Secretion of pleiotrophin stimulates breast cancer progression through remodeling of the tumor microenvironment

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Pleiotrophin (PTN, *Ptn*) is an 18-kDa secretory cytokine expressed in many breast cancers; however, the significance of *Ptn* expression in breast cancer has not been established. We have now tested three models to determine the role of inappropriate expression of *Ptn* in breast cancer. Mouse mammary tumor virus (MMTV) promoter-driven *Ptn* expressed in MMTV-polyoma virus middle T antigen (PyMT)-*Ptn* mouse breast cancers was first shown to induce rapid growth of morphologically identified foci of "scirrhous" carcinoma and to extensively remodel the microenvironment, including increased tumor angiogenesis and striking increases in mouse procollagens Iα2, IVα5, and XIα1, and elastin. Ectopic *Ptn* expression in MCF-7 (human breast cancer)-*Ptn* cell xenografts also was shown to markedly increase MCF-7-*Ptn* cell xenograft growth in nude mice; furthermore, it induced extensive remodeling of the microenvironment and tumor angiogenesis. In a coculture model of equal numbers of NIH 3T3 stromal fibroblasts and MCF-7-*Ptn* cells, PTN secreted from MCF-7-*Ptn* cells was then shown to induce a more malignant MCF-7-*Ptn* breast cancer cell phenotype and extensive remodeling of the MCF-7-*Ptn*/NIH 3T3 cell microenvironment; it up-regulated expression of markers of aggressive breast cancers, including PKCδ and matrix metalloproteinase-9 in both MCF-7-*Ptn* and NIH 3T3 cells. The morphological phenotypes of MCF-7-*Ptn* cell xenografts and MCF-7-*Ptn* cell/NIH 3T3 cell cocultures closely resembled breast cancers in MMTV-PyMT-*Ptn* mice. Inappropriate expression of *Ptn* thus promotes breast cancer progression in mice; the data suggest that secretion of PTN through stimulation of the stromal cell microenvironment alone may be sufficient to account for significant features of breast cancer progression.

angiogenesis | collagen | polyoma virus middle T | scirrhous carcinoma | stromal fibroblasts

Breast cancers progress through stages of increasing malignancy triggered by genetic and epigenetic mutations that promote their growth, invasiveness, and metastasis. Because mortality from breast cancer is most often due to growth of distant metastases that are not controlled by existing therapies, there is a vital need to better understand the molecular pathogenesis of breast cancer and to identify mutations in breast cancer cells that promote breast cancer progression and lead to metastasis (1).

Pleiotrophin [PTN (the protein), *Ptn* (the gene)] is a 136-aa heparin-binding cytokine (2, 3) frequently detected in human breast cancers (4). Its expression is constitutive in human breast cancer cells in which it has been tested (5). Furthermore, PTN stimulates functional responses that are known to stimulate breast cancer progression, such as proliferation (2, 3), cytoskeletal rearrangements, and loss of cell-cell adhesion (6, 7), transformation (8), induction of an epithelial to mesenchymal transition (6), and stimulation of tumor angiogenesis (9, 10), in cultured cells or in cells that ectopically express *Ptn*. These

functional responses to PTN are likely to be significant in progression of breast cancers, because interruption of constitutive PTN signaling in human breast cancer cells that inappropriately express *Ptn* reverses their transformed phenotype *in vitro* and *in vivo* (5); these PTN-stimulated functional responses thus support the possibility that mutations in breast cancer cells, which deregulate endogenous *Ptn* expression, may initiate similar functional responses and lead to breast cancer progression.

In the following studies, three models were tested to determine whether inappropriate expression of *Ptn* alone is sufficient to induce breast cancer or whether inappropriate expression of *Ptn* cooperates with different pathogenic mechanisms that stimulate breast cancer progression. Together, the studies demonstrate both *in vivo* and *in vitro* that inappropriate expression of *Ptn* promotes breast cancer progression; the studies also demonstrate that PTN secretion from human breast cancer cells alone is sufficient to promote progression of breast cancer to a more aggressive breast cancer phenotype through activation of stromal cells and extensive remodeling of the microenvironment.

