Investigation of the Role of Platelet-Derived Growth Factor (PDGF) in the Development of Breast Carcinomas

Christina Michele Griffin
B.S. Biochemistry, 2000 Xavier University of Louisiana

Submitted to the Department of Biology in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE
at the Massachusetts Institute of Technology,
June 2002

Copyright © 2002 by Christina M. Griffin. All rights reserved. The author hereby grants to MIT permission to reproduce and to distribute publicly copies of the thesis document in whole or in part.

Signature of Author:
Department of Biology, June 7, 2002

Certified by: Robert A. Weinberg, Professor of Biology, Thesis Supervisor

Accepted by: Alan Grossman, Chairman, Graduate Committee
Acknowledgements

I would like to thank my advisor, Robert A. Weinberg, for his encouragement and never-ending support. I would also like to thank the entire Weinberg lab especially, Dr. Lisa Spirio, Dr. Ittai Ben-Porath, Piyush Gupta, and Ben O’Conner for their smiles and constant supply of amusement. I have to give a special thanks to my family. Through so many years they have supported all of my ventures, from moving me across the country (several times) to always being empathetic and understanding even when I was at my worst. They are my foundation, my rock, and inspiration. Finally, I have to thank God for His eternal blessings of direction and strength. After this experience, I know that in Him I can do anything.
Contents

I. Tumorigenic Breast Model
II. Platelet-Derived Growth Factor (PDGF)
III. PDGF-A
IV. PDGF-B
V. PDGF-C and PDGF-D
VI. Receptor Expression and Effects of PDGF
VII. General Hypothesis
VIII. Theoretical and Experimental Plan: Stromal Activation
IX. PDGF is Not a “Lone” Activator
X. Results
XI. Figures

Table 1. PDGF in HMLER Tumor Formation in Nude Mice

Figure 1. Diagram of PDGF Receptors and Ligands

Figure 2. PDGF Ligand Gene Structures

Figure 3. Diagram of Stromal-Epithelial Interaction in Tumorigenesis

Figure 4a. Exon Structure of PDGF-B

Figure 4b. Protein Structure of Mature and Immature PDGF-B

Figure 5. Growth Curve of HMLERs With and Without PDGF-B Expression

Figure 6. RT-PCR of Several PDGF Ligand and Receptor mRNA Expression in Range of Breast Cancer Cell Lines

Figure 7. RT-PCR of PDGF-C Ligand mRNA Expression in Range of Breast Cancer Cell Lines

XII. Conclusions

XIII. References
Tumorigenic Breast Model

Breast tumors are composed of epithelial tumor cells combined with various stromal components, including extracellular matrix (ECM), endothelial cells, smooth muscle cells, immune system cells, and mainly fibroblasts (1, 2). These stromal components, which create the physiological environment of the tumor, often comprise the majority of the tumor mass. Relative to normal stroma, the stroma, that is specific to tumors, is characterized by specific changes in cellular morphology and extracellular matrix composition, such as increased expression of smooth muscle actin. This “activated” stroma is often defined as being desmoplastic (1, 2, 3).

There is evidence to suggest that the stroma, through paracrine interactions with the tumor cells, plays an essential role in tumor development (3, 4). Perhaps in the intermediate stages of multi-step tumor development, carcinoma-cells recruit stroma through paracrine signals that attract and also provide proliferative stimuli for fibroblasts and other stromal components. Factors released in turn by the stromal components in response to the carcinoma-derived signals may in the context of a tumor contribute to carcinoma cell survival, proliferation, and invasive capacity.

The fact that most human carcinoma cells cannot be easily propagated as pure populations in culture suggests that their growth may be dependent on stromal supporting factors. Experiments involving subcutaneous injections of human tumor cells into mice as tumor models demonstrate that fibroblasts, when co-injected with tumor cells, decrease the latency of tumor formation (3). Another indication that the stroma interacts with the tumor in promoting tumor formation comes from the finding that injection of
tumor cells into an orthotopic site, such as the mammary gland, promotes their tumorigenic abilities (5).

Unfortunately, little is known about the specific signals produced by carcinoma cells that recruit fibroblasts and other stromal components. In addition, current knowledge of the paracrine supporting signals which stromal components provide to the carcinoma cells is very limited. A major obstacle in the study of these paracrine interactions between the carcinoma cells and the stromal cells is the lack of experimental systems in which a stromal contribution can be studied independently of the contribution of the tumor (Fig. 3).

