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Identification of resistance mechanism to targeting of the ADAM 17 / EGFR axis in Triple negative breast cancer *in vivo*

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Neha

Abstract

Triple-negative breast cancers (TNBCs) (ER/PR/HER2 negative) represent 15% of invasive breast cancers and occur at a higher rate in young and African-American women. Exploration of novel therapeutic approaches is critical, since only 30% of woman with metastatic breast cancer will survive and virtually none with metastatic TNBC. The status quo as it pertains to the treatment of TNBCs can be summarized as: no effective therapies available. In part, the lack of therapeutic success is due to high genetic heterogeneity of TNBCs, challenging single drug approaches.

Many targeted strategies to treat TNBC are being explored, including the inhibition of kinase pathways (e.g. PI3K/Akt, MEK, VEGFR and PDGFR), the inhibition of DNA repair, of survival pathways and androgen receptor blockade. In most cases, such single-drug targeted therapy is combined with systemic genotoxic chemotherapy. For example, although about 60% of basal-like TNBCs over express EGFR, EGFR targeted therapy, including kinase inhibition, has been disappointing due to the development of resistance.

Various resistance mechanisms allow cancer cells to evade single-drug targeted therapies: mutations in the targeted molecules, extensive crosstalk/pathway redundancy and the up-regulation of alternate growth or survival pathways. Design of combinatorial approaches of therapeutics for TNBC that overcome resistance is therefore critical. The contribution of the proposed research is expected to be the identification of signaling network perturbations that occur in response to single targeted therapies, in particular in ADAM17/EGFR axis inhibition, and confer resistance. Based on published results, inhibition of the ADAM17/EGFR ligand axis in TNBC should provide therapeutic benefit with reduced tumor growth and decreased metastasis, if not possibly cure.

In our *in vitro* studies on PKCα and PPP1R14D gene knockout in MDA-MB-231 cells indeed led to decreases in cellular growth and migration. However, to our surprise, when the same cells were injected into mice through orthotropic fat pad transplantation, they produced aggressive, metastatic tumors that showed activation of alternate growth signaling pathways, namely of the mitogen-activated protein kinase ERK and of the PI3kinase target Akt, also a mitogen activated kinase. This suggested that TNBC cells were developing resistance to EGF ligand regulator knockdown by rewiring their growth factor signaling pathways. To determine where these additional growth signals come from, we first considered the tumor cells themselves.

In this context we discovered that when kept in culture, MDA-MB-231 cells expressing sh-RNAs targeting either PKCa or PPP1R14D maintained knockdown of the target for up to 35days tested. At the same time EGFR and ERK showed low activity as expected due to a decrease in EGF ligand cleavage; Akt activity was undetectable. Since we observed strong reactivation of ERK and new activation of Akt in tumors *in vivo*, we considered possible up-regulation of other growth factor receptors on the cell surface that would be engaged by factors released from the tumor stroma once cells are inserted *in vivo*. Indeed, we found that FGFR2 and Erbb4 were upregulated. It is therefore likely that reactivation of ERK and new activation of Akt was due to FGFR2 and Erbb4. This would suggest that combination therapy of EGF ligand release regulator inhibition and FGFR inhibition would decrease growth of these tumors *in vivo*.

Resumen

El cáncer de mama triple negativo (TNCB) es aquel que no expresa el receptor de estrógenos (ER), ni el de progesterona (PR) o el HER2. Esta patología representa el 15% de los tumores de mama invasivos y tiene una alta incidencia en mujeres jóvenes Afro-Americanas. Es responsable de una alta tasa de mortalidad por cáncer de mama ya que generalmente el TNCB causa metástasis; además, responde pobremente a las terapias con quimioterápicos a largo plazo y generalmente desarrolla resistencia a las terapias dirigidas, incluyendo las que implican al EGFR. Por todo ello, es fundamental el desarrollo de terapias alternativas, dado que solo el 30% de las mujeres con cáncer de mama metastásico sobrevive pero ninguna de las que presentan TNBC metastásico.

Actualmente, no existe una terapia adecuada y efectiva para el TNBC. En parte, esto se debe a la alta heterogeneidad genética que presentan estos tumores, lo cual redunda en la inefectividad de terapias basadas en una única droga. Terapias basadas en blancos terapéuticos específicos están en investigación y desarrollo, como aquellas basadas en la inhibición de quinasas implicadas en señalización (ejemplo: /Akt, MEK, VEGFR, PDGFR), reparación del DNA, supervivencia celular o acciones androgénicas. Mayormente, estas terapias específicas son combinadas con quimioterapia sistémica. Sin embargo, hasta el momento, los beneficios de tales propuestas terapéuticas no son claros. Aproximadamente el 60% de los TNBC de tipo basal sobreexpresan EGFR; sin embargo, las terapias que implican la inhibición del receptor son mayormente inefectivas debido al desarrollo de resistencia. Distintos mecanismos están involucrados en el desarrollo de resistencia a las terapias dirigidas, como ser mutaciones en la proteína blanco o la redundancia y sobreactivación de vías de señalización compartidas con otros factores de crecimiento.

Por lo tanto, es fundamental diseñar terapias combinadas para TNBC que contemplen el posible desarrollo de resistencia. El trabajo de investigación propuesto intenta identificar alteraciones de vías de señalización intracelular ocasionadas por las terapias dirigidas, particularmente en lo que

respecta al eje ADAM17/EGFR, con el fin de establecer su posible implicancia en el desarrollo de resistencia.

Dado que se desconoce como es regulada la actividad y selectividad de ADAM17, se realizó un amplio estudio mediante shRNA para dilucidar como se regula el clivaje de and PPP1R14D regulan el clivaje de TGFa, AREG y HB-EGF sin afectar la actividad proteasa de ADAM17. La inhibición del eje ADAM17/EGFR sería beneficioso para el tratamiento del TNBC. Nuestros estudios *in vitro* revelaron que células MDA MB 231 knockout para PKCα and PPP1R14D no presentan sobreactivación de RTKs, sugiriendo que en estos modelos podría verse potenciada la eficacia terapéutica de la inhibición del eje ADAM17/EGFR.

Sin embargo, cuando las mismas células fueron inyectadas a ratones, produjeron un fenotipo de tumor agresivo y metastásico, asociado a la reactivación de vías de señalización intracelular como las mediadas por ERK y PI3K/Akt. Ello se asoció a un aumento de la expresión y activación de distintas RTKs, incluido el EGFR como así también de Akt.

Estos resultados sugieren la activación alternativa de vías de señalización que permiten que las células tumorales proliferen y produzcan metástasis.

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1. Introduction

1.1 Triple negative breast cancer

Triple negative breast cancer (TNBC) represents a cluster of heterogeneous diseases showing distinct molecular, pathological and clinical features (1). Triple negative breast cancer (TNBC) is characterized by the absence of estrogen receptor (ER) and progesterone receptor (PR), as well as human epidermal growth factor receptor-2 (Her2) (2). TNBC is associated with poor prognosis, a high risk of local recurrence (LR), and poor disease-free survival (DFS) and cancer-specific survival (CSS) (2, 3). TNBCs are usually larger in size, higher in grade, with earlier lymph node involvement. They represent 15% of invasive breast cancers and occur at a higher rate in young and African-American women (4, 5).

Gene expression profiling and molecular pathology have revealed that BC naturally divides into luminal A and B, HER2-enriched, basal-like and claudin-low subtypes (6, 7). The claudin-low BC is characterized by loss of tight junction markers (notably claudins) and high expression of markers of epithelial-to-mesenchymal transition (EMT), in addition to being enriched for markers of mammary stem cells (7, 8). Basal-like tumors molecularly mostly identify as Triple-negative breast cancers (TNBCs) (ER/PR/HER2 negative). To date, the basal-like classification is available only in the research setting; thus, the triple-negative phenotype currently serves as a reliable surrogate in the clinical environment (1, 2).

Exploration of novel therapeutic approaches is critical, since only 30% of woman with metastatic breast cancer will survive and virtually none with metastatic TNBC. Therapeutic strategies to confront resistance are lacking and not one single targeted therapy is approved for TNBC (10).

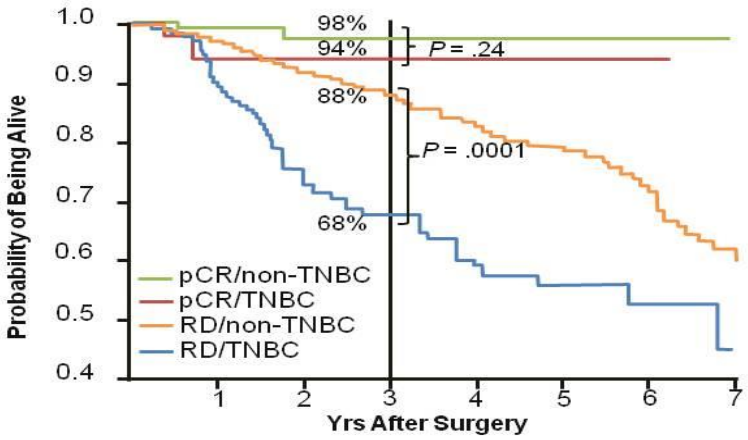
1.2 Metastasis

Metastasis involves a series of steps, dependent on the balance between the intrinsic properties of the tumor cells and the host response, each of which can be rate-limiting since a failure at any step may halt the process (11).

Due to the more aggressive phenotype of metastatic cells, the metastases of a tumor are often more difficult to treat than the primary tumor itself. These cells have gained the ability to leave the primary tumor, either via lymphatic tissue or blood vessels, and settled down in different organs. Historically metastasis was viewed as a linear progression based primarily on the malignant cells accrual of mutations, however, recent studies have demonstrated
the potential for dissemination is highly complex (12,13)

TNBC: Responsiveness to Neoadjuvant Conventional Chemotherapy

- TNBC is responsive to conventional NAC with good outcome similar to other subtypes
- < pCR = poorer outcome



Liedtke C, et al. J Clin Oncol. 2008;26:1275-1281.

Firstly the tumor cells start invading local host tissue and this process continues until the tumor cells intravasate systemic fluids. Systemic fluids help tumor cells to travel to distant organs and to start proliferating and inducing angiogenesis to supply their growth. Before extravasating the tumor cells attach to the capillary beds of the to be invaded organ. During this whole process, the tumor cells defend themselves from the immune system of the host and from other apoptotic signals produced in their environment. Metastastatic lesions can produce their own metastasis, called secondary metastasis (14).

The invasion process involves two main molecules from the cadherin family, E-cadherin and N-cadherin. E-cadherin basically helps in cell to cells adhesion and down regulation of this molecule leads to metastasis. N-cadherin is involved in epithelial-to-mesenchymal transition (EMT) during the gastrulation stage. EMT plays a major role in tumor progression by assisting invasion and intravasation into the bloodstream and by inducing proteases involved in the degradation of the extra cellular matrix (ECM)(14,15).

Integrins are the transmembrane receptors which help tumor cells to destroy ECM. The degradation of ECM is carried out mainly through metalloproteinases (MMPs) and the urokinase plasminogen activator (uPA) system (16).

