

Award Number: W81XWH-11-1-0029

TITLE: The Role of Platelet-Derived Growth Factor C and Its Splice Variant in Breast Cancer

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REPORT DATE: April 2014

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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REPORT DOCUMENTATION PAGE

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4. TITLE AND SUBTITLE The Role of Platelet-Derived Growth Factor C and its Splice Variant in Breast Cancer			5a. CONTRACT NUMBER W81XWH-11-1-0029		5b. GRANT NUMBER W81XWH-11-1-0029	
			5c. PROGRAM ELEMENT NUMBER		5d. PROJECT NUMBER	
			5e. TASK NUMBER		5f. WORK UNIT NUMBER	
6. AUTHOR(S) Alyssa Bottrell			7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Wayne State University School of Medicine, Detroit, Michigan 48201		8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSOR/MONITOR'S ACRONYM(S)		11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT The aim of this project is to elucidate the role of Platelet-Derived Growth Factor C (PDGFC) and its splice variant, t-PDGFC, in breast cancer. This study has resulted in novel findings: Results show PDGFC expression to be associated with more aggressive characteristics. Data <i>in vitro</i> and <i>in vivo</i> show that higher expression of the PDGFC isoforms in breast cancer cell lines is associated with higher proliferation, invasion, and metastatic potential. Furthermore, this study has shown PDGFC in the nuclear fraction of breast cancer cell lines, suggesting an uncharacterized novel function in the nucleus. Additionally, t-PDGFC was believed to be exclusively an intracellular protein. However, new results in this project show that t-PDGFC can be secreted from breast cancer cells presumably as a hetero-dimer with FL-PDGFC. Thus, both FL-PDGFC and t-PDGFC can be intracellular and extracellular proteins. This marks an important paradigm shift for PDGFC, once typically thought of as primarily an extracellular signaling molecule. Taken together, my study has potentially important implications in the functional significance of PDGFC in breast cancer and how to target aberrant PDGFC signaling. Future drug targeting may need to take into account the intracellular and extracellular roles of both PDGFC isoforms in order to combat cancer.						
15. SUBJECT TERMS Breast Cancer, Growth Factors, PDGFC						
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON	
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Introduction

With exception of basal cell and squamous cell skin cancers, breast cancer is the most commonly diagnosed cancer among women with an estimated one in eight United States women projected to be diagnosed with breast cancer in her lifetime [1]. This year alone 232,670 American women are predicted to be newly diagnosed with invasive breast cancer and approximately 62,570 are expected to be diagnosed with early stage *in situ* breast cancer [1]. There will be an estimated 40,000 American women who die due to breast cancer this year [1]. Due to breast cancer's significant morbidity and mortality of American women, it is important to understand the factors that drive the development, growth, and metastases of this prevalent and deadly disease.

PDGFC is a more recently discovered member of the PDGF family [2-4]. Our laboratory has implicated PDGFC in breast cancer progression [5]. As the result of differential splicing, there are two forms of the PDGFC messenger RNA; Full Length-PDGFC (FL-PDGFC), and the more recently discovered splice variant, Truncated PDGFC (t-PDGFC) is the result of a retained exon [6]. This retained exon creates a premature stop codon and an alternative start codon creates a truncated protein reported in the literature to be an intracellular protein [6]. In addition, one recent study reports nuclear PDGFC in human thyroid cells [7].

The aim of this project has been to further understand the role of PDGFC and its splice variant in breast cancer.

Body

In the past funding period I have continued to study the role of PDGFC isoforms in human breast cancer. Several new findings have been made in this research project, increasing our understanding of PDGFC and its splice variant in breast cancer. As stated in prior reports, we have established three sets of breast cancer cell line models:

Model 1, MCF-7 breast cancer cells which normally express little endogenous PDGFC were transfected with control vector or FL-PDGFC (His-tagged) to over-express FL-PDGFC or vector control. In addition, MCF-7 breast cancer cells transfected to over-express t-PDGFC/myc or FL-PDGFC/myc or vector control.

Model 2, MDA-MB-231 breast cancer cells which normally express high levels of both FL-PDGFC and t-PDGFC mRNA were transfected with control (shScramble) or PDGFC shRNA (shPDGFC) to knock down PDGFC expression.

Model 3, BT-549 breast cancer cells that express mostly endogenous FL-PDGFC were transfected with control vector or t-PDGFC myc tagged.

Specific Aim 1 is to establish the functional significance of PDGFC in phenotypic transformation of breast epithelial cells. We hypothesized that PDGFC induces transformation and an invasive phenotype. We further hypothesized that t-PDGFC enhances the oncogenic potential of FL-PDGFC in breast carcinoma cells. In a previous annual report we showed that knock down of PDGFC expression decreased anchorage-independent growth and cellular proliferation. During this funding period we show that expression of t-PDGFC increases the invasion of BT-549 breast cancer cells in an *in vitro* Matrigel invasion assay (Figure 1).

In addition, utilizing real time PCR we screened a panel of several human breast cancer cell lines to quantitate the total PDGFC RNA (total includes FL-PDGFC and t-PDGFC RNAs, Figure 2A). Designing one of the real time PCR primers within the unique retained exon of t-PDGFC allowed for quantification of only t-PDGFC RNA (Figure 2B). This data further confirms our laboratory's previous results via semi-quantitative PCR; Human breast cancer cell line MCF-7 has almost undetectable levels of total or t-PDGFC (Figure 2A & 2B). Additionally, human breast cancer cell line BT-549 has high levels of total PDGFC but relatively lower levels of specifically t-PDGFC making it an ideal model in which to over-express t-PDGFC (Figure 2A & 2B). While MDA-MB-231 human breast cancer cell line has relatively high levels of both total PDGFC and t-PDGFC (Figure 2A & 2B), making it an ideal model in which to knock down the expression of PDGFC.