One difficulty in studying the origin of cancer has been the lack of human tumor cell lines of defined genetic backgrounds. Our lab has previously developed a model for tumor progression through the introduction of defined genetic elements into normal, primary human cells. Using the approach of ectopically expressing SV40-Large T antigen, which inactivates p53 and Rb tumor suppressor proteins, small t-antigen (which inactivates the PP2A phosphatase), hTERT (catalytic subunit of telomerase), and H-ras V12 (constitutively active mutant) (3, 6) allows for the creation of genetically defined human tumor cell lines. SV40-Large T antigen inactivation of p53 and Rb allows cells to proliferate past replicative senescence. The precise role of PP2A in promoting tumorigenesis has still to be elucidated. Telomerase (hTERT) prevents the shortening of the telomeres on the ends of DNA and allows the cells to bypass crisis. H-ras V12, a human oncogene, encodes a protein that signals into various growth pathways, and when ectopically expressed in cells, constitutively signals for continuous cell proliferation. Together, this work has shown that perturbing these five genetic pathways is the
minimum requirement for tumorigenesis in human cells. The tumorigenicity of these cells depends on expression of oncogenic ras at high levels. However, they do not recruit a substantial stromal component. Perhaps the lack of a desmoplastic response reflects a lack of paracrine signaling between the carcinoma cells and stromal cells via growth factors like the platelet-derived growth factor (PDGF).

It is of major importance to develop tumor models that simulate in vivo breast tumors, where the interactions between the carcinoma cells and the stromal cells are recapitulated and can therefore be studied. One approach to achieve this end is to generate tumor cells that secrete factors that act in a paracrine manner to recruit the stroma and possibly initiate the desmoplastic response. One approach to this experimentally is to generate tumor cells that secrete PDGF. Since epithelial cells do not generally express the platelet-derived growth factor receptor (PDGFR), this factor should act as an attractant and mitogen specifically for stromal cells and not affect carcinoma cells (7, 8). Hence, PDGF can be utilized as a tool to recruit stroma and to create a model for the study of tumor paracrine interactions.

This focus on PDGF might appear arbitrary. However, an extensive body of research shows that PDGF is the most prominent factor known to induce attraction and proliferation of fibroblasts, smooth muscle cells and macrophages; all of these are important components of the stroma (9). Second, in most common human carcinomas such as breast, colon, prostate, and lung, PDGF seems to act largely in a paracrine manner, since upregulation of the receptors, PDGFR-α and PDGFR-β, is observed primarily in the stroma (9, 10). Specifically, in breast cancer, PDGF expression levels in carcinoma cells rise with tumor progression, while high receptor levels are observed in
peri-epithelial stromal cells adjacent to PDGF-secreting carcinoma cells (11). PDGF is thus a major candidate for playing a paracrine role in signaling from tumor cells to their environment, and also can be used as an experimental tool for the manipulation of the stroma by tumor cells.

**Platelet-Derived Growth Factor (PDGF)**

Early tissue culture work demonstrated the superiority of serum over plasma in stimulating the proliferation of fibroblasts in vitro (12). These observations suggested that a factor released from platelets during degranulation was probably responsible for this stimulatory activity. Subsequent investigations clearly demonstrated that a factor released from platelets upon clotting was capable of promoting the growth of various types of cells (13, 14). This factor was subsequently purified from platelets and given the name platelet-derived growth factor (PDGF) (15, 16). PDGF is now known to be produced by a number of cell types besides platelets and has been found to be a mitogen and/or chemoattractant for almost all mesenchymally-derived cells and connective tissue cells (17).

