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Review

Progranulin (Granulin-epithelin precursor, PC-cell derived growth factor, Acrogranin) in proliferation and tumorigenesis

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Summary. The granulin-epithelin precursor, progranulin, PC-cell-derived growth factor or acrogranin, is a high molecular weight secreted mitogen. It is abundantly expressed in rapidly cycling epithelial cells, in the immune system and in neurons, such as cerebellar Purkinje cells. Progranulin contributes to tumorigenesis in diverse cancers, including breast cancer, clear cell renal carcinoma, invasive ovarian carcinoma and glioblastoma. It regulates the rate of epithelial cell division in responsive epithelial cells, and confers an invasive phenotype on these cells. It is involved in the wound response. During embryogenesis, progranulin accelerates blastocyst formation, and is a growth factor for trophectodermal cells. In the neonate, progranulin, regulates the hormone-dependent virilization of the hypothalamus. It activates phosphorylation of Shc, and p44/42 MAPK (mitogen activated protein kinase) in the ERK (extracellular regulated kinase) signaling pathway; PI3K (phosophatidyl inositol-3-kinase), AKT/protein kinase B, and $p70^{S6kinase}$ in the phosophatidyl inositol-3-kinase pathway; and focal adhesion kinase in the adhesion/motility pathway. The signaling properties of progranulin are apparently similar to those of classic growth factors, but the functional properties of progranulin distinguish it from these molecules. Deleting the insulin-like growth factor I receptor from murine embryonic fibroblasts blocks proliferation in response to all classic growth factors, such as epidermal growth factor, or platelet-derived growth factor, whereas progranulin retains mitotic activity on these cells. The defined biological actions of progranulin probably represent a small fraction of its overall functions. Transcriptome analyses show that the *progranulin* gene is induced in numerous situations that vary from obesity to the transcriptional response of cells to antineoplastic drugs. Here, the biological roles of progranulin will be reviewed, with an emphasis on cancer and cell proliferation.

Key words: Progranulin, Proliferation, Tumorigenesis

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Introduction

Progranulin (pgrn) is a growth factor that is an important mediator of tumorigenesis and wound repair. Growth factors, such as pgrn, and their contingent signaling events, occupy a critical position in regulating the normal tissue dynamics of development, growth, and repair. Growth factors and their receptors are often over-expressed by cancer cells, and in other proliferative disorders, and are, therefore, attractive as potential therapeutic targets (reviewed in Favoni and Cupis, 2000). A better understanding of the pgrn system may suggest avenues for designing new therapies for neoplastic disease and wound management.

Pgrn is a glycoprotein of 576 amino acids in humans (reviewed in greater detail in Bateman and Bennett, 1998). It is composed of tandem repeats of a motif of 12 cysteine residues (Bhandari et al., 1992a, 1993; Plowman et al., 1992; Baba et al., 1993a) (Fig. 1). The individual domains are referred to as granulins or epithelins. The full-length protein, with seven and a half granulin domains, is referred to as pgrn (Bhandari et al., 1992a); the granulin-epithelin precursor (Xu et al., 1998), PC-cell-derived growth factor (Zhou et al., 1993), or acrogranin (Anakwe and Gerton, 1990). Molecular architectures of tandemly repeated cysteine-rich domains are common in membrane-associated regulatory proteins, such as the notch family, but there is no evidence that pgrn is membrane associated. Rather, it is either constitutively secreted (Zhou et al., 1993; Xu et al., 1998), or stored in vesicular organelles such as the acrosome (Anakwe and Gerton, 1990). The gene is located on chromosome 17 in humans (Bhandari and Bateman, 1992b), and chromosome 11 in mice (Bucan et al., 1996). The protein coding-region, is composed of 12

exons (Bhandari and Bateman, 1992b; Baba et al., 1993b).

Most recent work has focused on the intact precursor, which is a potent mitogen for many different cell lines, however individual granulin-epithelin domains have been isolated as discrete 6 kDa peptides from granulocytes (Bateman et al., 1990), kidney extracts (Shoyab et al., 1990) and urine (Sparro et al., 1997). One of these, granulinA/epithelin1, is mitogenic for human keratinocytes, and supports colony growth by NRK-SA6 (normal rat kidney) cells in the presence of transforming growth factor-ß (Shoyab et al., 1990). In other systems, granulinA/epithelin1 inhibits cellular proliferation, as does the related pgrn-derived peptide granulinB/ epithelin2 (Shoyab et al., 1990). High molecular weight forms of the granulin-epithelin precursor were identified in the acrosome of guinea pig sperm, as a glycoprotein called acrogranin (Anakwe and Gerton, 1990), and as an autocrine growth factor for the murine teratoma PC cell line, hence the name, PC-cell-derived growth factor or PCDGF (Zhou et al., 1993). A growth factor intermediate in size between the full length pgrn and the 6 kDa granulin-epithelin peptides, which is called epithelial transforming growth factor (TGFe), has an N-terminal sequence that is highly homologous with granulinA/epithelin1, and may be derived from the granulin-epithelin precursor (Parnell et al., 1992).

Granulin-like polypeptides, or their genes, have been demonstrated across many animal phyla, including mammals (Bateman et al., 1990; Anakwe and Gerton, 1990; Shoyab et al., 1990; Couto et al., 1992; Sparro et al., 1997), teleost fish (Belcourt et al., 1993; Uesaka et al., 1995), the urochordate sea squirt *Ciona intestinalis* (Dehal et al., 2002), the locust (Nakakura et al., 1992), marine worms (Deloffre et al., 1999), *Caenorhabditis elegans* (NCBI accession number NM_ 060580), as an anticoagulant in leech saliva (Hong and Kang, 1999), in the infective larval stage of the intestinal parasite *Toxocara canis* (Tetteh et al., 1999), and in the platyhelminthe *Schmitdea mediterranea* (NCBI AY067073 and AY067077). Granulin-like modules occur at the C-terminus of a family of plant thiol proteases (Schaffer and Fischer, 1988; Watanabe et al., 1991; Granell et al., 1992), suggesting an origin for the granulin domain of great antiquity. The conservation of the granulin/epithelin motif implies important functions, although there are species, such as *Drosophila*, which appear to have lost the *granulin* genes from their genome (NCBI *Drosophila melanogaster* genome website). The three dimensional structure of a granulin from fish was resolved by nuclear magnetic resonance as a stack of four beta hair pins (Hrabal et al., 1996), and a similar, but less rigid, spatial conformation was observed in synthetic human granulin peptides (Tolkatchev et al., 2000).The granulin fold can be partially overlain on the epidermal growth factor fold (Hrabal et al., 1996), but the overall conformation of granulin is unique (Hrabal et al., 1996), emphasizing that the granulin-epithelin system developed independently from any other growth factor family.

