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An oncogenic activity of PDGF-C and its splice variant in human breast cancer

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Abstract

Despite strong evidence for the involvement of PDGF signaling in breast cancer, little is known about the PDGF ligand responsible for PDGFR activation during breast cancer progression. Here, we found PDGF-C to be highly expressed in breast carcinoma cell lines. Immunohistochemical analysis of invasive breast cancer revealed an association between increased PDGF-C expression and lymph node metastases, Ki-67 proliferation index, and poor disease-free survival. We also identified a PDGF-C splice variant encoding truncated PDGF-C (t-PDGF-C) isoform lacking the signal peptide and the N-terminal CUB domain. While t-PDGF C homodimer is retained intracellularly, it can be secreted as a heterodimer with full-length PDGF-C (FL-PDGF-C). PDGF-C downregulation reduced anchorage-independent growth and matrigel invasion of MDA-MB-231 cells. Conversely, ectopic expression of t-PDGF-C enhanced phenotypic transformation and invasion in BT-549 cells expressing endogenous FL-PDGF-C. The present study provides new insights into the functional significance of PDGF-C and its splice variant in human breast cancer.

Keywords

Breast cancer; growth factors: structure and function; prognostic marker; oncogenic activity; tumor cell invasion

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

INTRODUCTION

Platelet-derived growth factor (PDGF) is a potent mitogen and a regulator of diverse cellular processes including chemotaxis, cell survival, and transformation (1,2). The discovery of PDGF-B chain (c-sis) as a cellular counterpart of the oncogene product ($p28^{v\text{-}sis}$) of the simian sarcoma virus (SSV) established a causative role for growth factors in human cancers more than three decades ago (3,4). Since then, studies have demonstrated a critical role of PDGF signaling in tumors of mesenchymal origin. In addition, increasing evidence suggests that PDGF also induces oncogenic signaling during cancer progression of epithelial origin via autocrine and paracrine mechanisms. Immunohistochemical analysis revealed increased expression/activation of PDGF receptor (PDGFR) in human breast tumor tissues (5–9). Moreover, an immunohistochemical study of 181 formalin-fixed, paraffin-embedded invasive ductal breast carcinomas demonstrated an association between α-PDGFR staining and lymph node metastasis, HER2/neu expression ($p=0.0265$), and Bcl-2 expression (10). In experimental models of breast carcinogenesis, a critical role of PDGF autocrine signaling is suggested for the regulation of oncogenic signaling networks. For instance, PDGF signaling is activated in MMTV-Neu/TGF-β transgenic mice as well as in Ras-mediated mammary tumors. Inhibition of PDGF/PDGFR autocrine signaling in these carcinoma cells with a PDGF-neutralizing antibody or a dominant negative PDGFR was sufficient to block TGF-β-induced epithelial-mesenchymal transition (EMT) or to reduce the metastatic potential in an *in vivo* metastasis assay (9). Despite strong evidence for the involvement of PDGF signaling in breast cancer, little research has been conducted to identify a PDGF ligand responsible for PDGFR activation in breast cancer.

The PDGF family consists of four members, PDGF-A, -B, -C, and -D. While the classical PDGF ligands A and B are secreted as active homodimers or heterodimer, the newly discovered PDGF-C and -D contain an N-terminal CUB domain and are secreted as latent homodimers (11,12). PDGF-A readily binds only α-PDGFR, whereas PDGF-B binds both α- and β-PDGFR, as reviewed in (2). Studies from our laboratory and others showed that the serine protease-mediated extracellular proteolytic removal of the N-terminal CUB domain is required for the C-terminal growth factor domain of PDGF-C and -D to activate their cognate receptor α- and β-PDGFR, respectively (11–16). Interestingly, unlike PDGF-A homodimer, PDGF-C dimer was shown to induce αβ-PDGFR heterodimerization and subsequent signal activation (17).

In the present study, we aim to identify a PDGF ligand whose expression is increased in human breast carcinoma cells, establish its causative role, and examine its association with clinical parameters. By screening a panel of breast epithelial cell lines *in vitro*, we found increased PDGF-C mRNA expression in breast carcinoma cell lines that represent malignant potential of breast cancer. More interestingly, we identified a PDGF-C splice variant encoding truncated PDGF-C protein lacking the CUB domain, referred to as t-PDGF-C hereafter. We show that t-PDGF-C enhances secretion of full-length PDGF-C (FL-PDGF-C) and promotes the oncogenic signaling of PDGF-C. Importantly, tissue microarray analysis of 171 patients with invasive breast cancer shows that increased cytoplasmic PDGF-C expression correlates with lymph node metastases, increased Ki-67 proliferation index, and poor disease-free survival (DFS).

MATERIALS AND METHODS

GOBO gene analysis

Using the database available at http://co.bmc.lu.se/gobo/, we performed Gene Based Outcome for Breast Cancer Online (GOBO) Gene Set Expression (GSE) analysis of PDGF family members in three subgroups (basal A, basal B, and luminal) of breast cancer, as defined in the literature (18).

Cell lines

MCF-7, MDA-MB-231, COS-1, T47D and BT-549 cell lines were purchased from ATCC (Manassas, VA). MCF10A cells were maintained as previously described (19). MCF-7, T47D, and BT-549 cells were maintained in DMEM/F12 medium with 10% fetal bovine serum (FBS), while MDA-MB-231 and COS-1 cells were cultured in DMEM high glucose media (Invitrogen, Carlsbad, CA) with 10% FBS.

RT-PCR analysis

cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA), as suggested by the manufacturer, followed by PCR with the Promega GoTaq Flexi DNA polymerase kit (Promega, Madison, WI). PCR cycling parameters were 2 minutes at 95°C, then 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 2.5 minutes at 72°C, followed by 5 minutes at 72°C and subsequent holding at 4°C. Primer sequences for the analysis of PDGF mRNA expression are as follows: PDGF-A (forward (F): 5'-ATACCTCGCCCATGTTCTGC-3'; reverse (R): 5'-CTTCTCGGGCACATGCTTA-3'), PDGF-B (F: 5'-CATTCCCGAGGAGCTTTATG-3'; R: 5'-CTCAGCAATGGTCAGGGAAC-3'), PDGF-C (F1: 5'-GCGGAATCCAACCTGAGTAG-3'; F2: 5'-AGAAGTTGAGGAACCCAGTG-3'; F3: 5'-TTCTTGGCAAGGCTTTTGTT-3'; F4: 5'-ATGTCAATGTGTCCCAAGCA-3'; R1: 5'-GGATAAGTATGAGGAAACCT −3'; R2: 5'-GCAGAACCCTGGTTCAGAAG-3'; R3: 5'-AACAAAAGCCTTGCCAAGAA-3'; R4: 5'-TGCTTGGGACACATTGACAT-3'; R5: 5'-AGAGCTGCTGGTGGTGATG-3'), PDGF-D (F: 5'-GAACAGCTACCCCAGGAACC-3'; R: 5'-CTTGTGTCCACACCATCGTC-3'), α-PDGFR (F: 5'-GAACTCACGGTGGCTGCTG-3'; R: 5'-ACTTTCATGACAGGTTGGG-3'), β-PDGFR (F: 5'-TTTTGCACCCACAATGACTC-3'; R: 5'-CCAATGGTGGTTTTGCAGAT-3'), cytokeratin (CK) 18 (F: 5'-ATCTTGGTGATGCCTTGGAC-3'; R: 5'-CCTGCTTCTGCTGGCTTAAT-3'), and β-actin (F: 5'-ACTCTTCCAGCCTTCCTTC-3'; R: 5'-ATCTCCTTCTGCATCCTGTC-3').

Real time RT-PCR analysis was performed using SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA, USA) and the Eppendorf Mastercycler® ep *Realplex*^{2} machine according to the manufacturer's protocol. Relative values of gene expression were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct=(\Delta Ct_{\text{target gene}} - \Delta Ct_{GAPDH})_{sample} - (\Delta Ct_{\text{target gene}} - \Delta Ct_{GAPDH})_{control}$. The fold change in relative expression was then determined by calculating $2^{-\Delta\Delta Ct}$. Samples were run in triplicate, and negative controls were included with no cDNA template for each primer pair in triplicate. The coefficients of variation of the technical replicates of all samples were less than 1.5%.

Primer sequences used for the real time PCR analysis are as follows: t-PDGF-C specific primers (F: 5'-TCCAAGCATCTGGACTGGCATAGA-3'; R: 5'-AGTATGAGGAAACCTTGGGCTGTGA-3'), total PDGF-C (F: 5'-AACGCTGTGGTGGGAACTGTGC-3'; R: 5'-TGCAATCCCCTGACACCGGTCT-3'), and GAPDH (F: 5'-ATCACCATCTTCCAGGAGCGA-3'; R: 5'-GCCAGTGAGCTTCCCGTTCA-3').

Cloning and sequencing of RT-PCR product containing an additional PDGF-C exon

PDGF-C transcripts were amplified by RT-PCR using PDGF-C-specific primers, (F: 5'-TCCAGCAACAAGGAACAGAA-3' R: 5'-GGGTCTTCAAGCCCAAATCT-3'). PCR products of PDGF-C were gel extracted using QIAquick Gel Extraction kit (Qiagen, Hilden, Germany) and cloned into the pCR4-TOPO cloning vector (Invitrogen, Carlsbad, CA). Inserts representing PDGF-C exons were sequenced using the M13 primers (ELIM Biopharmaceuticals).

