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THE ROLE OF PDGF C AND ITS SPLICE VARIANT IN BREAST CANCER

by

ALYSSA BOTTRELL

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2015

MAJOR: PATHOLOGY

Approved by:

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Advisor Date

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DEDICATION

To my family and friends who supported me through it all. Thank you for being there for me, thank you for understanding, and thank you for taking care of my daughter. Most important to my husband, Tony, for being there day in and day out. I can't thank you enough for everything you do for me and Maddie. And to my daughter, Madilyn, you brighten my day and make me so happy. Thank you for making me smile. And to my mother, Erica, thank you for all of your help. You are the best mom I could have ever hoped for. To my dad for always having an answer to my questions and teaching me to value education. To my brothers, Evan and Wes, for always making me laugh. To my maternal grandparents for being such inspirational people and an amazing couple.

To my friends Christie Williams and Sara Lugger, thank you for everything you've done and offer to do for me and Maddie. To my family and friends that I have lost to cancer: Great Aunt Lois, Ben Pirooz, Grandma Lucy, Uncle Mark, and "Don" Vettraino. I think of you often. I miss you. To my friends and family who are cancer survivors, especially my mom's best friend Jeanie Benson, I am so happy to have you in my life. Keep fighting Uncle Stu and Tom Williams.

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To the patients I have seen in the clinic, thank you for allowing me to be there. I have learned an immense amount from you all. I appreciate what each of you has done for me. To my future patients, I hope that I can be of help to you.

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PREFACE

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CHAPTER 1

INTRODUCTION

Breast Cancer

Cancer is a significant threat to the well-being of individuals worldwide. In 2008 it is estimated that 12.7 million new cancer cases were diagnosed and 7.6 million cancer deaths occurred worldwide [5]. Breast cancer is the most frequently diagnosed cancer in women worldwide with an estimated 1.38 million cases, and breast cancer is the leading cause of cancer deaths worldwide being responsible for over 458,400 deaths in 2008 [5]. Breast cancer accounts for 23% of cancer cases and 14% of cancer deaths worldwide in women making it the leading cause of cancer mortality for all women [5-7]. The majority of cases of breast cancer diagnosed in the United States are local or regional stage, while breast cancer diagnosed in developing countries is mostly advanced stage [8, 9]. Based on 5-year prevalence data, breast cancer is the most prevalent cancer with an estimated 4.4 million women living with breast cancer [6, 7].

In the United States there is predicted to be 1.7 million cases of cancer and there is expected to be 585,720 deaths due to cancer to occur in 2014 [10]. One in eight women in America will be diagnosed with breast cancer in her lifetime [11, 12]. Excluding skin cancers, breast cancer is the most common cancer diagnosed among American women with 232,670 women expected to be diagnosed with invasive breast cancer in 2014, accounting for 29% of female cancer diagnoses [10]. In addition, over 62,570 women in the United States will be diagnosed with carcinoma *in situ* of the breast

(defined as stage 0 breast cancer [13]) in 2014 [10]. For women, breast cancer is second only to lung cancer in the number of cancer deaths in the United States [10, 12]. However among women between 20 to 59 years of age breast cancer is the leading cause of cancer deaths [10]. An estimated 40,000 deaths due to breast cancer are expected to occur in 2014 accounting for 15% of cancer deaths in American women [8, 10, 14]. Evidence suggests that differences in breast cancer mortality amongst races are due to not only disparities in epidemiological factors such as educational level, socioeconomic status and access to care, but also due to a difference in tumor biology and etiology [12, 15-19]. Thus, breast cancer is not one disease, but many diseases emphasizing the need for individualized treatments. The number of people in the United States living after having been diagnosed with cancer, cancer survivors, continues to grow, reaching an estimated 11.9 million cancer survivors in 2008 [20]. Amongst American cancer survivors, breast cancer is one of the most common diagnoses [20]. The number of breast cancer survivors was expected to have reached approximately 1.85% of the population in the United States in 2010 and is predicted to continue growing to over 2% of the population by 2015 [21]. As the most prevalent cancer and the leading cause of cancer mortality of women in the world, there is no doubt of the importance of breast cancer research.

Increasing age is a risk factor for developing cancer [22, 23]. Increasing age is also a risk factor for the development of breast cancer [24], however breast cancer is unique in that most breast cancer cases are diagnosed in women under the age of 65 [20, 25]. Furthermore, amongst all primary cancers in women, breast cancer has the highest

tendency to metastasize [26]. Additionally, breast cancer had the broadest range of metastatic sites of any cancer according to a large autopsy study of cancers [26]. The most common sites of metastases of breast cancer primary tumors are to the axillary lymph nodes, the bone, the liver, and the lungs [27].

Breast Cancer Subtypes

Breast cancers can be categorized by various clinical, pathological, and molecular characteristics in an effort to determine prognosis and appropriate treatment. At the most basic level, histological analysis of tumor tissue involves determining the morphology of the breast cancer. Studies show that more than 80% of invasive breast cancers are ductal carcinoma, while the remaining invasive breast cancer cases are primarily lobular carcinoma, or mixed ductal and lobular carcinoma [28-32].

Individuals diagnosed with breast cancer are further evaluated such that the clinical and pathological stage are be determined [13]. In this process the breast cancer tumor tissue is assessed for important biological tumor features that are used to help guide treatment options and provide prognostic or predictive information [13]. Breast cancer is treated by various combinations of local treatment (surgery and/or radiation treatment) and systemic treatment (chemotherapy, endocrine treatment, biologic treatment, or combinations of those) depending upon the evaluation of the tumor tissue and the stage of the disease [13]. The current standard of care dictates that all cases of invasive breast cancer should be assessed for the expression of hormone receptors (estrogen and progesterone, [33-35]) and the expression of the HER2/neu oncogene [13,

36-39]. Treatment is then guided by tumor histology, disease stage, pathologic tumor characteristics, axillary lymph node status, multigene assay testing, patient illness, age, menopausal status, and patient preference [13]. Breast cancers that express the hormone receptors can be targeted with endocrine treatments thus improving survival (as reviewed in [40]). Breast cancers that overexpress HER2/neu, while generally thought of as having a poor prognosis, also have the benefit of targeted treatments available which has significantly improved their prognosis [41]. However, breast cancers that do not express the hormone receptors and do not express HER2/neu, known as "triple-negative" breast cancers, pose a special clinical problem in that there are no targeted treatments currently available. A better understanding of triple-negative breast cancers is needed in order to improve the outcome for those breast cancer patients, as targeted treatments have improved outcomes of hormone receptor positive breast cancer and HER2/neu overexpressing breast cancers.

Breast cancers have been known to be a heterogeneous group of tumors, however microarray analysis of gene expression and hierarchal clustering analysis identified five subtypes of breast cancer tumors: basal-like, HER2/neu overexpressing, luminal A, luminal B, and normal breast tissue-like subtype [42-44]. Basal-like breast cancers and triple-negative breast cancers are closely related, overlapping groups of breast cancers (as reviewed in [45]). Basal-like breast cancers have a triple-negative phenotype by lacking hormone and HER2/neu receptor expression, however they also express genes associated with myoepithelial (basal) cells, such as cytokeratin 5 and EGFR giving them their name "basal-like" (as reviewed in [45]). HER2/neu overexpressing (ER-negative) breast

cancer subtype, and basal-like breast cancer subtype had the worst prognosis of any of the molecular subtypes of breast cancer [46, 47]. With medical advancements since the discovery of HER2/neu overexpressing breast cancer, there are now the treatments that target HER2/neu, such as trastuzumab (Herceptin®) available which have significantly improved the disease-free survival and overall survival for those patients [48]. Basal-like breast cancers similarly having a natural disease history that confers a poor prognosis [46, 47] still have the added disadvantage of having no targeted therapies available.

Furthermore, Neve et al. analyzed the transcriptional profiles of a set of 51 breast cancer cell lines, and utilized hierarchical clustering to find clusters similar, but not exactly the same as the subtypes found in tumor tissue [1]. Their study initially found two distinct clusters, the Luminal subtype and a basal-like cluster [1]. The basal-like cluster was then found to have two major subdivisions, termed Basal A subtype and Basal B subtype [1]. The Basal B subtype was vimentin-positive with stem cell-like profiles and believed to contain the triple-negative clinical tumor subtypes [1]. HER2/neu overexpressing cell lines were found mixed throughout the Luminal and Basal A subtypes in this study [1]. This study demonstrated that breast cancer cell lines, like tumor tissue, are heterogeneous, however clusters with significant similarities have been found in an effort to identify a molecular basis for the development of future targeted therapies for breast cancer [1].

Platelet-Derived Growth Factor (PDGF) Family

PDGFs are members of the cysteine-knot growth factor superfamily (as reviewed in, [49]). The cysteine-knot superfamily of growth factors comprises several members: the four PDGF ligands (PDGF A, PDGF B, PDGF C, and PDGF D), the five vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, VEGF-D, and VEGF-E) along with placenta growth factors (PlGF-1 and PlGF-2), NGF, TGFβ, and v-Sis [49, 50]. The members of this superfamily contain eight conserved cysteine residues [49].

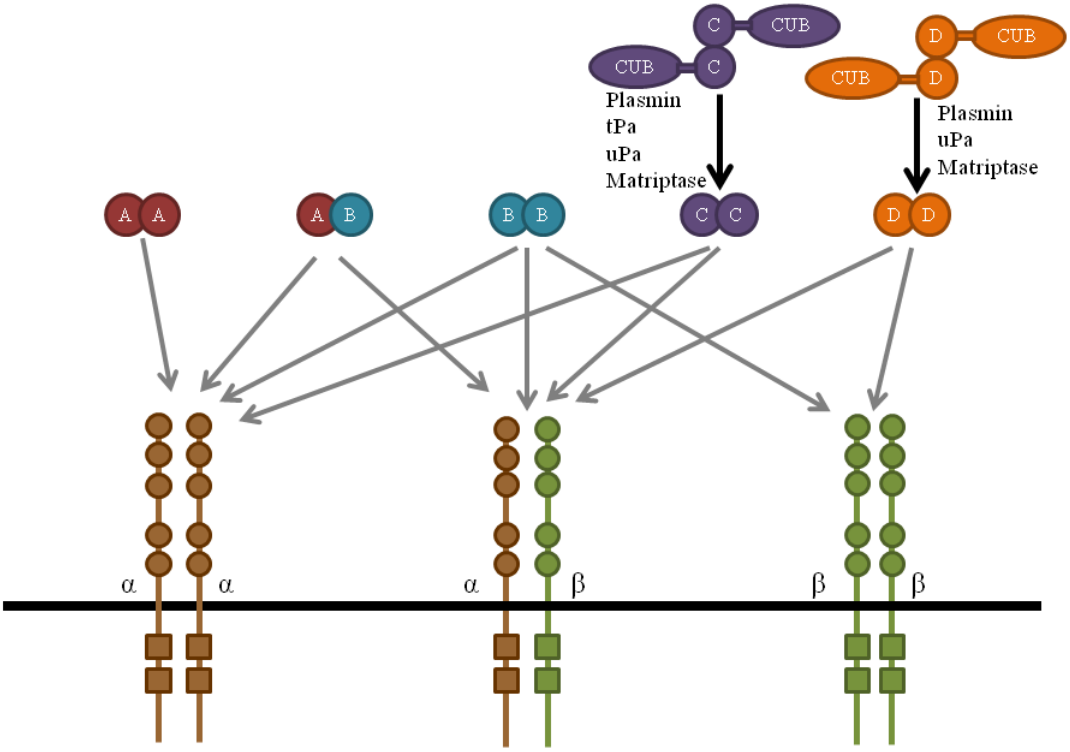


Figure 1. The PDGF family of ligands and receptors. Proteases capable of proteolytically processing and activating PDGF C and PDGF D are listed. The ligands and receptors homo-dimerize or hetero-dimerize as depicted.

The platelet-derived growth factor family consists of the four ligands, PDGF A, PDGF B, PDGF C and PDGF D, in addition to the two PDGF receptors, PDGF Receptor-

α (PDGFR-α) and PDGF Receptor-β (PDGFR-β) (Figure 1). Their name is derived from the fact that PDGF was first isolated from platelets ([51-58] as reviewed [59]). These growth factors form five sets of homo- or hetero-dimeric ligands, PDGF AA, BB, AB, CC and DD (as reviewed in [60]) (Figure 1). Platelet-derived growth factors are mitogens for an array of cell types such as smooth muscle cells, fibroblasts, and glial cells (as reviewed in [60-62]). The PDGF-Receptors (PDGFRs) are receptor tyrosine kinases. PDGFRs are known to bind homo- or hetero-dimeric PDGF ligands, inducing homo- or hetero-receptor dimerization (PDGFR-α/α, PDGFR-α/β, or PDGFR-β/β, Figure 1) resulting in auto-phosphorylation which then leads to signal transduction via several molecules including PI3K, PLC-γ, Raf-1, GAP, and PKC (as reviewed in [62, 63]). PDGFRs are expressed in many different cell types including endothelial, epithelial and neural cells, and are necessary for embryological development (as reviewed in [60]). Traditionally PDGFs have been believed to function through paracrine signaling with epithelial cells secreting ligands that act on adjacent stromal cells.

PDGFs & Breast Cancer

Since the simian sarcoma virus oncogene, *v-sis*, was discovered to be derived from the PDGF gene ([64, 65] as reviewed in [66]), PDGFs have been implicated in many cancers (as reviewed in, [67]). Several cancer cell lines express PDGFs and PDGFRs [68]. Autocrine signaling loops have been discovered in transformed cells [69, 70].

In addition, abnormal tumor stromal interactions have been implicated in the development of breast cancer ([71] as reviewed in [72]). Of note, PDGFs have been implicated in the desmoplastic response seen in some breast cancers [73, 74]. In addition, PDGF Receptor expression has been detected in a number of soft tissue tumors further implicating aberrant PDGF signaling in abnormal tumor growth [75]. Despite multiple reports of PDGF receptor expression in breast cancer [76-78], the PDGF ligand in breast cancer had yet to be determined.

This is of importance as aberrant PDGF signaling can be targeted with pharmacologic therapies. Currently there are several drugs approved by the United States Food and Drug Administration (FDA) which target aberrant PDGFR signaling ([79], as reviewed in [80]). Imatinib mesylate (Gleevec®, STI571, Novartis) is the most notable of these drugs and the first drug created utilizing rational drug design [81, 82]. Dasatinib (Sprycel®, BMS-354825, Bristol-Myers Squibb), sunitinib malate (Sutent®, SU11248, Pfizer), sorafenib (Nexavar®, Bayer and Onyx Pharmaceuticals) are other receptor tyrosine kinase inhibitors that target PDGFRs.

PDGF C & Breast Cancer

PDGF C and PDGF D are the newest members of the platelet-derived growth factor (PDGF) family [68, 83, 84]. PDGF C was identified in the year 2000 by three groups who designated it “fallotein” [85], “Spinal Cord-Derived Growth Factor (SCDGF)” [86], and finally “PDGF C” [87]. The PDGF C gene is located on chromosome 4q32 and consists of six exons [68, 88]. PDGF C mRNA expression was

analyzed in a myriad of human tissues and found to be highly expressed in heart, pancreas, kidney, liver and ovary human tissues with intermediate or no expression in several other human tissues analyzed [87]. Of note, this study did not analyze expression in human breast tissue [87]. A majority of the mRNA was of approximately 3.9-kilobases while there was a minor transcript of approximately 2.8-kilobases detected in human tissues [87].

PDGF C and PDGF D are unique from PDGF A and PDGF B in that they contain an amino-terminal CUB domain encoded by exons 2 and 3 [68, 87] which has been suggested to bind the pericellular matrix and block receptor binding [89]. Like the other members of the PDGF family, the carboxy-terminus of PDGF C contains the conserved cysteine knot growth factor homology domain [68]. PDGF C has 12 cysteine residues which form three intra-subunit disulfide bonds and two inter-subunit disulfide bonds [89]. PDGF C forms homodimers, and latent homodimers are secreted then requiring removal of the CUB domain in order to activate the PDGF receptor [87]. The growth factor domain of PDGF C stimulates receptor phosphorylation while full-length PDGF C does not [87]. The serine protease tissue plasminogen activator (tPA) is capable of proteolytically activating PDGF C by removal of the CUB domain, necessary for PDGFR activation [90, 91]. When secreted and proteolytically activated, PDGF C homodimers bind to and activate platelet-derived growth factor receptor alpha homodimers (PDGFR-α/α) [87, 92] or platelet-derived growth factor receptor-alpha/beta (PDGFR-α/β) heterodimers (Figure 1) [92-94]. PDGF D activates PDGFR-β receptors and when

present with PDGFR-β, PDGFR-α receptors are also activated likely by heterodimerization of the alpha and beta receptors [84] .

Since its discovery, PDGF C has been implicated in normal development and in many disease processes. Studies have shown that PDGF C plays an important role in palate formation [95], and also in the aberrant development in cleft lip/cleft palate (CL/P) [96]. In addition PDGF C is necessary for normal kidney development and likewise aberrant PDGDC signaling has been implicated in the development of glomerular diseases [97].

Several studies have implicated PDGF C in the development of fibrosis [98]. Aberrant expression of PDGF C in the heart of a transgenic mouse model resulted in cardiac fibrosis along with heart and vascular dysfunction [99]. An increase in PDGF C expression was seen in a strain of mice that suffer from bleomycin-induced lung fibrosis, while there was no increase in PDGF C expression in a bleomycin-resistant mouse strain [100]. Their results suggest that PDGF C expression plays a role in lung fibrosis. Furthermore, Interleukin-13 (IL-13) was shown to stimulate lung fibroblasts via PDGF signaling [101]. Later it was shown that IL-13 transgenic mice demonstrate increased PDGF C expression in the lungs suggesting PDGF C's role in lung disease and fibrosis [102]. In addition, showing a continuum of aberrant signaling in the liver, PDGF C causes liver fibrosis and later carcinoma development [103].

PDGF C's specific role in tumorigenesis was first demonstrated in 2001 when it was shown that the aberrant transcription factor in Ewing Family Tumors (EFTs)

increased PDGF C expression [104]. In addition it was demonstrated that NIH/3T3 cells could be transformed by expression of PDGF C, and that several Ewing Family Tumor cell lines and primary tumor samples expressed PDGF C [104]. Inhibition of PDGF C signaling was able to reverse the malignant phenotype, as demonstrated by anchorage-independent growth, and thus has been suggested as a potential therapeutic target in these tumors [105]. Later, PDGFR-α was demonstrated in medulloblastoma tumors [106]. Discovery of expression of PDGF C and activated PDGFR-α in medulloblastoma cell lines suggested the possibility of autocrine signaling being important in these tumors [106]. Further evidence of PDGF C functioning in an autocrine manner as a growth factor in cancer was demonstrated in glioblastoma brain tumor cells [107]. PDGF C has been shown to also signal in a paracrine manner within the tumor microenvironment causing blood vessel maturation and stromal cell recruitment [108, 109]. Expression of PDGF C in the liver of mice produced severe hepatic fibrosis which progressed to liver adenomas and finally lead to the development of hepatocellular carcinoma [103].

Increasing evidence in the literature has implicated aberrant PDGF signaling in breast cancer development and metastases [75-78, 110-113]. Thus our laboratory set out to determine the PDGF ligand most relevant in breast cancer. Utilizing Gene-expression Based Outcome for Breast Cancer Online (GOBO) Gene Set Expression (GSE) analysis [2] to assess gene expression in 51 human breast cancer cell lines [1] shows interesting results. 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, as defined in the literature [1]. A subset of 30 breast cancer cell lines- 16 Luminal, 4

Basal A, and 10 Basal B breast cancer cell lines- were tested for invasive potential *in vitro*, and it was found that Basal B cell lines were more often highly invasive cell lines as compared to Luminal or Basal A breast cancer cell lines [1]. Furthermore, the Basal B cell lines tended to appear more mesenchymal, less differentiated and Basal B cells also showed expression of the $CD44^{+}/CD24^{-/low}$ pattern associated with breast cancer stem cells [1, 114, 115]. Total PDGF C expression is shown 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 2A). The difference in PDGF C expression is significant amongst the three breast cancer cell line subtypes, $p \leq 0.00001$ (Figure 2A.)

As shown in Figure 2B, the relative expression of overall PDGF C varies widely in the panel of 51 human breast cancer cell lines, with the highest PDGF C expression being found in the MDA-MB-231 cell line. Such gene expression data in a large panel of human breast cancer cell lines helps identify which cell lines would be ideally suited for further *in vitro* and *in vivo* studies.