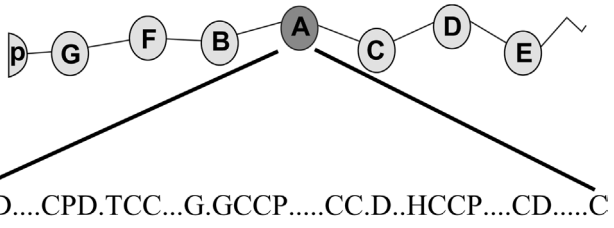

Fig. 1. Pgrn consists of seven (A-G) and a half (p) tandem repeats of a twelve cysteine motif (Bhandari et al., 1992a, 1993; Plowman et al., 1992; Baba et al., 1993a). The granulin motif is found in no other animal proteins, but occurs at the C-terminus of certain plant thiol proteases (Schaffer and Fischer, 1988; Watanabe et al., 1991; Granell et al., 1992). In many cases, mammalian pgrn is secreted as an intact mitogenic protein (Zhou et al., 1993; Xu et al., 1998). Granulin domains A, B, C, D and F have been isolated from biological tissues as discrete 6 kDa peptides (Bateman et al., 1990; Shoyab et al., 1990; Sparro et la., 1997). In some systems the granulin peptides stimulate cell proliferation but in other systems, they act as inhibitors of mitosis (Shoyab et al., 1990; Plowman et al., 1992; Liau et al., 2000).

Tissue distribution

The *pgrn* gene is expressed in most epithelial cell lines tested (Bhandari et al., 1992a; Plowman et al., 1992; Daniel et al., 2000), many hematopoietic cell lines, in some fibroblastic cell lines and in transformed smooth muscle cell lines (Bhandari et al., 1992; Daniel et al., 2000). At the tissue level, the rat spleen expresses the highest amount of pgrn mRNA, followed by reproductive tissues and adrenal. Expression was also detected in lung and kidney whereas skeletal muscle had very low levels of the transcript (Bhandari et al., 1993; Baba et al., 1993a).

In situ hybridization of healthy adult rodent tissue delineated the specificity of *pgrn* gene expression *in vivo*. The *pgrn* gene is constitutively expressed in rapidly cycling epithelium, especially in skin and gastrointestinal tract (Daniel et al., 2000). It is abundant in the male reproductive system including spermatids (Anakwe and Gerton, 1990; Baba et al., 1993a; Daniel et al., 2000) and the epididymis (Daniel et al., 2000). Strong signals were also obtained in immune cells and in specific neurons of the brain such as Purkinje cells of the cerebellum, pyramidal and granule cells of the hippocampus and in some neurons of the cerebellar roof nuclei. Very low levels, or no pgrn mRNA, were detected in unstimulated connective tissue, muscle or endothelia (Daniel et al., 2000). It has recently been reported, however, that pgrn is induced in dermal fibroblasts and endothelial cells in response to transcutaneous wounding (He et al., 2003). Pgrn immunoreactivity has been observed in capillary endothelia adjacent to invasive ovarian tumors (Jones et al., 2003), and together these observations are suggestive of a role for pgrn mRNA in active endothelial cells and connective tissue.

Progranulin in cell division

The diverse biological activities exhibited by mammalian cells are highly controlled; growth factors are central to this regulation and dictate a cell's ability to proliferate, survive, differentiate and to migrate. The initial demonstration that pgrn functions as an autocrine growth factor came from work on the PC murine-teratoma cell line. These cells originated from poorly tumorigenic insulin-dependent adipogenic teratomas (Serrero and Khoo, 1982; Serrero, 1985), and were rendered highly tumorigenic by successive passage *in vivo* (Serrero et al., 1991). In becoming aggressively tumorigenic, PC-cells acquired the ability to secrete a high molecular weight growth factor (PCDGF), that, upon isolation and structural characterization, was found to be indistingusihable from murine pgrn (Zhou et al., 1993). Pgrn/PCDGF confers elevated tumorigenicity to PC cells, since tumor growth of PC cells was markedly reduced when Pgrn/PCDGF mRNA was attenuated using an antisense strategy (Zhang and Serrero, 1998).

Early models of growth factor action characterized growth factors as "competence" factors, which prepare the cell for division, and "progression" factors, which enable competent cells to proceed through mitosis (Pledger et al., 1978; O'Keefe and Pledger, 1983; reviewed in Jones and Kazlauskas, 2001). Both competence and progression factors are required to sustain proliferation of non-transformed cells in serum-free media. The categorization of growth factors in this way is not always clear cut. It ignores many of their other functions, for example the regulation of apoptosis, but in the case of 3T3 murine embryo fibroblasts, platelet-derived growth factor (PDGF) acts as a competence factor while insulin-like growth factor-I (IGF-I) is a progression factor (O'Keefe and Pledger, 1983). By deleting the IGF-I receptor from mice by homologous recombination (Sell et al., 1994), it became possible to obtain murine embryo fibroblasts without an IGF-I-receptor (R-cells) i.e. lacking the progression arm of the competence-progression couple, and this revealed an unusual property for pgrn (Xu et al., 1998).

R- cells are unable to proliferate in response to IGF-I. Less predicatably, they do not divide in response to any other conventional growth factor (Sell et al., 1994), and could not be transformed by several oncogenes that are normally highly active in murine-embryonic fibroblasts (Sell et al., 1993, 1994; reviewed in Baserga, 2000). Nevertheless, R- cells continue to proliferate in serum (Sell et al., 1994), suggesting that extracellular factors exist that can overcome the mitotic block imposed on IGF-I-receptor deficient cells. This was confirmed when pgrn was isolated from the conditioned medium of BRL-3A (buffalo rat liver) cells, and shown to support the proliferation of R- cells (Xu et al., 1997, 1998). IGFs are overexpressed in many tumors, and the growth of several different cancers is blocked when the IGF-I system is targeted (reviewed in Moschos and Mantzoros, 2002). Pgrn may interfere with this suppression, and, in principle, prolonged anti-IGF therapy may select for cancer cells with an overactive pgrn-system.