Construction of a t-PDGF-C expression vector

To generate a t-PDGF-C expression plasmid, the 61 bp was inserted into wild type PDGF-C in pCR2.1 vector containing a myc tag at the C-terminus of PDGF-C (pCR2.1-PDGFC-myc) by using the QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) and insertion primers (F: 5'-GCAACAAGGAACAGAACGGTAGAACTATATCCAAGCATCT GGACTGGCATAGAAAAGAGGAGAAAGAACATTTAAAAGGA GTACAAGATCCTCAGC-3'; R: 5'-GCTGAGGATCTTGTACTCCTTTTAAATGTTC TTTCTCCTCTTTTCTATGCCAGTCCAGATGCTTGGATATAGTTCCTACCGTTCTGTT CCTTGTTGC-3'). After cloning, the sequences of t-PDGF-C were confirmed by DNA sequencing. The inserts in pCR2.1-PDGF-C-myc and pCR2.1-t-PDGF-C-myc plasmids were purified after restriction enzyme digestion with HindIII and BamH1, followed by subcloning into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA).

Generation of custom antibody against the PDGF-C growth factor domain

An antibody was raised in rabbits against PDGF-C using a synthetic peptide (N'-CGRKSRVVDLNLLTEEVRLYSC-C') representing amino acids 229–250 in the PDGF-C growth factor domain. The resultant antibody was affinity purified (Zymed Biomedical, So. San Francisco, CA) and is referred to as anti-PDGF-C/GFD Ab.

His-SpinTrap pull-down assay

COS-1 cells were transiently transfected with PDGF-C-His and/or t-PDGF-C-myc expression vectors using FuGENE 6 from Roche (Basel, Schweiz) and cultured for 48 hours. Conditioned medium (CM) was collected, and the cells were lysed in 25 mM Tris-HCl, pH 7.5, 1% NP40, 100 mM NaCl, and protease inhibitor cocktail. Imidazole was added to concentrated CM and lysates at a final concentration of 20 mM. Cell lysate or concentrated CM was loaded on His-SpinTrap column (GE Life Science, Piscataway, NJ), washed with the binding buffer (20mM sodium phosphate, 500 mM NaCl, 20 mM imidazole, pH 7.4),

and eluted with elution buffer (20mM sodium phosphate, 500 mM NaCl, 500 mM of imidazole), followed by immunoblot analysis.

Proteolytic cleavage of purified PDGF C proteins by recombinant human matriptase

Microcon® centrifugal filter devices were used to exchange the conditioned media containing PDGF C proteins with recombinant human Matriptase (rhMatriptase) buffer (50 mM Tris, 50 mM NaCl, 0.01% (v/v) Tween® 20, pH 9.0). Recombinant human matriptase/ST14 Catalytic Domain (R&D Systems) 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, followed by immunoblot analysis.

Subcellular fractionation

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

Construction of a PDGF-C shRNA vector

Three target shRNA sequences were created using Ambion's web-based protocol (PDGF-C shRNA Target 1: 5'-GGATACAACTTACGTTTGA-3'; Target 2: 5'-CCAGCTAGTTTCAGAGTTA-3'; Target 3: 5'-GTATGGTGCTAGAGTTAGA-3') and inserted into pSilencer-U6 hygro vector (Ambion, Foster City, CA). The subsequent vectors were designated pSilencer-PDGF-C shRNA1, shRNA2, and shRNA3.

shRNA-mediated PDGF-C knockdown in MDA-MB-231

To create a PDGF-C knockdown cell line, each pSilencer-PDGF-C construct was transfected into MDA-MB-231 breast cancer cells using Lipofectamine 2000 (Invitrogen), following the manufacturer's protocol and selected in the presence of 700μg/ml of Hygromycin for 10 days. Stable clones were analyzed for PDGF-C knockdown using RT-PCR and immunoblot analyses. The clone that demonstrated the best knockdown was utilized in subsequent experiments.

Soft agar assay

Breast cancer cells (BT-549, MDA-MB-231) were grown to sub-confluence and dissociated into single cell suspensions and 5,000 cells were embedded in 0.35% Bacto Agar (BD Biosciences, San Jose, CA) on top of a 0.6% agar bottom layer in triplicates. Cells were allowed to grow for 2 weeks then stained with 4% Giemsa Stain (Fischer Scientific, Pittsburg, PA). Colonies between the size of 0.2 and 0.5 mm were counted using a colony counter (Oxford Optronix, Oxford, UK) in the Translational Research Core of the Karmanos Cancer Institute.

Matrigel cell invasion assay

MDA-MB-231 and BT-549 cells were plated in serum-free media at a density of 3.75×10^5 cells/ml into Matrigel-coated transwells (BD, Franklin Lakes, NJ). Transwells were then placed in 24-well containing growth media. Invasion was permitted to occur over 5 hours

and cells on the upper surface of the filter were mechanically removed with a cotton swab. Cells migrated to the lower side of the filter were stained with 0.9% Crystal Violet and counted in 5 different high-powered fields using an inverted Nikon TMS microscope (Melville, NY).

Animal Study

1×10^6 cells in 50 μl of Matrigel™(BD Biosciences):media (1:1) mixture were orthotopically injected into the R4 mammary fat pad of 6-week old female NOD/SCID mice with a 25 gauge needle. Tumor measurements were taken at the indicated time points using digital calipers. Tumor volumes were calculated using the formula; tumor volume = a x $b^2/2$, where b is the smaller of the two measured tumor diameters.

Patients and samples

The tissue microarrays used for this study were constructed for a previous study (20) from paraffin-embedded tumor samples from 216 patients with invasive breast cancer (IBC) who underwent surgical resection at Seoul National University Bundang Hospital from May 2003 to November 2005. The histopathologic variables, immunohistochemical data of ER, PR, HER-2, Ki-67, and p53, and molecular subtypes were also used for the present study.

Immunohistochemical analysis of PDGF-C

Following optimization of staining conditions using positive and negative controls, immunohistochemical analysis was carried out using DAKO EnVision detection kit (Dako, Carpinteria, CA). Briefly, tissue array blocks were cut into 4 μm-thick sections, dried, deparaffinized, and rehydrated. Antigen retrieval was performed in a steamer for 40 minutes in 10 mM citrate buffer pH 6.0, and endogenous peroxidase activity was blocked with a 3% H_2O_2-methanol solution. The slides were blocked with 10% normal goat serum for 10 minutes and were incubated with anti-PDGF-C/GFD Ab (1:200) for one hour at room temperature. The slides were then incubated with HRP-labeled polymer conjugated to secondary antibody for 30 minutes. Diaminobenzidine was used as a chromogen, and the sections were counterstained with Mayer's hematoxylin.

Histological interpretation and statistical analysis

After omitting 45 cases with uninterruptable IHC results due to loss of tissue cores, a total of 171 IBCs were informative for PDGF-C expression and used for the analyses. Staining results were interpreted by a breast pathologist who was blinded to patient outcomes. PDGF-C expression was evaluated according to the percentage of positive cells and the intensity of staining in the nucleus and cytoplasm. The percentage of positive cells was scored as 0 (0% positive cells), 1 (< 10% positive cells), 2 (10–50% positive cells), 3 (>50% positive cells). The staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). For the immunoreactive score (IRS), the percentage of positive cells and the staining intensity were multiplied, resulting in a value between 0 and 9. For statistical analysis, patients who had an IRS of 0–3 were combined into one group with negative to weak PDGF-C expression (the "PDGF-C negative" group), and patients who had an IRS of 4–9 were combined as "PDGF-C positive" group.

All data were analyzed with SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). Pearson chi-square test or Fisher's exact test was used when comparing frequencies between PDGF-C positive group and PDGF-C negative group. Disease-free survival (DFS) was measured from the date of surgery to the date of loco-regional recurrence, distant metastasis, or death. At the time of the analysis, the median follow-up was 5 years (range, 3–6 years). There were three (2%) loco-regional recurrences as first events and 20 (12%) distant metastases. Eleven patients died of recurrent or metastatic breast cancer. Kaplan-Meier survival curves for DFS were constructed and differences were determined by log-rank test. P values were two sided, with $P<0.05$ considered as significant.

RESULTS

Increased PDGF-C expression and identification of a PDGF-C splice variant in breast carcinoma cells

Evidence has implicated aberrant PDGF signaling in breast cancer development and metastases (5–10,21,22). In this study, we set out to determine the PDGF ligand relevant in breast cancer. First, we performed GOBO Gene Set Expression (GSE) analysis to assess expression levels of the PDGF family members in 51 human breast cancer cell lines (18,23). 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 (Fig. 1A). GOBO allows grouping the breast cancer cell lines according to the clinical features assessed in breast cancers, such as hormone receptor expression and HER2/neu overexpression. Interestingly, PDGF-C expression is highest amongst the triple-negative (TN) breast cancer cell lines and lowest in the hormone receptor (HR)-positive breast cancer cell lines (Fig. 1B). There is no significant difference amongst the various clinical subtypes of breast cancer cell lines in the expression levels of PDGF A, PDGF D, or either of the PDGF receptors (Supplementary Fig. 1), whereas significant differences are found in PDGF B expression levels 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 (Supplementary Fig. 1). Analysis of the TCGA database showed that the expression levels of both PDGF-C and its cognate receptor α-PDGFR proteins are higher in TN breast cancer compared to HER2-positive or HR-positive breast cancer. However, it should be noted that the differences are not statistically significant (Supplementary Fig. 2).

