REVIEW

Roles of prolactin and related members of the prolactin/growth hormone/placental lactogen family in angiogenesis

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Abstract

Prolactin, growth hormone and placental lactogen are members of a family of polypeptide hormones which share structural similarities and biological activities. Numerous functions have been attributed to these hormones, among which stand out their recently discovered effects on angiogenesis, the process by which new blood vessels are formed from the pre-existing microvasculature. Prolactin, growth hormone and placental lactogen, along with two non-classical members of the family, proliferin and proliferin-related protein, can act both as circulating hormones and as paracrine/autocrine factors to either stimulate or inhibit various stages of the formation and remodeling of new blood vessels, including endothelial cell proliferation, migration, protease production and apoptosis. Such opposing actions can reside in similar but independent molecules, as is the case of proliferin and proliferin-related protein, which stimulate and inhibit angiogenesis respectively. The potential to exert opposing effects on angiogenesis can also reside within the same molecule as the parent protein can promote angiogenesis (i.e. prolactin, growth hormone and placental lactogen), but after proteolytic processing the resulting peptide fragment acquires anti-angiogenic properties (i.e. 16 kDa prolactin, 16 kDa growth hormone and 16 kDa placental lactogen). The unique properties of the peptide fragments versus the full-length molecules, the regulation of the protease responsible for specific protein cleavage, the selective expression of specific receptors and their associated signal transduction pathways are issues that are being investigated to further establish the precise contribution of these hormones to angiogenesis under both physiological and pathological situations. In this review article, we summarize the known and speculative issues underlying the effects of the prolactin, growth hormone and placental lactogen family of proteins on angiogenesis, and address important remaining enigmas in this field of research.

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Introduction

In the past three decades, a striking number of studies have been published on angiogenesis - the outgrowth of new blood vessels from pre-existing ones. This process is essential for tissue growth during development and normally stops at adulthood. Thus, with the exception of the female reproductive organs (i.e. ovary, uterus, and placenta), where angiogenesis occurs as a normal process, in most adult tissues capillary growth occurs only rarely and in association with tissue repair after injury by wounding or inflammation. However, the lack of proper spatial and temporal regulation of angiogenesis contributes to various pathological conditions, including tumor growth, ophthalmic and rheumatic diseases, psoriasis, hemangioblastoma and ischemic diseases (Folkman 1995). It is widely accepted that angiogenesis is regulated by the interplay of pro- and anti-angiogenic molecules and that the blood vessels remain quiescent when the effects of these factors are at equilibrium. This balance can be disrupted by the overproduction of an endogenous promoter or the underproduction of an endogenous inhibitor of angiogenesis, leading to the activation of the normally quiescent angiogenic process. Conversely, when the balance is shifted in favor of the anti-angiogenic factors, the angiogenic process is impaired, and growth of new blood vessels does not fulfil the tissue requirements.

Angiogenesis regulators can modulate the ability of endothelial cells to digest the basement membrane, proliferate, migrate and associate into a new capillary network. Members of the vascular endothelial growth factor (VEGF) and angiopoietin families are known to have a

predominant role as pro-angiogenic factors (Ferrara 1999, Ferrara & Alitalo 1999, Gale & Yancopoulos 1999, Holash *et al.* 1999, Marti & Risau 1999, Yancopoulos *et al.* 2000). Conversely, platelet factor 4, thrombospondin-1, and the recently discovered angiostatin and endostatin stand out as important endogenous anti-angiogenic molecules (O'Reilly 1997, O'Reilly *et al.* 1997, Cao 1998, Hagedorn & Bikfalvi 2000, Jiménez *et al.* 2000). Some of these mediators have received special attention since their action is specific for vascular endothelial cells. The control of the endogenous synthesis or the exogenous administration of specific factors in pathologies associated with exacerbated or impaired angiogenesis are considered promising approaches for therapeutically modulating angiogenesis, since these molecules would specifically affect endothelial cells and no other cell types. Some of the specific regulators of angiogenesis are already undergoing phase I, II and III clinical trials (for reviews see Twardowski & Gradishar 1997, Nelson 1998, Ferrara & Alitalo 1999, Carmeliet & Jain 2000, Hagedorn & Bikfalvi 2000, Kerbel 2000, Thompson *et al.* 2000).

While tremendous effort has been concentrated on the investigation of factors thought to be specific for angiogenesis, the role of 'broad action agents', such as hormones, remains obscure. The study of the first type of molecules has been facilitated because they have clearly identified cellular targets (vascular endothelium, smooth muscle and pericytes); however, the study of hormones is difficult to interpret owing to the quantity and diversity of their targets and actions. The effects of hormones on the angiogenic process may be much more complex since they could act directly on vascular cells, or indirectly by recruiting other cell types to produce other regulators.

One of the major advances in physiology over the past decade has been to realize the importance of local, paracrine, or autocrine actions of hormones, independent of their systemic effects. Just as significant has been the better understanding of the vasculature as an endocrine tissue (Samson 1997). The endothelium can produce many different hormones that locally regulate its function, and endothelial cells are ideally positioned to respond to circulating factors. It has recently been recognized that members of the family of hormones that include prolactin (PRL), placental lactogen (PL), and growth hormone (GH), along with two non-classical members of the family, proliferin and proliferin-related protein, can be locally produced by endothelial cells or neighboring cells and can act as pro- or anti-angiogenic factors. In the following sections we will briefly describe the effects of the PRL/GH/PL family members on angiogenesis. The possible role of these molecules as autocrine, paracrine and/or endocrine mediators will be discussed, with special reference to the proteolytic fragments of PRL which have anti-angiogenic effects. Clinical implications and future directions will also be addressed.