Like the IGFs, pgrn stimulates the phosphorylation of Shc, and p44/42 MAPK (mitogen activated protein kinase) in the ERK (extracellular regulated kinase) signaling pathway, and PI3K (phosophatidyl inositol-3-kinase), AKT/protein kinase B, and $p70^{S6kinase}$ in the phosophatidyl inositol-3-kinase pathway (Zanocco-Marani et al., 1999). It does not stimulate phosphorylation of the insulin-receptor substrate (IRS-1), suggesting that pgrn does not directly mimic IGF-I signaling. The intensity and duration of growth factor induced signaling is known to direct distinct functional outcomes (reviewed in Marshall, 1995), and this is reflected in the different biological responses of R-cells to pgrn and other growth factors. Pgrn activates a prolonged phosphorylation of the p44/42 MAPkinases in R-cells (Zanocco-Marani et al., 1999). EGF, in comparison, stimulates only a short, weak, activation (Zanocco-Marani et al., 1999), while PDGF elicits a longer, but still transient activation of p44/42 MAPkinase (Zanocco-Marani et al., 1999). Correspondingly, PDGF promotes entry into S-phase, but the R-cells do not enter M-phase. The levels of cyclin B1 decreases to prestimulation levels after 48 hours, whilst EGF fails to stimulate entry of R- cells into S-phase at all. Pgrn promotes cyclin B1 expression and passage through the complete cell cycle (Zanocco-Marani et al., 1999), so that from a functional viewpoint, it may be said to behave as both competence and progression factor in this case.

Progranulin receptors

The proliferative properties of pgrn raise the obvious question of the nature of its receptor. At present, the receptor for pgrn has neither been isolated nor cloned, but experiments utilizing chemical cross-linking methodology have provided some clues to the nature of the receptor for pgrn and its related peptides. A protein of about 120 kDa present on the cell surfaces of a mink lung epithelial cell line, CCL64, mouse fibroblasts, NIH3T3 and PC cells was identified as a putative receptor for pgrn by chemical cross-linking experiments. Through Scatchard plots using the membranes of the CCL64 cell line, it has been proposed that there are two classes of binding sites for pgrn: the high affinity and the low affinity binding sites (Xia and Serrero, 1998). In SW13 cells, a protein of molecular weight of 170-175 kDa present on the membrane was reported to cross-link to TGFe, a pgrn related peptide (Parnell et al.,1995). Culouscou and co-workers (1993) had shown that granulinA/epithelin1 binds to a 140-145 kDa protein on the cell surface of a human breast cancer cell line, MDA-MB 468. Scatchard analysis revealed the presence of high affinity and low affinity binding sites for granulinA/epithelin1 in this cell line (Culouscou et al., 1993).

The delta-like (dlk) protein was shown to interact with pgrn by screening a NIH 3T3 (mouse embryonic fibroblast) cell line cDNA library using the yeast two hybrid system (Baladron et al., 2002). Dlk is a member of the epidermal growth factor (EGF)-like homeotic family (Baladron et al., 2002) which is involved in several differentiation processes including adipogenesis, hematopoiesis and adrenal gland differentiation (reviewed in Laborda, 2000). In the presence of glucocorticoids, Balb/c 3T3 fibroblasts stably transfected with antisense *Dlk1* constructs exhibited the ability to form foci, had a higher growth rate and lost contact inhibition (Baladron et al., 2002). Although dlk may be part of the pgrn-signaling system it is unlikely that dlk could serve on its own as a receptor for pgrn for the following reasons. Firstly, in contrast to antisense *Dlk1* transfectants, mammalian cells stably transfected with antisense pgrn constructs exhibited impaired cell growth and a reduction in colony and tumor formation (Zhang and Serrero, 1998; He and Bateman, 1999; Lu and Serrero, 2000). Secondly, the dlk protein has a molecular mass of 41 kDa (Laborda et al., 1993) which is much smaller than the other cell surface proteins identified to date to bind to pgrn and its related peptides. Thirdly, in addition to pgrn, dlk has also been shown to interact with GAS1 (Baladron et al., 2002), an integral plasma membrane protein that induces growth arrest in the G0/S phase of cell cycle (Del Sal et al., 1992). Overexpression of *GAS1* gene in NIH 3T3 cells prevented cell proliferation (Del Sal et al., 1992), while stable tranfection of these cells with antisense GAS1 resulted in higher growth rates and loss of contact inhibition (Evdokiou and Cowled, 1998). These are similar biological characteristics seen with the antisense *Dlk1* transfectants. Given that dlk and GAS1 exert similar biological effects on the cell, this interaction may have a role in modulating cell growth. On the other hand, dlk's ability to interact with pgrn in which both proteins exert opposing biological effects on the cell suggests the intriguing possibility that dlk could modulate the interaction between pgrn and its receptor (Baladron et al., 2002). At this time the physiological significance of this interaction is not known.

Progranulin and tumorigenesis

There is considerable evidence that pgrn is important in tumor growth. This rests principally on three arguments; (i) the *pgrn* gene is specifically up-regulated in a number of human cancers; (ii) pgrn promotes tumor formation in experimental systems when it is over-expressed; (iii) attenuating pgrn expression blocks tumor formation. Pgrn (called in this context PCDGF) is highly expressed in estrogen-independent breast cancer cell lines, such as MDA-MB-468 (Lu and Serrero, 2000). In hormone-dependent breast cancer lines, such as MCF-7, pgrn mRNA expression is regulated by estrogen (Lu and Serrero, 1999), as are other growth factors such as transforming growth factor-α and IGF-II (Bates et al., 1988; reviewed in Westley and May, 1994). The association of *pgrn* gene expression with breast cancer was confirmed when the transcriptome profiles of normal and transformed mammary epithelia were compared (Leerkes et al., 2002). Using shotgun sequencing of open reading frame expressed sequence tags (ORESTES), global gene expression from a pool of 24 breast cancer cell lines was compared with that of purified normal mammary cells (Leerkes et al., 2002). The *pgrn* gene was detected with an incidence of 17:1 between tumor and normal breast epithelia, (from 37,980 tumor and 21,437 normal sequences). Tumor-related expression was observed for only 154 genes out of a genome of approximately 30,000 genes, and the only other growth factor that was detected as elevated in the tumor cells was transforming growth factor-ß1 (Leerkes et al., 2002). Subtracted membrane-associated polyribosomal cDNA libraries identify mRNAs whose translated products enter the endoplasmic reticulum. Many of these proteins are destined for the plasma membrane or secretory pathways. Using this approach with libraries generated from one normal breast, four breast cancer cell lines and a prostrate cancer cell line revealed that the pgrn transcript was among the top 15 endoplasmic reticulum-destined genes expressed specifically in breast cancers as opposed to normal tissue (Egland et al., 2003).

