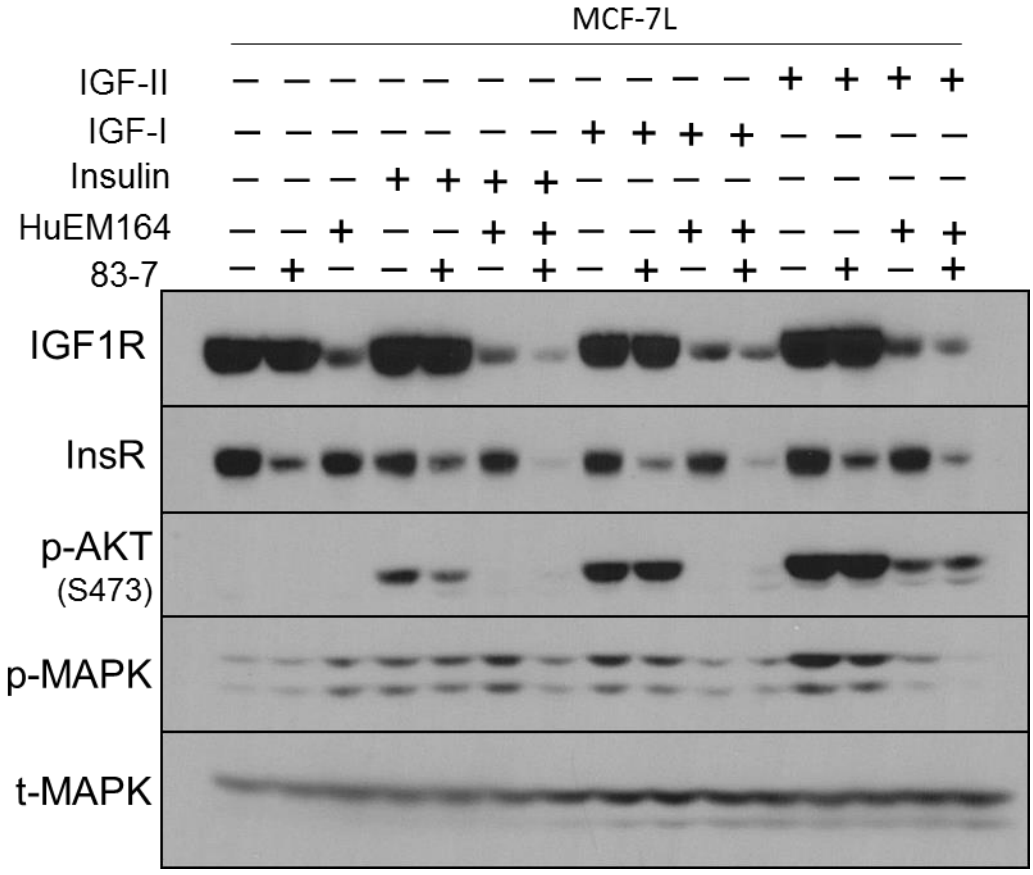


Figure 2.25. HuEM164 and 83-7 mAbs did not synergistically block ligand-mediated signaling in MCF-7L, compared to single targeting agent.
MCF-7L cells were plated, serum-starved overnight and pre-treated with 20 µg/mL of HuEM164 and 10 µg/mL of 83-7 antibodies overnight before exposing to either 20nM insulin, 10nM IGF-I and 20nM IGF-II for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

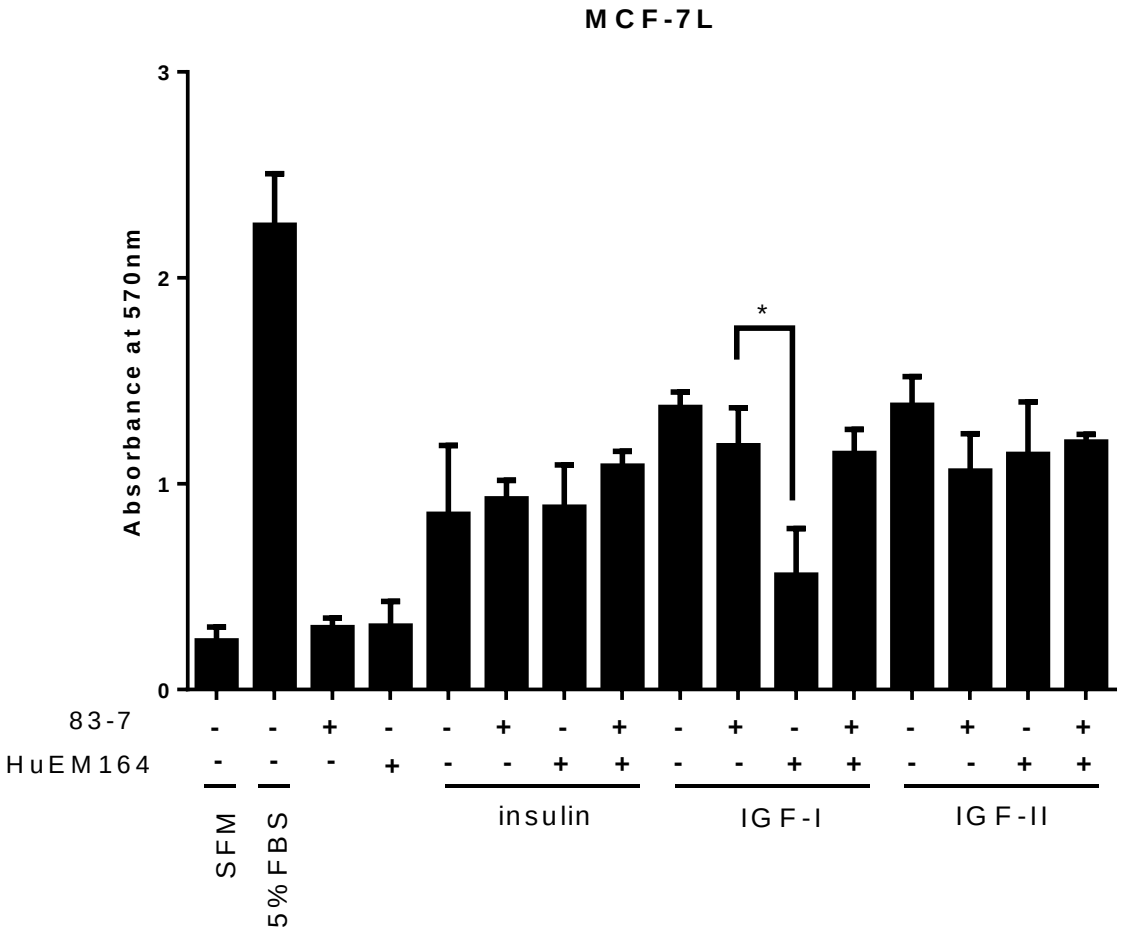


Figure 2.26. Co-treatments of 83-7 and HuEM164 mAbs provided no combined inhibition of ligand-driven monolayer proliferation in MCF-7L compared to HuEM164 alone.

MCF-7L cells were plated, serum-starved for 24 hours and treated with 20 µg/mL of HuEM164 or/and 10 µg/mL 83-7 antibodies in combination with either 10 nM insulin, 5 nM IGF-I or 10 nM IGF-II. Readings were taken 5 days later and the values were represented as mean ± standard deviation in triplicates. T-test analysis was performed to identify significance between HuEM164 treated vs. untreated group. *, p,0.05.

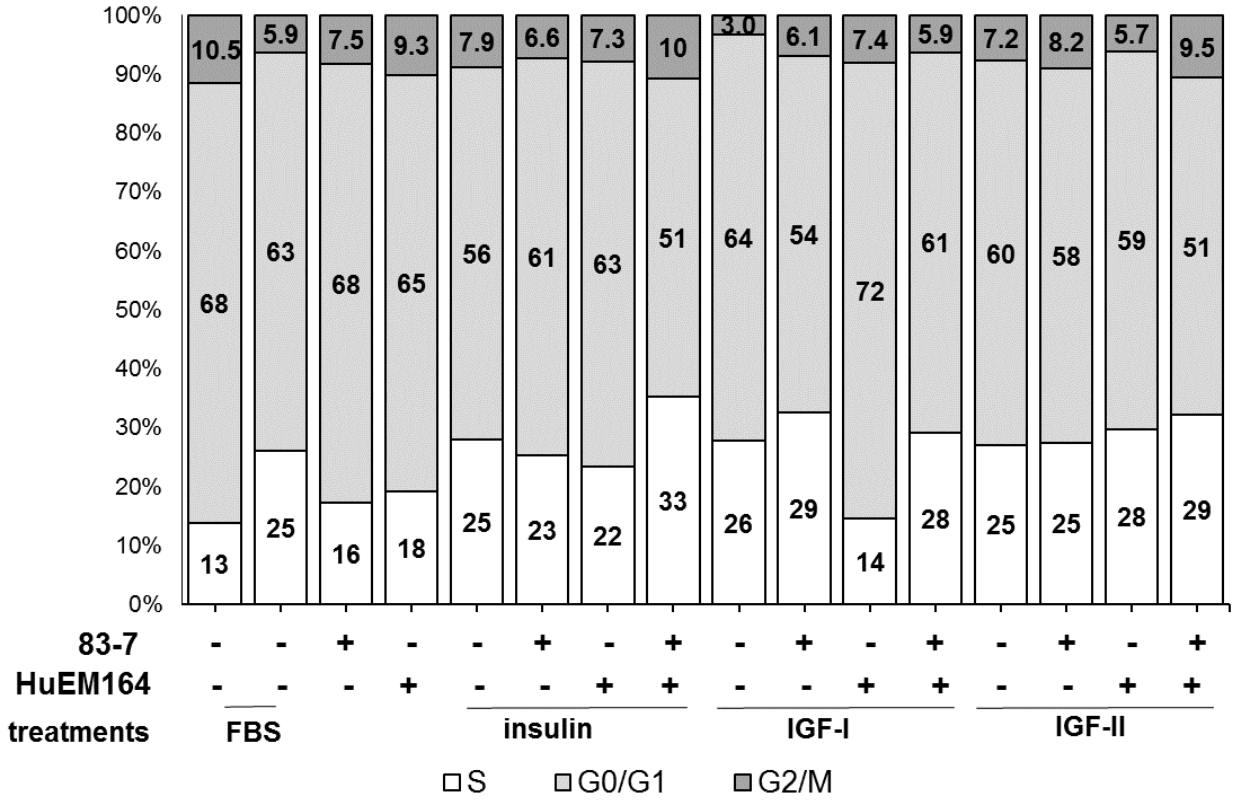


Figure 2.27. Co-treatments of 83-7 and HuEM164 mAbs provided no combined inhibition of cell cycle progression in MCF-7L, compared to HuEM164 alone.

Cells were plated, serum-starved for 24 hours and treated with 20 µg/mL of HuEM164 or/and 10 µg/mL 83-7 antibodies in combination with either 10 nM insulin, 5 nM IGF-I or 10 nM IGF-II overnight. Cell cycle analysis was performed using flow cytometry.

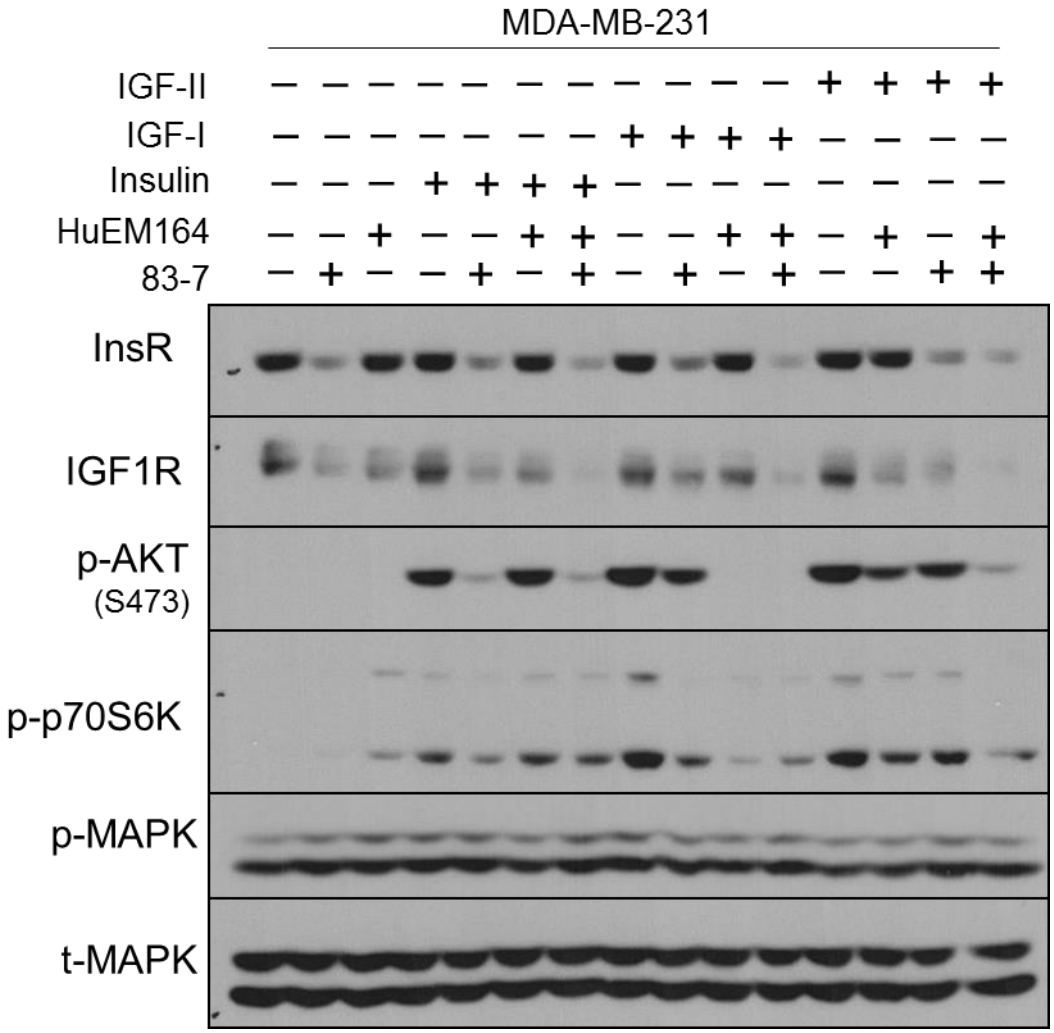


Figure 2.28. Co-treatments of 83-7 and HuEM164 mAbs synergistically blocked IGF-II-regulated signaling in MDA-MB-231.
Cells were plated, serum-starved overnight and pre-treated with with 20 µg/mL of HuEM164 and 10 µg/mL of 83-7 antibodies overnight before exposing to either 20nM insulin, 10nM IGF-I and 20nM IGF-II for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses.