Next, we screened for the expression levels of the PDGF family members using a panel of breast carcinoma cell lines that represent different degrees of tumorigenicity and invasiveness. We found increased PDGF-C mRNA expression in cell lines with malignant potential, whereas there was no correlation between the malignant potential of breast carcinoma cell lines and the expression levels of PDGF-A, -B, or -D (Fig. 1C). The non-tumorigenic MCF10A (24) and minimally invasive and estrogen-dependent MCF-7 cells (25,26) expressed relatively low levels of PDGF-C. In contrast, highly malignant and invasive BT-549 and MDA-MB-231 cells (27,28) expressed high levels of PDGF-C. Similarly, aggressive inflammatory breast cancer cell lines, SUM149 and SUM190 (29,30),

expressed higher levels of PDGF-C mRNA compared to the modestly invasive ductal carcinoma cell line SUM102 (31). Interestingly, a longer RT-PCR product of PDGF-C mRNA was detected at higher levels from malignant breast carcinoma cells (arrow in Fig. 1C), suggesting differential splicing of PDGF-C. Cloning and sequencing analysis of RT-PCR products for PDGF-C mRNAs from MDA-MB-231, SUM102, SUM149, and SUM190 identified a 61 base pair exon between the previously reported exons 1 and 2, depicted in Figure 1D as exon 2'. To examine whether additional splice variant(s) exist in these cells, we performed RT-PCR analysis using different combinations of primer pairs (Fig. 1D) and the sizes of resultant RT-PCR products are summarized in Figure 1E. Our results indicated that no other splice variant exists at a detectable level in these cells. The levels of the splice variant (t-PDGF-C) and total PDGF-C mRNAs were further quantified by real-time PCR (Fig. 1F). Interestingly, although total PDGF-C mRNA levels were comparable between T47D (lane 3 in Fig. 1C) and MDA-MB-231 (lane 5 in Fig. 1C), a much higher level of PDGF-C protein was detected in conditioned medium from MDA-MB-231 cells that express the PDGF-C splice variant (Fig. 1G). In addition, little PDGF-C protein was detected in BT-549 that expresses PDGF-C mRNA as well as its cognate receptor α-PDGFR at high levels (Fig. 1C lane 4 and Fig. 1G lane 4). The lack of accumulated extracellular PDGF-C protein in BT-549 cells is likely due to constitutively active autocrine signaling, resulting in internalization and degradation of the ligand-receptor signaling complex.

t-PDGF-C is retained intracellularly as a homodimer and its expression results in upregulation of the full-length PDGF-C (FL-PDGF-C) protein level

A sequence analysis of the PDGF-C splice variant containing the 61 bp exon 2' predicted a shift in the open reading frame, thus generating a stop codon at the 51st codon if the same AUG initiation codon is utilized as for FL-PDGF-C. Interestingly, there is a potential alternative translation initiation site at methionine^{164} that can generate a truncated isoform of PDGF-C (t-PDGF-C), lacking most of the N-terminal CUB domain as depicted in Fig. 1D. To determine whether this splice variant of PDGF C mRNA indeed produces t-PDGF-C proteins, we constructed a t-PDGF-C expression vector with a myc-tag at the C-terminus. After transfection into the COS-1 cells, t-PDGF-C expression at the RNA level was confirmed by RT-PCR analysis (Fig. 2A upper panel, lane 4). As expected from an alternative translation initiation at methionine^{164}, ~37 kDa PDGF-C protein was detected in COS-1 cells (Fig. 2A middle panel, lane 4). The t-PDGF-C dimer protein, lacking an N-terminal secretory signal peptide, was found only in cell lysates but not in conditioned medium (Fig. 2E), as shown by immunoblot analysis using both anti-c-myc and anti-PDGF-C/GFD antibodies (Fig. 2B&E). Next, we examined the effects of t-PDGF-C expression on FL-PDGF-C expression. To this end, COS-1 cells were transfected with a fixed amount of FL-PDGF-C expression vector DNA together with increasing amounts of t-PDGF-C expression vector DNA. Interestingly, t-PDGF-C expression resulted in increases in the levels of FL-PDGF-C proteins (Fig. 2A and B, lanes 1–3). This may provide an explanation as to the above findings that a much higher level of PDGF-C protein was detected in MDA-MB-231 cells that express the PDGF-C splice variant (Fig. 1G) compared to T47D cells, although total PDGF-C mRNA levels were comparable between those cell lines. It should be noted that those cancer cells express high levels of serine proteases that cleaves FL-PDGF-C into PDGF C GFD.

t-PDGF-C can form a heterodimer with FL-PDGF C which can be found both intracellularly and extracellularly

When PDGF-C dimer species were examined in a non-reducing condition, FL-PDGF-C homodimer (~85 kDa FL/FL in Fig. 2B lane 1) or t-PDGF-C homodimer (~52 kDa t/t in Fig. 2B lane 4) was detected upon transfection with FL-PDGF-C or t-PDGF-C expression vector, respectively. When cells were co-transfected with both vectors, an intermediate size of PDGF-C dimer was also detected, representing a heterodimer (~70 kDa FL/t in Fig. 2B, lane 3).

To further investigate the nature of PDGF-C dimer species, cells were transfected with either FL-PDGF-C expression vector containing a His-tag at the C-terminus [FL-PDGF-C-His] or t-PDGF-C-myc expression vector individually, or co-transfected with FL-PDGF-C-His and t-PDGF-C-myc vectors. Transfection of FL-PDGF-C and/or t-PDGF-C expression vector was confirmed by RT-PCR analysis as shown in Fig. 2C. PDGF-C dimers containing a FL-PDGF-C-His subunit were captured by His-tag pull down assay, followed by immunoblot analysis. As shown in Fig. 2D (top panel, lane 2), immunoblot analysis in a reducing condition detected ~48 kDa FL-PDGF-C monomer in His-tag pull down upon transfection with FL-PDGF-C-His vector only. When His-pull down samples from co-transfectants were analyzed, ~37 kDa t-PDGF-C-myc protein was detected by immunoblot analysis using anti-PDGF-C GFD or anti-c-myc Ab (Fig. 2D, lane 3, top and middle panel, respectively). As expected, no PDGF C protein was detected in His-pull down samples from t-PDGF-C-myc transfectants. In a control experiment, immunoblot analysis with anti-c-myc Ab was performed to detect t-PDGF-C-myc protein in total cell lysates of transfectants (Fig. 2D, bottom panel). These results confirm that t-PDGF-C can form a heterodimer with FL-PDGF C

Realizing that in human breast cancer cells, t-PDGF-C mRNA is not detected by itself, but instead it is expressed along with the full-length PDGF-C mRNA transcript (Fig. 1C), we investigated expression and function of t-PDGF-C protein using the BT-549 cell model which naturally expresses predominantly full-length PDGF-C. In BT-549 cells engineered to express t-PDGF-C with a myc tag, t-PDGF-C dimer proteins were detected both intracellularly and extracellularly (Fig. 2E). Interestingly, while the myc-tagged t-PDGF-C homodimer (t/t) is exclusively intracellular, myc-tagged t-PDGF-C can be secreted as a hetero-dimer with endogenous full-length PDGF-C (FL/t) as detected by immunoblot analysis of conditioned medium under non-reducing condition using anti-c-myc antibody (Fig. 2E). 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 dimer (GFD-D) (Fig. 2F), suggesting that secreted t-PDGF-C as a FL/t heterodimer may act as a reservoir for the formation of biologically active GFD-D of PDGF-C.

PDGF-C expression positively correlates with lymph node metastases, HER2/neu oncogene expression, and increased proliferation, and inversely correlates with patient disease-free survival rates

In a continuing effort to identify a PDGF ligand relevant to human breast cancer, breast tumor tissues were analyzed by immunohistochemical staining for the PDGF family members. Our previous study found no increases in PDGF-A or -B expression that correlated with patient clinicopathological characteristics (unpublished observation). Thus, in the present study, we focused on analyses of newly identified PDGF ligands, PDGF-C and PDGF-D. While PDGF-D expression was barely detected (data not shown), increased PDGF-C expression was observed in tumor tissues (Fig. 3A). PDGF-C was weakly expressed in the nucleus and/or cytoplasm of normal mammary gland. Stromal cells also showed weak to moderate cytoplasmic staining with occasional nuclear staining of PDGF-C. To determine the specificity of the IHC analysis, anti-PDGF C antibody was incubated with immunogenic peptides (used for the generation of this antibody) overnight then utilized as an IHC control. As shown in Supplementary Figure 3, immunoreactivity was abrogated when the antibody was pre-absorbed with immunogenic peptide. Correlations between the expression of PDGF-C and histopathologic features of the tumors was determined and summarized in Supplementary Table 1. Increased cytoplasmic PDGF-C expression was correlated with nodal metastasis ($p=0.002$), HER2 amplification ($p=0.015$) and high Ki-67 proliferation index ($p=0.007$). Kaplan-Meier analysis showed that patients with only cytoplasmic PDGF-C expression had significantly worse DFS compared to patients with nuclear or no PDGF-C expression ($p=0.024$; Fig. 3B). Moreover, in the luminal subtypes of breast cancer, only cytoplasmic PDGF-C expression revealed more significant difference in the DFS of the patients ($p=0.017$; data not shown). Next, we examined whether tumor tissues express increased levels of PDGF-C mRNA and its splice variant encoding t-PDGF-C. As shown in Supplementary Fig. 4, analysis of RNA samples collected from 20 pairs of invasive breast cancer tissues and adjacent normal tissue from the same patient revealed significant increases in both FL-PDGF-C and t-PDGF-C mRNA expression ($p=0.0115$). It should be mentioned that RT-PCR analyses for β–actin and cytokeratin 18 were included for the control of equal amount of RNA samples and the epithelial origin (32) of tissue samples, respectively.

