Identification of a Resistance Mechanism to IGF-IR Targeting in Triple Negative Breast Cancer Cells

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ABSTRACT

The insulin-like growth factor (IGF) axis regulates physiological development by promoting proliferation and survival signals. Aberrant IGF signaling has been reported in multiple malignancies including triple negative breast cancer (TNBC). TNBC patients are associated with poor prognosis due the lack of treatment options and aggressive nature of these tumors. Intrinsic and acquired resistance present a major challenge in TNBC treatment owing to the heterogeneity of the disease. No targeted therapy is currently approved for these patients and chemotherapy remains the mainstay treatment. The IGF-Trap is a novel anti-cancer drug candidate that limits IGF signaling by binding circulating and locally produced IGF-I and IGF-II. We previously observed varied tumor response when human TNBC MDA-MB-231 xenograft tumors were treated with the IGF-Trap, suggesting that these cells were heterogeneous in respect to sensitivity to IGF-IR signaling blockade. In this study, we examined the biomarker(s) predictive of response and resistance mechanism(s) to the IGF-Trap in MDA-MB-231 cells. Preliminary results on a small number of isolated clones showed that expression levels of IGF-IR in clonal subpopulations of MDA-MB-231 correlated with sensitivity to the IGF-Trap, suggesting IGF-IR expression level may be a determinant of response to the IGF-Trap. Chronic exposure of MDA-MB-231 cells to the IGF-Trap *in vitro* resulted in the selection of a resistant population (MDA-MB-231-R) that lost sensitivity to the IGF-Trap. We found that these cells had increased expression of WNT5A but the proliferation of these cells was not sensitive to WNT signaling inhibition. Importantly, we showed that MDA-MB-231-R cells had increased fibroblast growth factor receptor 1 (FGFR1) expression and activation levels, and this was accompanied by increased expression of FGF1. The proliferation and survival of MDA-MB-231-R cells were also more sensitive to FGFR1 inhibition than the parental cells, suggesting a role of FGFR1 signaling in providing a resistance mechanism

to the IGF-Trap. Combination of the IGF-Trap and FGFR1 inhibition suppressed the proliferation of MDA-MB-231 cells in an additive manner and was superior to either agent alone. Dual IGF-IR and FGFR1 inhibition may overcome resistance to IGF-axis targeting and could provide a valuable treatment strategy in TNBC.

RÉSUMÉ

Les IGFs (de l'anglais *insulin-like growth factors*) régulent le développement physiologique en stimulant la prolifération et favorisant la survie des cellules. Une signalisation IGF aberrante a été observée dans plusieurs types de tumeurs malignes, y compris le cancer du sein triple négatif (TNBC). Le pronostic associé au TNBC est de façon générale mauvais en raison du manque d'options de traitement et de la nature agressive de ces tumeurs. La résistance intrinsèque et acquise présente un défi majeur pour le traitement du TNBC en raison de l'hétérogénéité de la maladie. Aucun traitement ciblé n'est actuellement approuvé pour ces patients et la chimiothérapie demeure la principale option thérapeutique. La Trappe-IGF représente une nouvelle stratégie pharmaceutique anticancéreuse qui bloque la signalisation IGF en captant l'IGF-I et l'IGF-II produits localement. Nous avons précédemment observé une réponse thérapeutique variée chez des tumeurs de TNBC humaines MDA-MB-231 xenotransplantée traitées à la Trappe-IGF, indiquant que ces cellules étaient hétérogènes quant à leur la sensibilité au blocage de la signalisation IGF. Dans cette étude, nous avons examiné les biomarqueurs prédictifs d'une réponse thérapeutique et les mécanismes de résistance à la Trappe-IGF chez les cellules MDA-MB-231. Des résultats préliminaires sur un petit nombre de clones isolés ont montré que les taux d'IGF-IR dans les sous-populations clonales de MDA-MB-231 étaient corrélés avec la sensibilité à la Trappe-IGF, suggérant que le niveau d'expression IGF-IR pourrait être déterminant dans la Trappe-IGF. L'exposition chronique des cellules MDA-MB-231 à la Trappe-IGF *in vitro* nous a permis de sélectionner une population résistante (MDA-MB-231-R) qui a perdu de sa sensibilité à la Trappe-IGF. Nous avons observé que ces cellules avaient une expression accrue de WNT5A, cependant la prolifération de ces cellules n'était pas sensible à l'inhibition de la signalisation WNT. De plus, ces cellules MDA-MB-231-R avaient une expression et une activation accrue du récepteur

de facteur de croissance fibroblastes-1 (FGFR1), accompagné d'une expression accrue du FGF1. La prolifération et la survie des cellules MDA- MB-231-R étaient davantage sensibles à l'inhibition du FGFR1 comparé aux cellules parentales, suggérant un rôle de la signalisation FGFR1 en fournissant un mécanisme de résistance à la Trappe-IGF. La combinaison de la Trappe-IGF et de l'inhibition de FGFR1 a supprimé la prolifération des cellules MDA-MB-231 d'une manière additive et était supérieure à l'un ou l'autre agent seul. L'inhibition de deux IGF-IR et FGFR1 peut surmonter la résistance au ciblage de l'axe IGF et pourrait fournir une stratégie de traitement valable dans TNBC.

PREFACE

I have chosen to present a manuscript based thesis and have included the publications listed below as part of my thesis:

Chapter3

Tsui J., Vaniotis G, Fernandez M.C., Brodt P. Identification of a Resistance Mechanism to IGF-IR Targeting in Triple Negative Breast Cancer Cells. *In preparation*.

CONTRIBUTIONS OF AUTHORS

Tsui J.: Wrote this thesis and performed all the experiments included in this thesis with the exceptions below.

Chapter 3

Vaniotis G.: Expanded and stored parts of the clonal populations of MDA-MB-231 cells.

Fernandez M.C.: Expanded and stored parts of the clonal populations of MDA-MB-231 cells.

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LIST OF ABBREVIATIONS

4E-BP1	eukaryotic translation initiation factor 4E binding protein 1
ALK	anaplastic lymphoma kinase
ALS	acid-labile subunit
APC	adenomatous polyposis coli
ATR	Ataxia Telangiectasia Mutated and Rad3 Related
BL1	basal-like 1
BL2	basal-like 2
CDKs	cyclin-dependent kinases
CKs	cytokeratins
CREB	cyclic-AMP response element-binding protein
DVL	Dishevelled
eEF2K	eukaryotic elongation factor 2 kinase
EGFR	epidermal growth factor receptor
eIF4E	eukaryotic translation initiation factor 4E
EMT	epithelial to mesenchymal transition
ER	estrogen receptor
ERK1/2	extracellular signal-regulated kinase 1/2
FGFR	fibroblast growth factor receptor
FKHRs	forkhead transcription factors
FZD	frizzled
GEF	guanine nucleotide exchange factor
GH	growth hormone
GPCRs	G-protein-coupled receptors
GRB2	growth factor receptor bound protein 2
GSK3β	glycogen synthase kinase 3 beta
HDACs	histone deacetylases
HER2	epidermal growth factor receptor 2
ID4	inhibitor of DNA binding 4
IGF	insulin-like growth factor

IGF-IR	insulin-like growth factor I receptor
IGFBP	IGF-binding proteins
IM	immunomodulatory
IR	insulin receptor
IRS	insulin receptor substrate
Ki67	Marker of Proliferation Ki67
LAR	luminal androgen receptor
LID	liver specific IGF-I deficiency
M	mesenchymal
M6P	cation-independent mannose-6-phosphate
mAbs	monoclonal antibodies
MSL	mesenchymal stem-like
mTOR	mammalian target of rapamycin
NSCLC	non-small cell lung cancer
PARP	poly-ADP ribose polymerase
pCR	pathological complete response
PDGFR	platelet-derived growth factor receptor
PI3K	phosphatidyl inositol 3' kinase
PIP3	phosphatidylinositol-3,4,5-triphosphate
PORCN	Porcupine
PR	progesterone receptor
PTB	phosphotyrosine-binding
PTEN	phosphatase and tensin homolog
ROR	receptor tyrosine kinase like orphan receptor
rpS6	ribosomal protein S6
RTKs	receptor tyrosine kinases
RYK	RTK-like protein
S6K1	S6 kinase 1
SHC	Src homology/collagen domain protein
SOS	Son of Sevenless
TGFβ	transforming growth factor β
TKI	tyrosine kinase inhibitor
TNBC	triple negative Breast cancer

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CHAPTER 1:

THE IGF SYSTEM: AN OVERVIEW

1.1 The IGF ligands and binding proteins

The insulin-like growth factor ligands (IGF) -I and IGF-II are products of two distinct genes that are regulated independently. IGF-I is produced as a prohormone with a C-terminal E peptide, and matures when the peptide is enzymatically cleaved in the Golgi [1]. Both IGF-I and IGF-II are comprised of domains A, B, C, and D, and they circulate as 70- and 67-amino acid polypeptides, respectively [2]. The major source of plasma IGF-I is the liver but it is also produced locally in different organs [3]. Hepatic IGFs (endocrine) and locally derived IGF-I (autocrine/paracrine) have different regulatory mechanisms. For instance, production of hepatic IGF-I is regulated primarily by growth hormone (GH), although insulin and nutritional status also regulate serum IGF-I levels [4, 5]. IGF-I in turn regulates the production of GH through a negative feedback mechanism by acting on both the hypothalamus and pituitary [6]. In extrahepatic tissues, the expression of IGF-I is regulated by tissue specific factors in addition to GH [7-9]. IGF-II expression is imprinted and only the paternal allele is expressed while the maternal allele is silenced in most normal tissues [10]. IGF-I and IGF-II share a 62% homology and both have a 40% homology with proinsulin [11]. Mice with a liver specific IGF-I deficiency (LID) had a dramatic decrease in circulating IGF-I levels [3] yet did not demonstrate growth or development retardation. This mouse model confirmed that liver is the major production site for circulating IGF-I, and highlighted the functionality of locally-produced IGF-I.

There are five IGF-binding proteins (IGFBP1-5) that bind both IGF-I and IGF-II with similar affinities while IGFBP-6 preferentially binds IGF-II. IGFBP-3 is the predominant IGFBP in the circulation. Most of the circulating IGF-I is bound in a ternary complex with IGFBP-3 and an acid-labile subunit (ALS). This prolongs the IGF-I half-life and regulates the bioavailability of IGFs, and thereby IGF signaling [12]. Studies have also shown that IGFBPs have IGF-independent

functions in cell proliferation, migration, and apoptosis. In turn, the activity of IGFBPs are controlled by various proteases. Depending on physiological conditions, IGFBPs can either promote or suppress tumor cell growth. For instance, hypermethylation of the *igfbp-3* promoter was documented in heptaocellular carcinoma specimens [13]. Induction of IGFBP-3 expression resulted in growth inhibition in hepatoma and gastric carcinoma cells [14-16]. Downregulated expression of IGFBP-3/4 with resultant increased IGF bioavailability and IGF-IR signaling was identified as a potential resistance mechanism to genfitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI), and cisplatin in squamous and lung cancer cells, respectively [17, 18]. In contrast, some studies have suggested that IGFBP-4 and IGFBP-6 can stimulate cell migration and suppress apoptosis [19-21].

1.2 The IGF receptors

The insulin-like growth factor I receptor (IGF-IR) and insulin receptor (IR) are members of the receptor tyrosine kinases (RTKs) family and share a 84% homology in the tyrosine kinase domains [22]. The mature IGF-IR and IR are expressed as a heterotetramers, consisting two α-(130kDa), and two β-chains (95kDa) joined by α-α and α-β disulfide bonds. The α-subunits are entirely extracellular contain the ligand-binding domain, and the β-chains consist of an extracellular domain, a single pass transmembrane domain and intracellular domain [2, 22]. Within the cytoplasmic domain of IGF-IR, Tyr 950 is a binding site for phosphorylated substrates, while lysine 1003 servers as an ATP-binding site. The kinase domain consists of Tyr 1131, 1135, and 1136 that become phosphorylated upon ligand binding. In addition, Tyr 1250, 1251, and 1316 in the carboxyl domain are also key tyrosine residues and their phosphorylation is required for the receptor's signaling and biological functions [23]. IGF-IR binds IGF-I with the highest affinity, and also binds IGF-II and insulin, with 10- and 100-fold lower affinities, respectively [24].

Due to the high homology between IGF-IR and IR, an α and β chain of these receptors can heterodimerize forming IGF-IR/IR hybrid receptors. IGF-IR/IR hybrid receptors retain high affinity for IGF-I, but have a low affinity for insulin [25]. Due to alternate splicing, the IR has 2 isoforms, IR-A and IR-B, the former lacking the 11 amino acids encoded by exon 11. IR-A has high affinity for both IGF-II and insulin, and upon ligand binding can initiate mitogenic signaling. In contrast, IR-B does not bind IGF-II and mediates the metabolic effects of insulin [2, 23, 26, 27].

Lastly, the IGF-IIR, also known as a cation-independent mannose-6-phosphate (M6P) receptor, has much higher affinity to IGF-II than IGF-I, and shows no affinity to insulin. Unlike IGF-IR and IR, IGF-IIR is a single transmembrane glycoprotein that lacks intrinsic tyrosine kinase activity. In addition to mediating endocytosis and sorting of lysosomal enzymes, the receptor binds IGF-II and enhances its clearance and degradation, thereby limiting the bioavailability of IGF-II and IGF signaling [28].

1.3 Signal transduction by IGF

Ligand binding by the IGF-IR results in the activation of the Ras/Raf/MAPK and the PI3K/Akt pathways, ultimately resulting in cell growth, proliferation and survival. The binding of ligand to the extracellular domain of IGF-IR leads to a conformational change in the β-chains, and activation of the intrinsic tyrosine kinase activity of the β-chains, resulting in auto-transphosphorylation of key tyrosine residues on the intracellular, juxtamembrane, and carboxyl domain, including Tyr 1131, 1135, and 1136 in the kinase domain which result in receptor activation. Tyr 950 serves as a docking site for insulin receptor substrate 1-4 (IRS-1 to -4) and Src homology/collagen domain protein (SHC) to bind through the amino-terminal phosphotyrosine-binding (PTB) domain [23, 29, 30]. Phosphorylation of IRS-1 allows the recruitment and docking of the adaptor subunit (p85) of phosphatidyl inositol 3'-kinase (PI3K) via the SH2 domains [26].

This interaction results in allosteric activation of the catalytic subunit (p110) of PI3K and production of phosphatidylinositol-3,4,5-triphosphate (PIP3) on the cytoplasmic side of the plasma membrane. Protein kinase B (also known as Akt) is then recruited to the plasma membrane and interacts with PIP3, leading to the phosphorylation and activation of Akt (See Fig. 1.1) [31].

Akt can phosphorylate a range of targets that regulate cell cycle progression, survival and proliferation. Akt inactivates several pro-apoptotic proteins via phosphorylation, including BAD, procaspase-9, and forkhead transcription factors (FKHRs) [31]. In addition, Akt activates transcription factor cyclic-AMP response element-binding protein (CREB) and NF-κB to upregulate the expression of anti-apoptotic genes [32, 33]. Phosphorylation of the ubiquitin ligase mdm2 by Akt results in inhibition of p53 regulated processes, contributing to genome instability [31]. In addition to rescuing cells form apoptosis, Akt promotes cell cycle progression by activating the mammalian target of rapamycin (mTOR) [27, 30, 34]. mTOR regulates translation by modulating the activity of components of the protein synthesis machinery. Direct substrates of mTOR include eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1) [27, 31, 34]. Phosphorylation of 4E-BP1 by mTOR prevents its binding to eIF4E, which then allow eIF4E to form the cap-dependent translation complex with eIF4F for translation initiation [34]. Phosphorylation by mTOR is required for the activation of S6K1. Activated S6K1 promotes translation initiation, elongation, and ribosome biogenesis through transcriptional control by modulating various effectors, such as ribosomal protein S6 (rpS6) and eukaryotic elongation factor 2 kinase (eEF2K) [34, 35] (See Fig. 1.1).

In parallel, phosphorylated IRS-1 or SHC can also couple growth factor receptor bound protein 2 (GRB2) to the receptor, leading to the recruitment of the guanine nucleotide exchange factor (GEF) Son of Sevenless (SOS) from the cytosol to the plasma membrane. The interaction

of GRB2 and SOS activates Ras by stimulating the exchange of GDP to GTP, allowing Ras to activate Raf [36]. In turn, Raf activates the extracellular signal-regulated kinase 1/2 (ERK1/2). Activated ERK regulates gene expression by phosphorylating diverse responsive targets in the cytoplasm and nucleus. For example, ERK can directly phosphorylate and activate Ets-1, AP-1 and c-Myc [37]. Activated Ets-1 associates with other transcription factors, such as AP-1, to activate transcription of cell cycle progression factors such as cyclin D1, cyclin A, and c-Fos. c-Myc drives cell proliferation by directly inducing the expression of genes that promote cell cycle progression and increase cell size, including cyclins and cyclin-dependent kinases (CDKs), such as CDK1, CDK2 and CDK4. Additionally, Ras has been shown to interact with, and activate PI3K directly, thereby activating the PI3K/Akt pathway (See Fig. 1.1) [38].

1.4 The IGF axis in normal development

The IGF system is essential for pre- and postnatal growth. Studies using the homologous recombination system to ablate IGFs and IGF-IR expression have elegantly demonstrated their essential functions in normal somatic growth and physiology [39-42]. For example, mice with an *igf1* gene deletion exhibited severe prenatal growth retardation and lower birth weight and most did not survive after birth [39]. IGF-I deficient mice that survive are sterile, indicating that IGF-I is required for puberty. Indeed, in humans, the level of serum IGF-I gradually increases after birth and peaks during peripubertal age, then slowly declines with age [40]. In contrast, mice overexpressing IGF-I had a 1.3-fold increase in weight as compared to wild type mice [41] and had enlarged brains, pancreas, kidneys, and spleens, indicating that IGF-I plays a role in organogenesis. IGF-II knockout mice were smaller at birth, but were able to develop normally postnatally [42]. These findings suggest that, in mice, both IGF-I and IGF-II are essential for prenatal development, but only IGF-I is required for postnatal growth, which is consistent with

findings that serum IGF-II declines drastically after birth. However, IGF-II is more abundant than IGF-I in human adult serum, at approximately 700 ng/ml and 200ng/ml, respectively [26, 43], suggesting the divergent roles of IGF-II in rodent and human development.

1.5 The IGF axis in malignancy

In view of the critical and diverse roles that the IGF axis plays in development and growth, perhaps it is not surprising that abundant data from experimental models and clinical studies have shown altered IGF signaling in malignant progression. Aberrant IGF signaling has been documented in numerous types of cancers, including breast, colon, pancreatic and prostate carcinomas, rhabdomyosarcoma, Ewing's sarcoma and leukemias [44]. For example, in a study by Shimizu et al., IGF-IR was found to be overexpressed in 43.8% of the primary breast cancer specimens [45], and high IGF-IR levels were also found in non-small cell lung cancer (NSCLC) specimens [46].

Population studies documented an association between high serum IGF-I levels and cancer risk in colorectal, prostate, and breast cancer (the latter in premenopausal women only) [47]. Prospective study by Ma et al. [48] showed that men with high levels of circulating IGF-I had a 2.5-fold higher risk of developing colorectal cancer as compared to men with low IGF-I levels. Furthermore, premenopausal women with high serum IGF-I concentrations had a 2.3-fold higher risk of developing breast cancer as compared to women with low serum IGF-I concentrations [49]. Moreover, increased IGF-IR expression levels in primary prostate cancer specimens relative to benign prostatic epithelium have been reported [50]. However, it is important to note that some studies did not corroborate these findings [51, 52]. For instance, a prospective study by Schernammer et al. [53] did not detect association between serum IGF-I levels and breast cancer risk in premenopausal women.

There is an extensive body of experimental data implicating the IGF axis in malignancies. The oncogenic role of IGF-IR was first demonstrated in a study by Kaleko et al. [54] in which the authors showed that overexpression of IGF-IR in murine, human and rat fibroblasts resulted in their transformation in a ligand-dependent manner. The oncogenic role of IGF-IR was further demonstrated by studies showing the inability of mouse fibroblasts to transform in the absence of IGF-IR expression, despite overexpression of many, but not all oncogenes including SV40 T antigen, activated Ha-Ras and c-Src [55-57]. In addition, IGF-I was shown to induce proliferation, promote invasion and metastasis in different cancer cell types and experimental tumor models. For example, Wu et al. [58] reported that LID mice with decreased circulating IGF-I levels had an increased latency of mammary tumor development as compared to controls. The mammary tumors from control mice showed extensive metaplasia, while tumors from LID mice showed largely hyperplasia with low level of metaplasia. In another study, exogenous IGF-I treatments increased the growth rate and liver metastasis of colon cancer cells in control and LID mice as compared to vehicle-treated mice [59]. Moreover, there were significantly more liver metastases in the control as compared to LID mice. Lastly, IGF-II was shown to stimulate the growth and migration of rhabdomysarcoma cells, and high IGF-IR expression was associated with dependence on IGF-IR signaling for proliferation and sensitivity to IGF-IR inhibitors [60, 61].