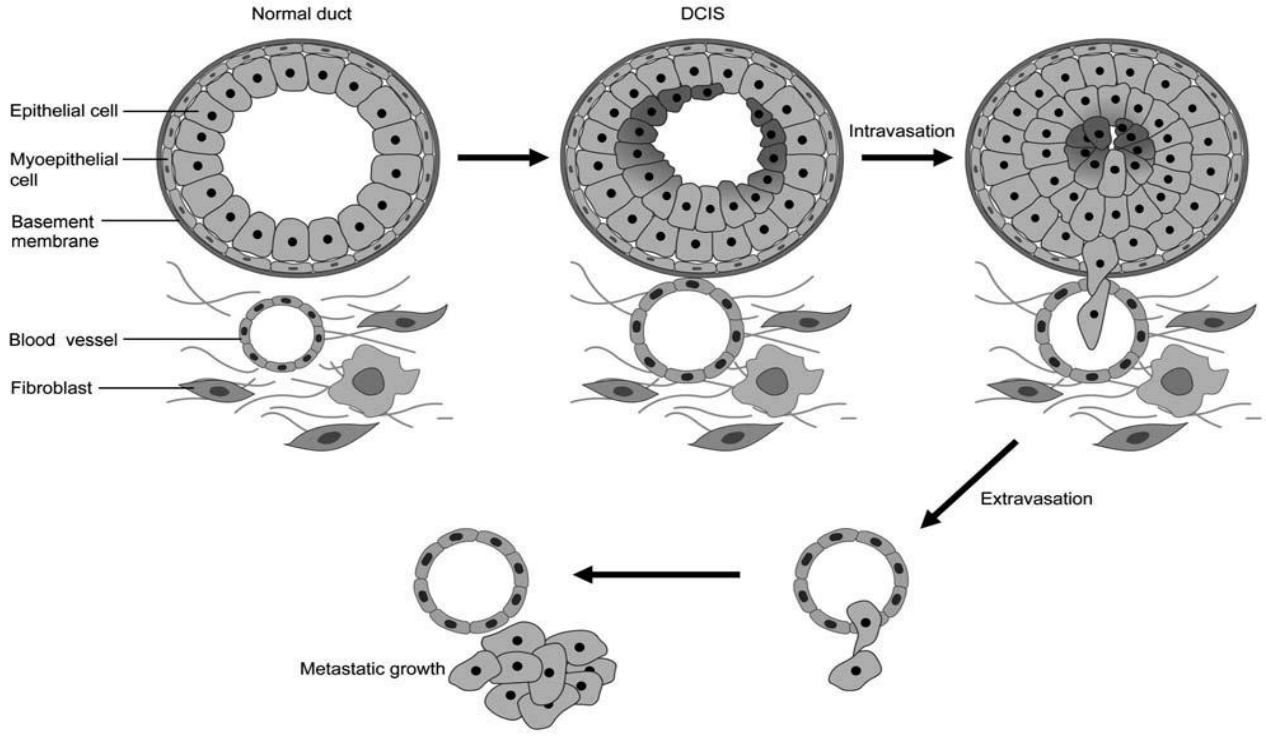
One important factor contributing to metastasis formation is the local tissue and its stiffness, which may impact the kinetics of metastasis and also the susceptibility of the tissue to be invaded by metastatic tumor cells (17, 18). Therefore a better understanding of extra cellular matrix (ECM) qualities that favor metastasis is crucial to develop approaches that target the metastatic potential of cancer cells.

The tumor cells travel inside the system either singly or coordinately. For coordinated movement they need intercellular junctions and they circulate in blood or lymphatic system as tumor cell emboli. The singly circulate mainly by protease-dependent mesenchymal movement or by

protease-independent amoeboid movement. The microenvironment is a very important factor for tumor growth and metastasis. The tumor microenvironment can be nourishing or aid in detection

and destruction of tumor cells, e.g. by the induction of apoptotic signals relayed by cells of the immune system. Many different specialized cells, including fibroblasts, immune cells, endothelial cells and mural cells of the blood and lymph vessels, together with the ECM make up the microenvironment which influences tumor progression (18).

It is also postulated that tumor cells themselves might secrete substances to prime the 'soil' prior to metastasis to establish a 'pre-metastatic niche' supporting future metastatic sites (11).



1.3 Targetable signaling pathways

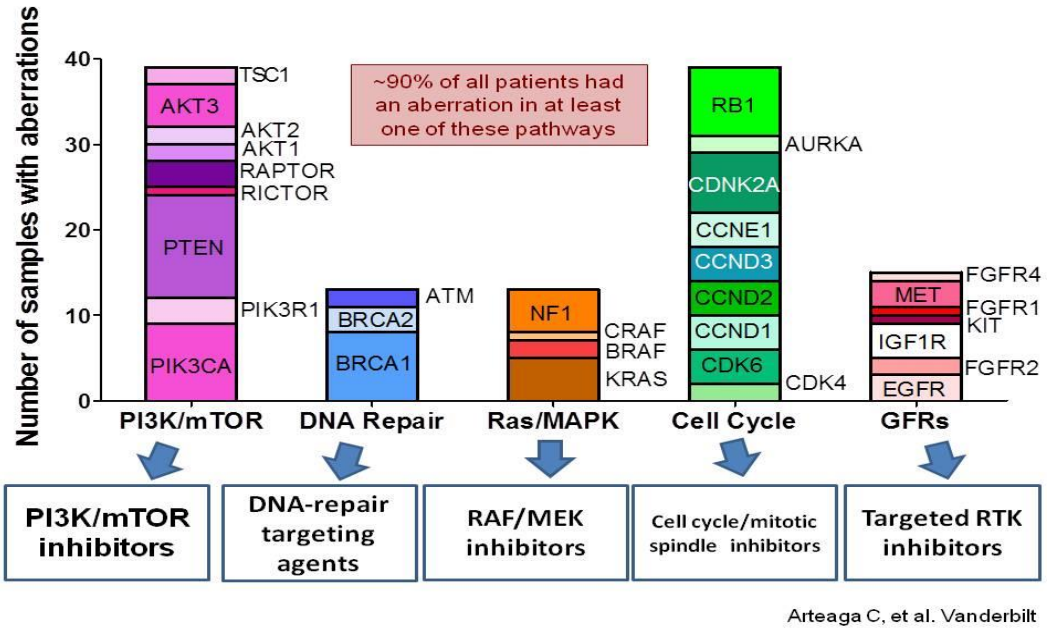
The basal-like breast cancers (BBCs) were found to be molecularly distinct from the luminal A, luminal B, and HER2 subtypes of breast cancer, but to share many characteristics with high-grade serous ovarian cancers (HGSOC), including loss of *TP53*, *RB1*, and *BRCA1*, as well as

MYC amplification. Several potential targets are currently investigated in TNBC/BBC, including *PTEN*, *INPP4B*, *PIK3CA*, *KRAS*, *BRAF*, *EGFR*, *FGFR1*, *FGFR2*, *IGFR1*, *KIT*, *MET*, *PDGFRA*, and the HIF1-α/ARNT pathway (19,20,21).

Other target agents under current investigation are Src, insulin-like growth factor (IGF)/IGF-receptor (IGFR), PI3K/AKT/mTOR and RAS/MEK/ERK inhibitors. as well as agents that promote apoptosis such as Poly ADP ribose polymerase (PARP) inhibitors or agents that target invasion and metastasis (Metalloprotease inhibitors) all of which interfere with critical signaling pathways that have been found upregulated in TNBC. Several different EGFR targeting agents are also in clinical trials (22).

1.4 Targeting EGFR

The epidermal growth factor receptor (EGFR) may be a potential target in the treatment of advanced TNBC. High expression of EGFR was noted in approximately 60% of TNBCs. Cetuximab, a monoclonal antibody that targets EGFR, have shown somewhat limited benefit. The combination of carboplatin and cetuximab is well tolerated for advance stages of TNBCs. But there is always reactivation of alternative signals inside the tumor cells that help them to survive inside the host system (20, 21).



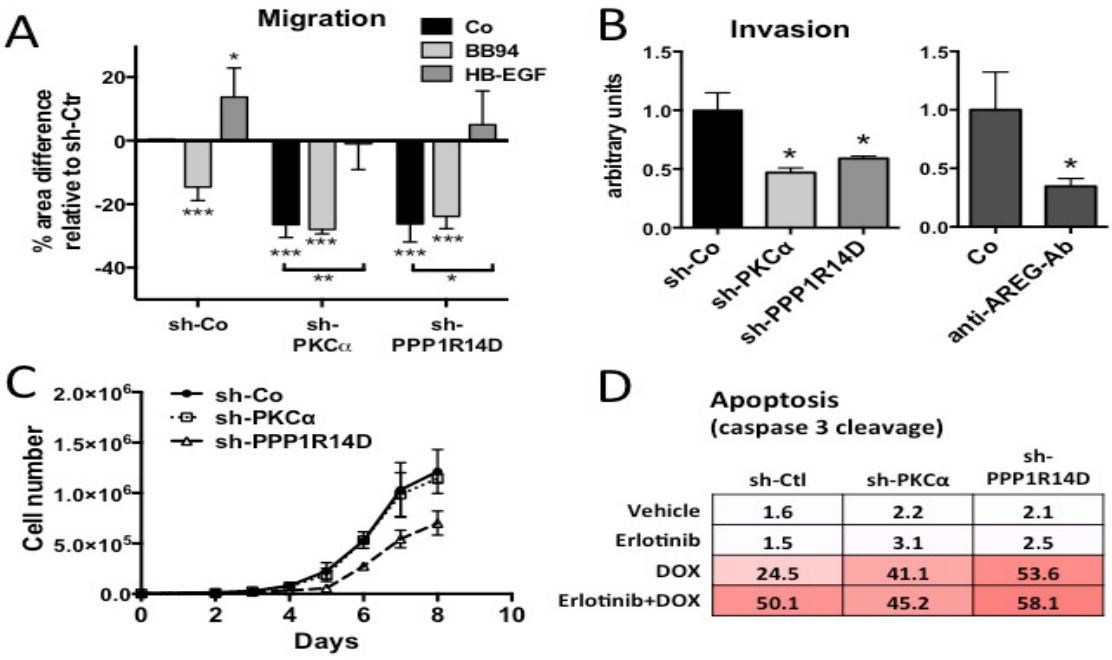
The treatment of TNBCs can be summarized as: no effective therapies available. In part, the lack of therapeutic success is due to high genetic heterogeneity of TNBCs (10, 23), challenging single drug approaches. In most cases, such single-drug targeted therapy is combined with systemic genotoxic chemotherapy; however, to date these studies have produced little tangible results (10). For example, although about 60% of basal-like TNBCs overexpress EGFR (24), EGFR targeted therapy, including kinase inhibition, has been disappointing due to the development of resistance (10,25).Various resistance mechanisms allow cancer cells to evade single-drug targeted therapies: mutations in the targeted molecules, extensive crosstalk/pathway redundancy and the upregulation of alternate growth or survival pathways (26).

The above-mentioned complications lead our lab to design combination therapy approaches which can circumvent the resistance generated due to single targeting of the EGFR. This resistance frequently involves the release of EGF ligands from tumor or stromal cells that overcome receptor inhibition in the tumor. Our added approaches would target the signaling pathways that mediate EGF ligand cleavage and are activated as a result of EGFR inhibition; thus counteracting one important resistance mechanism to EGFR targeting.

1.5 Previous work

Previously, our lab had performed a large scale shRNA screen for regulators of phorbol ester induced TGFα cleavage, targeting the human kinome and phosphatome (28). Our screen identified about 40 positive or negative kinase/phosphatase regulators of induced TGFα cleavage, including PKCα, PKCδ and PPP1R14D. PPP1R14D is a PKC-activated specific protein phosphatase 1 inhibitor (27, 29). Both genes selectively regulate the cleavage of TGFα, AREG and HB-EGF without affecting ADAM17 protease activity, and do not affect ADAM10 substrates (28). With these results in mind, we first investigated knockdown of PKCα, PPP1R14D in TNBC cells *in vitro and* observed decrease of TNBC relevant cellular phenotypes *in vitro (proliferation, migration, invasion, EGF ligand cleavage)*.

PKCα and PPP1R14D knockdown significantly reduced migration (**Fig. 1A) of MDA-MB-**231 TNBC cells (wound-closure scratch assay) and this inhibition was rescued by addition of EGF ligands such as HB-EGF. The metalloprotease inhibitor batimastat (BB94) was as effective as PKCα and PPP1R14D knockdown. PKCα and PPP1R14D knockdown also strongly inhibited invasion of MDA-MB-231 cells into collagen matrix (**Fig.1B). A neutralizing anti-amphiregulin (AREG) antibody** mimicked this effect (**Fig. 1B), suggesting that AREG** release plays a role in enhancing TNBC cells invasion. Only PPP1R14D but not PKCα knockdown reduced cell growth of MDA-MB-231 cells, suggesting substitution of this function by other PKCs (**Fig. 1C). However, knockdown of either** gene significantly sensitized MDA-MB-468 cells to doxorubicin (24% -> 41-53% apoptotic cell marker caspase 3 at 8hours) (**Fig. 1D). 6 hours pre-treatment with erlotinib** further enhanced apoptosis in control but not in knockdown cells, suggesting that regulator targeting mimics EGFR inhibition



	sh-Ctl	sh-PKCα	sh-PPP1R14D
Vehicle	1.6	2.2	2.1
Erlotinib	1.5	3.1	2.5
DOX	24.5	41.1	53.6
Erlotinib+DOX	50.1	45.2	58.1

Following these *in-vitro* studies, I performed *in-vivo* studies to confirm whether they perform in a similar manner. PKCa and PPP1R14D knockdown were maintained in MDA-MB 231 cells by continuous input of IPTG and injected in mice with fat pad mammary transplant.

2. Hypothesis

We will test hypothesis that PKCα and PPP1R14D knockdown MDA-MB-231cells shows no tumor growth and metastasis in vivo.