In order to confirm PDGF C expression in the nucleus, we analyzed the subcellular localization of endogenous PDGF-C proteins in human breast cancer cell line T47D by biochemical fractionation of cell lysates followed by immunoblot analysis (Fig. 3C). Interestingly, the proteolytically processed PDGF-C growth factor domain protein was found in the chromatin bound nuclear fraction of T47D breast cancer cells with a smaller amount found in the cytoskeletal fraction, supporting nuclear PDGF-C expression, in agreement with a previous report of the nuclear localization of PDGF-C in human thyroid tissue samples, papillary thyroid carcinoma, and cell lines (33).

Causative roles of PDGF-C and t-PDGF-C in the regulation of breast cancer cell growth and invasive phenotype

We asked if expression of t-PDGF-C modulates oncogenic effects of PDGF-C. To this end, we examined the phenotypes of BT-549 cells engineered to express t-PDGF-C. Expression

of t-PDGF-C was confirmed at both RNA (Fig. 4A) and protein (Fig. 2E) levels. Similar to the transient expression of t-PDGF-C in COS-1 cells (Fig. 2A), t-PDGF-C expression in BT-549 cells resulted in increases in PDGF-C levels in CM (Fig. 4A). It should be mentioned that breast carcinoma cells process FL-PDGF-C into a growth factor domain (GFD) (~18 kDa) by serine protease-mediated proteolytic cleavage (13,16), whereas PDGF-C is secreted mostly as a latent growth factor (FL-PDGF-C) by normal epithelial cells or COS cells (Fig. 2A). Interestingly, while an accumulation of FL-PDGF-C was prominent in CM from COS-1 cells upon t-PDGF-C expression, only a mild, but consistent, increase in the level of PDGF-C GFD was detected in CM from BT-549 cells upon t-PDGF-C expression, likely due to constitutive activation and degradation of PDGF-C GFD/α-PDGFR signaling axis. Importantly, t-PDGF-C expression significantly enhanced the ability of BT-549 cells to grow in an anchorage-independent manner (Fig. 4B and Supplementary Fig. 5) as well as to invade through Matrigel (Fig. 4C and Supplementary Fig. 6). These results clearly demonstrate the functional significance of expression of PDGF-C and its splice variant for potentially malignant phenotypic changes of breast carcinoma cells. It should be also noted that modulation of PDGF-C and/or t-PDGF-C in breast carcinoma cells had little effect on levels of other PDGF family members (Supplementary Fig. 8).

To further determine the functional significance of PDGF-C expression in breast cancer, we established an MDA-MB-231 cell line where PDGF-C expression is downregulated. Immunoblot analysis confirmed shRNA-mediated PDGF-C knockdown in these cells (Fig. 5A). PDGF-C knockdown significantly reduced both the ability of breast carcinoma cells to grow in an anchorage-independent manner as assessed by a soft agar assay as well as the invasive phenotype as determined by a Matrigel invasion assay (Fig. 5B–C and Supplementary Figs. 5–6). To address the off-target effects associated with the shRNA vector, PDGF C expression was also downregulated using two independent small interfering RNAs (siRNAs). As shown in Supplementary Figure 7, siRNA-mediated PDGF C knockdown also significantly reduced cell invasion. Importantly, PDGF-C knockdown greatly reduced MDA-MB-231 tumor growth *in vivo* when these cells were implanted orthotopically into the mammary fat pad of female NOD/SCID mice (Fig. 5D).

DISCUSSION

Emerging evidence suggests increased PDGF-C expression (as summarized in Supplementary Table 3) and diverse roles of PDGF-C in human cancers. A role for PDGF-C in human cancer was first suggested when Ewing sarcoma cell lines were shown to have increased expression and secretion of PDGF-C (34,35). A functional significance of PDGF-C was demonstrated by expression of a dominant negative PDGF-C construct that reduced anchorage independent growth in those cell lines (34,35). PDGF-C autocrine signaling for tumor progression has also been suggested in brain tumors such as glioblastoma and medulloblastoma (36,37). In addition to its autocrine effects, PDGF-C contributes to tumor progression through paracrine signaling, as it is critical for tumor angiogenesis and the recruitment of cancer-associated fibroblasts (38,39). Besides the role of PDGF-C role in tumor progression, recent studies suggest that PDGF-C expression may be among the critical determinants for the therapeutic response, as it is highly expressed in tumors refractory to anti-VEGF treatment or the chemotherapeutic agent cisplatin (40,41).

Importantly, the present study identified PDGF-C as a PDGF ligand relevant to human breast cancer. Increased PDGF-C expression was correlated with nodal metastasis (p=0.002), HER2 amplification (p=0.015), and high Ki-67 proliferation index (p=0.007), revealing remarkable similarity to the previously reported immunohistochemical study of α-PDGFR using 181 formalin-fixed paraffin-embedded invasive ductal breast carcinomas (10). Carvalho et al. found that α-PDGFR staining is associated with lymph node metastasis (p=0.0079), HER2/neu expression (p=0.0265), and Bcl-2 expression (p=0.0121). These two independent studies clearly suggest the significance of the PDGF-C/α-PDGFR signaling axis during human breast cancer progression. GOBO data, presented in this study, 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. This is of significance as the treatment of triple-negative breast cancer in the clinic has been challenging. A better understanding of triple-negative breast cancer biology is needed in order to develop drugs that can target that subset of breast cancers. It should be noted that immunohistochemical studies by us and Carvalho et al. showed association between the PDGF-C/α-PDGFR and HER2/neu expression, unlike GOBO data, warranting further investigation.

Zhao et al. (42) reported a splice variant of PDGF-C by database mining of human expression sequence tag (EST) clones and subsequent cloning and expression study; this variant turned out to be the same splice variant that we identified in the present study. The authors showed that the truncated PDGF-C isoform is a cytoplasmic protein able to heterodimerize with full-length PDGF-C, which is consistent with our data. By RT-PCR analysis of the two alternatively spliced PDGF-C transcripts in 30 pairs of renal cell carcinoma (RCC) and adjacent normal tissues, these authors reported increased FL-PDGF-C transcript in RCC tumors with no significant difference in t-PDGF-C transcript between RCC samples and matched normal renal tissues. Based on these observations, the authors proposed that the N-terminal truncated isoform of PDGF-C may act as a dominant negative molecule that regulates the secretion of FL-PDGF-C possibly through the heterodimerization (42). In contrast, our results demonstrated that t-PDGF-C increases the expression level and secretion of FL-PDGF-C. Moreover, 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 as a heterodimer. Furthermore, this study demonstrates that matriptase, a serine protease shown previously by our laboratory to process full-length PDGF-C into biologically active growth factor domain (GFD) protein (16), also has the ability to proteolytically process the secreted t-PDGF-C isoform to the GFD. This significant finding suggests that t-PDGF-C may serve as a repository for active PDGF-C GFD in the extracellular milieu, thus making it a possible source of extracellular pro-oncogenic signaling in cancer.

Importantly, our RT-PCR analysis showed both PDGF-C transcripts are frequently upregulated in human breast cancer tissues compared to the matching adjacent normal tissues (Supplementary Figure 4). Moreover, our functional study demonstrated that t-PDGF-C induces phenotypic transformation, suggesting an oncogenic activity of t-PDGF-C in breast cancer. These novel and exciting observations highlight the complexity in regulating biosynthesis, protein stability, and secretion of PDGF-C. Unlike FL-PDGF-C, t-

PDGF-C lacks the signal peptide and the CUB domain, raising a question as to how t-PDGF-C is inserted into rough endoplasmic reticulum for the dimerization. At present, the molecular mechanisms underlying the formation of FL/t PDGF-C heterodimer and its secretion are still unknown. Similarly, we do not understand the molecular mechanism by which t-PDGF-C promotes expression levels of PDGF-C. It would be of interest to examine whether t-PDGF-C competitively binds to the negative regulator(s) that modulate the protein stability and/or secretion of FL-PDGF-C, thereby indirectly promoting the secretion and oncogenic activity of FL-PDGF-C. These important biological questions and the molecular mechanisms remain to be fully investigated. Importantly, our preliminary study suggests that t-PDGF-C may be frequently expressed in human cancers as detected in several human prostate cancer as well as head and neck squamous carcinoma cell lines (Supplementary Figure 9).

A recent study reported nuclear localization of PDGF-C as larger molecular weight PDGF-C entities in human thyroid tissues as well as in cell lines (33). It was suggested that nuclear PDGF-C is post-translationally modified by reversible covalent binding to small ubiquitin-like modifiers (SUMO). Interestingly, the level of SUMO-1 modified nuclear PDGF-C was lower in thyroid papillary carcinomas compared to the non-neoplastic tissues. This study is the first to show the proteolytically-processed GFD of PDGF-C in the nucleus of human breast cancer cell lines that naturally express PDGF-C by a yet unknown mechanism. In an effort to determine whether nuclear translocation of PDGF-C GFD occurs via its binding to and activation of PDGFR, subcellular localization of PDGF-C was examined in the presence or absence of Imatinib Mesylate (PDGFR inhibitor). Our preliminary study indicated that PDGFR inhibition had little effect on the levels of PDGF-C GFD in the nucleus (data not shown). Interestingly, we also detected full-length PDGF-C in the nuclear fraction of MCF-7 cells transfected with full-length PDGF-C (data not shown). Since the full-length PDGF C fails to interact with PDGFR, this study also suggested PDGFR-independent nuclear subcellular localization of PDGF-C. However, it should be noted that in T47D human breast cancer cells that express endogenous PDGF-C, nuclear PDGF-C is mostly the proteolytically processed GFD. Interestingly, 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 (43). Lack of its nuclear localization sequences abolished mitogenic activity of the growth factor suggesting that nuclear translocation is necessary for its activity (43,44). The presence of growth factors in the nucleus has been suggested to play a role in transcriptional regulation. In fact, FGF-2, LEDGF and SDGF have been shown to play roles in transcriptional regulation. These growth factors were shown to bind to the promoters of stress-related genes such c-fos and NGFI-A, inducing their expression (45,46). PDGF-C being localized to the nucleus greatly complicates the possible roles of such versatile 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.