1.6 Targeting the IGF System for cancer therapy

Given the large body of evidence supporting the critical role of IGF signaling in cancer development and in view of the high IGF-IR expression in many cancer types, agents that target the IGF-axis have been developed for anti-cancer therapy. The IGF/IGF-IR targeting drugs that have entered clinical trials can be categorized into three classes: 1) humanized monoclonal anti-IGF-IR antibodies, 2) small molecule tyrosine kinase inhibitors, and 3) ligand neutralizing

antibodies. These classes of drugs have different mechanisms of action, target selectivity, and pharmacological properties. Below is a brief summary of several of the agents that have been extensively evaluated or are currently in human trials.

1.6.1 Monoclonal antibodies (mAbs) against IGF-IR

Anti-IGF-IR antibodies act by binding to the extracellular domain of the receptor thereby blocking ligand binding and inducing receptor internalization and degradation that result in decreased cell surface IGF-IR expression levels. These antibodies are generally specific to IGF-IR and do not cross react with the IR.

In preclinical studies, CP-751187 (Figitumumab), a fully human IgG_2 mAb to IGF-IR with ability to bind IGF-IR/IR heterodimers, showed activity as monotherapy in xenograft models of several cancer types including colon, breast, and lung cancer [62]. Additive effects were observed when this antibody was combined with chemotherapy or other targeted drugs [62]. Based on these findings, Figitumumab was the first IGF-IR inhibitor to enter clinical trials, and it was tested in multiple types of malignancies in early phase trials, including relapsed or refractory multiple myeloma, solid tumors, unselected sarcomas, and Ewing's sarcoma [63-66]. These trials were mainly positive, for instance, Lacy et al. [63] reported a 78% overall response rate in patients with relapsed or refractory multiple myeloma treated with Figitumumab. Moreover, 8 out of 29 patients with sarcoma or Ewing's sarcoma had stable disease, and 2 patients achieved objective responses (including one complete response) after receiving Figitumumab in a phase I study [65]. Of note, the phase II trial of carboplatin and paclitaxel with or without Figitumumab in patients with advanced NSCLC yielded some very exciting data [67]. In the Figitumumab plus carbolplatin and paclitacxel arm, objective response was seen in 54% of patients as compared to 42% in the carboplatin and paclitaxel alone arm [67]. These findings prompted a subsequent phase III trial

evaluating the same drug combination as a first-line treatment for advanced NSCLC [68]. However, this trial was discontinued early because the addition of Figitumumab to carboplatin and paclitacxel did not improve overall survival, and increased treatment-related adverse events were observed [68]. The initial manuscript reporting the phase II clinical trial data was retracted 3 years after its publication due to identified data collection and analysis errors [69]. Another phase III trial evaluating the combination of Figitumumab and erlotinib as compared to erlotinib alone in NSCLC patients was also terminated early because it was concluded that the addition of Figitumumab demonstrated no superior effects on patient survival [70]. The clinical development of Figitumumab was thereafter terminated due to these negative results.

AMG 479 (Ganitumab) is a fully humanized anti-IGF-IR monoclonal antibody (IgG_1) [71]. It acts by inhibiting the binding of IGF-I and IGF-II to IGF-IR, resulting in decreased ligand-induced IGF-IR phosphorylation [71]. AMG 479 also acts on IGF-IR/IR hybrids, with no cross-reactivity with IR homodimers [71]. AMG 479 monotherapy efficacy was observed in both *in vitro* and *in vivo* studies of pancreatic carcinoma models where it induced pro-apoptotic and anti-mitogenic effects [71]. AMG 479 and gemcitabine combinations had an additive effect as compared to either agent alone in pancreatic carcinoma bearing mice [71]. A randomized phase II study by Kindler et al. investigated the efficacy and safety of Ganitumab in combination with gemcitabine in patients with metastatic pancreatic adenocarcinoma [72]. It was concluded that the combination had tolerable toxicity, and demonstrated trends towards improved 6-months survival rate and overall survival [72]. The efficacy of this combination was also validated in another phase II trial in patients with Ewing's family tumors or desmoplastic small round cell tumors [73]. As these early trials were suggestive of Ganitumab activity in metastatic pancreatic cancer, a phase III trial of combination Ganitumab and gemcitabine as first-line therapy in unselected patients with

metastatic pancreatic cancer was initiated. However, the trial was halted early after a pre-planned interim evaluation revealed a lack of improvement in progression-free or overall survival as compared to gemcitabine alone [74].

1.6.2 Tyrosine kinase inhibitors (TKIs)

Several IGF-IR TKIs have been developed, and are currently being tested in the clinical setting. IGF-IR TKIs are either ATP-competitive or non ATP-competitive [75]. In the case of an IGF-IR ATP-competitive TKI, it competes for the ATP-bindings sites on the receptor, thereby preventing receptor autophosphorylation and signal transduction.

Because of the high homology between IGF-IR and IR, this class of inhibitors also shows activity against the IR. While the dual inhibition of IGF-IR and IR may confer additional anti-tumor activity, the inhibition of IR activity disrupts glucose metabolism and can have a higher toxicity than specific IGF-IR inhibitors.

OSI-906 (linsitinib) is an ATP-competitive TKI of IGF-IR and IR [76]. It showed potent anti-tumor activity in *in vitro* and *in vivo* studies in several cancer types, including pancreatic, colorectal and breast carcinomas and rhabdomyosarcoma [76]. These findings led to early phase trials that confirmed the safety and efficacy of linsitinib. In a phase I clinical trial with linsitinib for advanced metastatic solid tumors, 41% of the patients had stable disease [77]. Notably, partial responses were observed in two patients with adrenocortical carcinoma. These early positive trials fueled a phase III clinical trial comparing linsitinib to placebo in patients with advanced adrenocortical carcinoma. Although long-term partial response was observed in three patients, linsitinib failed to show benefit in overall survival and progression free survival in the overall population [78]. However, given that partial response was achieved in three patients, it was suggestive that IGF-IR/IR inhibition may be beneficial in a subset of adrenocortical carcinoma

patients. Unfortunately, no study comparing responders and non-responders was followed due to insufficient data collection. Future trials should therefore implement potential predictive markers data collection for retrospective biomarker studies. Lastly, supported by preclinical studies demonstrating additive anti-tumor effects with combination IGF-IR inhibitor and bortezomib in multiple myeloma patients [79], a phase I/II trial is currently ongoing to evaluate the combinatorial use of linsitinib, bortezomib and dexamethasone in the treatment of multiple myeloma [NCT01672736].

1.6.3 Ligand neutralizing antibodies

The therapeutic potential of IGF-IR mAbs and IGF-IR TKIs may be limited by several factors. Because IGF-IR mAbs do not have affinity to IR, they do not inhibit IR-A mitoegenic signaling induced by IGF-II and/or insulin. A study by Buck et al. [80] showed that increased IR signaling through IGF-II and insulin abrogated the anti-proliferation effects of an IGF-IR mAb in sarcoma cells. Moreover, upregulated growth hormone release and subsequent increased IGF production has been reported in human patients treated with IGF-IR mAbs [81]. On the other hand, while IGF-IR TKIs target both IGF-IR and IR, the inhibition of both receptors could confer more side effects, such as hyperglycemia and hyperinsulinemia [82]. Ligand (IGF-I and IGF-II) neutralizing antibodies may circumvent the shortcomings of IGF-IR antibodies and IGF-IR/IR TKIs, because they prevent IGF-II/IR-A signaling without inhibiting the activity of IR-B.

MEDI-573 is a fully human IGF-I/II neutralizing antibody [83]. It acts by limiting the bioavailability of both IGF-I and IGF-II without cross-reactivity with insulin, thereby inhibiting IGF-IR and IR-A signaling without disrupting IR-B-mediated glucose metabolism [83]. In a phase I clinical trial with solid tumor patients that have been heavily pre-treated, 30% of patients treated with MEDI-573 had stable disease, but no partial or complete responses were observed [84]. Only

1 of the 43 recruited patients experienced treatment-related hyperglycemia. Based on preclinical findings that hormone receptor-positive and epidermal growth factor receptor 2 (HER2)-negative breast cancer tumors had increased IR-A:IR-B mRNA ratios [85], a phase I/II study is currently ongoing to investigate the therapeutic potential of MEDI-573 in combination with an aromatase inhibitor for the treatment of metastatic hormone receptor-positive, HER2-negative breast cancer [NCT01446159].

BI 836845 (Xentuzumab) is the only other IGF-I/II neutralizing antibody [86] that is currently being evaluated in human trials. Only two phase I trials evaluating the activity and safety of BI 836845 in patients with advanced solid tumors have been completed so far. Publication of these trial results [NCT01403974, NCT01317420] are still pending. Several other phase I trials are currently ongoing with this drug [NCT02123823, NCT02145741, NCT0219189, NCT02204072, NCT03099174] in patients with NSCLC, breast cancer and prostate cancer.

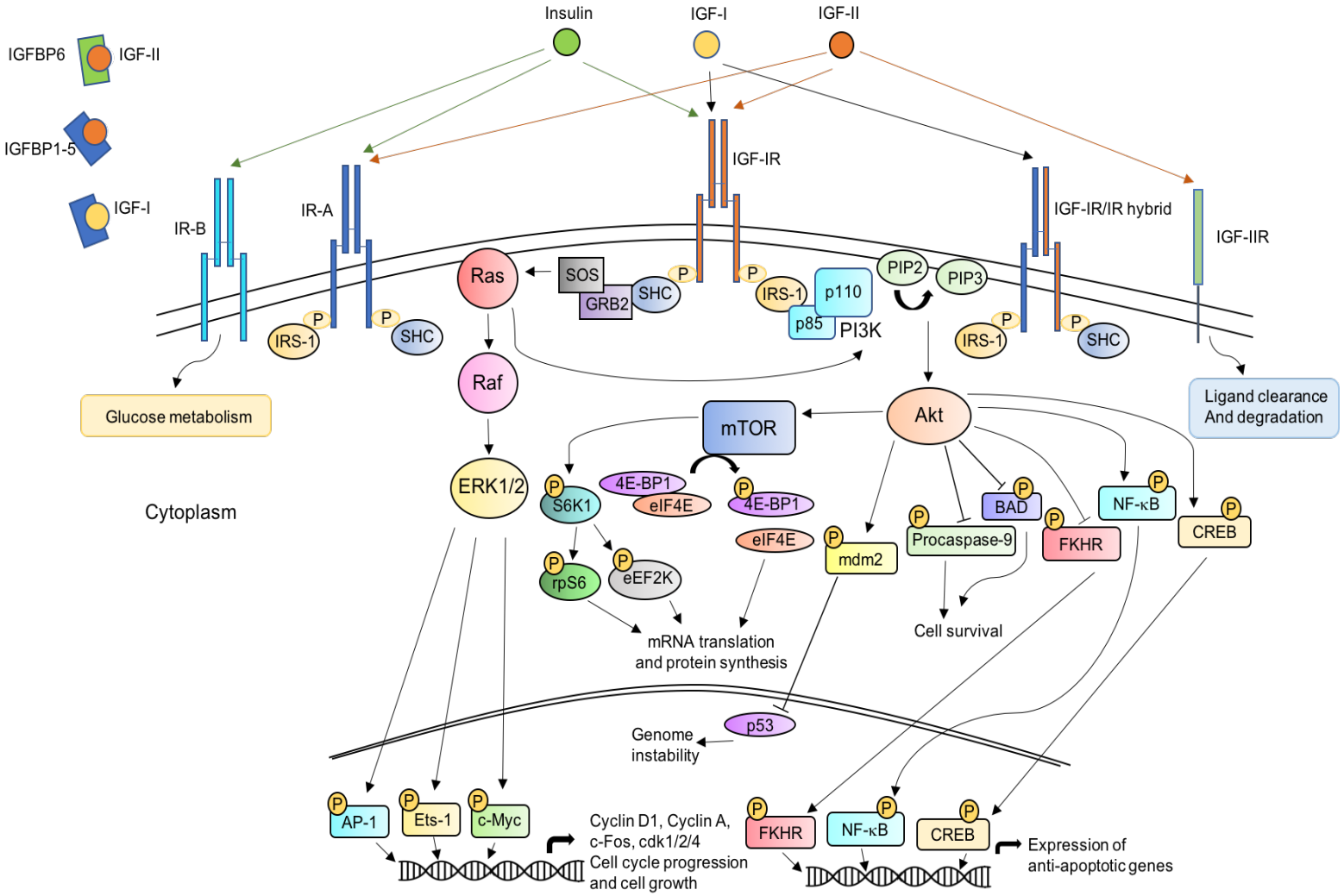

Fig. 1.1. The IGF system and signaling pathways. The IGF system consists of 3 ligands (IGF-I, IGF-II, and insulin), 5 transmembrane receptors (IGF-IR, IR-A, IR-B, IGF-IR/IR hybrid, and IGF-IIR), and 6 IGFBPs (IGFBP1-6). IGFBPs bind and regulate the bioavailability of IGFs. IGFBP1-5 have similar affinities for IGF-I and –II, while IGFBP6 preferentially binds IGF-II. IGF-IIR binds IGF-II with the highest affinity and it is responsible for ligand clearance and degradation, thereby regulating IGF signaling. IR-B is responsible for mediating the metabolic effects of insulin. IGF-IR has the highest affinity for IGF-I, while IR-A shows high affinity to both IGF-II and insulin. IGF-IR/IR hybrid has high affinity to IGF-I. Upon ligand binding, autophosphorylation of the tyrosine kinase domains of IGF-IR, IR-A, and IGF-IR/IR hybrid

results in the recruitment and phosphorylation of SHC and IRS-1, and subsequent activation of the Ras/Raf/MAPK and the PI3K/Akt pathways. The activation of these two pathways ultimately results in cell growth, proliferation and survival.

CHAPTER 2:

TRIPLE NEGATIVE BREAST CANCER: THE CHALLENGE

2.1 Molecular subtypes of breast cancer

Breast cancer is the second most common cancer worldwide accounting for 12% of all newly diagnosed cancer cases in 2012. It is, by far, the most common cancer in women, contributing to 25% (1.67 million cases) of the total new cancer cases in 2012 worldwide [87]. Breast cancer is a heterogeneous disease. Five molecular subtypes have been identified based on transcriptional profiles namely, the luminal A, luminal B, HER2-amplified, normal-like, and basal-like [88, 89]. Each subtype is associated with different histopathological and biological features, as well as therapeutic response and patient outcomes.

The luminal A subtype is the most common subtype, accounting 50-60% of all breast cancers. Luminal A tumors are hormone receptor-positive (estrogen receptor (ER) and/or progesterone receptor (PR)) and HER2-negative. They are often well differentiated with low Marker of Proliferation Ki67 (Ki67) expression [88]. These tumors are generally responsive to hormonal treatments, including ER modulators and down-regulators, such as tamoxifen and fulvestrant, respectively, as well as aromatase inhibitors, such as letrozole [90]. In general, luminal A breast cancer patients have the best prognosis and best overall survival of all subtypes. Moreover, these patients have significantly longer survival (median 2.2 years) relative to patients with other breast cancer subtypes in the metastatic setting [91].

Luminal B tumors represent 10-20% of all breast cancers. They are also hormone receptors-positive, but may or may not overexpress HER2. Compared to luminal A tumors, this subgroup is often presented as higher grade tumors that have a higher mitotic index with increased Ki67, cyclin E2, and cyclin D1 expression, rendering them more aggressive relative to the luminal A subtype [92]. Patients with luminal B tumors have higher pathological complete response (pCR) rates to chemotherapy relative to those with luminal A tumors [92]. These patients are also

amenable to hormonal therapies. However, they exhibit a poorer response to such treatments than luminal A cancer patients [93], and have a higher relapse rate and shorter relapse-free survival time [91].

HER2-amplifed breast cancers are seen in 15-20% of all breast cancer patients. HER2-amplified breast tumors are mostly hormone receptor-negative and characterized by HER2 overexpression and/or *HER2* gene amplification [94]. Although there is no known ligand, HER2 heterodimerizes with other members of the EGFR family to engage in growth promoting signal transduction. Heterodimers that contain HER2 are more stable and potent in transducing signals than those without HER2 [94, 95]. HER2-positive tumors are frequently poorly differentiated and have high expression of genes that regulate cell proliferation. *p53* mutations are seen in 72% of these tumours and are rarer in the luminal A (12%) and luminal B (29%) subtype [96]. Although patients with HER2-amplified tumors have a higher rate of pCR to chemotherapy as compared to patients with luminal tumors, the HER2 subtype was generally associated with poor prognosis before the development of targeted therapies, because those that did not achieve pCR had a higher relapse rate, due to the aggressive nature of these tumors [97, 98]. However, targeted therapies, including HER2 monoclonal antibodies, such as trastuzumab and pertuzumab and small-molecule kinase inhibitors, such as lapatinib, have become available as treatment options in addition to chemotherapy. These targeted agents have drastically improved the prognosis in patients with early or late stage disease [94].

Normal-like breast tumors account for 5-10% of all breast carcinomas. They are poorly defined and their gene expression profiles cluster with normal breast tissues and fibroadenomas [88]. Normal-like breast tumors have high expression of genes associated with adipose and basal epithelial cells. These tumors are often resistant to neoadjuvant chemotherapy. Some studies have

failed to identify the normal-like subgroup and suggested that the subtype could be derived from sample contamination with normal breast tissue resulting in high normal breast to tumor tissue ratios [99, 100]. Indeed, a study using microdissected breast tumor samples consisting of at least 90% tumor cells did not identify any normal-like cases [100].

2.2 Triple negative breast cancer (TNBC)

2.2.1 Triple negative breast cancer and basal-like cancer

Clinically, triple negative breast cancer (TNBC) is defined based on the absence of ER and PR expression, and the lack of HER2 overexpression or amplification in the cells as determined by immunohistochemistry and *in situ* hybridization, respectively [101-104]. Classification of basal-like breast cancer is based on the expression of several hundred genes using gene expression microarray. The majority (approximately 70%) of TNBCs are basal-like, therefore, the two terms have sometimes been used interchangeably [105]. However, although the characteristics of TNBCs and basal-like tumors overlap substantially, they each represent a distinct class of breast cancer. For example, approximately 10-35% of TNBCs did not classify as basal-like in a microarray analysis, and protein expression of basal cell markers is only seen in 40-80% TNBCs [103]. Correspondingly, some basal-like tumors (18-40%) are not of the triple negative phenotype. A subset (up to 20%) of basal-like tumors express ER or overexpress HER2 [102, 106]. Because gene expression profiling is not readily available in all centers, and there is no consensus on the definition of basal-like tumors, the diagnosis of triple-negative remains a surrogate for basal-like BC in the clinical setting [102].

2.2.2 Molecular features of TNBC

The molecular characteristics that are shared between the basal-like and triple negative subtypes are reviewed below. TNBCs are frequently high grade histologically and have higher

expression of proliferation-related genes (such as Ki67, TOP2A, and cyclin E) than other subtypes. These tumors are characterized by expression of basal cell cytokeratins (CKs) including CK5, 6, 14, and 17, loss of epithelial markers such as E-cadherin, and expression of myoepithelial markers such as caveolins 1/2 and P-cadherin. EGFR overexpression occurs more frequently in TNBCs than other subtypes [101-104, 107]. Histologically, these tumors are heterogeneous and although presenting predominantly as invasive ductal carcinoma, other types such as invasive lobular carcinoma, metaplastic, secretory, adenoid cystic carcinoma have also been reported [101, 102]. *p53* is the most commonly mutated gene and it is found in approximately 60% of TNBCs while *PI3KCA* mutations are present in about 10% of all TNBC cases. Other druggable gene mutations, such as *BRAF*, *RAS*, only occur at a very low incidence (<1-5%) in TNBC patients [108]. Gene amplifications and deletions have also been identified in TNBCs. For example, amplification of RTKs such as *IGF-IR, FGFR1, FGFR2*, EGFR, among others, has been reported in TNBCs [96]. Alterations in gene copy numbers such as loss of *phosphatase and tensin homolog* (*PTEN*) have been reported in 46.1% of basal-like tumors [109].

Of note, studies have reported an association between *BRCA1* germline mutations and basal-like/TNBCs. The majority of tumors that harbour *BRCA1* mutations have a basal-like and/or TNBC phenotype [110, 111]. BRCA1 is a tumor suppressor essential for DNA double-strand break repair through homologous recombination [112]. Loss of BRCA1 results in genomic instability and predisposes the cells to neoplastic transformation [112]. About 10% of TNBC patients are *BRCA1* germline mutation carriers [101]. On the other hand, deregulated BRCA1 expression has been reported in sporadic basal-like cancers, including downregulated BRCA1 expression, potentially due to promoter methylation and/or loss of heterozygosity, as well as overexpression of inhibitor of DNA binding 4 (ID4), a negative regulator of BRCA1 expression [101, 113, 114].

These findings implicate BRCA1 in the pathogenesis of basal-like/TNBCs, and this is supported by preclinical evidence that poly-ADP ribose polymerase (PARP) inhibitors have anti-tumor activity in BRCA-deficient cells *in vitro* and in mouse models [115, 116]. The combination of PARP inhibitors and loss of BRCA1 is synthetic lethal. Synthetic lethality occurs when loss of function of one gene permits cell survival but results in cell death in the event of combinatorial loss of both genes [117, 118]. PARP1 and PARP2 mediate DNA single-strand breaks and base excision repair [117, 118]. Inhibition of both of these enzymes prevents DNA damage repair, resulting in double-strand breaks when encountered by replication forks. Normal cells with functional BRCA1 undergo homologous recombination to repair double-strand breaks, allowing cell survival. In contrast, BRCA-deficient tumor cells are not able to perform homologous recombination, leading to genome instability and cell death [117, 118]. Currently, the utility of PARP inhibitors in TNBC patients is being evaluated in several clinical trials [NCT01818063], [NCT02032277], [NCT02158507], [NCT01074970], [NCT01104259].