Using an innovative antibody-based screening method, Liau and colleagues (2000) identified *pgrn* gene products as tumor-specific markers of human glioblastomas, and then demonstrated a proliferative response of glioblastoma cell lines to a granulin peptide (granulinD). Tumor-specific expression of the *pgrn* gene in glioblastomas was subsequently confirmed by gene microarray analysis (Markert et al., 2001). Pgrn was identified as one of the tumor antigens in a screen with sera of gastric cancer patients and its gene was demonstrated to be over-expressed in cancer tissues versus non-cancerous gastric tissues (Line et al., 2002). Pgrn is expressed at low levels in healthy renal epithelium (Donald et al., 2001), but it becomes highly elevated in clear cell renal carcinomas (Donald et al., 2001). Non-transformed renal epithelial cysts also show elevated pgrn expression (Ali et al., 1999). In ovarian cancer, a laser-microdissection technique identified *pgrn* as a major gene that is specifically up-regulated in invasive versus non-invasive ovarian tumors (Jones et al., 2003). *Pgrn* gene expression was also reported to be higher in Warthin's tumor, the second most common benign tumor of salivary glands than in non-tumor salivary gland tissue (Francioso et al., 2002). Neoplastic transformation of epithelia is associated with a transition to a more mesenchymal phenotype, and, in renal epithelia, this epithelial-mesenchymal transition is accompanied by a modest increase in *pgrn* gene expression (Kiemer et al., 2001).

The elevated pgrn expression in cancer cells is not sufficient, alone, to prove that pgrn is critical to tumor development, since it may be a secondary phenomenon

that is incidental to tumorigenesis. Pgrn does not transform murine 3T3 cells (Zanocco-Marani et al., 1999), suggesting that it is not, by itself, oncogenic. However, when a transformed, but anchorage-dependent and non-malignant, adrenal carcinoma line, SW-13, was forced to over-express pgrn, it became highly tumorigenic (He and Bateman, 1999). By increasing *pgrn* expression, the fraction of cells in S-phase increases, and the cells become anchorage-independent in soft agar (He and Bateman, 1999). The attenuation of *pgrn* expression using antisense cDNAs slows the proliferation of SW-13 by up to 80% in serum-free medium (He and Bateman, 1999). MDCK cells, which are non-transformed (but immortal) canine kidney epithelia (Madin and Darby, 1958; Gaush et al., 1966) show a similar response to pgrn (He and Bateman, 1999). Given that renal clear cell carcinomas demonstrate high pgrn expression (Donald et al., 2001), this may be clinically significant. In summary, the rate of proliferation in epithelial cells such as SW-13 and MDCK is regulated by their steady state level of *pgrn* gene expression, and, critically, pgrn supports the transition from a non-tumorigenic phenotype (parental SW-13), to a tumor-forming phenotype (SW-13 over-expressing *pgrn*). The dependence of SW-13 cells on pgrn for tumorigenicity provides a useful model to study pgrn action in tumor progression.

Carcinomal progression depends on many parameters besides accelerated cell division. Local invasion is critical. The cells must be motile and able to escape anoikis, a form of apoptosis that occurs when cells that are normally attached to a surface release their contact with the substratum (reviewed in Frisch and Screaton, 2001). Pgrn stimulated the motility of R-cells on plastic (Zanocco-Marani et al., 1999), and enhanced invasiveness of SW-13 cells through Matrigel (He et al., 2002). This is consistent with the observation that invasive, but not non-invasive, ovarian tumors show high levels of *pgrn* gene expression *in vivo* (Jones et al., 2003). Pgrn transcript levels were found to be two-fold lower in anchored human mammary carcinoma cells (MCF-7) relative to the unanchored cells, again suggesting that this gene plays an important role in invasion (Goldberg et al., 2001). Pgrn increased the expression of the matrix digesting metalloproteinases, MMP-13 and MMP-17, although the expression of other MMPs that are often associated with basement membrane digestion, such as MMP-2 and MMP-7 (He et al., 2002) were insensitive to pgrn stimulation. Intracellular signaling for motility employs several pathways, including the small GTPases and focal adhesion kinase (FAK)(Sieg et al., 2000). FAK is an essential intermediate in motility signaling for growth factors such as EGF and PDGF (Sieg et al., 2000), and becomes hyperphosphorylated in response to pgrn (He et al., 2002). Pgrn significantly reduced anoikic cell death in SW-13 (He et al., 2002), but not in R- cells (Zanocco-Marani et al., 1999). The difference in the anoikic response to pgrn of R- and SW-13 cells suggests either that SW-13 cells access anti-apoptotic pathways that are unavailable in R- cells, or reflects differences in the strength and duration of signaling pathways shared by both cell types. Each of the pro-tumorigenic activities of pgrn, i.e. proliferation, anchorage-independence, invasion and insensitivity to anoikis, depended on the p44/42 MAPkinase and phosphatidylinositol-3-kinase signal transduction cascades. Sensitivity towards pharmacological inhibitors of each of these signaling pathways vary between the different biological responses to pgrn suggesting that there is a partial division of labor between the two pathways (He et al., 2002).

Once a cancerous cell has negotiated the hazards of invasion, it faces the challenge of expansion in the interstitial extracellular matrix. Metastatic cells that have efficiently crossed the endothelial barrier show surprisingly low success rates in establishing tumor growths in the interstitium (Cameron et al., 2000), in part because the interstitial matrix suppresses the proliferation of normal and, in some cases, transformed epithelia (Keely et al., 1995; Alford et al., 1998). Parental SW-13 cells proliferate very slowly in type I collagen gels (but do not undergo large scale apoptosis). In contrast, SW-13 cells that overproduce pgrn, and are tumorigenic, proliferate well in type I collagen, but need additional signals from serum to do so (He et al., 2002). Thus pgrn may contribute to tumor progression by stimulating invasion, protecting against anoikis, and supporting tumor expansion in the unfavorable interstitial environment.