Results

Mouse Mammary Tumor Virus (MMTV)-*Ptn* Fails to Induce Breast Cancer in MMTV-*Ptn* Transgenic Mice. To test whether inappropriate expression of *Ptn* alone is sufficient to induce breast cancers in mice, MMTV-long terminal repeat-driven-*Ptn* (MMTV-*Ptn*) transgenic mice were generated [see supporting information (SI) *Materials and Methods* and SI Fig. 5]. Female MMTV-*Ptn* mice had normal mammary gland development and lactation and developed pups in normal numbers and in size equal to that of control mouse pups. Expression of the MMTV-*Ptn* transgene was detected in high levels in breast epithelial cells. In extensive microscopic examinations of breast tissues, dysplastic changes were not seen. MMTV-*Ptn* mice were free of detectable breast cancers for 18 months. Thus, in this context, inappropriate expression of *Ptn* in mouse breast epithelial cells fails to induce breast cancer, and *Ptn* thus is not an oncogene.

MMTV-*Ptn* Stimulates Breast Cancer Progression in MMTV-PyMT-*Ptn* Transgenic Mice. MMTV-*Ptn* mice were then bred with MMTV-polyoma virus middle T (PyMT) mice to generate MMTV-PyMT-*Ptn* (bitransgenic) mice. The MMTV-PyMT transgenic mouse

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Abbreviations: PTN, pleiotrophin; MMTV, mouse mammary tumor virus; IMD, intratumor microvessel density; MMP, matrix metalloproteinase.

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was chosen because *PyMT* is a potent oncogene; furthermore, the *PyMT* oncoprotein is known to coopt the Src family, ras, and phosphatidylinositol 3-kinase oncogenic signaling pathways (11). Four distinctly identifiable stages of tumor progression that range from premalignant to malignant invasive ductal carcinomas of the scirrhous subtype (hereafter “scirrhous carcinoma”) (12) occur within a single primary focus. The MMTV-*PyMT* mouse breast cancers express markers known to be significant in human breast cancers. They also have significant infiltration of inflammatory cells that frequently are found in human breast cancers. The MMTV-*PyMT* model thus closely recapitulates the many steps of human breast cancer progression.

MMTV-*PyMT-Ptn* mice developed breast cancers with striking variability in numbers and size, a characteristic of MMTV-*PyMT* breast cancers described previously (11). Statistically significant differences in time of onset, tumor volume, tumor burden, or average numbers of metastases per lung (recorded as metastasis index) were not found between MMTV-*PyMT-Ptn* and MMTV-*PyMT* littermate mice (data not shown). However, the areas occupied by the foci of scirrhous carcinoma in sections of MMTV-*PyMT-Ptn* mouse breast cancers were increased nearly 2-fold compared with breast cancers in MMTV-*PyMT* mice (39% vs. 22%) ($P < 0.05$) (SI Table 1), indicating that the scirrhous carcinoma grows significantly more rapidly in MMTV-*PyMT-Ptn* breast cancers than in MMTV-*PyMT* breast cancers, or, perhaps, progression of the breast cancers to the more aggressive scirrhous subtype is markedly accelerated in MMTV-*PyMT-Ptn* mice. In contrast, the areas occupied by invasive ductal carcinomas that lack the features of the scirrhous carcinoma were not much different in breast cancers in MMTV-*PyMT-Ptn* and MMTV-*PyMT* mice (19.2% vs. 16.8%) (SI Table 1).

When studied in H&E-stained sections, the nodular foci of scirrhous carcinoma in MMTV-*PyMT-Ptn* breast cancers were surrounded circumferentially by marked increases in matrix proteins interspersed with increased numbers of morphologically identified “activated stromal fibroblasts” compared with MMTV-*PyMT* breast cancers (compare Fig. 1 *A* and *B*). These matrix proteins surrounding the invasive nodules of scirrhous carcinoma were identified as collagen fibrils in sections of MMTV-*PyMT-Ptn* mouse breast cancers stained with Masson trichrome (Fig. 1*D*), whereas only a few collagen fibrils were seen to surround the invasive nodules of scirrhous carcinoma in MMTV-*PyMT* mice (Fig. 1*C*). A striking increase in mature elastin fibrils surrounding the invasive nodules of scirrhous carcinoma and surrounding the blood vessels (arrows) also was seen in MMTV-*PyMT-Ptn* breast cancers (Fig. 1*F*) compared with MMTV-*PyMT* breast cancers (Fig. 1*E*).