Our immunohistochemical analysis also showed nuclear staining of PDGF-C in some of human breast cancer tissues. When we separately analyzed nuclear vs. cytoplasmic PDGF-C staining in the 171 invasive breast carcinomas (IBCs), 32 cases (18.7%) demonstrated nuclear expression while 103 (60.2%) showed cytoplasmic expression of PDGF-C with IRS ≥4. Of the 103 cases with cytoplasmic staining of PDGF-C, 89 cases (52%) showed only cytoplasmic staining without nuclear staining. Supplementary Table 2 summarizes the correlations between the expression of PDGF-C in each compartment of tumor cells and histopathologic features of the tumors. Nuclear expression of PDGF-C was associated with low histologic grade (p=0.012), absence of nodal metastasis (p=0.011), expression of estrogen receptor (p=0.044), HER2 non-amplification (p=0.023), low Ki-67 proliferation index (p=0.013) and absence of p53 overexpression (p=0.016). Kaplan-Meier analysis showed that nuclear expression of PDGF-C had a trend toward good DFS (p=0.081). Notably, patients with only cytoplasmic PDGF-C expression had significantly worse DFS (p=0.024; Fig. 3B). These data suggest opposite effects between the cytoplasmic PDGF-C vs. nuclear PDGF-C during breast cancer progression. These intriguing observations open a largely unexplored area of PDGF biology in the nucleus. It would be of particular interest to examine the effects of t-PDGF-C on post-translational modification of FL-PDGF-C including SUMOlyation, subcellular localization including nuclear localization, and the functional consequences of PDGF-C expression at different subcellular locations.

Previous studies with PDGF-A and -B showed that a stretch of basic amino acid residues in the C-terminal region mediate the retention of PDGF-A and -B inside the cells or sequestration in the extracellular matrix. A splice variant of PDGF-A encoding the short isoform lacking this retention motif was shown to be secreted more efficiently, emphasizing the significance of alternative splicing for the regulation of growth factor subcellular localization and secretion (47,48). However, the consequence of t-PDGF-C expression encoded by the splice variant of PDGF-C is more than the exclusion of a particular domain for its own destiny. Our results clearly demonstrated that t-PDGF-C modulates the FL-PDGF-C expression level and secretion as well as the phenotypic transformation of breast carcinoma cells. Taken together, the present study demonstrated the functional significance and the clinical relevance of PDGF-C expression in breast cancer. This study also provided new insights into the regulation of FL-PDGF-C protein levels and secretion modulated by expression of its splice variant, a novel finding that appears to be unique to PDGF-C among the PDGF family members.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Rosenkranz S, and Kazlauskas A (1999) Evidence for distinct signaling properties and biological responses induced by the PDGF receptor alpha and beta subtypes. Growth Factors 16, 201–216 [PubMed: 10372961]
2. Yu J, Ustach C, and Kim HR (2003) Platelet-derived growth factor signaling and human cancer. J Biochem Mol Biol 36, 49–59 [PubMed: 12542975]
3. Doolittle RF, Hunkapiller MW, Hood LE, Devare SG, Robbins KC, Aaronson SA, and Antoniades HN (1983) Simian sarcoma virus onc gene, v-sis, is derived from the gene (or genes) encoding a platelet-derived growth factor. Science 221, 275–277 [PubMed: 6304883]
4. Waterfield MD, Scrace GT, Whittle N, Stroobant P, Johnsson A, Wasteson A, Westermark B, Heldin CH, Huang JS, and Deuel TF (1983) Platelet-derived growth factor is structurally related to the putative transforming protein p28sis of simian sarcoma virus. Nature 304, 35–39 [PubMed: 6306471]
5. Seymour L, and Bezwoda WR (1994) Positive immunostaining for platelet derived growth factor (PDGF) is an adverse prognostic factor in patients with advanced breast cancer. Breast cancer research and treatment 32, 229–233 [PubMed: 7865852]
6. Palman C, Bowen-Pope DF, and Brooks JJ (1992) Platelet-derived growth factor receptor (beta-subunit) immunoreactivity in soft tissue tumors. Laboratory investigation; a journal of technical methods and pathology 66, 108–115 [PubMed: 1309926]
7. Coltrera MD, Wang J, Porter PL, and Gown AM (1995) Expression of platelet-derived growth factor B-chain and the platelet-derived growth factor receptor beta subunit in human breast tissue and breast carcinoma. Cancer research 55, 2703–2708 [PubMed: 7780988]
8. Paulsson J, Sjoblom T, Micke P, Ponten F, Landberg G, Heldin CH, Bergh J, Brennan DJ, Jirstrom K, and Ostman A (2009) Prognostic significance of stromal platelet-derived growth factor beta-receptor expression in human breast cancer. The American journal of pathology 175, 334–341 [PubMed: 19498003]
9. Jechlinger M, Sommer A, Moriggl R, Seither P, Kraut N, Capodiecci P, Donovan M, Cordon-Cardo C, Beug H, and Grunert S (2006) Autocrine PDGFR signaling promotes mammary cancer metastasis. The Journal of clinical investigation 116, 1561–1570 [PubMed: 16741576]
10. Carvalho I, Milanezi F, Martins A, Reis RM, and Schmitt F (2005) Overexpression of platelet-derived growth factor receptor alpha in breast cancer is associated with tumour progression. Breast cancer research : BCR 7, R788–795 [PubMed: 16168125]
11. Li X, Ponten A, Aase K, Karlsson L, Abramsson A, Uutela M, Backstrom G, Hellstrom M, Bostrom H, Li H, Soriano P, Betsholtz C, Heldin CH, Alitalo K, Ostman A, and Eriksson U (2000) PDGF-C is a new protease-activated ligand for the PDGF alpha-receptor. Nat Cell Biol 2, 302–309 [PubMed: 10806482]
12. LaRochelle WJ, Jeffers M, McDonald WF, Chillakuru RA, Giese NA, Lokker NA, Sullivan C, Boldog FL, Yang M, Vernet C, Burgess CE, Fernandes E, Deegler LL, Rittman B, Shimkets J, Shimkets RA, Rothberg JM, and Lichenstein HS (2001) PDGF-D, a new protease-activated growth factor. Nat Cell Biol 3, 517–521 [PubMed: 11331882]
13. Fredriksson L, Li H, Fieber C, Li X, and Eriksson U (2004) Tissue plasminogen activator is a potent activator of PDGF-CC. The EMBO journal 23, 3793–3802 [PubMed: 15372073]
14. Ustach CV, Taube ME, Hurst NJ Jr., Bhagat S, Bonfil RD, Cher ML, Schuger L, and Kim HR (2004) A potential oncogenic activity of platelet-derived growth factor d in prostate cancer progression. Cancer research 64, 1722–1729 [PubMed: 14996732]
15. Ustach CV, and Kim HR (2005) Platelet-derived growth factor D is activated by urokinase plasminogen activator in prostate carcinoma cells. Mol Cell Biol 25, 6279–6288 [PubMed: 15988036]

16. Hurst NJ, Najy AJ, Ustach CV, Movilla L, and Kim HR (2012) Platelet-derived growth factor-C (PDGF-C) activation by serine proteases: implications for breast cancer progression. The Biochemical journal 441, 909–918 [PubMed: 22035541]

17. Gilbertson DG, Duff ME, West JW, Kelly JD, Sheppard PO, Hofstrand PD, Gao Z, Shoemaker K, Bukowski TR, Moore M, Feldhaus AL, Humes JM, Palmer TE, and Hart CE (2001) Platelet-derived growth factor C (PDGF-C), a novel growth factor that binds to PDGF alpha and beta receptor. The Journal of biological chemistry 276, 27406–27414 [PubMed: 11297552]

18. Neve RM, Chin K, Fridlyand J, Yeh J, Baehner FL, Fevr T, Clark L, Bayani N, Coppe JP, Tong F, Speed T, Spellman PT, DeVries S, Lapuk A, Wang NJ, Kuo WL, Stilwell JL, Pinkel D, Albertson DG, Waldman FM, McCormick F, Dickson RB, Johnson MD, Lippman M, Ethier S, Gazdar A, and Gray JW (2006) A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer cell 10, 515–527 [PubMed: 17157791]

19. Li G, Fridman R, and Kim HR (1999) Tissue inhibitor of metalloproteinase-1 inhibits apoptosis of human breast epithelial cells. Cancer research 59, 6267–6275 [PubMed: 10626822]

20. Park SY, Lee HE, Li H, Shipitsin M, Gelman R, and Polyak K (2010) Heterogeneity for stem cell-related markers according to tumor subtype and histologic stage in breast cancer. Clinical cancer research : an official journal of the American Association for Cancer Research 16, 876–887 [PubMed: 20103682]