Furthermore utilizing an online database repository of human breast cancer cell line gene expression data, Gene expression-based Outcome for Breast cancer Online (GOBO), total PDGFC expression was analyzed in many additional breast cancer cell lines [8, 9]. GOBO is an online tool that can be queried to assess the expression of a particular gene or genes of interest in large panel of 51 human breast cancer cell lines, and GOBO conveniently creates image output of the data (Figure 2C, Figure 3A and 3B). Although the gene expression data of GOBO tool does not distinguish between the expression of the PDGFC splice variants, this online tool still provides valuable data about total PDGFC expression in human breast cancer cell lines. As shown in Figure 2C, the relative expression of PDGFC varies widely in a panel of 51 human breast cancer cell lines, with the highest PDGFC expression being in MDA-MB-231 cell line. Such gene expression data in a large panel of human breast cancer cell lines helps identify which cell lines that would ideally suited for further *in vitro* and *in vivo* studies. Correlating with our laboratory's semi-quantitative data, GOBO data output shows relatively high levels of total PDGFC expression in human breast cancer cell lines such as MDA-MB-231 and BT-549 while there is relatively very low total PDGFC expression in the less aggressive, hormone receptor positive human breast cancer cell line MCF-7 (Figure 2C).

Furthermore, GOBO analyzes gene expression data in these human breast cancer cell lines grouped into breast cancer transcription profile subgroups Basal A, Basal B and Luminal defined in the literature [9]. Total PDGFC expression is found to be highest in Basal B breast cancer cell lines, and is also high in Basal A human breast cancer cell lines while it is comparatively lower in Luminal breast cancer cell lines (Figure 3A). Furthermore, the difference in PDGFC expression is statistically significant amongst the breast cancer subgroups ($p \leq 0.00001$ Figure 3A). In addition, GOBO analysis and graphical output displays gene expression by grouping the 51 breast cancer cell lines according to breast cancer receptor status such as hormone receptor expression and Her2/neu receptor over-expression (Figure 3B). PDGFC expression is highest amongst the triple negative breast cancer cell lines, and then second highest amongst the Her2 receptor over-expressing cell lines, with the lowest expression of PDGFC in the hormone receptor positive breast cancer cell lines ($p=0.00031$, Figure 3B). Taken together, this suggests that PDGFC expression tends to be higher in breast cancer cell lines with more aggressive features.

This project's Specific Aim 2 is to investigate t-PDGFC regulation of FL-PDGFC subcellular localization and PDGFR activation in human breast carcinoma cells. In previous annual reports we showed nuclear localization of PDGFC which suggests new roles of PDGFC in cancer cells. Nuclear localization of PDGFC in breast cancer cell lines is a novel finding and is potentially clinically relevant. In addition, in the literature our laboratory has shown mutation of amino acids in the hinge region of PDGFC to be important in proteolytic processing for activation of PDGFC (K225A/R231A/R234A) [5]. In a previous annual report of this project the same mutation of amino acids in the hinge region appear to be also important for secretion and nuclear localization of PDGFC. Furthermore, in a previous annual report expression of t-PDGFC by transfection increased secretion of PDGFC, which can then be proteolytically processed to the growth factor domain. This resulted in autocrine signaling as evidenced by the increase the downstream signaling of PDGFC through phospho-Akt and phospho-Erk shown previously. Further experiments during this funding period have shed new light on the subcellular localization of t-PDGFC. One study published recently in the literature describes t-PDGFC as an intracellular protein [6] and in previous annual reports due to technical difficulties detecting the myc-tagged t-PDGFC protein we mistakenly also believed t-PDGFC to be an exclusively intracellular protein. As seen in Figure 4, we are unable to detect myc-tagged t-PDGFC in the conditioned media of MCF-7 t-PDGFC transfected cells even in the presence of an effective irreversible serine protease inhibitor (AEBSF, 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), but we are able to detect FL-PDGFC in the condition media of MCF-7 FL-PDGFC myc-tagged transfected cells (Figure 4). In addition, AEBSF is able to inhibit the proteolytic processing of PDGFC in cells that naturally express PDGFC (Figure 5) suggesting that lack of detection of t-PDGFC myc-tagged in our MCF-7 over-expression model is not due to protease degradation of t-PDGFC in the conditioned media. Throughout these experiments, it was learned that western blot detection of our laboratory's myc-tagged antibody does not work as well under reducing conditions; however it does work well under non-reducing conditions. As seen in Figure 6 (Left blot), we now show that in BT-549 breast cancer cells

transfected cells, t-PDGFC myc-tagged protein can be not only secreted from breast cancer cells, but it is also of a unique molecular weight compared to intracellular t-PDGFC (non-reducing conditions). BT-549 cells naturally express FL-PDGFC while MCF-7 cells do not. The difference in molecular weight of the myc-tagged t-PDGFC intracellular as compared to extracellular myc-tagged t-PDGFC in the BT-549 model suggests that t-PDGFC is secreted presumably as a hetero-dimer with FL-PDGFC, while intracellular t-PDGFC is presumed to be a t-PDGFC homo-dimer. Importantly, myc-tagged t-PDGFC secreted into the conditioned media can be proteolytically processed by the serine protease matriptase to the growth factor domain (Figure 6, Right blot).