In addition, GOBO analysis groups and analyzes the breast cancer cell lines according to the clinical features assessed in breast cancers, such as hormone receptor expression and HER2/neu overexpression (Figure 3). According to GOBO analysis, PDGF C expression varies significantly amongst the clinical subtypes of breast cancer cell lines (Figure 3C). There is no significant difference amongst the various clinical subtypes of breast cancer cell lines in expression of PDGF A, PDGF D, or either of the

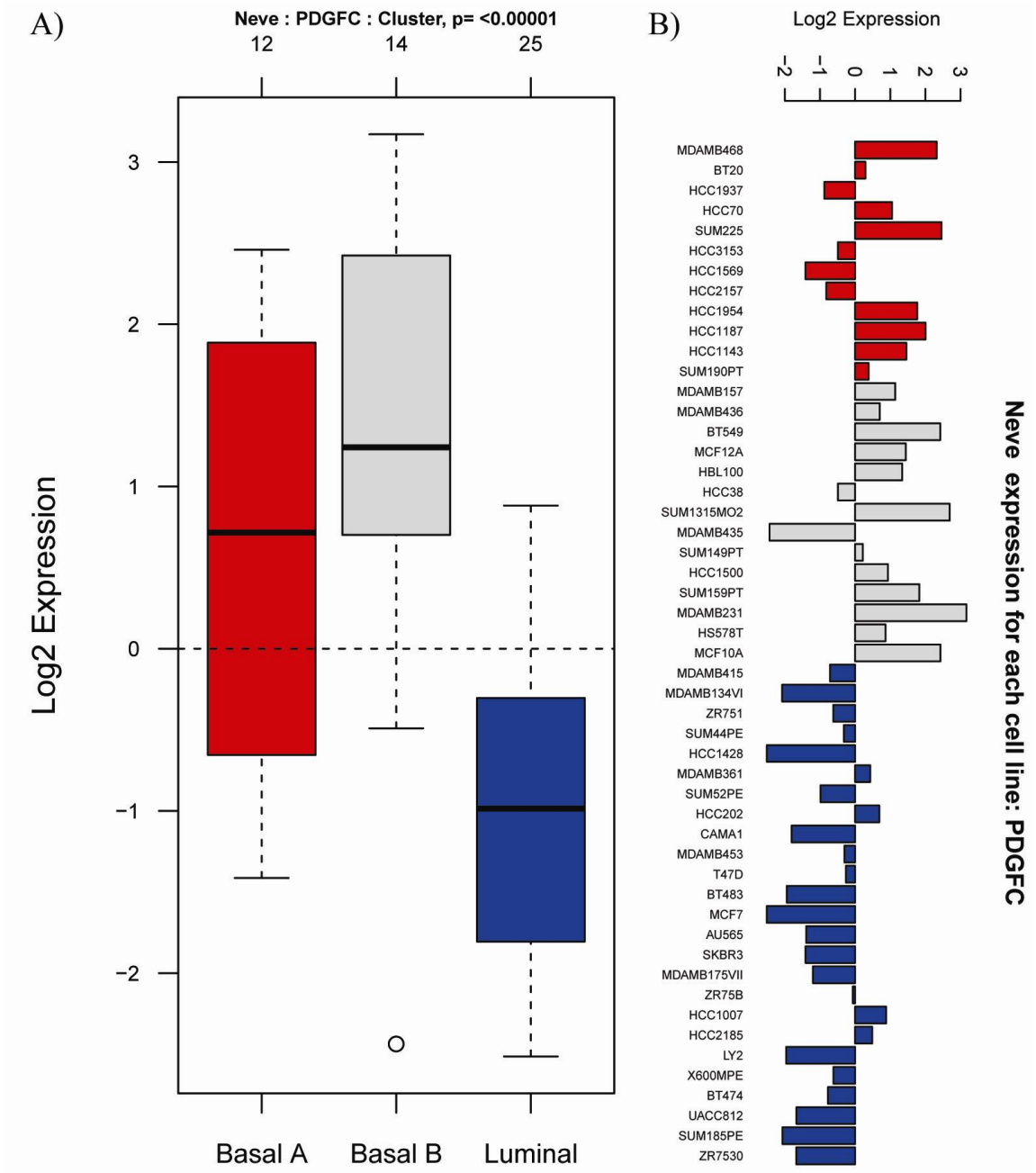


Figure 2. Gene set analysis in breast cancer cell lines for PDGF C expression. **A)** Box plots from Gene Expression-based Outcome for Breast Cancer Online (GOBO) of PDGF C expression in cell lines grouped into Basal A (red), Basal B (grey), and Luminal (blue) subgroups as described in Neve et al 2006 [1] and Ringner et al 2011 [2]) **B)** Gene Expression-based Outcome for Breast Cancer Online (GOBO) data output for relative (total) PDGF C expression in a panel of 51 breast cancer cell lines, color coded into Basal A (red), Basal B (grey), and Luminal (blue) subgroups. Based on data from Neve et al 2006 [1] and figures generated by GOBO Ringner et al 2011 [2]. http://co.bmc.lu.se/gobo

PDGF receptors (Figure 3A, D, E, & F). Interestingly, there is a significant difference in PDGF B expression amongst the clinical subtypes of breast cancer cell lines with the highest PDGF B expression being in hormone-receptor positive and HER2/neu overexpressing breast cancer cell lines, as compared to significantly lower PDGF B expression in the triple-negative breast cancer cell lines ($p = 0.01398$, Figure 3B). Most interesting, PDGF C expression is highest amongst the triple-negative (TN) breast cancer cell lines, and then second highest amongst the HER2/neu overexpressing (HER2) cell lines, with the lowest expression of PDGF C seen in the hormone receptor-positive (HR) breast cancer cell lines ($p = 0.00031$, Figure 3C). Taken together, this suggests that PDGF C expression tends to be higher in breast cancer cell lines with poor prognostic features such as triple-negative or HER2/neu expressing breast cancer.

Our laboratory screened a panel of human breast cancer cell lines and found that PDGF C expression appeared to increase in more aggressive breast cancer cell lines such as MDA-MB-231 cells, BT-549 cells and the SUM cell lines (Figure 4, data courtesy of NJH). Furthermore, a splice variant of PDGF C was discovered that is designated truncated PDGF C (Figure 4, data courtesy of NJH). Approximately 40 – 60% of genes have alternatively spliced forms [116]. With only approximately 30,000 genes in the human genome, alternative splicing is important for genetic diversity. Alternative splicing of a transcript can be the result of exon inclusion or exclusion, a new 5' site or a new 3' site which can produce an alternative amino-terminus translation initiation site, frameshift truncation or extension of the carboxy-terminus, or an in-frame insertion or deletion within the protein [116]. The PDGF C gene has two mRNA isoforms, full-

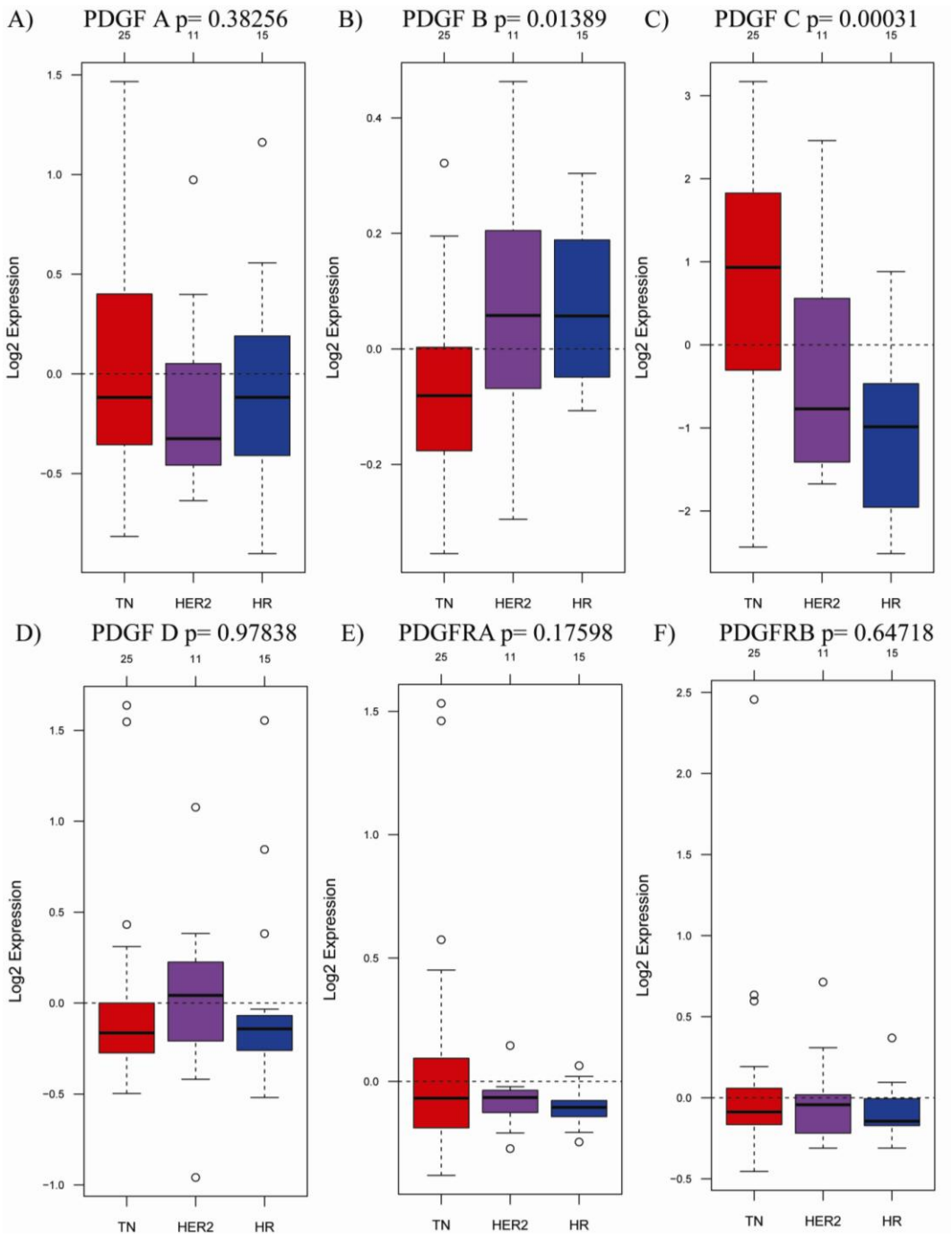
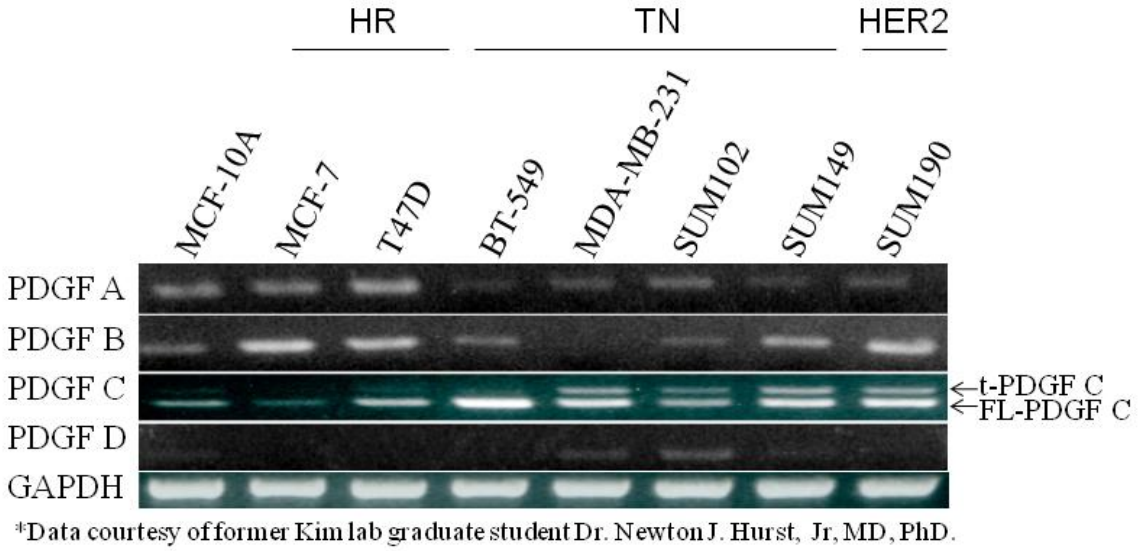


Figure 3. Gene set analysis in breast cancer cell lines for the PDGF family. Box plots from GOBO depicting **A)** PDGF A **B)** PDGF B **C)** PDGF C **D)** PDGF D **E)** PDGFRA **F)** PDGFRB gene expression in 51 breast cancer cell lines grouped into clinical subtypes: triple-negative (TN, red), HER2-positive (HER2, purple), and hormone receptor-positive (HR, blue). Based on data from Neve et al 2006 [1] and figures generated by GOBO Ringner et al 2011 [2] http://co.bmc.lu.se/gobo



*Data courtesy of former Kim lab graduate student Dr. Newton J. Hurst, Jr, MD, PhD.

Figure 4. PDGF family mRNA expression in human breast cancer cell lines and identification of a PDGF C splice variant. RT-PCR analysis of PDGF A, B, C and D in MCF-10A, MCF-7, T47D, BT-549, MDA-MB-231, SUM102, SUM149, and SUM190 cell lines. Data Courtesy of Dr. Newton J. Hurst, Jr, MD, PhD. HR= Hormone receptor positive, TN= Triple-Negative, HER2= HER2/neu overexpressing.

length PDGF C (FL-PDGF C) and the more recently discovered truncated PDGF C (t-PDGF C). The truncated PDGF C isoform is generated due to retention of a 61 base pair exon resulting in an amino-terminally truncated isoform of PDGF C (Figure 5). This PDGF C splice variant has been demonstrated in fetal brain [117] and in breast cancer cell lines (our laboratory's unpublished data, Figure 4, data courtesy of NJH).

Full-length PDGF C is 345 amino acids long. The expected molecular weight of this protein based on the number of amino acids would be approximately 38 kDa [118], while calculating based on the amino acid sequence estimates its size as 39 kDa [119]. A putative signal-peptidase cleavage site is located between amino acids 22 and 23 of PDGF C [87]. After removal of the signal sequence the expected molecular weight of

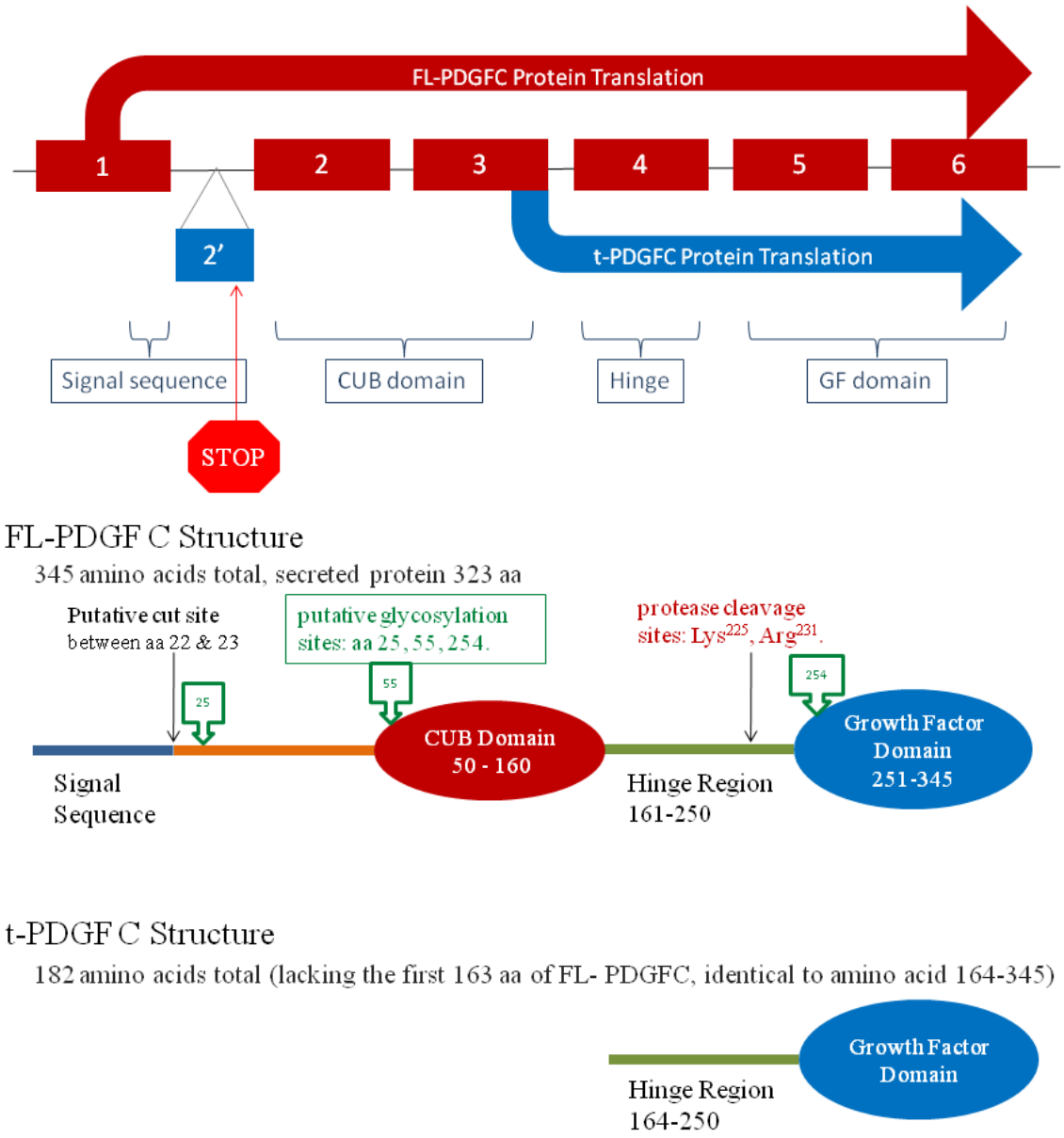


Figure 5. A diagram for the PDGF C mRNA and the protein domain structure. PDGF C gene diagram depicting exon locations (numbers), the translation initiation sites for FL-PDGF C (red arrow) and t-PDGF C (blue arrow).

full-length PDGF C is ~37 kDa [87]. The truncated form of PDGF C is predicted to be 182 amino acids in length and would be expected to be 20 kDa based on simple amino acid length [118] while based on the actual amino acid sequence is would be expected to

be 21 kDa in size [119]. In addition, the sequence of PDGF C contains three presumed N-glycosylation sites at amino acids 25, 55, and 254 [87] and treatment with glycosidase has been shown to alter the molecular weight of PDGF C [88]. In addition, SUMOylated PDGF C has been demonstrated in human thyroid tissue, papillary thyroid carcinomas and various cell lines [120]. In these tissues and cell lines PDGF C of various molecular weights was demonstrated. The ~55 kDa form appeared to be the SUMOylated form, while the ~39 kDa and ~43 kDa forms were not [120]. While a full-length PDGF C ~45 kDa species appears to be glycosylated in COS-1 cells and two species ~25 kDa and ~20 kDa are CUB domain-containing PDGF C entities [88]. Under non-reducing conditions medulloblastoma cells showed PDGF C entities at 80 kDa and 100 kDa that were 55 kDa under reduced conditions [106]. Thus, the molecular weight of PDGF C depends on the isoform, post-translational modifications and ligand dimerization.

Although the gene expression data of the GOBO tool does not distinguish between the expression of the PDGF C mRNA splice variants, this online tool still provides valuable data about total PDGF C expression in a large panel of human breast cancer cell lines. Correlating well with our laboratory's semi-quantitative PCR data (Figure 4, data courtesy of NJH), GOBO data output also shows relatively high level of total PDGF C expression in human breast cancer cell lines such as MDA-MB-231 and BT-549, while there is very low total PDGF C expression in the hormone receptor positive human breast cancer cell line MCF-7 (Figure 2B). Corroborating the GOBO data showing that PDGF C expression varies significantly amongst the clinical subtypes

of cell lines (Figure 3C), our laboratory finds that PDGF C expression appears to increase in breast cancer cell lines with poor prognostic features (Figure 4, data courtesy of NJH).

In addition to the recent discovery of a PDGF C splice variant, PDGF C has been recently reported to have nuclear localization. This was reported in the literature in 2006 when Reigstad, et al described nuclear localization of PDGF C in human papillary thyroid carcinoma samples and normal thyroid tissue samples from 14 patients by immunohistochemical (IHC) staining [120]. In addition, that study showed that several cultured cell lines also displayed nuclear localization of PDGF C and that inhibition of PI3K with Wortmannin treatment reduced PDGF C nuclear staining by indirect immunofluorescence in cell culture. Actinomycin-D treatment, serum starvation, and confluent cell cultures also acted to reduce the amount of nuclear PDGF C in cultured cells in their study. They also demonstrate that structural modeling indicates a potential nuclear localization sequence in the growth factor domain of PDGF C amino acids 312 – 322 with a putative SUMOlyation site on positively charged ^{314}lysine believed to be at the outer surface of the protein [120].

Statement of Hypotheses & Goals of this study

To determine the role of PDGF C and its splice variant in Breast Cancer:

- Analyze the expression of PDGF C isoforms in breast cancer cell lines
- Analyze the effect of alteration of PDGF C isoform expression on the behavior of breast cancer cells *in vitro* & *in vivo*

We hypothesize that in breast cancer cells:

- PDGF C expression increases the aggressiveness
- t-PDGF C expression modulates the function of FL-PDGF C

Aim 1: To establish the significance of PDGF C expression on the malignant characteristics of breast carcinoma cells.

Aim 2: To investigate t-PDGF C regulation of FL-PDGF C stability and subcellular localization in human breast carcinoma cells.

Aim 3: To elucidate the role of PDGF C isoforms in breast cancer progression *in vivo*.

CHAPTER 2

MATERIALS & METHODS

Cell culture

MCF-7, BT-549, and resulting transfectant cells were maintained in DMEM/F-12 (1:1) media (Gibco catalog # 11320), supplemented with 10% FBS (Gibco catalog # 16000) and 1% Pen/Strep (Hyclone® 10,000 U/mL of Pen, 10,000 U/mL of Strep; Thermo Scientific catalog # SV30010). T47D and resulting transfectant cells were maintained in DMEM/F-12 (1:1) media (catalog # 11320), supplemented with 10% FBS (Gibco catalog # 16000), 1% Pen/Strep (Hyclone® 10,000 U/mL of Pen, 10,000 U/mL of Strep; Thermo Scientific catalog # SV30010), 1% Glutamine (200 mM; Cellgro Cat No 25-005-CI), 1 mL fungizone (250 µg/mL; Gibco 15290-018), and 0.55 mL insulin (10 mg/mL; Sigma catalog # I0516-5mL). MDA-MB-231 cells, COS-1 cells and resulting transfectant cells were maintained in DMEM High Glucose media (Gibco catalog # 11995), supplemented with 10% FBS (Gibco catalog # 16000), and 1% Pen/Strep (Hyclone® 10,000 U/mL of Pen, 10,000 U/mL of Strep; Thermo Scientific catalog # SV30010).