As with many growth factors, PDGF belongs to a large family of similarly structured proteins (18, 19). In addition to PDGF, this family includes the homodimeric factors vascular endothelial growth factor (VEGF) and connective tissue growth factor (CTGF), a PDGF-like factor secreted by human vascular endothelial cells and fibroblasts (20). PDGF can also form heterodimers with VEGF (18). Relative to the various PDGF isoforms, VEGF is closely related to an isoform of PDGF-BB. CTGF shows little amino acid identity with PDGF-A or -B, but reacts with antiserum produced against PDGF (20).
Until recently, PDGF was thought to consist of two different but closely related chains (A and B chains) which assemble to form disulfide linked homo- or heterodimers (PDGF-AA, PDGF-AB, and PDGF-BB) (9, 21). Alternative splicing of the PDGF-A chain transcript can give rise to two distinct forms that differ only in their C-terminal end (22). The transforming protein of simian sarcoma virus (SSV), encoded by the v-sis oncogene, is derived from PDGF-B. Both PDGF-A and PDGF-B proteins have a retention domain at the carboxy-terminal regions that must be cleaved proteolytically to be a mature, soluble protein. In May of 2000, two more chains of PDGF (C and D) were discovered. In contrast to PDGF-A and PDGF-B, however, both PDGF-C and PDGF-D have so-called CUB domains in their amino-terminal region (23). CUB domains occur in a wide range of secreted proteins that regulate growth and development including VEGF (23). The CUB domains are thought to anchor proteins in the extracellular matrix. In addition, PDGF-C and PDGF-D can form homodimers (PDGF-CC and PDGF-DD) like PDGF-A and PDGF-B, however it is not known if they form heterodimers with each other or with PDGF-A and PDGF-B (21, 24).

Dimerization of the PDGF chains involves two inter-chain disulfide bonds (9, 25). PDGF is structurally related to a number of other growth factors that also form disulfide-linked homo- or heterodimers (Fig. 1). PDGFs act as dimers to cause dimerization and activation of their cell surface receptors, PDGFR-α and PDGFR-β (26). PDGF-AA, -BB, -AB, -CC all can activate PDGFR-αα receptors whereas -AB, -BB, -CC, and-DD dimers activate PDGFR-αβ and PDGFR-ββ receptors (9, 21, 24) (Fig. 2).
**PDGF-A**

The A chain is the product of a seven exon chromosomal 7 gene that gives rise to one of two differentially-spliced variants: the long and the short (144-162 aa; 16-18 kDa). The difference between the long and short forms is the result of alternative exon usage. Failure to remove the carboxy-terminal peptide, also known as the retention domain, results in a failure to release freely circulating PDGF because it remains retained in the intracellular space (27, 28). Retention implies binding to either cell-surface glycosaminoglycans or intercellular matrix (22). The short version contains no retention sequence and is secreted into the circulation (22). Expression of PDGF-A can be found in many mesenchymal types including fibroblast, endothelial cells, smooth muscle cells, macrophages, mammary epithelial cells and several tumor cell lines (HT-1080, CHRF) (9, 23).

**PDGF-B**

The B chain is the product of a six exon gene on chromosome 22. The B chain gene is related to the human c-sis gene, the normal human cell counterpart to the monkey v-sis (simian sarcoma) virus gene (9). The C-terminal cleavage of the immature B chain into the mature, soluble B chain occurs, resulting in a final mature product of 109 aa and a protein size of 28 kDa (28) (Fig. 4). This 28 kDa mature B chain can then homo- or heterodimerize with another B chain or an A chain. As is the case with the long form of chain A, a retention sequence of approximately 51 aa residues in length has also been identified in the B chain C-terminus (9). Failure to remove this peptide also results in B chain intracellular retention (28). Expression of PDGF-B can be found in several types of mesenchymal cells including fibroblasts, keratinocytes, endothelial cells and smooth
muscle cells. PDGF-B is even expressed in some epithelial mammary cell types and several carcinoma cell lines (BT-474, SW480, PC-3) (9, 23).

**PDGF-C and PDGF-D: New PDGF Ligand Members**

The C chain of a six exon gene on chromosome 4 and the D chain has 7 exons on chromosome 11-23. The N-terminal exon 2 and 3 for both PDGF-C and PDGF-D encode the CUB domain that anchors the translated protein to the extracellular membrane of the cell (Fig. 2). The PDGF/VEGF homology domain is the last two exons for both genes (23). Expression of these novel proteins can be found in both epithelial and mesenchymal type cells including some tumor cell lines (BT-474, PC-3, TF-1) (23).

**Receptor Expression and Effects of PDGF**

Binding of PDGF to its receptors is known to activate the intracellular tyrosine kinase domain of these receptors, leading to autophosphorylation of the cytoplasmic domain of the receptor as well as trans-phosphorylation of other intracellular receptors (26) (Fig. 1). Specific substrates that bind the SH2 groups on the cytoplasmic domain identified with the β-receptor include Src, GTPase Activating Protein (GAP), phospholipase Cγ (PLCγ) and phosphatidylinositol 3-phosphate (19, 26, 29, 30). Both PLCγ and GAP seem to bind with different affinities to the α- and β-receptors, suggesting that the particular response of a cell depends on the type of receptor it expresses and on the type of PDGF dimer to which it is exposed (30).