The prolactin/growth hormone/placental lactogen family

The classical members of this family of peptide hormones, PRL, GH, and PL, are homologous proteins thought to have arisen from a common ancestral gene. PRL and GH are mainly secreted by the anterior pituitary of all vertebrates, while PL is present only in mammals and is secreted by the placenta. These three hormones share many structural and biological features. Similarity at the mRNA and protein levels between GH, PL and PRL has been extensively reviewed (Nicoll *et al.* 1986, Goffin *et al.* 1996*b*) and clearly illustrated (Kelly 1990), with important differences noted among species. However, the relationship between structural homology and biological properties is not entirely clear. For example, there is 85% sequence identity between the peptide sequences of human GH and PL, while human PRL shares approximately 25% similarity with the other two hormones. Nevertheless, human PL has a very weak affinity for the GH receptor (Lowman *et al.* 1991) while all three human hormones bind with high affinity to the PRL receptor (Nicoll *et al.* 1986, Goffin *et al.* 1996*b*). Independent of species-related differences, all three hormones contain between 190–200 amino acids and the molecular mass of the mature proteins is ~22–23 kDa. Their tertiary structure is stabilized by intra-chain disulfide bonds and is basically composed of four anti-parallel α-helices (for reviews see Goffin *et al.* 1996*b*, Bole-Feysot *et al.* 1998). Moreover, PRL and GH receptors are structurally and functionally related to members of the class 1 superfamily of cytokine receptors (Bazan 1989, Kelly *et al.* 1991, Cosman 1993). These receptors are transmembrane proteins that share highly conserved sequences in their extracellular and intracellular domains (Murakami *et al.* 1991, Cosman 1993, O'Neal & Yu-Lee 1993, Bole-Feysot *et al.* 1998, Waters *et al.* 1999), and they all can activate the JAK/STAT (Janus kinases/signal transducers and activators of transcription) signal transduction pathway as a consequence of ligand binding-induced homodimerization of the receptors (Ihle & Kerr 1995, Yu-Lee 1997, Bole-Feysot *et al.* 1998, Waters *et al.* 1999).

PRL and GH were originally named after their first discovered functions, that is, the stimulation of milk production and linear body growth respectively. However, both hormones have a remarkable variety of biological activities. More than 300 functions have been described for PRL, including actions on reproduction, osmoregulation, behavior, immune regulation, growth, and metabolism (Ben-Jonathan *et al.* 1996, Bole-Feysot *et al.* 1998, Freeman *et al.* 2000). Likewise, GH actions include the stimulation of body and bone growth, the regulation of protein, carbohydrate and lipid metabolism, and modulation of reproductive and immune functions, to name a few (Ohlsson *et al.* 1998, Waters *et al.* 1999, Hull & Harvey 2001). On the other hand, PL was initially

discovered for its ability to bind the PRL receptor with high affinity and to mimic the action of PRL (Kelly *et al.* 1976). This hormone acts on the maternal compartment to stimulate mammary gland development and to maintain the corpus luteum and progesterone production (Talamantes & Ogren 1988). The biological actions and receptor-signaling events initiated by PRL, GH and PL have been extensively reviewed recently (Ben-Jonathan *et al.* 1996, Harvey & Hull 1997, Anthony *et al.* 1998, Bole-Feysot *et al.* 1998, Soares *et al.* 1998, Linzer & Fisher 1999, Freeman *et al.* 2000, Lewis *et al.* 2000, Hull & Harvey 2001), and only those effects related to angiogenesis will be described here in detail.

Effects of prolactin isoforms on angiogenesis

PRL exists in several molecular forms, some of which arise from alternative splicing of the PRL mRNA, but more from post-translational processing of the predominant 23 kDa form (named full-length PRL or 23K PRL) (Sinha 1995). In fact, PRL does not circulate as a single molecular species but as a family of related proteins (Smith & Norman 1990). In humans, circulating PRL appears to consist of five isoforms: the classical 23 kDa molecule, a glycosylated PRL of 25 kDa, a 16 kDa fragment of PRL, dimers of 50–60 kDa ('big PRL'), and aggregates of >100 kDa ('big big PRL') (for review see Smith & Norman 1990, Sinha 1992, 1995). In addition, a significant proportion of PRL molecules are phosphorylated on serine and threonine residues, which accounts for much of the charge heterogeneity observed for PRL (Walker 1994).

The functional diversity of PRL was thought to be explained, in part, by the molecular heterogeneity of the hormone (Sinha 1995), but there are few examples which clearly support this notion. The actions of different members of the PRL family on angiogenesis provide one of the clearest examples directly relating PRL functional diversity to its structural heterogeneity. In this regard, full-length PRL was considered to be inactive on blood vessel growth until recent data showed its potential as a pro-angiogenic factor. Conversely, the enzymatically cleaved 16 kDa N-terminal fragment of PRL has a well-defined anti-angiogenic effect.