21. Bronzert DA, Pantazis P, Antoniades HN, Kasid A, Davidson N, Dickson RB, and Lippman ME (1987) Synthesis and secretion of platelet-derived growth factor by human breast cancer cell lines. Proceedings of the National Academy of Sciences of the United States of America 84, 5763–5767 [PubMed: 3039506]

22. Lev DC, Kim SJ, Onn A, Stone V, Nam DH, Yazici S, Fidler IJ, and Price JE (2005) Inhibition of platelet-derived growth factor receptor signaling restricts the growth of human breast cancer in the bone of nude mice. Clinical cancer research : an official journal of the American Association for Cancer Research 11, 306–314 [PubMed: 15671560]

23. Ringner M, Fredlund E, Hakkinen J, Borg A, and Staaf J (2011) GOBO: gene expression-based outcome for breast cancer online. PloS one 6, e17911 [PubMed: 21445301]

24. Soule HD, Maloney TM, Wolman SR, Peterson WD Jr., Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, and Brooks SC (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. Cancer research 50, 6075–6086 [PubMed: 1975513]

25. Huseby RA, Maloney TM, and McGrath CM (1984) Evidence for a direct growth-stimulating effect of estradiol on human MCF-7 cells in vivo. Cancer research 44, 2654–2659 [PubMed: 6722798]

26. Schafer JM, Lee ES, O'Regan RM, Yao K, and Jordan VC (2000) Rapid development of tamoxifen-stimulated mutant p53 breast tumors (T47D) in athymic mice. Clinical cancer research : an official journal of the American Association for Cancer Research 6, 4373–4380 [PubMed: 11106256]

27. Sommers CL, Gelmann EP, Kemler R, Cowin P, and Byers SW (1994) Alterations in beta-catenin phosphorylation and plakoglobin expression in human breast cancer cells. Cancer research 54, 3544–3552 [PubMed: 8012979]

28. Dickson RB, Bates SE, McManaway ME, and Lippman ME (1986) Characterization of estrogen responsive transforming activity in human breast cancer cell lines. Cancer research 46, 1707–1713 [PubMed: 2418952]

29. Willmarth NE, and Ethier SP (2006) Autocrine and juxtacrine effects of amphiregulin on the proliferative, invasive, and migratory properties of normal and neoplastic human mammary epithelial cells. The Journal of biological chemistry 281, 37728–37737 [PubMed: 17035230]

30. Van den Eynden GG, Van Laere SJ, Van der Auwera I, Merajver SD, Van Marck EA, van Dam P, Vermeulen PB, Dirix LY, and van Golen KL (2006) Overexpression of caveolin-1 and −2 in cell lines and in human samples of inflammatory breast cancer. Breast cancer research and treatment 95, 219–228 [PubMed: 16244790]

31. Sartor CI, Dziubinski ML, Yu CL, Jove R, and Ethier SP (1997) Role of epidermal growth factor receptor and STAT-3 activation in autonomous proliferation of SUM-102PT human breast cancer cells. Cancer research 57, 978–987 [PubMed: 9041204]

32. Moll R, Franke WW, Schiller DL, Geiger B, and Krepler R (1982) The catalog of human cytokeratins: patterns of expression in normal epithelia, tumors and cultured cells. Cell 31, 11–24 [PubMed: 6186379]
33. Reigstad LJ, Martinez A, Varhaug JE, and Lillehaug JR (2006) Nuclear localisation of endogenous SUMO-1-modified PDGF-C in human thyroid tissue and cell lines. Experimental cell research 312, 782–795 [PubMed: 16443219]
34. Zwerner JP, and May WA (2001) PDGF-C is an EWS/FLI induced transforming growth factor in Ewing family tumors. Oncogene 20, 626–633 [PubMed: 11313995]
35. Zwerner JP, and May WA (2002) Dominant negative PDGF-C inhibits growth of Ewing family tumor cell lines. Oncogene 21, 3847–3854 [PubMed: 12032822]
36. Lokker NA, Sullivan CM, Hollenbach SJ, Israel MA, and Giese NA (2002) Platelet-derived growth factor (PDGF) autocrine signaling regulates survival and mitogenic pathways in glioblastoma cells: evidence that the novel PDGF-C and PDGF-D ligands may play a role in the development of brain tumors. Cancer research 62, 3729–3735 [PubMed: 12097282]
37. Andrae J, Molander C, Smits A, Funa K, and Nister M (2002) Platelet-derived growth factor-B and -C and active alpha-receptors in medulloblastoma cells. Biochem Biophys Res Commun 296, 604–611 [PubMed: 12176024]
38. di Tomaso E, London N, Fuja D, Logie J, Tyrrell JA, Kamoun W, Munn LL, and Jain RK (2009) PDGF-C induces maturation of blood vessels in a model of glioblastoma and attenuates the response to anti-VEGF treatment. PloS one 4, e5123 [PubMed: 19352490]
39. Anderberg C, Li H, Fredriksson L, Andrae J, Betsholtz C, Li X, Eriksson U, and Pietras K (2009) Paracrine signaling by platelet-derived growth factor-CC promotes tumor growth by recruitment of cancer-associated fibroblasts. Cancer research 69, 369–378 [PubMed: 19118022]
40. Crawford Y, Kasman I, Yu L, Zhong C, Wu X, Modrusan Z, Kaminker J, and Ferrara N (2009) PDGF-C mediates the angiogenic and tumorigenic properties of fibroblasts associated with tumors refractory to anti-VEGF treatment. Cancer cell 15, 21–34 [PubMed: 19111878]
41. Yamano Y, Uzawa K, Saito K, Nakashima D, Kasamatsu A, Koike H, Kouzu Y, Shinozuka K, Nakatani K, Negoro K, Fujita S, and Tanzawa H (2010) Identification of cisplatin-resistance related genes in head and neck squamous cell carcinoma. Int J Cancer 126, 437–449 [PubMed: 19569180]
42. Zhao J, Liu Z, Liu T, Nilsson S, and Nister M (2008) Identification and expression analysis of an N-terminally truncated isoform of human PDGF-C. Experimental cell research 314, 2529–2543 [PubMed: 18588873]
43. Imamura T, Engleka K, Zhan X, Tokita Y, Forough R, Roeder D, Jackson A, Maier JA, Hla T, and Maciag T (1990) Recovery of mitogenic activity of a growth factor mutant with a nuclear translocation sequence. Science 249, 1567–1570 [PubMed: 1699274]
44. Wesche J, Malecki J, Wiedlocha A, Ehsani M, Marcinkowska E, Nilsen T, and Olsnes S (2005) Two nuclear localization signals required for transport from the cytosol to the nucleus of externally added FGF-1 translocated into cells. Biochemistry 44, 6071–6080 [PubMed: 15835896]
45. Kimura H (1993) Schwannoma-derived growth factor must be transported into the nucleus to exert its mitogenic activity. Proceedings of the National Academy of Sciences of the United States of America 90, 2165–2169 [PubMed: 7681586]
46. Singh DP, Fatma N, Kimura A, Chylack LT Jr., and Shinohara T (2001) LEDGF binds to heat shock and stress-related element to activate the expression of stress-related genes. Biochem Biophys Res Commun 283, 943–955 [PubMed: 11350077]
47. Kelly K, Cochran BH, Stiles CD, and Leder P (1983) Cell-specific regulation of the c-myc gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35, 603–610 [PubMed: 6606489]
48. Ostman A, Thyberg J, Westermark B, and Heldin CH (1992) PDGF-AA and PDGF-BB biosynthesis: proprotein processing in the Golgi complex and lysosomal degradation of PDGF-BB retained intracellularly. The Journal of cell biology 118, 509–519 [PubMed: 1639841]