In general, PDGF isoforms are potent mitogens for connective tissue cells and for some epithelial and endothelial cells (31). In addition to its activity as a mitogen, PDGF is chemoattractant for fibroblasts, smooth muscle cells, neutrophils and mononuclear cells; these are cells that also respond mitogenically to PDGF (32). Because there are
differences between cells with respect to the amounts of α- and β-receptors they express, and because of the variability in PDGF isomer binding to receptors, there is a wide range of possibilities for the biological responses elicited by PDGF (8, 32).

Cells expressing only β-receptors include Central Nervous System (CNS) capillary endothelium, neurons and monocytes/macrophages (19). Cells showing coincident expression of α- and β- receptors include smooth muscle cells and fibroblasts (9). Vascular smooth muscle cells (SMC) and fibroblasts are both known to express both the α- and β-receptors. In SMC, PDGF-AA initiates increased protein synthesis, while -BB induces hyperplasia. In fibroblasts, the -BB isoform initiates chemotaxis, while -AA inhibits chemotaxis (29, 32). Cells known to express only α-receptors include oligodendroglial progenitors and platelets (33). There is also a considerable body of evidence to indicate that PDGF derived from macrophages, acting as a chemoattractant and mitogen for smooth muscle cells, contributes to the thickening of arterial walls characteristic of atherosclerosis (9).

**General Hypothesis**

It is my hypothesis that the stromal fibroblasts in tumors play a promoting role in tumor progression. These fibroblasts are actively recruited by the carcinoma cells through a paracrine signal like PDGF, and, in response, secrete factors such as TGF-β and VEGF that enhance tumor progression into the invasive and metastatic states.

**Theoretical and Experimental Plan: Stromal Activation**
The purpose of the following hypotheses and experiments is to utilize genetically defined breast cancer cells as a system to recruit and elicit a stromal contribution mimicking that observed in *in vivo* breast cancer. The effect of this recruited stroma on the tumorigenic properties of the tumor cells will be studied in this system. In addition, this system will be utilized to identify paracrine signals exchanged between tumor cells and the associated stroma and to study their role in tumor progression. These identified paracrine signals may give a more detailed account of the complex relationship between tumor cells and the surrounding stroma.

It has been difficult to appraise the role of stroma in tumor progression due to lack of defined experimental systems where stromal participation can be controlled. Primarily, I will attempt to create such a system using genetically defined human breast cancer cells developed in our lab as models. Thus, a series of genetic elements in virus-based expression vectors have been introduced into normal primary human mammary epithelial cells (HMECs) including the SV40 large T-antigen (disrupting the function of cellular p53 and Rb proteins), the small T-antigen (disrupting protein phosphatase PP2A function), the telomerase catalytic activity subunit hTERT, and the ras oncogene; these are named HMLER cells (3). The tumorigenic ability of these cells is dependent on relative ras expression levels compared with endogenous ras levels. Thus, cells with high-ras expression develop tumors upon subcutaneous injection into nude mice within 6-9 weeks, while cells expressing low-ras are non-tumorigenic upon injection but display anchorage-independent growth. Cells with no-ras are non-tumorigenic and anchorage-dependent. The tumors that develop from the high-ras expressing cells, in contrast to *in vivo* breast tumors, do not activate nor incorporate a large amount of the surrounding...
stroma. However, co-injection of these cells together with primary mammary fibroblasts decreases tumor formation latency, suggesting that stromal support can enhance tumor development (3).

In order to induce stromal activation, I will introduce a viral expression vector of the PDGF-B gene into the three forms of the described HMLER cells (no-ras, low-ras, and high-ras). PDGF-B is a strong attractant and mitogen for fibroblasts, smooth muscle cells and macrophages, which generally express the PDGF receptors (9). Human PDGF interacts with the mouse PDGF receptors. Hence, its expression is expected to have both a migratory and proliferative stimulus on these mesenchymal cell types adjacent to the developing carcinoma (8, 34, 35). In order to attain the full effect of PDGF-B in these carcinoma cells, I will introduce the PDGF-B gene into the HMLER cells both in its full cDNA (B-long) form and in a truncated form (B-short) where a retention domain 51 aa long at the carboxy-terminus, which acts to anchor the immature form of PDGF to the cell membrane or ECM, is deleted (28). This version encodes a soluble form of PDGF not requiring any additional proteolysis, and may exert longer distance effect (Fig. 4).