Prolactin (23K form)

The effects of PRL on angiogenesis were largely unrecognized since most studies failed to demonstrate any significant effect of PRL using *in vitro* and *in vivo* assays for angiogenesis (Ferrara *et al.* 1991, Clapp *et al.* 1993, Dueñas *et al.* 1999*a*). Nevertheless, recent evidence shows that PRL can stimulate the angiogenic process, but that its action may depend on the model utilized and the local conditions of the vascular endothelium (Struman *et al.* 1999, Merkle *et al.* 2000). This is perhaps best exemplified by studies aimed at identifying a role for PRL in the chick chorioallantoic membrane assay (CAM). This is an experimental approach traditionally used by embryologists that involves the analysis of the developmental potential of grafts implanted in the chorioallantoic membrane of the growing chicken embryo (Cockerill *et al.* 1995). The CAM appears on the yolk sac 48 h after incubation of the fertilized egg, becomes vascularized and grows rapidly over the next 6–8 days, and finally stops growing after day 11 (Ausprunk *et al.* 1974). Thus, the CAM assay can be performed in two different stages: before day 11, when the endothelial cells are actively dividing (early-stage bioassay) and after day 11, when endothelial cells divide infrequently and gradually acquire the characteristics of differentiated endothelial cells (late-stage bioassay). PRL has no effect on capillary outgrowth in the early-stage bioassay, that is, on the actively developing blood vessels (Clapp *et al.* 1993, Struman *et al.* 1999). Surprisingly, PRL stimulates the formation of new capillaries when tested on non-growing blood vessels during the late-stage CAM bioassay (Struman *et al.* 1999). These paradoxical results suggest that PRL may act to promote angiogenesis only in more advanced developmental stages, and hence that its action depends upon the local state of the vascular bed. PRL may act indirectly through the stimulation of angiogenic factors produced by non-endothelial cell types, or alternatively, the endothelial cells at this later stage of development may express the PRL receptor and respond directly to PRL. Thus, these findings may reflect the regulated expression of the PRL receptor in endothelial cells. Along with this possibility, the PRL receptor is not detected in all types of endothelial cells. For example, no specific binding sites for PRL were found on bovine brain capillary endothelial cell membranes (Clapp & Weiner 1992), and no PRL receptor mRNA was detected in rat retina capillary endothelial cells (Ochoa *et al.* 2001), bovine brain or in human umbilical vein endothelial cells (C Clapp & P A Kelly, unpublished observations). Likewise, studies in these cells failed to show direct effects of PRL on cell proliferation (Ferrara *et al.* 1991, Clapp *et al.* 1993, Struman *et al.* 1999, Ochoa *et al.* 2001), formation of capillary-like tubes in type I collagen gels (Clapp *et al.* 1993), and plasminogen activator inhibitor-1 (PAI-1) expression (Struman *et al.* 1999). PAI-1 is a known inhibitor of the urokinase type plasminogen activator (uPA), generally assumed to be involved in the stimulation of some of the early steps of angiogenesis, i.e. local proteolytic remodeling of matrix proteins and migration of endothelial cells (Bacharach *et al.* 1992).

However, in contrast to the above studies, a recent report demonstrated that bovine pulmonary artery endothelial cells express the mRNA for the PRL receptor and that these cells do respond to PRL (Merkle *et al.* 2000). In this study, monolayers of bovine pulmonary artery endothelium were subjected to mechanical injury and then

treated with PRL. PRL disrupted the actin cytoskeleton, produced changes in cell shape and reduced the adhesion of the cells to the substrate (Merkle *et al.* 2000). Whether PRL receptors and actions depend on the specific condition (mechanical injury) or the type of endothelium (bovine pulmonary artery) was not addressed. Likewise, the functional implications of these actions are unclear. Possibilities include an alteration of the barrier function and of the migration of endothelial cells, both of which are essential in blood vessel formation and thus PRL may have a role in the angiogenic process associated with tissue injury.

In summary, these data reveal that PRL may stimulate angiogenesis, but that its effects are limited by local conditions. It could be reasoned that specific developmental stages or stress conditions, such as injury, can induce the expression of the PRL receptor in vascular endothelium allowing PRL to promote angiogenesis or alter endothelial cell function. In addition, there are fundamental differences in the control, duration and extent of angiogenesis under physiological/pathological conditions that need to be contemplated when studying the action of putative regulators. Because *in vitro* studies disrupt these natural interactions and add artificial conditions (culture substrata and media, cell passage, etc) that further complicate analysis, more studies using intact physiological models should be performed and warrant further investigations.

16K prolactin

PRL can be proteolytically cleaved between amino acid residues Tyr^{145} and Leu^{146} and between Trp^{148} and Ser^{149} by a naturally occurring mechanism (Andries *et al.* 1992, Baldocchi *et al.* 1993). Excision of the tripeptide (Leu-Val-Trp) and reduction of the disulfide bonds yields N-terminal 16 364 Da and C-terminal 5808 Da fragments (Baldocchi *et al.* 1993) (Fig. 1). The 16 kDa fragment of PRL (16K PRL) retains PRL-like effects; it is mitogenic in the pigeon crop-sac and in the Nb2 lymphoma cell bioassays (Clapp *et al.* 1988), it has mammary mitogenic activity in the rat *in vivo* (Mittra 1980*a*), and it is both mitogenic and lactogenic in rat mammary cells in culture (Clapp *et al.* 1988). However, this proteolytic cleavage is a major posttranslational event that creates diversity in PRL actions, since the resulting 16K PRL displays biological actions not shared with the parent molecule. The specific effects of 16K PRL include inhibition of angiogenesis, both *in vivo* and *in vitro* (Fig. 2) (Ferrara *et al.* 1991, Clapp *et al.* 1993, Dueñas *et al.* 1999*a*, Struman *et al.* 1999) and 'proinflammatory' stimulation of the expression of the inducible isoform of nitric oxide synthase (iNOS) and nitric oxide (NO) production by rat pulmonary cells (Corbacho *et al.* 2000*b*).

