Progranulin (PC-Cell-derived Growth Factor/Acrogranin) Regulates Invasion and Cell Survival

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ABSTRACT

Progranulin (pgrn; PC-cell-derived growth factor, epithelin precursor, or acrogranin) has been identified recently as an autocrine regulator of tumorigenesis in several cancer cells including SW-13 adrenal carcinomas and some breast cancers, but how pgrn promotes tumor progression is not well understood. SW-13 cells do not form tumors in nude mice but become highly tumorigenic when their pgrn expression is elevated, and this provides a useful model in which to investigate the role of pgrn in tumorigenesis. Here we show that, in SW-13 cells, the level of pgrn expression is a major determinant of the intrinsic activity of the mitogen-activated protein kinase, phosphatidylinositol 3'-kinase, and focal adhesion kinase signaling pathways. Pgrn stimulates the invasion of SW-13 cells across Matrigel-coated filters, increases the expression of matrix metalloproteinase 13 and 17, protects against anoikis, and overrides the inhibition of cell growth imposed on SW-13 cells by interstitial type-I collagen. Inhibition of the mitogen-activated protein kinase and phosphatidylinositol 3'-kinase signaling pathways impairs each of the pgrn-dependent biological responses tested, but to different extents. The ability of pgrn to stimulate cell division, invasion, and survival demonstrates that pgrn regulates multiple steps in carcinogenic progression, and suggests that the pgrn system may be a possible future therapeutic target.

INTRODUCTION

Pgrn is a high molecular weight growth factor (1), which is also called PCDGF (2), epithelin precursor (3), or acrogranin (4). It is involved in tumorogenesis and the regulation of mitosis (2, 5–8), non-neoplastic proliferative disorders such as renal cysts (9), early embryonic development (10), hypothalamic differentiation (11), and acts as a cofactor for secreted HIV Tat protein (12). Pgrn activates both the PI3k (13) and ERK (13, 14) pathways, and promotes the expression of cyclin D1 (14) and cyclin B (13), cellular responses that are consistent with its proposed role as a growth factor. SW-13 cells, a nontumorigenic line from a human adrenal carcinoma, become tumorigenic in nude mice when they express elevated pgrn (5). Decreasing the intrinsic expression of pgrn mRNA in SW-13 cells by the use of antisense RNA impairs their proliferation (5). The highly tumorigenic murine teratoma PC-cell line requires pgrn (called PCDGF in this context) as an autocrine growth factor (2), and their tumor formation is markedly impaired when the level of pgrn/PCDGF mRNA is reduced (6). Pgrn/PCDGF gene expression is up-regulated by estrogens in hormone-sensitive breast cancer cell lines and is a major contributory factor to the mitogenic effects of estrogens in these cells (14, 15). Depletion of pgrn/PCDGF mRNA inhibits tumorogenesis in some breast tumor cell lines (15). High-grade renal cell carcinomas express elevated levels of pgrn (7). Pgrn is absent from normal glial cells (8, 16) but has been identified as a tumor-specific product of glioblastomas (8). The related, but distinct, epithelial-transforming growth factor has also been implicated in carcinogenesis (17).

Although pgrn is clearly an important determinant of tumor growth for many cells, its mitogenicity alone may not account for its actions in tumor promotion. Embryonic fibroblasts in which the insulin-like growth factor I receptor is deleted (R− cells) do not grow in response to classic growth factors (13, 18). Pgrn overcomes this block in cell proliferation, but, as judged by colony formation in soft-agar, it does not transform either R− cells or intact NIH-3T3 embryonic fibroblasts (13). Therefore, additional nonproliferative actions may be necessary for pgrn to support a transformed phenotype. Tumor formation is the culmination of many discrete biological events, but pgrn involvement in these processes is not well understood. Progression occurs only if cells become invasive. They must detach from and penetrate the basement membrane, and simultaneously escape anoikis, a form of cell death that occurs when adherent cells lose their normal attachment to the substrate (19). Proliferation is highly sensitive to the extracellular matrix (20), and invasion exposes cells to unfamiliar extracellular environments. For a tumor to progress, invasive cells must maintain an actively proliferating phenotype in the newly encountered matrix (21).