Given that pgrn stimulates several elements in the tumor progression cascade, does blocking the pgrn-system impede tumor growth? Reports from Serrero's laboratory strongly suggest that, at least for breast cancer cells, it does. Targeting pgrn/PCDGF mRNA expression in estrogen-independent MDA-MB-468 cells using an antisense pgrn/PCDGF expression vector, reduced colony formation in soft agar by up to 80%, and tumor incidence and weight in mice was reduced to approximately 90% that of the parental cells (Lu and Serrero, 2000). Immunoneutralization, or antisense attenuation of pgrn/PCDGF, significantly diminishes estrogen-stimulated DNA synthesis in MCF-7 cells (Lu and Serrero, 2001). The over-production of pgrn/PCDGF eliminates the requirement for estrogen in the proliferation of MCF-7 cells, suggesting that the estrogen-driven expression of pgrn/PCDGF contributes to the mitogenicity of estrogens in hormone-dependent breast cancer cells. The accumulation of cyclin D1 and hyperphosphorylated pRb, which are essential steps in the initiation of the cell cycle, is stimulated by pgrn/PCDGF. Blocking the p44/42 MAPkinase signaling cascade prevented the stimulation of cyclin D1 (Lu and Serrero, 2001). Not all of estrogen's effects on breast cancer cells require pgrn since the protooncogene c-myc, which is an established mediator of estrogen action in breast cancer cells, was not expressed in response to pgrn/PCDGF (Lu and Serrero, 2001). The estrogen

receptor can be phosphorylated and activated by MAPkinase, even in the absence of estrogenic ligand (reviewed in Weigel and Zhang, 1998; Klotz et al., 2002). Thus, the estrogen-mediated elevation of pgrn/PCDGF may, in principle, feed back on the estrogen receptor to reinforce the initial estrogen stimulus, via activation of MAPK, while estrogen-stimulated secretion of pgrn/PCDGF will transmit the proliferative response to adjacent cells that may have been uninvolved in the initial estrogen signal.

Selective estrogen response modifiers such as tamoxifen are used clinically to target estrogen receptors in the treatment of breast cancers (reviewed in Riggs and Hartmann, 2003). Over-production of pgrn/PCDGF blocks the anti-proliferative actions of tamoxifen on MCF-7 cells (Lu and Serrero, 2001). Indeed, pgrn-mediated resistance to antineoplastic drugs may be quite a widespread phenomenon. The cytotoxic drug doxorubicin dramatically elevates *pgrn* gene expression in MCF-7 cells, and cells that are resistant to doxorubicin show constitutively high levels of *pgrn* gene expression (Kudoh et al., 2000). Thus therapeutic targeting of the pgrn system may prove useful not only to impede tumor progression, but to sensitize pgrn-responsive tumors to certain antineoplastic drugs.

From the various strands of evidence reviewed above, a model emerges in which pgrn is recruited from normal processes of tissue homeostasis into tumor formation in solid cancers such as breast, glioblastoma, kidney and the ovary. The elevated expression of the *pgrn* gene in these cancers increases the rate of cell division, confers anchorage-independence, increases invasiveness and resistance to anoikis and decreases sensitivity to certain anti-tumor drugs (Fig. 2). Pgrn stimulates several important signaling pathways, notably the ERK and PI-3-kinase cascades and stimulates increases in the levels of cell cycle proteins, cyclin D1 and cyclin B. In these respects, pgrn resembles the classic growth factors, although, from a functional viewpoint, pgrn is atypical, since it is the only protein growth factor known to stimulate mitosis in murine embryo fibroblasts that lack functional IGF-I receptors. Clearly, however, pgrn does not exist to stimulate tumor progression, and in the remaining part of the review we will examine other physiological processes in which pgrn takes part.

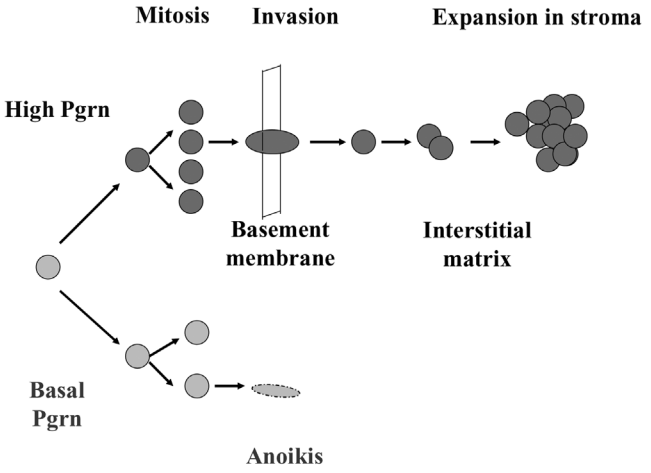

Fig. 2. : A schematic for some of the ways pgrn may promote tumor progression in the SW-13 carcinoma model. When SW-13 cells express basal levels of pgrn, they proliferate slowly, and when detached from the substrate, are poorly invasive, undergo detachment apoptosis or anoikis, and do not grow well in interstitial matrix environment. When the same cells express elevated levels of pgrn, their proliferation is enhanced, they become more invasive, resistant to apoptosis, and grow well in interstitial matrix environment, each of which contributes to epithelial tumor progression (He and Bateman, 1999; He et al., 2002).

Progranulin and development

The correlation between pgrn expression and epithelial tissue proliferation that was noted for adult tissue, clearly extends to the earliest stages of life. Pgrn is present in the oocyte (Diaz-Cueto et al., 2000; Suzuki et al., 2000b). Gerton and colleagues demonstrated that after fertilization pgrn (called acrogranin) mRNA levels decline to the 4 cell stage (Diaz-Cueto et al., 2000). It is re-expressed in the 8 cell embryo (Diaz-Cueto et al., 2000), and achieves highest pre-implantation levels in the trophectoderm of the blastocysts (Diaz-Cueto et al., 2000), which are the earliest cells to adopt an epithelial phenotype during development. Blastocysts in culture secrete pgrn/acrogranin into the medium (Diaz-Cueto et al., 2000). Adding pgrn/acrogranin to eight-cell embryos accelerates cavitation, and the expansion of the blastocoel, and acts as a growth factor for trophectodermal cells (Diaz-Cueto et al., 2000). Blocking the endogenously secreted pgrn/acrogranin with an immunoneutralizing antiserum significantly delayed the formation of blastocysts (Diaz-Cueto et al., 2000), demonstrating that pgrn/acrogranin is required in the critical transition from the morula to the fluid-filled blastocyst.