3. Objective

3.1 Primary Objectives

The purpose of this thesis is to investigate whether PKCα and PPP1R14D knockdown MDA-MB-231 cells shows similar reduction *in vivo* tumor growth and metastasis as it has shown previously in *in vitro* experiments.

3.2 Secondary objectives

- To perform mammary fat pad transplantation to insert PKCα and PPP1R14D knockdown MDA-MB-231 cells in mice
- To measure the tumor growth at different time points.
- To harvest the tumors and distant organs (Lungs and Liver) from mice at different at week 5, 4, 3.
- To take the pictures under fluorescent stereoscope to investigate the tumor growth and metastasis.
- To analyze the tumor samples by using western blot and q PCR.

4. Materials:

4.1 Chemical

Product	Vendor	Catalog Number
2-Propanol	Roth	AE73.1
Acrylamide 40%	Bio-rad	161-0148
Ammonium persulfate	Thermo Scientific	17874
Dimethyl-sulfoxide (DMSO)	Sigma-Aldrich	D2438
DMEM	GIBCO	41966
Ethanol	J. T. Baker	9401-33
Ethidum Bromide 1%	Fisher Scientific	BP1302-10
Fetal Bovine Serum (FBS)	Sigma-Aldrich	F1051
Formaldehyde	Sigma-Aldrich	F8775
Glacial Acetic Acid	Sigma-Aldrich	320099
Glycine	Fisher Scientific	BP381-5
Nitrocellulose membrane	Sigma-Aldrich	N8267
PBS	Life Technologies	10010-031
Penicillin/Streptomycin	GIBCO	15140
Protease inhibitor	Sigma-Aldrich	P8340
Stripping buffer	Thermo	48426
Temed	Fisher Bioreagents	112881
Tryptan blue 0,4%	Life Technologies	15250-061
Tris Base	Fisher Scientific	BP1525
Trypsin	Sigma-Aldrich	P8340
Tween 20	Fisher Scientific	BP337-100

4.2 Instruments and Equipments

Products	**Vendor**
Autoclave	Vakulal S 3000
ChemiDoc™ system	Bio-rad
Centrifuge	Eppendorf
Electric Pipettor	Integra Bioscience
Incubator	Heraeus
Ice machine	Ziegra
Light microscopes	Olympus CX2
Laminar flow hood	Heraeus LaminAir 2448
Microscope system	Zeiss Axioplan 2 Imaging
Nanodrop 1000,	Peqlab
Thermomixer	Eppendorf
Refrigerator (+4· C)	Summit®
Weighing scale	Sartorius CL420

4.3 Consumables

Products	**Vendor**
Cell culture 96 well round bottom plates	BD Discovery Labware
Cell culture round bottom plates, different Sizes	Greiner
Conical tubes, 15ml, 50ml	Greiner bio-one
Coverglasses for microscope slides	Langenbrinck
Eppendorf tubes, sterile, 1.5 ml, 2 ml	Eppendorf

Parafilm M	American National Can
Pipette tips without filter, diffenrent sizes	Corning
Single use syringes, sterile, different sizes	B. Braun
Tissue strainer 40µm, 70µm, 100µm	BD biosciences

4.4 Antibodies

Products	**Vendor**	**Catalog Number**
P EGFR (1068)	Cell Signaling	2234
Total EGFR	Cell Signaling	2232
P ERK	Cell Signaling	9101
Total ERK	Cell Signaling	9102
P Akt	Cell Signaling	9271
Total Akt	Cell Signaling	9272
P Stat3	Cell Signaling	9131
Total Stat3	Cell Signaling	9132
αTubulin	Cell Signaling	2144

4.5 Software

Products	**Vendor**	**Application**
Endnote X5	Thomson Reuters	Reference management
FlowJo	BD Biosciences	Analysis of flow cytometry data
GraphPad Prism 5.0	GraphPad Software, Inc.	Statistical analysis and database Management
Microsoft Office 2007	Microsoft	Documentation and Analysis Applications
Image Lab	Bio-rad	Analysis of western blots and gel images

4.6 Mice

Nude mice were used for in-vivo experiment (fat pad mammary transplant), injected MDA-MB 231 cells control, Pkcα and PPP1R14D knockout cells.

5. Methods

5.1 Cell culture

All cells have been cultivated at 37 C with a 5% carbon dioxide concentration, unless otherwise stated. Cells were kept in culture a maximum of 5 weeks, to limit genetic drift.

5.1.1 Counting of cells

Cells in suspension were stained with a 33% trypan blue solution at a ratio of 1:1 (10 µl Cell suspensions to 10 µl 33% trypan blue solution). Trypan blue stains dead/dying cells blue, while living/healthy cells are not stained. The cells were then counted using a Neubauer chamber immediately after staining. Cell concentration was calculated using the following equation.

2 x 10000 x Average of number of cells in four fields = number of cells per ml

5.1.2 Collection of cells for experiments

Adherent and semi-adherent cells were cultured in T-75 flasks until a max confluence of 90%. At confluence point, cells were washed with PBS, trypsinized at 37°C for 5 minutes, or until the cells detached from the surface of the flask. Trypsin was then neutralized with appropriate culture medium and cells were split into new culture flasks or counted before being used for experimentation. (Suspended semi-adherent cells were centrifuged at 400 x G for 8 minutes. Old media was removed from the cell pellet and cells were then combined to trypsinized cells for splitting or experimentation). Suspension cells were cultured the same as above. When confluence of 90% was reached, cells were split or used for experimentation.

5.1.3 Freezing and thawing cells

All long-term cell stores were kept in a cryogenic unit in liquid nitrogen at -196° C. Cells were collected from flasks, counted as mentioned above, centrifuged at 400xG for 8 minutes, after which culture media was removed. Cells were then re-suspended in freezing medium (90% FBS, 10% DMSO) at a concentration 1 x 106 – 1 x 107 and pipetted into cryogenic freezing tubes. Tubes were then placed into freezing vessels containing isopropanol and stored overnight in a freezer at -80° C. Cell tubes were then transferred to the cryogenic storage unit for indefinite long-term storage.

Thawing of long-term cell stores was done as quickly as possible to limit cell exposure to toxic levels of DMSO. Cells were immediately thawed and the freezing media diluted with normal culture media. Then cell suspensions were centrifuged at 400 x G for 8 minutes, followed by the removal of all media from the resulting cell pellet. Freshly thawed cells were then washed once with culture media to remove any residual freezing media and centrifuged again. Once the wash media was removed from the cell pellet, cells were re-suspended in culture media and place in the incubator to recover from the freezing/thawing process.

5.1.4 Triple Negative Breast Cancer cell line selection and acquisition

The MDA-MB-231 TNBC cell line was extracted from the human mammary gland/breast adenocarcinoma and was provided by Anna Starzinski- Powitz (Frankfurt, Germany) and Steve Palmer (EMD Serono).

5.1.5 Lentivirus Production and Infection

For virus production, the following protocol was used: 800,000 HEK293T cells were seeded in a 6-cm plate on day 0. On day 1, 50% confluent cells were transfected with 1 mg viral plasmid of choice, 0.9 mg of VSVG, and 0.1 mg pUMVC (retroviral polymerase) or 0.1 mg deltaPVR

(lentiviral polymerase) using 6 mL of FuGENE 6 (all premixed in serum-free medium and incubated 20 min at room temperature). On day 2, 18 h after transfection, medium was changed to 4 mL medium containing 30% (vol/vol) FCS in DMEM. On day 3, 48 h after transfection, viral supernatant was harvested and syringe-filtered through a 0.4-mM filter directly onto cells to be infected (50% confluent at point of infection). Polybrene 4 mg/mL was added to the virus, and infection was done by spin-infection at 750 × g for 30 to 60 min.

5.1.6 shRNA knockdown screen

IPTG inducible lentiviral shRNA vectors (pLKO904/905) were used. Lentivirally infected TNBC cells were grown for 7 days in IPTG-containing media (100mM) to induce strong gene knockdown (typically established within 3-5 days; returns to normal if IPTG is withdrawn). The knockdown cells *in vivo* were re-tested for their desired knockdown phenotype through qPCR. The gene knockdown of PKCa and PPP1R14D was done.

5.2 Subcutaneous mammary fat pad injections (Orthotopic transplantations)

On day 8, tumor cells were injected in cold medium containing growth factor reduced Matrigel into the mammary fat pad (1-5x105 cells/injection). The surgery was performed in a sterile hood to maintain a sterile atmosphere. The mouse was anesthetized by subcutaneously injecting Xylazin/Ketamine mix at a dose of 10mg/kg, 100 mg/kg body weight respectively. The mouse was fixed on a heating pad. The shaved area was cleaned by using the cotton swab dipped into 70% ethanol. A small incision was made between the fourth nipple and the midline with a scissor and makes a pocket by inserting the cotton swab moistened with PBS pH 7.4. The fat pad was squeezed with the tweezer from its base; by doing this, fully expose the fat pad to perform injections easily. The cell was Homogenized mixture by pipetting up and down. After surgery, an analgesic was injected, such as temgesic at 0.05-0.1 mg/kg bodyweight, subcutaneously in order to relieve the pain.

5.3 Tumor Harvest

At the day of harvest, 5 weeks after the implantation of the cells, 15 ml conical centrifuge tubes was filled with 3 ml Bouin's solution for each mouse. In addition, two 15 ml tubes filled with 5 ml formalin solution per mouse was used. The animals was Anesthetized by injecting Xylazin/Ketamine, at a dose of 10 mg/kg 100 mg/kg body weight subcutaneously. A long, vertical midline incision with scissors was made. Two horizontal incisions right below the front leg and above the rear leg were made. The tumor was exposed by pinning the skin to the base. The tumor volume was measured by using a caliper. The tumor dissociated from the skin using scissors. A part of the tumor was freezed in liquid nitrogen for RNA isolation. The other part placed into the conical centrifuge tube filled with formalin to perform immuno-histochemistry following paraffin embedding. Gently the lungs were taken out. The left lung placed into Bouin's solution. The lung Kept in solution for 3 days. Superficial metastatic foci clearly Observed to naked eye.

NOTE: Although, lung metastasis observed frequently in breast cancer, we wanted to collect liver and spleen to analyse metastasis. The cells at the metastatic area were denser and morphologically different and therefore could be distinguished easily from lung tissue. A day after harvest, the formalin solution was aspirated from 15 ml tubes and replace with 70% ethanol.

5.4 Immuno-precipitation and Western Blotting

This procedure is used to measure protein levels in a particular cell of interest for a given experiment.

5.4.1 Tumor Lysates

Tumor samples were taken in equal sized and TNE lysis buffer (200 ul per sample) containing

protease and phosphatase inhibitors was used to re-suspend and lyses the samples. Next mechanical homogenizer was used at moderate speed to make tissue lysates and separate protein and tissue. Sample always kept on ice to maintain low temperature. Before spinning down, the tumors are allowed to sit for 30 minutes in the TNE Lysis Buffer and put into 1.5 ml tubes. After the lysis incubation period, these tumor samples were centrifuged at 10,000 x G for 10 minutes at 40C to form a cell pellet. The tissue pellet was discarded and the suspension was aspirated off to another clean 1.5 ml tube. Volume of lysis buffer used was proportionate to cell number and plate size, typically 100-200 µl. The protein supernatant was stored for the protein assay.

5.4.2 Bradford Assay

Protein concentration of each lysate was measured to quantify specific sample concentrations based on colorimetric assay. 20 µl of Bio-Rad Protein Assay reagent S was combined with per ml of Bio-Rad Protein Assay reagent A. The working reagent A' (25 µl) was used on 5 µl of the sample or standard protein in a 96-well flat-bottomed plate. The plate was then incubated with Bio-Rad Protein Assay reagent B at 370C for 15 minutes. The colorimetric value was recorded using a photo spectrometer and analyzed with Microsoft Excel.

5.4.3 SDS PAGE Separation

SDS-PAGE gels had a concentration gradient from 4-12% of sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) for electrophoretic separation. Equal amounts of sample protein (50-80 µg) were mixed in a loading mixture with sample and TNE lysis buffer to a maximum volume of 80 µl to make up the loading sample and then heated to 950C for 5 minutes. Denatured loading samples were pipetted into gel wells alongside a pre-stained protein standard ladder. Gels were then enveloped in approximately 200 ml, 1x running buffer. An electric field of 130-150V was then applied to the gel and the gel run was sustained for 60-90 minutes.