This project's Specific Aim 3 is to elucidate the role of PDGFC isoforms in breast cancer progression *in vivo*. We hypothesized that PDGFC expression induces oncogenic cell signaling in autocrine and paracrine manners to increase proliferation and invasive ability of tumor cells as well as to mediate stromal responses critical for tumor cell invasion and metastasis. In order to investigate the role of t-PDGFC in human breast cancer we had previously established stable transfected cells, as described above, BT-549 Empty Vector (Figure 7A, Lane 3) and BT-549 t-PDGFC myc-tagged transfected (Figure 7A, Lane 4). These stable transfected BT-549 human breast cancer cells differentially expressing PDGFC isoforms were injected into the peri-mammary fat pad of mice and tumor growth was assessed. Unfortunately the BT-549 human breast cancer cells did not grow well in this animal model and the small palpable tumors regressed (Figure 7B). However, there may have been a slight growth for a short period in the BT-549 t-PDGFC over-expressing cells as compared to the controls. This is not conclusive data and will have to be assessed in a breast cancer cell line more capable of forming tumors in orthotopic mouse models than the BT-549 human breast cancer cells. In an orthotopic mouse model, transfected MDA-MD-231 human breast cancer cells were injected peri-mammary fat pad and tumor growth was assessed. Before injection PDGFC expression was assessed by PCR (Figure 7A, lane 1, shScramble. Lane 2, shPDGFC). MDA-MB-231 PDGFC knock down cells (MDA-MB-231 shPDGFC) grew smaller volume tumors than the control transfected cells (MDA-MB-231 shScramble Figure 7C). Finally, in a metastasis model, tail vein injection of MDA-MB-231 cells showed a trend for smaller lung metastases when PDGFC expression is knocked down, however the difference in lung weights were not significantly different between control transfected and PDGFC knock down (Figure 8B). DNA extraction purity problems prevented analysis of the amount of human DNA versus mouse DNA in the mouse lungs by quantitative Alu-PCR.

Key Research Accomplishments

- PDGFC expression tends to be increased in human breast cancer cell lines with more aggressive features (e.g. triple negative breast cancer cell lines).
- An irreversible serine protease inhibitor, AEBSF, effectively inhibits the proteolytic processing of PDGFC in breast cancer cells.
- t-PDGFC is capable of being secreted presumably as a hetero-dimer with FL-PDGFC.
- Secreted t-PDGFC myc-tagged is proteolytically processed by matriptase to the PDGFC growth factor domain.
- Over-expression of t-PDGFC in BT-549 cells may increase tumor size in mice; however this needs to be further investigated in a new cell line more suited for a mouse model.
- Knockdown of PDGFC expression in MDA-MB-231 human breast cancer cells decreases the size of tumors in mice in an orthotopic model.
- Knockdown of PDGFC in MDA-MB-231 cells may decrease lung weight in a tail vein injection model of lung metastases, however not significantly.

Reportable Outcomes

Wayne State University School of Medicine Department of Pathology Student Seminar Presentation
“Subcellular Localization of PDGFC Isoforms and Its Implications in Breast Cancer”
November 8, 2013
Detroit, Michigan

Wayne State University School of Medicine MD/PhD Program Presentation
“PDGFC & Breast Cancer”
October 18, 2013
Detroit, Michigan

Data Blitz Presentation at the Wayne State University School of Medicine MD/PhD Program Annual Retreat
“Subcellular Localization of PDGFC Isoforms and Its Implications in Breast Cancer”
March 25th, 2013
Detroit, Michigan

Attendance of the “Breast Cancer Symposium with Updates from the 2012 San Antonio Breast Cancer Symposium”
March 2, 2013
Troy, Michigan

Conclusion

My study supported by this Department of Defense Breast Cancer Research Program Pre-Doctoral Traineeship award has demonstrated expression of PDGFC and its splice variant (t-PDGFC) in breast cancer are associated with more aggressive features. Inhibition of PDGFC expression decreased the aggressiveness of breast cancer cells *in vitro* as well as *in vivo*, suggesting the functional significance of PDGFC in breast cancer. While it was thought that t-PDGF is exclusively intracellular, I found that t-PDGFC can be secreted, presumably as a heterodimer with FL-PDGFC, which can be proteolytically processed into the growth factor domain dimer. In addition, I made a novel finding that PDGFC is found in the nucleus of breast cancer cells, suggesting a potentially novel nuclear function of PDGFC. Taken together, we propose a new working model of PDGFC localization and processing in breast cancer (Figure 9).

Increasing evidence in the literature suggests that increased PDGFC expression is associated with therapy resistance: Doxorubicin-selected MCF-7 cells show a 39.3-fold increase in PDGFC expression by microarray and were more invasive in a Matrigel assay as compared to MCF-7 parental [10]. In addition, the doxorubicin-selected MCF-7 cells show an 126.0-fold decrease in (ESR1) estrogen receptor 1 expression as compared to MCF-7 parental [10]. Another recent study utilizing the estrogen receptor positive MCF-7 cell line showed that PDGFC expression increased significantly upon silencing of estrogen receptor expression, and there was induction of epithelial mesenchymal transition in the estrogen receptor-silenced MCF-7 cells as compared to the parental MCF-7 [11]. Currently available PDGF inhibitors target the receptor tyrosine kinase activity. Taking these reports with our findings above, we propose that PDGFC may present a therapeutic target to help overcome drug resistance. Future drug targeting may need to take into account the intracellular and extracellular roles of both PDGFC isoforms.

I am expecting to defend my PhD dissertation by June 2014. After which I will continue to complete the requirements of the MD/PhD program and plan to return to the third year of medical school in July 2014. I plan to complete medical school in 2016, fulfilling the requirements to earn both my Medical Degree and Doctorate of Philosophy Degree. After