Although the *pgrn* gene is widely expressed in both male and female tissue (Bhandari et al., 1993; Daniel et al., 2000), there is evidence that it plays a role in establishing the male phenotype. Sexually dimorphic pgrn-gene expression occurs during testicular development (Wertz and Herrmann, 2000) and in the sexual maturation of the hypothalamus (reviewed in detail in Suzuki and Nishiahara, 2002). The early bipotential urogenital ridge, from which the gonads, the renal system and the adrenal cortex develop (reviewed in Keegan and Hammer, 2002), does not express pgrn, but by 11.5 days post-conception (in mice), as the gonads differentiate in the genital ridge, pgrn expression is activated (Wertz and Herrmann, 2000). By 14.5 days post-conception, pgrn is expressed in the testis but not the ovary (Wertz and Herrmann, 2000). Pgrn function in testicular development is unknown, but its male-specific expression in the developing gonads suggests that it may be regulated downstream from male-determination genes

such as *Sry*, *Sox-9* or *AMH*.

The hypothalamus expresses a default female phenotype unless acted upon by estrogens in the neonatal stage, the estrogens being derived from circulating androgens by aromatization (reviewed in Suzuki and Nishiahara, 2002). Pgrn was identified by differential gene display as among the most androgen sensitive genes in the neonatal hypothalamus (Suzuki et al., 1998). It is expressed in the arcuate nucleus and the ventromedial hypothalamus in a gender-specific fashion (Suzuki et al., 1998). Lowering pgrn mRNA levels in the male neonatal hypothalamus using antisense oligonucleotides causes a general blunting of sexual activity in the mature animals, although this is not accompanied by changes in circulating sex hormones (Suzuki et al., 2000a). The precise mechanism by which pgrn regulates hypothalamic development remains uncertain, but these reports are the first to definitively establish functional outcomes for pgrn expression in the brain.

Regulation of the *progranulin* gene in the hematopoeitic system, inflammation and wound repair

Granulin/epithelin peptides have been isolated from human inflammatory cells, rat bone marrow (Bateman et al., 1990), and equine neutrophils (Couto et al., 1992) suggestive of roles in the hematopoietic system. Exposing neutrophils to *E.coli* resulted in the early immediate induction of pgrn mRNA level, although exposing the neutrophils to *Yersinia pestis* had no effect on the level of pgrn expression (Subrahmanyam et al., 2001). The pgrn transcript was identified as among the top 50 transcripts expressed in monocyte derived dendritic cells (Hashimoto et al., 1999). The *pgrn* gene has been identified as one of the genes that is up-regulated in acute myeloid leukemia (AML) blasts as compared to normal immature progenitor CD34+ cells (Virtaneva et al.,2001) and is also one of the genes that differentiate between acute lymphoblastic leukemia (ALL) and AML (Chow et al., 2001). On the other hand, Larramendy and co-workers demonstrated that in AML patients with white blood cell counts higher than $100x10^9$/L, the *pgrn* gene was under-expressed (Larramendy et al., 2002). Clark and co-workers provided further evidence demonstrating the up-regulation of pgrn mRNA levels in myeloid leukemic cells (Clark et al., 2002). It is known that high frequencies of mitochondrial DNA (mtDNA) dimers are found in myeloid leukemic cells (Clayton and Vinograd, 1969). Using hybrid cells containing dimer mtDNA made by fusing the LMTK- cells, a mouse fibroblast cell line which does not express the *thymidine kinase* gene, with enucleated mouse LA9 cells, another mouse fibroblast cell line which possess dimer mtDNA, they showed that *pgrn* gene is up-regulated 1.9 fold over that of LMTK- parental cell line in DNA microarray studies (Clark et al., 2002). Despite the association between pgrn expression and leukemia, the roles of pgrn in leukemia remain uncertain.

Recently, roles for pgrn in wound repair have been reported. In response to transcutaneous wounding, the pgrn transcript level is up-regulated relative to the levels in normal tissue (He et al., 2003). Pgrn mRNA remained high up to 10 days after injury, with the highest levels at the wound site itself, decreasing at sites away from the wound. Importantly, *pgrn* gene expression is induced in fibroblasts and endothelial cells, two cell types that do not express detectable pgrn transcripts in normal healthy skin. The up-regulation can be detected within one day following wounding. *Pgrn* gene expression is also detected in neutrophils and macrophages recruited to the site of the wound from day 1 but by day 7, the mRNA levels are lowered. In keratinocytes, the *pgrn* gene is constitutively expressed in the wounded skin as well as in normal healthy tissue (He et al., 2003).

The inflammatory response and formation of granulation tissue were enhanced in trancutaneous puncture skin wounds treated with recombinant pgrn versus untreated wounds. The number of macrophages and neutrophils were increased by day 4 following wounding. By day 7, the number of macrophages has decreased. On day 7, there were more fibroblasts and blood vessels within the pgrn treated wound site with many of the blood vessels being larger in size in treated wounds versus the untreated controls (He et al., 2003).

Pgrn was shown to be mitogenic for rat dermal fibroblasts (RF) and endothelial cells, human dermal microvascular endothelial cells (HMVEC), rat cardiac microvascular cells (RE) and a bovine adrenal medullary cell line (EJG). Pgrn induced the migration of the HMVEC and RF cell lines through collagen-coated filters. The migration of HMVEC was prevented by MAPK kinase inhibitor or PI-3 kinase inhibitor, which suggests roles for the ERK and PI-3 kinase signaling pathways in cell migration. Pgrn promoted the assembly of endothelial cells into tubule-like structures on Matrigel, a basement membrane-like substrate which is an angiogenic-like process (He et al., 2003). Thus the *in vivo* observations of increased fibroblast number and blood vessel count can be reproduced under defined conditions *in vitro*.

Using a different experimental approach, Zhu and co-workers (2002) also revealed a role for pgrn in wound healing. Mice whose *secretory leukocyte proteinase inhibitor* (*SLPI*) gene was knocked out exhibit a deficiency in wound repair (Ashcroft et al., 2000). The SLPI protein blocks the inflammatory responses elicited by macrophages and monocytes to microbial products (Jin et al., 1997, 1998). Based on these observations, it was rationalized that macrophages probably express a protein that binds to SLPI, and by screening a mouse bone marrow macrophage cell line cDNA library using the yeast two-hybrid technology with the mature SLPI peptide as bait, SLPI protein was shown to bind to pgrn. SLPI-pgrn interaction was confirmed through immunoprecipation *in vitro* and *in*

vivo, with binding mediated through the inter-granulin linker regions or to a site found only in pgrn's tertiary structure, since SLPI does not form stable complexes with the individual granulin/epithelin peptides (Zhu et al., 2002).