16K PRL is a potent inhibitor of *in vitro* angiogenesis (Fig. 2). It inhibits basal and basic fibroblast growth factor (bFGF)- or VEGF-stimulated proliferation of human

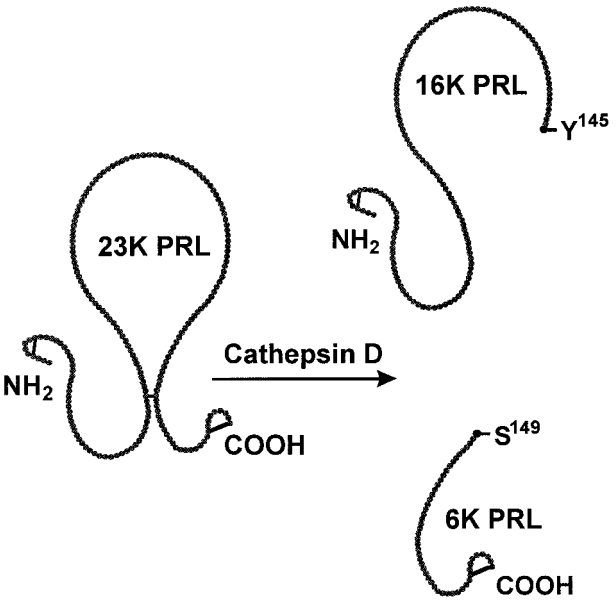

Figure 1 Diagram showing the linear sequence of a molecule of rat prolactin (PRL) with proteolytic cleavages between amino acids 145 and 149, which upon reduction of the intermediate disulfide bond generates an amino-terminal 16·4 kDa fragment and a carboxyl-terminal 5·8 kDa fragment.

(Clapp *et al.* 1993), bovine (Ferrara *et al.* 1991, Clapp *et al.* 1993, Struman *et al.* 1999) and rat (Ochoa *et al.* 2001) endothelial cells. Moreover, 16K PRL causes endothelial cell dissociation and disruption of the capillary-like structures formed when cells are cultured in three-dimensional type I collagen gels (Clapp *et al.* 1993). These capillary-like structures have a characteristic lumen and basal membrane, and they reflect the ability of endothelial cells to migrate, associate and modify the underlying extracellular matrix (Montesano *et al.* 1983). In this regard, 16K PRL also stimulates levels of PAI-1 mRNA and protein, and it inhibits uPA activity in endothelial cells (Lee *et al.* 1998, Struman *et al.* 1999). PAI-1 is the main inhibitor of uPA, and is known to prevent angiogenesis by limiting uPA-induced degradation of the extracellular matrix (Menashi *et al.* 1993), a requisite for angiogenesis. The formation of capillary-like structures in collagen gels requires uPA and is completely blocked by anti-uPA antibodies or by inhibiting the interaction of uPA with its receptor (Kollwijk *et al.* 1998). Therefore, 16K PRL is able to act directly on endothelial cells to inhibit processes that are essential for angiogenesis, such as endothelial cell growth, cell–cell and cell–extracellular matrix interactions, and the degradation of extracellular matrix.

Finally, the ability of 16K PRL to inhibit angiogenesis also appears to be related to its capacity to promote endothelial cell apoptosis. 16K PRL, but not full-length PRL, stimulates apoptosis of endothelial cells as revealed by induction of DNA fragmentation, activation of the