2.2.3 Clinical presentation of TNBC

TNBCs represent 15-20% of all breast cancer cases [119]. TNBC is more prevalent in African-American and Hispanic women, and in younger women (<40 years of age) [120-122]. Moreover, women with abdominal obesity are more likely to develop TNBC [123]. Accordingly, metformin, an antidiabetic drug, has been shown to have anti-proliferative effects in TNBC cells both *in vitro* and *in vivo* [124, 125]. Because of its aggressive nature and lack of treatment options, TNBC patients have poorer overall survival (5-year survival rates of TNBCs and non-TNBC patients are 77% and 93%, respectively [121]) and shorter disease-free survival with increased risk of relapse within the first 3 years following initial diagnosis [126]. Lastly, unlike other BC subtypes, TNBC cells frequently metastasize to the lungs and brain rather than to bone and liver

[91]. In the metastatic setting, TNBC patients have short median survival time (approximately 13 months) and duration of response to treatment, and practically all TNBC patients with metastasis eventually die of the disease [127, 128]. These unfavorable outcomes highlight the importance of developing better treatment strategy for TNBC patients.

2.2.4 Molecular subgroups of TNBC

To better understand the heterogeneity of TNBCs, Lehmann et al. [129] explored the molecular landscape of TNBCs using gene expression microarrays. The authors identified six intrinsic TNBC molecular subgroups based on their gene expression signatures namely: the basal-like 1 (BL1), basal-like 2 (BL2), immunomodulatory (IM), mesenchymal (M), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR) subgroups. Each of these subgroups has distinct pathway enrichments (See Table 2.1) and mutations that may be exploited as potential treatment targets. They are also correlated with different pCR and relapse-free survival rates following neoadjuvant chemotherapy [130].

The BL1 and BL2 subgroups are characterized by enrichment in cell cycle progression genes accompanied by high Ki67 expression. Additionally, DNA damage response pathway genes are upregulated in the BL1 subgroup, while the BL2 subgroup shows enrichment in genes associated with growth factor-mediated signaling such as the EGF, IGF and Met pathways. The increased expression of cell cycle progression genes suggests that these tumors may be more susceptible to antimitotic agents. Indeed, BL1 and BL2 tumors were shown to be more sensitive to treatment with taxanes than other TNBC subgroups as more patients with tumors in either one of these subgroups achieved pCR (63%) as compared to patients with the M (31%) or LAR (14%) tumor subtype [129, 131, 132]. However, this could not be confirmed by another study where only

BL1, but not BL2 tumors were associated high pCR rate (52% and 0%, respectively) in response to taxane and anthracycline-based therapies [130].

The M and MSL subgroups are both enriched in expression of genes involved in epithelial to mesenchymal transition (EMT), cell migration and growth factor receptor signaling, including IGF-IR, fibroblast growth factor receptor (FGFR), transforming growth factor β (TGFβ), and Wnt/β-catenin signaling. The MSL is distinct from the M subgroup in that it also has increased expression of angiogenesis-related genes and additional upregulated elements that are associated with growth factor receptor signaling such as EGFR and platelet-derived growth factor receptor (PDGFR). Moreover, the MSL subgroup is more "stem-like" with enriched stem cell markers such as ALDHA1, CD105, CD90, among others, and this "stemness" is accompanied with lower expression of proliferation-related genes. The M subgroup is more responsive to neoadjuvant chemotherapy than MSL tumors with pCR rates of 31% and 23%, respectively [130]. Cell lines that are classified into the mesenchymal-like subgroups (M and MSL) were more sensitive than LAR cell lines to dasatinib - an inhibitor of Src - a known mediator of cell migration and invasion [133]. Mesenchymal-like cell lines also exhibited sensitivity to PI3K/mTOR inhibition, likely due to their dependency on growth factor receptor signaling pathways [129].

The LAR subgroup is significantly enriched in expression of the AR and its downstream signaling components. *PI3KCA* is one of the most commonly mutated genes in TNBCs, but it is more frequently mutated in LAR tumors relative to other TNBC subgroups [134]. While being ER-negative, the expression signature of this subgroup resembles luminal ER-positive breast tumors. LAR cell lines were more sensitive than cell lines of other subgroups to AR signaling inhibition and dual PI3K/mTOR inhibitors as monotherapy [129]. Additive effects on suppression of proliferation were observed when the AR antagonist, bicalutamide was combined with a PI3K

inhibitor, demonstrating that these cells were dependent on both AR and PI3K signaling [134]. AR status may therefore be predictive of response to combination antiandrogen and PI3K inhibitors in TNBC patients.

Lastly, the IM subgroup is characterized by a gene signature characteristic of immune cell signaling. These tumors are enriched in antigen processing and T cell-related genes.

2.3 Current treatment and clinical development in TNBC treatment

Due to the extensive heterogeneity of TNBC, there is currently no targeted therapy approved for the treatment in TNBCs in the clinical setting. Chemotherapy remains the primary treatment for both early and advanced-stage TNBC patients [101, 104].

Chemotherapy can be beneficial in TNBC patients in both the neoadjuvant and adjuvant setting, including in patients with metastatic disease. Eighty eight percent of TNBC patients achieved pCR following a neoadjuvant platinum-based regimen, as compared to only 51% of patients with other BC subtypes [135]. Moreover, anthracycline alone, or in combination with taxanes resulted in pCR in 22% of TNBC patients as compared to 11% in non-TNBC patients [126]. Other studies have confirmed an increased response rate to anthracycline-based treatment in patients with TNBC [136]. However, those who have residual disease following a given chemotherapy regimen are more likely to experience early relapse and mortality due to the aggressive behavior of TNBC, contributing to the overall poor prognosis in TNBC patients [126, 136]. These findings suggest that a cohort of TNBC is chemosensitive, although biomarkers for selection of a specific class of chemotherapy remain to be defined. *BRCA1* mutation status appears to be predictive of response to cisplatin in BC patients; more *BRCA1* mutation carriers treated with cisplatin achieved pCR than those that were treated with another class of chemotherapy agents [137]. On the other hand, growth factors receptors and their downstream substrates (such as EGFR

and MAPK), as well as p*53* status were proposed as potential determinants for chemotherapy sensitivity in TNBC [107].

There are numerous active clinical trials investigating the efficacy of different treatment modalities in TNBC, including chemotherapy combinations, targeted therapy in combination with chemotherapy, targeted therapy alone, immunotherapy alone, or immunotherapy in combination with chemotherapy [101, 104, 138].

The involvement of RTK signaling in TNBC pathogenesis is well documented and RTK signaling components may therefore represent actionable targets. For example, blockade of EGFR signaling has been of interest in the treatment of TNBC. In addition to conveying growth and survival signals through the Ras/Raf/MAPK and the PI3K/Akt pathways as a transmembrane growth factor receptor, EGFR can also promote tumor progression by acting as a transcription factor in the nucleus [139]. EGFR overexpression is frequently seen in TNBC and high *EGFR* gene copy number is an independent adverse prognostic factor in TNBC patients [140, 141]. Preclinical studies demonstrated that inhibitors of EGFR signaling had anti-tumor activity in TNBC cell lines and xenograft models when used alone or in combination with chemotherapy [141, 142]. For example, enhanced suppression of tumor cell proliferation *in vitro* and xenograft tumor growth were observed when, cetuximab, an anti-EGFR human/mouse chimeric IgG_1 mAb that inhibits ligand-mediated EGFR phosphorylation [143], was combined with doxorubincin or other TKIs [144, 145]. These findings provided the rationale for trials targeting EGFR in TNBC patients.

A Phase II trial led by Baselga et al. [146] evaluated the efficacy of cetuximab in combination with cisplatin as compared to cisplatin alone in metastatic TNBC patients. Although the differences in objective response (20% vs. 10%) and overall survival (12.9 months vs. 9.4

months) in the two groups did not achieve statistical significance, both trended towards improvement in the combination arm relative to the cisplatin alone arm. Notably, the addition of cetuximab resulted in significantly longer progression free survival as compared to cisplatin alone (median 3.7 months vs. 1.5 months). However, the primary end point of having objective response in greater than 20% of patients was not met in this trial and no follow-up study was performed to identify predictive biomarkers to cetuximab in this trial.

The activity of EGFR TKIs in TNBC has also been explored in the clinical setting. Lapatinib is an EGFR/HER2 ATP-competitive TKI that binds the ATP-binding site of the receptors in inactive conformation, thereby inhibiting receptor phosphorylation [147]. A phase III trial was conducted to evaluate the activity of paclitaxel with either lapatinib or placebo in HER2-negative or HER2-untested metastatic breast cancer [148]. While HER2-positive patients that received lapatinib and paclitaxel showed significant improvement in various clinical end points relative to those in the placebo-paclitaxel arm, no differences in clinical end points were observed in HER2-negative patients in the two arms. A retrospective biomarker study of this trial evaluating expression of ER, PR, HER2, and EGFR showed that the addition of lapatinib did not improve event-free survival in TNBC patients as compared to the placebo arm [149]. Moreover, no association was detected between EGFR expression and response to lapatinib in the TNBC patients.

More trials investigating the efficacy of EGFR inhibitors in TNBC have been conducted, but the results have been at most modest [150-153]. Despite the reduced enthusiasm for EGFR inhibitors in TNBC, a single-arm phase II trial is ongoing to investigate the activity of the combination of panitumumab (a humanized IgG_2 EGFR mAb), carboplatin and paclitaxel in patients with localized TNBC [NCT02593175]. Another active single-arm phase II trial is

evaluating the addition of erlotinib (an ATP-competitive TKI against EGFR) to neoadjuvant carboplatin/docetaxel based chemotherapy [NCT00491816].

2.4 Other RTKs signaling as potential targets in TNBC

2.4.1 Role of IGF-IR signaling

Numerous preclinical and population studies have demonstrated the role of IGF-IR signaling in TNBC. Exogenous IGF-I was shown to promote the proliferation and survival of TNBC cells while IGF-IR inhibition impeded the proliferation of TNBC cells with high IGF-IR activity *in vitro* [154, 155]. IGF-IR TKI monotherapy was sufficient to reduce tumor size in a TNBC xenograft model with high IGF-IR activity. Combination of a small molecule TKI against IGF-IR and docetaxel caused complete tumor regression in this model. Moreover, co-inhibition of IGF-IR and other molecular targets (such as MEK and PI3K) was reported to have synergistic growth inhibition effects [156, 157]. Clinically, TNBC is more prevalent in African-American and Hispanic women [120-122]. Interestingly, Kalla et al. [158] reported elevated IGF-IR and IGF-II expression in in normal breast tissue obtained from African-American women as compared to Caucasian-Americans. Although no increase was observed in IGF-IR expression levels in malignant tissue relative to normal breast tissue obtained from African-American women, the phosphorylation levels of IGF-IR, IRS-1, and SHC were significantly increased in the tumor samples. Additionally, amplification of *IGF1R* has been reported in residual disease following neoadjuvant chemotherapy in patients with TNBC, suggesting a potential contribution of IGF-IR signaling to chemotherapy resistance [159]. Lastly, expression of IGF-IR in TNBC was found to correlate with shorter disease-free survival [160]. These findings demonstrate that IGF-IR signaling promotes tumor progression in TNBC, and that IGF-IR signaling inhibition may be a valuable target in TNBC.

2.4.2 Role of FGFR signaling

FGFR1 amplification was identified in approximately 10% of all breast cancers [161]. FGFR1 is the most commonly amplified receptor of the FGFR family in TNBC, documented in approximately 9% of cases studied [96]. Moreover, about 2% and 4% of breast cancer and TNBC, respectively, harbour *FGFR2* amplifications [162]. FGFR signaling has been shown to promote proliferation in TNBC cells lines [163]. Accordingly, inhibition of FGFR resulted in tumor growth reduction in a TNBC xenograft model, supporting the oncogenic role of FGFR signaling in TNBC. FGFR signal transduction can induce EMT via the PI3K/Akt pathway to downregulate the activity of glycogen synthase kinase 3 beta (GSK3β), resulting in stabilization and nuclear translocation of β-catenin [164]. Blockade of FGFR signaling resulted in decreased invasiveness and migration of TNBC cells *in vitro* [165]. In the clinical setting, increased FGFR1 expression, as determined by IHC has been reported to associate with worse overall survival in TNBC patients [165]. Furthermore, *FGFR1/2* amplifications were identified in TNBC residual disease after chemotherapy [159]. Interestingly, FGFR-driven resistance to therapy has also been described in other breast cancer subtypes. Hanker et al. [166] reported copy number gain of *FGF3/4/19* and increased FGFR signaling as a mechanism of resistance to combination lapatinib and trastuzumab therapy in HER2-positive xenograft models.

Overcoming intrinsic and acquired resistance to targeted therapies in TNBC patients remain a challenge due to the complexity of the disease. Unsuccessful trials investigating targeted therapies, such as EGFR inhibitors, highlight the identification of response biomarkers to specific drugs is critical to selecting appropriate patient populations.

Subgroups	Gene ontologies
Basal-like 1 (BL1)	Cell cycle progression and proliferation High Ki67 DNA damage response (Ataxia Telangiectasia Mutated and Rad3 Related (ATR)/BRCA pathway)
Basal-like 2 (BL2)	Growth factor signaling (EGF, Met, IGF-IR, nerve growth factor (NGF), canonical wnt, pathway) Glycolysis and gluconeogenesis
Mesenchymal (M)	EMT and cell migration (TGFβ, Rac1/Rho, Wnt/β-catenin signaling) Cell differentiation Growth factor signaling (e.g. IGF-IR, FGFR, anaplastic lymphoma kinase (ALK) pathway) Proliferation-associated genes
Mesenchymal stem-like (MSL)	EMT and cell migration Cell differentiation Growth factor signaling } Similar to the M subgroup Additional growth factor signaling (e.g. EGFR, PDGFR) Angiogenesis (e.g. VEGFR2) Stem cell markers (e.g. ALDHA1, CD105, and CD90) Low levels of proliferation genes
Luminal androgen receptor (LAR)	High AR expression and its downstream signaling components Luminal cytokeratin expression
Immunomodulatory (IM)	T-cell associated genes Antigen processing and presentation Cytokine signaling

Table 2.1. Molecular subgroups of TNBC and their gene expression characteristics.

CHAPTER 3:

UPREGULATED FGFR1 SIGNALING IS ASSOCIATED WITH RESISTANCE IN TRIPLE NEGATIVE BREAST CANCER CELLS TREATED WITH AN IGF-TRAP

Identification of a Resistance Mechanism to IGF-IR Targeting in Triple Negative Breast Cancer Cells

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Running title: Resistance mechanisms to IGF-IR targeting

3.1 Abstract

Triple negative breast cancer (TNBC) is associated with poor prognosis. TNBC does not respond to targeted therapy currently used for other BC subtypes and chemotherapy remains the primary treatment option. There is therefore an unmet need to develop effective therapy for TNBC. The type I insulin-like growth factor (IGF-I) axis plays a critical role in breast cancer progression by conveying survival and growth signals. Our laboratory reported on the production of the IGF-Trap, an IGF-I receptor (IGF-IR) decoy that reduces the bioavailability of IGF-I and -II, thereby limiting IGF signaling. When human TNBC MDA-MB-231 cells were xenotransplanted into nude mice and treated with the IGF-Trap, we observed variability in the response, as it ranged from complete tumor regression to disease stabilization and tumor progression in some mice. This suggested that MDA-MB-231 cells are heterogeneous in respect to their sensitivity to IGF-IR signaling blockade. The aim of the present study was to identify predictive marker(s) of response and acquired resistance mechanism(s) to the IGF-Trap in human TNBC MDA-MB-231 cells. We show here that MDA-MB-231 cells express multiple tyrosine kinase receptors (RTKs) and corresponding growth factors. We also show that MDA-MB-231 cells were heterogeneous in respect to IGF-IR expression levels and that IGF-IR expression levels may be a determinant of sensitivity to the IGF-Trap. Prolonged exposure of MDA-MB-231 cells to the IGF-Trap *in vitro* resulted in the selection of a resistant population with the ability to sustain proliferation in the presence of the IGF-Trap. Using an activated RTK array, we identified increased expression of activated fibroblastic growth factor receptor 1 (FGFR1) in the resistant MDA-MB-231 cells and increased expression and activation of FGFR1 in these cells was confirmed by qPCR and Western blotting. Moreover, IGF-Trap resistant cells had an increased sensitivity to the FGFR1-specific tyrosine kinase inhibitor (TKI), PD166866, as compared to the parental cells, and the addition of

the IGF-Trap had an additive effect in reducing cell viability in parental MDA-MB-231 cells. Collectively, our data suggest that levels of IGF-IR expression may predict sensitivity to the IGF-Trap and that dual IGF-IR and FGFR1 targeting may overcome TNBC cells resistance to IGF-axis inhibition.

Key words: triple negative breast cancer, IGF signaling, the IGF-Trap, predictive marker, acquired resistance, FGFR1

3.2 Introduction

Breast cancer is the second most common cancer overall, and the most common cancer type in women worldwide, with 1.67 million cases diagnosed in 2012 alone [1]. Fifteen to twenty percent of all breast cancer cases are diagnosed as TNBC. TNBC is more frequently diagnosed in younger (<40 years of age), African-American and Hispanic women [2-5]. Clinically, TNBC refers to tumors that show absence of ER and PR expression, and lack of HER2 overexpression or amplification, as determined by immunohistochemistry and *in situ* hybridization, respectively [6-9]. Six distinct transcriptional molecular subtypes that harbor different pathway enrichments have been identified in TNBC [10], reflecting the complexity of this disease subtype. Unlike hormone receptor-positive and HER2-overexpressing breast cancers, there is currently no approved targeted therapy for TNBCs due to their intra- and inter-heterogeneity, and chemotherapy remains the primary treatment option [6, 9]. Due to its aggressive nature and lack of treatment options, TNBC patients in general have poorer overall survival, shorter disease-free survival and increased risk of relapse within the first 3 years following initial diagnosis than patients with other breast cancer subtypes [4, 11]. Therefore, there is an unmet need to develop improved therapy for TNBC.

The IGF axis is critical in normal development and physiology by conveying survival and

growth signals through the Ras/Raf/MAPK and the PI3K/Akt pathways [12-14]. Dysregulated IGF-IR signaling has been implicated in malignant progression in multiple cancer types [15], including TNBC. A study by Davison et al. [16] showed that IGF-I alone was sufficient to induce growth and survival in TNBC cells, and tumor regression was observed in TNBC xenografts treated with an IGF-IR/IR TKI. Additionally, increased phosphorylation of IGF-IR and activation of its downstream substrates, IRS-1 and Shc was documented in TNBC specimens compared to normal breast tissues [17]. A study by Law et al. [18] reported that 41.9% of TNBC specimens analyzed expressed activated IGF-IR and IR. Moreover, IGF-I and IGF-IR overexpression were associated with increased incidence of metastasis and decreased survival rate in TNBC patients [19]. Together, these studies provided evidence for the involvement of IGF-IR signaling in TNBC pathogenesis and identified the IGF axis as a potential target in this disease.

Results from clinical trials evaluating the efficacy of IGF-IR inhibitors, including mAbs against IGF-IR and IGF-IR/IR TKIs, have been discouraging. Studies have revealed that the therapeutic effects of these two classes of inhibitors may be limited by several factors. For instance, the former could induce compensatory IR-A growth signaling elicited by IGF-II [20, 21], and the latter could disrupt insulin uptake and glucose metabolism resulting in hyperinsulinemia and hypoglycemia due to the cross-reactivity with IR [22].

Our laboratory reported on the production of the IGF-Trap, a soluble fusion protein comprised of the extracellular domain of human IGF-IR fused to the Fc portion of human IgG_1 [23]. The IGF-Trap reduces the bioavailability of circulating and locally produced IGF-I/II, thereby limiting IGF-IR signaling [23]. IGFs-neutralizing agents may have superior therapeutic potential to IGF-IR mAbs and IGF-IR/IR TKIs because they prevent IGF-II/IR-A signaling without inhibiting the metabolic function of IR-B. We found that the response of human TNBC

MDA-MB-231 xenografts in nude mice to the IGF-Trap was variable ranging from complete tumor regression to disease stabilization and tumor progression in some mice [23]. This suggested that MDA-MB-231 cells are heterogeneous in respect to sensitivity to IGF-IR signaling blockade.

The objective of the present study was to identify molecular marker(s) that could predict sensitivity to the IGF-Trap in clonal populations of MDA-MB-231 cells, as well as potential resistance mechanisms that provide TNBC cells with alternate survival pathways in the presence of IGF-IR signaling blockade.

3.3 Material and Methods

Cells. The human breast cancer cell line MDA-MB-231 was a generous gift from Dr. Peter Siegel's (The Goodman Cancer Center, McGill University, QC, Canada). Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (10,000 U/ml).