The PDGF-B-expressing carcinoma cells and their corresponding control cells lacking PDGF-B will be injected subcutaneously into immuno-compromised mice (NOD-SCID). In addition, injections will be performed orthotopically into the mammary fat pads of mice to provide a stromal environment more like the human breast. This environment may well simulate more effectively the mammary stromal environment. All cells will be co-injected subcutaneously and orthotopically in a mixture with a medium containing ECM components (Matrigel), which has been shown to enhance cell survival following injection (3). It is expected that in at least one of the forms of injection,
preferably into an orthotopic site, developing tumors will incorporate a significant amount of stroma, thereby recapitulating the development of spontaneously arising human breast tumors. Latency and size of tumors will be monitored and will be histologically examined for incorporation of stromal components compared to controls. Immunostaining for SV40 large T-antigen will allow visual separation of carcinoma cells from stromal cells. Also, markers characteristically expressed in activated breast tumor stroma, such as α-smooth muscle actin and MMP9, will be used to stain tumor sections (36).

Even if PDGF increases the amount of incorporated stroma in HMLER tumors, can PDGF expression enhance tumorigenesis? The hypothesized strictly paracrine role of PDGF may or may not be a sufficient step in the tumorigenic process to bypass the requirement of high-ras in our system (37). To address this potential insufficiency, the kinetics of tumor development using the HMLER system will be monitored. I hypothesize that stromal participation, induced by PDGF expression, will enhance the tumorigenicity of tumorigenic, high and low-ras expressing cells, resulting in lower tumor latency and possibly in the formation of highly invasive and metastatic tumors (38, 39). Also, stromal participation may confer tumorigenicity on the non-tumorigenic cells, expressing no-ras. Such a result will indicate that high-ras expression can be replaced by supporting signals supplied by the incorporated stroma and will suggest that a similar interactive dependence occurs in in vivo breasts cancers.

I would have to demonstrate that non-tumorigenic carcinoma cells, low-ras HMLERs, become tumorigenic when they secrete PDGF. While PDGF secreting cells do not demonstrate any advantage in growth in vitro in comparison to control cells, upon
injection into nude mice they rapidly develop into tumors, while the control cells do not
(Ben-Porath, unpublished) (Table 1).

It is essential that any enhancement in tumorigenicity of PDGF-expressing cells is
a result of a paracrine effect and not of an autocrine effect. I have verified using Reverse
Transcription-Polymerase Chain Reaction (RT-PCR) that HMLERs, unlike most
epithelial cells, express a small amount of the PDGF-β receptor, yet are not expected to
respond to ectopically expressed PDGF-B, especially in the short form (Fig. 6) (9, 40).
Hence, the in vitro growth characteristics in high and low conditions (with and without
serum) and anchorage-independent growth ability (soft agar) of the cells will be studied
to rule out any effects that PDGF autocrine secretion may exert on the carcinoma cells
themselves (Fig. 5). Also, the possible activation of PDGF β-receptors will be assayed
by any tyrosine-phosphorylation of the PDGF-β receptor. If the PDGF β-receptor
expression prevails as a problem, use of a dominant-negative PDGF β-receptor
(truncated) will be used to eliminate this effect (41, 42).

However, another approach may be taken to solve any paracrine versus autocrine
problem. Instead of expressing PDGF-B chain in the HMLERs, expressing the PDGF-C
chain may be implemented. PDGF-C has been shown to be a transforming mediator of
the formation of pediatric solid tumors characterized by aberrant transcription factors also
known as Ewing’s Sarcomas (EWS/FLI) driven oncogenesis (10). PDGF-C is expressed
in HMLERs can be oversxpressed over endogenous levels in a similar fashion as PDGF-
B (Fig. 7). Furthermore, PDGF-C has an equally strong, if not stronger, mitogenic effect
on fibroblast compared to PDGF-B but does not bind to the PDGF-ββ receptor (21).
Expressing PDGF-C in the same manner as PDGF-B should elicit the same stromal response as PDGF-B.