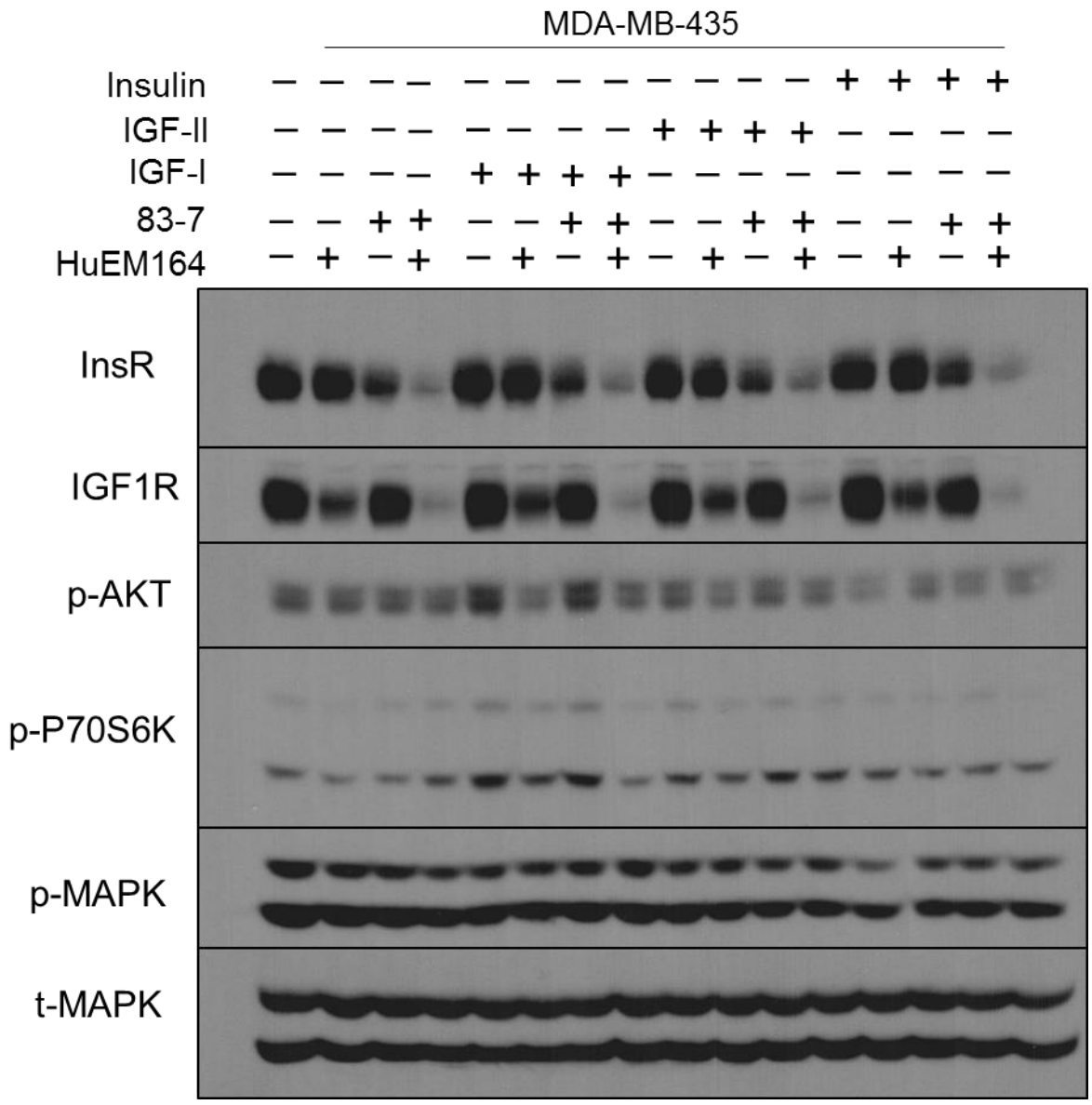


Figure 2.29. Co-treatments of 83-7 and HuEM164 mAbs synergistically down-regulated IGF1R and InsR expression levels in MDA-MB-435.
Cells were plated, serum-starved overnight and pre-treated with with 20 µg/mL of HuEM164 and 10 µg/mL of 83-7 antibodies overnight before exposing to either 20nM insulin, 10nM IGF-I and 20nM IGF-II for 15 minutes. Whole cell lysates were collected, separated by SDS-PAGE and subjected for indicated immunoblotting analyses

Chapter 3

Generation and characterization of InsR-evolved small protein scaffolds – the engineered T7 phage protein 2 protein

This work has been accepted for publication in *Molecular Cancer Therapeutics*

Introduction

Personalized medicine has become the next focus in cancer therapy. A one-treatment-fits-all approach is no longer appropriate in treating complex heterogenous disease such as breast cancer. Many of the distress from unrewarding clinical evaluations and the hinder in pharmaceutical industry could directly attributed to a lack of understanding and appreciation of human disease complexity and variability. It was only recently that the industry started to realize the need for tailoring drug to certain groups of populations and the importance of phenotyping efforts. This has stimulated the demand for protein engineering to develop effective protein drugs. Molecular profiles of tumors may provide insights and opportunities for the development of therapies to improve clinical outcomes for breast cancer.

While systemic inhibition of InsR may disrupt metabolism in an unselected patient group, certain subpopulations of cancer patients including non-diabetic endocrine-resistant breast cancer patients with increased InsR expression or insulin sensitivity may benefit from InsR inhibition. Yet, there are no reliable tools available for InsR quantification or specific InsR inhibition. The only drugs that inhibit InsR – linsitinib (OSI-906) and BMS-754807 – exist as dual IGF1R/InsR tyrosine kinase inhibitors. Evidence suggested OSI-906 was well tolerated and antitumor activity was observed in phase I clinical trial [178]. A phase II trial testing BMS-754807 in ER+ breast cancer patients resistant to aromatase inhibitors has completed (NCT01225172) but the results have not been disclosed.

Antibodies, the most commonly studied target specific binding molecules have long been used as therapeutic and diagnostic tools in medicine as well as in basic and applied research. Numerous antibodies have been approved against many diseases from cancer to viral and immunologic diseases [179-181]. Despite their valuable and widespread applicability, antibodies suffer from several limitations including limited tissue penetration [182-184], complex bispecific generation, suboptimal pharmacokinetic clearance for diagnostic imaging [183, 185], reduced ease to target select epitopes [186], modest instability and high production cost [187]. Non-immunoglobulin protein scaffolds have arisen as an alternative strategy including single domains that are relatively small (<15 kDa), structurally stable, tolerable to mutation, and do not require disulfide bonds and post-translational modification for activity [188]. A number of non-immunoglobulin scaffolds have been developed and tested in clinical trials [188]. Adnectin CT-322, an engineered 10^{th} type III domain of human fibronectin targeted against VEGFR-2, showed tolerable side effects and promising antitumor activity in patients with variety of solid tumors in phase I [189] although phase II trial of CT-322 did not show efficacy in recurrent glioblastoma [190]. More studies are underway with other adnectins (NCT02515669). ABY-025, an engineered affibody targeting HER2 receptor was able to discriminate HER2 status in metastatic breast cancer in patients under positron emission tomography (PET) imaging [191]. These and other examples have shown that small protein scaffolds can be engineered and selected against a wide array of targets and

their applications can range from basic science to diagnostic and therapeutic functions.

In this study, we used yeast surface display [192, 193], a platform for library screening and binder isolation to develop binders to InsR based on the T7 gene 2 protein (Gp2) scaffold [194]. Gp2 is a 45-amino-acid domain with two solvent-exposed loops (Figure 3.1A) capable of mutation to generate new binding function while retaining structural and thermal stability with midpoints of thermal denaturation of 65-80°C [194]. A Gp2 domain engineered to bind epidermal growth factor receptor was effectively used as a PET probe for *in vivo* imaging in murine xenograft tumor models [195]. Although Gp2 domain has been engineered against various targets, no Gp2 binder has so far been shown to exhibit biological activity on cancer cells. Here, we evolved three Gp2 variants exhibiting strong binding affinity against cell-surface InsR. The domains inhibit insulin-regulated growth in endocrine-resistant and –sensitive breast cancer models and exhibit differential inhibition of receptor and downstream signaling. Notably this inhibition is observed even though one of the variants exhibits enhanced binding upon insulin co-treatment. These agents are the first non-immunoglobulin proteins that inhibit InsR function in breast cancer.

Results

Discovery and evolution of InsR-specific Gp2 binders through yeast surface display

InsR-specific Gp2 domains were discovered and evolved through yeast surface display and various enrichment and depletion technologies including

magnetic bead selection and fluorescence activated cell sorting (FACS) with recombinant InsR ectodomain (rInsR) and detergent solubilized lysate of cells expressing InsR-GFP fusion (Figure 3.2A). Yeast displaying the naïve Gp2 library underwent two sorts of rInsR binding enrichment via magnetic beads and one FACS for full-length Gp2 before parallel error-prone PCR targeting the loops and the genes of enriched Gp2 variants. The resultant Gp2 population was further enriched with two sorts on rInsR-coated magnetic beads, one FACS with rInsR and one FACS with InsR-expressing cell lysate before undergoing a second round of mutations. During the final evolutionary round, the mutagenized InsR-enriched Gp2 population was sorted against cell lysate containing InsR-GFP and the top 2% of InsR binders were isolated via FACS (Figure 3.2B, middle). Appreciable binding was not observed for the enriched Gp2 population to GFP-only cell lysates or yeast displaying non-enriched Gp2 control incubated with InsR-GFP lysates, indicating that the population evolved a Gp2—InsR interaction rather than binding between other elements of the selection process (Figure 3.2B, left and right).

Among twenty selected top InsR binders from FACS, eight clones had unique protein sequences and proceeded for protein production in *E. coli*. Six variants had suitable protein yield in the soluble fraction of *E. coli* and were evaluated for binding to InsR overexpressing HEK293T cells. Two variants showed weak binding against cell-surface InsR; while a third variant showed no InsR specificity as it was not able to differentiate binding between $InsR^{high}$ and $InsR^{low}$ cell lines. Three variants, name Gp2 #1, #5 and #10 exhibited strong

binding to cell-surface InsR (Figure 3.3). Gp2 #1 has unique loop sequences whereas Gp2s #5 and #10 have equivalent loops but a single amino acid difference at framework position 24 (Figure 3.4B). The purity and molecular weights (7 kDa with C-terminal His_6 tag) of purified proteins were verified using SDS-PAGE (Figure 3.4A).

Affinity titration of Gp2 variants

To measure the affinity of soluble Gp2 variants against cell-surface InsR and further evaluate specificity, two cell types (Figure 3.5A) — InsR-GFP overexpression of HEK293T ($InsR^{high}$) and GFP-control transduced HEK293T ($InsR^{low}$) – were labeled with ranging concentrations of soluble Gp2 proteins. Titration curves indicate strong binding of soluble Gp2 variants against cell-surface InsR with low nanomolar affinity. The K_D values for Gp2 variants #1, #5 and #10 are 13 ± 14 nM, 2.4 ± 0.4 nM and 12 ± 9.0 nM, respectively (Figure 3.3). Unevolved Gp2 control does not bind InsR overexpressing cells (Figure 3.5B). Interestingly, Gp2 #1 reduced binding capacity after reaching a saturating point at very high concentration (1 μM), perhaps due to a high-dose “hook-effect” limiting antibody-based detection [196, 197] or a reversible conformational change at high concentration. Notably, other functional assays in the study were performed at nanomolar concentrations where this phenomenon is not observed. Moreover, this scenario was not observed with Gp2 #5 and #10.

Minimal binding of Gp2 variants to IGF1R

Since InsR is closely related to IGF1R, sharing 45-65% domain dependent sequence and activating almost identical downstream signaling pathways [4, 5,

17], it is important to examine whether the Gp2 variants are able to distinguish IGF1R from InsR. The tamoxifen resistant MCF-7L TamR and T47D TamR cell lines were shown to have mild to moderate increased levels of InsR expression compared to their parental cell lines, but lack IGF1R expression [148], making them a good model for this study. Cells were labeled with either anti-InsR antibody, anti-IGF1R antibody or 100 nM soluble Gp2, followed by AF647-conjugated anti-His antibody to better understand the binding capacity of these Gp2 relative to the commercially available antibodies. Gp2#5 and Gp2#10 bound greater to the TamR cells compared to their parental cells (Figure 3.6B and 3.7B), which is consistent to that of anti-InsR antibody (Figure 3.6A and 3.7A). While antibody labelling demonstrated relatively similar IGF1R and InsR expressions in both MCF-7Ls and T47Ds, Gp2#1 also showed comparable binding to the parental and TamR in both cell lines, suggesting that Gp2#1 may have some weak cross-reaction with IGF1R.

Inhibition of IGF-II/insulin-stimulated cell proliferation in breast cancer cells

TamR cells derived from estrogen receptor positive cell lines not only lack IGF1R expression but also depend on InsR signaling for cell growth compared to their parental cells [198]. To elucidate how these Gp2 variants affect biological function in these breast cancer cells, an MTT assay was used to access cell monolayer growth in MCF-7L (Figure 3.8A) and MCF-7L TamR cells (Figure 3.8B). All three Gp2 variants inhibited insulin-stimulated growth in both MCF-7L and MCF-7L TamR cells in a dose-dependent manner, whereas non-binding Gp2 control elicited no effect (Figure 3.10). All three Gp2 variants inhibited IGF-II-

stimulated monolayer growth in MCF-7L TamR cells but insignificant inhibition – even at 200 nM – was observed on IGF-I regulated growth in MCF-7L cells. In the absence of IGF1R in MCF-7L TamR cells, IGF-II signals through InsR, whereas IGF-I signals via IGF1R in MCF-7L [199]. These results suggest that the inhibitory effects of Gp2 variants are specific to InsR-regulated growth. Gp2 variants yield insignificant reduction on the basal growth in MCF-7L and MCF-7L TamR cells.