Neutrophil elastase and pancreatic chymotrypsin, two SLPI-targeted serine proteinases, digest pgrn, and given that neutrophil elastase is produced at sites of inflammation, it may be the proteinase that cleaves pgrn physiologically. Amino-terminal sequencing of pgrn fragments obtained following elastase treatment demonstrated that the enzyme hydrolyses in the inter-granulin regions. Using an SLPI mutant that is inactive as an inhibitor but is still capable of binding to elastase, it was shown that this mutant could prevent pgrn from being digested by elastase. Thus SLPI inhibits pgrn digestion by elastase via two mechanisms- binding to elastase and binding to pgrn (Zhu et al., 2002).

The differential processing of pgrn by elastase in the presence or absence of SLPI may have important consequences. Pgrn and the granulin/epithelin peptides exert contrasting effects on the recruitment and activation of neutrophils. GranulinB/epithelin2 but not pgrn was demonstrated to induce epithelial cells to release interleukin 8 (IL-8), which is the major chemotactic cytokine that recruits neutrophils. In contrast, pgrn, but not granulinA/epithelin1 or granulinB/epithelin2, blocked the tumor necrosis factor alpha (TNFα)-induced respiratory burst in neutrophils and this effect was enhanced by the presence of SLPI. Degranulation of neutrophils as well as their spreading but not their adherence following stimulation by TNFα were also inhibited by pgrn (Zhu et al., 2002). Thus pgrn blocks later rather than early events in inflammation. At the molecular level, neutrophil cell spreading and respiratory burst induced by TNFα is mediated via the proline rich tyrosine kinase 2 (Pyk2) (Fuortes et al., 1999) and its activation through phosphorylation was inhibited in cells treated with pgrn (Zhu et al., 2002). The wound healing deficiency in the SLPI knock-out mice was normalized by the administration of recombinant pgrn to the wound sites (Zhu et al., 2002).

Taken together, these studies clearly demonstrate a role for pgrn in tissue repair. Pgrn-derived polypeptides may enter a wound with inflammatory cells, or be induced in the wound fibroblasts and endothelia. The proliferation and migration of dermal fibroblasts and endothelial cells is stimulated by pgrn, as is the formation of new blood vessels in the wound. There is a balance between granulin peptides and pgrn maintained by the elastase inhibitor SLPI. The granulin peptides appear to promote inflammation, while the precursor protein impairs the action of the inflammatory cytokine TNFα.

Regulation of the *progranulin* gene in other disease and physiological states

Transcript profiling studies are suggestive of roles for pgrn in many physiological and pathological states, including a number that are associated with inflammation or tissue injury. Overexpression of pgrn mRNA was detected in the synovial tissue of patients with rheumatoid arthritis, an autoimmune disease characterized by chronic inflammation, but not in osteoarthritis, a non-inflammatory joint disease (Justen et al., 2000). The *pgrn* gene appears often to be up-regulated in a variety of brain disorders. Pgrn mRNA levels were up-regulated in rat hippocampus after 24 hours following traumatic brain injury (Matzilevich et al., 2002) and in spinal cords of patients with amyotrophic lateral sclerosis (ALS) (Malaspina et al., 2001). In microglia cells infected with Creutzfeldt-Jakob disease (CJD), there was an induction in *pgrn* gene expression as compared to normal microglia (Baker and Manuelidis, 2003). Examination of pgrn mRNA levels in the cerebral cortex of mouse models of mucopoly-saccharidoses I and IIIB where a subset of microglia cells are phagocytic revealed that *pgrn* gene was induced 1.8 fold over that of control mice (Ohmi et al., 2003).

When endothelial cells are subjected to turbulent sheer stress, pgrn mRNA levels are decreased as compared to cells undergoing laminar sheer stress (Garcia-Cardena et al., 2001). *Pgrn* gene expression was shown to be higher in adipose tissue of obese mice versus their lean counterparts (Nadler et al., 2000). Healthy muscle in young adult rodents shows little if any pgrn expression (Daniel et al., 2000), but the *pgrn* gene was elevated in skeletal muscle of rhesus monkeys during aging (Kayo et al., 2001). This observation, together with the ability of retinoids to increase *pgrn* expression in smooth muscle (Chen et al., 2001), is suggestive of undefined roles for pgrn in muscle physiology. In some studies pgrn levels declined. Transcription profiling studies in mesanglial cells exposed to either sphingosine-1-phosphate (S1P), dihydro-S1P (DHS1P) or platelet derived growth factor (PDGF) showed a reduction in pgrn mRNA levels relative to untreated cells (Katsuma et al., 2003).

In PC12 pheochromocytoma cells, pgrn was demonstrated to interact with the N-terminal half of type III hexokinase through yeast two hybrid and coimmunoprecipitation studies. Both proteins were also coimmunoprecipitated as part of a macromolecular complex which included four other proteins- insulin-like growth factor binding protein-4, Miz-1, leptin and prostaglandin D synthase (Sui and Wilson, 2000). At the present time, the physiological significance of this interaction is not known.

Is there a role for progranulin in the nucleus?

Classically, growth factors and cytokines act extracellularly by binding their cognate receptors on the cell surface and transducing signals across the plasma membrane. However, it has now been shown that some growth factors as well as some cytokines could translocate to the nucleus or cytosol and exert their

biological activities within the cell. Among others these include acidic and basic fibroblast growth factor (aFGF and bFGF), interferon gamma (IFN-γ), parathyroid related protein (PTHRP) and vascular endothelial growth factor (VEGF) (reviewed in Olsnes et al., 2003). In the case of pgrn, its ability to bind to intracellular molecules was first demonstrated by Trinh and co-workers. Using the yeast two hybrid technology they reported that pgrn binds to the cysteine rich domains encoded by exon1 of HIV-1 and HIV-2 Tat proteins (Trinh et al., 1999).