Another way to test the idea that stromal support is required for tumor development and/or continuous stromal recruitment and feedback is the use of PDGF inhibitors or antibodies. In the manner of a previous experiment, mice will be treated with PDGF dominant-negative ligand following initial formation of small tumor nodules (44). Tumor development will be monitored and tumor histology will be analyzed to determine whether inhibiting PDGF signaling decreases the stromal activation as assayed by increased expression of smooth muscle actin and/or any effects on tumor progression (36). An alternative approach that can be taken to address the necessary involvement of PDGF in tumorigenesis involves the development of an inducible PDGF expression system, such as an inducible PDGF system using a tetracycline-controlled system allowing for elimination of PDGF expression by treatment of mice with tetracycline.

**PDGF is Not a “Lone” Activator**

I have hypothesized that during tumor progression carcinoma cells and stromal cells interact through various paracrine signals, leading to a highway of feedback signals from carcinoma cells to stromal cells and vice versa that is sufficient for tumor development and outgrowth (45). The above described tumor model will serve as a system where stromal participation is experimentally induced by PDGF and is shown to enhance tumor development. Therefore, although the initial paracrine signal emanating from the epithelial cells is PDGF, it is necessary to assume that the signal interactions
established between the stroma and epithelium in this system consist of multiple paracrine signals mimicking the signals found in in vivo tumors.

Based on others’ experiments and the known activity of PDGF in tumors, it is likely that PDGF will induce stromal participation in tumor formation (29). However, if that is not the case, additional secreted factors that are candidates for stromal recruitment will be incorporated into this model. Possibly, a combined effect of several factors is required. The main candidates are transforming growth factor-beta (TGF-β) and the vascular endothelial growth factor (VEGF). The former is implicated in the activation of stroma, while the latter is a well-established angiogenic factor (46, 47, 48). Both of these factors are currently being studied in the laboratory in the context of other projects, and expression vectors and expressing cells exist. To form a more comprehensive picture of these interactions, there is a need to examine PDGF in conjunction with other stimulating paracrine factors, such as VEGF and TGF-β (49, 50). Many papers have described the expression of these growth factors and their corresponding receptors on both cell populations in primary breast tumors and breast tumor cell lines (51, 52). And others have implicated VEGF and TGF-β in cell transformation and angiogenesis (53, 54).

In order to begin looking at the relationship of TGF-β and VEGF with PDGF, there are several simple experiments that can be done. First, Western blot analyses and RT-PCRs for VEGF and TGF-β protein and RNA will be performed on PDGF-expressing HMLERs along with their controls to determine any increase of the expression of these factors. The vascular endothelial growth factor receptor (VEGFR) is known to be expressed by platelets and TGF-β has been implicated in chemotaxis along
with PDGF (50, 55). However, although VEGF and TGF-β may be expressed by HMLERs, they may not be secreted in an active form. Thus, ELISAs for VEGF and TGF-β will be performed in the PDGF-expressing and control cells to quantify the amount of active and inactive forms of these factors. In addition, ELISAs can be performed for PDGF in VEGF- and TGF-β-expressing cells (which can be supplied by colleagues in the lab). Correlations between the expression of these factors and the tumorigenic ability of the PDGF-expressing cells in the previously mentioned experiments would help to narrow the scope of secreted factor involved in tumorigenesis.

In addition, the previous references to PDGF, VEGF, and TGF-β roles in angiogenesis and metastasis lead the way to more experiments (50, 54). Perhaps two or three of these factors can be co-expressed in HMLERs so that in vitro and in vivo studies as described in the series of PDGF experiments above can be undertaken to elucidate a role that these three factors have in tumor neo-vascularization and metastasis (38).
Results

*PDGF-B Expression in HMLERs*

To investigate if the expression of PDGF-B in HMLER carcinoma cells can confer a stromal reaction, HMLERs were infected with human PDGF-B long chain or short chain ligands. For the PDGF-B long chain a 241 aa cDNA insert containing the entire length of human PDGF-B (28 kDa) was inserted into the EcoRI site of the pBlast mammalian vector. For the PDGF-B short chain a 109 aa cDNA insert of human PDGF-B was inserted into the same EcoRI site of the pBlast mammalian vector (Fig. 4). Blast-PDGF-B(short) and Blast-PDGF-B(long) were grown up in competent bacteria and selected with ampicilin. Selected plasmids were then transfected into 293T cells with pCL101 viral packager and Fugene (Roche). Mock transfections were performed with pGFP and the pBLAST vector lacking an insert. Supernatants were then used to infect HMLE, HMLE-HR and HMLE-PR cells. Positively infected cells were selected with Blast (1ug/mL) for five days. Infected cells were assayed for horizontal transfer by infecting 10T-1/2 with HMLER supernatants and selecting with Blast (1ug/ml). HMLER-PDGF-B cells will be checked for PDGF-B chain expression by Western Blot Analysis and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR).