The basis of the increased collagen staining was then examined; procollagen I α 2 transcripts were ≈ 34 -fold increased in MMTV-*PyMT-Ptn* breast cancers when measured by RT-PCR and compared with expression levels in MMTV-*PyMT* breast cancers. Furthermore, procollagen IV α 5 transcripts were increased ≈ 53 -fold, and procollagen XI α 1 transcripts were increased ≈ 54 -fold; however, the levels of procollagen I α 1 mRNA were decreased to $\approx 22\%$ of the levels of procollagen I α 1 in MMTV-*PyMT* breast cancers. Tropoelastin expression levels were nearly 4-fold increased in breast cancers of MMTV-*PyMT-Ptn* mice (SI Fig. 6). These findings thus substantiate the striking increases in collagen and elastin as described above in sections of breast cancers of MMTV-*PyMT-Ptn* mice. A surprising finding, however, was that the dramatic increases in collagen expression is mouse collagen-subtype specific.

Sections of breast cancers were then stained with anti-CD31 antibodies to identify microvascular endothelial cells; it was found that MMTV-*PyMT-Ptn* breast cancers had an ≈ 1.5 -fold increase in the intratumor microvessel density (IMD) compared with the IMD in breast cancers in MMTV-*PyMT* mice (SI Table 2). Surprisingly, the average microvessel area in MMTV-*PyMT*-

Fig. 1. Histological properties of MMTV-*PyMT-Ptn* breast cancers compared with MMTV-*PyMT* breast cancers. Representative images are shown. (*A* and *B*) Sections of breast cancers from MMTV-*PyMT* (*A*) or MMTV-*PyMT-Ptn* mice (*B*) stained with H&E. Note the striking increase in extracellular matrix proteins and stromal fibroblasts (arrow) that surround the foci of breast cancer cells in MMTV-*PyMT-Ptn* mice. (*C* and *D*) Masson trichrome-stained sections of breast cancers from MMTV-*PyMT* (*C*) or MMTV-*PyMT-Ptn* mice (*D*). Arrows point to the collagen fibrils that surround the foci of invasive nodules of breast cancer cells in MMTV-*PyMT-Ptn* mice contrasted with the far more limited fibrils and largely fat globules surrounding carcinoma cells in MMTV-*PyMT* mouse breast cancers. (*E* and *F*) Resorcin/fuchsin-stained sections of invasive nodules of breast cancer cells from MMTV-*PyMT-Ptn* mice (*F*) and MMTV-*PyMT* mice (*E*). Arrows point to the elastin fibrils surrounding the blood vessels. (*G* and *H*) *In situ* hybridization of *Ptn* mRNA in paraffin sections of tumors from MMTV-*PyMT-Ptn* and MMTV-*PyMT* transgenic mice. (*G*–*J*) The antisense human *Ptn* RNA probe was generated as described in *Materials and Methods* and used for *in situ* hybridization of sections of MMTV-*PyMT-Ptn* and MMTV-*PyMT* breast cancers. (*G*) Focus of the scirrhous subtype of invasive ductal carcinomas. (*H*) Early stage in development of scirrhous subtype of invasive ductal carcinoma. (*I*) Nonscirrhous invasive ductal carcinomas of MMTV-*PyMT-Ptn* breast cancers. (*J*) MMTV-*PyMT* breast cancers. (Scale bars: 150 μ m.)

Fig. 2. PTN secretion from MCF-7-Ptn cells stimulates s.c. xenograft growth *in vivo*. (A) MCF-7-mock cell, MCF-7-Ptn cell, and MCF-7-Ptn/NIH 3T3 cell xenograft growth in flanks of nude mice. Day 1 is the time of detection of the first palpable xenograft. (B) Histological phenotype of sections of MCF-7-mock cell xenografts (H&E). (C) Histological phenotype of sections of MCF-7-Ptn cell xenografts (H&E). (D) Histological phenotype of sections of MCF-7-Ptn Xenografts (Masson trichrome). (Scale bar: 100 μ m.)