The analogous experiment was repeated with T47D (Figure 3.9A) and T47D TamR cells (Figure 3.9B). Overall, T47D TamR cells have a higher basal growth compared to T47D parental cells and yield results consistent to those of MCF-7L TamR, where the Gp2 variants completely blocked insulin-regulated cell growth. Since IGF-II-stimulated growth was not strong in these cells, the inhibitory effects of Gp2 #5 and #10 were not significant compared to the Gp2 untreated groups. In contrast, toxicity was observed in T47D TamR cells treated with 200 nM of Gp2 #1 as the growth dropped below the basal level of the comparable untreated group. The T47D parental cell line exhibited some analogous behavior to MCF-7L in that Gp2 #1 inhibited insulin-driven growth and Gp2 #5 and #10 did not significant inhibit IGF-I-driven growth. However, T47D also showed several divergent outcomes relative to MCF-7L. Gp2 #1 had a strong inhibitory effect on IGF-I-regulated growth and Gp2 # 5 and #10 were not appreciable inhibitors of insulin-driven growth. Consistent to MCF-7L's growth result, Gp2 variants blocked insulin-driven, but not IGF-I-driven, cell cycle progression in MDA-MB-231 cells and kept the cells arrested at G_0/G_1 phases

(Figure 3.11). To further examine if these variants inhibited growth by inducing cell death, cleaved PARP was measured by flow cytometry. Results showed that Gp2 variants did not significantly induce apoptosis in MCF-7L nor MDA-MB-231, suggesting that Gp2 itself is not cytotoxic (Figure 3.12).

Differential effects of Gp2 variants on insulin/IGF signaling in breast cancer cells

To better understand the underlying mechanism of Gp2-mediated growth inhibition on breast cancer cells, receptor and effector phosphorylation analysis was carried out after various treatments with growth factors and Gp2 variants in MCF-7Ls (Figure 3.13), T47Ds (Figure 3.14) and their TamR derivatives. None of the Gp2 variants exhibited receptor activation in any of the cell lines. Overall, despite inhibiting growth, Gp2 #1 did not block insulin-mediated signaling in MCF-7L and T47D parental cells, as measured by phosphorylation of InsR/IGF1R, IRS (pY-20), AKT, p70S6K and MAPK. Similarly, in TamR cells, Gp2 #1 inhibits insulin-driven growth but is predominantly passive towards the tested signaling pathways with the exception of p70S6K inhibition in T47D TamR. Conversely, inhibition of InsR/IGF1R and MAPK phosphorylation are in accord with growth inhibition in MCF-7L TamR with IGF-II treatment. IGF-II driven signaling in T47D TamR was below the detection limit.

In T47D expressing IGF1R, Gp2 #1's inhibition of IGF-I-driven growth is in accord with AKT and p70S6K inhibition. Despite not impacting growth, Gp2 #1 strongly blocked IGF-I-mediated InsR/IGF1R and MAPK activation, and weakly inhibited AKT activation in MCF-7L parental cells. Gp2 #5 and #10 inhibited insulin- and IGF-II-driven growth in both TamR cell types in accord with blocking

phosphorylation of most tested molecules and exhibited minimal inhibition of insulin-mediated MAPK activation in MCF-7L TamR. IGF-II-driven phosphorylation in T47D TamR cells was below the detection limit. In parental cells, Gp2 #5 and #10 did not impact insulin-driven signaling, which coordinated with sustained growth in T47D but countered the observed growth inhibition in MCF-7L. Consistent with growth experiments, both Gp2 #5 and #10 had little effect on IGF-I signaling in MCF-7L parental cells except that Gp2 #10 blocked receptor phosphorylation at higher concentration and both Gp2s modestly inhibited AKT. Both variants inhibited IGF-I-mediated signaling to MAPK, AKT and p70S6K activation in T47D parental cells.

As the signaling results of MCF-7L and T47D were varying, two other triple negative cell lines, MDA-MB-231 and MDA-MB-435 were tested. The Gp2 variants blocked insulin-, but not IGF-I-stimulated AKT activation in MDA-MB-435 cells. Surprisingly, an opposite result was observed in MDA-MB-231. The Gp2 variants strongly blocked IGF-I-mediated signaling, but exhibited little to no inhibition on insulin-mediated signaling (Figure 3.15). These diverse signaling outcomes in different cell lines are consistent with different abundance of IGF1R/InsR hybrid receptors measured by immunoprecipitation (Figure 3.16). When the cells express predominantly holo-InsR and minimal hybrid receptors – as in MDA-MB-435 – the inhibition of InsR signaling is straightforward. In contrast, in MCF-7L and MDA-MB-231 cells that express an abundance of hybrid receptors, Gp2 may have minimal inhibitory effects on insulin but overlapping impacts on IGF-I signaling, as IGF-I is the preferred ligand for hybrid receptors.

Therefore, in term of signal transduction, it is not surprise that InsR-evolved Gp2 variants failed to block InsR existed in a hybrid context.

Positive cooperativity of insulin on binding of Gp2 #1, but not Gp2 #5 and #10

A competitive binding assay was carried out to assess the impact of natural ligands on Gp2 binding. HEK293T cells overexpressing InsR were treated with either insulin or IGF-II for 1 hour prior to Gp2 treatments. Gp2 binding was evaluated by flow cytometry. Results indicated that insulin, but not IGF-II enhanced Gp2#1 binding (Figure 3.17). Neither insulin nor IGF-II impacted binding by Gp2 #5 and #10. These results are additional evidence that Gp2 #1 has a different mode of action than Gp2 #5 and #10.

Discussion

Gp2 is a new protein scaffold that is a promising candidate for ligand engineering as it offers a smaller size in a robust, stable structural framework with a large available binding surface amenable to mutation [194]. Directed evolution yielded three Gp2 variants directed against InsR. Notably, all three carry a pair of cysteines, located at sites adjacent to each other in the wild-type structure, suggesting a disulfide bond in the proteins (Figure 3.1B and 3C). The Gp2 variants were able to be produced in T7 express *E. coli* (Figure 3.4) and their monomers showed InsR-specific binding (Figure 3.3) and InsR inhibition in breast cancer cells (Figure 3.8 and 3.9).

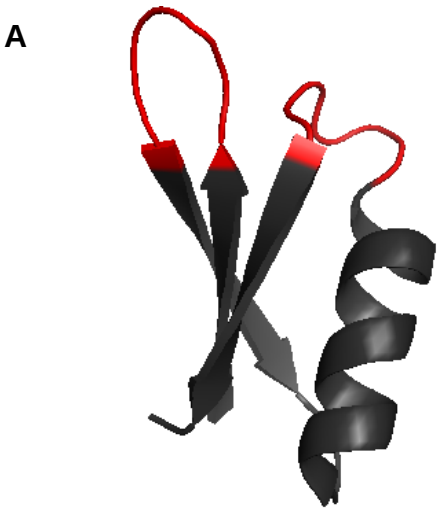
Our previous study showed that InsR inhibition alone (using either shRNA, small molecule inhibitory insulin analog or anti-InsR antibody) was only effective in blocking insulin-stimulated signaling and growth in MCF-7L TamR and T47D

TamR, but not their parental cells [198]. In this study, Gp2 variants inhibited growth in TamR cells and cell cycle progression in MDA-MB-231. The Gp2 variants were also able to efficiently block insulin-stimulated cell growth in MCF-7L parental with some variation in T47D parental cells. However, the signaling data in Figure 3.13A and 3.14A suggest that growth effects were not due to short term inhibition of InsR biochemical signaling or apoptosis induction. In parental cells, single chain of the InsR can exist as holoreceptors or InsR/IGF1R hybrid receptors. If InsR was predominantly contained in a hybrid form, then a Gp2 InsR specific binder may not inhibit the hybrid. Since the hybrid receptors have high affinity for IGF-I, but not insulin, this could explain why the Gp2 binders do not blocked IGF action. Additional studies need to be performed to determine if the Gp2 binders affect signaling in the TamR cells after long term exposure to explain the differences between the signaling and growth results we have shown here.

Gp2 #5 and #10 exhibit similar functional impact on growth, which is consistent with their almost identical sequences (an E24K framework mutation in #5 is the only difference). Both demonstrated moderate signaling inhibition and strong cell growth inhibition. Gp2 #1 appears to operate via a different mechanism of action than Gp2 #5 and #10. Unlike Gp2 #5 and #10, Gp2 #1 had three significant differences. Gp2 #1 completely blocked IGF-I and insulin-mediated growth in T47D parental cells, inhibited signaling in a different fashion and exhibited positive cooperative binding with insulin, which suggests a unique insulin/InsR binding mechanism. An insignificant reduction on the basal growth

in the MCF-7L and MCF-7L TamR cells could possibly be due to indirect effects of InsR inhibition on glucose metabolism (Figure 3.8).

InsR exists in two isoforms: isoform A and B. InsR-A, which lacks 12 amino acid via alternative splicing of exon 11 is predominantly expressed in fetal and cancer cells with high affinity to insulin and IGF-II; whereas, InsR-B is mainly expressed in metabolic organs such as adult muscle, liver and fat and binds only insulin at physiological concentration [167, 168]. In the cells we used, the predominant isoform is InsR-A [130] and further study will be needed to determine if the molecules we have described also have high affinity for InsR-B. Since Gp2 is one of the smaller protein scaffolds, it may be at advantage in the design strategy for targeting against a challenging epitope to develop isoform-specific binders.



Wild type:
KFWATVESS--EHSFEVPIYAETLDEALELAEWQYVPA--GFEVTRVRP

Naïve library:
KFWATVXXXXXXXXFEVPVYAETLDEALELAEWQYXXXXXXXXVTRVRP

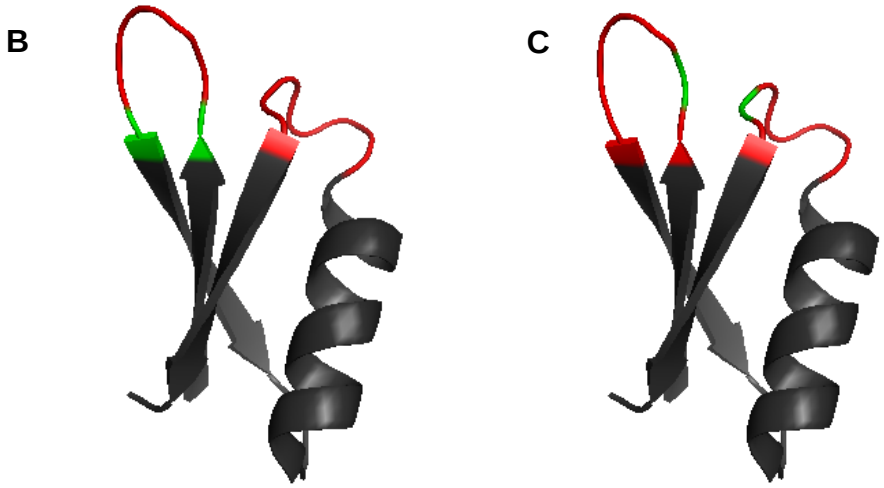


Figure 3.1. Structure of Gp2 domain, PDB: 2WNM. (A) The naïve Gp2 library was generated by diversifying the two adjacent loops highlighted in red. Segments of six, seven, or eight amino acids – randomized using a custom degenerate codon (15% A, 15% C, 25% G, 45% T at site 1; 45% A, 15% C, 25% G, 15% T at site 2; and 0% A, 45% C, 10% G, and 45% T at site 3) – were permitted. A framework mutation (I17V) is also noted. The InsR-evolved Gp2 variants – **(B)** Gp2 #1, **(C)** Gp2 #5 and #10 contain a pair of cysteines in the loops at site highlighted in green, suggesting a potential disulfide bond.

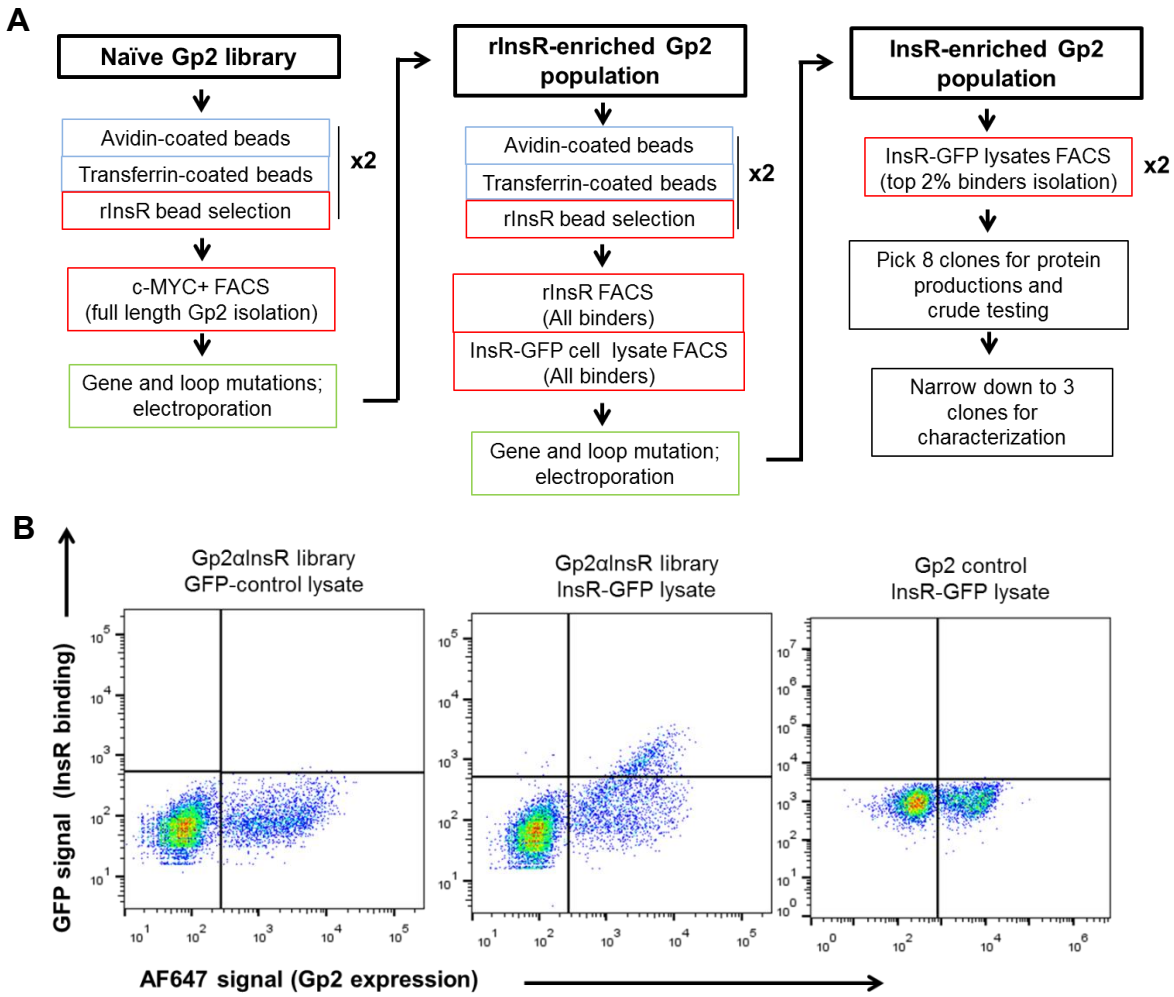


Figure 3.2. Identification of InsR-specific Gp2 through yeast surface display and directed evolution. (A) Schematic diagram of Gp2 scaffold engineering. Blue indicates negative depletion; red indicates InsR positive selection; green indicates gene and loop mutations of Gp2, followed by electroporation via homologous recombination with linearized yeast surface display pCT vector. **(B)** Yeast surface display of Gp2 libraries against mammalian cell lysates containing GFP-conjugated InsR. HEK293T cells were plated, transfected with either InsR-GFP or GFP-control plasmids and collected as whole cell lysates. Yeast library displaying evolved population (–Gp2αInsR) was pre-incubated in either InsR-GFP or GFP-control contained lysates for 1 hour before labeling for a mouse c-MYC antibody, followed by AF647-conjugated anti-mouse antibody (Gp2 expression). Binding of Gp2 to InsR-GFP was detected and isolated by flow cytometry. Gp2 against rabbit IgG was used as a Gp2-control and was tested against InsR-GFP contained lysates under similar conditions.