5.4.4 Protein transfer to Nitrocellulose membrane

From the gel, the proteins were blotted onto a nitrocellulose membrane using a 230 mA electric current for 60-90 minutes to force the proteins onto the membrane. This was done in a 1x transfer buffer solution with 10% methanol. Transfer chamber was placed in a cool water bath to prevent overheating.

5.4.5 Antibody Incubation

After transfer to a nitrocellulose membrane, the membrane was blocked with 5% milk/Tris-buffered saline solution (TBST; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Triton) for 1 h at room temperature. The primary antibody was incubated overnight at 4 °C in 5% BSA/ TBST. This is done to prevent background staining. The membranes were washed three times in TBST and then incubated with the respective secondary horseradish peroxidase (HRP) labeled antibody (1:5,000) for 1 h at room temperature in 5% (wt/vol) milk/TBST to detect the primary antibody. Membranes were washed 3 more times with TBST to remove excess secondary antibody.

5.4.6 Chemiluminescence and Quantification

The membranes were washed once more to remove excess antibody, then incubated with a chemiluminescent substrate (ECL) for the horseradish peroxidase for 5 minutes at room temperature. The membranes were exposed to the ECL and packed into a plastic film. Then the membranes were exposed inside the Chemiluminescence machine in the dark. The enzymatic reaction emits light in proportion to the amount of protein present when the HRP oxidized the substrate. Thus revealing the location and the quantity of the proteins, both primary and secondary antibodies, and the HRP on the membrane. Images of protein bands are analyzed for intensity using ImageLab software. Each protein band is normalized by dividing the value of its area by the same value of the associated loading control protein. Finally normalized protein

expression values are combined to form a graphic in GraphPad Prism for analysis.

5.4.7 Phorbol ester stimulation of signaling pathways

Cellular signaling pathways affected by phorbol ester were studied by western blot. Initially, cells were cultured with phorbol ester (R&D system, cat # 2667- CM) at doses of 0.05-0.2 µg/ml, then 0.2 µg/ml was used for time periods of 1-4 hours. The cells were injected into mice and tumors were isolated. Western blots of these whole-tumor protein lysates were incubated with antibodies targeting the signaling molecules Erk, phosphorylated Erk, Akt, phosphorylated Akt, phosphorylated EGFR, stat3 and phosphorylated stat3 .

5.5 qPCR

5.5.1 RNA extraction

The tumor samples were kept on dry ice. 1ml of Tri-reagent were used for each sample of tumor. After homogenization with mechanical homogenizer kept it for 5 minutes at room temperature. 0.2ml of chloroform was added in per ml of Tri-reagent. Covered the samples tightly and shake vigorously for 15 second and again kept on room temperature for 2-15 minutes. Then centrifuged at 12,000xg for 15 minutes at 2-8 C. The sample separated into three phases: 1) red organic phase (contained protein) 2) inter-phase (DNA) 3) Color less upper aqueous phase (contained RNA).The aqueous phase were taken into new fresh tube and 0.5ml of 2-propanol per ml of Tri-reagent was added and mixed. It was kept for 5-10 minutes at RT. Centrifuged at 12000xg for 10 minutes at 2-8 C. RNA will form a pellet on the side and bottom of the tube. Supernatant was removed and washed pellet by adding 1 ml of 75% ethanol per 1 ml of Tri reagent. Sample was vortex and centrifuges at 7500xg for 5 minutes at 2-8 C. Then RNA pellet was dried at room temp until it became transparent. 10-20ul of DNA, RNA free water was added and RNA concentration was measured using nano-drop. Testing samples were prepared (2ul of sample+8ul

Millipore water) and stored at -20. Simultaneously 396 well qPCR plate was prepared with 24 different primers (1ul each) in triplicates with spinning down occasionally in between. It kept for drying over night.

5.5.2 Reverse transcription

RT-PCR can be done in two steps, first with the reverse transcription and then the PCR. The two-step protocol is usually more sensitive than the one-step method; yields of rare targets may be improved by using the two-step procedure.

Before it started, RNase Inhibitor and Reverse Transcriptase placed on ice directly from the box. 10x reaction buffer, random decamers, and dNTP mix thawed quickly in your hands and place on ice; small 0.25ml PCR tubes used.

Small 0.25ml PCR tubes were used.

Component	Stock	Final amount	Experiment (+RT)	Control (-RT)
Total RNA			~1-2 □g	~1-2 □g
Random Decamers	50□M	5□M	2 □l	2 □l
10X RT Buffer	10X	1X	2 □l	2 □l
dNTP mix	2.5mM	0.5mM	4 □l	4 □l
RNase Inhibitor	10 U/□l	10U	1 □l	1 □l
Reverse Transcriptase	100 U/□l	100U	1 □l	0 □l
Nuclease-free water			x □l	x □l

Mixed gently, spin briefly. Incubated in the thermacycler at: 44°C for 1 hr, 92°C for 10 min to inactivated the reverse transcriptase, Stored reaction at –20°C or proceed to the PCR. We considered making master mixes as we were testing multiple sets of primers at once. A master mix contained everything except the PCR primers. We tested n set of primers, made a master mix enough for n+ 1 test. The components mixed gently but thoroughly. 22.5µl of your master

mix aliquot to each tube. 1.25µl of each of the appropriate primer added at 5µM working stock concentration.

Reactions assembled on ice and incubated in Thermacycler. Initial denaturation: 94°C for 4 min 30 cycles: Denatured at 94°C for 30 sec. Annealed at 55°C for 20–30 sec. Extended at 72°C for 45 sec. Final extension: 72°C for 5 min

cDNA were mixed with sybr green and 20 ul each sample added to well in triplicates. Immediately it kept in machine for readings and it took around one and half hour for full cycle. Calculations were done using sds software

5.6 Statistical Analysis

Student's t test was used for comparisons between two groups. $p<0.05$ was considered significant

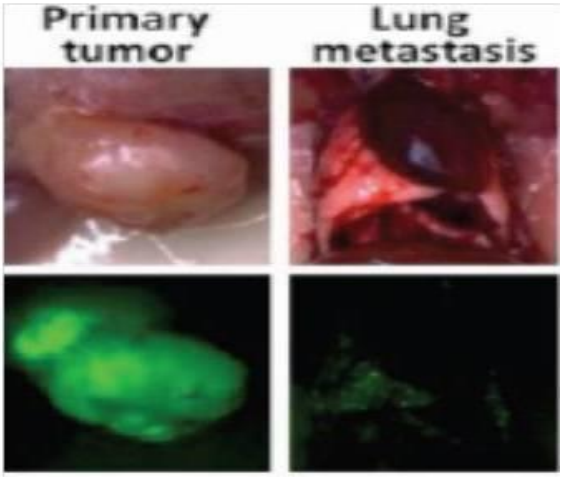
6. Results:

6.1 Confirmation of PKCa and PPP1R14D knockdown in MDA-MB231 cells *in vivo*

To study the role of PKCα and PPP1R14D in TNBC *in vivo* we used the previously described in introduction section MDA-MB-231 cells with the inducible knockdown system in an orthotopic transplantation mouse model. MDA-MB-231 cells (5x105) expressing a lenti-viral IPTG-inducible shRNA vector system (pLKO-904) and GFP were treated with IPTG for 5 days and then injected into the mammary fat pad of mice. The mice received IPTG in water after transplantation. 5 weeks after transplantation of the cells, the tumor size was measured and the mice were sacrificed. The tumors as well as the metastasis to the lungs and liver could be easily visualized and quantified using a fluorescent stereoscope **(Fig. 1A).**

In order to verify the knockdown of PKCα or PPP1R14D in the tumors *in vivo*, western blot and q PCR were performed **(Fig. 1B and 1C).** We found that indeed the protein levels as well as mRNA of both genes were significantly reduced in the respective tumors.

A.



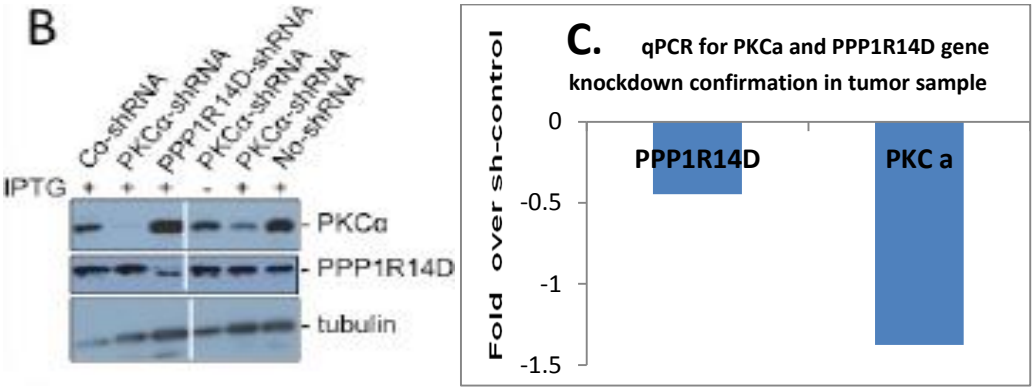


Figure 1: A) Picture is showing primary tumor and metastasis in lungs under fluorescent stereoscope as the cells injected were GFP+ and reflect green fluorescent light. **B)** Western blot results to examine the protein levels of both PKCa and PPP1R14D in tumor sample **C)** q PCR result to confirm the knockdown tumor in both PKCa and PPP1R14D at week 5.

6.2 *In vivo* measurements of tumor size of PKCα and PPP1R14D knockdown MDA-MB231 tumor cells

In order to test the effect of PKCα or PPP1R14D knockdown on tumor size tumors were measured by using a caliper at 5 weeks after transplantation. The formula used was length (cm) x width (cm) / 2 = tumor volume (cm2). We found that the tumor size of both PKCα and PPP1R14D knockdown tumors was significantly increased compared to control tumors as shown below in Figure 2. This result was unexpected, since we have previously seen that the knockdown of these genes causes reduced migration, invasion and/or growth of MDA-MB-231 cells *in vitro* (see previous results). To further examine the effects of the knockdown *in vivo*, we measured the extent of metastasis in the lung and liver.

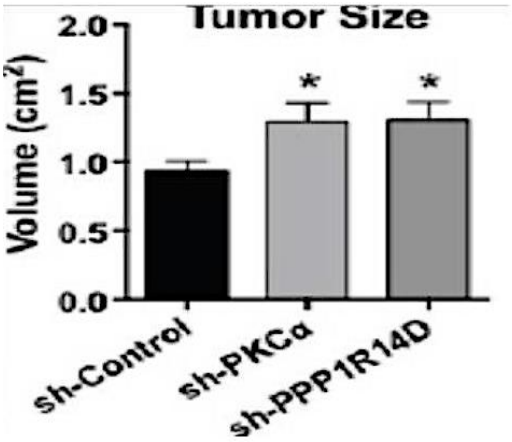


Figure 2: Quantification of tumor sizes. The PKCα and PPP1R14D knockdown tumor has shown significant tumor growth compared to sh-control tumor. *: P<0.05