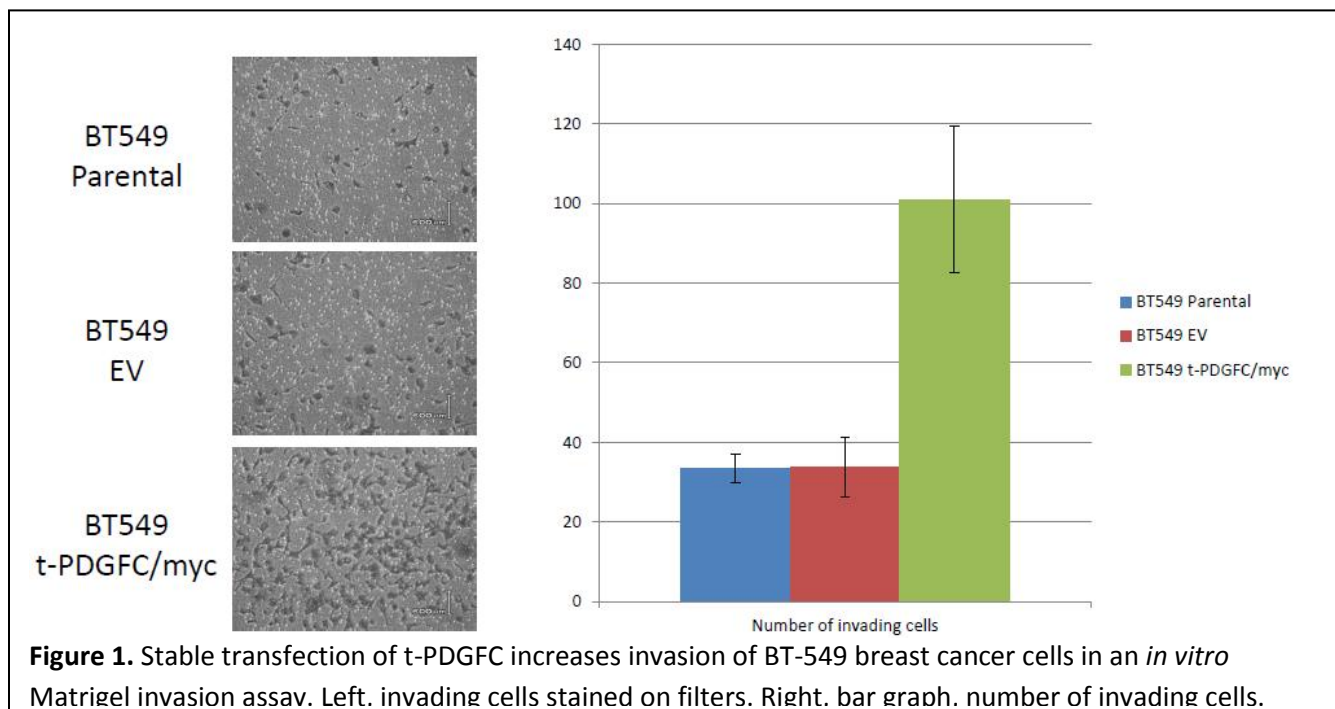
medical school I hope to secure a position as a resident physician in Internal Medicine followed by a fellowship specializing in Hematology and Oncology so that I can treat cancer patients.

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Appendices (None)