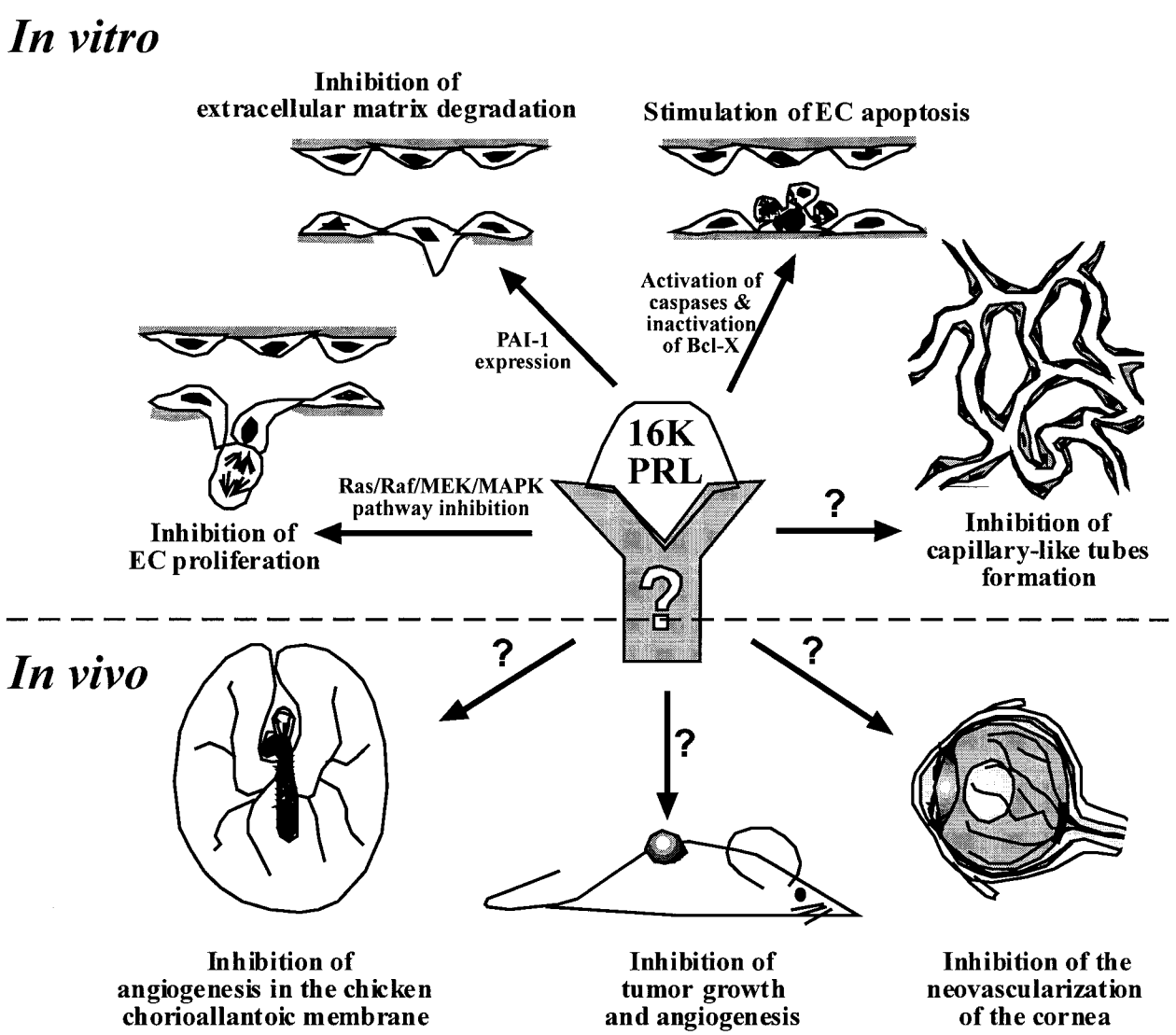

Figure 2 Anti-angiogenic actions of 16K PRL have been demonstrated using multiple *in vitro* and *in vivo* models of angiogenesis. *In vitro* models included assays for endothelial cell (EC) proliferation, apoptosis, and capillary-like formation in a collagen three-dimensional matrix. Studies *in vivo* included analysis of the effect of 16K PRL on angiogenesis of chicken chorioallantoic membrane, tumor growth in mice, and neovascularization of rat cornea. The identity of 16K PRL receptor and its mechanism of action remain unknown. However, 16K PRL inhibition of endothelial cell proliferation has been shown to involve the inactivation of the Ras/Raf/MEK/MAPK signaling pathway. The induction of apoptosis by 16K PRL occurred through the activation of the caspases cascade and the regulation of members of the Bcl-2 family (Bcl-X). Finally, 16K PRL induced the expression of plasminogen activator inhibitor 1 (PAI-1), contributing to the inhibition of extracellular matrix protease activity.

caspase cascade and inhibition of the anti-apoptotic action of the Bcl-2 family of proteins (Martini *et al.* 2000). Thus, 16K PRL action is not limited to the inhibition of the initial stages of angiogenesis, but may also affect apoptosis-related events such as vascular remodeling processes and blood vessel regression (Dimmeler & Zeiher 2000). Similarly, the anti-angiogenic factors angiostatin (Claesson-Welsh *et al.* 1998, Lucas *et al.* 1998), endostatin (Dhanabal *et al.* 1999) and thrombospondin-1 (Guo *et al.* 1997, Jiménez *et al.* 2000) induce endothelial cell apoptosis.

The anti-angiogenic properties of 16K PRL have been analyzed *in vivo* using the rat cornea neovascularization assay and the CAM assay (Fig. 2). To investigate the effect of 16K PRL, corneal angiogenesis was stimulated with bFGF in the absence or presence of 16K PRL, using PRL as a control. While full-length PRL had no effect, 16K PRL reduced the magnitude of the angiogenic bFGF-induced response by more than 65% (Dueñas *et al.* 1999*a*). Similarly, when tested on the early-stage CAM bioassay, 16K PRL inhibited the proliferation of actively growing

Table 1 16K prolactin (PRL) formation upon cleavage of endogenous PRL

	Tissues	References
Tissues *in vivo*	Serum	Sinha *et al.* (1985), Warner *et al.* (1993),
	Pituitary	Mittra (1980*a*), Sinha & Gilligan (1984), Sinha *et al.* (1985), Pellegrini *et al.* (1988), Shah & Hymer (1989), Warner *et al.* (1993)
	Amniotic fluid	Aston *et al.* (1984), Fukuoka *et al.* (1991)
	Cornea, iris and retina	Dueñas *et al.* (1999*b*)
Cells *in vitro*	Rat pituitary cells	Andries *et al.* (1992)
	Human umbilical vein endothelium	Corbacho *et al.* (2000*a*)
	Rat pulmonary fibroblasts	Corbacho *et al.* (2000*b*)

capillaries (Clapp *et al.* 1993, Struman *et al.* 1999). Remarkably, 16K PRL had no effect on the outgrowth of capillaries of the late-stage CAM bioassay (Struman *et al.* 1999), suggesting that 16K PRL might not promote the regression of an already established capillary network. This observation contrasts with data showing that endothelial cell capillary-like structures in collagen gels are disassembled in the presence of 16K PRL (Clapp *et al.* 1993), and with the proposal that 16K PRL induces apoptosis (Martini *et al.* 2000) as a means of blood vessel regression (Dimmeler & Zeiher 2000). Therefore, *in vivo* conditions present in specific tissues or developmental stages, such as the CAM, may influence the effect of 16K PRL as an anti-angiogenic factor.