Reagents. The IGF-IR rabbit monoclonal antibody was from Abcam (Cambridge, MA, USA). Rabbit monoclonal antibodies to pIGF-IR and pAKT were from Cell Signaling Technology (Danvers, MA, USA). The monoclonal mouse antibody to β-actin was from Sigma Aldrich (Oakville, Ontario, Canada). The rabbit polyclonal antibodies to pERK, ERK, AKT, pFGFR1, FGFR1 were from Cell Signaling Technology. Construction of the IGF-Trap was described in detail previously [23]. PD166866, a small molecule TKI specific to FGFR1, was from Sigma Aldrich.

Establishment of MDA-MB-231 clonal populations. Clonal subpopulations of MDA-MB-321 cells were isolated by limiting dilution cloning. Parental MDA-MB-231 cells were diluted to 0.8 cell/well and seeded into 96-well plates. Colonies that emerged from single cells were isolated and

expanded for subsequent experiments.

Development of IGF-Trap-resistant MDA-MB-231 cells. MDA-MB-231 cells were cultured in 24-well plates in the presence of increasing concentrations of 40-315 μg/ml IGF-Trap in complete DMEM. The IGF-Trap was replenished every 48 hours. When the cells reached 90% confluency, they were trypsinized and re-plated. When the remaining cells were able to reach confluency under the same dose of the IGF-Trap for 3-4 passages, the dose was doubled. The process was repeated until resistance to 315 μg/ml of the IGF-Trap was observed. A batch of cells that developed resistance to this dose over time was frozen and aliquots used in the experiments described. Hereafter, this subline is referred to as MDA-MB-231-R.

RNA isolation. RNA was isolated using TRIzol Reagent (Ambion, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. 1ml of TRIzol was added to homogenized samples and incubated until the solution became viscous. 0.2 ml chloroform were added and the solution mixed using a shaker. The extracts were then centrifuged at 13,000 rpm for 15 minutes in 4°C. RNA was precipitated with isopropanol and the pellets were washed with 75% ethanol. The pellets were air-dried and suspended in RNase-free water.

RT-PCR. Reverse transcription was performed using a standard protocol and the primers listed in Table 3.1 [23]. cDNA was amplified using 40 cycles, each consisting of 30 seconds at 94°C, 30 seconds at 55, 58, or 60°C (depending of specific primers), and 1 minute at 72°C. Amplified products were analyzed using electrophoresis (2% agarose gel) and visualized using the ImageQuant LAS 4000 imager (GE Healthcare Life Sciences, Baie d'Urfe, QC, Canada).

Quantitative Real time PCR. RNA isolation and reverse transcription were performed as described above. qPCR was performed using a standard protocol as previously described [24]. Amplification

of the cDNA was performed using FastStart Universal SYBR Green Master (Rox) (Roche, Laval, Quebec, Canada) and a PCR mixture containing 0.8μM of each of the indicated primers (Table 3.1) and 2μl of cDNA in a LightCycler (Bio-Rad Laboratories, Mississauga, ON, Canada). The data were analyzed using the iQ5 software (Bio-Rad Laboratorties).

Western blotting. Proteins (100 μg) were resolved in 6 or 8% SDS-polyacrylamide gels under reducing conditions. The proteins were transferred onto PDVF membranes and blocked in 5% skim milk-TBST at room temperature for 1 hour. Membranes were then probed, first at 4°C overnight with the indicated primary antibodies and then at RT for 2 hours with HRP-conjugated secondary antibodies (1:10000). Bands were visualized using the Amersham ECL prime/select Western blotting detection reagent (GE Healthcare Life Sciences). Primary antibody concentrations used were as follow: pIGF-IR, IGF-IR, pFGFR1 and FGFR1 were diluted 1:500, pERK, ERK pAKT, AKT were diluted 1:1000, β-actin was diluted 1:2000.

RTK arrays. The Proteome Profiler™ Human Phospho-RTK antibody arrays kit (R&D Systems, Burlington, Ontario, Canada) was used to identify activated receptor tyrosine kinases in the MDA-MB-231 cells. Cells of the parental MDA-MB-231 line and MDA-MB-231-R cells were seeded in 6-well plates and allowed to adhere overnight. The cells were starved overnight then treated with the IGF-Trap (315 μg/ml) in complete medium for 72 hours. Cells were lysed and 300 μg of cell lysates were used in each array. Cell lysis, and analysis of the lysates were performed according to the manufacturer's instructions.

IGF-I stimulation. MDA-MB-231 cells were seeded in complete medium and allowed to adhere overnight. The cells were serum starved the next day for 24 hours, then stimulated with 100 ng/ml IGF-I for the indicated time intervals. Cells were then lysed with RIPA buffer (50mM Tris, 150mM

NaCl, 0.1% SDS, and 1% Triton) containing protease inhibitor cocktail (Roche), sodium orthovanadate and sodium pyrophosphate.

Cellular proliferation assays. Cell proliferation was measured using the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay and by quantifying viable cell using dye exclusion with trypan blue. Cells were seeded in 48 or 96 well plates ($1x10^4$ cells/well for trypan blue exclusion and $3x10^3$ cells/well for the MTT assay, respectively) and allowed to adhere in complete DMEM overnight. Cells were then starved overnight and treated with the indicated concentrations of the IGF-Trap and/or PD166866 in the presence of serum for the indicated time intervals. The agents and medium were replenished every 48 hours. For the MTT assay, MTT (5 mg/ml) was added to each well and incubated for 2 hours at 37°C. Medium was then removed and the formazan crystals were dissolved with DMSO. Absorbance was measured at 565 nm. For cell counting, cells were trypsinized and counted using a hemocytometer.

Cell Cycle analysis. Cell cycle alterations in MDA-MB-231 and MDA-MB-231-R cells treated with PD166866 were determined by flow cytometry. The cells were seeded in 6-well plates ($5x10^4$ cells/well) and allowed to adhere overnight. The cells were then starved for 24 hours before treated PD166866 (10μM) or vehicle in the presence of serum for 72 hours. Subsequently, the medium and cells were collected, and centrifuged at 1000rpm for 5 minutes. The pelleted cells were washed twice with PBS and fixed with 70% ethanol at 4°C for 20 minutes. The DNA of the fixed cells was stained with proprium iodide (20 μg/ml) and treated with RNase A (100 μg/ml) for 2 hours at 4°C in dark. The cells were then passed through cell strainers (100μm) before analysis. Cell cycle distribution was determined by analyzing at least 10000 events with FACSCanto (BD Biosciences, San Jose, CA, USA). The data were analyzed using ModFit software (Verity Software House, Topsham, ME, USA).

Statistical Analysis. The results were expressed as mean ± SE of at least three experiments. Statistical analyses were performed either with the two-tailed student's t-test or with one-way ANOVA using. $p<0.05$ was considered as statistically significant.

3.4 Results

3.4.1 MDA-MB-231 cells express various tyrosine kinase receptors and ligands.

MDA-MB-231 cells are classified as the MSL subtype of TNBC with enriched expression of genes associated with growth factor signaling [10]. To identify RTKs in these cells that could potentially provide survival and growth signaling in the presence of IGF-IR blockade, we first profiled the receptor tyrosine kinase and ligands repertoires in these cells using RT-PCR. We found that in addition to IGF-IR, MDA-MB-231 cells also express EGFR, c-Met, and FGFR1 (Fig. 3.1A), a detectable level of EGF and relatively high level of FGF1 (Fig. 3.1B). RTKs and ligands expressed in these cells are summarized in Table 3.2.

3.4.2 IGF-I activates ERK and Akt signaling in MDA-MB-231 cells, and IGF-IR signaling inhibition by the IGF-Trap reduces the proliferation of these cells.

Signaling of MDA-MB-231 cells in response to IGF-I was next investigated. Serum-starved cells were stimulated with 100 ng/ml IGF-I for 5-20 min at which time the cells were lysed and the receptor and downstream signaling analyzed. Ligand-induced IGF-IR activation was observed within 5 minutes, peaking at 10 minutes and still detectable at 20 min post stimulation (Fig. 3.2A and B). Correspondingly, ERK phosphorylation could also be observed at 5 minutes, peaking at 15 min and declining at 20 minutes post stimulation (Fig. 3.2A and C). Interestingly, a basal level of constitutive ERK activation was observed in these cells, although IGF-I stimulation

resulted in a further increase in ERK phosphorylation. This constitutive activation is consistent with the presence of *BRAF* and *KRAS* mutations in these cells [10]. A low constitutive level of phosphorylated Akt was also observed and the addition of IGF-I resulted in further Akt activation that was maximal at 20 minutes post stimulation (Fig. 3.2A and D). Together, these results confirmed that MDA-MB-231 cells were responsive to IGF-I, triggering signaling through the Raf/MEK/ERK and PI3K/Akt pathways in these cells.

Next, the effect of the IGF-Trap on the proliferation of MDA-MB-231 was examined. MDA-MB-231 cells were incubated with increasing concentrations (20, 40, 80, 160, and 315 μg/ml) of the IGF-Trap in the presence of serum for 3 days before analysis using the MTT assay. MDA-MB-231 cells were sensitive to the IGF-Trap as evidenced by the significant reduction in proliferation when treated with concentrations of ≥80 μg/ml with up to 40% reduction in proliferation as compared to the vehicle-treated control at a concentration of 315 μg/ml (Fig. 3.3A and B). The growth inhibitory effect of the IGF-Trap was dose-dependent, suggesting that MDA-MB-231 cells were dependent on IGF-IR signaling for proliferation.

3.4.3 Clonal subpopulations of MDA-MB-231 cells express different levels of IGF-IR, and IGF-Trap sensitivity correlates with IGF-IR expression levels.

To determine whether the divergent sensitivity to the IGF-Trap was related to intrinsic heterogeneity of IGF-IR expression levels in clonal subpopulations of MDA-MB-231 cells, we isolated clonal populations of MDA-MB-231 cells using limited dilution cloning. We first investigated IGF-IR expression levels in 12 clones using RT-PCR (Fig. 3.4A and B). This revealed a range of IGF-IR expression levels in MDA-MB-231 clones. Clone 17 (C17) and clone 50 (C50) showed lower and higher level of IGF-IR expression, respectively, as compared to the parental

cells (Fig. 3.4A and B). This was confirmed using qPCR and Western blotting (Fig. 3.4C and D), identifying C17 as a "low IGF-IR expressor" and C50 as a "high IGF-IR expressor". These results suggested that MDA-MB-231 cells were heterogeneous in respect to IGF-IR expression level.

To determine whether IGF-IR expression levels were predictive of sensitivity to the IGF-Trap, we measured cellular proliferation of these clones in the absence or presence of the IGF-Trap. Treatment of parental MDA-MB-231 and C50 cells with the IGF-Trap (160 μg/ml) for 72 hours resulted in a significant growth inhibition of both cells (Fig. 3.5), indicating that proliferation of these cells was IGF-IR-dependent. Interestingly, however, the proliferation of C17 cells was not inhibited by IGF-Trap treatment (Fig. 3.5). While these findings suggest that IGF-IR expression levels may be correlated with intrinsic sensitivity to the IGF-Trap in MDA-MB-231 cells, these results need to be confirmed with a larger number of clonal populations.

3.4.4 Sustained ERK activation contributes to acquired resistance to the IGF-Trap in MDA-MB-231 cells.

To further investigate potential mechanisms that underlie the emergence of resistant cells over time as a result of long-term treatment with the IGF-Trap, we exposed MDA-MB-231 cells *in vitro* to gradually increasing concentrations of the IGF-Trap in the presence of serum for 3-4 months. This resulted in a population of MDA-MB-231 cells that was no longer sensitive to the IGF-Trap (MDA-MB-231-R) as the IGF-Trap failed to inhibit proliferation of these cells under conditions that significantly blocked the proliferation of parental MDA-MB-231 cells (Fig. 3.6A). Western blotting revealed sustained basal ERK activation levels in MDA-MB-231-R cells cultured in the presence of the IGF-Trap (Fig. 3.6B and C). In contrast, in parental cells treated with the IGF-Trap ERK phosphorylation was diminished relative to controls (Fig. 3.6B and C). The results

indicated that the loss of IGF-IR signaling dependency in MDA-MB-231 cells subjected to long-term exposure to the IGF-Trap was associated with upregulated ERK activation, likely providing IGF-independent growth signals.

3.4.5 FGF1 and FGFR1 expression and FGFR1 activation are upregulated in MDA-MB-231-R cells.

Activation of alternate RTKs has been identified as a mechanism of resistance to targeted therapies that block specific RTK signaling [25-27]. We therefore subjected MDA-MB-231-R and parental cell lysates to analysis by a phospho-RTK array to identify potential changes in activation levels of RTKs in the MDA-MB-231-R relative to the parental cells. No difference was observed in phosphorylation levels of majority of RTKs arrayed. The exception was FGFR1 as it was preferentially phosphorylated in MDA-MB-231-R cells as compared to the parent line (Fig. 3.7A). This was further confirmed by Western blotting, which FGFR1 activation was upregulated (1.8-fold) in the resistant population relative to the parental cells when treated with the IGF-Trap (Fig. 3.7B-C). FGFR1 and FGF1, a ligand of FGFR1, mRNA expression were significantly upregulated in MDA-MB-231-R (1.96- and 1.6-fold, respectively) as compared to parent MDA-MB-231 cells (Fig. 3.7D and E). Lastly, we examined whether the level of IGF-IR expression was different between the selected and unselected populations. MDA-MB-231-R had a significantly lower level of IGF-IR expression than MDA-MB-231 cells (Fig. 3.7F), lending support to our finding that IGF-Trap sensitivity may be correlated with IGF-IR expression levels. Collectively, these data suggest that acquired resistance to the IGF-Trap in MDA-MB-231 cells was associated with increased FGFR1 expression and activation, possibly through an autocrine FGFR signaling pathway.

3.4.6 The FGFR inhibitor PD166866 decreases the proliferation of MDA-MB-231 and MDA-MB-231-R cells in a dose-dependent manner.

To evaluate the effect of FGFR1 signaling inhibition on cell proliferation, we treated MDA-MB-231 and MDA-MB-231-R cells with increasing concentrations (0.1-75 μM) of the FGFR1-specific TKI, PD166866, in the presence of serum for 72 hours. PD166866 reduced the proliferation of both populations in a dose-dependent manner (Fig. 3.8A and B). However, while proliferation was suppressed in both MDA-MB-231 and MDA-MB-231-R cells, the latter were more sensitive to FGFR1 inhibition as evidenced by the more marked decrease in proliferation and lower IC_{50} value as compared to the parental MDA-MB-231 cells (Fig. 3.8A and B). Namely, we observed a 2-fold reduction in the IC_{50} relative to MDA-MB-231 cells from 31.4 μM to 60.6 μM, respectively (Fig. 3.8A). These results suggest that the proliferation of MDA-MB-231 and MDA-MB-231-R cells was promoted by FGFR1 signaling, and the IGF-Trap-resistant population was more dependent on this pathway for growth.

3.4.7 FGFR1 signaling inhibition induces cell cycle arrest and apoptosis in MDA-MB-231-R cells.

To further examine the role of FGFR1 signaling in resistance to the IGF-Trap, we investigated the effects of FGFR1 signaling inhibition on cell cycle progression. MDA-MB-231 and MDA-MB-231-R cells were treated with PD166866 (10 μM) for 72 hours before they were subjected to cell cycle analysis, using proprium iodide staining and flow cytometry. PD166866 induced significant G0/G1 phase arrest in both MDA-MB-231 (Fig. 3.9A-B, E) and MDA-MB-231-R (Fig. 3.9C-D, F) cells relative to the respective vehicle-treated controls. Correspondingly, significantly lower proportion of cells transitioned to S phase when treated with PD166866 in both cell lines (Fig. 3.9A-F). This suggests that G0/G1 to S phase transition was promoted by FGFR1

signaling in these cells. Importantly, PD166866 alone induced apoptosis in MDA-MB-231-R, but not in the parental cells (Fig. 3.9G), suggesting an increased dependency on FGFR1 signaling for survival in MDA-MB-231-R cells. Taken together, these results showed that both MDA-MB-231 and MDA-MB-231-R cells were dependent on FGFR1 signaling for S phase entry and FGFR1 signaling was a survival factor for MDA-MB-231-R cells.

3.4.8 The combination of IGF-Trap and a FGFR1 signaling inhibitor increases MDA-MB-231 growth suppression in an additive manner.

We next tested whether combining the IGF-Trap with FGFR1 inhibition could enhance the growth inhibitory effect of the IGF-Trap. MDA-MB-231 and MDA-MB-231-R cells were treated with the IGF-Trap and PD166866, alone or in combination, and their effects on proliferation were analyzed. PD1668866 (10 μM) and the IGF-Trap (160 μg/ml) as single agents had modest growth inhibitory effects on MDA-MB-231 cells (25% and 20% reductions, respectively) as compared to the vehicle-treated controls (Fig. 3.10). When PD166866 and the IGF-Trap were combined, an additive anti-proliferative effect was seen in MDA-MB-231 cells resulting in a marked and significant reduction in their proliferation (Fig. 3.10). MDA-MB-231-R cells were more sensitive to PD166866 than MDA-MB-231 as their proliferation was significantly suppressed (50%) in the presence of this inhibitor alone (Fig. 3.10), consistent with our findings that these cells had an increased dependency on FGFR1 signaling for proliferation. However, PD166866 did not re-sensitize MDA-MB-231-R cells to the IGF-Trap (Fig. 3.10), possibly because of the reduced IGF-IR expression in these cells. These data showed that the combination of the IGF-Trap and FGFR1 inhibition was efficacious and superior to either agent alone in suppressing the growth of MDA-MB-231 cells.

3.5 Discussion

The IGF-Trap is a novel cancer treatment candidate, a soluble fusion protein comprised of the extracellular domain of human IGF-IR fused to the Fc portion of human IgG that binds bioavailable IGF-I/II, thereby inhibiting IGF-IR signaling [23]. We previously reported that human TNBC MDA-MB-231 xenografts showed varied susceptibility when treated with the IGF-Trap [23], suggesting that subpopulations within the tumor may have different levels of dependency on IGF signaling for growth and some may rely on alternate growth-promoting pathways. The aim of this study was to identify biomarker(s) of response to the IGF-Trap and elucidate mechanism(s) of resistance to IGF-IR signaling inhibition by the IGF-Trap in TNBC cells. We found that human TNBC MDA-MB-231 cells expressed several growth factors (EGF and FGF1) and TK receptors (IGF-IR, EGFR, c-Met, FGFR1) (Fig. 3.1 A and B). IGF-I, HGF, and FGF2 mRNA could not, however, be detected. We also showed that MDA-MB-231 were responsive to IGF-I, as ERK and PI3K/Akt signaling were activated in these cells in response to IGF-I stimulation (Fig. 3.2). We further demonstrated that the IGF-Trap suppressed the growth of MDA-MB-231 cells in a dose-dependent manner (Fig. 3.3). Similar findings have been reported by Davison et al. [16] who showed that the addition of IGF-I resulted in ERK and Akt activation in multiple TNBC cell lines including MDA-MB-231 cells. In that study, IGF-I was also identified as a survival factor for TNBC cells in the presence of staurosporine.

Disappointing results from clinical trials with agents that target the IGF axis [28-30] highlight the need for predictive markers for patient selection. Here, we showed that MDA-MB-231 clonal populations had a range of IGF-IR expression levels at both mRNA and protein levels (Fig. 3.4). Furthermore, we showed a potential correlation between IGF-IR expression levels in clonal populations of MDA-MB-231 cells and intrinsic sensitivity to the IGF-Trap. The IGF-Trap induced greater growth inhibitory effect in MDA-MB-231 clonal populations with high IGF-IR

expression than in those that expressed low levels of IGF-IR (Fig. 3.5). TNBC subtypes express different levels of IGF-IR and it is especially enriched in the BL2, M and MSL subtypes [10]. Whether IGF-IR expression levels correlate with sensitivity to IGF-IR signaling inhibition in subtypes of TNBC remains unclear. Our data suggest that IGF-IR expression levels may have predictive value in intrinsic sensitivity to the IGF-Trap in TNBC cells and warrant further investigation of this correlation. Therefore, tumor IGF-IR levels may have value in stratifying TNBC patients for IGF-Trap treatment.

Considerable effort has been made to identify biomarkers of sensitivity to therapies targeting the IGF-axis. IGF-IR expression level has been proposed as one of the predictors of response to such treatments. In agreement with our findings, a study identified an association between IGF-IR mRNA levels and response to an IGF-IR mAb in a large panel of breast cancer cell lines [31]. Importantly, IGF-IR mRNA expression alone was not indicative of sensitivity. Similar data have been reported in rhabdomyosarcoma cells tested both *in vitro* and *in vivo* [32, 33]. Consideration of other molecules that contribute to IGF signaling such as IGF-II and IRS-1 was shown to improve response prediction when considered together with IGF-IR expression levels in rhabdomyosarcoma cells [33]. Another study found that IGF-IR protein but not mRNA levels predicted response to an IGF-IR/IR TKI in breast cancer cell lines [34]. Litzenburger et al. [35] examined the correlation between response to an IGF-IR/IR TKI and IGF-IR activity, as determined by a panel of genes regulated by IGF-I, in TNBC cell lines. The authors reported that IGF-IR signaling was active in most of the TNBC cells and level of IGF-IR activity was associated with response. However, it is important to note that IGF-IR expression and/or activation levels have been reported to have no predictive value in some studies that utilized patient samples, possibly due to the differences in sample preparation and analytical methods [36, 37]. Our data

show that while IGF-IR expression per se does not predict response, cells with high expression levels do show greater sensitivity and high expression may therefore be a useful predictor of response.