Ptn breast cancers measured 658 μ m², whereas in breast cancers in *MMTV-PyMT* mice, it measured 393 μ m² ($P < 0.01$) (SI Table 2). The blood vessels in *MMTV-PyMT-Ptn* mice had a normal appearance and were not dysplastic as is characteristic of many epithelial malignancies. Furthermore, as cited above, elastin fibrils that surround the new blood vessels in *MMTV-PyMT-Ptn* breast cancers were much increased. As for increased collagens and elastin, the increase in IMD and the blood vessels of larger size were consistently localized in close proximity to the foci of scirrhous carcinoma (Fig. 1 *E* and *F*, arrows), and, as discussed below, these foci are the sites in *MMTV-PyMT-Ptn* breast cancers that express the highest levels of the *Ptn* transgene.

Very high-level expression of the human *Ptn* transgene was then demonstrated within foci of scirrhous breast carcinomas in sections of *MMTV-PyMT* breast cancers analyzed by *in situ* hybridization using human antisense *Ptn* RNA probes (Fig. 1*G*). Expression of PTN also was highest within these foci when analyzed by immunohistochemistry (data not shown). High-level expression of *Ptn* also was seen in later stages of progression of breast cancers with features approaching the scirrhous phenotype (Fig. 1*H*). Lesser intensity staining was seen in areas of *MMTV-PyMT-Ptn* breast cancers that were not scirrhous (Fig. 1*I*); as anticipated, the antisense human *Ptn* RNA probe failed to hybridize with mRNAs in *MMTV-PyMT* mouse breast cancers (Fig. 1*J*), thus establishing the specificity of the probe to the *MMTV-Ptn* human transgene. It is important that foci of invasive scirrhous carcinoma with the highest levels of expression of *Ptn* correlated directly with the sites of highest density of collagens and elastin, numbers of morphologically identified activated stromal fibroblasts, and levels of new blood vessels of larger diameter.

PTN Secretion from MCF-7-Ptn Cells Stimulates s.c. Xenograft Growth *in Vivo*: A Second Model of Breast Cancer Progression. To test the possibility that secretion of PTN may significantly stimulate breast cancer progression independently of its effect on the breast cancer cell itself, we analyzed a second model in which xenografts of MCF-7 (human breast cancer) cells that express an

ectopic *Ptn* gene (MCF-7-Ptn cells) were compared with xenografts of MCF-7-mock (vector alone) cells in nude mice. MCF-7 cells do not express the receptor protein tyrosine phosphatase β/ζ , the PTN receptor (13), and thus MCF-7-Ptn cells are not activated by PTN through autocrine or paracrine mechanisms. MCF-7-Ptn cell xenografts were first palpated for 7–10 days (plotted as day 1 in Fig. 2*A*); they then grew rapidly thereafter. MCF-7-Ptn cells coinjected with equal numbers of “surrogate” NIH 3T3 cell stromal fibroblasts xenografts grew even more rapidly. MCF-7 cells alone failed to grow at sites of implantation (Fig. 2*A*). These data thus suggest that PTN secreted from MCF-7-Ptn cells alone is sufficient to stimulate rapid progression of the MCF-7-Ptn cell xenograft model; furthermore, the data suggest that the stromal fibroblast is a key element in the PTN secretion-dependent progression of MCF-7-Ptn cell xenografts.

In sections of MCF-7-Ptn cell xenografts stained with H&E, the MCF-7-Ptn cells were fibroblast-like and more invasive in appearance (Fig. 2*C*) than MCF-7-Ptn cells in monolayer culture. The nuclei were pleomorphic in shape and the nuclear chromatin appeared to be more densely condensed. The cells formed invasive nodules surrounded by activated stromal fibroblast-like cells intermixed with abundant extracellular matrix proteins identified as collagen in sections stained with Masson trichrome (Fig. 2*D*). New blood vessels surrounding the invasive nodules of MCF-7-Ptn cells were abundant. In contrast, MCF-7-mock cells were difficult to detect at sites of implantation, and, where found, they were interspersed with fat vacuoles, fibroblasts, and different inflammatory cells (Fig. 2*B*). The MCF-7-Ptn cell xenografts thus phenotypically resemble the foci of scirrhous carcinoma in *MMTV-PyMT-Ptn* breast cancers (compare Figs. 1*D* and 2*D*). The data thus support the possibility that secretion of PTN from breast cancer cells that inappropriately express *Ptn* stimulates breast cancer progression.