*PDGF β-receptor expression in Breast Cancer Cell Lines*

Now that these HMLERs express the PPDGF-B chain ligands, the PDGF-B could possibly have an autocrine effect on the HMLERs. Thus, we examined the expression of PDGF β-receptor mRNA in several breast cancer cells lines varying in range of tumor formation and invasive ability. RT-PCR assays were performed with GAPDH control.
Indeed, low levels of PDGF -receptor mRNA were detected in MCF10A, HMLE-HR, and HMLE-PR cells (Fig. 6). However, this preliminary data would not be enough to determine the occurrence of an autocrine effect. So, in addition to the mRNA data, protein expression data of the PDGF -receptor would need to be elucidated by Western Blot Analysis or ELISA. In addition, to determine any effect the expression of PDGF-B may have on the HMLER PDGF-B-expressing cells, in vitro data would have to be obtained. Growth curves at both high conditions (10% DME) and low growth conditions (no DME) would have to be done (Fig. 5). Also phospho-kinase assays to determine PDGF -receptor activation in HMLER-PDGF cells.

**PDGF-C Expression Profile in Breast Cancer Cell Lines**

Since, the PDGF-B expression in the HMLERs may activate the PDGF β-receptors in the HMLERs, it would be advantageous to find another PDGF ligand that does not react with the PDGF β-receptor. To find the best-suited ligand for our model, we checked not only for PDGF β-receptor expression, but also PDGF α-receptor expression. Only the MD-MBA-468 cells express PDGF α-receptor mRNA, any ligand that binds the PDGF α-receptor would be ideal for our system (Fig. 6). Thus, we chose PDGF-C ligand to express in HMLERs as an alternative to PDGF-B. We profiled PDGF-C mRNA expression in several breast cancer cell lines by RT-PCR and found that it was expresses in all except SKBR3 and T177 (primary breast carcinoma; gift of Charlotte Kuperwasser) (Fig. 7).
<table>
<thead>
<tr>
<th>Low-ras PDGF cells:</th>
<th></th>
<th>With Matrigel:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Take</td>
<td>Latency</td>
<td>Take</td>
</tr>
<tr>
<td>Hygro</td>
<td>0/9</td>
<td></td>
<td>0/6</td>
</tr>
<tr>
<td>Hygro-L</td>
<td>3/9</td>
<td>40-90</td>
<td>6/9</td>
</tr>
<tr>
<td>Hygro-S</td>
<td>5/9</td>
<td>40-50</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High-ras PDGF cells:</td>
<td></td>
<td>With Matrigel:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Take</td>
<td>Latency</td>
<td>Take</td>
</tr>
<tr>
<td>Puro</td>
<td>2/9</td>
<td>90</td>
<td>5/9</td>
</tr>
<tr>
<td>Puro-L</td>
<td>3/9</td>
<td>50-70</td>
<td>4/9</td>
</tr>
<tr>
<td>Puro-S</td>
<td>3/9</td>
<td>45-60</td>
<td>2/9</td>
</tr>
</tbody>
</table>