We hypothesize that pgrn may be able to promote tumor formation by acting on some or all of the postmitotic steps outlined above, and it is clearly important to investigate which aspects of tumor progression are modulated by pgrn. Using pgrn-dependent SW-13 cells as a model, we have investigated the ability of pgrn to confer invasiveness on epithelial cells, to protect against anoikis, to support proliferation in interstitial matrices, and the relative importance of ERK and PI3k signaling pathways in regulating pgrn responses.

MATERIALS AND METHODS

Cell Culture and Establishment of Stable Transfectants. All of the cells were maintained in DMEM (Life Technologies, Inc., Rockville, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 400 μg/ml G418 (Life Technologies, Inc.), and maintained at 37 °C, 5% CO2, absolute humidity. SW-13 cells were purchased from American Type Cell Culture (Manassas, VA). Three stable transfectants were used as described previously (5); SW-13/pgrn, transfected with full-length pgrn cDNA placed in the sense orientation in the PCDNA3 vector, SW-13/vec, transfected with pCDNA3 (empty vector), and SW-13/AS, transfected with pgrn cDNA inserted in the antisense orientation in pCDNA3. The relative expression of pgrn, and the mitotic and tumorigenic properties of these cell lines have been reported elsewhere (5).

Transient Transfection and Purification of Recombinant Pgrn. Recombinant pgrn was generated by transient transfection of COS-7 cells and purified by reverse-phase-high-performance liquid chromatography as reported previously (5).

Western Blots. Cells (1 × 106) were grown in 10-mm tissue culture Petri-dishes (Sarstedt, St-Laurent, Quebec, Canada) and were serum-deprived for 48 h. Cells were lysed in a solution of 0.5% Triton X-100, 50 mM HEPES...
(pH 7.5), 150 mM NaCl, 100 mM sodium fluoride, 10 mM sodium PF, 5 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride. One hundred µg of total cellular protein was immunoprecipitated following the manufacturer’s recommended protocols with either P44/42 MAPK antibody, PKB/Akt antibody, or FAK antibody (Santa Cruz Biotech, Santa Cruz, CA) diluted 1:100. The immunoprecipitates were electrophoresed in 10% SDS-PAGE and the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Mississauga, Ontario, Canada). After blocking, the membranes were hybridized with rabbit phospho-p44/42 MAPK, phospho-PKB (residue S473; Cell Signaling, Beverly, MA), or mouse phospho-tyrosine antibody (Santa Cruz Biotech) at 1:1000 dilution to determine the phosphorylation of these kinases. The membranes were subsequently incubated with antirabbit IgG secondary antibodies (1:2000) conjugated with alkaline phosphatase (phospho-p44/42 MAPK and phospho-PKB/PKB) and visualized using a Sigma FAST 5-bromo-4-chloro-3-indoylphosphate/Nitro Blue tetrazolium system (Sigma-Aldrich) or for the detection of phospho-tyrosine, with antiouso IgG antibody conjugated to horseradish peroxidase (Sigma, St. Louis, MO) visualized using an enhanced chemiluminescence kit (Amersham Life Science, Buckinghamshire, United Kingdom). The equal loading of the proteins was confirmed by Western blotting analysis using antibodies against p44/42 MAPK, Akt/PKB, and FAK, respectively (Santa Cruz Biotech) following the manufacturer’s recommended protocols.