Cell counting

To count cells using the Reichert Bright Line® Hemacytometer 0.1 mm deep, cells are washed once with Phosphate Buffered Saline (PBS) and trypsanized, then spun down and resuspended in media. The cell suspension is diluted 1:10 in media (10 µL of cell suspension + 90 µL of media). To this 20 µL of 0.4% Trypan Blue Solution (Sigma

T8154) is added (a 1.2 dilution) and mixed by pipetting. To count cells, 10 µL of the cell/trypan blue solution is pipetted into the hemocytometer/coverslip. The bright cells (live cells) in all four 1.00 sq mm corner quadrants of the hemacytometer are counted. The number of cells per milliliter is calculated using the following formula:

Number of cells/mL = total number cells in all four corner quadrants /4 * 1.2* 10* 10^4

Cell lines

Table 1. Human Breast Cancer Cell Line Characteristics. [1, 121, 122]

	Origin: 1° Tumor?	Origin: Metastatic Site?	ER	PR	HER2	1° tumor histology
MCF-7	N	Y, PE	+	+	–	IDC
T47D	N	Y, PE	+	+	–	IDC
BT-549	Y	N	–	–	–	IDC
MDA-MB-231	N	Y, PE	–	–	–	IDC
SUM-102PT	Y	N	–	–	–	IDC
SUM-149PT	Y	N	–	–	–	Inflammatory
SUM-190PT	Y	N	–	–	+	Inflammatory

ER = estrogen receptor, PR = progesterone receptor, + indicates expression of receptor, – indicates lack of receptor expression. HER2 = HER2/neu, + indicates overexpression of HER2/neu. Y = yes, N = no. PE = pulmonary effusion. IDC = invasive ductal carcinoma.

MCF-10A cells are a spontaneously immortalized non-tumorigenic breast epithelial cell line isolated from a 36-year-old woman with fibrocystic disease of the breast [123, 124]. MCF-7 cells are hormone receptor positive human breast cancer cells isolated from the

metastatic pleural effusion of a patient [125, 126]. BT-549 cells are a hormone receptor negative cell line isolated from a primary breast cancer [1]. MDA-MB-231 breast cancer cell line was established from the pleural effusion of a 51-year-old woman with a poorly differentiated breast cancer [127]. T47D is a hormone receptor positive breast cancer cell line isolated from a 54-year-old patient with breast cancer [128]. SUM102, SUM149, SUM190 are human breast cancer cell lines [1, 121, 122]. COS-1 cells are a simian cell line [129].

Transfection

Cells were transfected using Lipofectamine™ 2000 Transfection Reagent (Invitrogen Cat. No. 11668-019) according to the manufacturer's protocol. For transient trasfection, following transfection cells were grown overnight in normal serum-containing growth media and the following day cells were serum-starved for 48-hours before collecting RNA or protein. For stable transfection, cells were kept under antibiotic selection for 2-weeks. Proper expression was confirmed by PCR and then liquid nitrogen stocks were made.

Vaccinia virus infection transfection

MCF-7 cells were infected with vaccinia virus. After 30 minutes, the cells were washed with PBS and transfected with plasmid using Effectene reagent (Qiagen, Valencia, CA). After 48 hours of co-infection/transfection with vaccinia virus and expression plasmid, the serum-free conditioned medium and cellular fractionation protein was collected.

In vitro cleavage by recombinant human matriptase

Microcon® centrifugal filter devices were used to exchange the conditioned media in the sample buffer with rhMatriptase buffer (50 mM Tris, 50 mM NaCl, 0.01% (v/v) Tween® 20, pH 9.0). Recombinant Human Matriptase/ST14 Catalytic Domain (rhMatriptase) (R&D Systems, Catalog # 3946-SE) was diluted 1:10 in PBS and 6 µL of the diluted rhMatriptase was added to 20 µL of concentrated conditioned media. Samples were then incubated at 37°C for the times indicated. Samples were resolved by SDS-PAGE and Western blot for analysis of proteolysis.

Plasmid DNA preparation

Invitrogen™ Subcloning Efficiency™ DH5α™ Competent Cell (Cat. No. 18265-017) were transformed with plasmid according to the manufacturers protocol. Transformed cells were spread on Invitrogen™ imMedia™ Amp Agar plates (Cat. No. Q601-20) and grown overnight at 37 °C. Isolated colonies were picked and grown up overnight at 37°C with shaking 225 rpm in LB broth (Sigma Cat. No. L3022) supplemented with the appropriate selective antibiotic. Maxi prep kit (Qiagen HiSpeed® Plasmid kit, Cat. No. 12663) or QIAprep® Miniprep kit (Qiagen, Cat No. 27106) was used to isolate plasmid DNA. This DNA was used for transfections of cell lines in cell culture.

Mutagenesis

10 nanograms of template were used for the mutagenesis reactions using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, catalog #200524). The

mutagenesis reaction was incubated at 95°C for 30 seconds and then run for 18 cycles, each cycle consisting of: 30 seconds at 95°C, 1 minute at 55°C and 7 minutes at 68°C.

Table 2. Mutagenesis Primers.	
PDGF C NLS RKRK/AAAA Forward	5'-CCTTCAGTTGGCACCAGCGACCGGTGTCGCGGGATTGCACGCATCACTCACC -3'
PDGF C NLS RKRK/AAAA Reverse	5'-GGTGAGTGATGCGTGCAATCCCGCGACACCGGTCGCTGGTGCCAACTGAAGG -3'
PDGF C ΔSignal Sequence Forward	5'-GCTGGCTAGCGTTTAAACTTAAGATGGAATCCAACCTGAGTAG -3'
PDGF C ΔSignal Sequence Reverse	5'-CTACTCAGGTTGGATTCCATCTTAAGTTTAAACGCTAGCCAGC -3'

Then mutagenesis reactions were transformed into XL1-Blue supercompetent cells according to the manufacturer's protocol, then grown for 1 hour at 37°C in SOC media and finally spread on LB-ampicillin agar plates incubated overnight at 37°C. To make agar plates a 2X agar solution (3 grams of Bacto™ Agar, BD Bioscience REF 214010, in 100 mLs of dH_2O makes a 2X solution), mixed with Invitrogen imMedia™ Amp Blue (Part No. 45-0038) 1 packet in 100 mLs of dH_2O gives a 2X solution of the liquid media, to give a 1X Blue/White agar screening solution and was then poured out into plates. These Blue/White screening plates were used to grow the transformation control reactions, the mutagenesis control reactions, and mutagenesis reactions. Plasmid DNA was isolated as described previously. Mutation was confirmed by sequencing of the plasmid DNA.

Sequencing of mutagenesis constructs

Constructs were sent out for sequencing using GENEWIZ Molecular Biology.

Table 3. PDGF C Sequencing Primers.	
"C Seq 1R"	5'- GTATGAGGAAACCTTGGGCTG -3'
"C Seq 2F"	5'- CTGGTGTGGTTCTGGTACTG -3'
"C Seq 3R"	5'- GTCTTCCAAGGTACTAAAGGCAG -3'
"C Seq 4F"	5'- TGCACACCTCGTAACTTCTC -3'

GENEWIZ standard T7 promoter Primer and BGH Primer where used to sequence the beginning and the end of the sequence since pcDNA3.1 vectors contain those primer annealing sequences.

Conditioned media

A 100 mm dish of cells were grown under normal serum-containing conditions until confluent. The media was aspirated, and then the cells were washed twice with PBS and replaced with 5 mLs of serum-free media. Cells were kept at 37°C 5% CO_2 for 48 hours under these conditions. Media was collected and spun at 2000 rpm @ 4°C for 5 minutes to pellet any cells/cellular debris. To concentrate the conditioned media was transferred to Amicon® Ultra-4 Centrifugal Filter Units (Millipore UFC801024), spun at 4000 rpm at 4°C for 20 minutes. The flow-through was discarded and phenol red was exchanged out by washing with PBS, spinning at 4000 rpm at 4°C for 20 minutes and discarding the flow-through. Washing with PBS was repeated two more times to remove all residual phenol-red. Protein level of concentrated conditioned media was determined using the

Pierce BCA assay as detailed below using PBS as the blank. Dr. Julie Boerner kindly provided conditioned media from SUM102, and SUM149 cell lines.

Proliferation assay

Cells were seeded into 96-well tissue culture plates in the appropriate cell culture media. For serum-containing conditions (10% serum) and low serum conditions (1% serum), 1,000 cells were seeded per well. After the indicated incubation times, WST-1 reagent (Roche Cat No. 1 644 807) was added to each well according to the manufacturer's protocol. The absorbance at 450 nm was read by microplate reader (Bio-Rad Microplate Reader, Model No. Benchmark) and adjusted using cell-free media as the blank. Samples were done in six replicates.

Invasion assay

Growth Factor-Reduced Matrigel™ coated transwells (BD Biocoat™ 24 well plate, 0.8 micron, Product Number 354483) were rehydrated with serum-free media. Cells were seeded at $5x10^4$ cells/mL in serum free media. Chemoattractant (500 µL of serum containing media) was added to the bottom chamber of the invasion assay. The re-hydrated transwell was placed over the chemoattractant and 500 µL of cells ($5x10^4$ cells/mL) were added to the top chamber. Cells were allowed to migrate while incubating in humidified 37°C, 5% CO_2 atmosphere. Matrigel™ and non-invading cells were scrubbed from the inside of the invasion chambers with moistened cotton swabs. Invading cells were stained with crystal violet and visualized under the microscope and counted.

Soft agar assay

BactoAgar (BD catalog # 214050) combined with the cells' normal growth media (1:1) was used to make a bottom layer of 0.6% agar, and a top layer of 1.2% agar. The top layer also contained 5,000 cells/mL. These were grown in 6-well plates with 2 mL of normal growth media per well. The media was changed every third day. Colonies were analyzed using the colony counter (Oxford Optronix) in the Translational Research Core of the Karmanos Cancer Institute. Utilizing the strictest of parameters looking at the shape of a colony as well as its relative tightness may have led to dismissal of colonies visually observed rather than inclusion. Samples were done in triplicates.

Lysis and RNA isolation

RNA was isolated with the RNeasy® Mini Kit (Qiagen, Cat. No. 74106) according the manufacturer's protocol.

Reverse transcription

The iScript cDNA Synthesis Kit (BioRad, Catalog #170-8891) was used for reverse transcription using 1 µg of RNA and the reaction protocol of 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, then hold at 4°C to create cDNA. Samples of cDNA from several human breast cancer cell lines were a gift from former Kim Laboratory member, Dr. Newton J. Hurst, Jr., MD, PhD. This cDNA was used to assess PDGF C levels by Real Time PCR.

Semi-quantitative RT-PCR

Table 4. Human Semi-Quantitative PCR Primers.	
Target	**Sequence**
PDGF A	Forward: 5’- TGAGGATTCTTTGGACACCA -3’ Reverse: 5’- GGGCCAGATCAGGAAGTTG -3’
PDGF B	Forward: 5’- CATTCCCGAGGAGCTTTATG -3’ Reverse: 5’- CTCAGCAATGGTCAGGGAAC -3’
PDGF C	Forward: 5’- TCCAGCAACAAGGAACAGAA -3’ Reverse: 5’- GGGTCTTCAAGCCCAAATCT -3’
PDGF D	Forward: 5’- GAACAGCTACCCCAGGAACC -3’ Reverse: 5’- CTTGTGTCCACACCATCGTC -3’
PDGFR-α	Forward: 5’- GAACTCACGGTGGCTGCTG -3’ Reverse: 5’- ACTTTCATGACAGGTTGGG -3’
PDGFR-β	Forward: 5’- TTTTGCACCCACAATGACTC -3’ Reverse: 5’- CCAATGGTGGTTTTGCAGAT -3’
GAPDH	Forward: 5’- ATCACCATCTTCCAGGAGCGA -3’ Reverse: 5’- GCCAGTGAGCTTCCCGTTCA -3’

The GoTaq® Flexi DNA Polymerase kit (Promega, catalog #M8291) was used for semi-quantitative RT-PCR using 1 µL of reverse transcription reaction. Cycling parameters were 95°C for 2 minutes, then 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds,

72°C for 2 minutes 30 seconds. Then at the end there is one 72°C step for 5 minutes followed by holding at 4°C. PCR reactions were separated and visualized on agarose gels with ethidium bromide with an UV transilluminator. Samples were run alongside DNA size standards (Invitrogen TrackIt™ 1 kb Plus DNA ladder, catalog #10488-085) to verify size.

Real Time PCR

Table 5. Real Time PCR Primers.

Target	Sequence
Total PDGF C	Forward: 5'-AACGCTGTGGTGGGAACTGTGC-3' Reverse: 5'-TGCAATCCCCTGACACCGGTCT-3'
t-PDGF C	Forward: 5'-TCCAAGCATCTGGACTGGCATAGA-3' Reverse: 5'-AGTATGAGGAAACCTTGGGCTGTGA-3'
GAPDH	Forward: 5'-ATCACCATCTTCCAGGAGCGA-3' Reverse: 5'-GCCAGTGAGCTTCCCGTTCA-3'

Reverse transcription reaction was prepared and mixed with primers according to the Stratagene Brilliant SYBR® Green QPCR Master Mix (Catalog# 600548). Samples were run on the Eppendorf Realplex^2 Mastercycler epgradient machine. Cycling parameters were 10 minutes at 95°C, then 40 cycles of 30 seconds at 95°C, 1 minute at 55°C and 1 minute at 72°C. Samples were run in triplicate. Differences in RNA levels were calculated by the $\Delta\Delta C_T$ method and error bars were calculated by propagation of error from the replicate samples as described elsewhere [130, 131].

Protein extraction

Cell cultures were washed twice with PBS and then whole cell lysate was isolated by adding 1x RIPA buffer (Millipore RIPA lysis buffer 10x product # 20-188 diluted to 1X with dH_2O) including freshly added protease inhibitors (Roche cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets, Product Number 11873580001; Phenylmethanesulfonyl fluoride, PMSF) and phosphatase inhibitors (Sodium Fluoride, Sodium Orthovanadate) to cells and scraping with a rubber policeman. The lysate was transferred to tubes and incubated on ice for 30 minutes. Samples were then centrifuged at 4°C at 12,000 rpm for 20 minutes. The supernatant, the whole cell lysate, was transferred to new tubes and saved for analysis.

Nuclear and cytosolic fractionation

Cells are rinsed twice with PBS and then cell lysis buffer (10 mM HEPES pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.3% NP-40, 1x Roche cOmplete, EDTA-free Protease Inhibitor Cocktail, 1 mM DTT, 0.5 mM PMSF) was added to the tissue culture dish. The tissue culture plates are incubated on ice for approximately 10 minutes. The cells are then scraped with a rubber policeman and samples were transferred to tubes. The tubes were incubated on ice for 10 minutes. The tubes were spun at high speed for two minutes at 4°C. The supernatant, the cytoplasmic fraction, was transferred to new tubes and the protein quantitated. To extract the nuclear fraction the remaining pellet was washed with cold PBS and then spun at full speed for two minutes at 4°C. The supernatant PBS was discarded. Nuclear extraction buffer (20 mM HEPES pH 7.9, 400

mM NaCl, 1 mM EDTA, 1 mM EGTA, 1x Roche cOmplete, EDTA-free Protease Inhibitor Cocktail, 1 mM DTT and 1 mM PMSF) was added to the pellet and incubated on ice for 30 minutes with intermittent vortexing to mix. The tubes were then spun at maximum speed for 10 minutes at 4°C. The supernatant, the nuclear fraction, was transferred to new tubes and the protein quantitated.

Subcellular fractionation

Subcellular fractions: cytoplasmic extract, membrane extract, soluble nuclear extract, chromatin-bound extract and cytoskeletal extract were isolated using the Subcellular Protein Fractionation Kit for Cultured Cells (Thermo Scientific Product Number 78840) according to the manufacturer's protocol.

Protein quantitation

Quantifying the amount of protein in whole cell lysates, nuclear or cytosolic fractionations, or subcellular fractions was done using the Thermo Scientific Pierce BCA (bicinchoninic acid) Protein Assay kit (Product Number 23225) using Thermo Scientific Pierce Bovine Serum Albumin (BSA) Standards.

Western blotting

Protein was separated on custom made Tris-Glycine Sodium Dodecyl Sulfate (SDS) Polyacrylamide gels (PAGE). Protein was transferred to Whatman® Optitran® BA-S83 0.2 µm pore size reinforced nitrocellulose membranes (REF 10439396), blocked in 5% milk in Tris Buffered Saline with Tween® 20 (TBST: 50 mM Tris Base, 150 mM sodium

chloride, 0.2 % Tween® 20) and incubated in primary antibody 5% milk in TBST. Blots were washed and incubated in the appropriate secondary antibody and then washed three times with TBST and incubated with Western Lightning® Plus-ECL, Enhanced Chemiluminescence Substrate (Perkin Elmer Product No. NEL103001) and then developed to Hyblot CL® Autoradiography film (Denville Scientific, Inc., Cat No E3012).

Primary antibodies

PDGF C (R&D Systems, product number AF1560), GAPDH (Santa Cruz, product number sc-25778), Phospho-Erk (Thr202/204) (Cell signaling, product number 9101), Total Erk (Cell signaling, product number 9102), Phospho-Akt (Ser473) (Cell Signaling, product number 9271), Total Akt (Cell Signaling, product number 9272), Histone H1 + core proteins (Millipore, product number MAB052), SP1 (Cell Signaling, product number 5931), Cytokeratin 18 (Millipore product number CBL 185), Hsp90 (Santa Cruz, product number sc-13119), Transferrin Receptor (BD Transduction, product number 612124), Myc-Tag (Cell Signaling, product number 2276) antibodies were used according to manufacturers' protocol.

Secondary antibodies

Goat anti-Rabbit peroxidase conjugated secondary antibody (Jackson ImmunoResearch, product 111-035-003) was diluted 1:5000. Rabbit anti-Mouse peroxidase conjugated secondary antibody (Sigma, product A9044) was diluted 1:5000. Donkey anti-Goat

peroxidase conjugated secondary antibody (R&D Systems, product HAF109) was diluted 1:1000.

PDGF C custom antibody

A custom antibody against the growth factor domain of PDGF C (amino acids 230-250) was created using a peptide (N'-CGRKSRVVDLNLLTEEVRLYSC-C'). The resultant antibody was affinity purified (Zymed Biomedical, San Francisco, CA, USA) as described previously [132].

Antibody specificity test

40 µL of conditioned media or 40 µg of whole cell lysate from stably transfected MCF-7 cells was run under reducing conditions. The resulting western blots were cut into slices. A custom peptide Hs PDGF C #230-250 peptide (Zymed® Laboratories, Inc, South San Francisco, CA) used to generate a custom PDGF C antibody was diluted in solution with 1:1000 PDGF C custom antibody in 5% milk in TBST and the preservative sodium azide. Peptide:Antibody ratios of 2:1, 10:1 and 50:1 molar competition (peptide to antibody ratio) were prepared. Peptide was pre-incubated with the custom antibody 1 hour at 4°C. Western blot membranes of control transfected MCF-7 Neo cell line conditioned media which have undetectable levels of PDGF C, and PDGF C overexpressing transfected MCF-7 cell line conditioned media which have very high levels of PDGF C, were blocked for 1 hour at room temperature in 5% milk in TBST and then incubated with antibody alone or peptide:antibody solutions at 4°C overnight, then developed to film.

Cell culture protease inhibition experiments

Cells were washed twice with PBS and serum free media was added containing the appropriate concentration of protease inhibitors. Cells were incubated for 48 hours under otherwise normal culture conditions. Protease Inhibitor Cocktail was used in a 1:200 dilution according to manufacturers specifications (Sigma, product number P1860). Aprotinin was used at 10 µg/mL (Sigma, product number A3428), and AEBSF was used at 0.25 mM concentration (Sigma, product number A8456) in serum free cell culture media for protease inhibition experiments.

Animal studies

NOD/SCID Mouse

An animal model is necessary to simulate all the steps of tumor metastasis: intravasation, survival in the blood and lymphatics, extravasation, and colonization in a new organ. No *in vitro* model can simulate all of these sequential steps necessary for tumor metastasis. Immunodeficient animal models allow implanted human tumor cell growth. There are various immunodeficient animal models available including the nude mouse [133], SCID mouse [134, 135], NOD/SCID mouse [136] and Rag-1-deficient mouse [137]. NOD/SCID mice stand out as particularly well suited to study primary tumor growth and tumor metastatic spread [138].

Tumor measurements

$1x10^6$ cells in 50 µl of Matrigel™ (BD Biosciences):media (1:1) mixture were injected into the R4 mammary fat pad of 6-week old female NOD/SCID mice with a 25 gauge needle by Dr. Fred Miller (Karmanos Cancer Institute). Tumor measurements were taken at least weekly using digital calipers. Tumor volumes were calculated as described elsewhere using the formula tumor volume = a x $b^2/2$, where b is the smaller of the two measured tumor diameters [139, 140]. Miss Lisa Movilla helped with the animal handling.