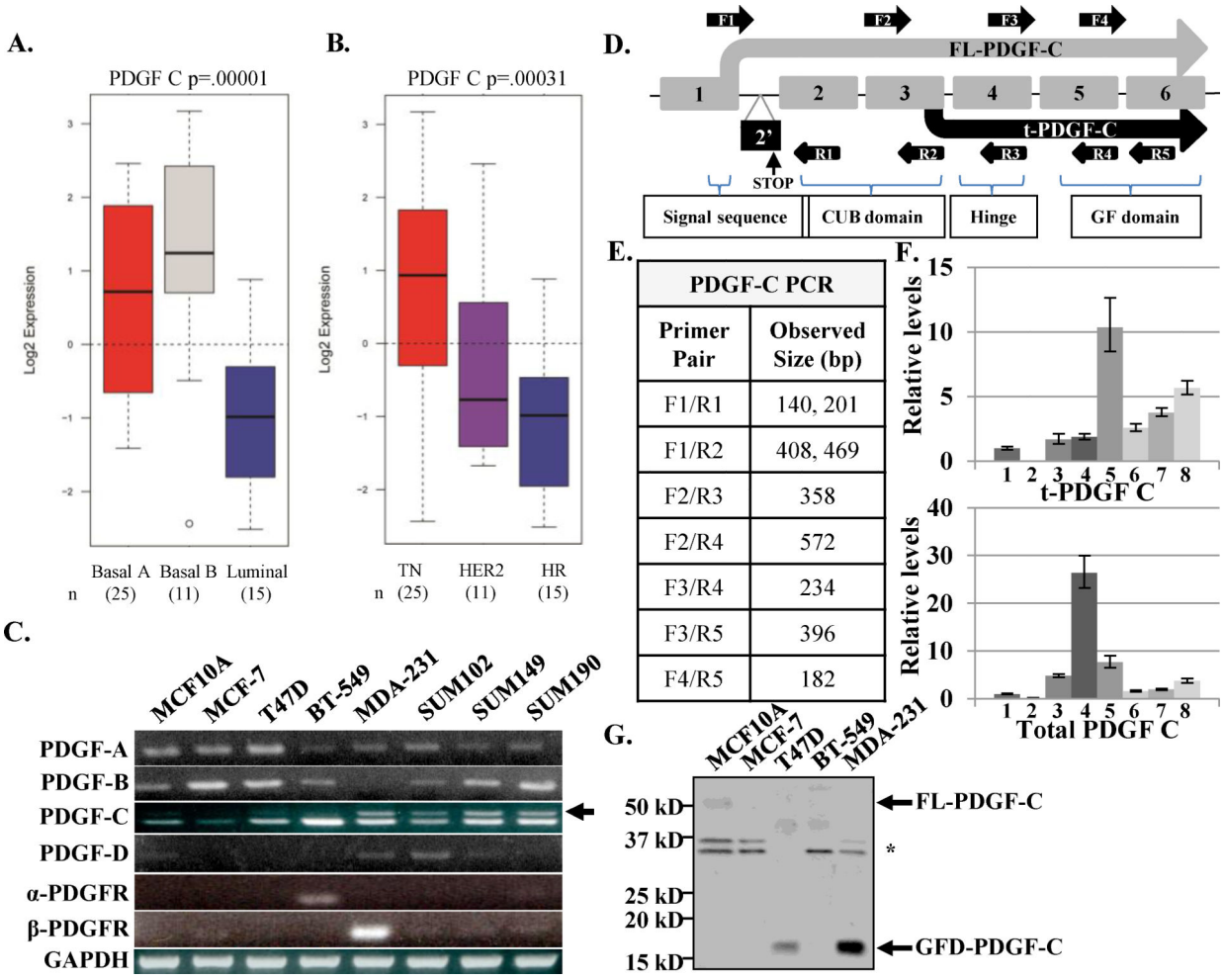

Figure 1. Expression of PDGF family members and identification of a PDGF-C splice variant in breast cancer cell lines.

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 [18] and Ringner et al 2011 [23]) **B)** Box plots from GOBO depicting PDGF C 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). (**C**) RT-PCR analysis of PDGF-A, -B, -C, and -D, α-PDGFR, and β-PDGFR in breast cancer cell lines. (**D**) A diagram of the PDGF-C gene depicting the locations of PDGF-C exons (numbers), the translation initiation sites for FL-PDGF-C (gray arrow) and t-PDGF-C (black arrow), and locations of primers (small black arrows) used in Panel E. **(E)** Primer pairs and the observed size of RT-PCR products of PDGF-C are shown. (**F**) Real-time RT-PCR analysis of t-PDGF-C and total PDGF-C mRNA in MCF-10A (lane 1), (MCF-7 (lane 2), T47D (lane 3), BT-549 (lane 4), MDA-MB-231 (lane 5), SUM102 (lane 6), SUM149 (lane 7), and SUM190 (lane 8) cell lines. The PDGF-C mRNA level in MCF10A was arbitrarily given as 1, and the error bars represent S.D. of triplicates. (**G**) Immunoblot analysis of PDGF-C using conditioned medium collected from indicated cells.

*: Non-specific; GFD-PDGF-C: Growth factor domain of PDGF-C; FL-PDGF C: Full length of PDGF C.

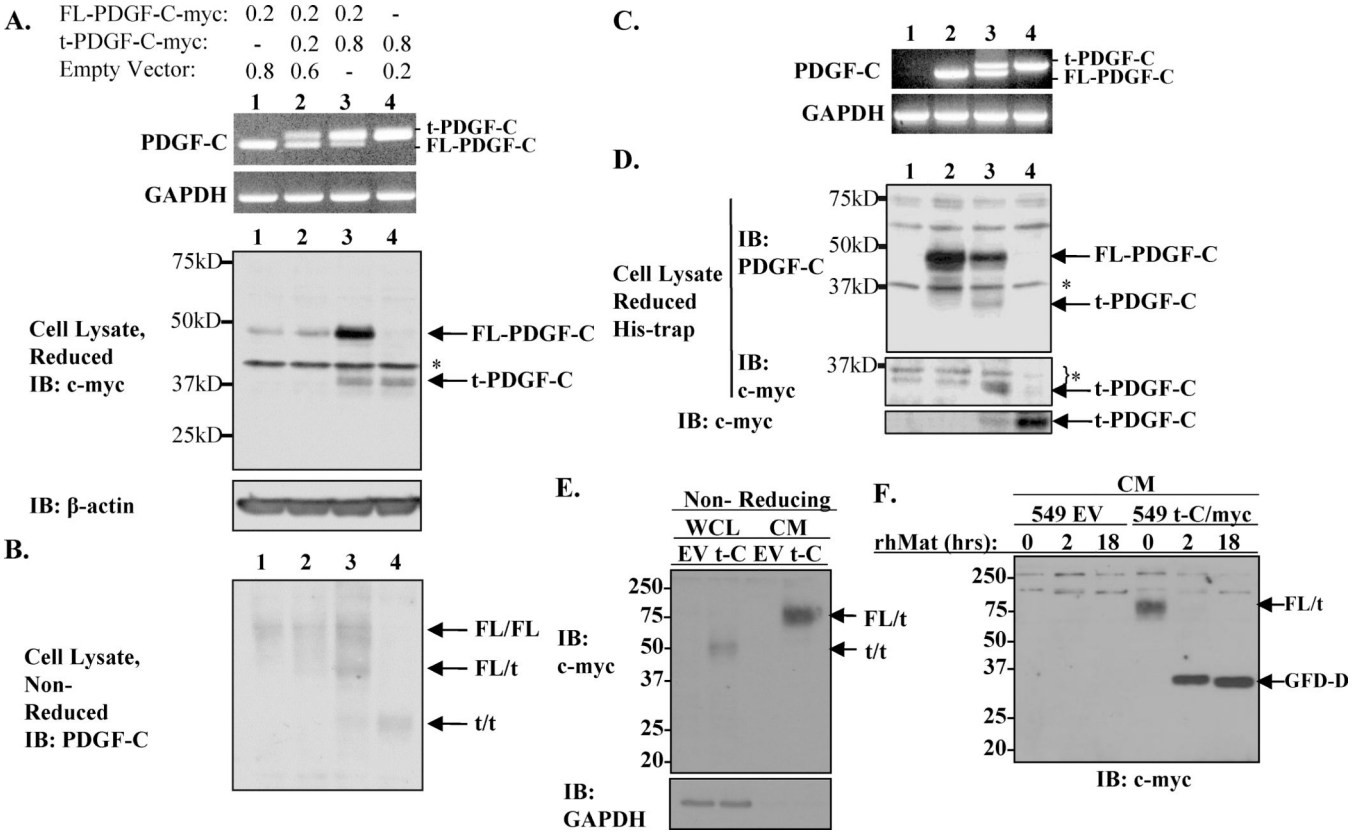

Figure 2. t-PDGF-C can heterodimerize with FL-PDGF-C, and FL-PDGF-C-containing dimer can be secreted.

(A) RT-PCR analysis of PDGF-C in COS-1 cells transiently transfected with different amounts (μg) of FL-PDGF-C-myc, t-PDGF-C-myc, and/or control vector. Immunoblot analysis of transfected PDGF-C in cell lysates using anti-c-myc Ab in a reducing condition (middle panel) and in cell lysates using anti-PDGF-C/GFD Ab in a non-reducing condition **(B)**. **(C)** RT-PCR analysis of PDGF-C in COS-1 cells transiently transfected with control vector (lane 1), FL-PDGF-C-His (lane 2), or FL-PDGF-C-His and t-PDGF-C-myc (lane 3), or t-PDGF-C-myc (lane 4). **(D)** Immunoblot analysis of PDGF-C in His-tag pull down samples of cell lysates in a reducing condition using anti-PDGF-C/GFD Ab (top panel) or anti-c-myc Ab (middle panel). Immunoblot analysis of total cell lysates in a reducing condition using anti-c-myc Ab (bottom panel). Immunoblot (IB) analysis of **(E)** Whole Cell Lysate (WCL) 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 t-C/myc). **(F)** Immunoblot analysis of conditioned media samples after incubation with recombinant human matriptase for the indicated times. Asterisks indicate non-specific bands.