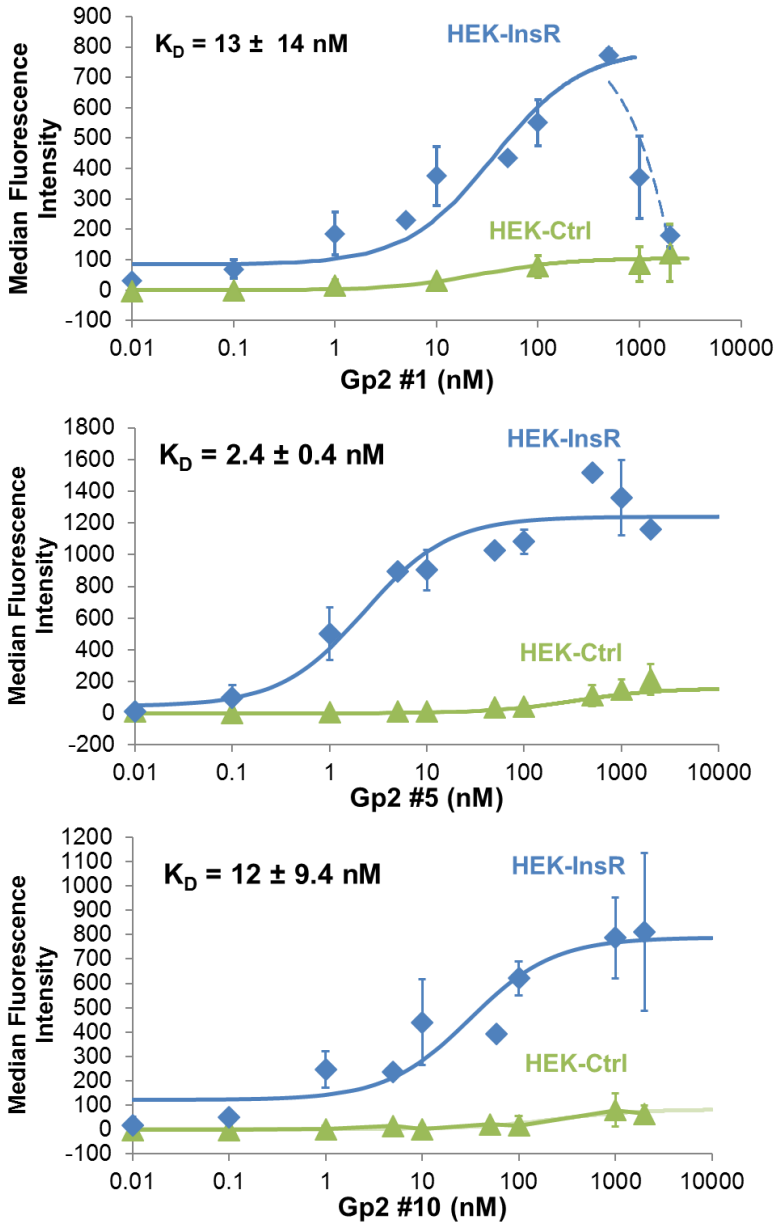
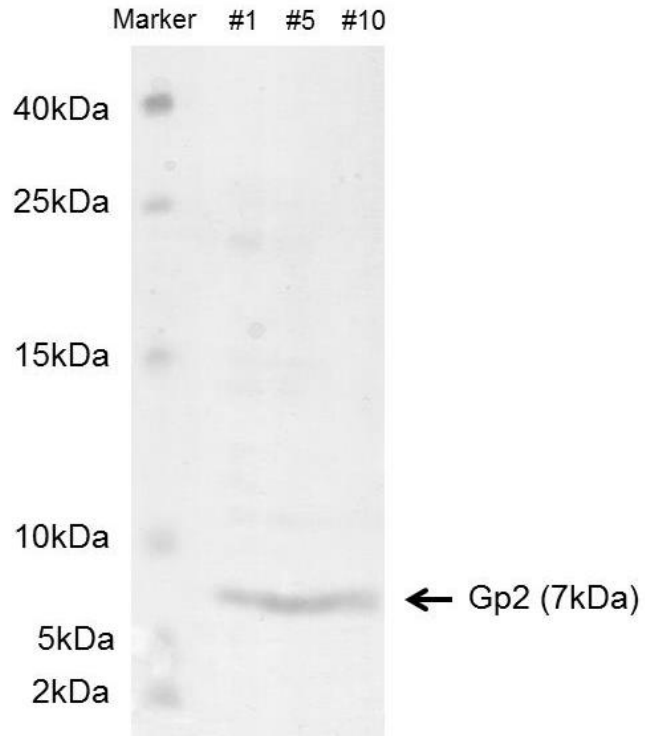


Figure 3.3. Affinity titration of Gp2 variants. HEK293T lentivirus transduced with either pLenti-InsR-GFP or pLent-GFP-ctrl were labeled with increasing concentrations of indicated soluble Gp2. Binding was detected by AF647-conjugated anti-His antibody via flow cytometry. Fluorescence signal was subtracted from the basal signal. K_D values represented median ± standard deviation of three independent experiments. Some data points were not repeated and thus no standard deviation indicated. Note that HEK-Ctrl cells express modest levels of InsR (Supplementary Figure 2A), which accounts for the non-zero signal at higher concentrations.

A



B

Gp2	Protein sequences
WT	ASKFWATVESSEHSFEVPIYAETLDEALELAEWQYVPAGFEVTRVRPGSHHHHHH
#1	ASKFWATVCSGHDGYCFEVPVYAETLDEALELAEWQYDSTYYDYAVTRVRPGSHHHHHH
#5	ASKFWATVDCLYNDTAFEVPVYAETLDKALELAEWQYDPNYCIVTRVRPGSHHHHHH
#10	ASKFWATVDCLYNDTAFEVPVYAETLDEALELAEWQYDPNYCIVTRVRPGSHHHHHH

Blue: framework mutation, Red: loop mutation, underline: Gp2 loops

Figure 3.4. Verification of Gp2 variants by size and sequencing (A) Coomassie blue staining of soluble Gp2 clones. Identified Gp2 variants were purified using Ni-NTA resin and size exclusion filter, separated by 15% SDS-PAGE. The gel was stained with Coomassie blue. **(B)** Protein sequences of Gp2 variants. **Red** indicated as loop mutation; **blue** indicated as framework mutation relative to initial library.

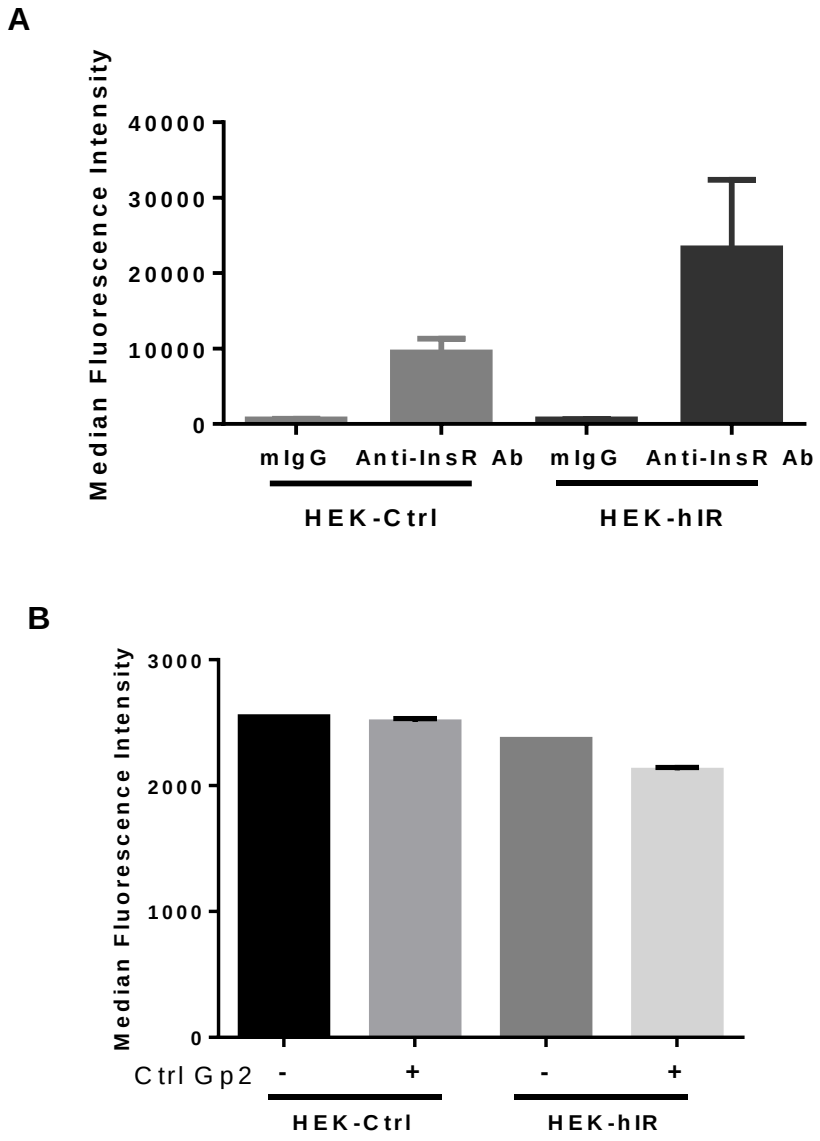


Figure 3.5. Minimal binding of Gp2 control to InsR overexpressed HEK293T cells. (A) InsR expression levels in the indicated cells were evaluated with antibody via flow cytometry. Cells were labeled with a mouse anti-InsR antibody (clone 83-7), followed by a secondary anti-mouse antibody conjugated with AF647. Results represent median ± standard deviation of three independent experiments. **(B)** Cells were labeled with 0 µM (-) or 1 µM (+) non-binding control Gp2, followed by a secondary fluorescein-conjugated anti-His antibody and detected via flow cytometry. Results represented median ± standard deviation, n=3 for Gp2-Ctrl labeled cells, n=1 for fluorescein-ctrl labeled cells.

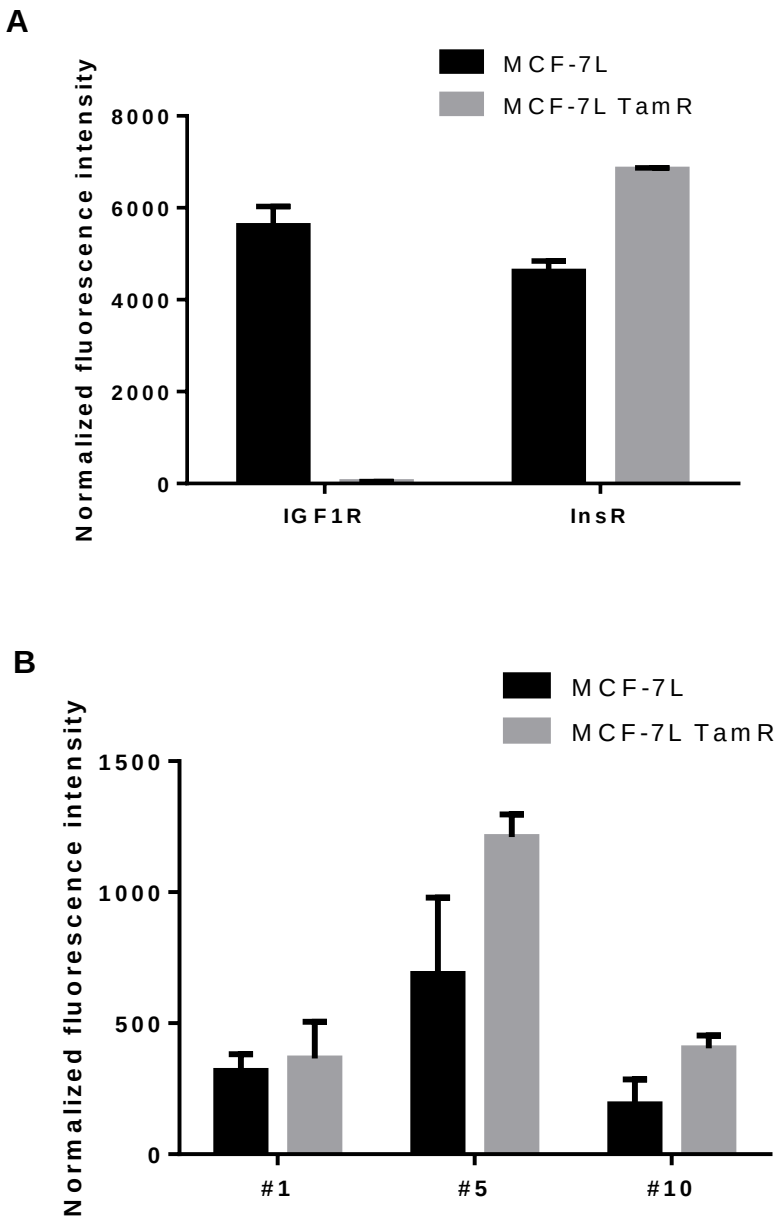


Figure 3.6. Minimal binding of InsR-evolved Gp2 variants to IGF1R in MCF-7Ls. (A) Cells were labeled with a mouse PE-conjugated anti-IGF1R antibody or a mouse anti-InsR antibody followed by an AF647-conjugated anti-mouse antibody for an hour each at 4°C. **(B)** Cells were labeled with 100 nM of indicated Gp2 variants and AF647-conjugated anti-His antibody for an hour each at 4°C. Bindings were measured via flow cytometry. Values were normalized to their respected controls and represented as geometry mean ± standard deviation in duplicates.