The anti-angiogenic properties of 16K PRL make it a potential factor to limit angiogenic-dependent diseases such as tumor growth. A recent study clearly demonstrates that 16K PRL can inhibit tumor vascularization and growth from human colon cancer cells implanted in T- and B-cell-deficient mice (Bentzien *et al.* 2001). Cancer cells stably transfected with an expression vector coding for 16K PRL secreted large amounts of the biologically active PRL fragment. When injected into mice, these cells resulted in tumors 63% smaller and 44% less vascularized than those produced by control non-transfected cancer cells (Bentzien *et al.* 2001).

The signaling mechanisms mediating 16K PRL actions are not well understood. The observation that 16K PRL has unique effects not shared with the full-length PRL is consistent with the fragment acting via a specific receptor, different from the known PRL receptor. In agreement with this notion, a specific, high-affinity, saturable binding site for ^{125}I-labeled 16K PRL has been described on capillary endothelial cells, and 23K PRL does not compete for this site (Clapp & Weiner 1992). Moreover, no evidence of specific binding sites for ^{125}I-labeled PRL (Clapp & Weiner 1992) nor for the expression of known PRL receptor transcripts, has been obtained in any of the endothelial cell cultures in which 16K PRL inhibited endothelial cell proliferation (Ochoa *et al.* 2001, C Clapp & P A Kelly, unpublished observations). Although the identity of the 16K PRL receptor remains unknown, it has been shown that 16K PRL can inhibit the mitogenic actions of both bFGF and VEGF on endothelial cells by acting distally to their receptors and proximally to the mitogen-activated protein kinases (MAPKs), specifically by inhibiting the activation of Raf-1 (D'Angelo *et al.* 1995, 1999). However, the molecular mechanisms through which 16K PRL inhibits the activation of the Ras/Raf/MEK/MAPK pathway, resulting in compromised proliferation and stimulated apoptosis, remains unclear.

In summary, results from both *in vivo* and *in vitro* studies indicate that 16K PRL acts as a potent and specific anti-angiogenic factor, while PRL is either inactive or may function to promote angiogenesis under certain restricted conditions. The dichotomous actions of 16K PRL and PRL appear to be mediated by distinct receptors. Finally, the relative contribution of each hormone to angiogenesis would ultimately be determined by the activity of the enzyme responsible for PRL cleavage and by the local expression of the specific receptors in the endothelium, and potentially other cell types, of different tissues.

Endogenous 16K prolactin

Using immunoblotting methodologies, a 16K immunoreactive PRL has been detected in human serum (Sinha *et al.* 1985, Warner *et al.* 1993), and some evidence suggests that its concentration is elevated in pregnant women close to the day of delivery (Sinha *et al.* 1985) (Table 1). The levels in serum of this 16K PRL-like molecule have not been routinely analyzed under different physiological or pathological conditions. In serum, PRL levels are commonly detected by assays (RIA, ELISA, IRMA) that depend on antibodies raised against the unmodified monomeric form of the hormone (23 kDa PRL). It has been shown that 16K PRL has low affinity for such antibodies (Clapp *et al.* 1988), and thus these immunoassays may underestimate multiple forms of the hormone in serum.

16K PRL could reach the circulation from different sources, including the pituitary gland and extra-pituitary tissues (Table 1). A 16K immunoreactive PRL is detected

Table 2 PRL cleavage activity and 16K PRL synthesis

	Tissues	References
PRL cleavage by tissue homogenates	Mammary gland, prostate, liver, kidney, spleen	Compton & Witorsch (1984), Clapp (1987)
	Rat brain	De Vito *et al.* (1992)
	Hypothalamo–neurohypophysis	Torner *et al.* (1999)
	Rat pulmonary fibroblasts	Corbacho *et al.* (2000*a*)
PRL cleavage by tissue explants	Mammary gland, liver, kidney, spleen	Baldocchi *et al.* (1992)
PRL cleavage by serum	Lactating rat and pups serum	Baldocchi *et al.* (1992)