Supporting Data



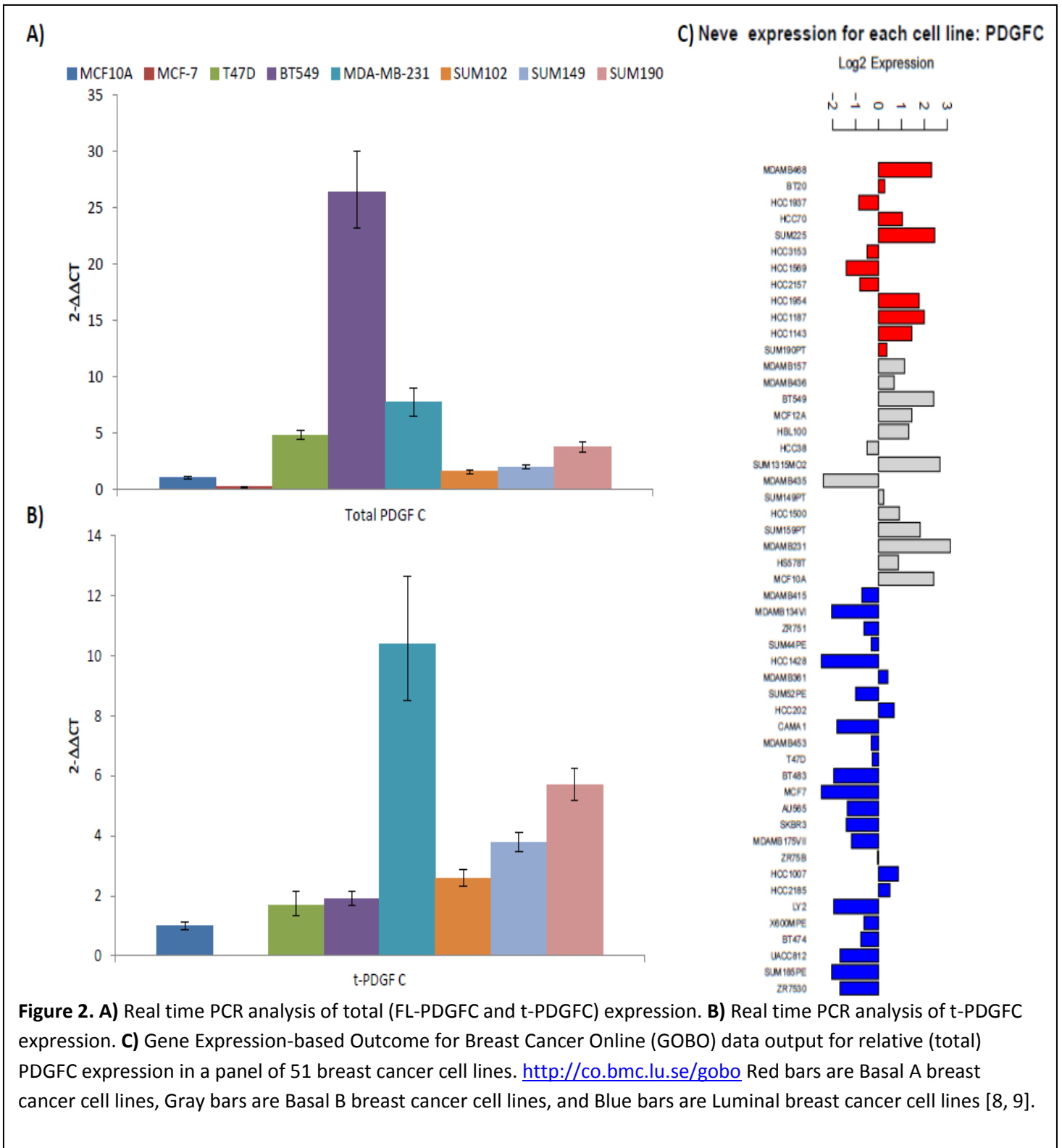
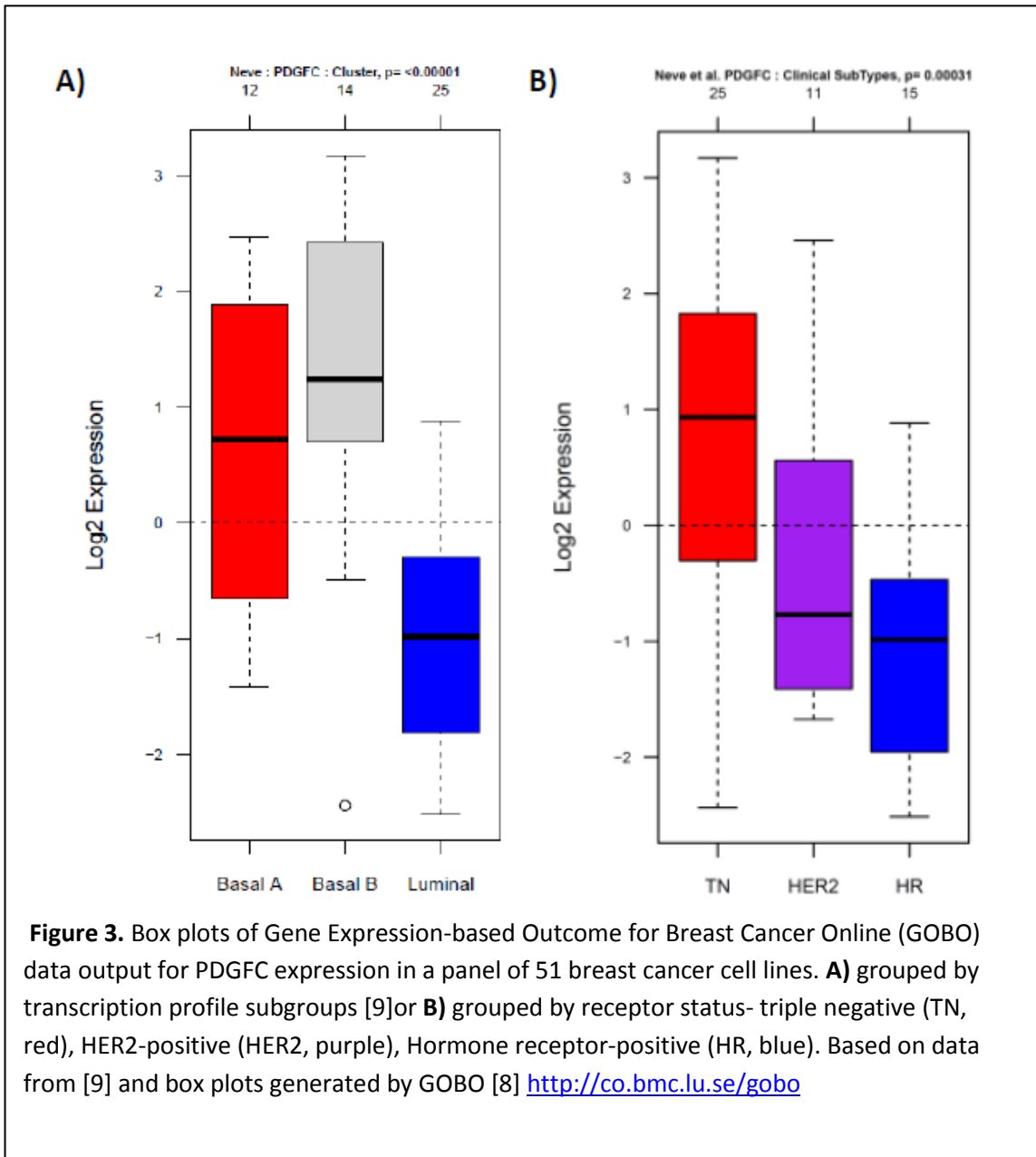


Figure 2. A) Real time PCR analysis of total (FL-PDGFC and t-PDGFC) expression. **B)** Real time PCR analysis of t-PDGFC expression. **C)** Gene Expression-based Outcome for Breast Cancer Online (GOBO) data output for relative (total) PDGFC expression in a panel of 51 breast cancer cell lines. <http://co.bmc.lu.se/gobo> Red bars are Basal A breast cancer cell lines, Gray bars are Basal B breast cancer cell lines, and Blue bars are Luminal breast cancer cell lines [8, 9].