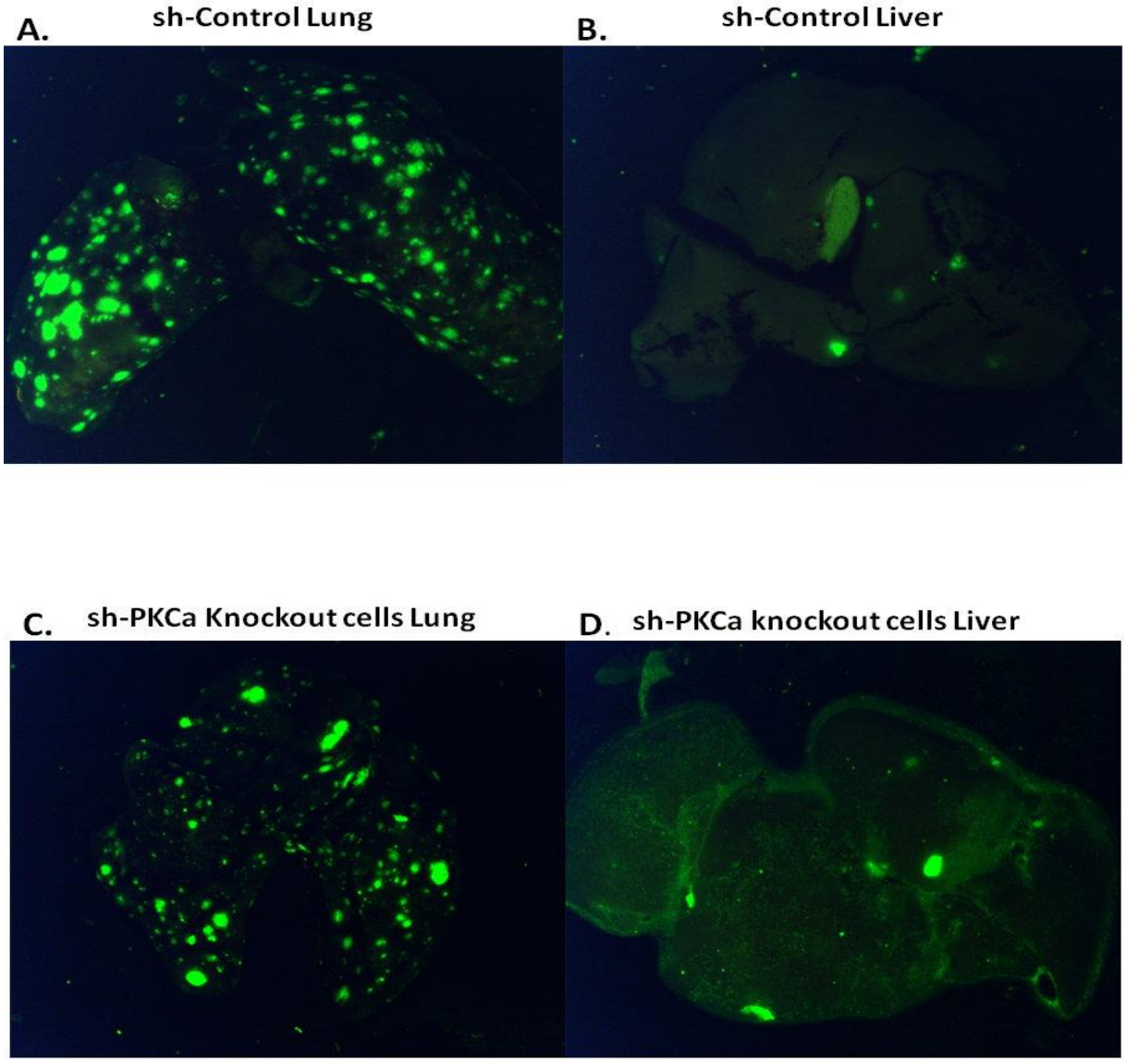
6.3 *In vivo* analysis of tumor metastasis in lungs and liver of mice in both PKCα and PPP1R14D knockdown tumors.

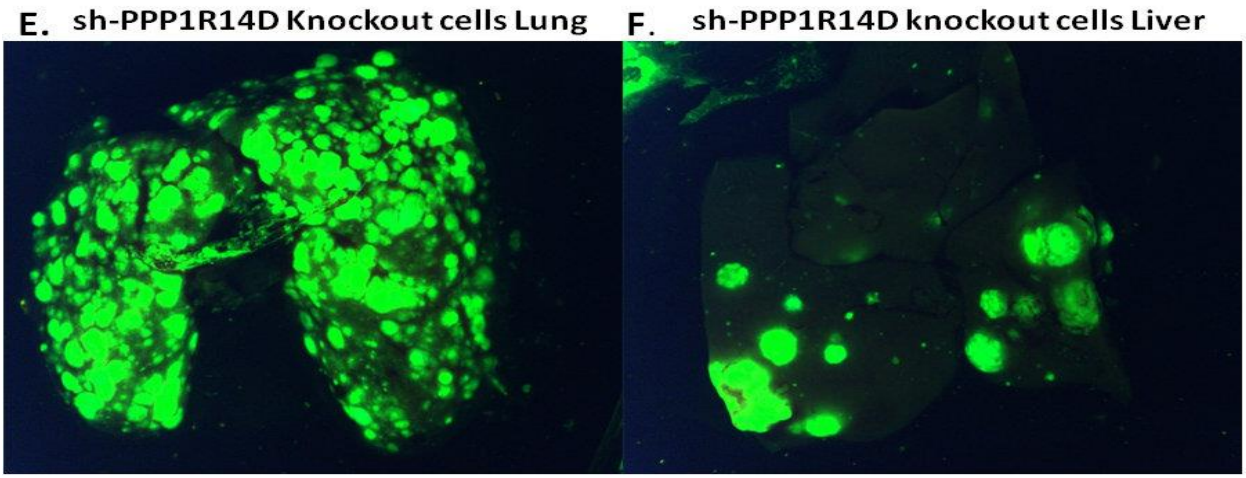
Tumor metastasis was analyzed in lungs and liver of mice at 5 weeks after transplantation using a fluorescent stereoscope (as MDA-MB-231 cells used in the xenograft model are GFP+). Epifluorescence pictures were taken for each tumor sample in each histological preparation and fluorescence was quantified using Image J software and by applying the following formula:

Corrected total cell fluorescence =
Integrated density – (Area of selected cell x mean fluorescence of background reading)

At the time point examined (5 weeks) the cells expressing the control shRNA already show moderate level of metastasis in the lungs (Fig. 3A) and lower levels in the liver (Fig. 3B). PKCα knockdown resulted in slightly increased but not statistically significant metastasis in both lung

and liver (Fig. 3C and 3D, quantified in Fig 3G and 3H). On the other side, PPP1R14D knockdown resulted in more aggressive metastasis compared to sh-control tumor in both lung and liver (Fig. 3E and 3F, quantified in Fig. 3G and 3H). In order to further examine the unexpected tumor growth and metastasis to distant organs, we assessed the activation status of EGFR-dependent signaling pathways as well as of other cellular growth pathways.





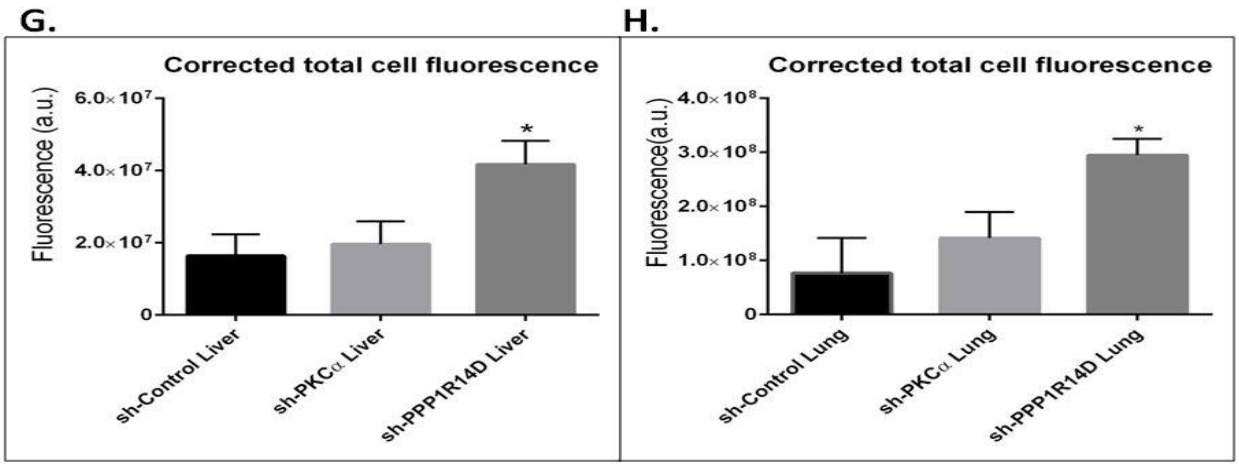


Figure 6.3: Analysis of PKCa and PPP1R14D knockdown on metastasis. **A-F**: Fluorescent representative images, **G, H**: quantifications Fluorescent image of Lung metastasis in mice with MDA-MB-231 GFP+ cells with sh-control at 5 weeks.

The metastasis was not expected, since we have previously seen that the knockdown of these genes causes reduced migration, invasion and/or growth of MDA-MB-231 cells *in vitro*.

Since we found that the knockdown of both genes PKCα and PPP1R14D, resulted in increased tumor size and metastasis, we subsequently examined whether there was re-activation of the EGFR pathway in these tumors *in vivo*. In addition, we examined the activity of major mitogenic pathways in the tumors that are downstream of EGFR or other RTKs.

6.4 PKCα and PPP1R14D knockdown effect on signaling pathway activation *in vivo* at week 5

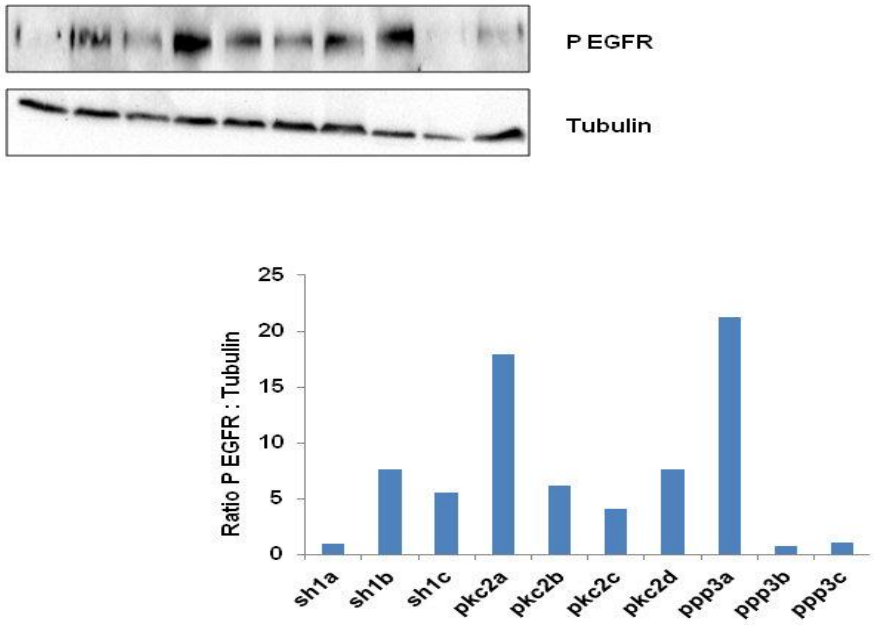
In order to examine possible reasons for aggressive tumor growth and metastasis *in vivo* at 5 weeks despite EGF ligand cleavage regulator knockdown, activity state of EGFR, ERK1/2, Akt and Stat3 were measured.

These pathways lie downstream of EGFR and involved in cancer cell growth and metastasis (30). Lysates were prepared from tumors and the phosphorylation levels of EGFR, ERK1/2, Akt and Stat3 were examined by western blot using phosphor-specific antibodies.

Phosphorylation levels of EGFR were variable in Control tumors **(Fig.A).** However, all PKCα tumors and one PPP1R14D knockout tumor sample showed reactivation of EGFR. These data suggest that stromal cells might be involved in the up regulation of phosphor-EGFR in some tumor samples.

On the other side, two PPP1R14D knockdown samples had much lower levels of phosphor-EGFR, suggesting that there is probably no involvement of EGFR in the aggressive tumor phenotype in these mice.

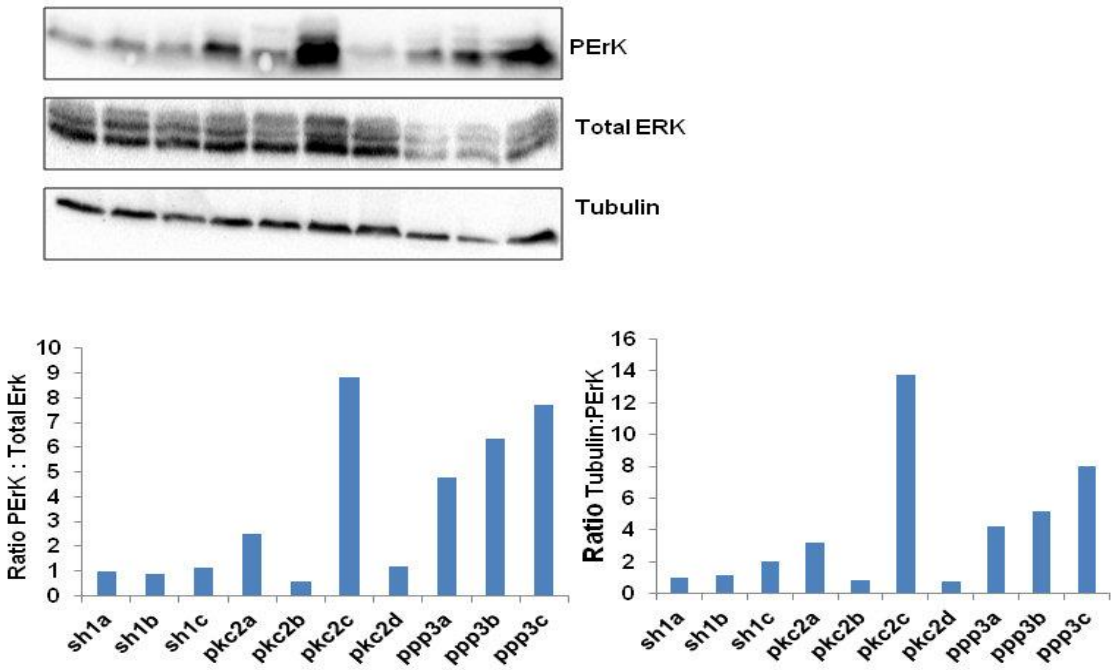
A.