Because anti-cancer therapies that target a single RTK may result in rapid emergence of resistant cells, it is essential to identify underlying molecular mechanisms that could render cells resistant to RTK targeting. To this end, we generated a MDA-MB-231 subline resistant to the IGF-Trap by continuous long-term incubation of the cells with increasing concentrations of the IGF-Trap *in vitro*. We found that in response to chronic exposure to the IGF-Trap, dependence on IGF-IR signaling was lost in MDA-MB-231-R cells as evidenced by the ability to proliferate and sustained ERK activation in the presence of the IGF-Trap (Fig. 3.6A-C). This loss of dependence was associated with downregulated IGF-IR expression in MDA-MB-231-R cells (Fig. 3.7F), further evidence that IGF-IR expression levels may determine sensitivity to the IGF-Trap. Furthermore, upregulated FGFR1 and FGF1 expression (Fig. 3.7D-E), as well as upregulated FGFR1 signaling were observed in MDA-MB-231-R cells (Fig. 3.7A-C). Whether the increased FGFR1 activation was autocrine, possibly through FGF1, or a ligand-independent mechanism remains to be explored. FGFR1 signaling inhibition was able to induce cell cycle arrest in MDA-MB-231 and MDA-MB-231-R cells as evidenced by the significantly higher proportion of cells arrested in G0/G1 phase accompanied by significant lower fraction of cells in S phase when treated with PD166866 (Fig. 3.9A-F). Furthermore, both MDA-MB-231 and MDA-MB-231-R cells were sensitive to FGFR1 inhibition (Fig. 3.8). However, the ability of PD166866 to induce apoptosis and have a more marked inhibitory effect on proliferation, also reflected in reduced IC_{50} in MDA-MB-231-R relative to MDA-MB-231 cells (Fig. 3.8, 3.9G and 3.10) provide evidence that the IGF-Trap resistant cells were more sensitive to FGFR1 inhibition. This suggests that FGFR1 signaling

was involved in providing a growth-promoting and survival mechanism to these cells rendering them, in turn, resistance to the IGF-Trap (Fig. 3.11). PD166866 had similar ability in suppressing cell cycle progression in MDA-MB-231-R and the parental cells, the increased anti-proliferation effects observed in MDA-MB-231-R cells (Fig. 3.8 and 3.10) may be partly due to the ability of this inhibitor to trigger apoptosis in these cells (Fig. 3.9G). Lastly, PD166866 did not re-sensitize MDA-MB-231-R cells to the IGF-Trap (Fig. 3.10). This may be a result of downregulated IGF-IR expression, and consequently, insensitivity to the IGF-Trap.

The mechanism underlying the emergence of a FGFR1-enriched and IGF-Trap resistant subpopulation of MDA-MB-231 cells remains unclear. To our knowledge, no direct crosstalk between IGF-IR and FGFR1 has been reported and IGF-IR has not been identified as a regulator of FGFR expression, although IGF-IR signaling was reported to induce expression of genes that positively regulate FGFR in breast epithelial cells [38]. The potential direct communication between the two receptors will therefore be interesting to explore. Alternatively, it is possible that clonal selection occurred under the selection pressure of the IGF-Trap, where subpopulations with low IGF-IR expression and constitutive FGFR1 signaling were more likely to survive. Supporting this hypothesis, divergent FGFR1 expression was observed in clonal subpopulations of MDA-MB-231 cells (Supp. Fig. 3.1).

The oncogenic role of FGFR signaling in TNBC has been described in several studies. Of interest, a recent study by Sharpe et al. [39] demonstrated that autocrine FGFR signaling could promote growth of TNBC cells both *in vitro* and *in vivo*. Moreover, amplification of *FGFR1/2* was proposed to confer chemotherapy resistance in TNBC [40]. Clinically, FGFR1 expression was reported to be a predictor of poor overall survival in TNBC patients [41]. Importantly, FGFR signaling was reported to mediate resistance to various cancer treatments in different cancer types.

Huang et al. [27] identified amplification, overexpression, and constitutive activation of PDGFRα as the major acquired resistance mechanism to BMS-754807, an IGF-IR/IR TKI, in rhabdomyosarcoma Rh41 cells. In addition to PDGFRα, however, upregulated FGF9 and FGFR2 expression were also observed in Rh41 cells with acquired resistance to BMS-754807 relative to parent Rh41 cells, suggesting that FGFR signaling may also have a role in acquired resistance to IGF-IR targeting therapies in rhabdomyosarcoma. Amplification and overexpression of FGFR1 have been linked to *de novo* tamoxifen-resistance in luminal type breast cancers cell lines [42]. Increased ligand-dependent and ligand-independent activation of FGFR1 and downstream signaling were observed in FGFR1-overpexressing cell lines, and re-sensitization to tamoxifen was achieved when FGFR1 expression was silenced, providing evidence that resistance to hormonal therapy could be mediated by overexpression and/or activation of FGFR1. Recently, Manchado et al. [43] identified upregulated FGFR1 expression and activation as an adaptive compensatory mechanism of proliferation and survival in lung cancer cells with KRAS mutations treated with the MEK inhibitor trametinib *in vitro*. Furthermore, when patient-derived xenografts of KRAS-mutated lung adenocarcinomas were treated with trametinib, increased activation of fibroblast growth factor receptor substrate 2 (FRS2), a major downstream substrate of FGFR was observed.

Finally, we observed modest growth suppression in MDA-MB-231 cells treated with the IGF-Trap or PD166866 alone at concentrations of 160 μg/ml and 10 μM, respectively (Fig. 3.8 and 3.10). This may reflect the intra-clonal heterogeneity of the cell line and indicate that different MDA-MB-231 subpopulations are dependent on different signaling pathways for growth. The concept of intra-tumor heterogeneity is well accepted, and combination therapies targeting multiple pathways may therefore be required to improve therapeutic efficacy. Our results showing

additive and significant anti-proliferation effects in MDA-MB-231 cells treated with combination IGF-Trap and PD166866 (Fig. 3.10) suggest that subpopulations of the parental MDA-MB-231 cells may be dependent on IGF-IR, FGFR1 or both for their proliferation.

In the present study, we showed that IGF-IR expression levels is a potential determinant of intrinsic sensitivity to the IGF-Trap in human TNBC MDA-MB-231 cells and this is worthy of further study. We also showed that upregulated and increased dependency on FGFR1 signaling were associated with IGF-Trap treatment resistance in these cells, and that combining the IGF-Trap with a FGFR1 inhibitor increased the growth inhibitory response seen with either agent alone. The therapeutic efficacy of this combination therapy remains to be verified *in vivo* and may provide a promising therapeutic strategy in TNBC patients.

3.6 Figure legends

Fig. 3.1. MDA-MB-231 cells express multiple growth factor receptors. Shown in (A) and (B) are results of RT-PCR analyses of growth factor receptors (A) and growth factors (B) expressed in MDA-MB-231 cells. Human liver tissue (huLiver) was used as positive control in (B). Two primer sets were used for each growth factor receptor. The expected transcript size is shown on the right.

Fig. 3.2. MDA-MB-231 cells are responsive to IGF-I. Cells were serum starved for 24 hours then stimulated with 100 ng/ml IGF-I for the indicated time intervals. Shown in (A) is a representative Western blot for IGF-IR and downstream signaling following IGF-I stimulation. Shown in the bar graphs are mean (and SE) of 3 experiments expressed as pIGF-IR/IGF-IR (B), pERK/ERK (C), and pAkt/Akt (D) ratios normalized to levels in unstimulated cells that were assigned a value of 1.

Fig. 3.3. The IGF-Trap inhibits the proliferation of MDA-MB-231 cells. Cells were treated

with the indicated concentrations of the IGF-Trap in the presence of serum for 3 days. Proliferation was measured by the MTT assay. The anti-proliferative effect of the IGF-Trap is shown as a (A) dose response curve and (B) as a bar graph. Shown are the means ± SE (n=3) expressed as % (A) and fold change (B) relative to control, vehicle-treated cells that were assigned a value of 100 and 1, respectively. *p<0.05, **p<0.01, ***p<0.001.

Fig. 3.4. Clonal populations of MDA-MB-231 express divergent IGF-IR levels. IGF-IR expression levels in clonal subpopulations of MDA-MB-231 cells were initially analyzed by RT-PCR. Shown in (A) are representative RT-PCR results of 2 independent experiments. (B) Levels of IGF-IR expression were normalized to GAPDH and expressed as relative IGF-IR expression levels to parental MDA-MB-231 cells that were assigned a value of 1. IGF-IR expression levels in clones C17 and C50 relative to the parental cells were also analyzed by qPCR (C) and Western blotting (D). The IGF-IR expression levels of each clone were based on 2 separate immunoblots (D). Data Shown in (C) were normalized to GAPDH and are expressed as means ± SE, n=4. ***p < 0.001.

Fig. 3.5. The sensitivity of two clonal sublines of MDA-MB-231 to the IGF-Trap correlates with IGF-IR expression levels. Parent MDA-MB-231, C17, and C50 cells in complete medium were incubated with the IGF-Trap (160 μg/ml) for 72 hours. Cell proliferation was determined by the MTT assay. Shown are means ± SE of 3 experiments expressed as fold change relative to control vehicle-treated cells that were assigned a value of 1. *p<0.05, **p<0.01.

Fig. 3.6. MDA-MB-231-R cells are resistant to the growth inhibitory effects of the IGF-Trap. (A) MDA-MB-231 and MDA-MB-231-R cells in complete medium were treated with the IGF-Trap (315 μg/ml) for 72 hours. Cell proliferation was measured by the MTT assay. Shown are means ± SE of based on 5 experiments and expressed as fold change relative to the respective

vehicle-treated controls that were assigned a value of 1. ERK activation in MDA-MB-231 and MDA-MB-231-R cells treated with the IGF-Trap or vehicle for 3 days was analyzed by Western blotting. Shown in (B) is a representative immunoblot of 3 separate experiments and in (C) means ± SE of the results of 3 immunoblots expressed as fold change in ratio of pERK/ERK relative to vehicle-treated MDA-MB-231 and MDA-MB-231-R cells that were assigned a value of 1. *p<0.05, **p<0.01, ***p<0.001.

Fig. 3.7. FGF1 and FGFR1 expression and FGFR1 activation levels are upregulated in IGF-Trap resistant MDA-MB-231-R cells. (A-B) MDA-MB-231 and MDA-MB-231-R cells were treated with the IGF-Trap at the indicated concentrations in the presence of serum for 72 hours. A phopsho-RTK array was then used to profile phosphorylated RTKs in total cell lysates derived from these cells, revealing a specific increase in FGFR1 activation in MDA-MB-231-R cells (A). Shown in (B) is an immunoblot of pFGFR1 and FGFR1 levels in these cells and in (C) a bar graph expressed as pFGFR1/FGFR1 ratios normalized to the level in vehicle-treated MDA-MB-231 cells that was assigned a value of 1. qPCR (D-F) was also used to analyze the expression levels of FGFR1 (D), FGF1 (E) and IGF-IR (F) in these cells. Data in (D-F) were normalized to GAPDH and are expressed as means ± SE (n=4) of fold change in transcript expression in MDA-MB-231-R cells relative to MDA-MB-231 cells that were assigned a value of 1. *p<0.05, **p<0.01, ***p<0.001.

Fig. 3.8. The anti-proliferative effect of the inhibitor PD166866 on MDA-MB-231 and MDA-MB-231-R cells is dose-dependent. MDA-MB-231 and MDA-MB-231-R cells in complete medium were treated with PD166866 at the indicated concentrations for 72 hours. Cell proliferation was measured by the MTT assay. Shown in (A) is a dose response curve and in (B) a bar graph of the growth suppression effect of PD166866. Shown are means ± SE of the results of

3 experiments expressed as % (A) and fold change (B) relative to the respective vehicle-treated controls that were assigned a value of 100 and 1, respectively. IC_{50} values were calculated by nonlinear regression analysis using GraphPad Prism 5.0. *p < 0.05, ***p<0.001.

Fig. 3.9. Effects of PD166866 on cell cycle progression in MDA-MB-231 and MDA-MB-231-R cells. Shown in (A-B) are representative flow cytometry data for cell cycle phase and apoptosis analysis performed on MDA-MB-231 and MDA-MB-231-R (C-D) cells treated with PD166866 (10μM) or vehicle in complete medium for 72 hours. The cells were fixed after treatment and cell cycle progression was analysed after proprium iodide staining using flow cytometry. Shown are the proportions of MDA-MB-231 (E) and MDA-MB-231-R cells (F) in each of the cell cycle phases. Shown in (G) are proportions of apoptotic cells in each treated population. Results in (E-G) are expressed as means ± SE (n=3) of percent of total cells in G0/G1, S, and G2 phase (E-F) and in (G) percent of all cells analyzed .*p<0.05, **p<0.01, ***p<0.001.

Fig. 3.10. Effect of tumor cells treatment with the FGFR1 inhibitor PD166866 in combination with the IGF-Trap. MDA-MB-231 and MDA-MB-231-R cells in complete medium were treated for 48 hours with the IGF-Trap (160 μg/ml), PD166866 (10 μM), or a combination of both. Cell proliferation was determined by cell counting using the exclusion dye trypan blue. Results are expressed as means ± SE (n=3) of fold change in cell number per well, relative to the respective untreated control cells that were assigned a value of 1. *p<0.05.

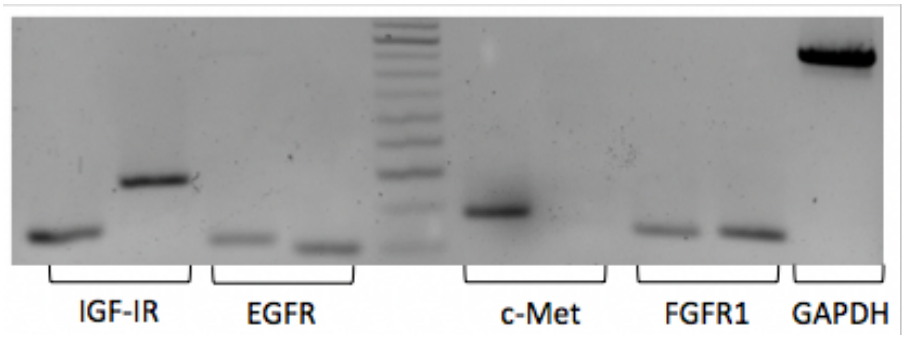
Fig. 3.11. A proposed model for the mechanism of IGF-Trap resistance in MDA-MB-231 cells based on our data. The IGF-Trap inhibits IGF-IR signaling, and consequently, decreases ERK phosphorylation and cell proliferation in parent MDA-MB-21 cells (Left). MDA-MB-231 cells continuously incubated with the IGF-Trap lose their sensitivity to the growth inhibitory

effects of the Trap, due to increased autocrine FGFR1 signaling and FGFR1 mediated ERK activation, likely through increased FGF1 expression (Right).

Supp. Fig. 3.1. MDA-MB-231 clonal populations have different levels of FGFR1 expression. FGFR1 expression levels in clonal populations of MDA-MB-231 were measured by RT-PCR. Shown are representative results of 2 analyses.

3.7 Figures

A



B

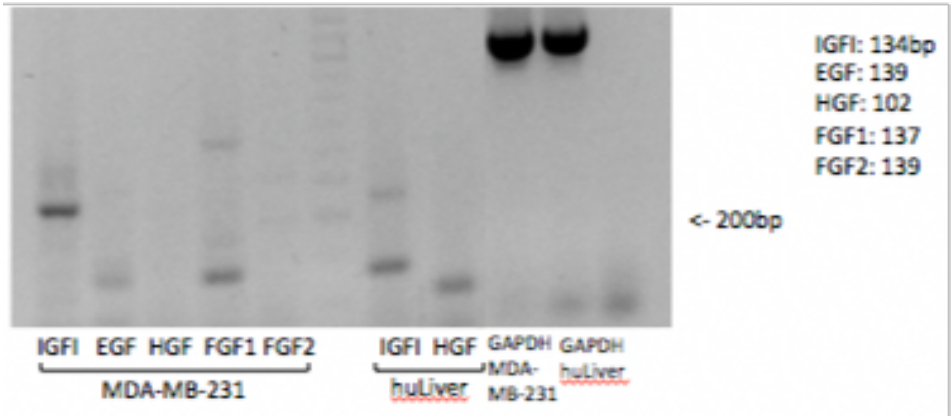


Fig. 3.1.

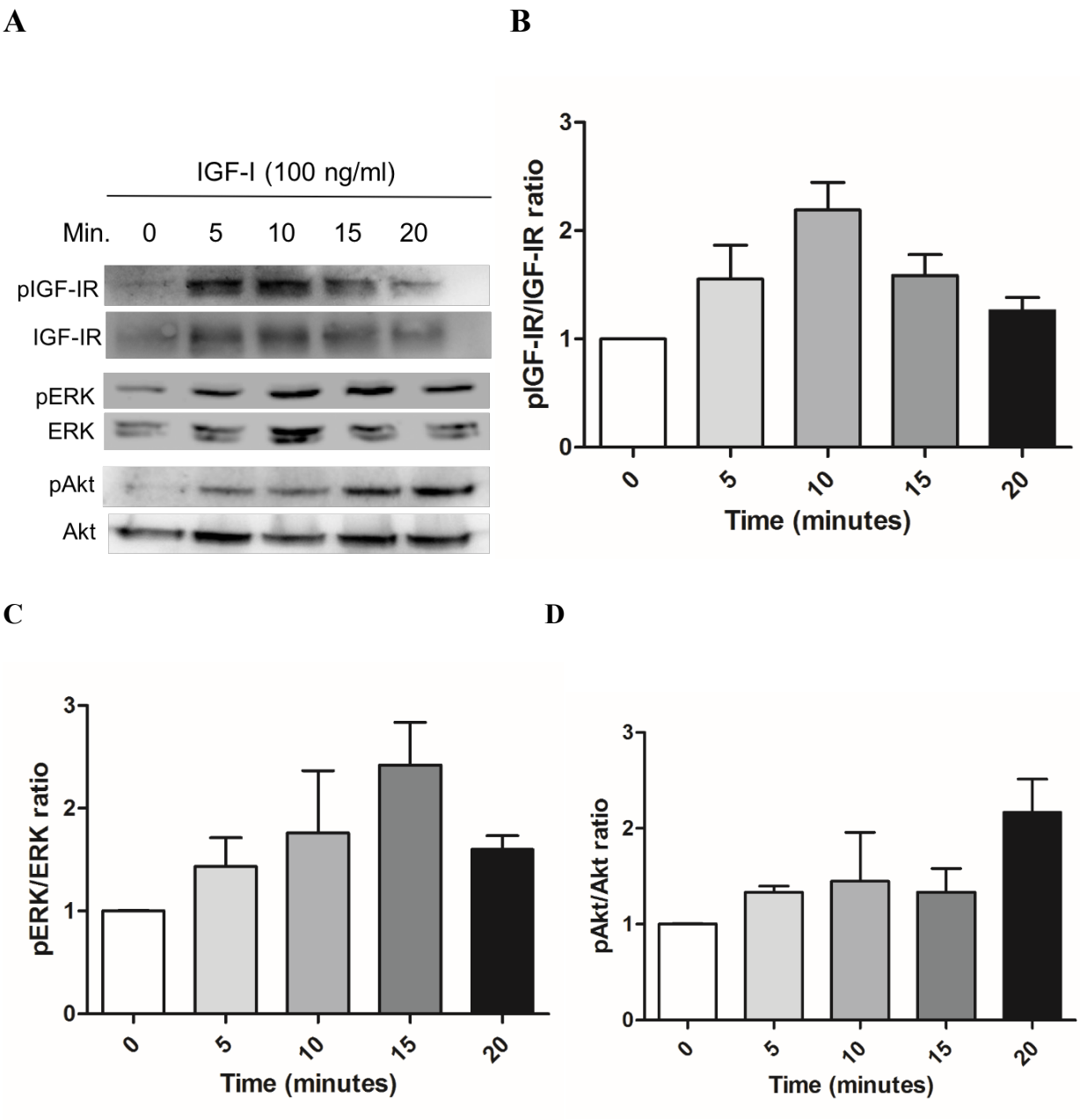
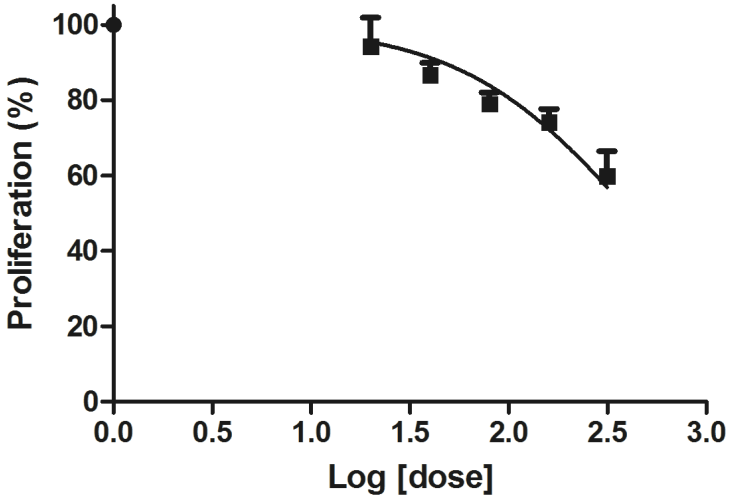

A
B
IGF-I (100 ng/ml)
Min. 0 5 10 15 20
pIGF-IR
IGF-IR
pERK
ERK
pAkt
Akt
pIGF-IR/IGF-IR ratio
Time (minutes)
C
D
pERK/ERK ratio
pAkt/Akt ratio
Time (minutes)
Time (minutes)

Fig. 3.2.