in the anterior pituitary of rats (Mittra 1980*b*, Shah & Hymer 1989, Andries *et al.* 1992), mice (Sinha & Gilligan 1984) and humans (Sinha *et al.* 1985, Pellegrini *et al.* 1988, Warner *et al.* 1993) and may be a portion of the PRL secreted into the bloodstream. Moreover, 16K PRL may be generated by proteases present in the circulation. The serum of lactating rats specifically cleaves PRL, generating the 16K isoform (Baldocchi *et al.* 1992). In addition, circulating PRL may be proteolytically processed to 16K PRL in target tissues (Tables 1 and 2). In this regard, PRL cleaving activity has been demonstrated in homogenates of mammary gland (Wong *et al.* 1986, Clapp 1987), brain (DeVito *et al.* 1992, Clapp *et al.* 1994), posterior pituitary (Clapp *et al.* 1994), prostate, liver, kidney and spleen (Compton & Witorsch 1984, Clapp 1987, Baldocchi *et al.* 1992). Finally, several extra-pituitary cell types express the PRL gene (Ben-Jonathan *et al.* 1996) and can cleave locally produced PRL. For example, human endothelial cells (Corbacho *et al.* 2000*a*) and rat pulmonary fibroblasts (Corbacho *et al.* 2000*b*) express PRL mRNA and produce a 16K protein that may correspond to the N-terminal part of the PRL molecule, as it is recognized in Western blots by 16K PRL-directed polyclonal antibodies (Corbacho *et al.* 2000*b*) and by monoclonal antibodies against the N-terminal end of PRL (Corbacho *et al.* 2000*a*). Also, incubation of exogenous PRL with a fibroblast lysate results in the formation of 16K PRL (Corbacho *et al.* 2000*b*). Finally, a 16K immunoreactive PRL has been detected in eukaryotic cells that express a transfected PRL gene (Cole *et al.* 1991, Yamamoto *et al.* 1992), indicating the existence of proteolytic activity to generate 16K PRL from PRL.

Whereas 16K PRL is observed under many physiological/pathophysiological conditions, the identity of the proteolytic enzymes responsible for this posttranslational modification has remained unresolved. However, different lines of evidence suggest that cathepsin D, a lysosomal aspartyl protease, may be one such enzyme responsible for PRL cleavage into 16K PRL. Cathepsin D has been demonstrated to cleave PRL to give the corresponding fragments (Baldocchi *et al.* 1993). PRL cleavage by tissue homogenates occurs at the same pH optimum (pH 3–5) as that for cathepsin D activity (Compton & Witorsch 1984, Wong *et al.* 1986, Clapp 1987, Baldocchi *et al.* 1992). Finally, PRL remains intact in the presence of pepstatin-A, an inhibitor of cathepsin D activity (Baldocchi *et al.* 1993).

The ability to cleave PRL and generate 16K PRL appears to differ among tissues and to change according to various physiological states. Mammary gland homogenates are able to generate more 16K PRL than the liver or kidneys from the same rats (Baldocchi *et al.* 1992), and result in more 16K PRL when extracted from lactating rats than from virgin or pregnant rats (Clapp 1987). Interestingly, estrogen treatment reduces the PRL cleaving activity of neurohypophyseal enzymes (Torner *et al.* 1999), suggesting that generation of 16K PRL can be regulated. This is especially important in view of the unique properties of 16K PRL, because a regulated enzymatic activity could constitute an on/off regulatory switch for 16K PRL bioactivity. In support of this possibility, the expression of cathepsin D can be regulated by estrogen and progesterone in the uterus (Elangovan & Moulton 1980, Maudelonde *et al.* 1990) and by estrogen in breast cancer cells (Westley & May 1987, Wang *et al.* 2001).

It should be noted that whereas the sequence of the 16K fragment of PRL produced by incubation of PRL with rat mammary gland extracts or cathepsin D corresponds to the N-terminal portion of the molecule (Baldocchi *et al.* 1993), proteolytic enzymes can generate other PRL fragments with the same molecular weight. Khurana *et al.* (1999) have demonstrated that thrombin cleaves PRL between amino acid residues Lys^{53} and Ala^{54} resulting in the formation of a C-terminal 16 kDa fragment that retains little PRL mitogenic activity and lacks the specific anti-angiogenic action of the N-terminal 16K fragment. Although it is not known whether thrombin cleaves PRL *in vivo*, or whether a C-terminal 16K PRL occurs normally, these results raise reasonable concerns, and the nature of endogenous 16K PRL fragments needs to be carefully examined in future studies.

Neurohypophyseal and endothelium-derived prolactin

The number of PRL isoforms with effects on angiogenesis increased with the discovery of two novel sites of hormone

Table 3 PRL expression and action in endothelial cells

	PRL mRNA	PRL-immunoreactive proteins	Effect of anti-PRL antibodies	Autocrine effects
RRCEC	Full-length	23 kDa PRL	None	None
HUVEC	Full-length + smaller mRNA	23, 21, 16, 14 kDa PRLs	Stimulatory	Inhibitory
BBCEC	Full-length + smaller mRNA	23, 21, 14 kDa PRLs	Inhibitory	Stimulatory

The expression of PRL mRNAs and proteins by different types of endothelial cells is summarized. Moreover, autocrine actions of endothelial-derived PRLs are predicted based on the effect of anti-PRL antibodies on endothelial cell proliferation. Antibodies had no effect on rat retinal capillary endothelial cells (RRCEC), stimulated human umbilical vein endothelial cells (HUVEC) and inhibited bovine brain capillary endothelial cells (BBCEC). This indicates that RRCEC-derived PRL has no autocrine effect, while HUVEC and BBCEC-derived PRLs have autocrine and anti-mitogenic and pro-angiogenic effects, respectively, which were neutralized by the antibodies.