Tumors showed significant up regulation of Phospho-Erk, likely explaining their unharnessed growth potential. ERK-P activation in tumors varied and was most pronounced in PPP1R14D knockdown cells **(Fig. B)**.

This coincided with their more aggressive growth and metastasis as compared to PKCα knockout cells.

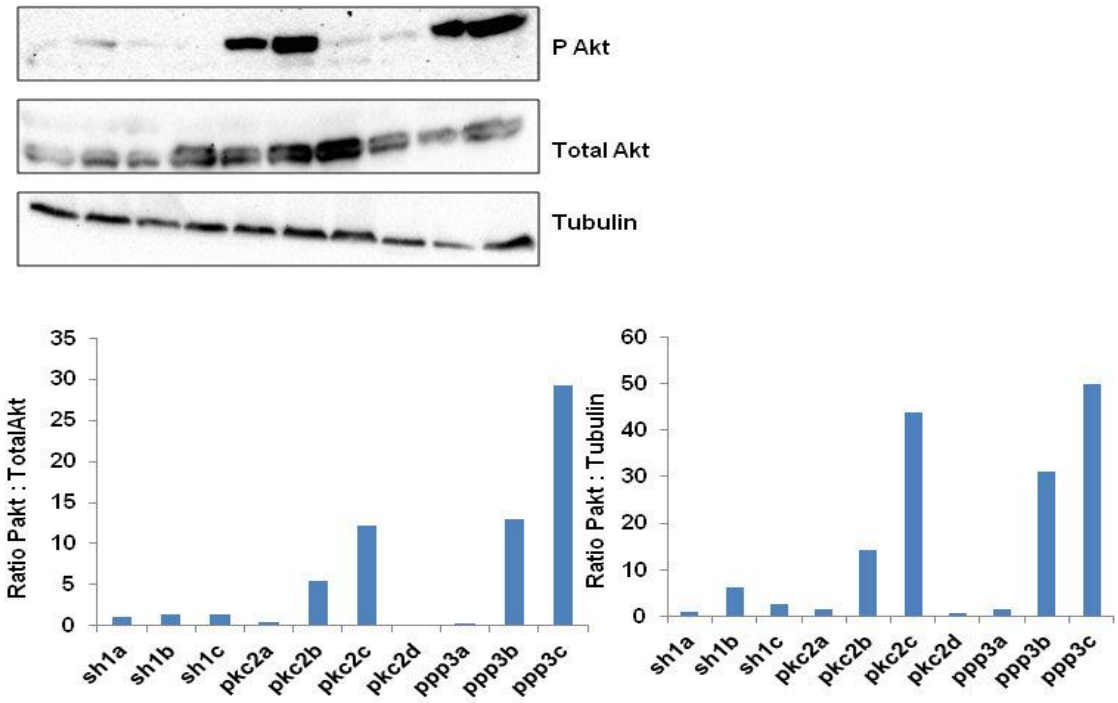
B.



In the case of Akt **(Fig. C)** which is also downstream effector of EGFR and can participate in resistance mechanism for cancer cells. Two samples of PKCα showed significant upregulation of phosphorylation levels of Akt.

On the other hand, two of PPP1R14D knockdown tumor samples showed also the same behavior. This indicates that there is activation of one or more other RTKs which have as a Akt downstream effector.

C.



Further Phosphorylation levels of stat3 **(Fig.D)** was examined as it is also a downstream effector of EGFR and regulates cell proliferation and growth. In PKCα knockdown tumor cells, all of them showed significant increase in phosphorylation levels of stat3 except one sample.

On the other hand, PPP1R14D knockdown tumor samples also showed significant upregulation of phosphorylated levels of stat3 compared to control tumor samples.

D.

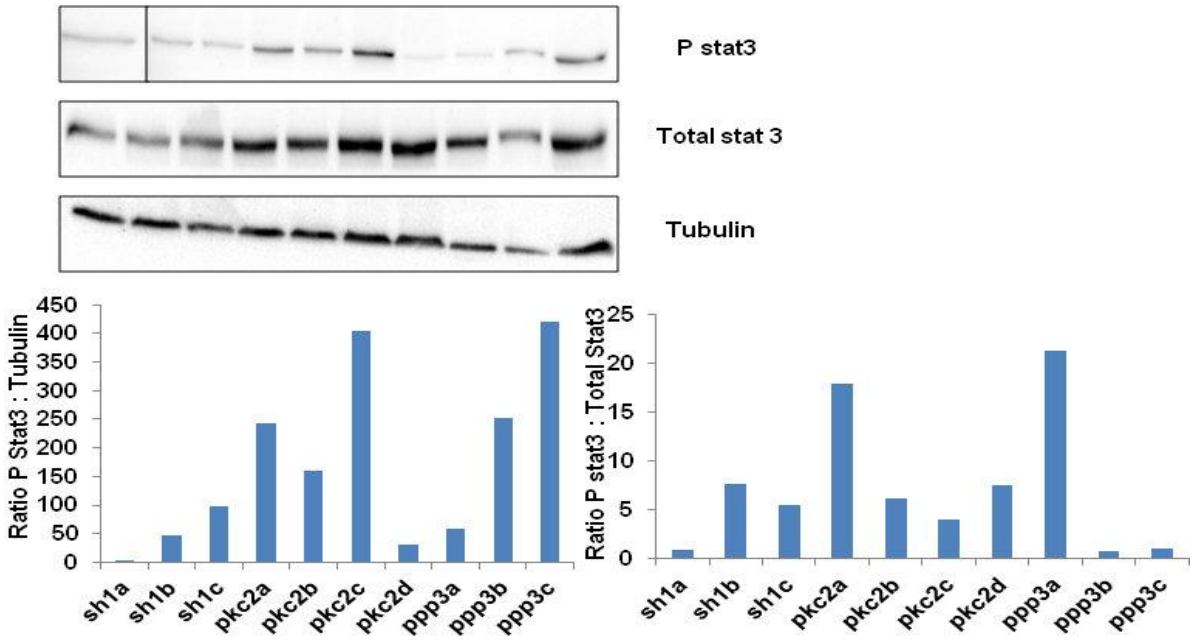


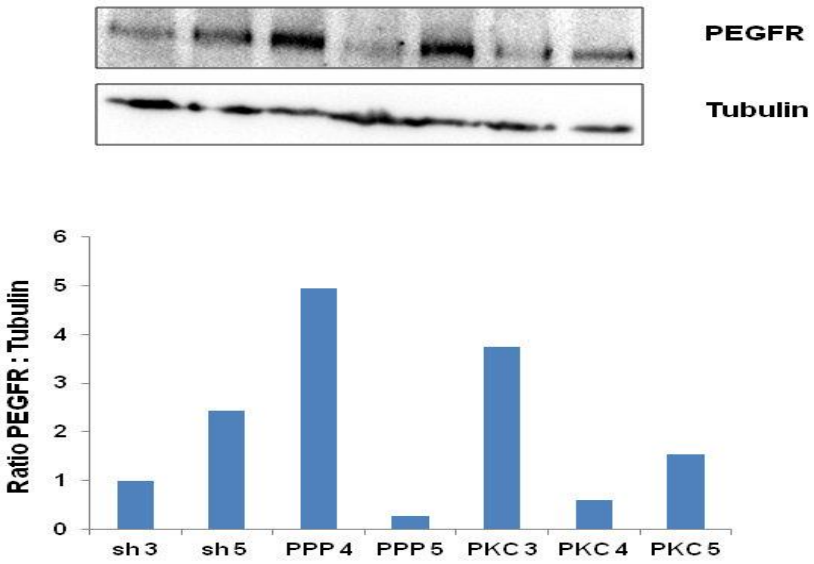
Figure 6.4 A. western blot showing phosphorylated levels of EGFR in week 5 PKCa and PPP1R14D knockdown tumor cells **B**. western blot showing phosphorylated levels of ERK ½ in week 5 PKCa and PPP1R14D knockdown tumor cells **C.** western blot showing phosphorylated levels of Akt in week 5 PKCa and PPP1R14D knockdown tumor cells **D.** western blot showing phosphorylated levels of stat3 in week 5 PKCa and PPP1R14D knockdown tumor cells. sh = sh-control, PPP = PPP1R14D knockout cells, PKC = PKC a knockout cells

Overall the above data show a reactivation of EGFR and a profound increase of ERK1/2 and Akt activation in some of the PKCα and PPP1R14D knockdown tumor samples. In order to examine whether these are early tumor responses or if they only appear in the advanced stages of tumor growth, we further examined the activation levels of these pathways at earlier time points.

6.5 PKC a and PPP1R14D knockdown effect on signaling pathway activation *in vivo* at week 4

At week 4, phosphorylation levels of EGFR showed variable results **(Fig. A).** In PPP1R14D knockdown tumor samples, one sample has significant upregulation of phospho-EGFR levels. Other sample didn't show any EGFR involvement. However, PKCα knockdown tumor samples followed the same pattern. One tumor sample showed significant increase in phospho-EGFR levels and others result were not significant.

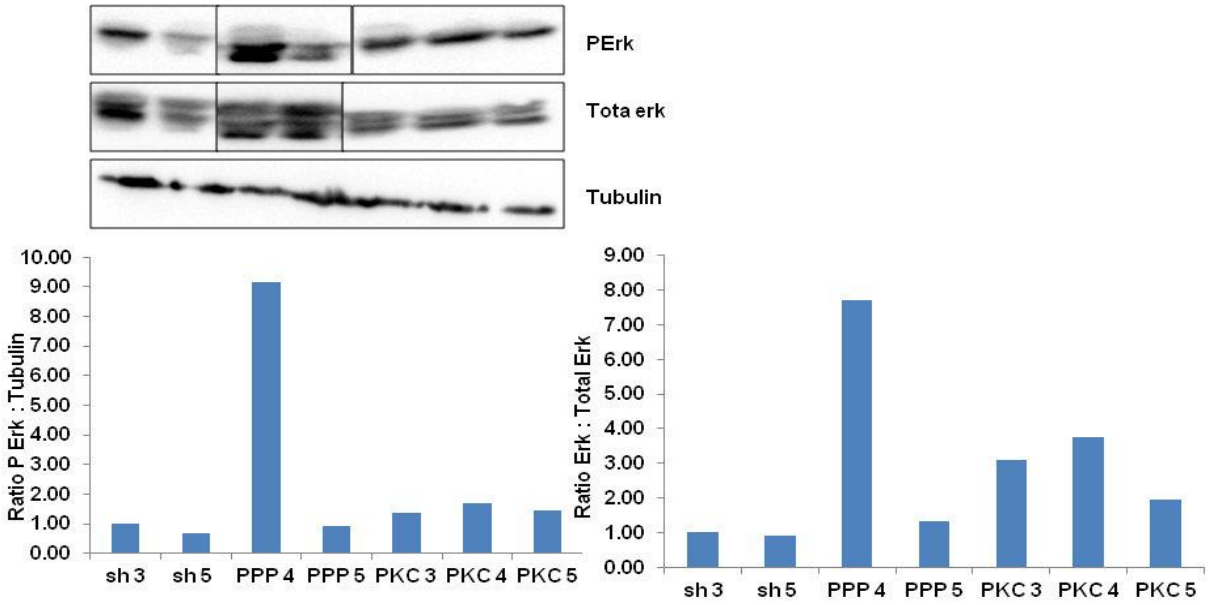
A.



At week 4, the phosphorylated levels of Erk showed upregulation. In PPP1R14D knockdown tumor samples, one of the tumor sample showed significant increase in phospho-ErK levels compare to other tumor samples of the same group **(Fig.B).** Other also showed upregulation but not significant compared to control tumor samples.