A


Proliferation (%)
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Log [dose]

B

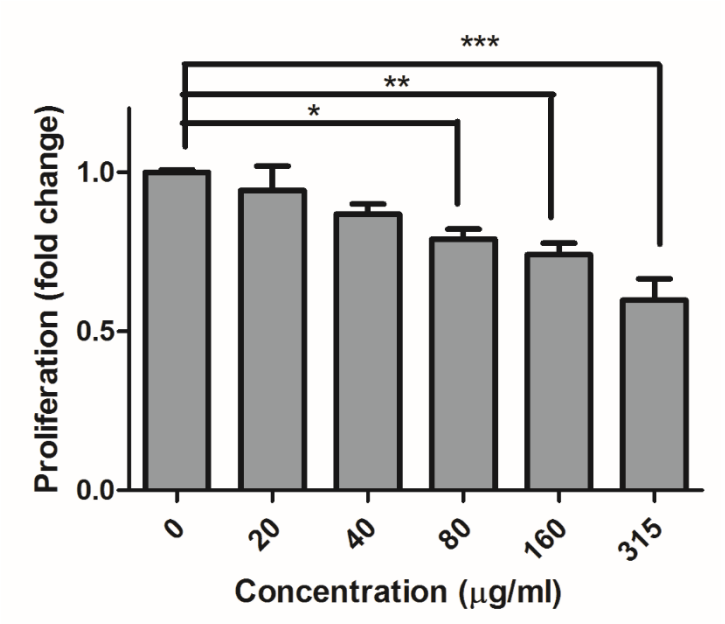

***
**
*
Proliferation (fold change)
1.0
0.5
0.0
0
20
40
80
160
315
Concentration (μg/ml)

Fig. 3.3.

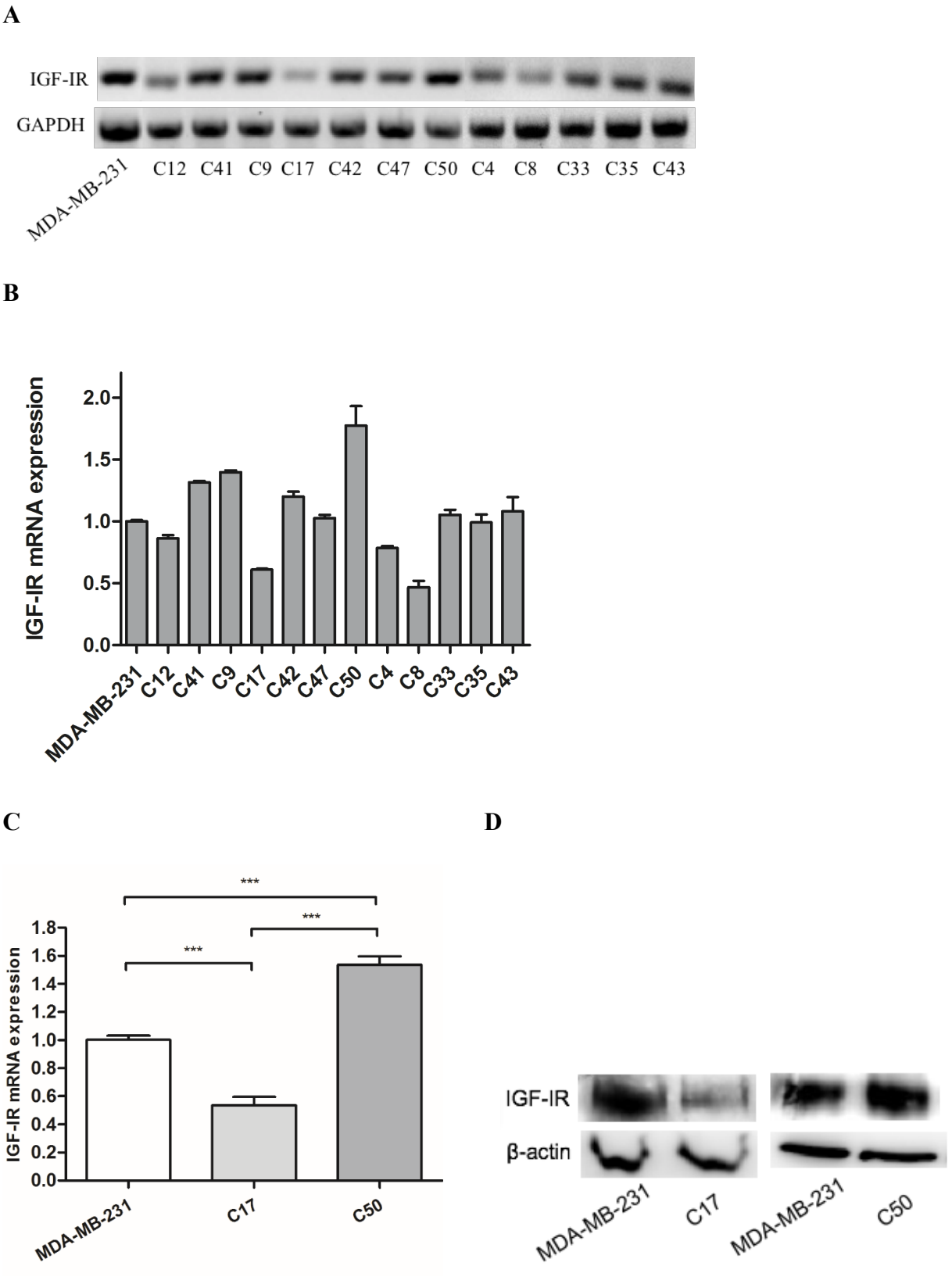

A
IGF-IR
GAPDH
MDA-MB-231
C12
C41
C9
C17
C42
C47
C50
C4
C8
C33
C35
C43
B
IGF-IR mRNA expression
2.0
1.5
1.0
0.5
0.0
MDA-MB-231
C12
C41
C9
C17
C42
C47
C50
C4
C8
C33
C35
C43
C
IGF-IR mRNA expression
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
***
***
MDA-MB-231
C17
C50
D
IGF-IR
β-actin
MDA-MB-231
C17
MDA-MB-231
C50

Fig. 3.4.

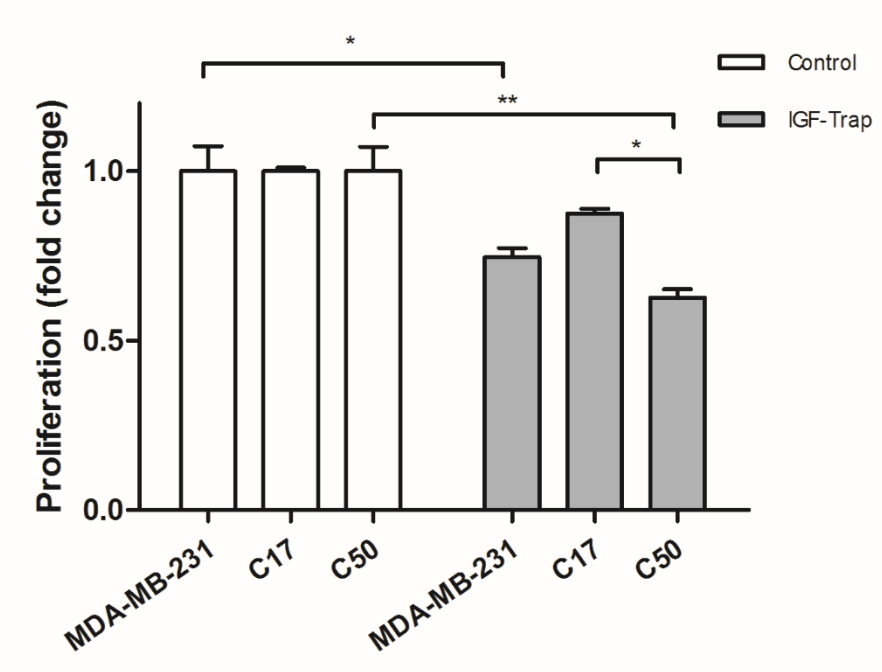

*
**
*
Control
IGF-Trap
Proliferation (fold change)
1.0
0.5
0.0
MDA-MB-231
C17
C50
MDA-MB-231
C17
C50

Fig. 3.5.

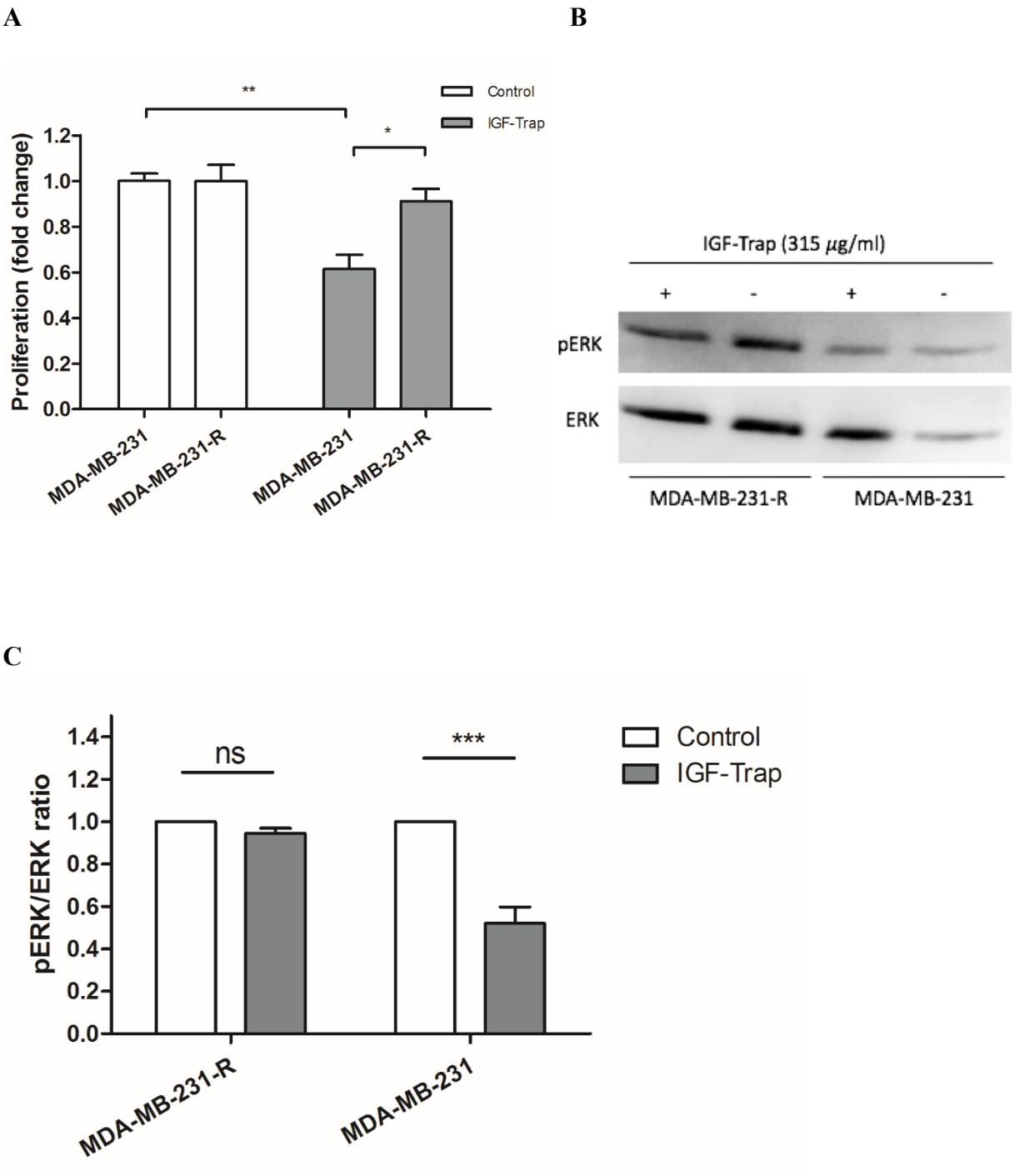
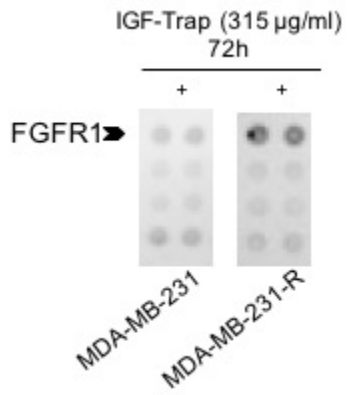
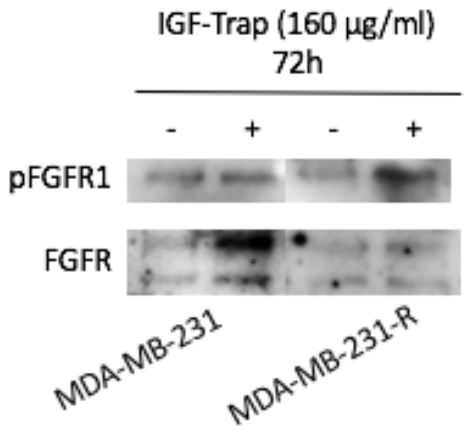

A
B
C
Control
IGF-Trap
Proliferation (fold change)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
*
MDA-MB-231
MDA-MB-231-R
MDA-MB-231
MDA-MB-231-R
IGF-Trap (315 μg/ml)
+
-
+
-
pERK
ERK
MDA-MB-231-R
MDA-MB-231
pERK/ERK ratio
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ns
***
Control
IGF-Trap
MDA-MB-231-R
MDA-MB-231

Fig. 3.6.

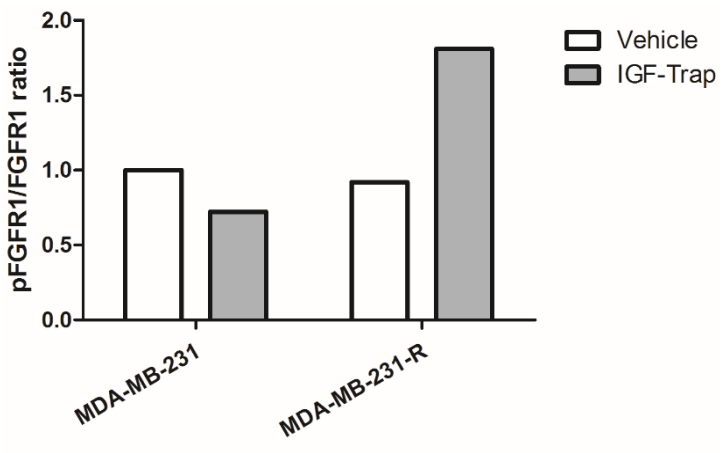
A


IGF-Trap (315 µg/ml)
72h
+
+
FGFR1
MDA-MB-231
MDA-MB-231-R

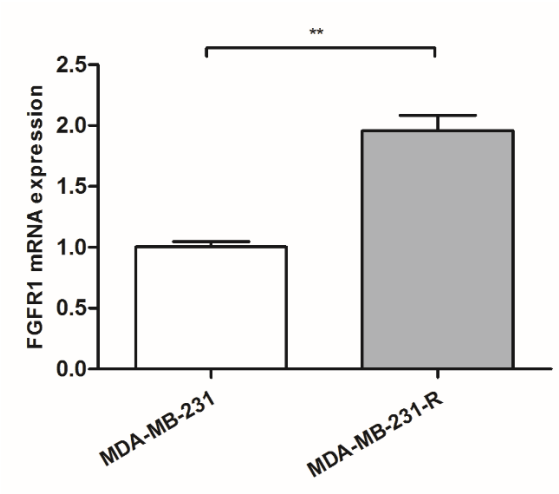
B


IGF-Trap (160 µg/ml)
72h
-
+
-
+
pFGFR1
FGFR
MDA-MB-231
MDA-MB-231-R

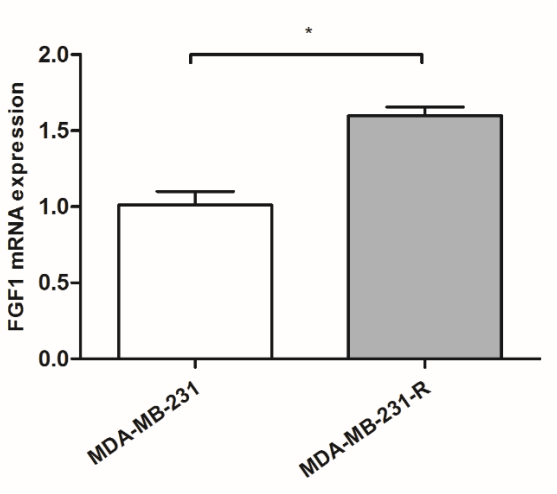
C


pFGFR1/FGFR1 ratio
2.0
1.5
1.0
0.5
0.0
Vehicle
IGF-Trap
MDA-MB-231
MDA-MB-231-R

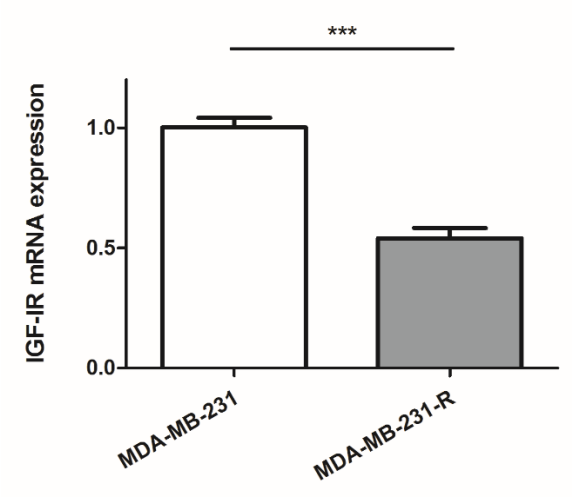
D


**
FGFR1 mRNA expression
2.5
2.0
1.5
1.0
0.5
0.0
MDA-MB-231
MDA-MB-231-R

E


*
FGF1 mRNA expression
2.0
1.5
1.0
0.5
0.0
MDA-MB-231
MDA-MB-231-R

F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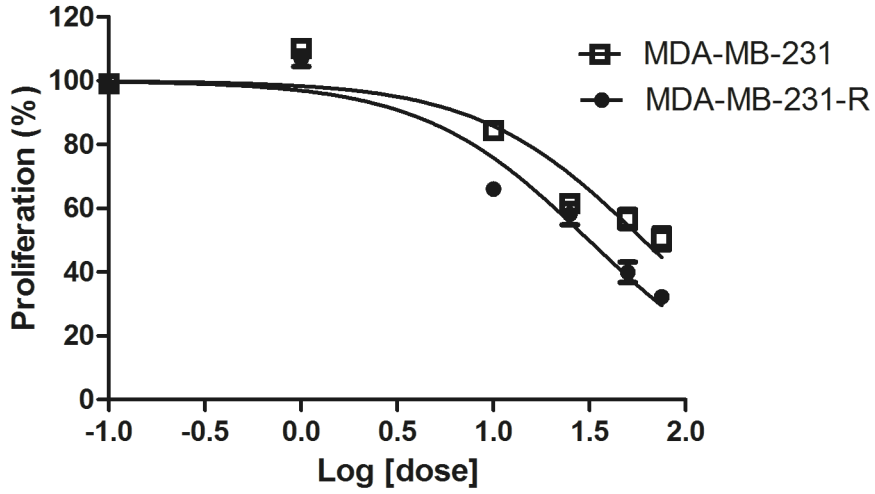
IGF-IR mRNA expression
1.0
0.5
0.0
MDA-MB-231
MDA-MB-231-R

Fig. 3.7.