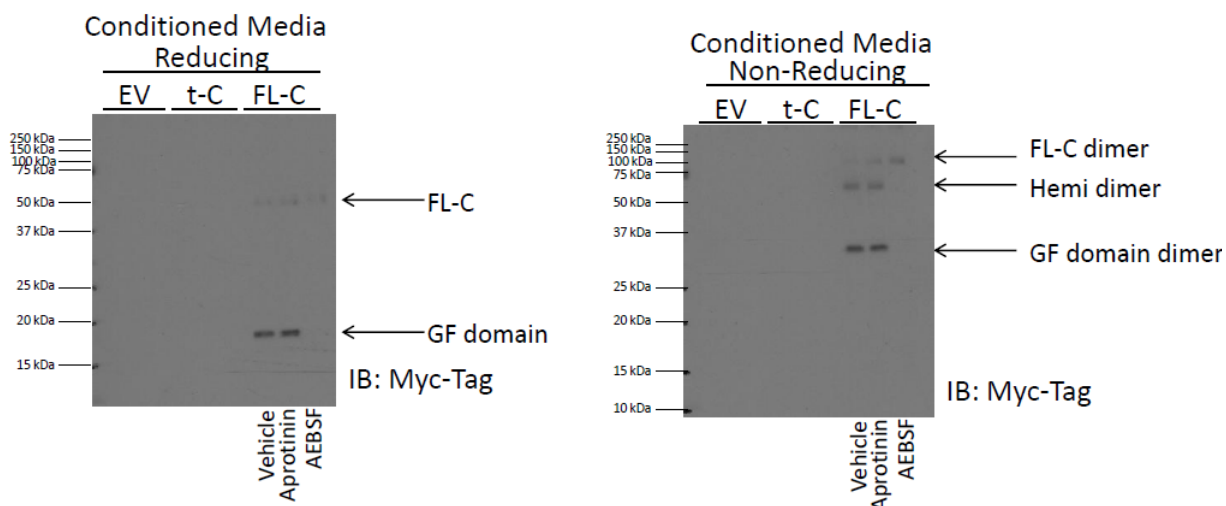


Figure 4. Immunoblot (IB) of conditioned media from MCF-7 stable transfected with empty vector control (EV), t-PDGFC myc-tagged (t-C) or FL-PDGFC myc-tagged (FL-C) and treated with vehicle control, Aprotinin a reversible serine protease inhibitor, or AEBSF an irreversible serine protease inhibitor. **Left panel**, reducing blot. **Right panel**, non-reducing blot. GF = growth factor domain. Hemi-dimer = FL-PDGFC/Growth Factor domain dimer.

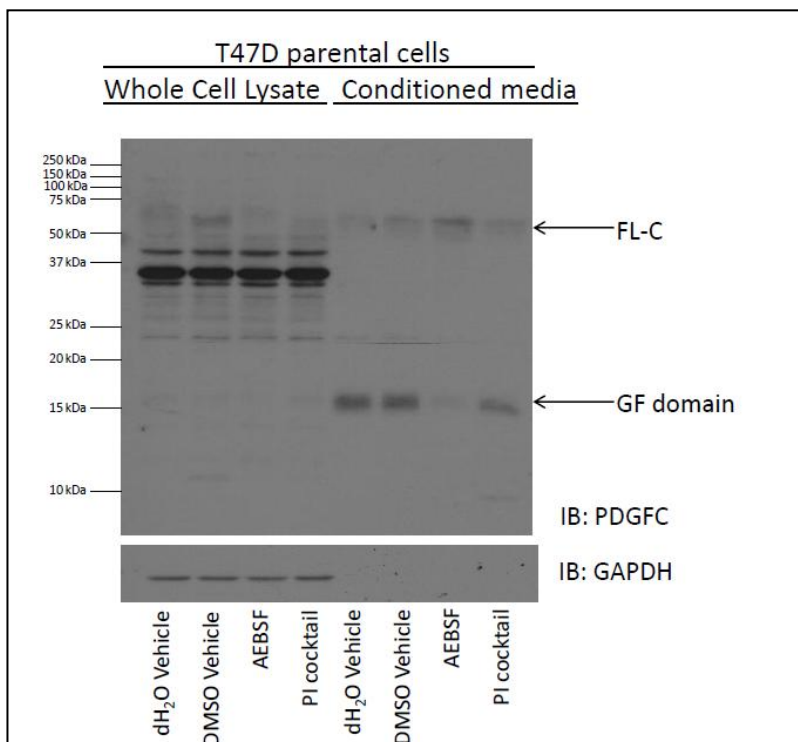


Figure 5. Reducing Immunoblot (IB) probed with PDGFC antibody. Whole cell lysate (left) or conditioned media (right) in T47D breast cancer cells which naturally express PDGFC. dH₂O Vehicle = deionized, distilled water control treatment, DMSO Vehicle = Dimethyl sulfoxide control treatment, AEBSF = 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride dissolved in dH₂O, PI Cocktail = Protease Inhibitor Cocktail dissolved in DMSO.

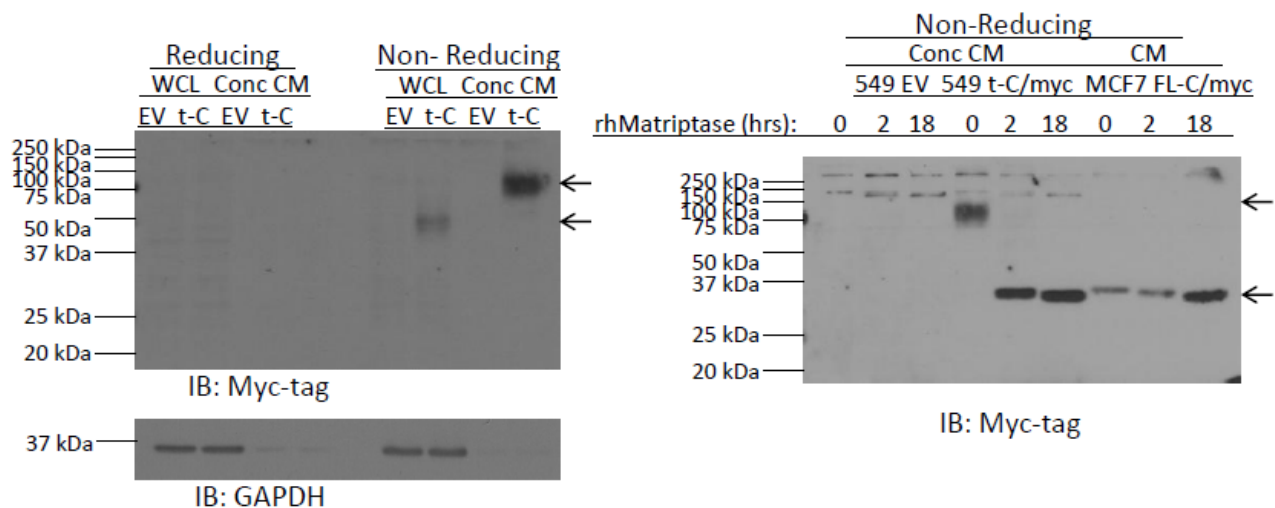


Figure 6. Immunoblot of Whole Cell Lysate (WCL), Concentrated conditioned media (Conc CM), or Conditioned Media (CM) in BT-549 cells stable transfected with empty vector control (EV) or t-PDGFC myc-tagged (t-C). Right blot shows incubation times of the conditioned media samples after collection with recombinant human Matriptase for various time points (hours). MCF-7 FL-PDGFC myc-tagged CM is a positive control.