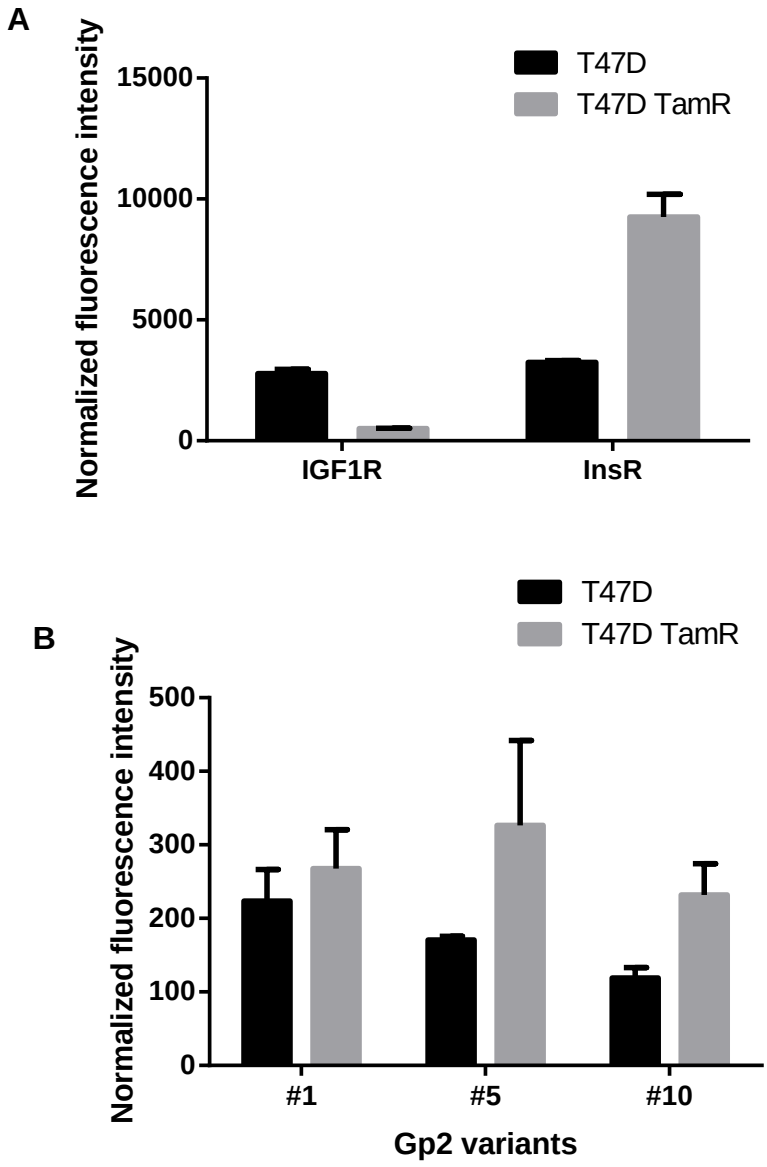


Figure 3.7. Minimal binding of InsR-evolved Gp2 variants to IGF1R in T47Ds. (A) Cells were labeled with a mouse PE-conjugated anti-IGF1R antibody or a mouse anti-InsR antibody followed by an AF647-conjugated anti-mouse antibody for an hour each at 4°C. **(B)** Cells were labeled with 100 nM of indicated Gp2 variants and AF647-conjugated anti-His antibody for an hour each at 4°C. Bindings were measured via flow cytometry. Values were normalized to their respected controls and represented as geometry mean ± standard deviation in duplicates.

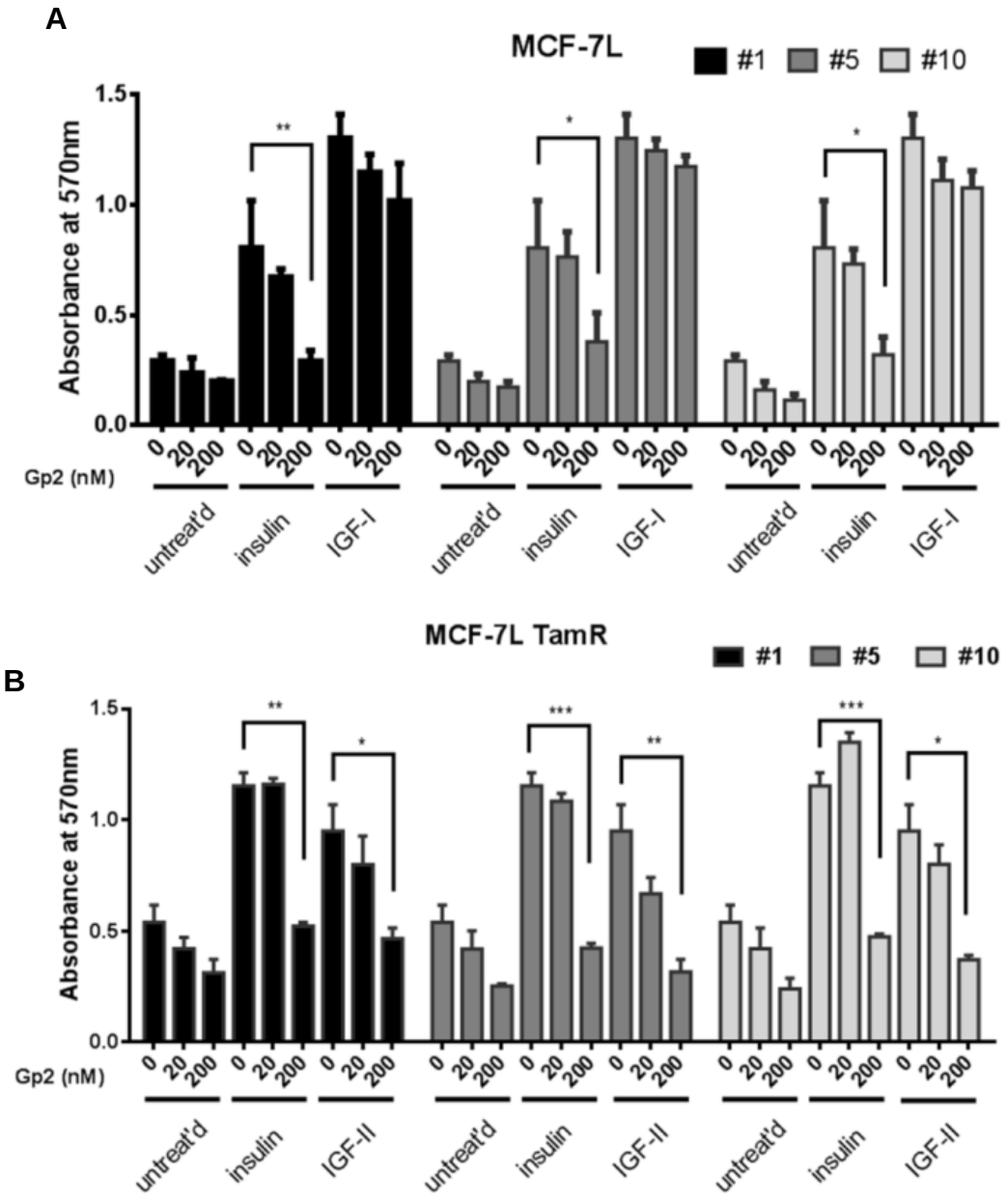


Figure 3.8. Inhibition of IGF-II/insulin-stimulated monolayer proliferation in MCF-7Ls by Gp2 variants. Cell monolayer growth of **(A)** MCF-7L and **(B)** MCF-7L TamR were measured using MTT proliferation assay. Cells were plated, starved for 24 hours and then treated with indicated concentrations of soluble Gp2 in combination with either 10 nM insulin, 10 nM IGF-1 or 10 nM IGF-II. Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated versus treated groups. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

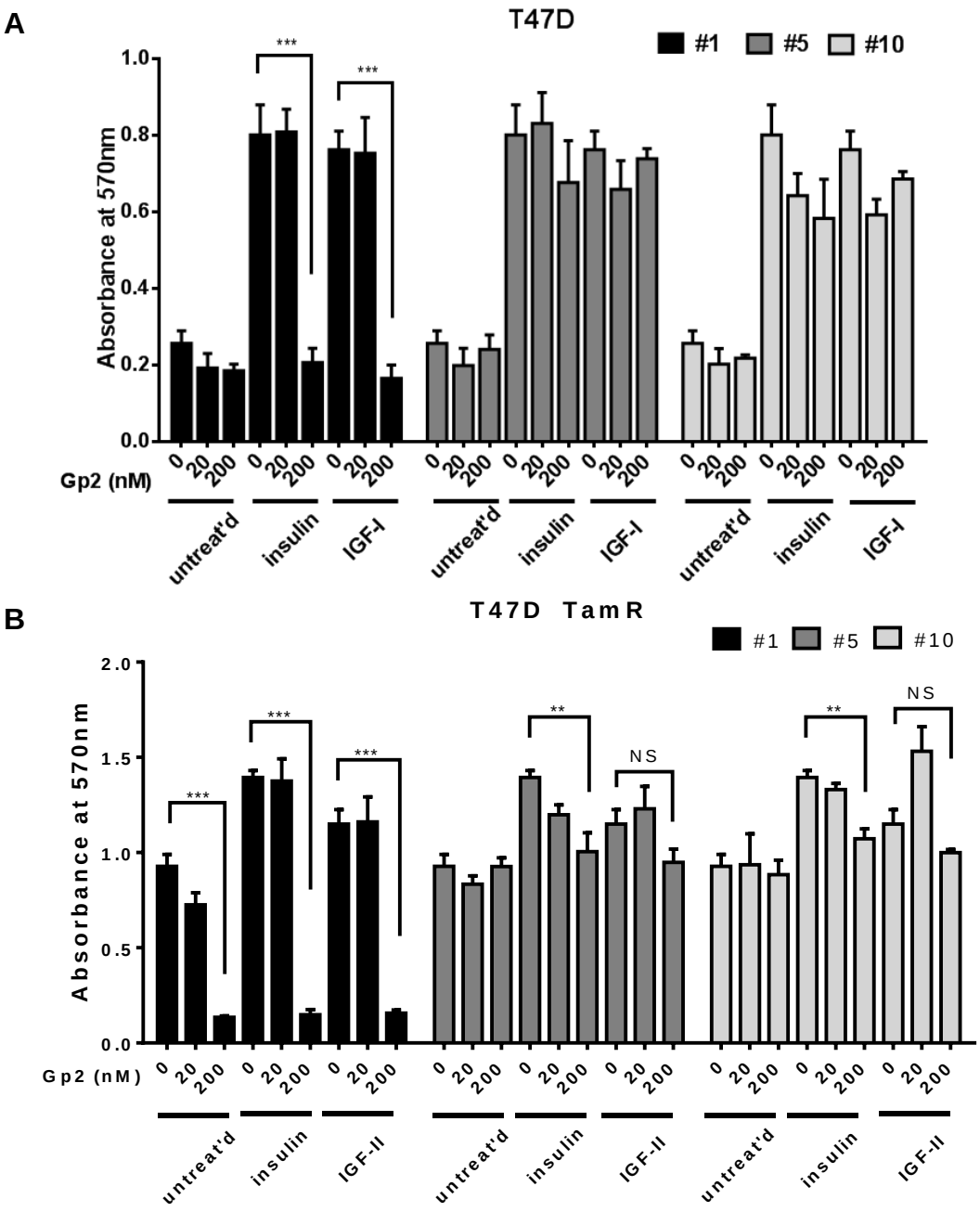


Figure 3.9. Differential effects of Gp2 variants on monolayer proliferation of T47Ds. Cell monolayer growth of **(A)** T47D and **(B)** T47D TamR. Cells were serum-starved for 24 hours and treated with indicated concentration of soluble Gp2 in combination with either 10 nM insulin, 10 nM IGF-I or 10 nM IGF-II. Readings were taken 4 days later and the values were represented as mean ± standard deviation in triplicates. Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated versus treated groups. **, $p<0.01$; ***, $p<0.001$.

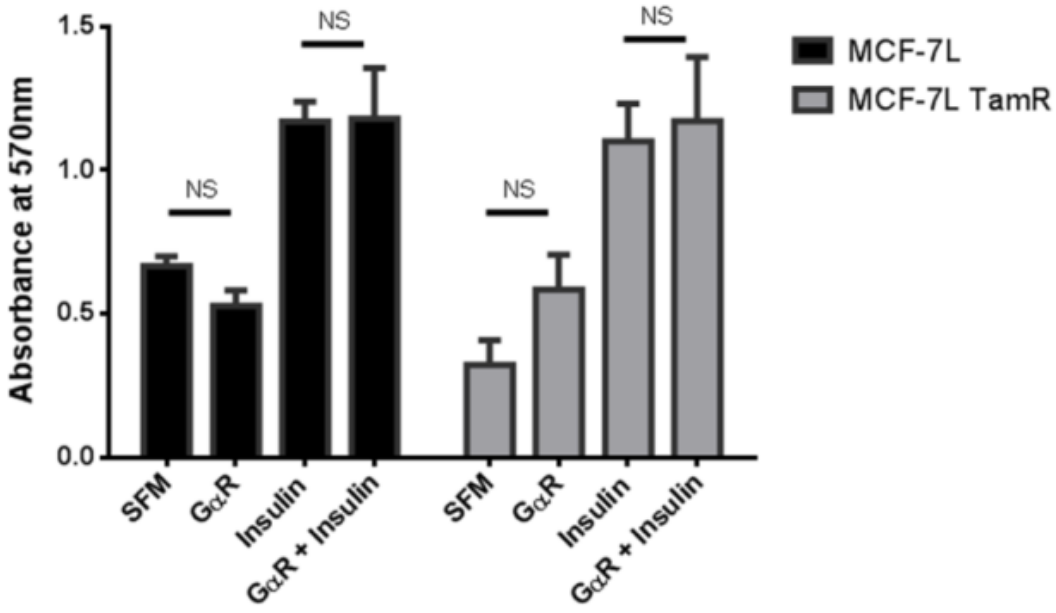


Figure 3.10. Minimal effects of Gp2 control on monolayer proliferation growth. Gp2 against rabbit IgG was used as a Gp2 control in affecting cell growth in MCF-7L and MCF-7L TamR. Readings were taken 5 days later and the values were represented as mean ± standard deviation in triplicates. Two-way ANOVA with Bonferroni comparison was performed to identify significance among untreated versus treated groups. NS, not significant;