Table 1 Low-ras and high-ras tumor formation in nude mice with and without Matrigel. Also, with and without PDGF-B (long) or (short). Take is tumor/injections and latency is in day.
Fig. 1 Processing and action of platelet-derived growth factor (PDGF) isoforms. A-, B-, C-, D- chains of PDGF are synthesized as precursor molecules that form dimers by disulfide-bonds and undergo proteolytic processing: cleavage of the C-terminal end for A- and B- and excision of CUB domain for C- and D-. Different PDGF isoforms bind to and dimerize α- and β-receptors with different specificities. Receptors extracellular parts contain 5 Ig-like domain; ligands bind to 3 outer-most domains.
Fig. 2 The gene structure for all known PDGF ligands. Black, N polypeptide; red: CUB domain; white: dimerization domain; grey: retention domain. For PDGF-A there are alternative splice isoforms: one at exon 6 (211) and one at exon 7 (196).
Fig. 3 Diagram of the stromal-epithelial interaction that may happen in vivo. Carcinoma cells export increasing amounts of PDGF ligand which activates PDGF receptors on peri-epithelial stromal cells. The increased activation of PDGF receptors, turn, promotes growth factor synthesis (VEGF, TGF-β, etc) which then stimulates the carcinoma cell proliferation, migration, and increase invasive ability.
Fig. 4a Exon structure of PDGF-B.
Fig. 4b Protein Structure of PDGF-B. Red: signalling peptide; black: N-polypeptide; white: dimerizing region of PDGF-B; grey: retention domain with a stop codon at 241 aa. As a monomer, mature PDGF-B is 28 kDa and as a dimer is 56 kDa.
Fig. 5 Growth curve of Blast-PDGF-B(short) and Blast-PDGF-B(long) and controls without PDGF *in vitro* and at high conditions (10% DME).

Ren-Porath unpublished
Fig. 6 Reverse-Transcription Polymerase Chain Reaction showing levels of PDGF A-chain, PDGF α-receptor, and PDGF β-receptor mRNA expression in several breast cancer cells line. 10ug of cDNA were used GAPDH primers used to standardize. Exposure times vary.
<table>
<thead>
<tr>
<th>Cell Line</th>
<th>PDGF-C</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMLE3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMLE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF-7 ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD-MBA 453</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MD-MBA 468</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT-474</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKBR3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T47D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCF10A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMLE-HR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HMLE-PR</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S229</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 7 Reverse Transcription-Polymerase Chain Reaction showing levels of PDGF C-chain mRNA expression in several breast cancer cell lines. 10ug of cDNA were used GAPDH primers used to standardize.
Conclusions

Previously published evidence suggests the occurrence of stromal activation or a desmoplastic response in in vivo epithelial tumors (3). Many growth factors, including PDGF, have been implicated in the induction of a desmoplastic response in stromal cells surrounding the tumor via paracrine interactions with carcinoma cells (29). The work presented here demonstrates a very probable role of PDGF in the development of human breast carcinomas by stromal activation.

There are two conclusions that can be deduced from the preliminary experiments that have been performed and presented in this work. First, PDGF appears to have no affect on HMLER no-ras, low-ras, or high-ras carcinoma cell in vitro growth (Fig. 5). This suggests that despite the expression of PDGF-βR mRNA, there is little to no autocrine activation of PDGF-βR by the HMLERs. If there was autocrine activation, we would expect an increase in cell number compared to the control. This experiment would need to be repeated at high and low conditions (high serum and low serum).

Second, PDGF does appear to affect HMLER tumor formation by decreasing tumor latency and increasing tumor size. There was a dramatic difference in the PDGF-expressing HMLER low-ras carcinoma cells compared to the non-expressing PDGF-HMLER low-ras carcinoma cells (Table 1). This suggests that PDGF appears to have a paracrine effect on the tumor associated stroma. However, to determine whether PDGF is instrumental in causing stromal activation as proposed by the model in Figure 3, histology would have to be performed on all tumor sections. Staining tumor sections for α smooth muscle actin is one assay that can also be used to infer stromal activation (36). It would be interesting to see in PDGF-expressing HMLER high-ras carcinoma a change
in the stromal incorporation and/or morphology although we do not see a change in tumor latency and size.

However, if it appears that there is no change in the stromal component of our PDGF-expressing HMLER tumors, there are other experiments that can be performed in order to elucidate what may be the role of PDGF in human breast carcinomas. Correlations between the expression PDGF and other growth factors have been published in several papers (51, 53). The vascular endothelial growth factor (VEGF) and transforming growth factor-β (TGF-β) are two growth factors highly correlated with PDGF expression in carcinomas. PDGF, VEGF, and TGF-β are determined to have roles in tumorigenesis and angiogenesis in in vivo carcinomas (50, 54). It would be advantageous if the relationship between TGF-β and PDGF or VEGF and PDGF were investigated. Perhaps the role PDGF plays in human breast carcinoma development lies in cooperating with these other growth factors via a paracrine highway between carcinoma cells and stromal cells.
References