Tail vein injection

MDA-MB-231 cells ($1x10^6$ cells in 100 µL of sterile PBS) were injected in the tail vein of CB17 SCID mice using a 27-gauge needle by Dr. Fred Miller (Karmanos Cancer Institute). Eight mice were injected with MDA-MB-231 shScramble, 8 mice were injected with MDA-MB-231 shPDGF C and three mice were left uninjected. Mice were monitored weekly and euthanized if in apparent distress. Shortly after injection one mouse from the MDA-MB-231 shScramble injected group died and two mice from the MDA-MB-231 shPDGF C injected group died. On day 30 after injection, one mouse from the MDA-MB-231 shScramble group was euthanized for apparent respiratory distress. On Day 32 all remaining mice were sacrificed and organs were harvested. Lung volumes were weighted and half of the lung tissue was processed for paraffin embedding, the other half was frozen at -80°C for later DNA analysis. At day 32 there remained 6

mice injected with MDA-MB-231 shScramble, 6 mice injected with MDA-MB-231 shPDGF C and 3 uninjected mice.

Tissue fixation & staining

The tissue samples were allowed to incubate overnight at 4°C in 4% PFA with gentle agitation (80 rpm). The tissue samples were dehydrated by incubating for 30 minutes in 70% ethanol, then 30 minutes in 80% ethanol, then 30 minutes in 90% ethanol, then 30 minutes in 95% ethanol, then 20 minutes in 100% ethanol, 20 minutes in 100% ethanol again, 20 minutes in 100% xylene, 20 minutes in 100% xylene, 1 hour in melted paraffin (at 55°C), then at last overnight in melted paraffin (55°C). Tissue was then placed in molds and embedded in melted paraffin for later staining. Fixed & embedded tissue was hematoxylin & eosin (H&E) stained courtesy of Dr. Larry Tait. Dr. Cher and Dr. Bonfil's laboratory allowed use of their microscope to capture images.

Statistical analysis

P-values <0.05 were considered statistically significant. Real Time PCR error bars represent standard deviation of triplicates. Error bars were calculated by propagation of error from the replicate samples as described elsewhere. Data from WST-1 proliferation were analyzed using IBM® SPSS® Statistics version 22 using repeated measures ANOVA. Invasion assay bar graph indicates the average number of invading cells assessed in three high-power fields. Error bars indicate the standard deviation of the values. T-tests were performed to determine between averages to determine significance. Soft agar assay bar graph indicates the average number of colonies formed in three wells.

Error bars indicate the standard deviation of the three wells. T-tests were performed to determine between averages to determine significance. For *in vivo* tumor growth analysis, Tumor growth was measured at the indicated time points following peri-mammary fat pad orthotopic injection of female NOD/SCID mice (6 and 7 mice in shScramble and shPDGF C groups, respectively). Data are mean ± SEM. Lung weight bar graph indicates average weight (grams). Data are averages with error bars indicating standard deviation. Box-whisker plots represent the quartiles (25%, median, and 75%). Dr. Park performed statistical analysis of Kaplan-Meier survival curves showing probability of disease-free survival with log-rank test, two sided P value.

CHAPTER 3

RESULTS

3.1 PDGF C gene expression in human breast cancer cell lines.

PDGF signaling has been shown to play an important role in the development and progression of several types of cancers (as reviewed in [67]). Previous studies have shown expression of PDGF receptors in human breast cancers and also associated PDGF receptor staining with poor prognostic features of breast cancer such as metastasis to lymph nodes and bone metastasis, in addition to association with overexpression of the oncogene HER2/neu [75-78, 110, 112]. Furthermore, our laboratory's data and one paper in the published literature have shown that PDGF C has an alternatively spliced mRNA variant ([117] and Figure 4, data courtesy of NJH). This newly discovered PDGF C splice variant mRNA contains an additional 61 base pair retained exon, shifting the translation start codon thus creating an N-terminally truncated PDGF C variant that our laboratory designates as truncated-PDGF C (t-PDGF C) while the other group refers to it as PDGF C-Cb [117].

Since the discovery of the splice variant of PDGF C [117] its function has not yet been elucidated. In addition, since our laboratory had previously determined by RT-PCR analysis that PDGF C expression level is correlated with the malignant potential of human breast cancer cell lines (Figure 4, data courtesy of NJH) we further analyzed the expression levels of PDGF C and its splice variant in human breast cancer cell lines. Total PDGF C expression (both FL-PDGF C and t-PDGF C) was analyzed by real time

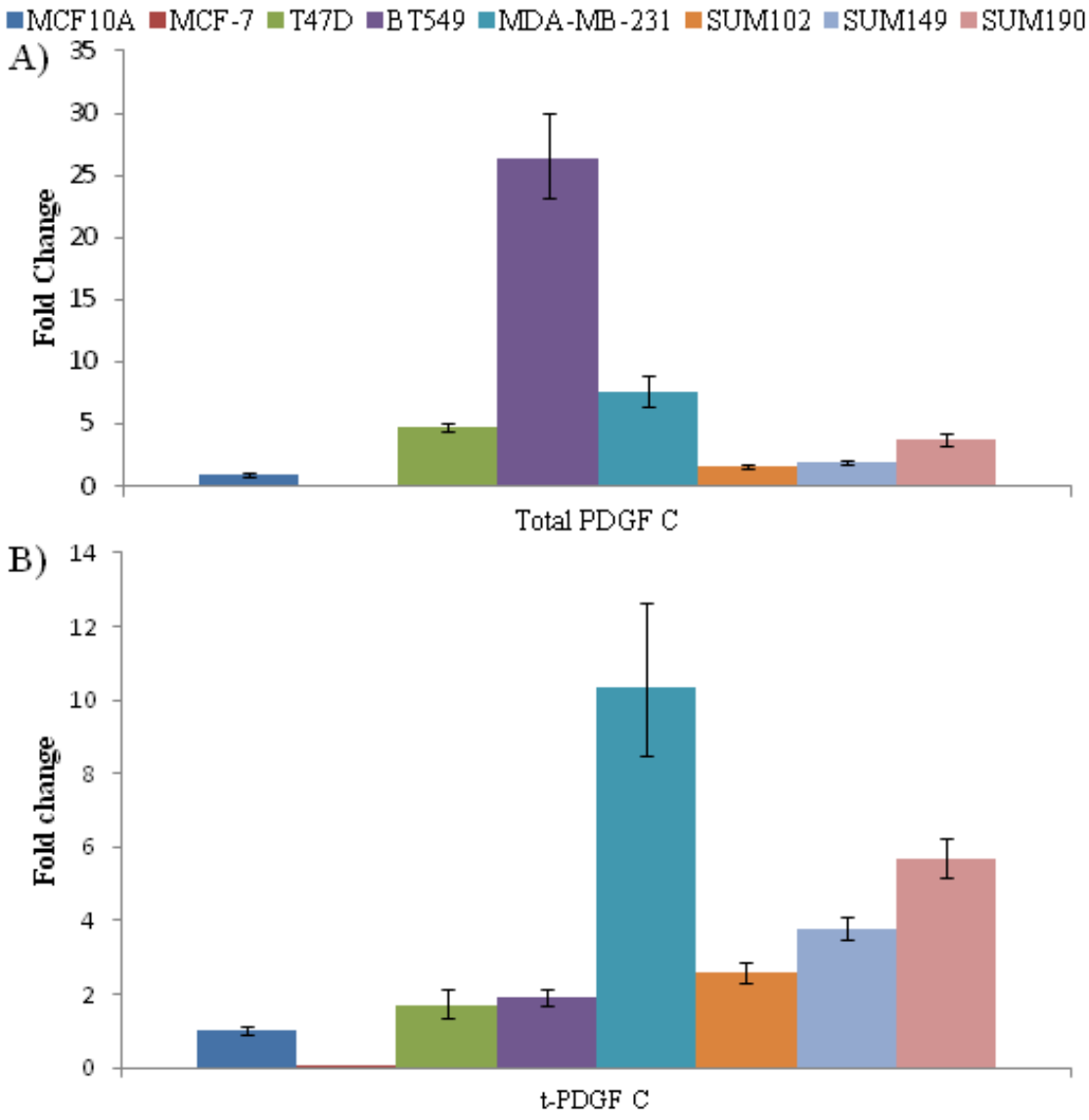


Figure 6. PDGF C mRNA expression in breast cancer cell lines. Real-time RT-PCR analysis of **A)** Total PDGF C (both FL-PDGF C and t-PDGF C) and **B)** t-PDGF C in MCF-10A, MCF-7, T47D, BT-549, MDA-MB-231, SUM102, SUM149, and SUM190 cell lines. MCF-10A PDGF C RNA level was arbitrarily set as 1 and the error bars represent standard deviation of triplicates. Error bars were calculated by propagation of error from the replicate samples.

PCR in a panel of human breast cancer cell lines using primers capable of annealing to both splice variants (Figure 6A). In addition, by designing one primer within the unique

retained exon 2' of t-PDGF C (Figure 5), the level of specifically t-PDGF C was assessed by real time PCR in the panel of human breast cancer cell lines (Figure 6B). These breast cancer cells lines were selected for analysis because they cover a range of breast cancer subtypes, from the non-malignant MCF10A breast cell line to highly aggressive breast cancer cell lines such as the triple-negative MDA-MD-231 isolated from the metastatic pleural effusion of breast cancer, and the inflammatory breast cancer cell lines SUM149PT and SUM190PT [121, 122, 127, 141]. As shown in Figure 6, the non-malignant MCF10A cell line and the hormone-receptor positive MCF-7 breast cancer cell line show the lowest levels of total PDGF C expression and t-PDGF C expression. Of note, there is almost undetectable expression of either total PDGF C or t-PDGF C in the MCF-7 breast cancer cell line (Figure 6). While the hormone-receptor positive T47D breast cancer cell line ([121, 122, 128]) has low to intermediate expression of total PDGF C and t-PDGF C (Figure 6). The more malignant and triple-negative papillary invasive ductal carcinoma BT-549 breast cancer cell line [121, 122] showed the highest levels of total PDGF C (Figure 6A) while it had intermediate levels of specifically t-PDGF C expression (Figure 6B). The very aggressive and invasive triple-negative MDA-MB-231 breast cancer cell line showed the second highest level of total PDGF C expression and the highest expression of specifically t-PDGF C amongst the panel screened (Figure 6). Amongst the SUM breast cancer cell lines, the SUM102PT breast cancer cell line had the lowest expression of total PDGF C or t-PDGF C appearing to express PDGF C similar to perhaps slightly higher than the T47D breast cancer cell line. The more aggressive

inflammatory breast cancer cell lines, SUM149PT, and SUM190PT, had higher levels of total PDGF C and t-PDGF C expression than SUM102PT cells (Figure 6).

This data confirms and quantifies our laboratory's previous qualitative results via semi-quantitative PCR (Figure 4, data courtesy of NJH).

3.2 Experimental models.

Very little research has been done to investigate the role of PDGF C in breast cancer and since it was so recently discovered, often the PDGF C splice variant is not addressed in studies of PDGF C. In order to better understand the role of PDGF C isoforms in human breast cancer, this study was undertaken. To that end, breast cancer cell lines transfected to differentially express FL-PDGF C and t-PDGF C were created. Semi-quantitative RT-PCR confirms the differential expression of the FL-PDGF C mRNA and the t-PDGF C mRNA (Figure 7).

The human breast cancer cell line MCF-7 has almost undetectable levels of total PDGF C or t-PDGF C (Figure 6A & 6B) making this cell line an ideal model in which to overexpress the PDGF C isoforms (Figure 7). Additionally, human breast cancer cell line BT-549 has high levels of total PDGF C, but relatively lower levels of specifically t-PDGF C RNA (Figure 6A & 6B), making this cell line an ideal model in which to overexpress the t-PDGF C RNA (Figure 7). While MDA-MB-231 human breast cancer cell line has relatively high levels of both total PDGF C and t-PDGF C (Figure 6A & 6B), making it an ideal model in which to knockdown the expression of PDGF C by shRNA (Figure 7).

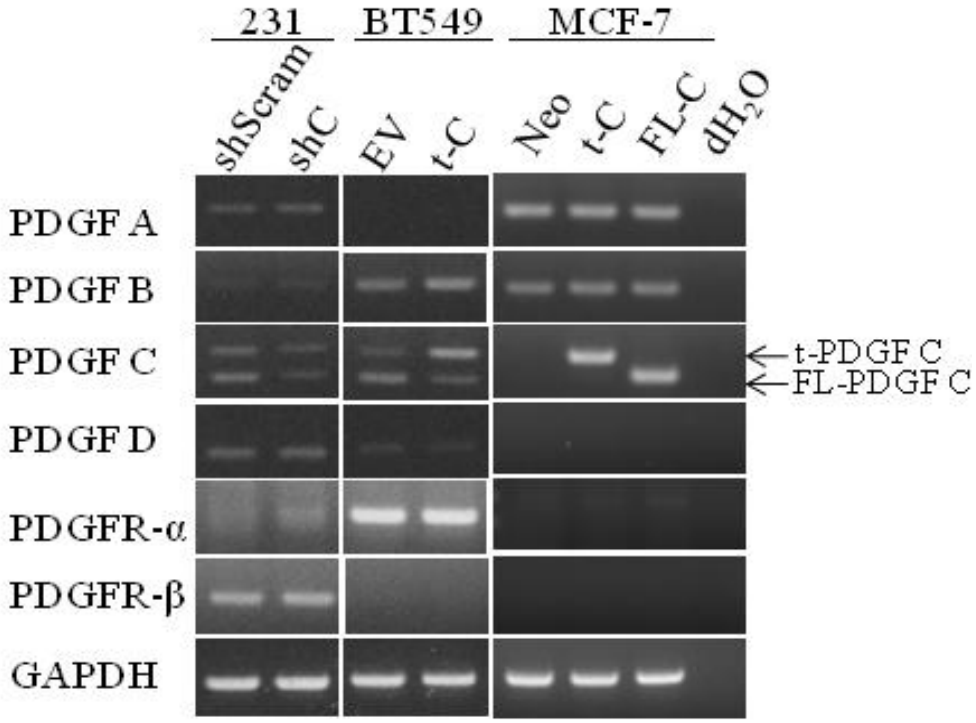


Figure 7. The mRNA expression levels of the PDGF family members in breast cancer cell models that differentially express PDGF C isoforms. RT-PCR analysis of PDGF A, B, C, and D, as well as their receptors PDGFR-α, and PDGFR-β in stably transfected experimental models. 231= MDA-MB-231 transfected cells. ShScram= scramble shRNA control transfected. ShC= PDGF C shRNA knockdown. EV= empty vector control transfected. t-C= truncated PDGF C transfected. Neo = control. transfected. FL-C= Full-length PDGF C transfected. Negative control= dH_2O.

Model 1: MCF-7 breast cancer cells which normally express little endogenous PDGF C were transfected with control vector or full-length-PDGF C (His-tagged) to overexpress FL-PDGF C or vector control (as described elsewhere, [132]). In addition, later another set of MCF-7 transfectants were created in order to investigate both PDGF C splice variants. These MCF-7 breast cancer cells were transfected to overexpress t-PDGF C/myc or FL-PDGF C/myc or vector control (Figure 7).

Model 2: MDA-MB-231 breast cancer cells which normally express high levels of both FL-PDGF C and t-PDGF C mRNA were transfected with control (shScramble) vector or PDGF C shRNA (shPDGF C) to knockdown PDGF C expression (Figure 7).

Model 3: BT-549 breast cancer cells that express predominantly full-length-PDGF C were transfected with control vector or t-PDGF C/myc tagged (Figure 7).

PDGF C expression and the entire PDGF family expression was confirmed in the cell line models by RT-PCR analysis (Figure 7). This demonstrates that the stably transfected cells express PDGF C isoforms as desired.

3.3 PDGF C custom antibody is specific to PDGF C growth factor domain.

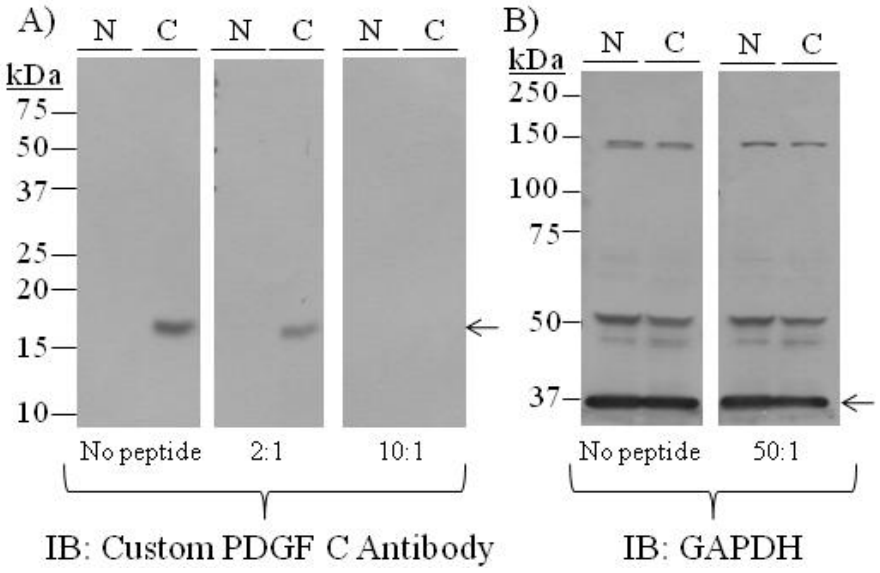


Figure 8. Specificity of PDGF C custom antibody in immunoblot analysis. Reducing immunoblot (IB) of MCF-7 Neo control vector stable transfected (N) and MCF-7 FL-PDGF C stable transfected (C) **A)** conditioned media probed with our laboratory's custom PDGF C antibody, and **B)** whole cell lysate probed with GAPDH control antibody. Ratios depict Peptide:Antibody ratios of 2:1, 10:1 and 50:1 molar competition with no peptide as the control.

Our laboratory's custom PDGF C antibody is a polyclonal antibody that was raised in rabbits against a synthetic peptide epitope of the PDGF C growth factor domain amino acids 230 to 250 (as described in [132]). As demonstrated in Figure 8, detection of PDGF C protein on western blot can be blocked by incubating the blot with an excess of PDGF C peptide, while detection of GAPDH is not blocked by an excess of PDGF C peptide. This demonstrates the specificity of our laboratory's custom antibody for detecting PDGF C via western blotting.

3.4 PDGF C is a secreted growth factor.

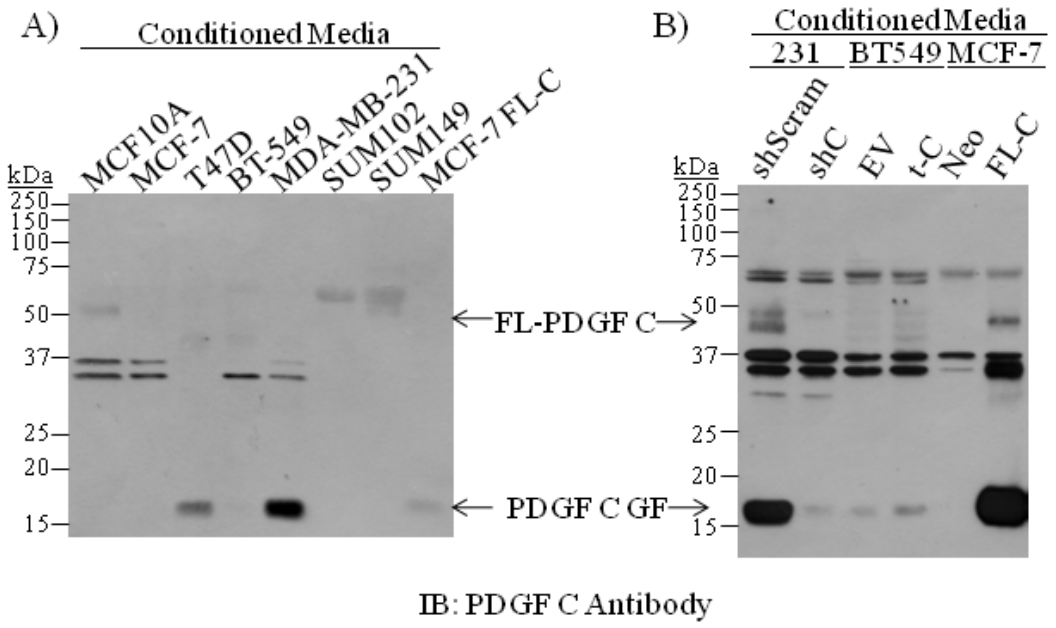


Figure 9. PDGF C is secreted from breast cancer cells. Reducing immunoblot (IB) analysis of PDGF C using conditioned media collected from indicated cells **A)** human breast cancer cell lines, and **B)** in transfected experimental models. All conditioned media is concentrated, except that of MCF-7 FL-PDGF C transfected cells. FL-C or FL-PDGF C= Full-length PDGF C. 231 = MDA-MB-231. shScram = control shRNA. shC = PDGF C shRNA. EV = empty vector control. t-C = truncated PDGF C. Neo = empty vector control. PDGF C GF= PDGF C Growth Factor domain.