PTN Secretion from MCF-7-Ptn Cells Stimulates Epithelial Island Formation, Activation of Stromal Fibroblasts, Extensive Remodeling of Microenvironment, and Activation of Markers of Aggressive Breast Cancer in Cocultures of MCF-7-Ptn Cells and NIH 3T3 Cells: A Third Model of Breast Cancer Progression. The MCF-7-Ptn cells were then cocultured with equal numbers of NIH 3T3 cells and analyzed by phase-contrast microscopy. The MCF-7-Ptn cells became loosely clustered during logarithmic growth and tightly clustered “epithelial islands” (14) at confluence (Fig. 3*B* and *D*), whereas MCF-7-mock cells in coculture with NIH 3T3 cells failed to form epithelial islands either during logarithmic growth or at confluence (Fig. 3*A* and *C*). In hematoxylin/eosin-stained sections, MCF-7-Ptn cells that expressed an siRNA shown to effectively “knock down” *Ptn* expression (see Western blot, SI Fig. 7*A*) failed to form epithelial islands in coculture with NIH 3T3 cells (Fig. 3*F*). In contrast, MCF-7-Ptn cells that expressed a control (scrambled) siRNA effectively formed epithelial islands (Fig. 3*E*). Furthermore, by using an anti-human-*Ptn* probe to detect MCF-7-Ptn cells and distinguish epithelial islands by *in situ* hybridization, it was shown that MCF-7 cells that expressed *Ptn* with a C-terminal KDEL endoplasmic reticulum retention sequence (15) (MCF-7-Ptn-KDEL cells), which effectively blocked secretion of PTN from the MCF-7-Ptn-KDEL cells (see Western blot, SI Fig. 7*B*), failed to form epithelial islands in coculture with NIH 3T3 cells (Fig. 3*G*). However, the MCF-7-Ptn cells that express *Ptn* with a C-terminal KDAS (control) sequence readily formed epithelial islands (Fig. 3*H*); thus, in this model, epithelial island formation in cocultures of MCF-7-Ptn cells with NIH 3T3 cells is MCF-7 cell-*Ptn* expression-dependent, MCF-7 cell PTN secretion-dependent, and surrogate NIH 3T3 stromal cell-dependent (coculture).

The MCF-7-Ptn cells in cocultures of MCF-7-Ptn cells and NIH 3T3 cells within epithelial islands were found to be more fibroblast-like (Fig. 3*B* and *D*) than MCF-7-Ptn cells in mono-

sion also was readily detected in the MCF-7-*Ptn* cells within epithelial islands (Fig. 4D) but not in MCF-7-mock cells in coculture with NIH 3T3 cells (Fig. 4C) nor in MCF-7-*Ptn* cells in monolayer culture (data not shown). A slight increase in anti-tropoelastin immunoreactivity was found in PTN-stimulated NIH 3T3 cells (Fig. 4B), compared with nonstimulated NIH 3T3 cells (Fig. 4A). Protocollagen gene expression also was seen in high levels in NIH 3T3 cells in coculture with MCF-7-*Ptn* cells; however, the appearance of fibrillar collagen in cocultures was delayed, perhaps, it is speculated, because collagens were degraded by metalloproteinases such as matrix metalloproteinase (MMP)-9 (see below).

PKC δ is a marker of aggressive breast cancers; it is known to be activated in both breast cancer cells and activated stromal fibroblasts (16); PTN furthermore is known to activate PKC activity in PTN-stimulated cells (7). Activated PKC δ in turn activates pro-MMP-9 (17), and activated MMP-9 degrades collagens, releasing collagen peptides that themselves are growth stimulatory (18). Activated PKC δ was readily identified with immunofluorescent microscopy in both NIH 3T3 stromal fibroblasts and the MCF-7-*Ptn* cells transfected with the control “scrambled” siRNA in coculture with NIH 3T3 cells (Fig. 4E). In contrast, markedly reduced levels of activated PKC δ were found in cocultures of MCF-7-*Ptn* cells transfected with the *Ptn*-specific siRNA and NIH 3T3 cells (Fig. 4F). The data thus support the fact that secretion of PTN from MCF-7-*Ptn* cells in coculture with NIH 3T3 cells leads to a marked activation of PKC δ , a known marker of aggressive breast cancers, in both MCF-7-*Ptn* cells and NIH 3T3 cells.