Cell Growth Assay. Cell proliferation was determined as described previously (5). Briefly, 1.5 × 10^5 cells were seeded in 12-well plates (Corning Costar, Cambridge, MA) and maintained in DMEM with or without 10% fetal bovine serum for 7 days, with the medium replaced every 3 days. After 7 days, the cells were trypsinized and an aliquot counted in trypan blue (Sigma) in a hemocytometer. P0990859, an inhibitor of p44/42 MAPK (Tocris, Avonmouth, Bristol, United Kingdom), and wortmannin, an inhibitor of PI3k (Calbiochem, La Jolla, CA), were used to examine the role of signal pathways in the pgm response. Preliminary experiments were performed to establish the approximate ID_{50} for both agents and were found to be 10 µM P0990859 and 10 nM wortmannin in serum-free medium. The final concentration of DMSO, the solvent for the inhibitors, was 0.1% and had no effect on the viability of the cells (not shown).

Colony Formation Assay in Soft Agar. Soft agar assays of SW-13 were performed using a modified protocol from Ref. 17 as described previously (5). Briefly, 150 µL × McCoy medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum was mixed with an equal volume of 1.2% Sea Plaque agar (Mandel Scientific, Guelph, Ontario, Canada) and solidified in a 24-well plate. Cells (2 × 10^4) were mixed well with 0.2 ml of 1 × McCoy’s medium with 5% serum, 0.1 ml of 2 × McCoy’s medium with 10% fetal bovine serum and 0.1 ml agar, and overlayed on the bottom gel, allowed to set at room temperature, and then grown in a 37°C incubator. Colony formation was monitored under the microscope.

Matrigel Invasion Assay. Cell invasion was assayed by a Matrigel invasion assay (22) using Transwell chambers (Costar) with 5-µm pore polycarbonate filters. The filters were coated with 50 µg of Matrigel (VWR Canlab, Montreal, Quebec, Canada). Cells (5 × 10^4) in 200 µL of serum-free DMEM were placed on each filter, and 800 µL of serum-free medium supplemented with 25 µg/ml fibronectin (Sigma) placed in the lower chamber. Twenty-four h later the filters were washed, fixed, and stained as described (22). Cells on the upper surface of the filters were removed with cotton swabs. Cells that had invaded to the lower surface of the filter were counted by microscopy selecting 10 random fields per filter (×400 magnification). To evaluate the effects of recombinant pgm on cell invasion purified pgm was added to the upper chamber containing SW-13/vector cells.

RT-PCR. Reverse transcription was carried out using 3 µg of total RNA, 20 pmol oligodeoxythymidylic acid, and 200 units Moloney murine leukemia virus reverse transcriptase enzyme according to the instructions of the enzyme manufacturer (Life Technologies, Inc.). The PCR reaction was carried out using 3 µl of the reverse transcription reaction product. Primer sequences and reaction conditions were as described in the following references: MMP-1, MMP-2, MMP-3, MMP-7, MMP-10, MMP-13, MMP-14, MMP-15 and MMP-16 (23); MMP-17 (24); UPA and UPAR (25); and GADPH (26). Thirty amplification cycles were performed for each set of primers except MMP-2, MMP-3 and MMP-7, which required 35 cycles for good detection. Preliminary experiments were performed to ensure that the amplifications were not saturating under these conditions.

Anoikis Assay. Anoikis was determined using a protocol modified from Ref. 13. Cells (10 × 10^4) were resuspended in 1 ml of serum-free DMEM and grown in polypropylene tubes (Sarstedt) at 37°C, 5% CO2. After 48 h, 20 µl from each suspension were mixed with 20 µl of trypan blue (Sigma) and counted in a hemocytometer. The numbers of unstained living cells and heavily stained dead cells were recorded, and cell death was expressed as percentage of control cells treated with 4 µg/ml camptothecin (100% cell death).