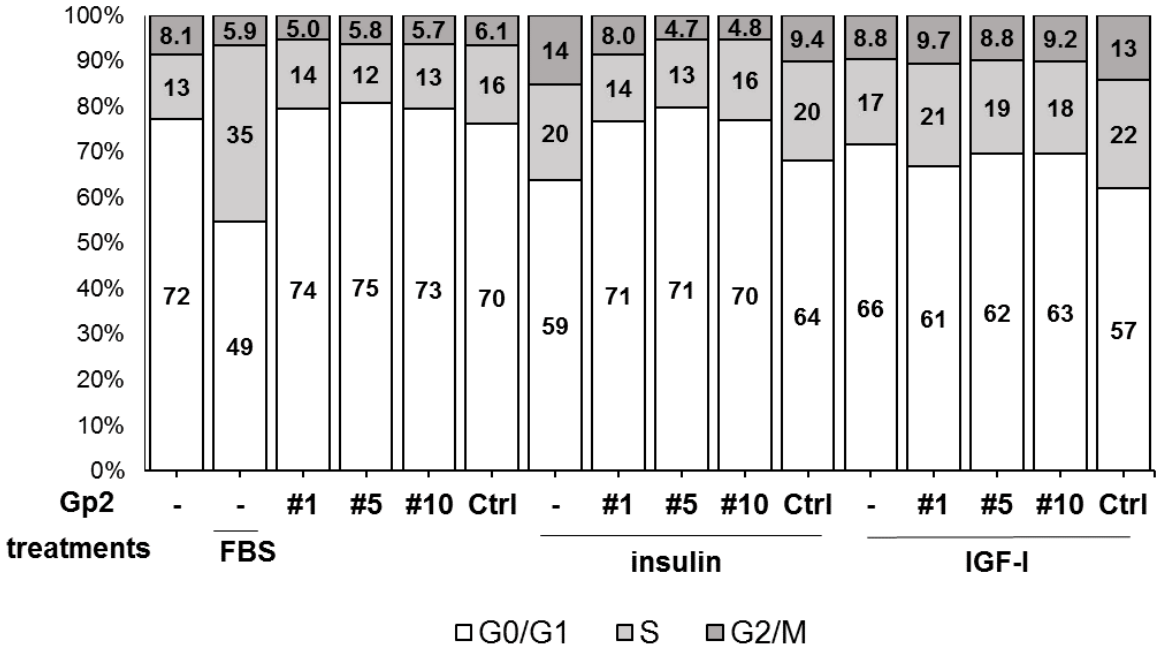


Figure 3.11. Inhibitory effects of Gp2 variants on the distribution of cell cycle phases in MDA-MB-231.

Cells were serum-starved for 24 hours and treated with either 200 nM of indicated soluble Gp2 alone or in combination with either 20 nM insulin or 10 nM IGF-I overnight. Cell cycle analysis was performed using flow cytometry. Two independent experiments were carried out and both showed consistent outcomes. Representative data is presented.

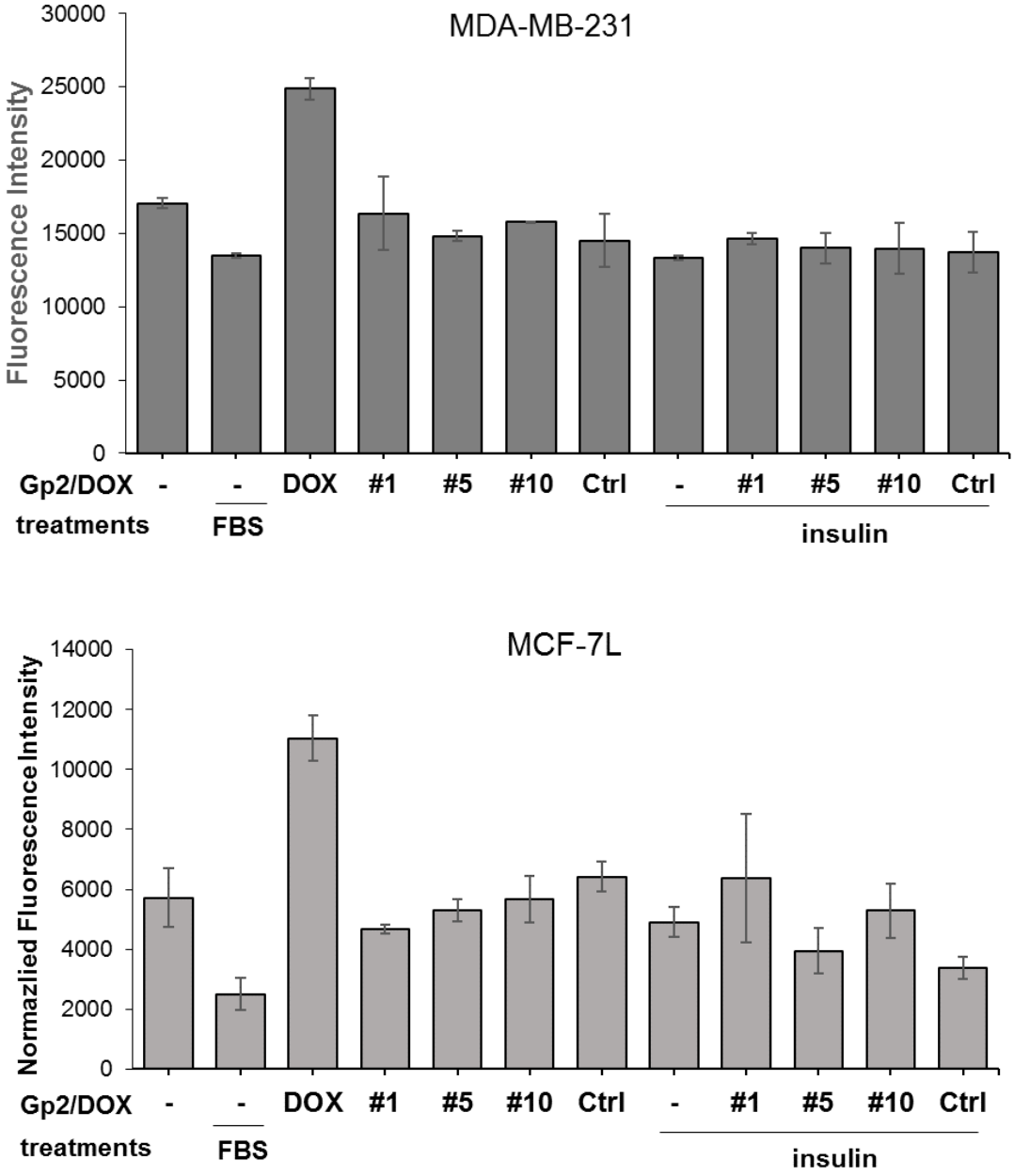


Figure 3.12. Minimal apoptosis effects of Gp2 variants in breast cancer cells. Cells were serum-starved overnight and treated with either 200 nM of indicated soluble Gp2 alone, in combination with 20 nM insulin or doxurubicin (DOX) as a positive control for 2 days. Cleaved PARP was measured using flow cytometry and the values represented as geographic mean ± standard deviation of duplicates from two independent experiments.

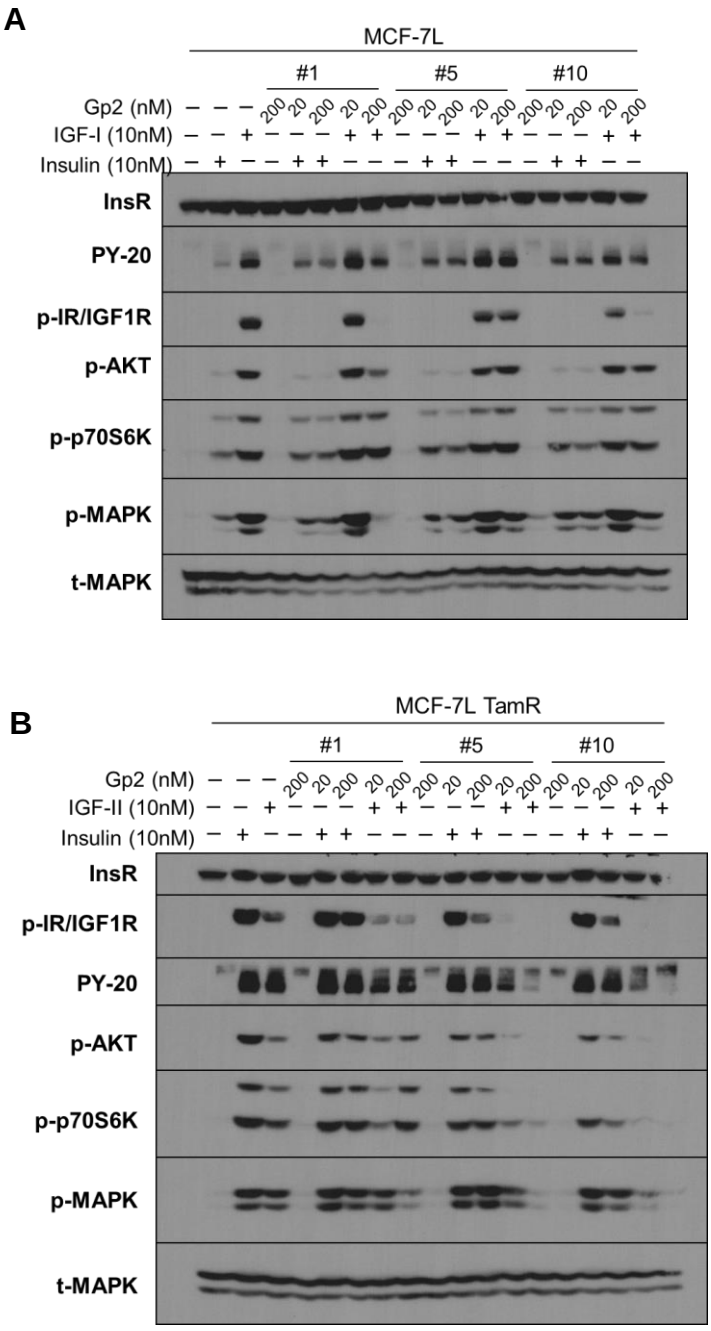


Figure 3.13. Differential signaling effects of Gp2 variants on insulin/IGF signaling in MCF-7L. (A) MCF-7L and **(B) MCF-7L TamR** cells were plated, serum-starved overnight, pre-treated with indicated concentrations of Gp2 overnight before treating with either 10 nM IGF-I, 10 nM IGF-II or 10 nM insulin for 15 minutes. Whole cell lysates were separated by SDS-PAGE and immunoblotted with indicated antibodies.

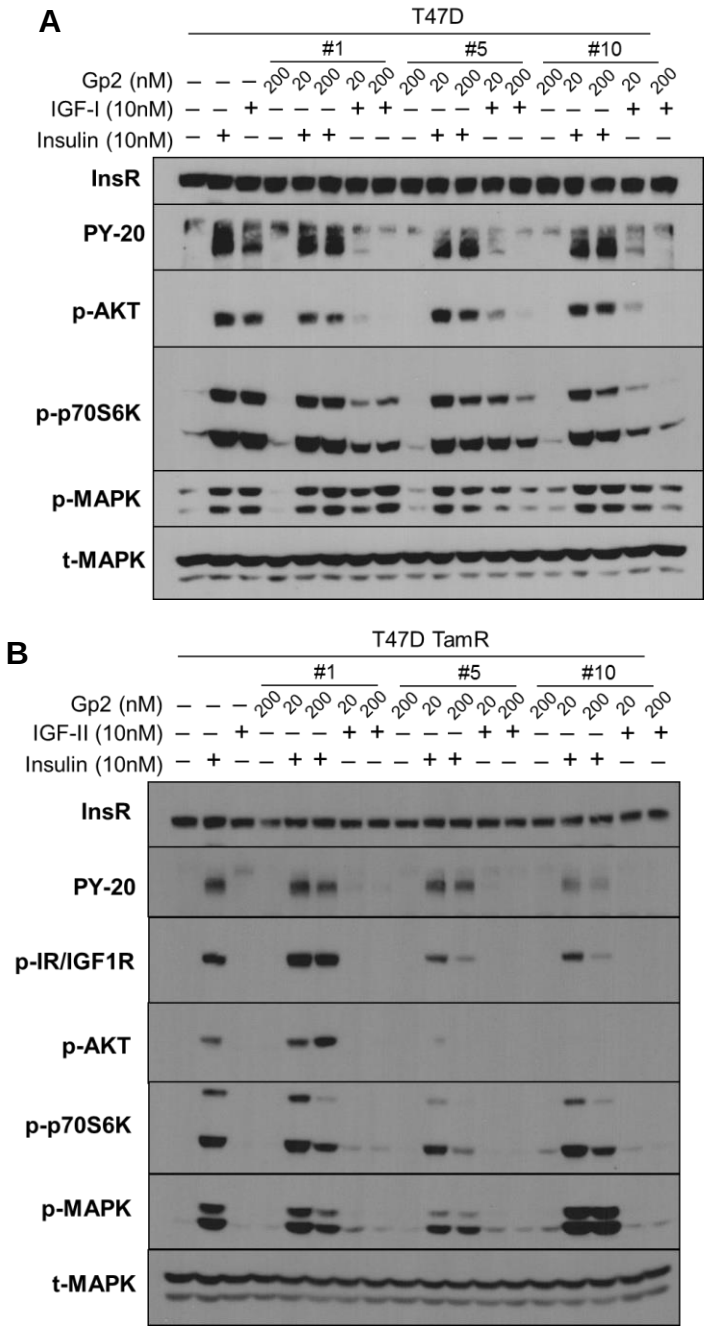


Figure 3.14. Differential signaling effects of Gp2 variants on insulin/IGF signaling in T47Ds.

(A) T47D and **(B)** T47D TamR cells were plated, serum-starved overnight, pre-treated with indicated concentrations of Gp2 for 4 hours before treating with either 10 nM IGF-I, 10 nM IGF-II or 10 nM insulin for 15 minutes. Whole cell lysates were separated by SDS-PAGE and immunoblotted with indicated antibodies.