Conditioned media from MCF-7, MCF-7-mock, and MCF-7-*Ptn* cells in coculture with NIH 3T3 cells were then examined by gelatin zymography (19). Bands of ≈ 92 and ≈ 105 kDa that correspond to human pro-MMP-9 and murine pro-MMP-9 were detected in gels of conditioned media from MCF-7-*Ptn*/NIH 3T3 cell cocultures (Fig. 4G, lane 3). Human pro-MMP-9 also was readily detected by immunohistochemistry using anti-pro-MMP9 antibodies in MCF-7-mock and MCF-7-*Ptn* cells cocultured with NIH 3T3 cells (Fig. 4J and K) but not when these cells were examined in monoculture (Fig. 4H and I). The highest levels of pro-MMP-9 were detected in MCF-7-*Ptn* cells cocultured with NIH 3T3 cells (Fig. 4K); the levels were significantly higher compared with MCF-7-mock cells in coculture with NIH 3T3 cells (Fig. 4J). Importantly, human and murine pro-MMP-9 were found in very limited amounts in conditioned media of cocultures of MCF-7 (Fig. 4G, lane 1) or MCF-7-mock cells with NIH 3T3 cells (Fig. 4G, lane 2) in comparison with levels of murine and human pro-MMP-9 in media from MCF-7-*Ptn* cells with NIH 3T3 cells (Fig. 4G, lane 3). Thus, the high level of expression of MMP-9 in both MCF-7-*Ptn* and NIH 3T3 cells in cocultures of MCF-7-*Ptn* and NIH 3T3 cells leads to export of MMP-9 in media; export of MMP-9 is minimal if it occurs at all from MCF-7-mock or MCF-7 cells in coculture with NIH 3T3 cells.

Discussion

These studies demonstrate that inappropriate expression of *Ptn* alone in MMTV-*Ptn* transgenic mice is not sufficient to induce breast cancer; thus, in this context, PTN is not an oncogenic protein. However, inappropriate expression of *Ptn* driven by MMTV in MMTV-*PyMT-Ptn* mice induced more rapid growth of foci of breast cancers with the histological phenotype of scirrhous carcinoma; it induced truly remarkable increases in new collagen and elastin deposition, extensive extracellular matrix remodeling, increased tumor angiogenesis, and increased size of healthy-appearing new blood vessels. The foci of scirrhous carcinoma in MMTV-*PyMT-Ptn* mice express very high levels of the MMTV-*Ptn* transgene and, thus, high-level expression of MMTV-*Ptn* correlates directly with foci of scirrhous carcinoma

and, furthermore, with the remarkable increases in collagen, elastin, and tumor angiogenesis and increased size of new blood vessels within the MMTV-*PyMT-Ptn* breast cancers. Secretion of PTN from MCF-7-*Ptn* cells alone in two other models used in this study also was shown to be sufficient alone to stimulate progression of breast cancer cells and induce a remarkable remodeling of the microenvironment with striking increases in collagens, elastin, and, in the case of MCF-7-*Ptn* xenografts in nude mice, tumor angiogenesis. Surprisingly, the histological patterns of the MCF-7-*Ptn* epithelial islands in MCF-7-*Ptn* and NIH 3T3 cell cocultures and xenografts of MCF-7-*Ptn* cells in nude mice closely resemble the histological pattern of breast cancers in MMTV-*PyMT-Ptn* mice, namely scirrhous carcinomas. Thus, constitutive PTN-signaling in MMTV-*PyMT-Ptn* mice promotes progression of mouse breast cancer and induces a breast cancer phenotype in MMTV-*PyMT-Ptn* mice akin to scirrhous carcinoma in humans. Because the scirrhous subtype of invasive ductal carcinoma is among the most aggressive human breast cancers (20), the study raises the possibility that inappropriate expression of *Ptn* may be an important factor in the pathogenesis of scirrhous invasive ductal carcinoma.