Cell Proliferation in Collagen. Type I collagen was obtained from BD Biosciences, Bedford, MA. Three-hundred µl of collagen (30 µM 1.4 M NaHCO3, 60 µl 5 × serum-free DMEM, and 210 µl collagen at 1.71 mg/ml) was placed per well of a 24-well culture plate. The gel was overlaid with 500 µl of collagen containing 3000 cells/well and after setting an additional 500 µl of DMEM with or without 10% fetal bovine serum. Proliferation was measured by counting cells in 10 randomly selected fields of view (×100 magnification). To assess the proliferation of SW-13 cells on collagen surfaces 1.2 × 10^4 cells in 500 µL DMEM with or without serum were plated onto the 300-µl collagen gels (1 mg/ml) equilibrated previously with DMEM with or without 10% fetal bovine serum. After 20 h all of the cells were attached and displayed a predominantly elongated morphology. After 6 days of incubation the gels were digested with 200 units/well collagenase I (Life Technologies, Inc., Burlington, Ontario, Canada) in trypsin-EDTA, and the cell number was determined using a hemocytometer.

Statistical Analysis. The difference in the mean value of different groups was measured by ANOVA followed by Welch test. The calculation was carried out using a licensed software package Instat (GraphPad Software V2.04a). Significance is reported as two-sided P.

RESULTS

Pgm Activates the ERK, PI3k, and FAK Cascades in SW-13 Cells. Phosphorylation of p44/42 MAPK, and PKB/Akt and FAK were used to investigate the activation of the ERK, PI3k, and focal adhesion signal transduction cascades, respectively (Fig. 1). All three of the pathways are active in SW-13/vector cells in the absence of serum (Fig. 1). The elevated expression of pgm by SW-13/pgm cells increases the phosphorylation of p44/42 MAPK, PKB/Akt, and FAK relative to that of SW-13/vector cells. Reduced pgm expression in SW-13/AS cells lowers intrinsic activation of each pathway. Kinase levels did not change relative to total protein levels in the three cell lines. The addition of the p44/42 MAPK kinase inhibitor P0990859 and the PI3k inhibitor wortmannin to SW-13/pgm cells inhibits their proliferation implying that both pathways are active in pgm-stimulated proliferation in monolayer culture (Fig. 2). P0990859 inhibited the monolayer (Fig. 2A) and anchorage-independent growth (Fig. 2B) of SW-13/pgm cells to an equivalent extent, but concentrations of wortmannin that elicit only partial inhibition of monolayer growth fully blocked anchorage-independent growth (Fig. 2B). To facilitate the comparison between different assay systems, in all of the additional experiments the concentration of each of these agents that causes an ~50% reduction in cell proliferation after 7 days in monolayer culture (defined as ID_{50} (mono)) was used throughout. Tyrosine-phosphorylation of p44/42 MAPK and PKB/Akt is reduced but not eliminated at these concentrations of inhibitors (Fig. 2D). Higher concentrations of both of these agents completely prevent cell proliferation in SW-13/pgm cells and elicited an apparent loss of cells in SW-13/vector cells. Inhibition of the PI3k and ERK pathways significantly increases cell death in SW-13/vector but not SW-13/pgm cells (Fig. 2C).

Pgm Regulates Cancer Cell Invasion. Matrigel-coated filters have been widely used to investigate invasive migration through basement membrane-like matrices (27). SW-13/pgm cells show a greater ability to pass through Matrigel-coated filters than do SW-13/WT cells (Fig. 3). The enhanced invasiveness can be duplicated by the addition of 1 nM pgm to the SW-13/vector cells. P0990859 and
wortmannin at their ID$_{50}^{(mono)}$ completely block the invasiveness of SW-13/pgrn cells measured over a 24-h period.