A


MDA-MB-231
MDA-MB-231-R
Proliferation (%)
120
100
80
60
40
20
0
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
Log [dose]

B

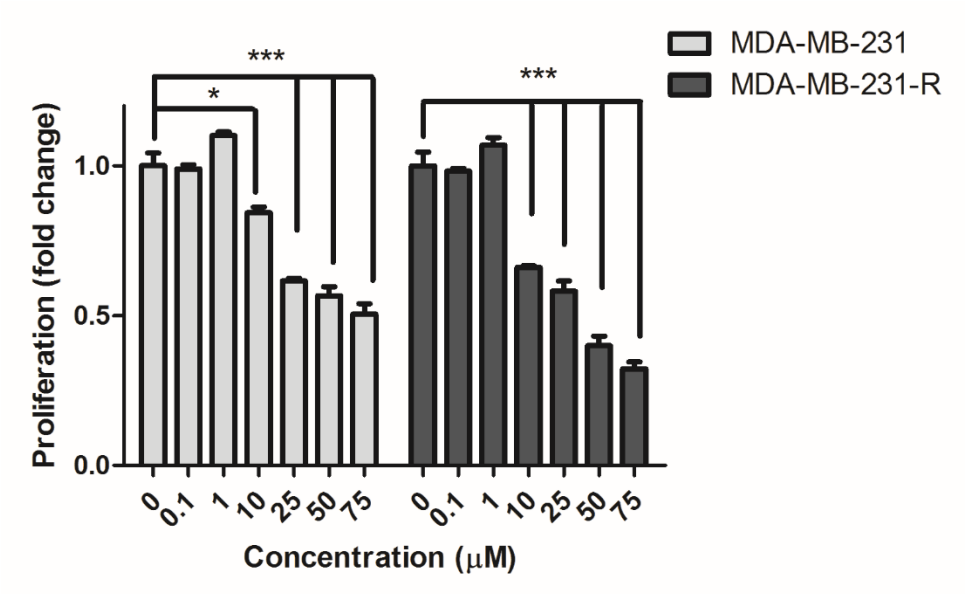
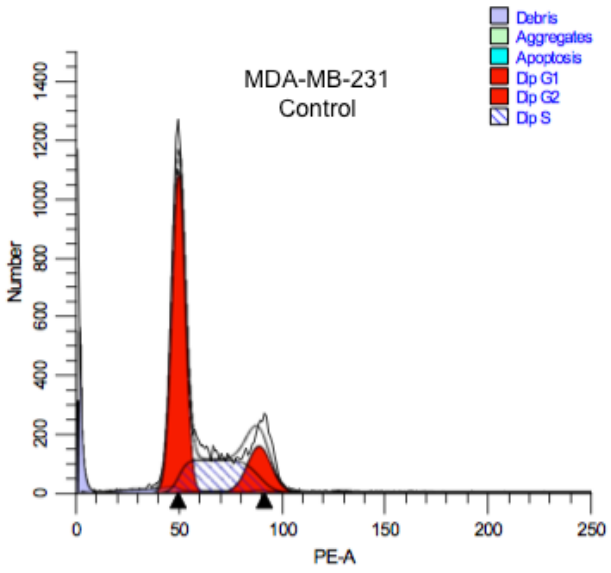
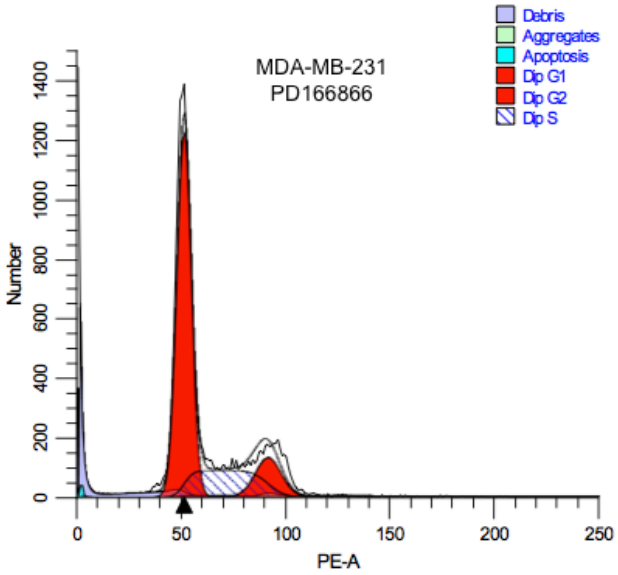

MDA-MB-231
MDA-MB-231-R
***
*
***
Proliferation (fold change)
1.0
0.5
0.0
0
0.1
1
10
25
50
75
0
0.1
1
10
25
50
75
Concentration (μM)

Fig. 3.8.

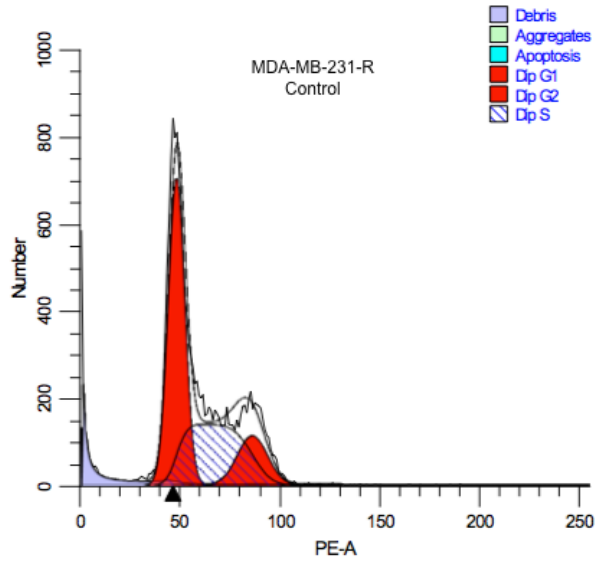
A


MDA-MB-231
Control
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
PE-A

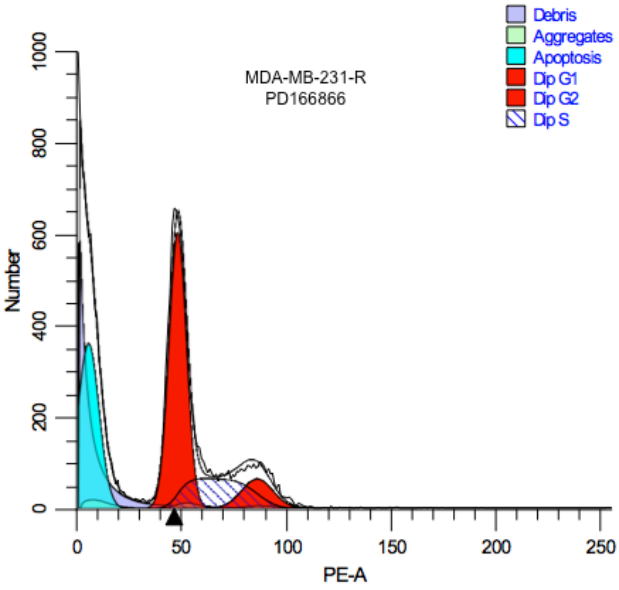
B


MDA-MB-231
PD166866
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
PE-A

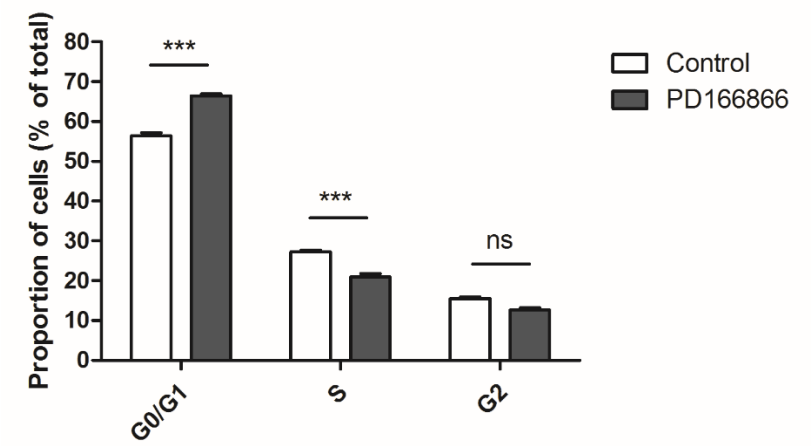
C


MDA-MB-231-R
Control
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
PE-A

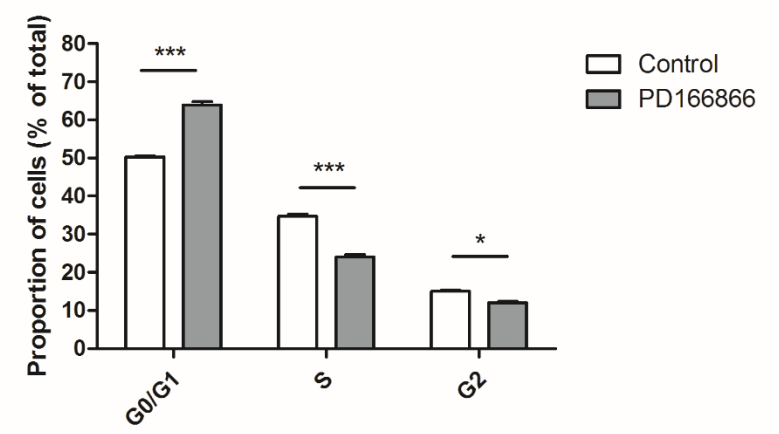
D


MDA-MB-231-R
PD166866
Debris
Aggregates
Apoptosis
Dip G1
Dip G2
Dip S
Number
PE-A

E


Proportion of cells (% of total)
80
70
60
50
40
30
20
10
0
***
***
ns
G0/G1
S
G2
Control
PD166866

F


Proportion of cells (% of total)
80
70
60
50
40
30
20
10
0
***
***
*
G0/G1
S
G2
Control
PD166866

G

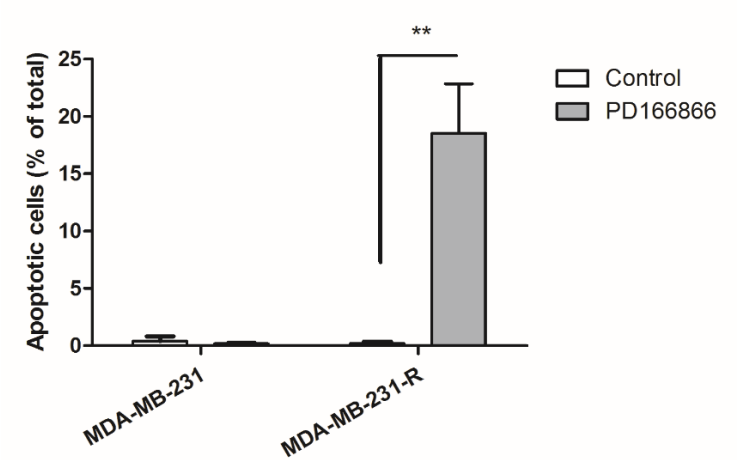

Apoptotic cells (% of total)
25
20
15
10
5
0
**
MDA-MB-231
MDA-MB-231-R
Control
PD166866

Fig. 3.9.

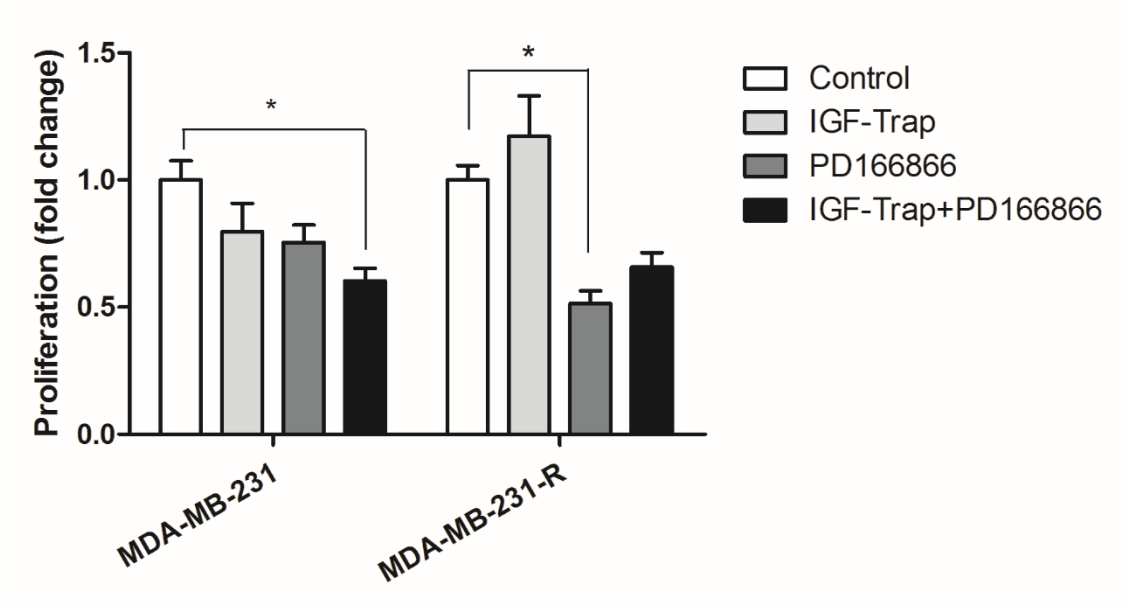

Proliferation (fold change)
1.5
1.0
0.5
0.0
*
*
MDA-MB-231
MDA-MB-231-R
Control
IGF-Trap
PD166866
IGF-Trap+PD166866

Fig. 3.10.

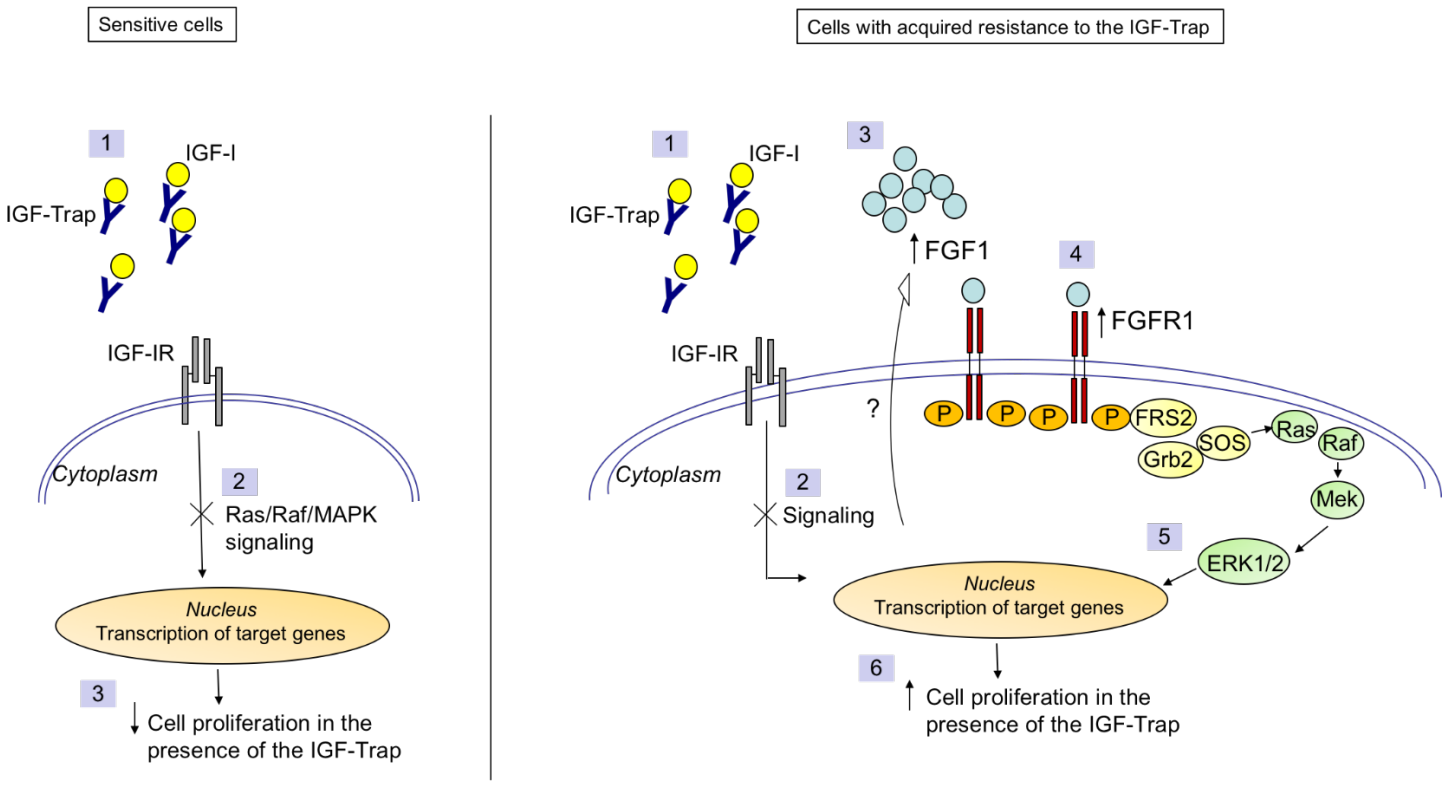
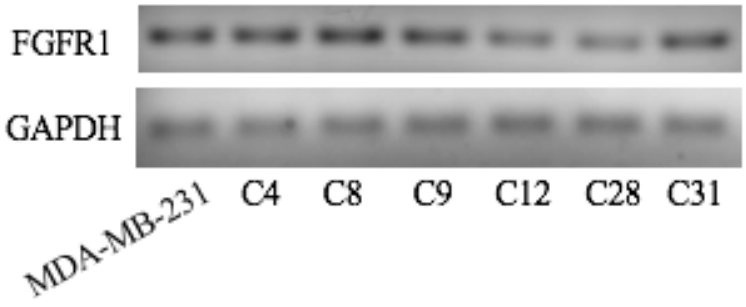


Fig. 3.11.



Supp. Fig. 3.1

Gene	Primer Sequence
IGF-IR Forward (pair 1)	CCTGCACAACTCCATCTTCGTG
IGF-IR Reverse (pair 1)	CGGTGATGTTGTAGGTGTCTGC
IGF-IR Forward (pair 2)	ACGCCAATAAGTTCGTCCACAGAGACCT
IGF-IR Reverse (pair 2)	GAAGACTCCATCCTTGAGGGACTCAG
EGFR Forward (pair 1)	AACACCCTGGTCTGGAAGTACG
EGFR Reverse (pair 1)	TCGTTGGACAGCCTTCAAGACC
EGFR Forward (pair 2)	ACCTGCGTGAAGAAGTGTCC
EGFR Reverse (pair 2)	CGTCTTCCTCCATCTCATAGC
Met Forward (pair 1)	TGCACAGTTGGTCCTGCCATGA
Met Reverse (pair 1)	CAGCCATAGGACCGTATTTCGG
Met Forward (pair 2)	ATTTTGCTTTGCCAGTGGTGG
Met Reverse (pair 2)	GAGCGATGTTGACATGCCACT
FGFR1 Forward (pair 1)	GCACATCCAGTGGCTAAAGCAC
FGFR1 Reverse (pair 1)	AGCACCTCCATCTCTTTGTCGG
FGFR1 Forward (pair 2)	CACCCGAGGCATTATTTGAC
FGFR1 Reverse (pair 2)	AAGTTCCTCCACAGGCACAC
IGF-I Forward	CTCTTCAGTTCGTGTGTGGAGAC
IGF-I Reverse	CAGCCTCCTTAGATCACAGCTC
EGF Forward	TGCGATGCCAAGCAGTCTGTGA
EGF Reverse	GCATAGCCCAATCTGAGAACCAC
HGF Forward	GAGAGTTGGGTTCTTACTGCACG
HGF Reverse	CTCATCTCCTCTTCCGTGGACA
FGF1 Forward	ATGGCACAGTGGATGGGACAAG
FGF1 Reverse	TAAAAGCCCGTCGGTGTCCATG
FGF2 Forward	AGCGGCTGTACTGCAAAAACGG
FGF2 Reverse	CCTTTGATAGACACAACTCCTCTC
GAPDH Forward (PCR)	GGTGAAGGTCGGTGTGAACG
GAPDH Reverse (PCR)	AATGCCAAAGTTGTCATGGA
GAPDH Forward (qPCR)	TGCACCACCAACTGCTTAGC
GAPDH Reverse (qPCR)	GGCATGGACTGTGGTCATGAG

Table 3.1. Primer sequences.

Receptors		Growth factors	
IGF-IR	+	IGF-I	-
EGFR	+	EGF	Low
Met	+	HGF	-
FGFR1	+	FGF1	+
		FGF2	-

Table 3.2. Summary of receptors and growth factors expression in MDA-MB-231 cells.

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CHAPTER 4:

ADDITIONAL RESULTS

4.1 Summary

In addition to changes in the level of FGFR1 phosphorylation, decreased phosphorylation of the RTK-like protein (RYK) was observed in MDA-MB-231-R cells. Therefore, the role of RYK and WNT signaling in conferring resistance to the IGF-Trap in MDA-MB-231 cells was examined in this chapter. We showed that although the level of RYK phosphorylation was decreased, WNT5A expression was increased in MDA-MB-231-R cells. However, WNT signaling inhibition did not suppress proliferation in either MDA-MB-231 or MDA-MB-231-R cells.

4.2 Introduction

The family of WNT proteins consists of 19 secreted glycoproteins [167] that have diverse roles in regulating different cellular processes, including cell fate, stem cells renewal, cell proliferation, survival, and migration. Several classes of WNT receptors have been identified including 10 members of the frizzled (FZD) family of G-protein-coupled receptors (GPCRs), and several RTKs, including receptor tyrosine kinase like orphan receptor 1/2 (ROR1/2) and RYK. The binding of WNTs to their receptors activates signal transduction through either β-catenin-dependent ('canonical') or β-catenin-independent ('non-canonical') signaling [168, 169].

In the absence of canonical WNTs, such as WNT3A and WNT1, a β-catenin destruction complex containing adenomatous polyposis coli (APC), GSK3β, and Axin is stabilized in the cytoplasm, β-catenin is phosphorylated and this results in β-catenin ubiquitination and proteosomal degradation [170, 171]. In the absence of β-catenin translocation to the nucleus, the TCF/LEF transcription factor associates with other repressive proteins, such as histone deacetylases (HDACs) and the transcription of WNT target genes is repressed. The binding of WNTs to FZD and co-receptors LRP5/6 induces transmembrane signal transduction, and leads to phosphorylation and

activation of Dishevelled (DVL). DVL sequesters the destruction complex to the plasma membrane, preventing β-catenin phosphorylation and enabling its nuclear translocation where it can associate with TCF/LEF transcription factors, recruit transcriptional co-activators, and initiate transcription of genes, such as c-myc and cyclin D1, that regulate cell proliferation, survival, and differentiation. Non-canonical WNTs such as WNT5A and WNT11 do not mediate their effects through the β-catenin-dependent pathway. In addition to FZD receptors, β-catenin-independent signaling is also mediated through ROR1, ROR2, and RYK. Instead of regulating β-catenin, the activation of these receptors results in signaling cascades that regulate cell migration, polarity, and proliferation. The β-catenin-independent signaling has been reported to activate the planar cell polarity, MEK/ERK, and JNK/AP-1 pathways, as well as the activation of calcium-dependent signaling cascades [170, 171].