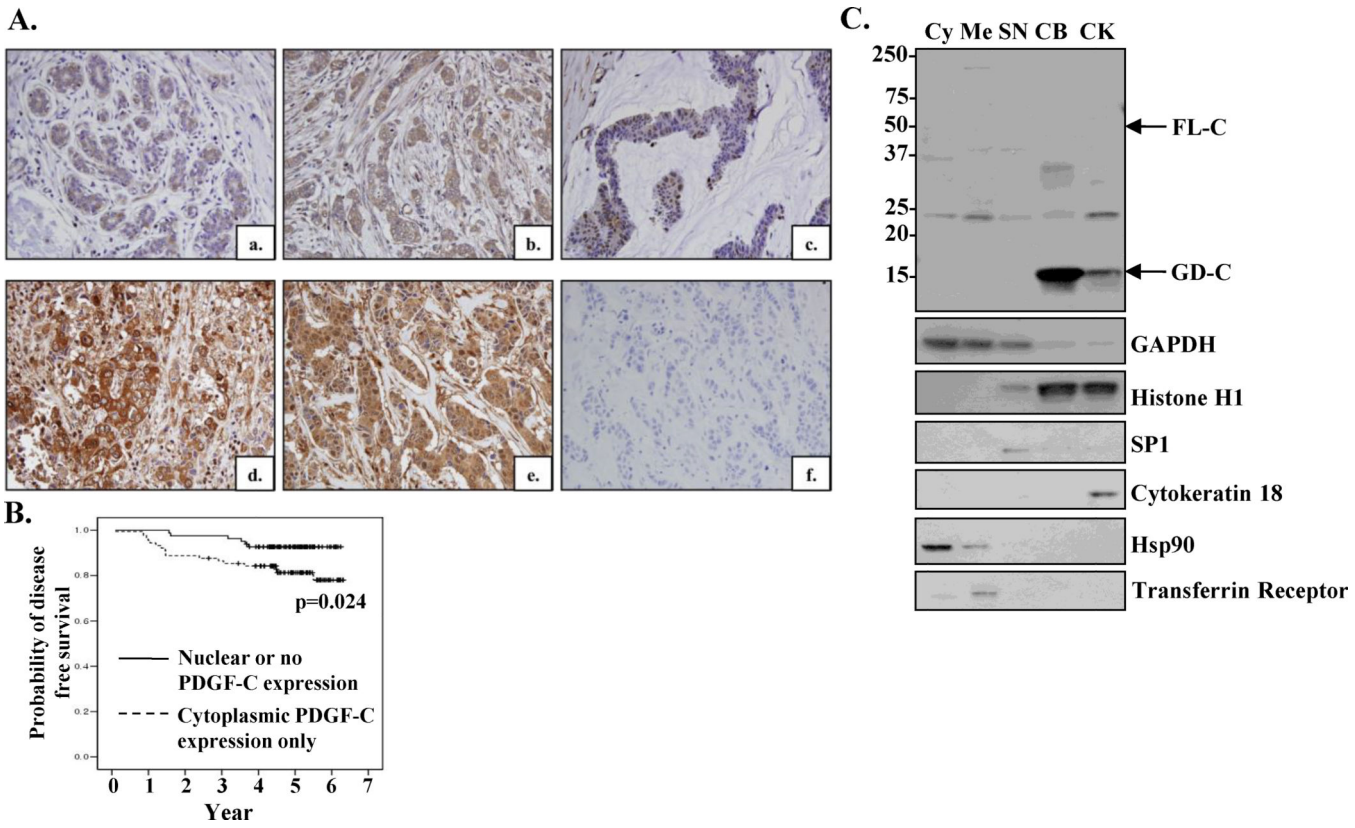

Figure 3. Nuclear localization of PDGF C.

A) (a) A representative image of PDGF-C expression in normal breast tissue. Epithelial cells show weak cytoplasmic expression [immunoreactive score (IRS), 2]. (**b-e**) Representative images of PDGF-C expression in breast cancers: (**b**) An invasive breast cancer with weak cytoplasmic expression (IRS, 3). (**c**) A case of mucinous carcinoma exhibiting moderate nuclear expression (IRS, 4). (**d**) An invasive breast cancer showing strong cytoplasmic expression with rare nuclear expression (IRS, 9). (**e**) An invasive breast cancer with moderate nuclear and cytoplasmic expression (IRS, 4 for nucleus, 6 for cytoplasm). (**f**) Negative control of (e). **B**) Kaplan-Meier survival curve showing the probability of disease-free survival for patients with invasive breast cancer in relation to the expression of PDGF-C. **C)** Reducing immunoblot of T47D parental subcellular fractions probed with the indicated antibodies. Cy= Cytoplasmic, Me= Membrane, SN= Soluble Nuclear, CB= Chromatin Bound Nuclear, CK= Cytoskeletal.

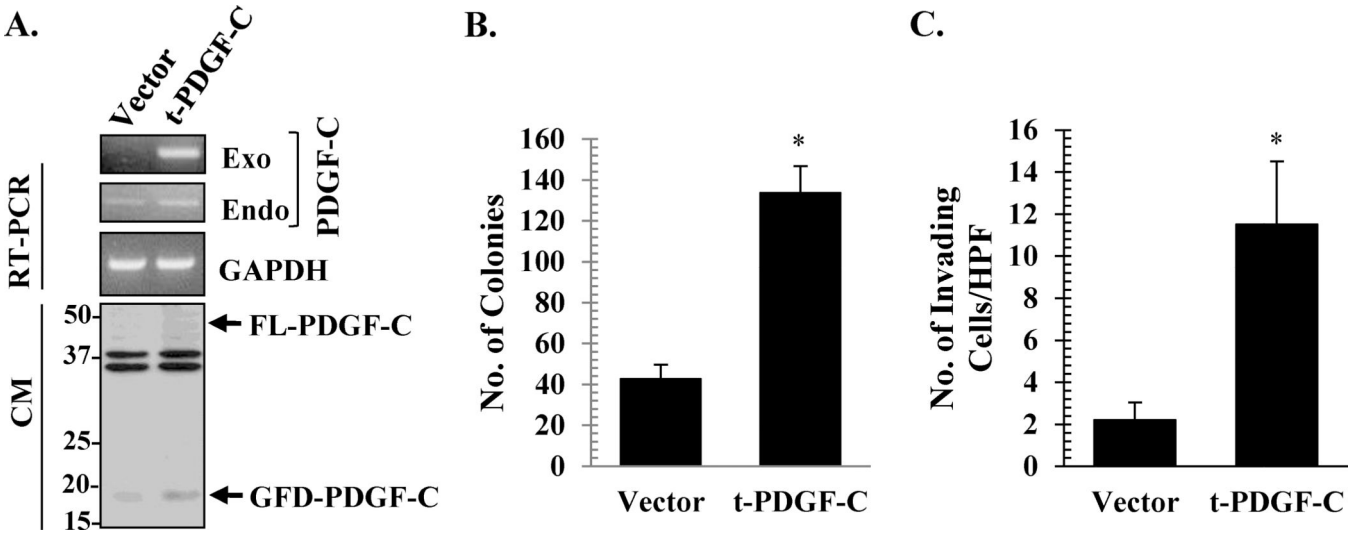

Figure 4. t-PDGF-C promotes phenotypic transformation of breast carcinoma BT-549.

A) RT-PCR analysis of PDGF-C mRNA expression using t-PDGF-C-specific primers, endogenous PDGF-C, or GAPDH (top panels) and immunoblot analysis of the PDGF-C protein levels in conditioned media (CM, bottom panel) in BT-549 cells transfected with control or t-PDGF-C expression vector. GFD-PDGF-C: Growth factor domain of PDGF-C; FL-PDGF-C: Full length of PDGF-C. Soft agar analysis **(B)** and Matrigel invasion **(C)** were performed on BT-549 breast cancer cells. Data are means of triplicates of three independent experiments ± SD. * p-value<0.05.

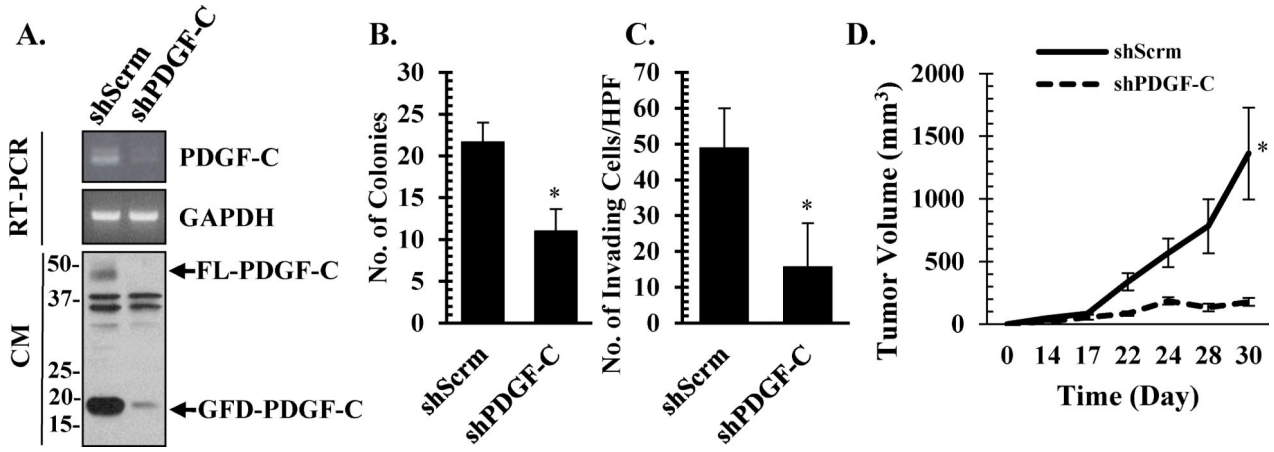

Figure 5. PDGF-C knockdown abrogates transformed phenotypes of MDA-MB-231 cells.

A) RT-PCR (top panel) and immunoblot (bottom panel) analyses of PDGF-C in control (shScrm) and PDGF-C knockdown (shPDGF-C) MDA-MB-231 cells. GFD-PDGF-C: Growth factor domain of PDGF-C; FL-PDGF-C: Full length of PDGF-C. Soft agar **(B)** and Matrigel invasion **(C)** assays were performed on control (shScrm) and PDGF-C knockdown (shPDGF-C) MDA-MB-231 cells. Data are means of triplicates of three independent experiments ± SEM. * p-value<0.05. **D)** *In vivo* tumor growth of shScrm- and shPDGF-C-MDA-MB-231 cells were measured at the indicated time points following orthotopic injection into the mammary fat pad of female NOD/SCID mice (6 and 7 mice in shScrm and shPDGF-C groups, respectively). Data are mean ± SEM. * p-value<0.05.