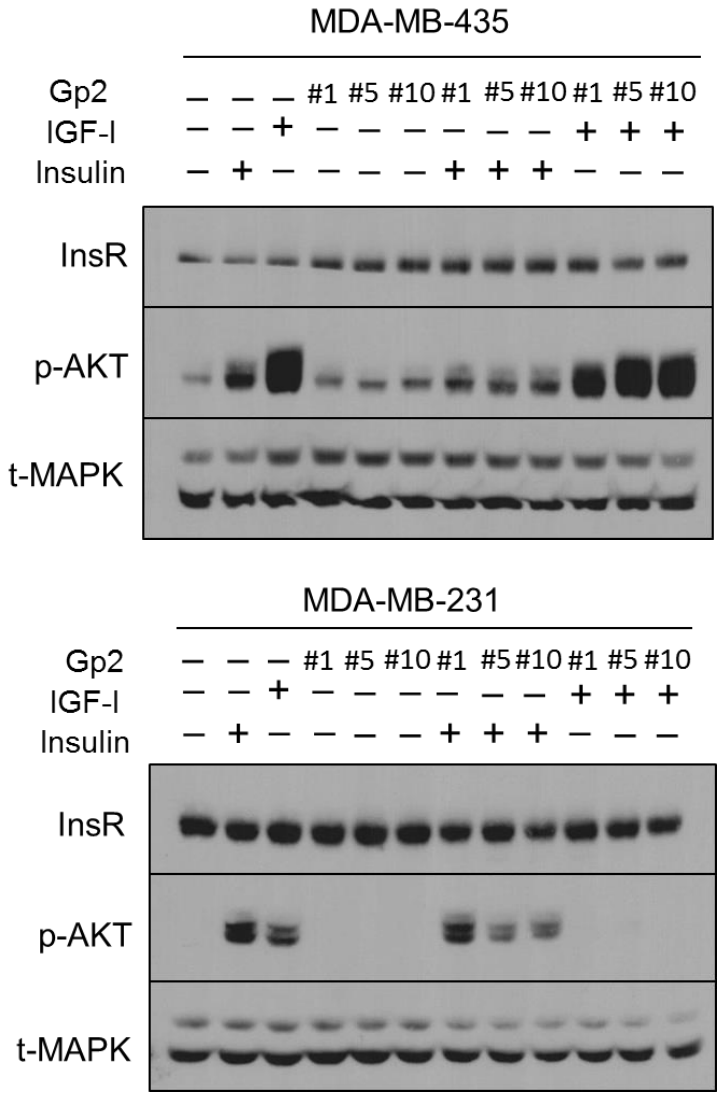


Figure 3.15. Differential signaling effects of Gp2 variants on triple negative breast cancer cells.

MDA-MB-435 and MDA-MB-231 cells were plated, serum-starved and pre-treated with 200 nM of indicated soluble indicated Gp2 overnight before treating with either 10 nM insulin or 5 nM IGF-I for 15 minutes. Whole cell lysates were collected, separated by 8% SDS-PAGE and blotted for indicated antibodies.

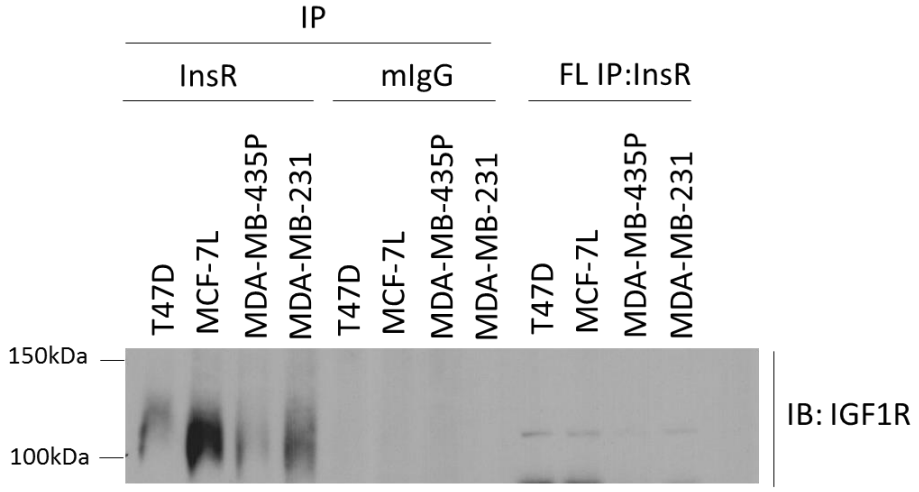


Figure 3.16. The abundance of IGF1R/InsR hybrid receptors attributed to the differential signaling effects on Gp2 variants in different breast cancer cell lines.

Whole cell lysates of indicated cell lines were collected and immunoprecipitated with either a mouse anti-InsR antibody or a control mouse IgG overnight. Immunoprecipitation were resolved using SDS-PAGE and subjected for IGF1R immunoblotting. FL stands for “flow through”, representing the supernatant of anti-InsR immunoprecipitation.

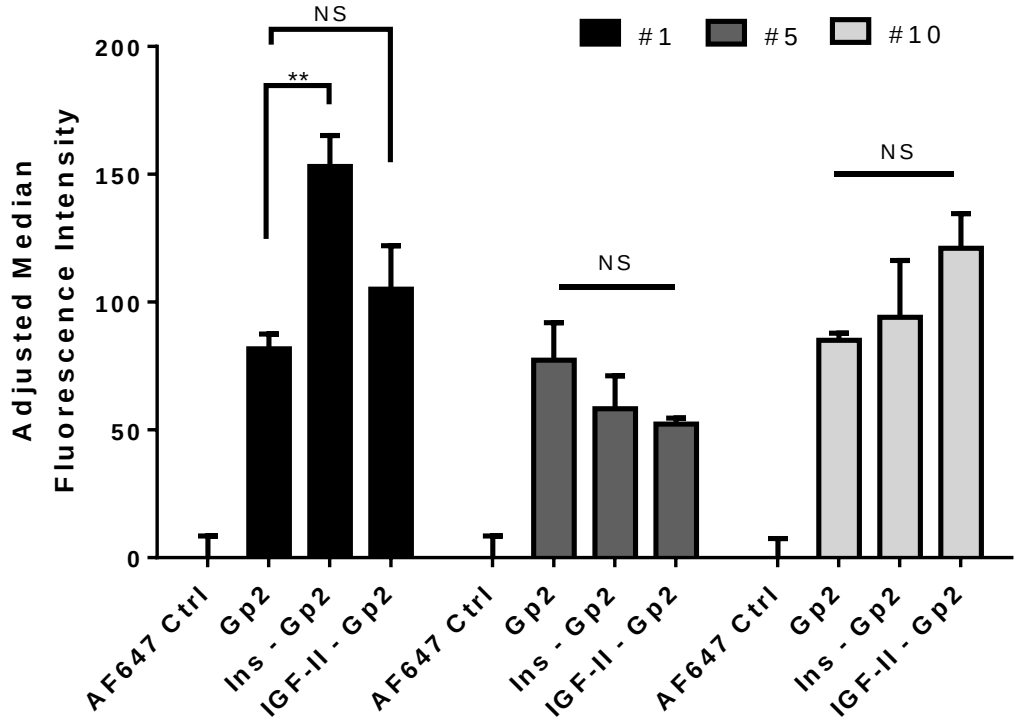


Figure 3.17. Non-competitive binding of Gp2 variants against InsR.
HEK293T pLenti-InsR-GFP cells were trypsinized and pre-incubated with either 1 µM of insulin or IGF-II for 1 hour before labeling with 100 nM Gp2 variants, followed by AF647-conjugated anti-His antibody. Binding was measured using flow cytometry. Results represented adjusted median ± standard deviation in triplicates, where the basal values were subtracted from all samples. Paired two-tailed t tests were used to compare Gp2-labeled versus ligands-Gp2-labeled samples. NS, not significant; **, $p<0.01$.

Chapter 4

Concluding remarks and future studies

Conclusion

In summary, the generation of endocrine resistant models by a former colleague in the lab, Dedra Fagan, Ph.D. provided evidence for the inefficacy of IGF1R blocking agents in treating endocrine resistant breast cancer patients due to the lack of IGF1R expression. The highly homologous receptor, InsR which has been neglected as a potential target remains intact and can fully compensate for IGF1R signaling. Data from the first part of the work highlighted the role of InsR in breast cancer biology, especially in the context of resistance to endocrine therapies in breast cancer. Several InsR-selective agents, from a short hairpin RNA, small peptide, antibody to engineered small scaffolds have consistently shown effective blockage of endocrine resistant breast cancer growth. InsR inhibition may potentially be beneficial in suppressing triple negative breast cancer, especially in cancers expressing IGF1R/InsR receptors. As mentioned, hyperinsulinemia resulted from anti-IGF1R blockage may promote insulin-driven growth and thereby partially contributed to the disappointing clinical results testing anti-IGF1R monoclonal antibodies.

The second part of the work demonstrated small InsR-evolved Gp2 variants not only exhibited strong binding affinity to InsR, but also promising biological outcomes in breast cancer cells. The data also indicated some interesting characteristics of Gp2#1 that worth pursuing in the future, including the possibility of binding a second receptor other than InsR and also the capability to bind the activated Insulin/InsR complex. This observation is, however, not noticeable with Gp2#5 and #10, suggesting that Gp2#1 might have

some unique functions, while Gp2#5 and #10 might work similarly to other InsR-selective inhibitors.

Overall, targeting InsR is needed to completely disrupt the IGF1R/InsR network, which is especially evident in endocrine resistant breast cancers. Whether InsR inhibition could overcome primary (*de novo*) endocrine resistance or treat secondary (acquired) resistance, it is not certain. The continued function of InsR may explain why targeting IGF1R was unsuccessful in phase III clinical trials. InsR inhibition is slowly being recognized as an important pathway to inhibit as cumulative studies, including those from our group, have emphasized the expression of this receptor in breast cancer. The emergence of precision medicine also increasingly recognizes the development of new cancer interventions that may one day improve clinical outcomes.

Future directions

While this thesis mainly focuses on the generation of InsR-binding scaffolds and *in vitro* studies, many opportunities are awaiting to be discussed and pursued. This section presents some of the directions for future investigations:

<u>Choices of combinatorial treatments</u>

Studies from my first part of work suggested the use of HuEM164 and 83-7 mAbs did not show synergistic inhibitory effects. Non-specific agonism of 83-7 mAb was also observed in endocrine-sensitive breast cancer cells. This observation needs to be verified in the TNBC lines. It is also unsure whether 83-7 mAb interferes with HuEM164 efficacy or its non-specific agonism is sufficient to

overcome HuEM164 inhibition. As an alternative, Gp2#5 or Gp2#10 could be used in combination with anti-IGF1R mAb. Single agent inhibition of Gp2 variants has only been tested so far and their combination efficacy with anti-IGF1R remains elusive.

Epitope mapping

The binding capability of these Gp2 variants to InsR has been demonstrated, however their sole specificity has yet to be determined. Current data suggested that Gp2#5 and Gp2#10 may be specific against InsR, while Gp2#1 may have weak affinity to IGF1R. This speculation can be confirmed in $InsR^{null}$ cells with either $IGF1R^{high}$ or $IGF1R^{low}$ cell lines. In addition, the epitope of these Gp2 variants are unknown. Gp2#5 and Gp2#10 do not seem to share the same epitopes as the natural ligands, suggesting allosteric inhibitory actions of Gp2#5 and #10. Competitive binding assay also showed an unexpected binding phenomenon of Gp2#1 with the possibilities to bind either receptor-ligand complex or the activated conformation of InsR. Thus, epitope mapping will shed light on some of these questions. Some experiments can be done including competitive binding assay among these Gp2 variants in the presence or absence of natural ligands (mainly to determine whether activated conformation of InsR is required for Gp2#1 binding). Crystal structures of these Gp2/InsR can certainly identify the exact epitopes and other physical properties. Theoretically, mammalian cell surface panning could potentially be explored, where a library of mammalian cells displaying InsR with few mutations on every 10-amino-acid segment on the ectodomain of the receptors can be used for binding screening.

The loss of binding function due to the mutations at certain regions could indicate potential epitopes of the binders.

Mechanism of action

The differential signaling outcomes from these Gp2 variants in different breast cancer cell lines could due to two main reasons: 1.) the differential mechanistic action of these Gp2 variants and 2.) abundance and stoichiometry of holoreceptors versus hybrid receptors in different cell lines. Although some InsR downstream signaling studies have been conducted, the story is not fully understood. Do these Gp2 involve additional pathways other than MAPK and PI3K pathways? How do they affect insulin/InsR gene signature and downstream signaling cascades?

In vivo studies

Few studies have shown InsR inhibition in animal models. Rats treated with S961 has shown hyperinsulinemia, hyperglycemia and insulin-mediated glucose intolerance, however these side effects were restored by an anti-diabetic drug, peroxisome-proliferator-activated receptor gamma (PPARγ) agonist – pioglitazone [200]. While the IGF1R/InsR tyrosine kinase inhibitor has been evaluated in a mouse model of type 2 diabetes, this drug clearly made glucose control worse [129]. If InsR inhibitors are to be translated into the clinic, some of the questions including dose, schedule, potential systemic and metabolic toxicities need to be answered in an *in vivo* setting. Since metabolic toxicity is the main concern, the combination use of metformin may address hyperglycemia condition resulted from InsR inhibition. Perhaps, an intermittent treatment

between metformin and InsR inhibitor may be a better dosing approach to prevent uncontrolled hyperglycemia.

Protein engineering against InsR-A or hybrid receptor

In the long run, it would be valuable to target the mitogenic form of InsR – InsR-A, leaving the metabolic isoform B InsR unperturbed. No InsR isoform specific antibody or binding agent has been reported. It is certainly challenging from a protein engineering perspective as InsR-A lacks only 21 amino acids at a less prominent region. Nevertheless, it is feasible if we have the representative target system and reliable/stable protein library. These Gp2 variants may be a useful platform for the evolution of isoform-specific binders. Mammalian cell lines expressing either InsR-A or InsR-B conjugated to respective fluorescent tags need to be generated to serve as a necessary reagent to begin screening. Biotinylated recombinant receptors may be used initially to select binders from a library, however this approach has a chance of losing potential translatable binders. Once the cell lines are generated, the screening/panning process using yeast surface display will need to be less stringent at the beginning until we are confident with the enriched population of the binders. Similarly, there are no efficient methods to quantitatively or qualitatively measure the level of IGF1R/InsR hybrid receptors. Thus, it will certainly be a motivation in the future to engineer and identify a molecular binder against IGF1R/InsR hybrid receptor.

Materials and Methods

Cell lines and culture

MCF-7L and T47D are human ER-positive breast cancer cell lines. MCF-7L (parental cell line) 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 cell line (data not shown). T47D (parental cell line) was obtained from ATCC and maintained in MEM supplemented with 5% FBS, 1X nonessential amino acids and 6 ng/mL insulin. MCF-7L TamR and T47D TamR cells were generated as described [148]. MCF-7L TamR cells 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; while T47D TamR cells were maintained in phenol-red free IMEM supplemented with 6 ng/mL insulin, 1X nonessential amino acids, 5% charcoal/dextran-treated FBS and 100 nM 4-OH tamoxifen. MDA-MB-231, MDA-MB-435 and HEK293T cells were obtained from ATCC and maintained in HyClone™ Dulbecco's Modified Eagle Medium (GE Healthcare Life Science) supplemented with 10% FBS. 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®.