Our data furthermore suggest the potential that PTN expression may account for many of the features of scirrhous carcinoma seen in the breast cancers in MMTV-*PyMT-Ptn* mice. PTN stimulates new collagen of different subtypes and new elastin synthesis (L. Ezquerro, G. Herradon, and T.F.D., unpublished data). Collagen fragments are known to stimulate growth of carcinoma cells and to stimulate antiapoptotic pathways, favoring growth of the carcinoma cells (21). Through activation of integrins and “outside-in” signaling, both elastin and collagens are known to activate the ERK pathway in epithelial cells and function to prevent caspase 8 activation and apoptosis (18). Thus, the data generated suggest the possibility that the increased synthesis of extracellular matrix proteins is an important contributor to the increased growth of these foci of scirrhous carcinoma in MMTV-*PyMT-Ptn* breast cancers. PTN also is known to stimulate new blood vessel formation and tumor angiogenesis (10, 22), and, in these studies, secretion of PTN leads to up-regulation of two well recognized markers of aggressive breast cancers, PKC δ and MMP-9, in both breast cancer cells and stromal fibroblasts.

Activated stromal fibroblasts are often identified by characteristic morphological features and different biomarkers, such as α -smooth muscle actin; however, more recently, activated stromal fibroblasts have been characterized by different functional responses that result from their activation, such as expression of the extracellular matrix proteins collagens, elastin, and MMPs and release of growth factors and cytokines (23). These studies thus identify PTN as one factor that activates stromal fibroblasts to induce many features of aggressive breast cancers, and, thus, it is suggested that inappropriate expression of *Ptn* may be very important in breast cancer progression. The importance of the epithelial cancer cell-stromal fibroblast interactions is well established (24), but only limited progress has been made in identifying the factors that activate stromal cells to initiate cross-talk and tumor progression. These studies thus demonstrate that activated stromal cells are central in the models used in this study; they are responsible for the synthesis, deposition, and remodeling of the extracellular matrix protein and release of factors that stimulate the growth and malignant phenotype of the carcinoma cells themselves (25). The studies thus indicate that PTN secreted from the breast cancer cells is the mechanism of stromal cell activation; the studies also identify the fact that PTN alone is sufficient to stimulate in activated fibroblasts many of the critical signaling pathways needed to aggressively promote breast cancer progression.

Materials and Methods

Cell Culture, Transfections, and Tissue and Cell Staining. Human breast cancer MCF-7 and murine fibroblast NIH 3T3 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in minimum essential medium or Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37°C and 5% CO₂.

Cells were transfected with the different constructs described in *SI Materials and Methods* by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations.

MCF-7 and NIH 3T3 cell cocultures were fixed with 4% paraformaldehyde and either stained with H&E or blocked with PBS containing 1% inactivated FBS and 0.1% Triton X-100 and incubated with antitropoelastin antibodies, anti-phospho-PKC δ (Thr⁵⁰⁵) antibodies (Cell Signaling, Danvers, MA) or anti-pro-MMP-9 antibodies that recognize human and mouse pro-MMP-6 (Calbiochem, San Diego, CA) and, subsequently, with FITC-conjugated or HRP-conjugated secondary antibodies.

The tissues excised from euthanized animals were fixed in 10% zinc formalin and embedded in paraffin. The specimens were sectioned and stained with H&E, Masson trichrome for collagen, and resorcin/fuchsin for elastin.

Transgenic Constructs, Transgenic Mice. See *SI Materials and Methods* and SI Fig. 5. All mice were housed in the animal facility of

The Scripps Research Institute and maintained according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

IMD and *in Situ* Hybridization. *In situ* hybridization and IMD were performed as described (10, 26). For details, see *SI Materials and Methods*.

Western Blot Analysis and Gelatin Zymography. Western blot analysis and gelatin zymography were performed as described (19, 27). See *SI Materials and Methods*.

Statistics. Results are presented as means $\pm$ SEM. Student's *t* test was used to determine the statistical significance of tumor onset time, the time of tumor detection, tumor size, and scirrhous area of scirrhous foci in breast cancers.

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