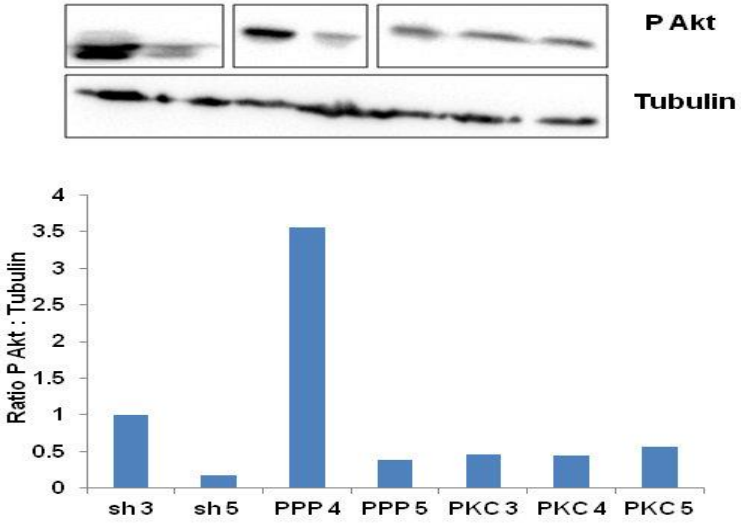
In case of PKCα knockdown tumor samples, all of the samples showed upregulation of phospho-ErK levels compared to controls which is indicating the involvement of other RTKs are having same downstream effector which is ErK **(Fig. B).**

B.



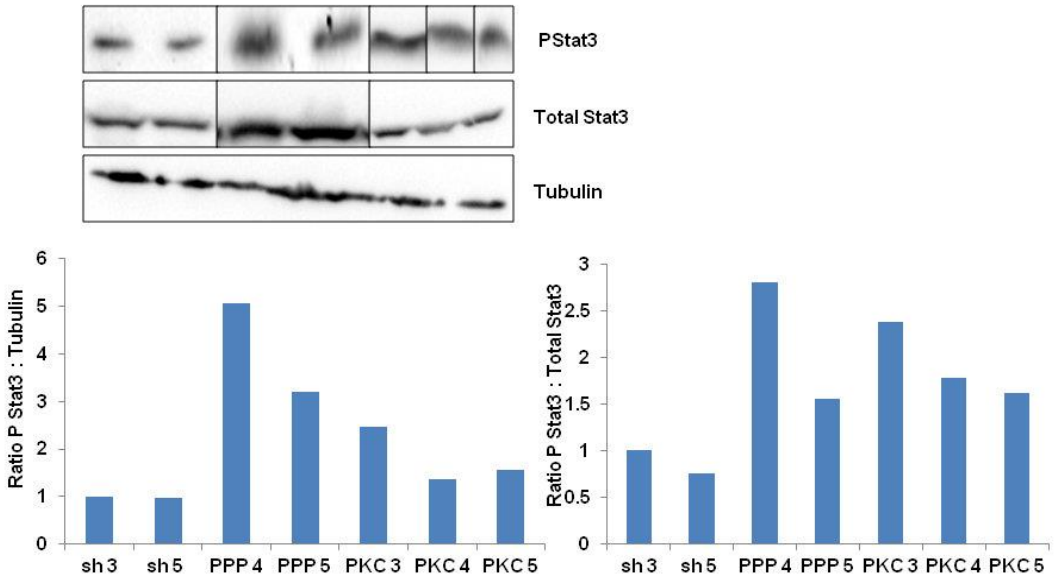
At week 4, only the one sample from PPP1R14D knockdown tumors showed significant up-regulation of Phospho-Akt levels compare to control tumor samples **(Fig. C).** However, PKCa knockdown tumor samples did show upregulation in phosphorylated levels but it's not significant compared to control tumor samples **(Fig. C).**

C.



At week 4, the phospho-stat3 levels in PPP1R14D knockdown tumor samples showed significant up-regulation compared to control tumor samples. However, PKCα knockdown tumor samples also showed upregulation of phospho-stat3 in all the tumor samples. The phospho-stat3 levels were highest increased in PPP1R14D.

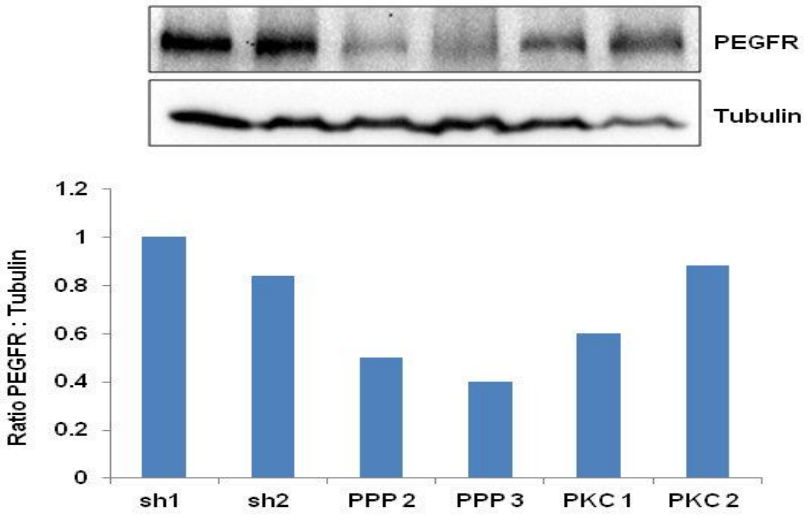
D.



6.6 PKCα and PPP1R14D knockdown effect on signaling pathway activation *in vivo* at week3

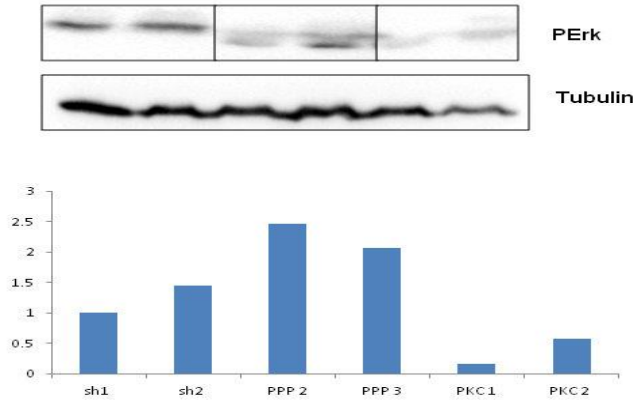
At week 3, the phosphorylation levels of EGFR didn't upregulation in both PPP1R14D and PKCα tumor samples **(Fig. A).** It suggests that there is no involvement of EGFR in tumor growth and metastasis at week 3 time point. This result motivated us to look for other downstream effectors of EGFR to check their phoshphorylated levels as those effectors are also target for other RTKs.

A.



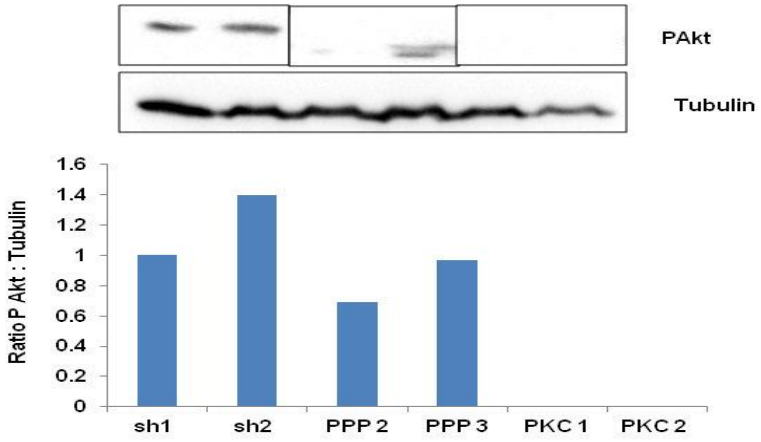
The phospho ERK ½ did show upregulation in PPP1R14D knockdown tumor samples but not signaficant increase **(Fig. B).** On the other hand, PKCa didn't show upregulated phospho-ERK levels **(Fig. B),** suggesting no involvement in tumor growth and metastasis at week3.

B.



At week 3, Phospho-Akt levels were also not upregulated in both PKCα and PPP1R14D knockdown tumor samples, indicated no involvement of Akt in tumor growth and metastsis at this time point **(Fig. C).**

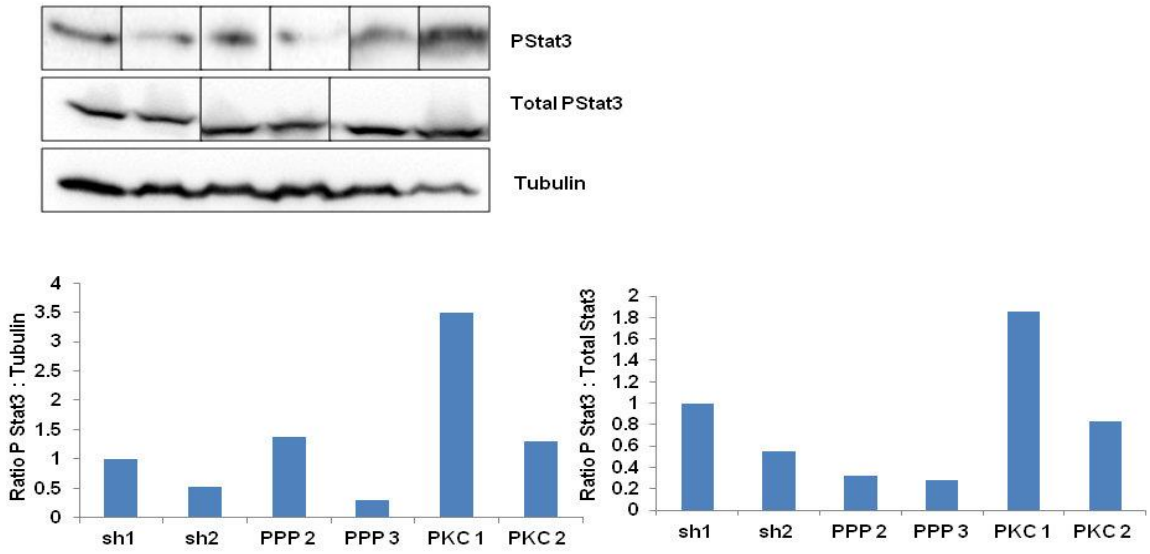
C.



At week 3, the stat3 phosphorylation levels were upregulated in PPP1R14D knockdown tumor samples but not significant increase (**Fig. D**). On other side, one of the PKCα knockdown tumor sample showed signaficant increase in phospho-stat3 levels and other one didn't show significant upregulation of phospho-stat3 levels of the same group **(Fig. D).**

This suggest that some of the samples from both knockdown genes showed involvement of stat3 at week 3 time point and few other didn't show.

D.



In summary, I found up-regulation of phosphorylated levels of Erk and reactivation of Akt pathway in week 5 and week 4 mainly. The stat3 is consecutively up-regulated all three weeks and EGFR showed variable signals which are assuming because of stromal cells involvement.

The reactivation of mitogenic pathways described above is reminiscent of the effects previously observed when specific kinase inhibitors were used *in vivo* and caused the activation of alternative growth pathways, leading to tumor cell survival and resistance to treatments (30, 31). In order to examine the possibility of alternative pathway activation in our *in vivo* model, we examined the upregulation of a panel of RTKs that have been previously involved in cancer resistance mechanisms (32).

6.7 PKCa and PPP1R14D knockdown effect on receptor tyrosine kinases (RTKs) Expression *in vivo*:

In order to test the mRNA expression levels of different receptor tyrosine kinases, we performed qPCR. The RNA was extracted from tumors and the concentration was measured with nano drop. A Qiagen kit was used for reverse transcription and sample preparation for qPCR.

The expression levels of each RTK were calculated using the Delta-Delta Ct method. Data were plotted as log 2 fold change of control samples values. The RTKs, I studied, are well cited literature for having involvement in cancer cell growth and acquiring resistance to single targeted therapies.

At week 5 **(Fig. A)**, In PKCα knockdown cells predominantly family members, DDR family, HER 3 and HER4 are showed significantly increased mRNA expression levels. FGFR has been involved in tumor angiogenesis. DDR play important role cell differentiation and homeostasis by communicating with cell-matrix. This function is disrupted by tumor cell by destroying the communication and which lead to DDR mutation and alter in receptor function (31, 33, 39).

HER 3 has mainly involved in dimerization with other family members and alter the response of cancer therapies. The role of HER4 is not very well understood in tumor biology and in normal cell, it helps in cell differentiation.