Reagents

IGF-I, IGF-II and insulin were purchased from Gemini and Eli Lily, respectively. Puromycin solution was purchased from Sigma-Aldrich. Geneticin

(G418 sulfate) solution was purchased from Life Technologies. Humanized anti-IGF1R monoclonal antibody HuEM164 was generously provided by Immunogen Inc. Anti-InsR monoclonal antibody, alpha subunit clone 83-7 was purchased from EMD Millipore. S961 peptide was generously provided by Novo Nordisk, Denmark [158].

Antibodies

Antibodies for total IGF1R (#3027), phosphorylated IGF1R tyrosine 1135 (#3918), phosphorylated AKT serine 473 (#9271) and threonine 308 (#9275), total p44/42 MAPK (#9102), phosphorylated p44/42 MAPK (#4376), phosphorylated p70 S6 kinase (#9205) used in immunoblotting were purchased from Cell Signaling Technology. Normal mouse IgG (sc-2025), InsR antibody for immunoprecipitation (sc-57342) and InsR antibody for immunoblotting (sc-711) were purchased from Santa Cruz Biotechnology. Horseradish peroxidase-conjugated anti-phosphotyrosine (pY-20) was purchased from BD Transduction Lab. (#610012). Anti-rabbit horseradish peroxidase-conjugated secondary antibody was purchased from Pierce.

Generation of stable InsR knockdown with shRNA

Lentiviral pLKO.1 vectors encoding either InsR mRNA specific short hairpin RNA (shRNA) sequences or vector controls were purchased from Open Biosystems through BioMedical Genomics Center at the University of Minnesota, Minneapolis, MN. Two different constructs of shRNA against InsR are described as shIR#2 and shIR#6. Their full sequences are respectively CCGGCACTGATTACTTGCTGCTCTTCTCGAGAAGAG

CAGCAAGTAATCAGTGTTTTT and

CCGGCTCAGGATTCTCACGACTCTACTCGAGT

AGAGTCGTGAGAATCCTGAGTTTTT. Lentivirus production was carried out in HEK293T packaging cells and the viral particles were used to transduce MCF-7L and T47D cells. Final concentration of 10 µg/mL polybrene was used to increase transduction efficiency. Cells underwent puromycin selection and were maintained in 2 µg/mL for MCF-7L and MCF-7L TamR; 1.5 µg/mL for T47D parental cells and 15 µg/mL for T47D TamR.

Overexpression of insulin receptor

pEGFP-N2 vector containing full length human *InsR* cDNA was obtained from Addgene (#22286) [201] and transiently transfected into MCF-7L and MCF-7L TamR using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol. Cells were selected and maintained in 500 µg/mL G418 for a week before re-plating for immunoblotting analysis or monolayer growth assay.

Immunoblotting analysis

Cells were plated at a density of 3 x 10^5 cells in 60 mm diameter dishes and allow to equilibrate overnight. Full medium was replaced with serum-free medium (SFM) for 24 hours. Cells were then treated, washed twice with ice-cold PBS and lysed with lysis 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 with complete proteases inhibitor cocktail (Roche Diagnostics). Lysates were centrifuged at 12,000 g for 30 minutes at 4°C. Protein concentrations were measured using 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 according to manufacturer guidelines. For Immunoprecipitation (IP), whole-cell lysates were incubated with either anti-InsR antibody or mIgG overnight at 4°C. Protein A/G PLUS-Agarose bead slurry was added into the samples and incubated for 4 hours at 4°C. Beads were washed with lysis buffer 5 times and boiled in 5X Laemmli loading buffer. Samples were resolved by 8% SDS-PAGE, transferred to PVDF membrane and immunoblotted.

Reverse transcription and quantitative real-time PCR

Cells were seeded at a density of 2 x 10^5 cells in 6-well plates in growth media until reaching 80% confluent. Cellular RNA was isolated using TriPure Reagent according to the manufacturer (Roche). For RNA quality verification, a ratio of 260 nm to 280 nm was determined. A total of 1 µg of RNA was reverse transcribed using qScript cDNA synthesis kit (Quanta Biosciences), and quantitative PCR was performed using the University SYBR Green Kit according to the manufacturer's protocol (Roche) on an Eppendorf Mastercycler Realplex machine. The relative abundance of *InsR* mRNA was calculated using cycle threshold values that were derived from a standard curve and normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) level as an internal control. The forward and reverse primers are as followed: InsR 5'-CAACGTGGTTTTCGTCCCC-3' and 5'-AGATGACCAGCGACTCCTTG-3'; GAPDH 5'-TGAAGGTCGGAGTCAACGATTTGGT-3' and 5'-GAAGATGGTGATGGGATTTC-3'

Monolayer growth assay

Cells were plated at a density of 15,000 cells per well in 24-well plates and allowed to attach overnight. Full media were replaced and starved with SFM for 24 hours. After 5 or 6 days of treatment, growth was assessed via MTT assay. Each well was added 60 µL of 5 mg/mL thiazolyl blue tetrazolium bromide solution (MTT) from Sigma-Aldrich in SFM for 4 hours at 37°C in dark. Media were aspirated and purple formazan crystals were lyzed with 500 µL of solubilization solution (95% dimethylsulfoxide and 5% Improved MEM). Absorbance was measured with a plate reader at 570 nm using a 650nm differential filter to access growth.

Anchorage-independent growth assay (soft agar)

A 1-mL layer of 0.8% SeaPlaque-agarose (bioWhittaker) in 1% FBS-containing growth media was solidified into each well of a 6-well plate. The bottom layer was overlaid with 1mL of 0.5% top agar mixture for 15,000 cells per well with appropriate treatment. All plates were incubated at 37°C. The number of colony formation was assessed on a light microscope with an ocular grid. Only colonies exceeding two thirds of grid square were scored. Five random fields were counted per well.

Cell cycle analysis

Cells were plated at density of 8×10^5 cells in 60 mm dishes, starved with SFM for 24 hours and treated overnight. Cells were trypsinized, washed twice with ice-cold PBS containing 1% bovine serum albumin (BSA) and re-suspended with staining buffer (PBS containing 0.1 mg/mL propidium iodide, 0.5% triton X-

100, 16 µg/mL RNase, 1% BSA) for 2 hours. Cell cycle analyses were performed for DNA content using BD Accuri C6 flow cytometry. Single cells were gated and 10,000 events were collected. The proportion of cells in G_0G_1, S and G_2/M phases was quantified using FlowJo software.

Binder Selection and Directed Evolution

The naïve Gp2 library contains $4x10^8$ unique variants in which the sequences of two adjacent loops are diversified to six to eight amino acids with composition biased to mimic natural antibody repertoires (17% tyrosine, 13% serine, 11% aspartic acid, 9% asparagine, 6% alanine and histidine, 5% cysteine and threonine, 4% glycine and proline, 3% phenylalanine, arginine, valine,2% leucine, isoleucine, glutamic acid, lysine, 1% glutamine, tryptophan and <1% methionine) [194]. Binder selection and directed evolution were generally adopted from [194, 202]. Recombinant insulin receptor ectodomain (rInsR) (R&D Systems, Minneapolis MN), which served as a target antigen for screening, was biotinylated with EZ-link® NHS-PEG_4-Biotin (Thermo Scientific, Rockford IL) resulting in nine biotin moieties per receptor. Yeast displaying the naïve Gp2 library underwent magnetic sorting, which consisted of two negative depletions (first with avidin-coated beads, then beads with immobilized biotinylated transferrin) to remove any non-specific binders, followed by a positive selection to enrich binders to immobilized biotinylated rInsR. Magnetic sorts on the naïve library were performed at 4°C with two washes during rInsR selections. Bound yeast were grown, re-induced and sorted more stringently with another round of magnetic sorting (two depletions and positive selection, as above) at room

temperature with three washes. The resultant population was sorted by flow cytometry to isolate full length Gp2 mutants. Induced yeast were labeled with 10 µg/mL mouse anti-c-MYC antibody (9E10, BioLegend, San Diego CA) followed by 10 µg/mL fluorescein-conjugated goat anti-mouse antibody (Sigma Aldrich, St. Louis, MO). All fluorescein-positive yeast were collected via BD FACS Aria II. Sorted yeast were grown and plasmid DNA was extracted using Zymoprep yeast plasmid miniprep kit II (Zymo Research Corp., Irvine CA). The enriched Gp2 population was diversified by random mutagenesis to the full gene and the loops of Gp2 in parallel via error-prone PCR using nucleotide analogs 2'-deoxy-P-nucleoside-5'-triphosphate and 8-oxo-2'-deoxyguanosine-5'- triphosphate (Trilink Biotechnologies, San Diego CA) [203]. Mutant genes and gene fragments were retransformed into yeasts via electroporation with homologous recombination with linearized pCT vector. The process results in shuffling of mutated Gp2 loops.

The mutagenized Gp2 population underwent two rounds of magnetic sorting (both with three washes at room temperature), one flow cytometry sort against biotinylated rInsR and one flow cytometry sort against mammalian cell lysates expressing GFP-conjugated InsR. For first flow cytometry sort, the yeast library was labeled with anti-c-MYC antibody 9E10 and 50 nM biotinylated rInsR, followed by fluorescein-conjugated goat anti-mouse antibody and Alexa Fluor 647 (AF647)-conjugated streptavidin (Life Technologies, Eugene, OR). Yeast clones that showed double positive signals for fluorescein and AF647, indicating full length Gp2 expression and rInsR binding, were collected. To ensure selected Gp2 clones showed consistent binding capacity to cell surface InsR, the yeast

population underwent another round of flow cytometry against mammalian cell lysates [204]. HEK293T cells were plated, transiently transfected with InsR-GFP plasmid (Addgene #22286) using Lipofectamine 2000 (Life Technologies) according to manufacturer's protocol and lysed (1% Triton-X, 2 mM EDTA, 1x protease inhibitor in PBS). Plasmid expressing GFP alone (Addgene #19319) was used to create a GFP control. The induced yeast population was incubated in the cell lysate expressing either InsR-GFP or GFP-control for one hour, washed twice with cold 1% PBSA (1% bovine serum albumin in PBS) and labeled with 9E10 and AF647-conjugated anti-mouse antibodies. All yeast clones that showed GFP and AF647 signals were collected.

This enriched Gp2 population underwent mutagenesis and two rounds of flow cytometry sorts against cell lysates (two fold diluted relative to the first cell lysate sort) for affinity maturation. For each sort, yeast exhibiting the top 2% of GFP:AF647 ratio (InsR binding per displayed Gp2) were collected. These cells were grown and zymoprepped to isolate plasmid DNA. Clonal plasmid was obtained by transforming extracted DNA into *E.coli* One Shot® TOP10 *E. coli* (Invitrogen, Carlsbad, CA). Single colonies were grown in LB medium and miniprepped (Invitrogen, Carlsbad, CA). Purifed DNA was sequenced by Eurofins Genomics (Huntsville, AL).

Gp2 production and purification

NheI and BamHI restriction enzymes were used to transfer the Gp2 gene from pCT yeast surface display vector into a pET vector with a C-terminal His_6 tag (Novagen, EMD Milipore, Billerica, MA). The resultant plasmids were

transformed into T7 express competent *E. coli* and grown in LB medium containing 100 µg/mL kanamycin. One liter of LB medium was inoculated with 5 mL of overnight culture, grown at 37°C to an optical density at 600nm of 0.6-1.0 units, and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside overnight at 20°C. Cells were pelleted, resuspended in 20 mL of lysis buffer (50 mM sodium phosphate, pH 8.0, 0.5 M sodium chloride, 5 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 25 mM imidazole) and underwent five freeze-thaw cycles. The soluble fraction was isolated by centrifugation at 10,000 g for 40 min at 4°C and filtered through a 0.45 µm syringer filter. Gp2 was purified by nickel-nitrilotriacetic acid affinity chromatography (Qiagen, Hilden, Germany) and by size exclusion/gel filtration chromatography using PD-10 column packed with Sephadex G-25 (GE Healthcare Bio-Sciences, Pittsburgh, PA)

Affinity titration

Lentiviral pLenti-InsR-GFP containing full length human *InsR* cDNA and control vector were purchased from Applied Biological Materials Inc. (British Columbia, Canada). They were referred to InsR-GFP and GFP-ctrl, respectively and were used to generate stable InsR overexpressing cell lines. Lentivirus production and transduction were carried out in HEK293T cells with 10 µg/mL polybrene to increase transduction efficiency. Cells underwent selection and were maintained in 2 µg/mL puromycin (Sigma-Adrich, St. Louis, MO). Stable HEK293T cell lines with either GFP-tagged InsR overexpression or GFP-control were labeled with soluble Gp2 at increasing concentrations for one hour at 4°C,

washed with PBSA and labeled with AF647-conjugated anti-His_5 antibody (Qiagen, Valencia, CA) for one hour at 4°C. Fluorescence was analyzed on a BD Accuri C6 flow cytometer and quantified using FlowJo software. The equilibrium dissociation constant, K_D, was determined by minimizing the sum of squared errors assuming a 1:1 binding interaction.

Cell surface binding assay

Cells were detached using trypsin, washed with PBS, labeled with soluble Gp2 at varying concentrations for one hour at 4°C, washed with cold PBSA and labeled with AF647-conjugated anti-His_5 antibody for one hour at 4°C. An anti-InsR antibody, clone 83-7 (hybridoma was kindly provided by Ken Siddle from University of Cambridge, UK) was used as a positive control for cell surface receptor binding. Fluorescence was analyzed on a BD Accuri C6 flow cytometer and quantified using FlowJo software.

Detection of cleaved Poly ADP-Ribose Polymerase (PARP)

Cells were plated at density of 6×10^5 cells in 60 mm dishes, serum-starved overnight and treated for 2 days. Cells were trypsinized, washed twice with ice-cold PBS with 1% BSA and resuspended in fixation/permealization solution (BD cytofix/cytoperm™, BD Bioscience, San Diego CA) for 30 minutes. These fixed cells were stained with cleaved PARP, Asp214 rabbit antibody (#5625, Cell Signaling Technology), followed by an AF647-conjugated anti-rabbit antibody (Life Technologies) for an hour each at 4°C. Fluorescence was analyzed on a BD Accuri C6 flow cytometry and quantified using FlowJo software.

Competitive binding assay

HEK293T cells transduced with pLenti-InsR were trypsinized, washed with PBS and pre-treated with or without insulin or IGF-II in 1% PBSA at 4°C for one hour. Cells were then labeled with Gp2 followed by AF-647 conjugated anti-His_5 antibody at 4°C for one hour each. Fluorescence signal was detected using BD Accuri C6 flow cytometer and analyzed using FlowJo software.

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