**Pgrn Elevates the Transcription of Specific Matrix-degrading Enzymes.** Movement through the basement membrane and into the stroma is highly dependent on the expression of MMPs. SW-13/vector cells exhibit expression of several MMPs known to be associated with cellular invasiveness. High levels of transcript expression (Fig. 4A) were detected for MMP-1 (collagenase 1), MMP-10 (stromelysin 2), MMP-14 (membrane-type 1 MMP), MMP-15 (membrane-type 2 MMP), and MMP-16 (membrane-type 3 MMP). The relative MMP gene expression levels in SW-13 vector are close to those reported for SW-13 (23) except for MMP-7, which is poorly expressed in our experiments. The elevated expression of the pgrn gene in SW-13/pgrn cells leads to an up-regulation of MMP 13 (collagenase 3) and MMP-17 (membrane-type 4 MMP) mRNA levels (Fig. 4B). MMP-2, MMP-3, and MMP-7 transcripts were low (Fig. 4), requiring 35 cycles of amplification to be easily detected, and were not regulated by pgrn expression. UPA is also expressed but at low levels (not shown), UPAR was not detected (data not shown), and neither transcript was regulated by pgrn expression. The weak signals obtained with primer pairs for UPA and UPAR, and MMP-7 are not artifacts of the primers used or the amplification conditions, because we successfully amplified these transcripts using identical conditions from unrelated cell lines (data not shown).

**Pgrn Protects SW-13 Cells against Anoikis.** The ability of the cells to resist anoikis after suspension for 48 h in serum-free medium was determined. All of the results are expressed as percentage cell death relative to camptothecin (100%). Elevated expression of pgrn

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**Fig. 1.** The activation of the ERK, PI3k, and FAK pathways is proportional to the level of pgrn expression. The levels of tyrosine phosphorylation of (A) p44/42 MAPK, (B) Akt/PKB, and (C) FAK were determined in SW-13/Pgrn, SW-13/vector, and SW-13/AS cells. In each case the top panel is the corresponding phosphoprotein, and the bottom panel is the corresponding total target protein. The adjacent plots show the mean of three independent determinations of phosphorylation for each protein normalized against the corresponding levels of total kinase and expressed relative to SW-13/vector cells; bars, ±SD. The relative expression of pgrn mRNA in each cell line is shown in D from two independent RNA extractions and compared against the total RNA loading using the 18S RNase.

**Fig. 2.** A comparison of the effects of inhibiting ERK and PI3k signaling pathways on the growth of SW-13/pgrn cells in monolayer (A) and anchorage-independent growth (B). Ten μM PD098059 and 10 nM wortmannin were used corresponding approximately to the ID$_{50}$s for monolayer growth; n = 4; bars, ±SE. In C, the effects of inhibitor treatment on cell death is shown for SW-13/vector and SW-13/pgrn cells (n = 3; bars, ±SE). The action of wortmannin and PD098059 on Akt/PKB and p44/42 MAPK is shown in D.
protects SW-13/pgrn cells from anoikis (Fig. 5A). SW-13/WT, SW-13/vector, and SW-13/AS are equally susceptible to anoikis. PD098059 and wortmannin at their respective ID$_{50}$ (mono) eliminate the protection provided by increased expression of pgrn, and cell death becomes comparable with that of SW-13/vector and SW-13/AS cells (Fig. 5, B and C). Higher concentrations of these drugs do not cause additional cell death, which does not reach the 100% death obtained with camptothecin (Fig. 5, B and C). Cell survival can be restored by coincubating PD098059 and wortmannin treated SW-13/pgrn cells with increasing concentrations of pgrn; however, even at pgrn concentrations of 140 nM, which are supramaximal for monolayer growth, there is no cellular proliferation (Fig. 5B and C).