Recently, Hoque and co-workers presented evidence supporting pgrn's interaction with intracellular Tat proteins as well as with cyclin T, an intracellular binding target of Tat proteins. In the hunt for molecules that might regulate cyclin T1 activity, Hoque and co-workers had found that pgrn as well as pgrn fragments containing CDE and PGFBA could bind to cyclin T1 in the yeast two hybrid system (Hoque et al., 2003). Cyclin T1 is expressed in all tissues. It associates with cyclin dependent kinase 9 (CDK9), to form the positive transcription elongation factor b (P-TEFb) complex and this complex plays a role in transcriptional elongation by phosphorylating the carboxyl terminal domain (CTD) of the largest subunit of RNA polymerase II (reviewed in De Luca et al., 2002). Pgrn however does not bind to CDK9. The binding between pgrn and cyclin T1 has been shown to occur *in vitro* and *in vivo* through the histidine domain of cyclin T1. When coexpressed in Cos7 cells, both pgrn and most of the cyclin T1 were colocalized to the cytoplasm. On the other hand, coexpression of pgrn fragments containing CDE with cyclin T1 resulted in the colocalization of cyclin T1 and most of the pgrn fragments containing CDE in the nucleus. It has been proposed that the interaction between pgrn and cyclin T1 of the P-TEFb complex prevents CDK9 from phosphorylating the carboxy terminal domain (CTD) of the largest subunit of RNA polymerase II, thereby suppressing transcription elongation. However, pgrn itself was reported to be a substrate for P-TEFb complex (Hoque et al., 2003). Cyclin T1 interacts with the HIV-1 Tat protein (Wei et al., 1998). Pgrn also binds to the HIV-1 and HIV-2 Tat proteins (Trinh et al., 1999; Hoque et al., 2003) with the interaction between pgrn and HIV-1 Tat occurring via the activation domain of HIV-1 Tat (Hoque et al., 2003), and pgrn suppresses transactivation mediated by HIV-1 Tat (Hoque et al., 2003).

These findings raise the possibility that within the cell, pgrn plays a role as a repressor of Tat trans-activation and transcriptional elongation mediated by P-TEFb complex (Hoque et al., 2003). Tat proteins may act outside the cell, and it has been suggested that when Tat is secreted from HIV infected cells, it interacts with granulin peptides and that these granulin peptides may serve as extracellular Tat co-factors in mediating Tat's mitogenic and angiogenic activities (Trinh et al., 1999).

Regulation of *progranulin* gene expression

Given the range of biological situations in which pgrn appears to take part, it is important to establish what regulates its expression, both in physiological and pathological states. Towards this end the human (Bhandari et al., 1996) and mouse (Baba et al., 1993b) promoters have been isolated and characterized. Structural analysis of the promoter did not reveal the presence of a conventional TATA box (Baba et al., 1993b; Bhandari et al., 1996). The human gene has two major transcriptional start sites that are about 100 bp apart. It has four potential CCAAT boxes and a number of regulatory sequences that have been implicated in inflammatory responses such as four copies of IL-6 response element (RE) and three nuclear factor (NF) IL-6 binding sites (Bhandari et al., 1996). Several of these putative regulatory elements occur also in the mouse promoter (Baba et al., 1993b). Transcriptional profiling demonstrated that proinflammatory cytokines, TNFα and IL-1 can activate pgrn gene transcription through the nuclear factor kappa B(NF-κB) signalsome complex in mouse embryonic fibroblasts (MEFs) (Li et al., 2002). The human *pgrn* gene promoter has three potential NF-κB binding sites, suggesting that the regulation by TNFα and IL-1 in these cells is probably direct.

Nuclear receptors and steroid hormones have been implicated in regulating pgrn mRNA levels in a number of tissues and cells. As was discussed above, in the breast cancer cell line MCF-7, 17ß-estradiol induces pgrn mRNA levels (Lu and Serrero, 1999). Both androgen (Suzuki et al., 1998) and estrogen (Suzuki et al., 2001) were reported to up-regulate *pgrn* gene expression in neonatal rat hypothalamus. The levels of transcript encoding pgrn were also increased by treatment with all-trans retinoic acid (ATRA) in HL-60, human promyelocytic leukemic cells (Kohroki et al., 1999, 2001) and in rat vascular smooth muscle cells (Chen et al., 2001). On the other hand, a different mode of regulation was observed in a mouse promyelocytic (MPRO) cell line that was derived by transducing a dominant-negative retinoic acid receptor into normal bone marrow cells. These cells are developmentally arrested at the promyelocytic stage, but unlike leukemic models of granulopoiesis such as HL-60 and NB4, which do not express secondary granule proteins, superphysiological concentrations of ATRA induces the full expression of genes for both neutrophil primary and secondary granule proteins in MPRO cells (Lawson et al., 1998). Microarray studies revealed that upon ATRA exposure, the pgrn transcript levels in MPRO cells remained unchanged up to 48 hours but were lowered at 72 hours (Lian et al., 2001), by which time all the cells have differentiated to polymorphonuclear neutrophils (Lawson et al., 1998). Although nuclear hormone receptors clearly regulate *pgrn* gene expression, an analysis of the promoter of the *pgrn* gene did not reveal the presence of any canonical sex steroid or retinoic acid response elements (Baba et al., 1993b; Bhandari et al., 1996). This suggests that the regulation of the *pgrn* gene by sex steroids and ATRA is secondary to the induction of other nuclear hormone-responsive regulatory factors.

Transcript profiling studies revealed that the levels

of pgrn transcript increased in human fibrosarcoma cells, HT1080, that were growth arrested by induction of p21 (Chang et al., 2000). This is an apparently contradictory observation, since p21 is an inhibitor of cyclin-dependent kinases, and causes growth arrest, while pgrn promotes mitosis. The secretion of growth promoting factors following cell-cycle arrest by p21 may prevent growth arrested cells from progressing into apoptosis, since medium from the arrested cells contains mitotic activity, and expression of other growth promoting or anti-apoptotic cytokines besides pgrn is also elevated by the induction of p21 (Chang et al., 2000).

Conclusions

Pgrn emerges in numerous biological contexts. In many cases it is a positive actor, contributing to physiological processes of development, normal tissue dynamics, and repair. Nevertheless, the combined observations that: i.) pgrn is specifically expressed in several cancer types, ii.) attenuation of pgrn expression blocks tumor formation in some cells, and iii.) over-expression of pgrn in non-tumor forming cells such as SW13, enables them to form tumors *in vivo*, argues forcefully for a deleterious role for pgrn as a tumor progression factor. Signaling studies show that pgrn activates secondary messenger cascades that are similar to those of well-established growth factors, although it remains unknown whether the pgrn receptor is a tyrosine kinase-type receptor, or if pgrn signaling is initiated in other ways. Pgrn has been traced back to the unfertilized egg and spermatozoon, and takes part in the development of the preimplantation embryo. It is involved in sexual differentiation of the neonatal hypothalamus, and its presence in adult neuronal cells is suggestive of other as yet unknown roles in brain function. It will be important now to ask whether, and in what ways, the pgrn system can be manipulated for therapeutic ends in cancer or tissue repair. Despite the increase in our understanding of pgrn function and its mechanism of action in recent years, clearly much more remains to be discovered.

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