The involvement of WNTs in cancer pathogenesis was first described in mouse mammary tumor models. Nusse et al. [172, 173] reported that overexpression of WNT1 was sufficient to induce spontaneous mammary hyperplasia and tumorigenesis in mice. Additionally, inherited inactivating mutations in APC, a negative regulator of β-catenin, predispose carriers to familial adenomatous polyposis that could progress to colon carcinoma when combined with other gene mutations, such as *KRAS* and *p53* [174]. Studies have reported that the consequence of aberrant WNT signaling was context-dependent, that it could both promote and inhibit tumor initiation and progression, depending on the type and stage of cancer. For example, high nuclear β-catenin expression was associated with decreased patient survival in colorectal cancer, but cytoplasmic or nuclear β-catenin expression was predictive of increased patient survival in lung cancer [175, 176]. Additionally, increased WNT1 expression was reported in early stage breast tumor specimens compared to normal breast tissues. However, WNT1 expression levels declined in high grade

breast tumors, suggesting that WNT1 may be important only in the early stage of breast cancer progression [177]. The results of these studies highlight the complexity and the context-dependency of WNT signaling. Using a phospho-RTK array, we observed a decreased level of RYK phosphorylation in MDA-MB-231-R cells. Therefore, we investigated the role of RYK and WNT signaling in providing a compensatory mechanism to the IGF-Trap in MDA-MB-231 cells.

4.3 Material and Methods

RTK arrays. The procedures performed for the Proteome Profiler™ Human Phospho-RTK antibody arrays kit was described in Chapter 3.

Reagents. LGK974, a Porcupine-specific inhibitor, was from Cayman Chemical Company (Ann Arbor, MI, USA).

Quantitative Real time PCR. RT-qPCR was performed as described in Chapter 3.

Cellular proliferation assays. Cell proliferation was measured using the MTT assay, as described in Chapter 3. Cells were treated with the indicated concentrations of LGK974 in the presence of serum for 72 hours.

4.4 Results

4.4.1 RYK phosphorylation is decreased, but WNT5A expression is increased in resistant MDA-MB-231 cells.

The phosho-RTK array revealed a decrease in RYK phosphorylation in MDA-MDA-231-R cells as compared to the parental population (Fig. 4.1A). The involvement of WNT5A in resistance to anti-cancer therapies has been reported in several studies [178, 179]. We therefore sought to investigate the expression level of RYK and WNT5A, a ligand of RYK, in MDA-MDA-231-R cells. RYK expression was unchanged in MDA-MB-231-R cells, as determined by qPCR (Fig. 4.1B). Surprisingly, we observed a significant increase (approximately 2.65-fold) in WNT5A

mRNA expression in MDA-MB-231-R cells relative to the parent cells (Fig. 4.1C), suggesting that WNT5A may have a role in contributing to the acquired resistance to the IGF-Trap in these cells.

4.4.2. WNT signaling inhibition does not suppress proliferation in MDA-MB-231 and MDA-MB-231-R cells.

Porcupine (PORCN) is a membrane bound O-acyltransferase that facilitates post-translational palmitoylation of WNT proteins [180]. These modifications are required for the processing of WNT ligand secretion. It has been reported that knockout of *Porcn* in mouse embryonic stem cells abolished WNT secretion, thereby reducing WNT signaling [181]. Here, we investigated whether inhibiting WNT secretion using a small molecule PORCN inhibitor, LGK974, could suppress the proliferation of MDA-MB-231 and MDA-MB-231-R cells. We found that LGK974 (at 1 and 10 μM) did not inhibit the proliferation of either MDA-MB-231 or MDA-MB-231-R cells relative to the respective vehicle-treated control as measured 72 hours post treatment (Fig. 4.2). A modest, but insignificant suppression was seen when the cells were treated with 25 μM LGK974. Importantly, MDA-MB-231-R cells did not show increased sensitivity to LKG974 relative to the parental cells, suggesting that WNT signaling likely did not contribute to the differential proliferation of these cells.

4.5 Discussion

We observed upregulated WNT5A expression (Fig. 4.1C) but decreased RYK phosphorylation (Fig. 4.1A) in MDA-MB-231-R cells. This may be due to decreased expression of other RYK ligands, such as WNT11. The underlying mechanisms and functional implications of these changes remain unclear. We chose to investigate WNT5A because aberrations in WNT5A and RYK expression/signaling have been associated with drug resistance in several malignancies.

For instance, a recent study by Anastas et al. [178] showed that WNT5A expression was upregulated when melanoma cells were chronically treated with a BRAF inhibitor. The authors further demonstrated that WNT5A promoted Akt signaling through FZD7 and RYK in these cells. Knockdown of either WNT5A or RYK led to decreased cell proliferation, Akt signaling, and BRAF inhibitor re-sensitization, suggesting a role of WNT5A/RYK signaling in promoting acquired resistance to BRAF inhibition in melanoma. Moreover, Hung et al. [179] observed WNT5A overexpression in breast cancer MCF-7 cells resistant to doxorubicin *in vitro*. These findings were supported by analysis of breast cancer biopsies where WNT5A expression was found to be upregulated in post-chemotherapy specimens relative to pre-chemotherapy specimens, suggesting a role for WNT5A in chemotherapy resistance. We found increased WNT5A expression in IGF-Trap resistant MDA-MB-231 cells, but LGK974 - an inhibitor of WNT signaling did not reduce cell proliferation (Fig. 4.2). This may be explained by the fact that LGK974 is not specific for WNT5A. The inhibition of other WNTs may have masked the functions of WNT5A in this context. Selectively silencing WNT5A using siRNA in MDA-MB-231-R cells may provide more specific information on the role of this ligand in contributing to the acquired resistance to the IGF-Trap. Collectively, the role of RYK and WNT5A in acquired resistance to IGF-IR signaling inhibition by the IGF-Trap in MDA-MB-231 cells remains to be elucidated and warrants further investigation.

4.6 Figures

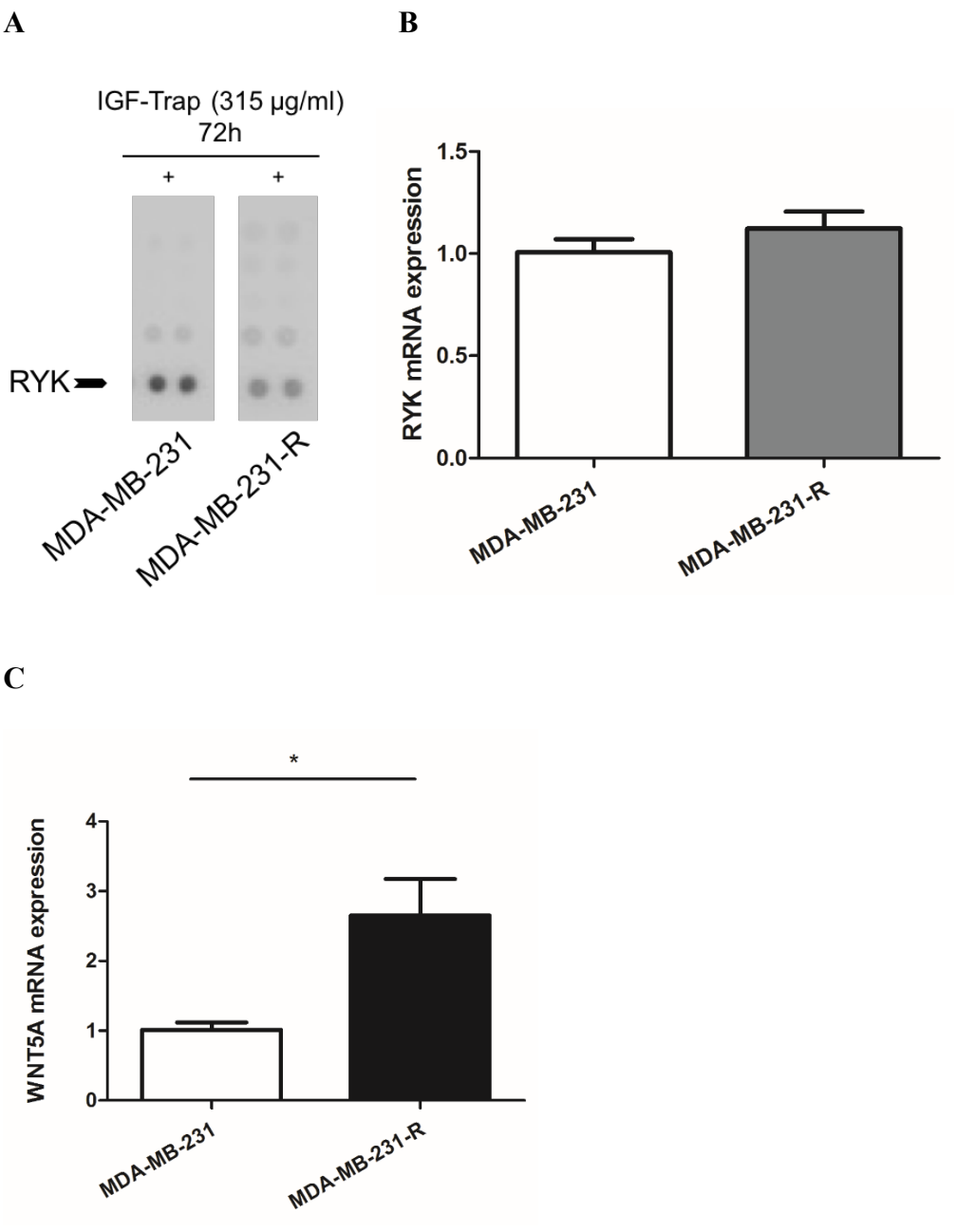

Fig. 4.1. MDA-MB-231-R cells have decreased RYK phosphorylation and upregulated WNT5A expression. (A) MDA-MB-231 and MDA-MB-231-R cells were treated with the IGF-Trap (315 µg/ml) in the presence of serum for 72 hours. Total cell lysates were then subjected to a phopsho-RTK array to assess the phosphorylation of RTKs. The array revealed decreased RYK

phosphorylation in MDA-MB-231-R cells relative to the parent cells. qPCR (B-C) was then used to examined the expression level of RYK (B) and WNT5A (C) in these cells. Data in (B-C) were normalized to GAPDH and are expressed as means ± SE (n=4) of fold change in transcript expression in MDA-MB-231-R cells relative to MDA-MB-231 cells that were assigned a value of 1. *$p < 0.05$

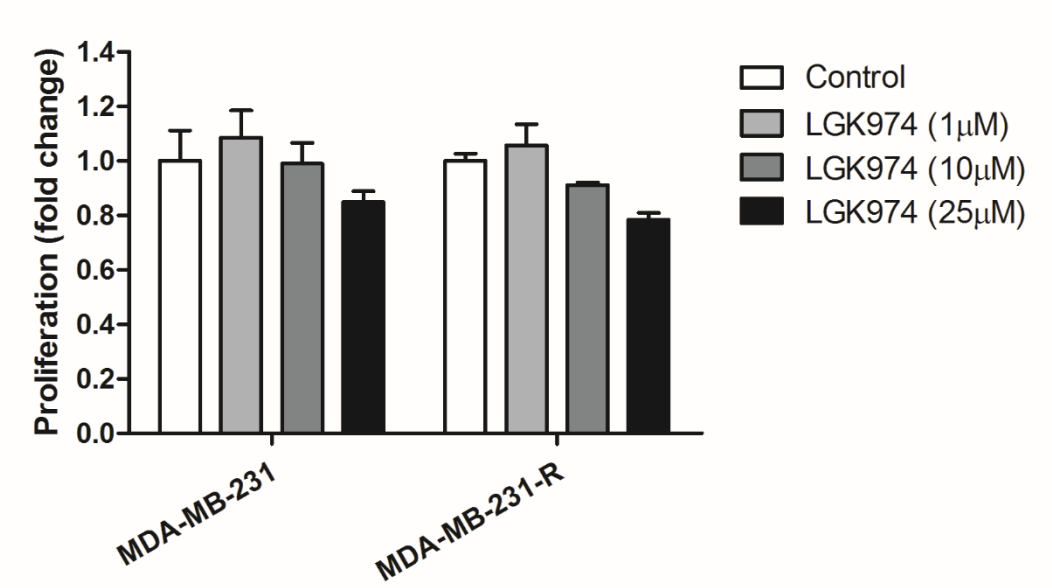

Fig. 4.2. LGK974 does not reduce the proliferation of MDA-MB-231 and MDA-MB-231-R cells. MDA-MB-231 and MDA-MB-231-R cells were treated with LGK974 (1, 10, and 25 μM) for 3 days in complete medium. Cell proliferation was determined by the MTT assay. Results are presented as means ± SE (n=3) expressed as fold change relative to control, vehicle-treated cells that were assigned a value of 1.

CHAPTER 5:

GENERAL DISCUSSION

5.1 Summary of data

In the current study, we aimed to identify predictive marker(s) of response to the IGF-Trap as well as resistance mechanism(s) in human triple negative breast cancer cells. We found that MDA-MB-231 cells expressed a range of TK receptors and corresponding ligands, including the RTKs IGF-IR, EGFR, c-Met, and FGFR1and the ligands EGF and FGF1. The Raf/MEK/ERK and the PI3K/Akt pathway were activated in response to IGF-I stimulation in these cells. Additionally, clonal subpopulations of MDA-MB-231 cells were isolated using limiting dilution cloning and we showed that these subpopulations were heterogeneous in respect to IGF-IR expression levels. Data based on 2 clones analysed to date suggested that sensitivity to the IGF-Trap may correlate with the levels of IGF-IR expression in these clonal populations. We developed an MDA-MB-231 subline resistant to the IGF-Trap by chronically exposing these cells to increasing concentrations of the IGF-Trap. While proliferation of parental MDA-MB-231 cells was significantly inhibited by the IGF-Trap, the resistant MDA-MB-231-R cells lost dependence on IGF-IR signaling and were able to sustain proliferation in the presence of the IGF-Trap. Upregulated expression and activation of FGFR1, as well as increased FGF1 expression were observed in MDA-MB-231-R cells. FGFR1 inhibition by PD166866 was sufficient to induce significant cell cycle arrest in MDA-MB-231 and MDA-MB-231-R cells. However, MDA-MB-231-R cells showed increased sensitivity to FGFR1 signaling inhibition relative to the parent cells, as evidenced by a lower IC_{50} value and reduced survival of these cells when treated with PD166866 alone. Finally, we showed that combination of the IGF-Trap and FGFR1 signaling inhibition was additive in growth inhibition and superior to either agent alone in MDA-MB-231 cells.

In addition to alteration in FGFR1 signaling, we observed a decrease in RYK phosphorylation in MDA-MB-231-R cells. However, WNT5A expression was upregulated in

these cells. LGK974 alone did not induce significant growth inhibition in MDA-MB-231 and MDA-MB-231-R cells, and these two cell lines showed similar sensitivity to this inhibitor.

5.2 Implications of the data

The clinical potential of drugs targeting the IGF axis is yet to be unlocked. A lesson to learn from the unsuccessful late phase clinical trials with this class of agents is the importance of identifying biomarker(s) predictive of response. The value of predictive markers(s) to specific drugs has been demonstrated in different cancer types, such as the use of Herceptin in HER2-positive breast cancer [182] and Vemurafenib in melanoma with BRAF V600E mutation [183]. One of the patient selection criteria in trials with Vermuafenib in melanoma patients was expression of the BRAF V600E mutation [183]. This criterion was based on preclinical studies showing that Vermurafenib had anti-tumor activity only in melanoma cells lines expressing the BRAF V600E mutation but not in cell lines with wild-type BRAF [184, 185]. Therefore, the inclusion of potential predictive biomarkers as part of patient selection criteria should be taken under consideration in future trials exploring IGF axis-targeting drugs. Trials should also collect data for potential biomarkers to be identified retrospectively. Here, we showed that response to the IGF-Trap correlated with IGF-IR expression levels in 2 clonal populations of TNBC, although confirmation of these data in a larger number of clones is still pending. IGF-IR expression levels may therefore have value in predicting response to the IGF-Trap and selecting TNBC patients for the IGF-Trap treatment.

Intrinsic and acquired resistance to anti-cancer therapies remain a challenge in the treatment of cancer patients. Activation of alternate RTKs was shown to confer resistance to RTK targeting drugs [166, 186, 187]. For instance, upregulated c-Met signaling and/or c-Met amplification has been identified as a mechanism of intrinsic and acquired resistance to drugs

that target EGFR in NSCLC [188]. Increased HER3 expression and activation, and downstream PI3K/Akt activation was reported to confer resistance to lapatinib in HER2-postive breast tumors [189]. Preclinical studies have shown that targeting multiple pathways could prevent or delay the development of drug-resistance [190]. For example, Herrea-Abreu et al. [191] showed that the addition of a PI3K inhibitor to a CDK4/6 inhibitor prevented the acquisition of resistance to the latter agent in an ER+ patient-derived tumor xenograft model, suggesting that combination therapies may be beneficial in patients, although potential increased toxicities need to be carefully considered. Furthermore, a landmark study by Gerlinger et al. [192] showed extensive intratumoral heterogeneity in all the tumors analyzed in the study, highlighting that subpopulations of a tumor may have different dependencies on different pathways and drugging multiple targets may be required to improve therapeutic efficacy. We found that increased FGFR1 signaling was associated with acquired resistance to the IGF-Trap in TNBC cells. We also showed that the MDA-MB-231 cell line was composed of heterogeneous populations in respect to IGF-IR and FGFR1 expression levels, reflecting the heterogeneity within this cell line. Combining the IGF-Trap and FGFR1 inhibition had an additive growth suppression effect in MDA-MB-231 cells, suggesting that these cells were dependent on both IGF-IR and FGFR1 for proliferation.

In addition to alteration in FGFR1 signaling, decreased phosphorylation of RYK, but upregulated expression of WNT5A were observed in MDA-MB-231-R cells. The involvement of WNT5A and RYK in mediating resistance to various types of anti-cancer therapy has been reported [178, 179]. Lehmann et al. [129] showed that multiple molecular subgroups of TNBC harboured enriched gene expression in molecules associated with WNT signaling. Moreover, activation of the β-catenin-dependent pathway was shown to be associated with the triple

negative/basal subtype of breast cancer [193, 194], suggesting a role for this pathway in the pathogenesis of these breast cancer subtypes. A recent study by Solzak et al. [195] showed that components of WNT signaling were upregulated in MDA-MB-231 cells treated with a PI3K inhibitor. The combination of a PI3K inhibitor and LGK974 was synergistic in reducing cell viability in TNBC cell lines *in vitro*. LGK974 as monotherapy is currently under investigation in a phase I trial in various cancer types including TNBC [NCT01351103]. The results of the trial are pending. We found that LGK974 alone did not inhibit the proliferation of MDA-MB-231 and MDA-MB-231-R cells under the conditions used. The potential involvement of RYK and WNT5A in resistance to the IGF-Trap in TNBC cells is presently unclear and will require further investigation.

5.3 Suggestions for future studies

To verify that the increased basal ERK activation was a consequence of increased FGFR1 signaling in MDA-MB-231-R cells, thereby providing an escape mechanism to the IGF-Trap, experiments investigating the effects of PD166866 alone and in combination with the IGF-Trap on ERK phosphorylation in MDA-MB-231 and MDA-MB-231-R cells are underway. Moreover, the anti-proliferation effect of a MEK inhibitor in these cells is under investigation. To assess the relevance of the IGF-Trap resistance profile identified *in vitro* to tumor growth and IGF-Trap resistance *in vivo*, MDA-MB-231 tumors that progress in the presence or absence of IGF-Trap treatment *in vivo* should be analyzed and their gene expression profiles compared. Additionally, to verify the additive growth suppression effect of dual IGF-IR and FGFR1 inhibition *in vivo,* the growth rate of MDA-MB-231 tumors treated with the IGF-Trap and PD166866 alone and in combination should be examined.

We also showed increased WNT5A expression in MDA-MB-231-R cells, but these cells did not have an increased sensitivity to LGK974. This may be due to the pan-WNT signaling inhibition by LGK974. Silencing WNT5A and analyzing the consequent proliferation and survival in MDA-MB-231-R and MDA-MB-231 cells may elucidate the role of this ligand in resistance to the IGF-Trap in TNBC cells.

5.4 Conclusion

In conclusion, we identified IGF-IR expression levels as a potential predictor of sensitivity to the IGF-Trap in TNBC cells. Upregulated FGFR1 signaling was associated with resistance to IGF-Trap treatment in MDA-MB-231 cells. Dual inhibition of FGFR1 and the IGF axis had an additive inhibitory effect on cell proliferation. Our data suggest that this combination may overcome resistance to IGF-axis targeting and may be an effective strategy for TNBC. The data provide a rationale for further investigation of this drug combination *in vivo*.

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