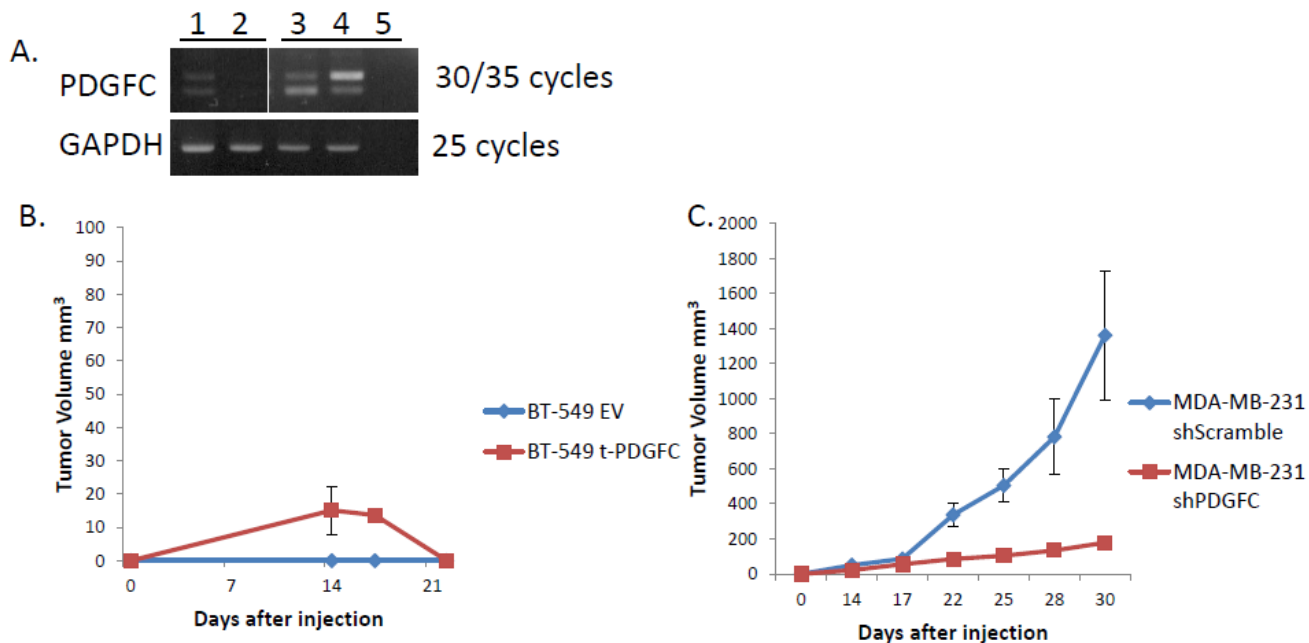
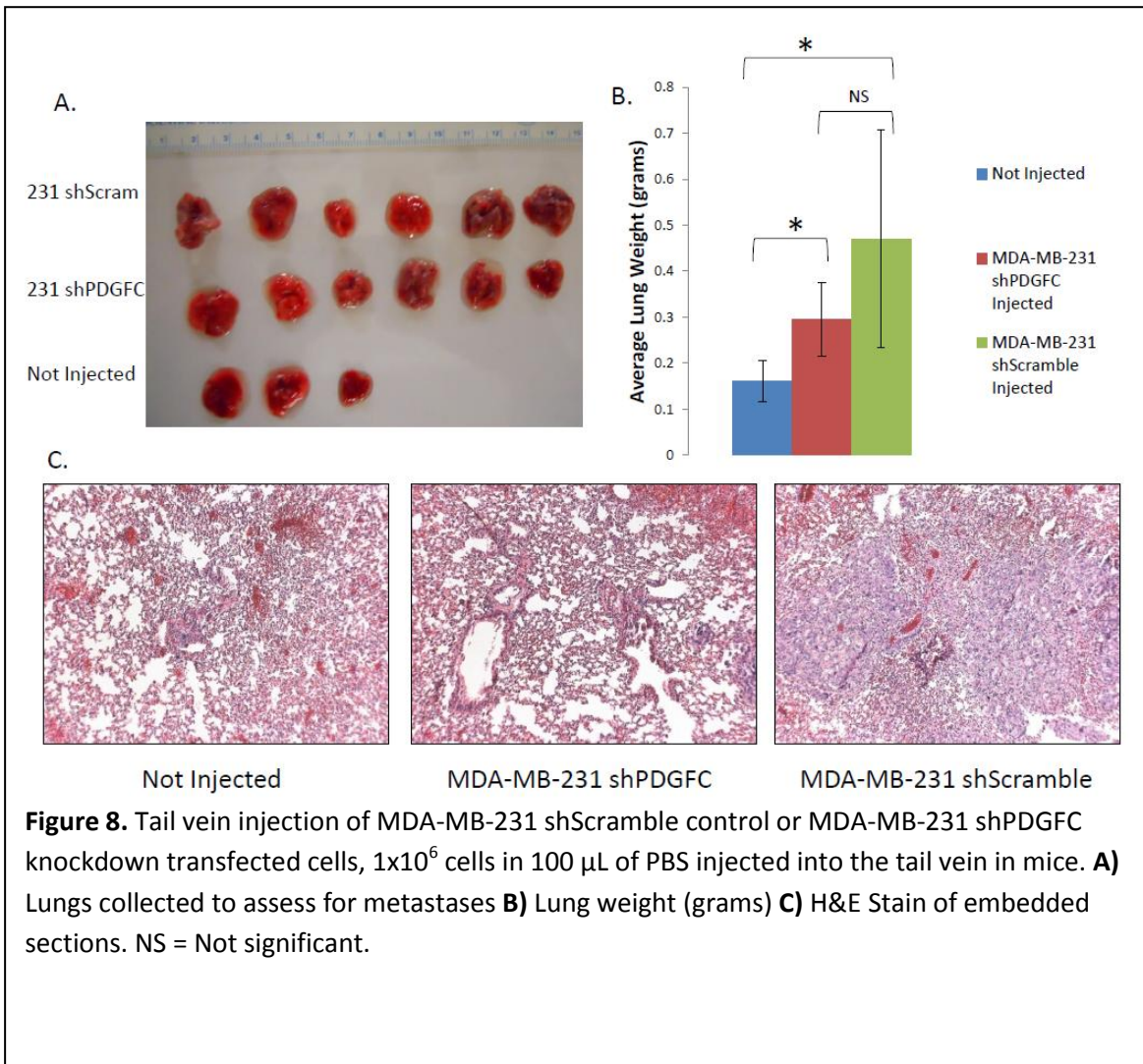