Traditionally PDGF ligands are thought of as secreted growth factors whose mechanism of action is to act on extracellular PDGF receptors in a paracrine, juxtacrine or autocrine matter (as reviewed in [79]). Thus it is natural to assess the secretion of PDGF C in human breast cancer by western blot analysis of the conditioned media of several breast cancer cell lines (Figure 9A). As shown, there is detectable PDGF C growth factor domain protein (PDGF C GF) expressed and secreted in some of the analyzed breast cancer cell lines' conditioned media, including high levels in the aggressive triple-negative MDA-MB-231 cells which express both full-length PDGF C and the splice variant mRNA (Figure 9A). Additionally in our cell line model of MDA-MD-231 cells, downregulation of PDGF C expression by stable shRNA knockdown significantly decreases the amount of PDGF C growth factor domain protein detected on western blot (Figure 9B). Relatively high levels of PDGF C growth factor domain protein is also seen in the conditioned media of T47D cells (Figure 9A). Surprisingly despite high levels of full-length PDGF C mRNA levels, there appears to be relatively much lower PDGF C growth factor domain protein levels found in BT-549 breast cancer cells, requiring longer film exposure to detect via western blot analysis (Figure 9A). Lower than expected protein levels in these cells are presumably due to the high levels of the PDGF Receptor-α in these cells as seen at the mRNA level via PCR analysis (Figure 7). Presumably autocrine signaling and receptor endocytosis causes there to be rapid uptake of PDGF C ligand and thus not as much is detected on western blot. Upon stable transfection and overexpression of the splice variant, t-PDGF C, there is a slight but consistent increase in the PDGF C growth factor domain protein when equal amounts of

concentrated conditioned media protein are loaded on western blot (Figure 9B). MCF-7 breast cancer cell line expressed no PDGF C growth factor domain protein as assessed by western blot (Figure 9A), making it a good model for overexpression of PDGF C. As seen in Figure 9B, MCF-7 cells stably transfected to overexpress full-length PDGF C shows very high levels of PDGF C growth factor domain protein in unconcentrated conditioned media.

In order to investigate possible autocrine signaling the BT-549 cell line model, downstream signaling molecules and their phosphorylation was assessed in the whole cell lysates of serum-starved cells. As shown in Figure 10, there is higher phospho-Akt and higher levels of phospho-Erk in BT-549 cells stably transfected to overexpress t-PDGF C/myc as compared to control empty vector transfected BT-549 cells. This data in addition to the lower than expected PDGF C GF protein detected in the conditioned media supports the notion that PDGF C protein is involved in autocrine

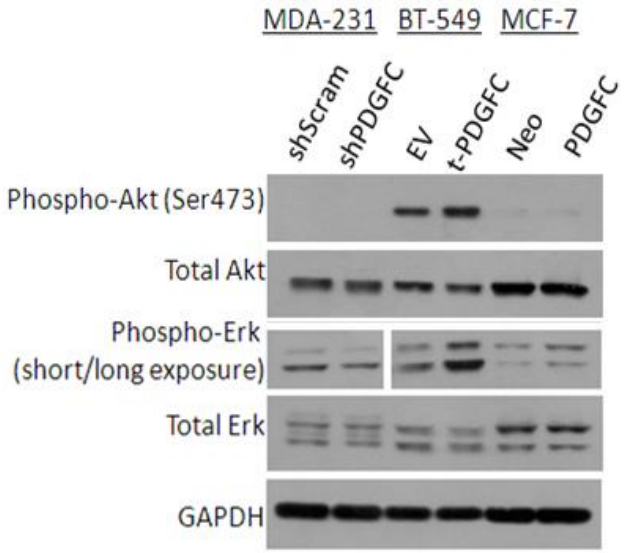


Figure 10. Autocrine signaling in BT-549 cell line model. Whole cell lysates in the indicated stable transfected cell lines were immunoblotted with phospho-Akt, total Akt, phospho-Erk, total Erk and GAPDH. MDA-231= MDA-MB-231. ShScram = ShRNA scramble control transfected, ShPDGFC= ShPDGF C knockdown. EV= Empty vector transfected, Neo= Neo control transfected.

signaling in cells that express PDGF receptors that PDGF C can active. This data suggests that PDGF C ligand is secreted into the conditioned media, then quickly taken up activating the PDGFR-α receptors and causing downstream signaling.

3.5 PDGF C is also a nuclear protein.

A previous study described the nuclear localization of PDGF C in human thyroid tissue samples, papillary thyroid carcinoma samples, and cell lines [120]. It was found that nuclear PDGF C was post-translationally modified with the small ubiquitin-like modifier (SUMO) [120]. This led our laboratory to likewise investigate the cellular localization of PDGF C in human breast cancer cell lines. Subcellular localization of PDGF C was analyzed by simple nuclear and cytosolic fractionation in the MCF-7 model expression cell lines. Full-length PDGF C that has not been proteolytically processed to the growth factor domain is seen at approximately 50 kilodaltons (kDa) in the cytosolic fraction, nuclear fraction and secreted into the conditioned media of the stably transfected MCF-7 cells (Figure 11A). In addition, as expected, full-length PDGF C is secreted into the conditioned media where it can be proteolytically processed to the PDGF C growth factor domain (PDGF C GF) and seen at approximately 18 kDa (Figure 11A). In order to analyze the subcellular localization of endogenous PDGF C in human breast cancer cell lines, T47D human breast cancer cells were separated into several subcellular fractions and subjected to western blot analysis. Full-length PDGF C not proteolytically processed (~50 kDa) can be seen in the conditioned media of the positive control, MCF-7 FL-PDGF C/myc transfected lane (Figure 11B). Also, there is a slightly higher molecular weight

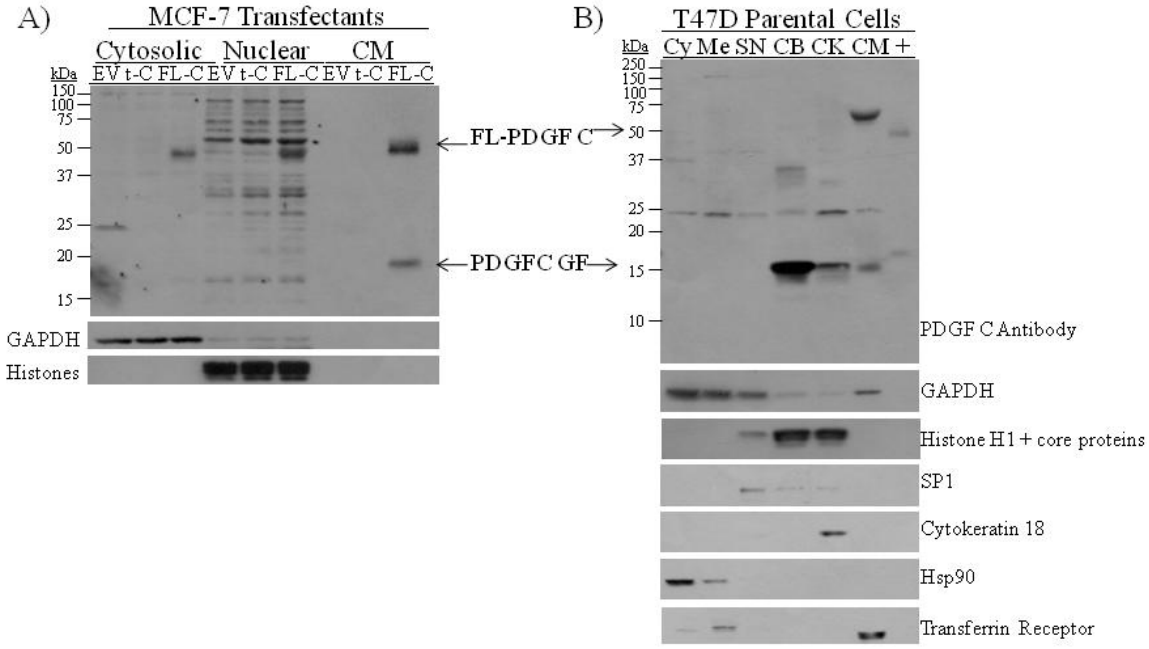


Figure 11. Nuclear localization of PDGF C in breast cancer cells. **A)** Reducing immunoblot of MCF-7 stable transfectant cells probed with PDGF C, GAPDH and histones antibodies. **B)** Reducing immunoblot of T47D parental subcellular fractions probed with the indicated antibodies. EV= MCF-7 Empty Vector transfected. t-C= MCF-7 t-PDGF C/myc transfected. FL-C= MCF-7 FL-PDGF C/myc transfected. Cy= Cytoplasmic, Me= Membrane, SN= Soluble Nuclear, CB= Chromatin Bound Nuclear, CK= Cytoskeletal, CM= T47D concentrated conditioned media, and + indicates MCF-7 FL-PDGF C/myc conditioned media, as a positive control.

band (~60 kDa) in the conditioned media of T47D cells that may represent full-length PDGF C unprocessed in that cell line (Figure 11B). Interestingly the proteolytically processed PDGF C growth factor domain protein is found predominantly in the chromatin bound nuclear fraction of T47D breast cancer cells (Figure 11B) with a smaller amount found in the cytoskeletal and concentrated conditioned media (Figure 11B). This is in agreement with published literature showing nuclear PDGF C can be detected in the chromatin bound fraction of cultured NIH/3T3 cells [120]. Additionally, in T47D cells there are multiple faint bands detected between the 25 kDa and 37 kDa molecular weight

markers in the chromatin bound nuclear fraction (Figure 11B). This may represent endogenous t-PDGF C, as the PDGF C splice variant protein product has been shown to be expressed in a similar manner, as multiple bands of a similar size, in both transfected COS-7 cells and endogenous in a human malignant glioma cell line in the literature [117]. Nuclear localization of PDGF C in breast cancer cell lines is a novel finding and is potentially clinically relevant.

3.6 AEBSF, an irreversible serine protease inhibitor, prevents PDGF C processing.

Since it was difficult to detect t-PDGF C in our overexpression cell line models (Figure 11A), we undertook experiments aimed at inhibiting the processing and degradation of protein in case t-PDGF C was being rapidly subjected to proteases thus making it difficult to detect. Our laboratory's previous study identified aprotinin, a serine protease, as an inhibitor of PDGF C processing in human breast cancer cell lines [132]. The inhibitors diisopropyl fluorophosphate (DFP) and phenylmethanesulfonylfluoride (PMSF) irreversibly inactivate serine proteases by reacting with serine 195 generating a stable transition state adduct (as reviewed in [142]). However due to toxicity DFP is not considered safe to handle and although less toxic, PMSF is not considered stable. Sulfonyl Fluorides were discovered to be inhibitors of serine proteases and began to be characterized by Fahrney and Gold ([143], and as reviewed in [144]). The sulfonyl fluoride known as 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) is known by the trade name Pefabloc® SC (Pentapharm, [145]). It is an irreversible serine protease inhibitor that is water soluble and less toxic than the serine protease inhibitor

phenylmethanesulfonyl fluoride (PMSF), and considered stable in aqueous solutions making it particularly useful for cell culture use [145-147]. AEBSF is relatively small molecular weight of 239 Daltons [145], and is cell permeable [148-150]. It has been shown to inhibit a range of serine proteases including ones relevant to activation of PDGF's by proteolytic processing such as plasmin, tPA, uPA and recombinant human matriptase [151]. In an *in vitro* experiment, human recombinant matriptase was inhibited by AEBSF [151]. In order to investigate the inhibition of endogenous PDGF C, T47D cells were serum starved for 48 hours with a commercially available protease inhibitor cocktail, AEBSF, or vehicle control treated (Figure 12). Only the irreversible serine protease inhibitor AEBSF acted to

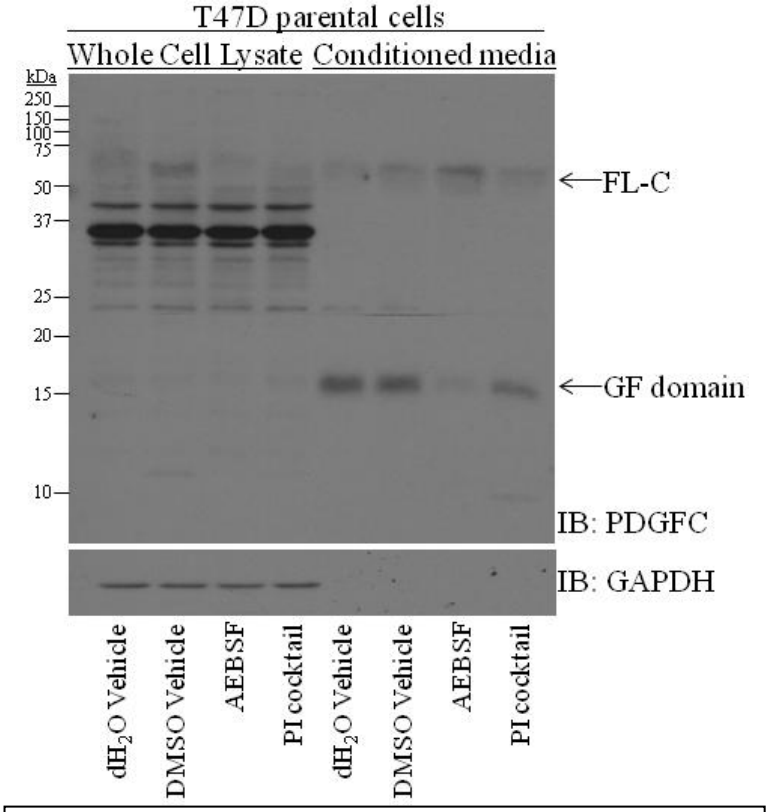


Figure 12. Serine proteases proteolytically process PDGF C. Reducing immunoblot (IB) probed with PDGF C antibody (top), GAPDH (bottom). Whole cell lysate (left) and conditioned media (right) from T47D parental breast cancer cells. dH_2O Vehicle = deionized, distilled water vehicle control treatment, DMSO = dimethyl sulfoxide vehicle control treatment, AEBSF = 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF) dissolved in dH_2O treatment, PI Cocktail = Protease inhibitor cocktail dissolved in DMSO. All treatments 48-hrs in serum free media.

sufficiently inhibit the full-length PDGF C proteolytic processing of PDGF C to the growth factor domain (GF domain, Figure 12). In addition, AEBSF treatment appears to allow for the accumulation of full-length PDGF C in these cells as evidenced by a darker band at ~60 kDa in the AEBSF treated condition (Figure 12). The commercially available protease inhibitor cocktail did not inhibit the processing of PDGF C as well as AEBSF inhibition did (Figure 12). This may be explained by the fact that the

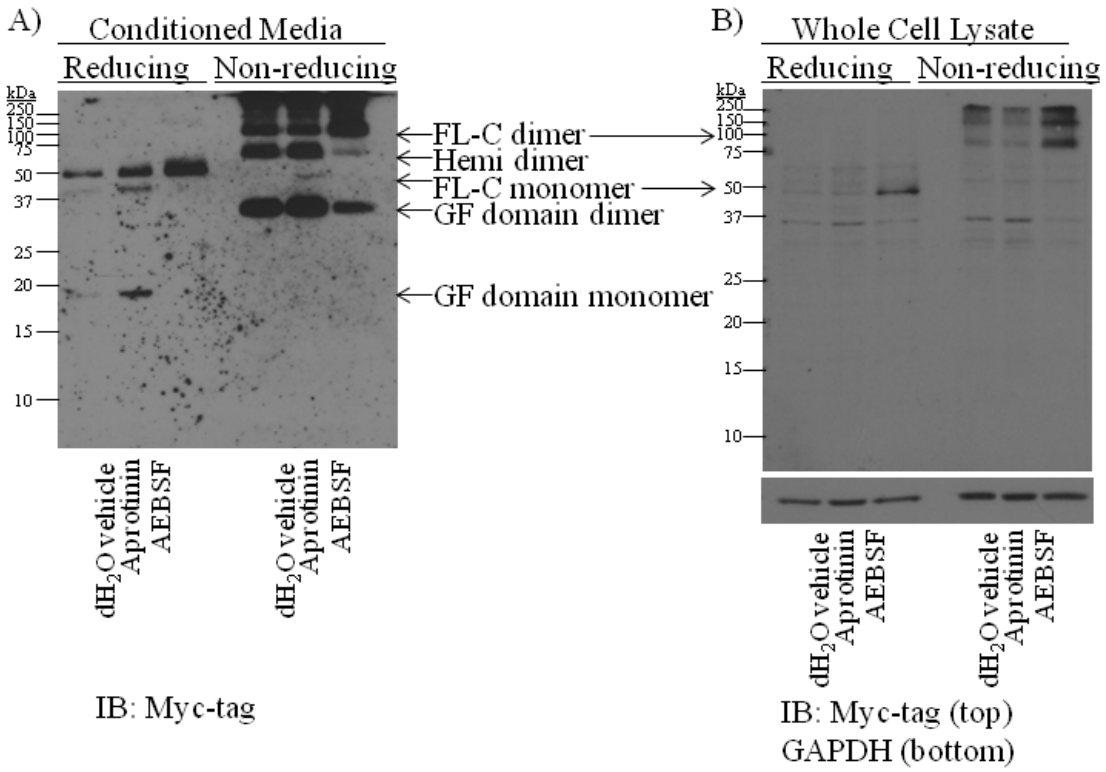


Figure 13. AEBSF is a potent inhibitor of PDGF C processing. Immunoblot (IB) analysis of MCF-7 cells stable transfected with FL-PDGF C/myc **A)** conditioned media, and **B)** whole cell lysates. Each blot has reducing samples (left) and non-reducing samples (right). dH_2O vehicle indicates, vehicle control of serum-free media with dH_2O added in lieu of a protease inhibitor. FL-C= Full-length PDGF C, Hemi dimer = Full length PDGFC dimer with one CUB domain removed. GF domain= growth factor domain.

commercially available protease inhibitor cocktail contains the reversible serine protease inhibitor, aprotinin, while AEBSF is an irreversible serine protease inhibitor. Aprotinin alone appears to be unable to completely inhibit PDGF C processing to the active growth factor domain (~18 kDa, Figures 13 and Figure 14). However, Aprotinin appears to stabilize the hemi-dimer (as seen on non-reducing blots) and the growth factor domain (as seen on both reducing and non-reducing blots) (Figure 13 and Figure 14). This is seen to accumulate, possibly due to inhibition of the further proteolytic processing, or degradation, of the PDGF C growth factor domain. Interestingly, the irreversible serine protease inhibitor AEBSF is found to be very effective at inhibiting the processing of full-length PDGF C to the hemi-dimer and inhibiting to processing to the subsequent growth factor domain dimer (Figure 13 and Figure 14). Thus the two step processing of full-length PDGF C appears to be predominantly due to serine proteases in the breast cancer cell lines MCF-7 cells and T47D cells. Unfortunately the t-PDGF C/myc tagged protein was not detected via western blot analysis even with irreversible serine protease inhibition of the MCF-7 stably transfected overexpressing cell lines (Figure 14). Thus we conclude that the difficulty in detecting t-PDGF C is likely not due to it being subjected to proteolytic degradation.

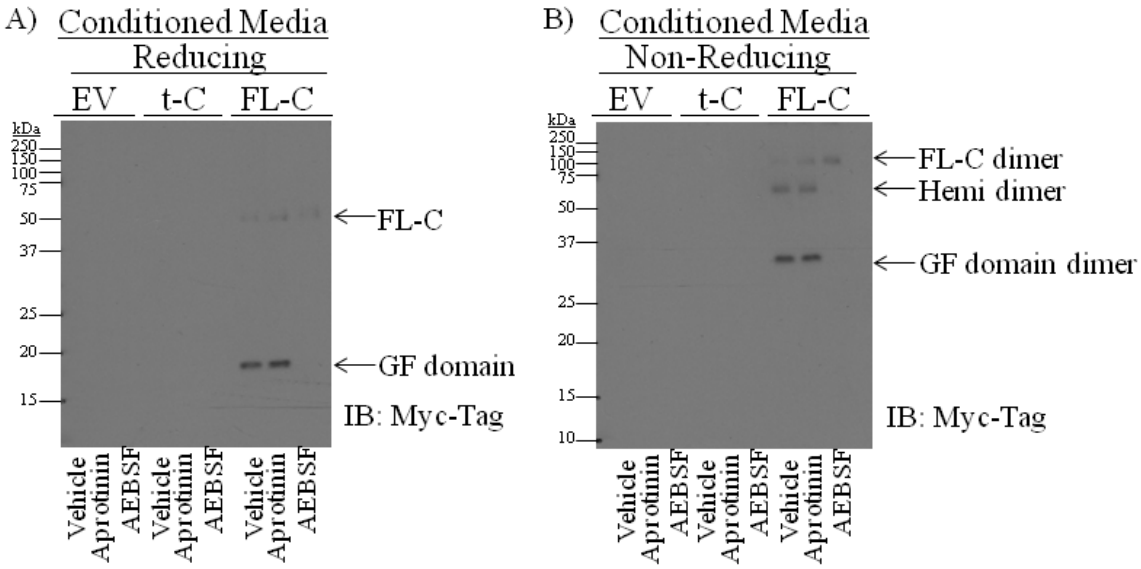


Figure 14. Serine protease inhibition effectively prevents proteolytic processing of PDGF C. Immunoblot (IB) analysis of conditioned media from MCF-7 cells stable transfected with Empty Vector (EV), Truncated PDGF C/myc-tagged (t-C), or Full-length PDGF C/myc-tagged (FL-C). Samples were treated with vehicle control, Aprotinin (a reversible serine protease inhibitor), or AEBSF (an irreversible serine protease inhibitor). **A)** Reducing blot, **B)** non-reducing blot, probed with myc-tag antibody. FL-C = Full-length PDGF C. GF= growth factor domain. Hemi-dimer = Full-length PDGF C and growth factor domain hetero-dimer.

3.7 Truncated PDGF C can be secreted as a dimer with full-length PDGF C and proteolytically processed by the serine protease matriptase into an active growth factor domain dimer.