On the other hand, PPP1R14D knockdown cells showed with significantly increased mRNA expression level of FGFR family and HER4 just similar with PKCα knockdown cells. There is also increase in VEGFR family, DDR family and HER3, but it was not significant.

In order to examine whether these are early tumor responses or if they only appear in the advanced stages of tumor growth, we further examined the mRNA expression levels of these RTKs at earlier time points.

At week 4 **(Fig. B)**, the PKCα knockdown tumor samples showed upregulation of FGFR 2 which is similar with week 5 results. However PPP1R14D tumor samples showed upregulation

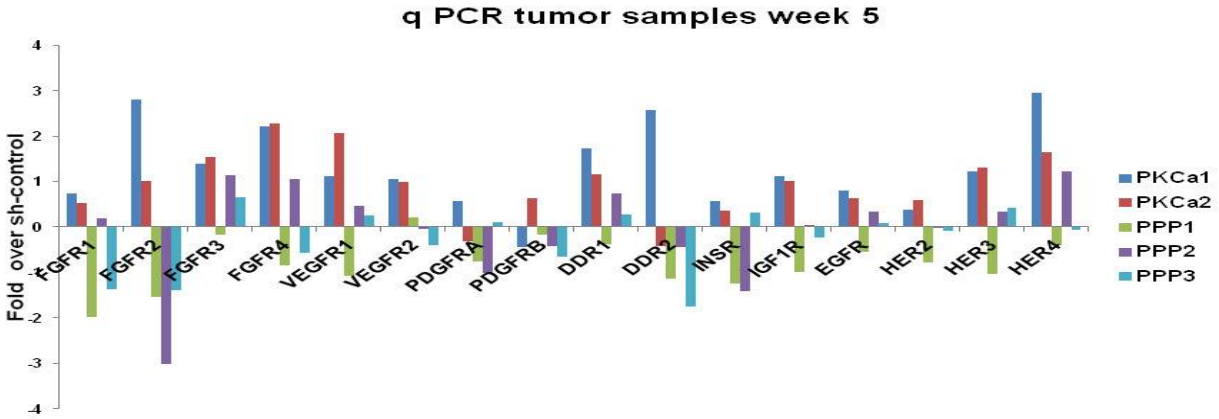
of FGFR2 and also one of the samples showed upregulation of other RTKs which are PDGFR family and ERBB4.

At week 3(**Fig. C),** one of the PKCα knockdown tumor sample showed significant up-regulation in mRNA expression levels of FGFR2, ERBB3 and ERBB4 and one of the PPP1R14D tumor sample showed high expression level of mRNA of ERBB2.

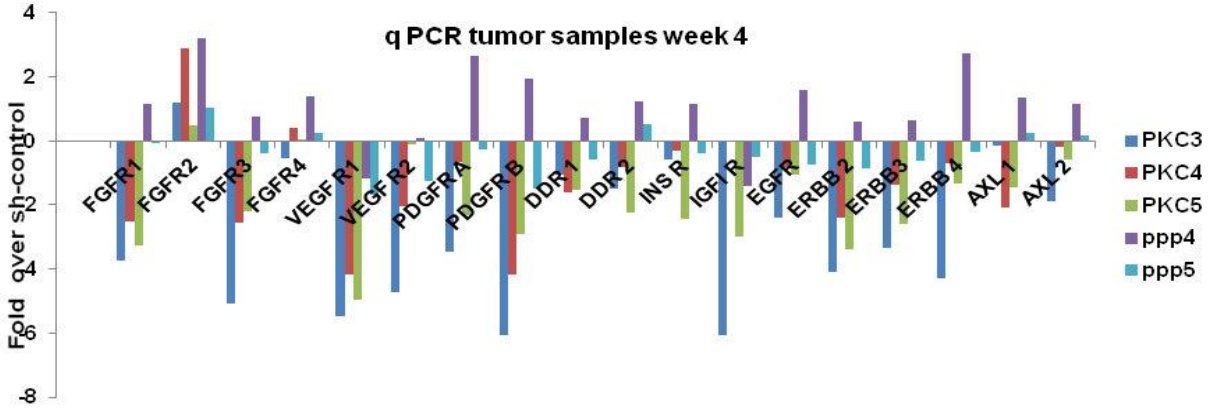
In qPCR data from three weeks, I found upregulation of mRNA expression levels of FGFR and EGFR family members which was common in all the three weeks. DDR family was only upregulated in week 5.

In summary, the FGFR, DDR, EGFR , VEGFR and PDGFR family has been upregulated in both PKCα and PPP1R14D knockdown tumor samples at different time points. These RTKs played major role in activation of alternative pathways, which lead to excessive tumor growth and metastasis in later time points.

A.



B.


q PCR tumor samples week 4
Fold over sh-control
4
2
0
-2
-4
-6
-8
FGFR1
FGFR2
FGFR3
FGFR4
VEGF R1
VEGF R2
PDGFR A
PDGFR B
DDR 1
DDR 2
INS R
IGF I R
EGFR
ERBB 2
ERBB3
ERBB 4
AXL 1
AXL 2
PKC3
PKC4
PKC5
ppp4
ppp5

C.

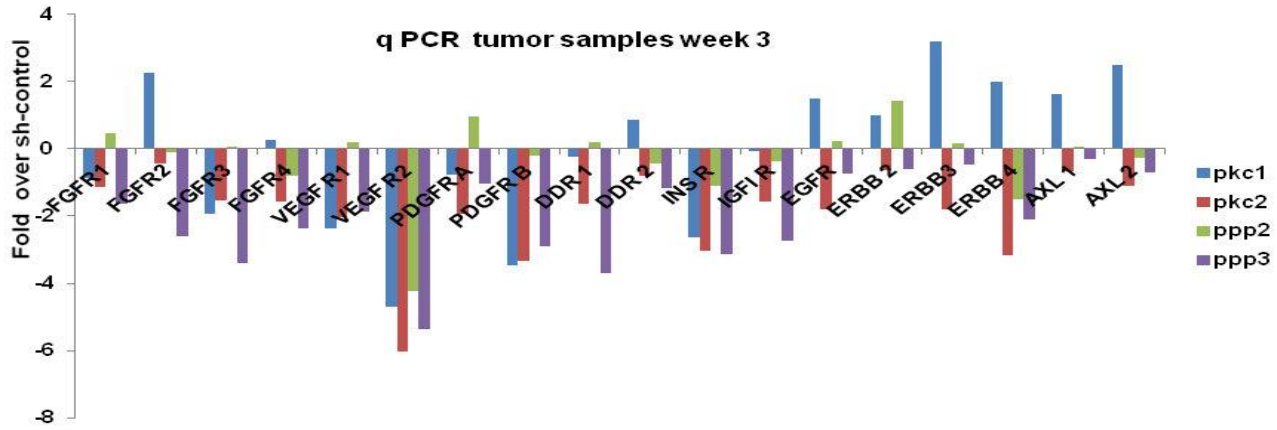

q PCR tumor samples week 3
Fold over sh-control
4
2
0
-2
-4
-6
-8
FGFR1
FGFR2
FGFR3
FGFR4
VEGF R1
VEGF R2
PDGFR A
PDGFR B
DDR 1
DDR 2
INS R
IGF I R
EGFR
ERBB 2
ERBB3
ERBB 4
AXL 1
AXL 2
pkc1
pkc2
ppp2
ppp3

Figure 6.6 (A, B, C) pkc =PKC a, ppp = PPP1R14D, FGFR = Fibroblast growth factor receptor, VEGFR = vascular endothelial growth factor, PDGFR = *Platelet-derived growth factor receptor, DDR =* Discoidin domain receptor, INSR = Insulin receptor, IGFI R = Insulin receptor, EGFR = Endothelial growth factor receptor, ERBB = Endothelial growth factor receptor also named HER, AXL = AXL receptor.

Discussion:

Triple negative breast cancer is an aggressive breast cancer subtype with poor prognosis and no successful treatment options. The underlying causes for triple negative breast cancer and its high metastatic potential continue to be tirelessly studied. Both environmental and genetic factors play critical roles in this disease. Prevention, diagnosis, and treatment must consider the specific factors in order to effectively quell metastatic manifestations (31, 34, 35). This thesis proposes that combination therapies may play a critical role in triple negative breast cancer and metastasis by circumventing resistance to EGFR targeting, for example as proposed here by targeting of the ADAM17/EGF ligand axis. I discuss here why knockdown of PKCα and PPP1R14D, ADAM 17 cleavage regulators studied in my master's thesis, did not successfully prevent tumor growth and metastasis *in vivo* although it inhibited EGF ligand release *in vitro*.

The first experiment to confirm knockdown of PKCα and PPP1R14D in tumor samples provided us with the solid data that knockdown worked in our tumor samples. However, tumor growth and metastasis was not blocked as expected. Phosphorylation levels of EGFR measured in the tumors at different time points revealed that there is variability in phospho-EGFR levels and not all tumors show inhibited EGFR activation, as would be expected for ADAM17 cleavage regulator knockdown. Interestingly, at week 3 there is no up-regulation of phospho-EGFR levels in any of the tumors. At week 4, one tumor sample from each gene knockdown showed up-regulation. At week 5, however, all tumors with PKCα knockdown and one of the PPP1R14D knockdown tumor samples showed phospho-EGFR up-regulation. This lead us to conclude that over time some of the tumors manage to turn back on the primary downstream target of our intervention, EGFR. This could occur due to ligand-independent activation of the receptor or by compensatory release of ligands from the uninhibited tumor stroma.

We also found that there is significant up-regulation of phospho-Erk levels at week 4 and 5 in PPP1R14D knockdown tumor samples compared to control tumor samples. At week 3 PPP1R14D knockdown tumors didn't show significant up-regulation of phospho-Erk. The PKCα knockdown tumor samples had up-regulation but this was not significant compared control

tumor samples in all three time points. This suggests that PPP1R14D knockdown tumors reactivate the major downstream target of EGFR, ErK. This could occur due to EGFR reactivation, as mentioned above, or alternate activation of ErK secondary to other growth factor receptors that the tumor cell up regulates in response to EGFR inhibition.

ErK is a downstream part of an evolutionarily conserved signaling module that is activated by the Raf serine/threonine kinases. Raf activates the MAPK/ErK kinase (MEK) ½ duel specificity for protein kinase and it activates Erk1/2 and can be activated by numerous growth factor receptors, many of them receptor tyrosine kinases. Mutation of Raf leads to cancer. The small GTPase Ras lies upstream of the Raf-MEK-ErK pathway, and represents the most frequently mutated oncogene in human cancers. Finally, Ras is a key downstream effector of the epidermal growth factor receptor (EGFR), which is mutationally activated and/or overexpressed in a wide variety of human cancers. ErK activation also promotes upregulated expression of EGFR ligands that lead to tumor growth (31, 36, 37).

The phosphorylation levels of Akt were also up-regulated at week 5 in both PPP1R14D and PKCα knockdown tumor samples. At week 4, only one PPP1R14D tumor knockdown samples showed upregulation of Akt-Phospho levels and no upregulation at the week 3 time point. These results lead us to conclude that next to ErK, tumors also upregulated the activity of other mitogen activated kinases like Akt, in response to EGFR inhibition. The phosphorylation of Akt could be the result of crosstalk between MAP kinase and PI3k pathways. The variable phospho-EGFR, upregulation of ERK and Akt activity could be the result of limited crosstalk between all three of them (34, 38).

Different RTKs were checked for their up-regulation with qPCR and surprisingly, the FGFR family and ERBB4 were strongly up-regulated in week 3, 4 and 5 time points. Few others, DDR family members and VEGFR showed up-regulation in few tumor samples but this was not consistent across tumors. FGFR2 indeed is known to activate ERK1/2. The VEGFR family in contrast is known to activate PI3K/AKt.

Conclusion:

This investigation examined the knockdown effects of both PKCα and PPP1R14D cleavage regulators of ADAM17 in triple negative breast cancer. The *in-vitro* studies showed reduced invasion, migration and proliferation in MDA-MB-231 cells. Contrary to *in-vitro* results, *in-vivo* studies showed excessive tumor growth and metastasis.

In summary, I have identified resistance mechanisms in TNBC that occur in response to EGFR targeting, or targeting of EGF ligand release by knockdown of ADAM17/EGF ligand cleavage regulators. My results identify FGFR and VEGFR family member as alternate targets for combination therapy with EGFR inhibitors. It also suggests that biopsies during the course of treatment and in particular of metastasis can meaningfully influence treatment decisions.

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