Figure 7. Perimammary fat pad injection of mice (MDA-231 shscramble, MDA-231 shPDGFC, BT549 EV, and BT549 t-PDGFC myc-tagged stable transfected cells). 1×10^6 cells in 50 μ l of matrigel/media mix injected perimammary fat pad. **A)** Semi-quantitative PCR. Lane 1: MDA-MB-231 shScramble, Lane 2: MDA-MB-231 shPDGFC, Lane 3: BT-549 Empty Vector, Lane 4: BT-549 t-PDGFC, Lane 5: dH₂O negative control. **B)** BT-549 Empty Vector (EV) or BT-549 t-PDGFC transfected cell tumor growth. **C)** MDA-MB-231 shScramble control or MDA-MB-231 shPDGFC knockdown transfected cell tumor growth.



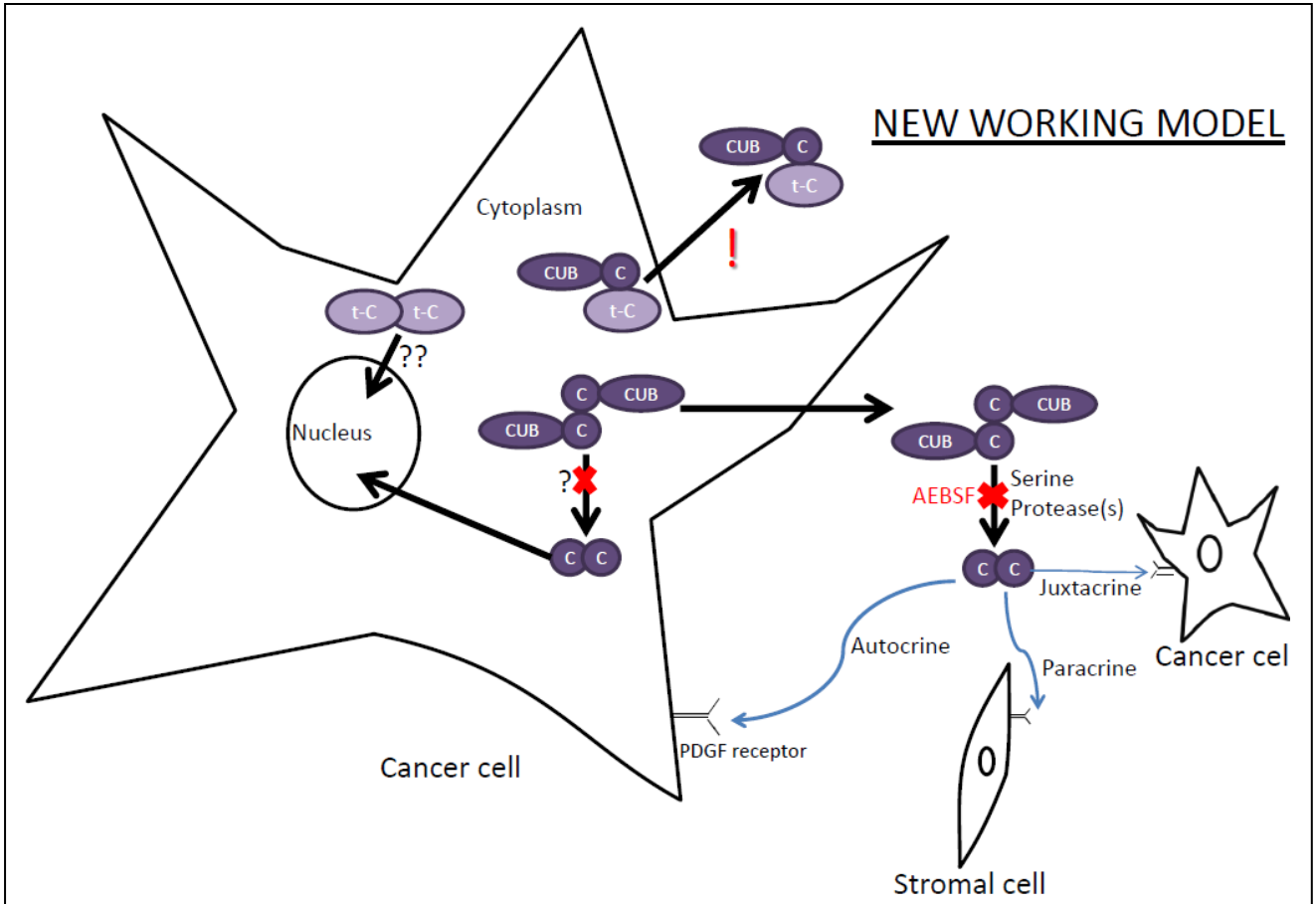


Figure 9. New Working Model. FL-PDGFC = dark purple C + CUB domain. t-PDGFC = light purple t-C. dark purple C-C = Growth factor domain dimer. AEBSF = 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, irreversible serine protease inhibitor.