The only study published in the literature describing the PDGF C splice variant, describes the PDGF C splice variant protein product, t-PDGF C, as an intracellular protein [117]. Due to technical difficulties in detecting t-PDGF C in our breast cancer cell line models, we were unable to detect t-PDGF C when overexpressed in MCF-7

breast cancer cells despite using an expression vector with a myc-tag and inhibiting proteases. Realizing that in human breast cancer cell lines, t-PDGF C mRNA does not appear to be expressed alone in cells, but instead it is expressed along with the full-length PDGF C mRNA transcript (Figure 4, data courtesy of NJH). These observations lead us to believe that protein stability of t-PDGF C may be a problem when t-PDGF C is expressed without full-length PDGF C. We further hypothesized that t-PDGF C may need to be expressed with full-length PDGF C in our cell line models in order to be able to detect the protein. Since MCF-7 breast cancer cells do not express PDGF C at the RNA level, we focused our efforts to detect t-PDGF C protein on the BT-549 cell model which naturally expresses predominantly full-length PDGF C. In BT-549 cells transfected to overexpress t-PDGF C/myc tagged, after much troubleshooting, it was discovered that the myc-tag antibody used in an attempt to try to detected t-PDGF C/myc in the stably transfected BT-549 cells did not work well under reducing conditions (Figure 15A). However, the myc-tagged antibody worked much better under non-reducing SDS-PAGE western blotting conditions (Figure 15A). To our surprise when conditioned media samples from the BT-549 t-PDGF C/myc transfected cells were run under non-reducing conditions, t-PDGF C/myc can be detected secreted from the cells (Figure 15). Furthermore, this secreted t-PDGF C/myc is of a unique molecular weight as compared to the intracellular t-PDGF C/myc protein detected under non-reducing conditions (Figure 15). The difference in molecular weight of the intracellular myc-tagged t-PDGF C as compared to the molecular weight of the extracellular myc-tagged t-PDGF C in this BT-549 model system suggests that t-PDGF C may be secreted as a

hetero-dimer with a larger protein, presumably endogenous full-length PDGF C, while

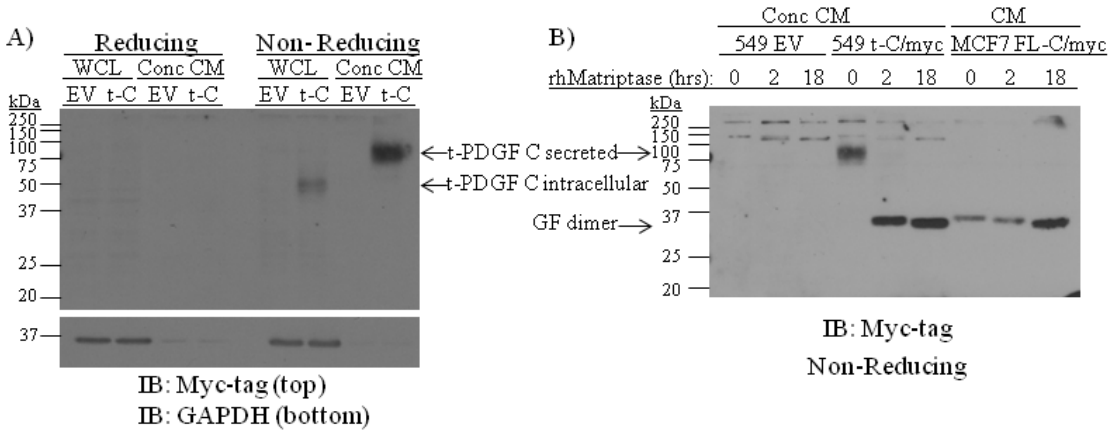


Figure 15. Truncated PDGF C is secreted into the conditioned media as a heterodimer with FL-PDGF C. Immunoblot (IB) analysis of **A)** Whole Cell Lysate (WCL), Concentrated Conditioned Media (Conc CM), or Conditioned Media (CM) from BT-549 cells stable transfected with empty vector (EV or 549 EV) or t-PDGF C/myc-tagged (t-C or 549 r-C/myc) and MCF-7 transfected with Full-length PDGF C (MCF7 FL-C/myc) conditioned media. Immunoblot analysis of **B)** conditioned media samples after incubation with recombinant human matriptase for the indicated times.

intracellular t-PDGF C is presumed to be a t-PDGF C homo-dimer. Importantly, the myc-tagged t-PDGF C secreted into the conditioned media can be proteolytically processed by the serine protease matriptase to produce the PDGF C growth factor domain (Figure 15B). The PDGF C splice variant being capable of being secreted is a novel finding. Zhao, et al concluded that the t-PDGF C protein (designated PDGF-Cb in their publication) is an intracellular protein [117]. Here we show that t-PDGF C/myc tagged protein can be secreted when expressed in a human breast cancer that also expresses endogenous full-length PDGF C. This suggests that t-PDGF C is capable of being an intraceullular, and extracellular protein. Furthermore, here we show that the serine protease matriptase is able to proteolytically process this secreted t-PDGF C/myc. Thus

presumably, secreted t-PDGF C could act as a reservoir of more active PDGF C growth factor domain and it may be capable of extracellular activation of PDGF receptors.

In order to further analyze the PDGF C isoforms detected, samples were run on a low percent acrylamide gel by SDS-PAGE for an extended period of time in order to better resolve the molecular weights of the various protein entities under non-reducing conditions (Figure 16). The full-length PDGF C homodimer can be detected by both the myc-tag antibody and our laboratory's custom PDGF C antibody (Figure 16A & B). Furthermore, the partially proteolytically processed hemi dimer (one CUB domain removed) can be seen in the full-length PDGF C transfected conditioned media. This protein entity is distinct from the newly discovered t-PDGF C heterodimer. Finally, the whole cell lysates show predominantly protein of the t-PDGF C homodimer molecular weight. Of note, the PDGF C antibody does not appear to detect the t-PDGF C protein species (Figure 16B). This may explain the previous difficulty in detecting t-PDGF C protein in transfected cells. The three dimensional conformation of t-PDGF C protein may be altered such that the PDGF C antibody is not able to access the epitope, resulting in poor detection via western blot. Thus, creation of a t-PDGF C custom antibody may be helpful in order to be able to better detect endogenous t-PDGF C in the future.

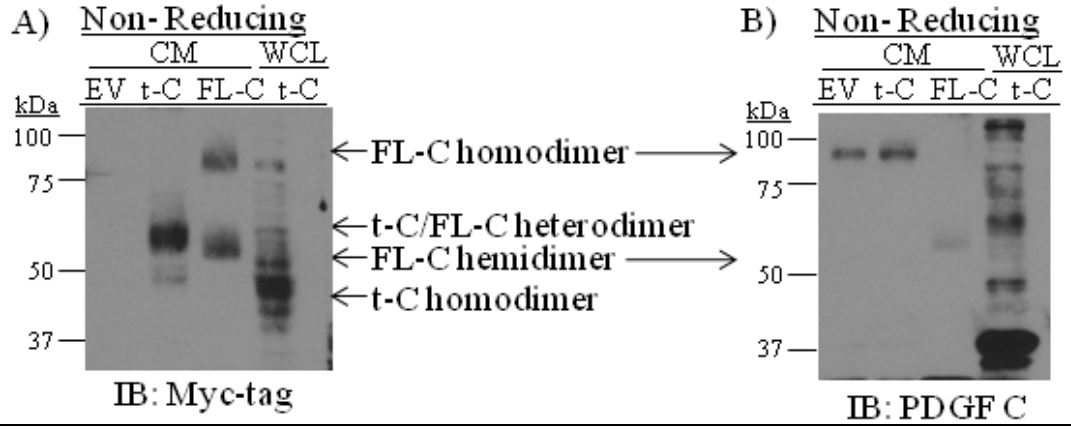


Figure 16. Heterodimerization of truncated PDGF C. Non-reducing immunoblot (IB) analysis of BT-549 empty vector transfected (EV), BT-549 t-PDGF C/myc transfected (t-C), and MCF-7 Full-length PDGF C/myc transfected (FL-C) CM = conditioned media, and WCL= whole cell lysates. **A)** Probed with myc-tag antibody, and **B)** probed with PDGF C antibody. BT-549 conditioned media is concentrated.

3.8 Mutating a putative nuclear localization sequences does not abolish nuclear localization of PDGF C.

A previous report demonstrating nuclear PDGF C suggested a putative nuclear localization sequence in the amino acids of PDGF C [120]. In order to investigate if this putative nuclear localization sequence is responsible for PDGF C nuclear localization, we mutated four basic amino acids of the putative nuclear localization sequence to alanine (R312A/K314A/R318A/K322A) and subsequently transiently transfected COS-1 cells (Figure 17). RT-PCR analysis shows that the cells express PDGF C after transient transfection (Figure 17A). Mutating the nuclear localization sequence does not affect the secretion of PDGF C into the conditioned media, nor does it appear to affect the proteolytic processing of PDGF C into the growth factor domain (Figure 17B). Furthermore, the mutated putative nuclear localization sequence does not appear to

inhibit the nuclear localization of PDGF C. In the nuclear fraction of the cells, not only is full-length PDGF C seen at ~50 kDa, but also the proteolytically processed PDGF C growth factor domain is seen at ~18 kDa (Figure 17C). Thus we conclude that the putative nuclear localization sequence published in the literature does not appear to be crucial for PDGF C nuclear localization.

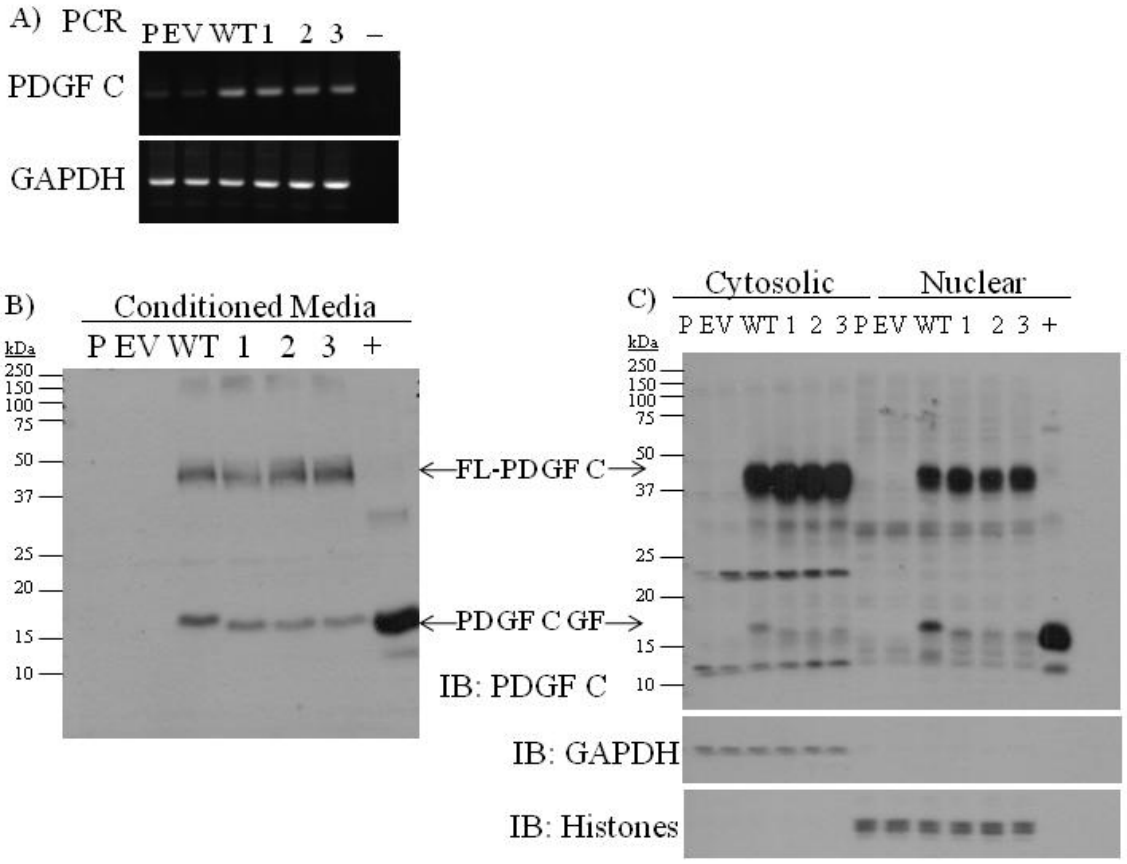


Figure 17. Mutation of putative nuclear localization sequences does not diminish nuclear localization of PDGF C. **A)** PCR, **B)** immunoblot analysis of conditioned media, and **C)** immunoblot analysis of cytosolic and nuclear fractions. COS-1 Parental (P), COS-1 transfected with Empty Vector (EV), Wild Type PDGF C/His expression vector (WT) or mutant PDGF C expression vectors (three independent expression plasmid preparations; colony 1, colony 2, or colony 3). – indicates dH_2O negative PCR control. + designates conditioned media from MCF-7 stably transfected with FL-PDGF C/His.

3.9 Amino acids in the hinge region are important in PDGF C subcellular localization.

In our laboratory's previous study, the cleavage site of PDGF C by serine proteases was identified as arginine and lysine amino acids in the hinge region [132]. A cleavage-deficient mutant was generated and expressed in a vaccinia virus expression system [132]. By mutation of those key amino acids in the hinge region of PDGF C (K225A/R231A/R234A, "cleavage-deficient PDGF C") our laboratory showed that those residues are important in the proteolytic processing and activation of PDGF C [132]. In order to investigate the importance of these crucial amino acids on the subcellular localization of PDGF C, MCF-7 cells which do not naturally express PDGF C were infected-transfected with either empty vector, wild type PDGF C, or the cleavage-deficient mutant PDGF C vaccinia vectors and expression was confirmed (Figure 18A). Wild type PDGF C is secreted and proteolytically processed in the conditioned media while the cleavage-deficient mutant PDGF C protein is not secreted (Figure 18B). Wild type PDGF C is predominantly found as full-length PDGF C (~50 kDa) in the cytosol while it is predominantly the proteolytically processed growth factor domain in the nuclear fraction (Figure 18C). The cleavage-deficient PDGF C appears to accumulate as full-length PDGF C (~50 kDa) in the cytosol, but the proteolytically processed growth factor domain is absent from the nuclear fraction (Figure 18). This suggest that amino acids in the hinge region are crucial for not only proper proteolytic processing by serine proteases as our previous study indicated [132], but they may also play an important role in the subcellular localization of PDGF C.

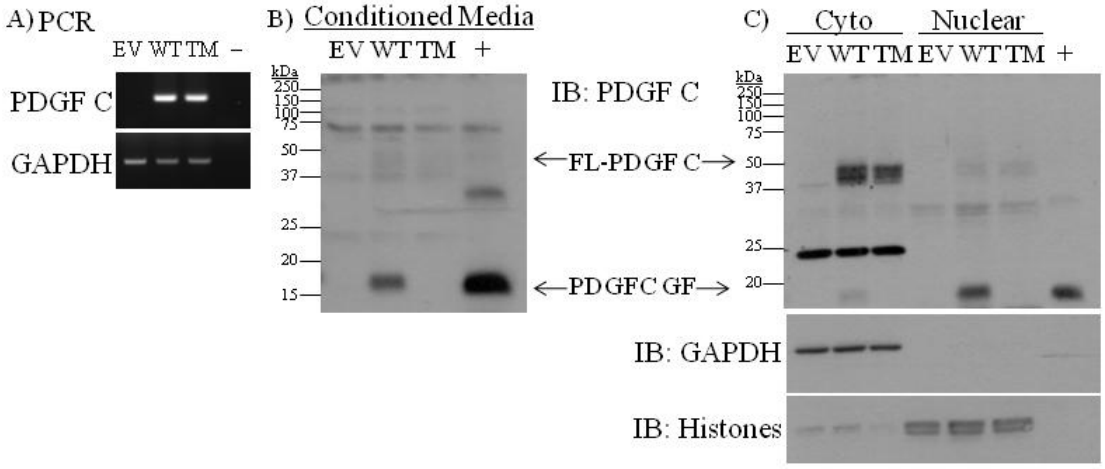


Figure 18. The serine protease cleavage site in the hinge region is critical for the subcellular localization of PDGF C. **A)** RT-PCR, immunoblot analysis in the reducing condition using **B)** conditioned media and **C)** cytosolic (cyto) or nuclear fractionation of MCF-7 cells engineered to express Empty Vector (EV), PDGF C wild type (WT), or PDGF C cleavage-deficient triple mutant (TM) using vaccinia virus expression system. – indicates dH_2O PCR negative control, + indicates conditioned media from MCF-7 stably transfected with FL-PDGF C/His-tag as a positive control.

3.10 Deletion of the putative PDGF C signal sequence abolishes secretion and proteolytic processing.

Since previous experiments indicate that both full-length PDGF C and proteolytically processed PDGF C growth factor domain can be found in the nuclear fraction of cells, we further investigated where the proteolytic processing takes place. To this end, a mutant PDGF C vector was constructed that lacks the putative signal sequence of PDGF C. Transient transfection of the deleted signal sequence PDGF C mutant vector in COS-1 cells shows RNA expression of the vectors (Figure 19A) and shows that secretion is abolished when the signal sequence is deleted (Figure 19B). Furthermore, it appears that there is no PDGF C growth factor domain detected in the whole cell lysate of

the deleted signal sequence PDGF C mutant (Figure 19B). This experiment was carried out with or without the presence of the irreversible serine protease inhibitor AEBSF in the event that proteolytic degradation might affect detection of mutant PDGF C (Figure 19 +/- AEBSF lanes). It does not appear that the mutant protein is being degraded. Furthermore, it does not appear that the mutant protein is capable of being proteolytically processed as no growth factor domain is detected (Figure 19B). Thus we conclude that the putative signal sequence is in fact important in the secretion of full-length PDGF C. Furthermore, secretion is likely necessary for proteolytic processing of PDGF C.

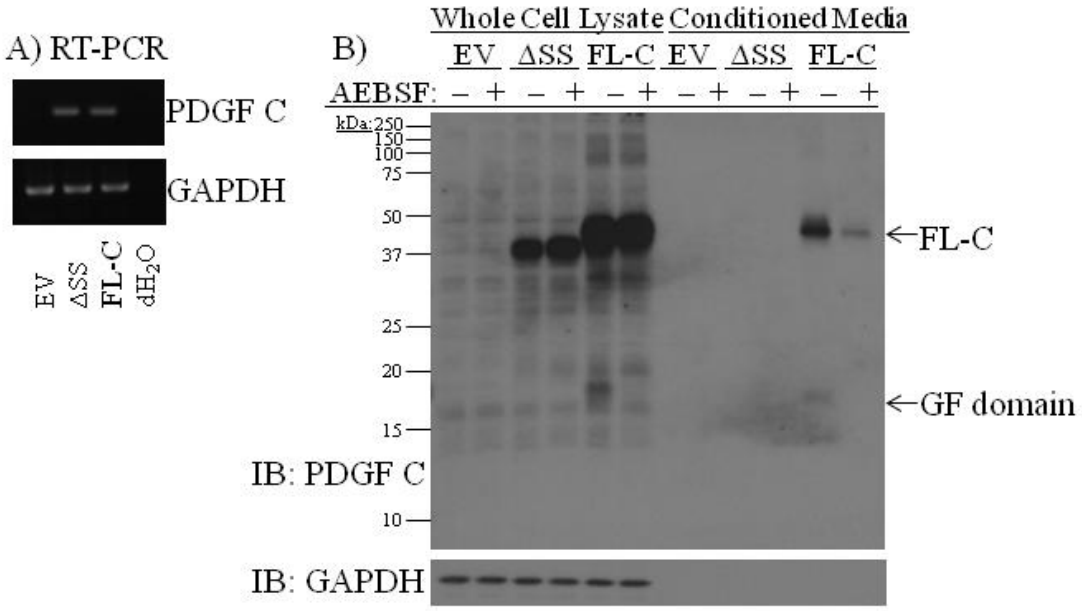


Figure 19. Deletion of the putative PDGF C signal sequence prevents PDGF C secretion. **A)** RT-PCR analysis and **B)** reducing immunoblot analysis (IB) in COS-1 cells that are transient transfected with empty vector (EV), PDGF C expression vector lacking the signal sequence (ΔSS) or wild type PDGF C (FL-C). dH_2O= PCR negative control. + indicates addition of AEBSF protease inhibitor, – indicates no AEBSF.