**Proliferation in Collagen.** SW-13/pgrn cells grow faster than SW-13 vector cells when plated as monolayers on collagen surfaces, but only in the presence of serum (Fig. 6A). The growth of SW-13/pgrn cells is slower on collagen than on plastic (Fig. 6A). In three-dimensional gels of interstitial type I collagen (Fig. 6B) the elevated expression of pgrn confers a growth advantage for SW-13/pgrn over SW-13/vector cells in the presence of 10% fetal bovine serum of ~6-fold. SW-13/pgrn cells grow in collagen gels as clusters of individual cells that do not form cell-to-cell contacts (data not shown), whereas in soft agar cell-cell contact is retained, and discrete colonies are formed. In the absence of serum there is no difference between the growth of SW-13/pgrn cells, SW-13/WT, SW-13/vector, or SW-13/AS cells. Serum confers a small increase in cell number on SW-13/WT, SW-13/vector, and SW-13/AS cells. At its ID$_{50}$ (mono) PD098059 completely eliminates the growth advantage in collagen of SW-13/pgrn cells (Fig. 6C). In contrast, wortmannin at its ID$_{50}$ (mono)
causes a partial impairment in cell growth in collagen gels that is similar to that seen in monolayer culture (Fig. 6C). When followed over time all of the SW-13 cell variants showed increased cell number in collagen gels regardless of their relative pgrn expression even in the presence of pathway inhibitors or in the absence of serum, although at levels much lower than for SW-13/pgrn in serum (Fig. 6C). Thus, each of the SW-13 variants remains viable throughout the experiment.

**DISCUSSION**

Pgrn stimulates cell division and promotes tumor formation *in vivo*, but how it contributes to the individual stages in tumor progression is less well established. SW-13 cells are unable to form tumors in nude mice (5), although they are carcinomal in origin, but tumorigenicity is restored when the cells are engineered to express pgrn at elevated levels (5). Therefore, SW-13 cells provide a valuable model in which to investigate the role of pgrn in tumor progression.

The ERK, PI3k, and integrin-associated FAK pathways are critical in tumor progression (28, 29) and, as shown here, the growth factor pgrn is an essential intrinsic determinant of their activity in SW-13 cells. All three of the pathways are active in SW-13/vector cells (Fig. 1) although not to an extent that supports tumorigenicity (5). Their phosphorylation is greatly reduced in SW-13/AS cells and elevated in the tumorigenic SW-13/pgrn cells relative to SW-13/vector controls (Fig. 1). Pharmacological inhibitors were used to probe the significance of ERK and PI3k pathways in regulating pgrn-dependent cell activities. All of the biological responses that we investigated, namely, monolayer growth, anchorage-independent colony growth, invasion, protection against anoikis, and proliferation in collagen, were sensitive to the pharmacological inhibition of both pathways. However, the inhibitory profiles showed considerable differences. In SW-13/pgrn blocking the PI3k pathway is more effective at inhibiting anchorage-independent than monolayer growth. The activation of both pathways is independently essential for pgrn-stimulated invasion because at their ID_{50} (mono) both drugs completely inhibited invasion across Matrigel (Fig. 3). PKB/Akt is an important antiapoptotic signal (30, 31), and inhibition of the PI3k pathway impairs the enhanced survival of SW-13/pgrn cells in anoikis assays, but pgrn-stimulated p44/42 MAPK activity is equally important in blocking anoikis in SW-13 cells (Fig. 5, B and C) as reported recently for lung adenocarcinomas (31). Overall, the differential sensitivity of the various pgrn responses to pathway inhibitors suggests that pgrn stimulates a network of intracellular interactions, each interaction contributing to the protu-

![Fig. 5. Overexpression of pgrn protects SW-13 cells against anoikis. A, the percentage of cell death obtained by suspending SW-13/pgrn, SW-13/AS, SW-13/vector, and SW-13/WT cells in serum-free medium for 48 h is compared. Camptothecin was used to provoke 100% cell death (Cam). PD098059 (B) and wortmannin (C) impair cell survival by SW-13/pgrn cells. Portions SW-13/pgrn cells coculated with 10 μM PD098059 (B) or 10 nM wortmannin together with pgrn (140 nm) at a concentration that is supra-maximal for cell proliferation in monolayer cultures (n = 4; bars, ±SE).