3.11 The functional significance of PDGF C isoform expression in breast cancer.

We hypothesized that higher PDGF C expression induces transformation and a more invasive phenotype. We further hypothesized that t-PDGF C enhances the oncogenic potential of FL-PDGF C in breast cancer cells. Overexpression of the splice variant, t-PDGF C, in BT-549 cells which naturally express predominantly full-length PDGF C, causes phenotypic transformation. In an *in vitro* invasion assay, the t-PDGF C/myc overexpressing BT-549 cells were more invasive than parental BT-549 cells or empty vector transfected BT-549 cells (Figure 20). Furthermore, *in vitro* soft

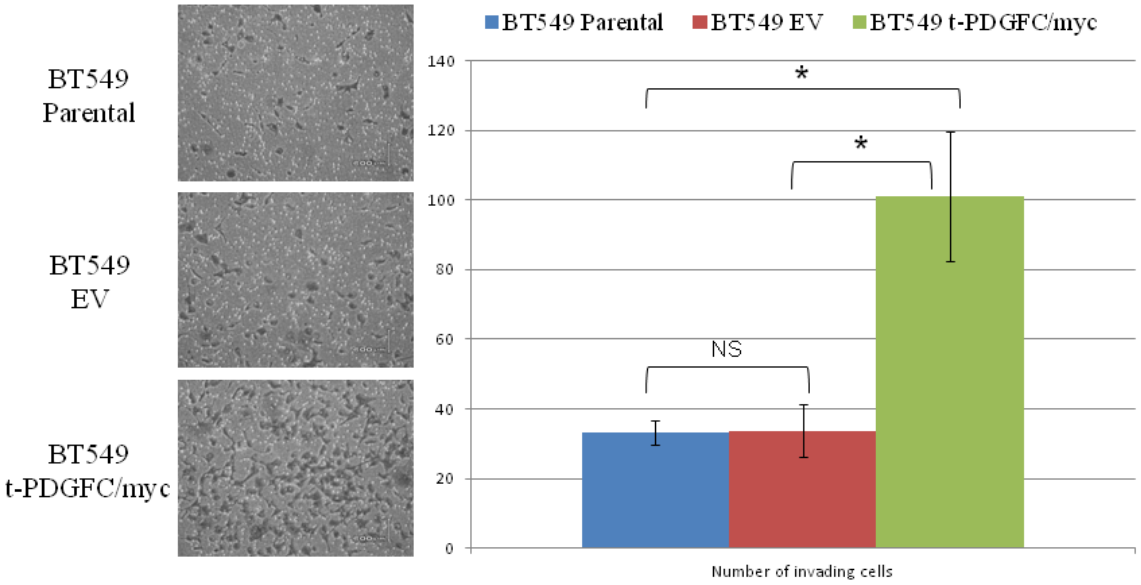


Figure 20. Truncated PDGF C overexpression increases the invasiveness of breast cancer cells. *In vitro* Matrigel™ invasion assay of parental and BT-549 stable transfected with empty vector (EV) or t-PDGF C/myc. Left, invading cells stained on filters. Right, bar graph indicates an average of the number of invading cells in triplicates. The bars indicate the standard deviation of the values. NS= not significant. *= significant.

agar colony analysis shows significantly more colonies in the t-PDGF C overexpressing

BT-549 cells when analyzing for colonies 75-300 µm in size (Figure 21). In order

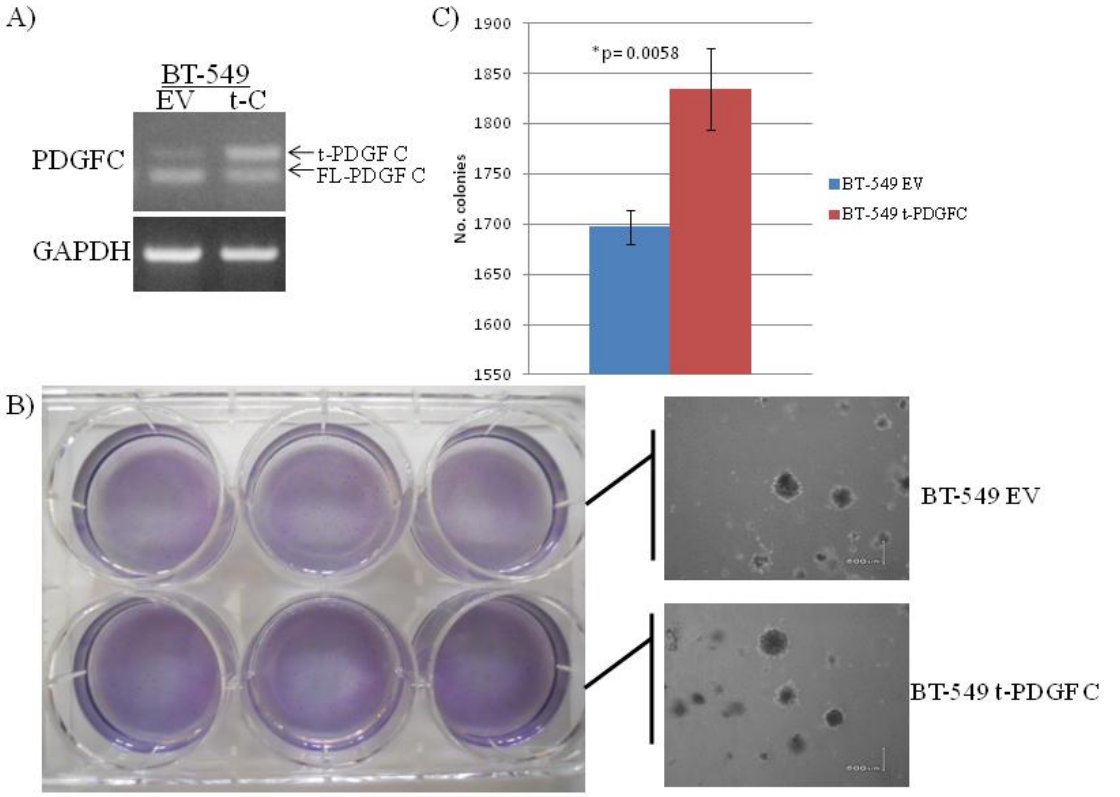


Figure 21. Truncated PDGF C overexpression increases soft agar colony formation of breast cancer cells. **A)** PCR of BT-549 Empty Vector stable transfected (EV) and BT549 t-PDGF C/myc stable transfected (t-C) cells, **B)** Soft agar colony formation, representative microscopic images of colonies were obtained at 40X magnification, **C)** Quantitation of the average number of colonies form three wells, with standard deviation depicted by error bars.

to analyze the overall effect of knockdown of PDGF C expression, soft agar colony formation was also analyzed in the MDA-MB-231 cell model (Figure 22). Knockdown of PDGF C expression significantly decreases soft agar colony formation (Figure 22B). In addition, knockdown of PDGF C expression significantly reduces cellular proliferation under both serum-containing and low-serum conditions, as compared to control transfected cells (Figure 22C & D).

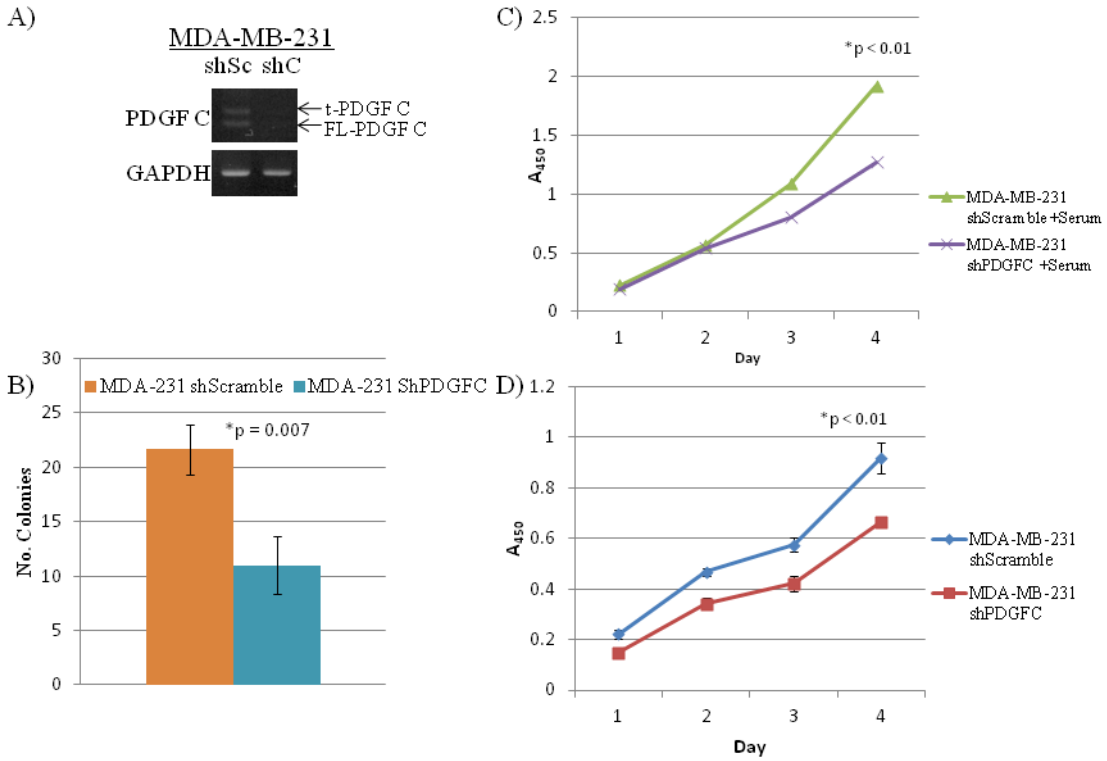


Figure 22. PDGF C knockdown decreases proliferation & soft agar colony formation. MDA-MB-231 scramble transfected (shSc, shScramble) and MDA-MB-231 PDGF C knockdown (shC, shPDGF C) **A)** PCR analysis of PDGF C expression, **B)** soft agar colony formation, average of colonies formed. Error bars indicate standard deviation of triplicate replicates. **C)** proliferation in under normal serum-containing conditions, and **D)** proliferation in low serum (1%) conditions.

3.12 PDGF C expression *in vivo*.

We hypothesized that PDGF C 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-PDGF C in human breast cancer, the stably transfected BT-549 cells were injected into the peri-mammary fat pad of mice and tumor growth was assessed (Figure 23A). Unfortunately the BT-549 human breast cancer cells did not grow well in this animal model and the small palpable tumors began to regress after approximately 14 days (Figure 23A). However, the slight growth that was detected in the period before tumor regression of the t-PDGF C/myc overexpressing cells as compared to control empty vector transfected suggests that the overexpression of t-PDGF C may be pro-oncogenic. Because the BT-549 cells were not able to form lasting tumors, injection of more cells, such as $5x10^6$ cells may be necessary for lasting xenograft tumor formation with BT-549 cells as one study in the literature has since successfully done [152], or a more tumorigenic breast cancer cell line should be selected to further investigate the *in vivo* effects of t-PDGF C overexpression on breast cancer tumor growth. Using the GOBO data (Figure 2) and our laboratory's previous semi-quantitative PCR analysis of PDGF C expression in cell lines (Figure 4, data courtesy of NJH) a cell line shown in the literature to be more tumorigenic in orthotopic animal models could be selected for future animal studies.

MDA-MB-231 cells successfully form tumors in mice [153]. Furthermore, increased tumor take is observed when MDA-MB-231 cells are injected in the mammary fat pad as opposed to subcutaneous tumor cell injection [153]. In an orthotopic mouse model, MDA-MB-231 human breast cancer cells were injected peri-mammary fat pad and tumor growth was assessed. Before injection, PDGF C expression was assessed by PCR (Figure 23B). MDA-MB-231 PDGF C knockdown cells (MDA-MB-231 shPDGF

C) grew smaller tumor volumes that the control transfected cells (MDA-MB-231 shScramble, Figure 23B).

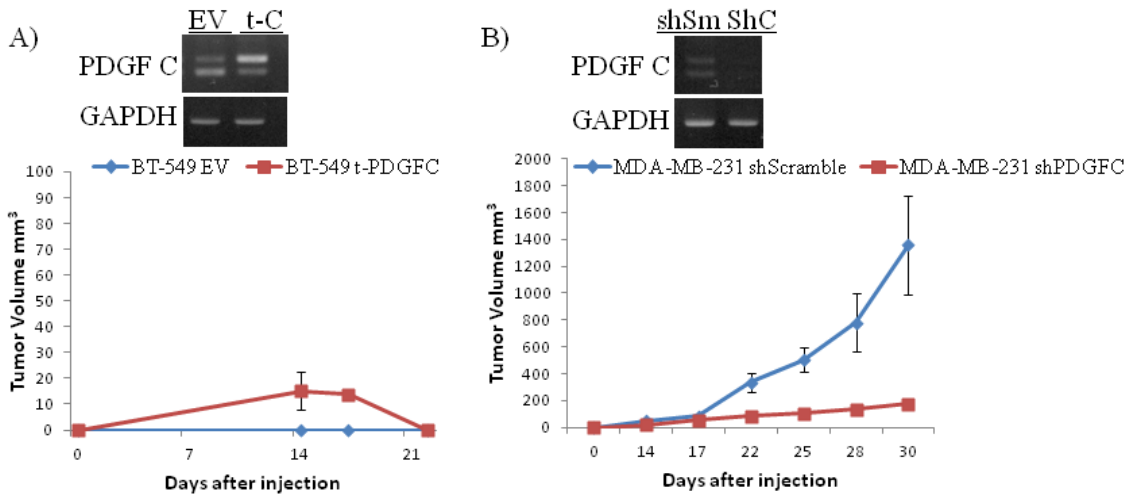


Figure 23. PDGF C knockdown decreases tumor growth *in vivo*. 1x10^6 cells in 50 µL of Matrigel™/media mix of BT-549 Empty Vector (EV), BT-549 t-PDGF C/myc-tagged (t-C), or MDA-MB-231 shScramble (ShSm), MDA-MD-231 shPDGF C (ShC) stable transfected cells were injected into the perimamary fat pad of NOD/SCID mice. **A)** RT-PCR analysis of PDGF C expression (top), and tumor growth of BT-549 Empty Vector or BT-549 t-PDGF C transfected (bottom) measured at the indicated time points. **B)** RT-PCR analysis of PDGF C expression (top), and tumor growth measured at the indicated time points (bottom) of MDA-MB-231 shScramble control transfected or MDA-MB-231 shPDGF C knockdown transfected cells. Data are mean ± SEM.

Metastases are a serious threat to cancer patients and particularly breast cancer patients are at risk of spread to vital organs [26, 27]. In order to investigate alteration of PDGF C expression on breast cancer metastases, MDA-MB-231 stably transfected breast cancer cells were used in a metastasis animal model experiment. After tail vein injection of MDA-MB-231 control transfected or PDGF C knockdown cells, lungs were harvested

and analyzed (Figure 24). There was a trend for smaller lung weight when PDGF C expression is knocked down (MDA-MB-231 shPDGF C, Figure 24B) however the difference in lung weights were not significantly different between control transfected and PDGF C knockdown (Figure 24B). Harvesting of the lungs at an earlier time point may be necessary in the future in order to have the ability to detect a significant difference in the metastatic ability of MDA-MB-231 PDGF C knockdown versus the control transfected cells.

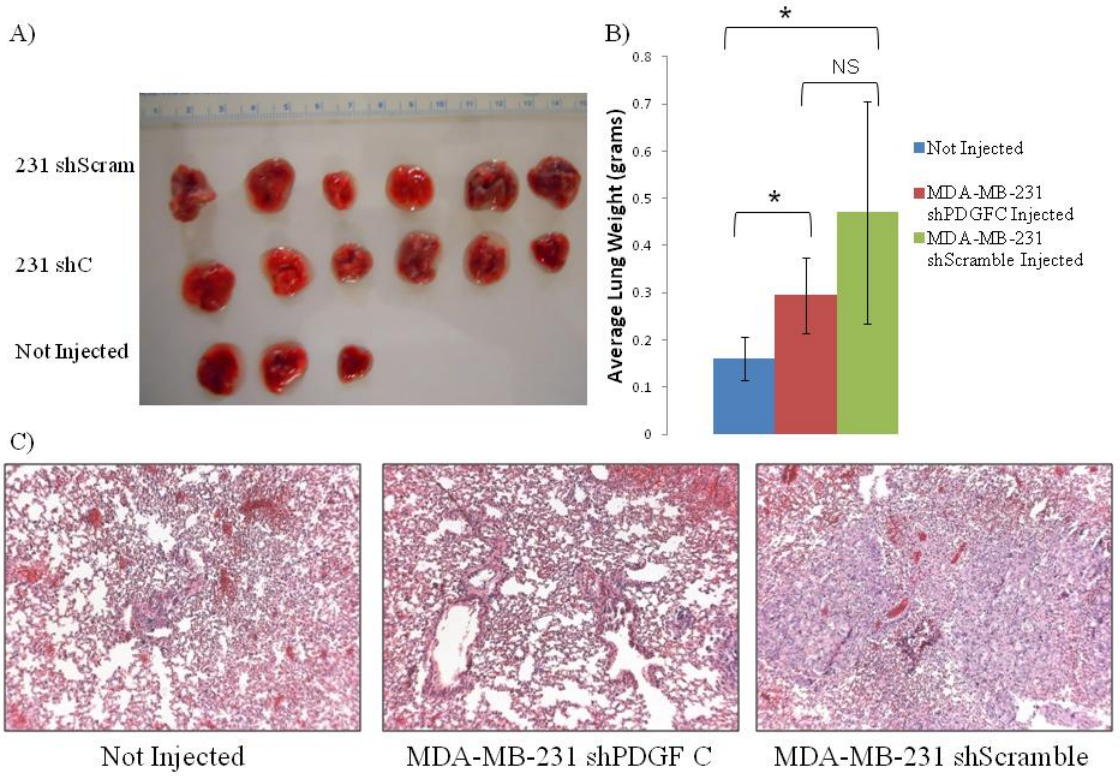


Figure 24. PDGF C knockdown may decrease lung metastases. MDA-MB-231 shScramble control (shScram) transfected cells or MDA-MB-231 shPDGF C knockdown (shC) transfected cells, $1x10^6$ cells in 100 µL of PBS injected into the trail vein of SCID mice. **A)** Lungs collected **B)** Lung weight (grams) **C)** H&E stain of embedded sections 100X magnification. Data are averages with error bars indicating standard deviation. NS= not significant. *= significant.

CHAPTER 4

Discussion & Future Directions

Summary of Findings

This study further supports PDGF C as the significant PDGF ligand in human breast cancer. In human breast cancer cell lines increased PDGF C expression is correlated with clinical and pathologic features that confer a poor prognosis, as demonstrated by higher PDGF C expression in triple-negative breast cancer cell lines (Figure 4, data courtesy of NJH, Figure 3, & Figure 6). Additionally, PDGF C expression was high in HER2/neu overexpressing cell lines (Figure 4, data courtesy of NJH, Figure 3, & Figure 6) and may be increased in the inflammatory breast cancer cell lines (Figure 4, data courtesy or NJH). This study's findings demonstrate that higher expression of PDGF C isoforms are associated with more aggressive characteristics in breast cancer cells *in vitro* and *in vivo*, including higher cellular proliferation, invasion, tumor growth, and possibly metastatic growth. Knockdown of PDGF C expression lead to a reduction in tumor growth, and cellular proliferation.

Furthermore, this study demonstrates the complexity of PDGF C in human breast cancer due to not only its recently discovered splice variant, and proteolytic processing of PDGF C, but also due to the varying subcellular localization of PDGF C isoforms in human breast cancer cells. According to the published literature, the novel PDGF C isoform, truncated PDGF C, generated from the PDGF C splice variant is described as an intracellular protein [117]. Initially this finding made sense as the t-PDGF C protein was

believed to lack a signal peptide due to the premature stop codon in the retained exon, exon 2' of t-PDGF C (Figure 5). However, of note, the current study demonstrates for the first time to our knowledge the ability of the PDGF C splice variant isoform to be secreted into the conditioned media of transfected cells (Figure 15A). Furthermore, this study demonstrates that matriptase, a serine protease shown previously by our laboratory to process full-length PDGF C [132], also has the ability to proteolytically process the newly discovered secreted truncated PDGF C isoform to the growth factor domain *in vitro* (Figure 15B). This significant finding suggest that the protein product of the PDGF C splice variant, truncated PDGF C, could serve as a repository for more active PDGF C growth factor domain in the extracellular milieu, thus making it a possible source of extracellular pro-oncogenic signaling in cancer.

In addition, to our knowledge, never before has PDGF C been localized to the nuclear fraction in human breast cancer cell lines. This study is the first to show the proteolytically-processed growth factor domain of PDGF C in the nucleus of human breast cancer cell lines that naturally express PDGF C (Figure 11). The only study known in the published literature demonstrates PDGF C as larger molecular weight PDGF C entities in the nuclear fraction human thyroid cells [120]. In the current study we show full-length PDGF C in the nuclear fraction of human breast cancer cells transfected to ectopically express PDGF C, however in T47D human breast cancer cells that express endogenous PDGF C, nuclear PDGF C appears to be in the proteolyically processed growth factor domain (Figure 11). In addition, here we show that amino acids in the hinge region of PDGF C appear to be crucial to subcellular localization, while a putative

nuclear localization sequence described in the literature did not appear as important (Figure 18 and Figure 17).

Heterodimerization of FL-PDGF C and t-PDGF C

The mechanism for secretion of truncated PDGF C is unknown and warrants further investigation. The premature stop codon in the second exon unique to the PDGF C splice variant encoding truncated PDGF C occurs after the signal sequence of PDGF C, previously presumed to lead to an alternative translational initiation. Lacking the signal peptide important for secretion of proteins, one can only speculate how truncated PDGF C may be secreted into the conditioned media of human breast cancer cell lines. One hypothesis possibly explaining the secretion of truncated PDGF C involves a pause in the translation of the truncated PDGF C mRNA while it is being translated. It has been shown that ribosomes translate mRNA at different speeds for various reasons including to allow for protein folding, and frameshifts (as reviewed in [154]). When the ribosome reaches the stop codon in the retained exon of PDGF C, it could be possible that the ribosome does not disengage translation upon reaching the stop codon, but pauses before it resumes translation at the alternative start codon with the nascent signal peptide still attached. This could allow the t-PDGF C protein to have a signal peptide and be secreted from the cell through the traditional secretory pathway.

Further studies are needed to understand how t-PDGF C is secreted from the cell as seen in transfected cells (Figure 15). In addition, further studies are needed to analyze endogenous t-PDGF C localization. With the development of better detection techniques,

endogenous t-PDGF C expression and localization should be assessed to answer these questions.

Technical Challenges

Initially the lack of an antibody specific to the truncated PDGF C isoform proved very challenging when attempting to detect t-PDGF C expression in human breast cancer cell lines. In the future specific antibodies developed for truncated PDGF C peptide versus full-length PDGF C, would help immensely in detection of the t-PDGF C and facilitate teasing apart the fucntions of the PDGF C isoforms from each other.

Another significant challenge to fully understanding the role of each PDGF C isoform in the field of PDGF research is a lack of expression data for each PDGF C mRNA variant. Microarray results prevalent in current research only show overall PDGF C mRNA expression and do not distinguish between the truncated PDGF C splice variant expression from the full-length PDGF C expression level.

Significance & Future Directions

PDGF C has been shown to play an important role in normal development while its aberrant expression has been implicated in many pathological conditions including several types of cancer. Evidence of the importance of PDGF signaling in breast cancer development and metastases has been accumulating [75-78, 110-113]. Here we provide further evidence for the importance of PDGF C signaling in human breast cancer. Our laboratory's previous findings found no correlation of PDGF A, PDGF B, or PDGF D

expression with the clinical or pathologic characteristics of breast cancer cell lines (Figure 4, data courtesy of NJH and manuscript in preparation). Furthermore, recent findings with collaborators found little to no expression of PDGF D by immunohistochemical staining of invasive breast cancer tissue samples (unpublished observation). However, PDGF C immunohistochemical staining was found to be significantly correlated with poor prognostic clinical and pathological characteristics such as lymph node metastasis, HER2/neu overexpressing tumors, and high Ki-67 staining, a marker for proliferation (manuscript in preparation).

GOBO data further demonstrates that PDGF C expression is significantly different amongst breast cancer cell lines grouped by clinical and pathological features, with the highest PDGF C expression in triple-negative breast cancer cell lines (Figure 3). This is of significance as the treatment of triple-negative breast cancer in the clinic has been challenging. Expression of the hormone receptors, estrogen and progesterone, or expression of HER2/neu oncogene allows for more treatment options such as the use of hormone targeting therapies or treatment that targeting the HER2/neu receptor. However triple-negative breast cancers do not express any of those receptors, and thus lack targeted treatment options. A better understanding of triple-negative breast cancer biology is needed in order to develop drugs that can target that subset of breast cancers.

Furthermore, a significant problem in the treatment of all cancers is the development of treatment resistant cancer. Additionally, despite the appearance of being cured of a cancer, there is always the possibility of cancer recurrence months or years

later in any cancer patient. Cancer stem cells are believed to be a cause of tumor recurrence, treatment resistance, and metastases (as reviewed in [155]). These cancer stem cells are believed to be a subpopulation of cells within cancerous tumors that are resistant to treatments and thus remain after "curative" treatment, then capable of driving the recurrence of the cancer [155]. In breast cancer, cancer stem cells have been determined to express $CD44^{+}/CD24^{-/low}$ cell surface proteins [114]. Thus in order to be able to permanently cure cancer patients it is important to understand the differences in these therapy resistant subpopulations of tumors to specifically eradicate those cancer stem cells along with the bulk of remaining tumor cells [155]. Recent studies provide mounting evidence for the importance of PDGF signaling, including specifically PDGF C, in therapy resistant cancer cells and in cancer stem cells [156]. In an effort to identify ways to target breast cancer stem cells, one study used proteome analysis to demonstrate an increase in PDGF signaling in a population of HMLE cells that had undergone EMT (epithelial-mesenchymal transition) by forced expression of twist, while the non-cancer stem cell epithelial-like population of cells demonstrated higher EGFR signaling [156]. Furthermore, utilizing quantitative PCR this study also showed that Basal B subtype breast cancer cell lines expressed higher RNA levels of the PDGF receptors, PDGFRA (encoding PDGFRα) and PDGFRB (encoding PDGFRβ), along with increased RNA expression of the ligand PDGF C, thus the authors suggest that PDGF C autocrine signaling may play a role in EMT [156]. Increased expression of PDGF C in Basal B breast cancer cell lines demonstrated in that study is further corroborated by the GOBO analysis of PDGF C expression (Figure 2A).

Recently several studies have shown that therapy resistant tumors express high levels of PDGF C suggesting as a possible mechanism of escape. One such study examining tumors resistant to anti-VEGF treatment, found that there was an increase in PDGF C expression to compensate for a loss of VEGF signaling [157]. In another study of therapy resistant tumors, PDGF C was found to be differentially expressed in squamous cell carcinoma cell lines resistant to cisplatin chemotherapy as compared to derivative cell lines that were sensitive to cisplatin [158]. Furthermore, this study also showed that PDGF C expression was one of five genes that predicted patient outcomes after chemotherapy treatment, suggesting its importance in drug resistance mechanisms [158].

In another study of chemotherapy resistant breast cancer cells, doxorubicin-selected MCF-7 breast cancer cells, showed a 39.3-fold increase in PDGF C expression by microarray analysis (Figure 25A). The doxorubicin-resistant MCF-7 cells were found to be both more motile and more invasive in an invasion assay as compared to the MCF-7 parental cells [4]. This study further showed that MCF-7 doxorubicin-resistant cells had significantly more breast cancer stem cells ($CD44^{+}/CD24^{-}$ cells) than the parental MCF-7 cells (more than 30% in doxorubicin-resistant cells versus less than 0.05% in the parental) [4]. Furthermore, this study also showed that that the doxorubicin-resistant MCF-7 cells had a 126.0-fold decrease in estrogen receptor 1 (ESR1) expression as

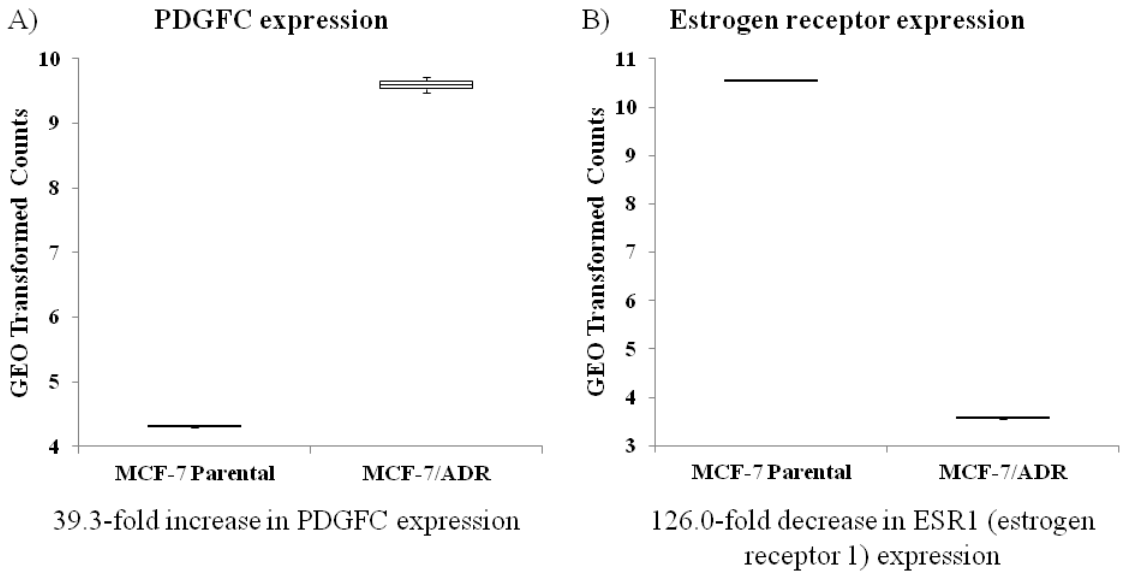


Figure 25. Drug resistant breast cancer cells show increased PDGF C expression and decreased estrogen receptor expression. Box-whisker plots of GEO Data for **A)** PDGF C expression, and **B)** Estrogen Receptor Expression. Graph generated using GEO data [3] from GEO Data Set 4084 [4].

compared to MCF-7 parental cells (Figure 25B), and were capable of forming tumors in mice without estrogen supplementation [4]. Normally MCF-7 breast cancer cells require estrogen supplementation for *in vivo* tumor growth [159]. Thus confirming that the drug resistant cells had also become estrogen independent while also having increased PDGF C expression [4]. Thus doxorubicin-resistance was associated with an increase in breast cancer stem cell population, an increase in PDGF C expression and it was also associated with hormone (estrogen) independent disease [4].

In another study, siRNA-mediated silencing of estrogen receptor expression in the breast cancer cell line MCF-7 caused an approximately 50-200 fold increase in the PDGF C expression via microarray analysis, again supporting a possible link between hormone receptor expression and PDGF C expression [160, 161]. These studies bolster the case

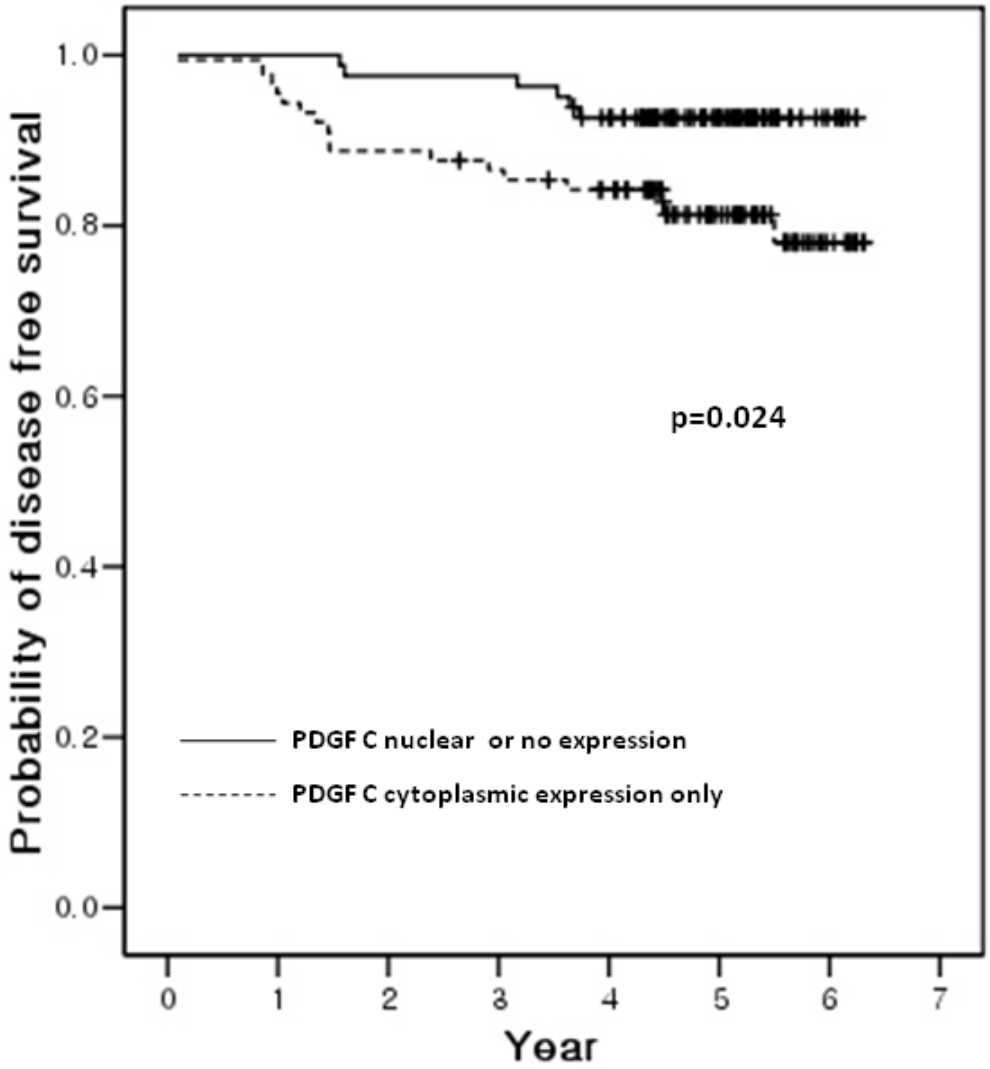
for PDGF C playing an important role in therapy resistant breast cancer in addition to suggesting a possible interaction between PDGF C expression and estrogen receptor expression in breast cancer. The GOBO analysis findings above further corroborate this inverse relationship between PDGF C expression and estrogen receptor expression, as the hormone receptor positive breast cancer cell lines had the lowest expression levels of PDGF C (Figure 3) as do our laboratory's findings that hormone receptor positive breast cancer cell lines such as MCF-7 have little to no PDGF C expression while triple-negative (i.e. hormone receptor negative, by definition) breast cancer cells lines such as MDA-MB-231 tend to have much higher PDGF C expression (Figure 6).

Finally, nuclear localization promotes PDGF C to the elite status of growth factors with nuclear functions. Other growth factors have recently been discovered to have nuclear localization and nuclear functions. Heparin-binding growth factor-1, also known as fibroblast growth factor-1 (FGF-1) is a mitogen for mesenchymal cells that was found to localized to the nucleus [162]. Lack of its nuclear localization sequences abolished mitogenic activity of the growth factor suggesting that nuclear translocation is necessary for its activity [162, 163].

PDGF C being localized to the nucleus greatly complicates the possible roles for such multi-tasking growth factors in the development and progression of cancer, as their effects are not necessarily exclusively extracellular actions on their respective receptors more easily capable of being blocked by currently available therapeutics. Instead their

effects can then also be due to a multitude of intracellular roles that may prove more difficult to modulate with drug targeting.

Nuclear PDGF C found in this study raises multiple questions. First, do one or both forms of PDGF C, the full-length unprocessed and the processed growth factor domain, have a biological function in the nucleus? Additionally, is the function of nuclear PDGF C that of a pro-oncogenic or anti-oncogenic role? Nuclear PDGF C seen in the chromatin bound nuclear fraction of breast cancer cells suggests that it may play a role, either directly or indirectly in gene transcription. Further studies such as chromatin immunoprecipitation (ChIP) could potentially identify genes that PDGF C may interact with in the nucleus. However because of a current lack of a PDGF C antibody suitable for immunoprecipitation, PDGF C expressed with tags such as myc-tag may need to be utilized for ChIP studies until more suitable PDGF C antibodies are available. A collaborative study involving our laboratory found that a 171 invasive breast cancer sample tissue microarray analyzed by immunohistochemical staining for PDGF C and interpreted by a breast pathologist blinded to patient outcomes, shows that absence of PDGF C staining or exclusively nuclear staining confers a significantly better disease-free survival in breast cancer patients than cytoplasmic PDGF C staining (manuscript in preparation, Figure 26). Further studies are needed to tease out the pro-oncogenic versus anti-oncogenic signaling of PDGF C isoforms based on its subcellular localization.



Data courtesy of collaborator Dr. So Yeon Park (Seoul, Korea).

Figure 26. PDGF C expression and disease-free survival. Kaplan-Meier survival curve depicting disease-free survival measured from date of surgery to date of loco-regional recurrence, distant metastasis, or death. Statistical analysis with log-rank test, two sided P value. Data courtesy of Dr. So Yeon Park.

Conclusion

This study has shown that increasing expression of PDGF C isoforms leads to more aggressive characteristics in breast cancer cells *in vitro* and *in vivo*. Furthermore,

this study reconfirms the importance of serine proteases in the processing of PDGF C isoforms, and identifies a potent, irreversible inhibitor of PDGF C processing in cells *in vitro*. Additionally this study identifies the amino acids in the hinge region of PDGF C as crucial to subcellular localization. This is the first time to our knowledge that the splice variant, t-PDGF C, has been shown to be secreted from transfected cells and presumably as a heterodimer with full-length PDGF C. In addition, this study suggests that the homodimer of t-PDGF C is an intracellular protein. This is of importance because of its implications on paracrine signaling in cancer. As demonstrated in the proposed working model in Figure 27, t-PDGF C can be secreted from the cell, and it can be proteolytically processed to the growth factor domain dimer. Thus the secreted t-PDGF C may serve as an important reservoir for PDGF C signaling. When processed to the active growth factor domain in the extracellular milieu, it may be able to act on surrounding cells similar to full-length PDGF C (Figure 27). Finally this study confirms a recent discovery in the published literature, that PDGF C is also a nuclear protein (Figure 27). PDGFs were primarily thought of extracellular signaling molecules and these recent discoveries of PDGF C in the nuclear fraction of cells hints at a multiplicity of functions intracellular and extracellular.

In order to fully the understand the nuances of PDGF C's effects in disease it is important for the research community to recognize the PDGF C mRNA splice variant and its protein, t-PDGF C, and not simply lump the expression changes of both full-length PDGF C and t-PDGF C into one. It is possible that important alterations in the expression of one or the other PDGF C mRNA splice variant, or important alterations in

the ratio of the two are lost when treated as one in data analysis of PDGF C expression. Additionally, further studies are needed to understand the subcellular localization of both PDGF C isoforms, and the effects of such on the balance between pro-oncogenic and anti-oncogenic signaling. This has important clinical implications as future drug targeting of aberrant PDGF C signaling in disease may need to take into account the intracellular and extracellular roles of both PDGF C isoforms.

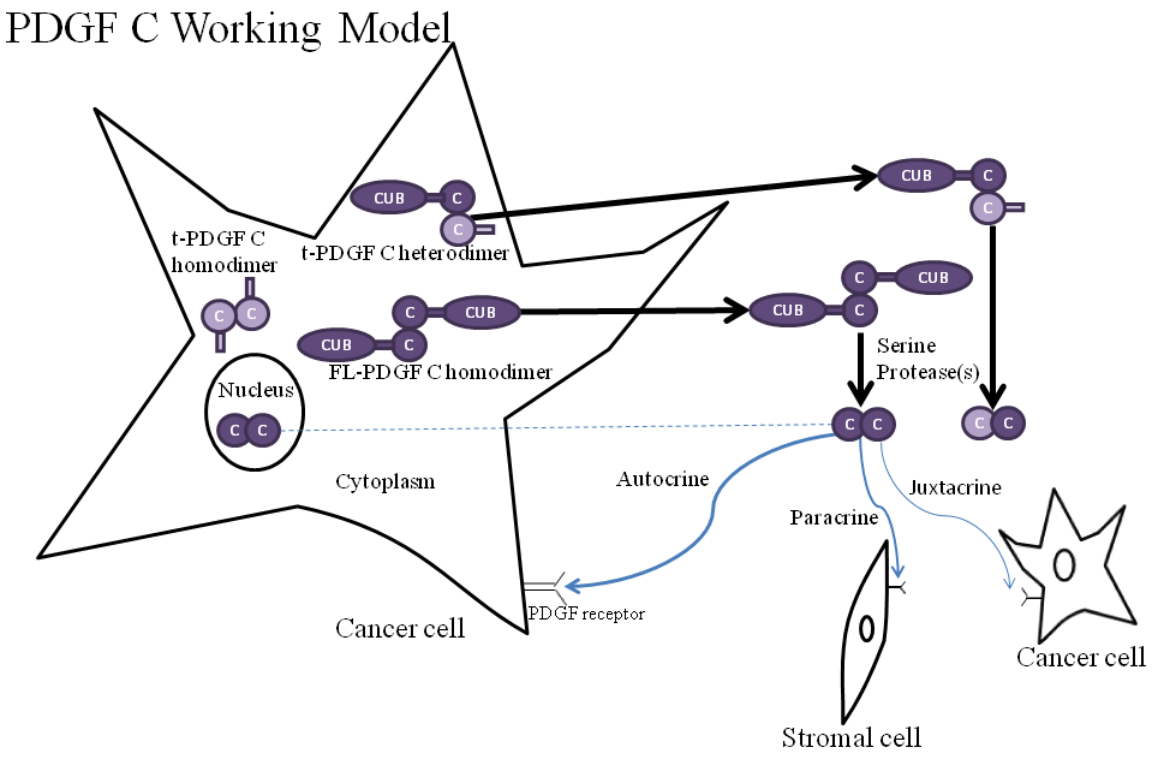


Figure 27. A working model for PDGF C's role in breast cancer.

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ABSTRACT

THE ROLE OF PDGF C AND ITS SPLICE VARIANT IN BREAST CANCER

by

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The PDGF family consists of four members; while PDGF A and B are secreted as active dimers, PDGF C and D are secreted as latent dimers that undergo serine protease-mediated extracellular proteolytic activation. Gene expression analysis of breast cancer cell lines showed that PDGF C expression is associated with Basal B subtype breast cancer cells which have cancer stem cell-like characteristics. Furthermore, PDGF C expression is associated with triple-negative (estrogen receptor-, progesterone receptor- and HER2/neu-negative) breast cancer cells, a challenging type of breast cancer to treat. During the course of our study, we discovered a splice variant of PDGF C encoding the truncated PDGF C protein (t-PDGF C). Specific aims of this dissertation are to determine the role of full-length PDGF C (FL-PDGF C) and t-PDGF C in breast cancer and to characterize their subcellular localizations. This study found that although t-PDGF C presumably lacks the signal peptide, it is secreted as a heterodimer with FL-PDGF C which can undergo extracellular proteolytic activation. Furthermore, PDGF C was found in the nuclear fraction of breast cancer cells, suggesting an uncharacterized

function in breast cancer. A putative nuclear localization sequence in PDGF C showed little effect on its nuclear localization as determined by the point mutagenesis assay. Interestingly, we found that the serine protease cleavage site in the hinge region plays a critical role for both extracellular proteolytic processing and nuclear accumulation of PDGF C, suggesting that the biochemical processing and the subcellular localization are co-regulated. For the functional study, we established *in vitro* cell models engineered to overexpress PDGF C isoforms or inhibit its expression. This study found that PDGF C expression correlates with cell proliferation, invasive phenotype and anchorage-independent growth *in vitro*. Importantly, t-PDGF C expression further promoted PDGF C-induced phenotypic transformation. PDGF C downregulation decreased tumor growth and metastatic potential *in vivo*. Taken together, this study identified PDGF C and its splice variant as key signaling molecules in breast cancer. In addition, once thought of as primarily an extracellular signaling molecule, nuclear localization marks a potentially important paradigm shift in PDGF C biology.

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