![Fig. 6. The proliferation of SW-13 cells in type I collagen requires pgrn expression. In A, 1.2 × 10^5 SW-13/pgrn, SW-13/vector, and SW-13/AS cells were plated on collagen monolayers and grown for 6 days with (■) or without (□) 10% fetal bovine serum (n = 3; bars, ±SE). Portions SW-13/pgrn cells cultured with 10 μM PD098059 (PD) or 10 nM wortmannin (W) represent three independent experiments in duplicate. The proliferation of SW-13 cells in collagen matrices was assessed by colony formation in agarose gels. The effect of the inhibitors was assessed by examining colony formation in gels prepared with 10% bovine serum and grown for 10 days. The numbers of colonies were compared by using the Student’s t test. In B, SW-13/pgrn, SW-13/AS, and SW-13/WT cells were grown in the presence or absence of 10% fetal bovine serum for 10 days. The numbers of colonies were compared by using the Student’s t test. In C, SW-13/pgrn cells were grown in the presence or absence of 10% fetal bovine serum for 10 days. The numbers of colonies were compared by using the Student’s t test.](cancerres.aacrjournals.org)
morphic biological responses of pgrn, but to different extents for each response.

Invasion across the basement membrane is among the earliest steps of epithelial tumor progression. SW-13/pgrn cells are considerably more invasive across Matrigel-coated filters than SW-13/vector cells, and this may be important in their enhanced tumorigenicity in vivo. Purified pgrn (1 nm) stimulated cell invasiveness to an extent equivalent to that of SW-13/pgrn cells. Pgrn is not strongly mitogenic for SW-13 cells at this concentration (5), indicating that pgrn is more active as a stimulus for invasion than for proliferation. In addition to the requirement for ERK and PI3k pathways, the increase in FAK phosphorylation (Fig. 1C) may contribute to the invasive phenotype of the SW-13/pgrn cells, because FAK is important in growth factor-mediated motility (32).

Because limited pericellular digestion of the extracellular matrix is essential for invasion to occur (33, 34) we investigated the expression of genes for matrix-degrading enzymes in pgrn-stimulated tumor progression. SW-13 cells express high levels of mRNA for several matrix-degrading enzymes including collagenase 1, stromelysin 2, and four membrane-type MMPs (Fig. 4A). Other MMPs often associated with invasive epithelial cells, such as MMP-2, -3, and -7 (34), are poorly expressed in SW-13 and are not up-regulated by pgrn expression, although these proteinases may be supplied by the stroma in vivo (34). These results are consistent with the low but detectable intrinsic invasive property of SW-13/vector cells (Fig. 3). Increased expression of pgrn increases the transcript levels of MMP-13 (collagenase 3) and MMP-17 (Fig. 4, A and B). MMP-17 is expressed in all breast carcinomas and breast cancer cell lines (35), but its role in tumor growth is not well understood. MMP-13 degrades several components of the extracellular matrix including fibronectin, tenascin C, and collagens I, II, III, and IV (36, 37), and can activate latent transforming growth factor β (38). MMP-13 is secreted as a zymogen, which is activated, in part, by membrane-bound MMP-14 (39) and MMP-1 (36), enzymes that are also expressed by SW-13 cells (Fig. 4). MMP-13 is strongly associated with invasive cancer, being expressed in the epithelial compartment of highly malignant head and neck carcinomas, vulvar squamous cell carcinomas, late stage melanomas, but not in premalignant or Clark grade I and II early stage melanomas (40). It is also highly expressed in breast tumors, but mainly by the tumor stroma (40). Clearly, therefore, collagenase 3 is a good candidate for continued investigation as a putative pgrn-regulated progression factor; however, the expression of high levels of MMP-1, MMP-10, and MMP-15 by both SW-13/vector and SW-13/pgrn cells suggests that matrix degradation may not be the major limiting factor that determines the low malignancy of SW-13/vector cells.

Nontumorigenic SW-13/vector cells are highly susceptible to anoikis (Fig. 5), but importantly, the increased expression of pgrn in SW-13/pgrn cells protects against anoikis. Therefore, pgrn may contribute to the invasive phenotype by enabling cells to evade detachment cell death. The normal expression level of pgrn in nontumorigenic SW-13 cells confer no protection against anoikis, because SW-13/vector and SW-13/WT cells were as susceptible to anoikis as were SW-13/AS cells. Insulin-like growth factor I receptor-deleted R+ fibroblasts are not protected from anoikis by pgrn, although it stimulates their proliferation (13). The differential protection afforded to SW-13 and R+ cells by pgrn indicates that additional unidentified signals, which are available to SW-13 cells but not R+ cells, are required for pgrn to prevent anoikic cell death. It is unlikely that these signals act solely through the ERK and PI3k pathways, because both of these signaling pathways are activated by pgrn in anoikis-susceptible R+ cells (13).

Three-dimensional collagen gels, which model the interstitial matrix, may be growth inhibitory for epithelial cells (41) and may promote the formation of differentiated structures even among some transformed cells (42, 43). This is important during tumor invasion because the ability of invasive epithelial cells to proliferate in the newly encountered interstitial matrix environment determines their capacity to expand into the stroma. The proliferation of SW-13/pgrn cells is inhibited when they are grown as monolayers on collagen as compared with plastic surfaces (Fig. 6A). In three-dimensional collagen gels, nontumorigenic SW-13/WT and SW-13/vector cells multiply very slowly but they do not become highly apoptotic (Fig. 6C). In contrast, tumorigenic SW-13/pgrn cells grow well in collagen gels (Fig. 6B). The growth of SW-13/pgrn cells in collagen and soft agar are distinct biological processes, producing dispersed clusters in collagen gels rather than discrete colonies, and displaying different sensitivity to pathway inhibitors (Fig. 6C). The ability of pgrn to overcome collagen inhibition is likely to be important in determining its tumorigenicity in vivo. Serum is essential for pgrn-mediated proliferation on collagen surfaces (Fig. 6A) or when embedded in collagen gels (Fig. 6B), whereas the proliferation of SW-13/pgrn cells is serum-independent on plastic surfaces (5). Neither serum or pgrn alone are sufficient to support SW-13 proliferation in collagen gels, thus at least two soluble extracellular signals are needed to overcome the growth-inhibitory effects of collagen, one from pgrn and another, presently unidentified, from serum. Blocking either of these signals may prevent early postinvasive tumor growth.

In summary, pgrn restores malignancy to SW-13 cells (5) and is required for tumor growth in some breast cancer lines (14, 15). It initiates a complex series of biological responses that are important in tumor development. It is proinvasive, protects against anoikis, and confers a proliferative phenotype in interstitial-type collagen matrices. Increased expression of pgrn elevates the expression of the genes for the matrix-degrading protease collagenase 3 and MMP-17. Cells that express high levels of pgrn show elevated phosphorylation of signaling molecules in the ERK, PI3k, and FAK pathways. All of the biological responses to pgrn that were tested are sensitive to pharmacological inhibition of the ERK and PI3k pathways, but the contribution of these two pathways to pgrn stimulation differs for each response. The differential contribution of the MAPK and PI3k pathways to pgrn stimulation of these activities, as well as the activation of the pgrn-dependent phosphorylation of FAK, opens a window on pgrn signaling as a complex network of intracellular interactions.

Therapeutic blockade of the pgrn system in appropriate cells, whether at the ligand or receptor level, would target this network, inhibit the pgrn signaling as a complex network of intracellular interactions. The blockade of the pgrn system in appropriate cells, whether at the ligand or receptor level, would target this network, inhibit the cell cycle, decrease activation of FAK, lower the expression of certain proinvasive matrix-degrading enzymes, restore the sensitivity of cells to anoikis, and inhibit their postinvasive growth in stromal matrices. Given the diversity of the effects of pgrn on tumor development, the blockade of the pgrn system may be useful as a potential target for cancer therapy in pgrn-sensitive tumors.

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