production: the hypothalamo–neurohypophyseal system and the vascular endothelium. The hypothalamo–neurohypophyseal system consists of neurons of the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei, whose axons end in the neurohypophysis. These neurons are classically known to produce vasopressin and oxytocin (Brownstein *et al.* 1980). PVN and SON neurons also express PRL mRNA and contain PRL-like immunoreactive and biologically active proteins of 23 and 14 kDa (Clapp *et al.* 1994, López-Gomez *et al.* 1995). The absence of smaller PRL mRNAs and the presence of a cleaved, non-reduced PRL-like protein support the idea that the 14K protein in these neurons is generated not by alternative splicing but by the proteolysis and reduction of PRL (Clapp *et al.* 1994). Presumably, the neurohypophyseal 14 kDa PRL corresponds to the N-terminal part of the PRL molecule, as it is recognized by 16K PRL-directed polyclonal antibodies (Clapp *et al.* 1994), and by monoclonal antibodies directed against the N-terminal end of PRL (Torner *et al.* 1995). Consistent with it being derived from the N-terminal portion of PRL (like 16K PRL), the neurohypophyseal 14K PRL-like protein displays inhibitory actions on endothelial cell proliferation (Clapp *et al.* 1994, López-Gomez *et al.* 1995). The possibility of the 14K PRL-like protein being either a proteolytically processed product of 16K PRL or an independent product of PRL proteolysis needs to be addressed. During recombinant synthesis of primate PRL a PRL fragment of approximately 14 kDa that may arise from proteolysis at Ile^{133} was observed (Cole *et al.* 1991).

The 14K PRL-like protein is localized within the secretory granules of vasopressin-containing cells (Mejía *et al.* 1997) and is released by cultured neurohypophyseal endings (Torner *et al.* 1995). Moreover, because an immunoreactive 14K PRL-like protein is detected in rat (Torner *et al.* 1995) and human (Fukuoka *et al.* 1991) serum, the hypothalamo–neurohypophyseal system may be a source of this protein in the circulation (Clapp & Martínez de la Escalera 1997). In addition, a 14K PRL-like protein has also been detected in human amniotic fluid (Aston *et al.* 1984, Fukuoka *et al.* 1991), and it is synthesized by mammary epithelial (Lkhider *et al.* 1997) and endothelial cells (Clapp *et al.* 1998, Corbacho *et al.* 2000*a*), suggesting other putative sources for this PRL.

With reference to endothelium-derived PRL, PRL gene expression has been demonstrated *in vitro* in endothelial cells from different species and vascular beds, i.e. rat retinal capillary endothelial cells (Ochoa *et al.* 2001), human umbilical endothelial cells (Corbacho *et al.* 2000*a*), and bovine brain capillary endothelial cells (Clapp *et al.* 1998) (Table 3). Although all cells expressed the full-length PRL mRNA and synthesized 23 kDa PRL, differences were observed regarding the production of lower molecular weight PRL forms. While retinal cells only expressed the full-length mRNA, and synthesize 23 kDa PRL (Ochoa *et al.* 2001), umbilical vein and brain capillary cells also expressed a small mRNA and PRL-immunoreactive proteins of lower molecular weight. Sequencing of the small mRNA indicated that it corresponded to an alternatively spliced PRL mRNA with deletion of the third exon of the gene (Clapp *et al.* 1998). Theoretically, the translation of such a small mRNA would correspond to a protein of about 20 kDa with reduced capacity to activate the cloned PRL receptors (Goffin *et al.* 1995). In this regard, a 21 kDa PRL-immunoreactive protein is found in both brain capillary and umbilical vein endothelial cells, along with 16 and 14 kDa PRL-like isoforms (Clapp *et al.* 1998, Corbacho *et al.* 2000*a*). In addition to the heterogeneous expression of PRL mRNA and protein, the amounts of PRL secreted also varied between the different endothelial cell types. Analysis of PRL-like bioactivity in the conditioned media indicated that retinal endothelial cells released at least 300 times the amount of PRL secreted by brain capillary or umbilical vein endothelium (Clapp *et al.* 1998, Corbacho *et al.* 2000*a*).

The observation that endothelial cells produce and release PRL suggests the possibility that PRL isoforms may

function as autocrine regulators of angiogenesis. This possibility was investigated by culturing endothelial cells in the presence of anti-PRL antibodies to sequester the endothelial-derived PRLs and block their possible autocrine activity. Results of these studies again reflected the heterogeneous nature of endothelial cells. While no effect was observed on the growth of retinal capillary endothelial cells (Ochoa *et al.* 2001), anti-PRL antibodies stimulated human umbilical vein endothelial cells (Corbacho *et al.* 2000*a*) but inhibited bovine brain capillary endothelial cell proliferation (Clapp *et al.* 1998). The lack of effect observed on rat retinal endothelial cells is consistent with the fact that these cells only produce 23 kDa PRL, a form of PRL shown to have no effect on their proliferation *in vitro* (Ochoa *et al.* 2001). In human umbilical vein endothelial cells, the stimulatory effect of anti-PRL antibodies suggested the recognition and neutralization of PRLs that would otherwise inhibit endothelial cell proliferation. Such anti-mitogenic autocrine function could be attributed to the 16K and 14K immunoreactive proteins secreted by human umbilical vein endothelial cells, as they were the major proteins recognized by PRL antibodies (Corbacho *et al.* 2000*a*) and correspond to the PRLs with antiangiogenic properties. Finally, the observation that anti-PRL antibodies inhibit the growth of bovine brain capillary endothelial cells was consistent with the secretion of PRLs with an autocrine pro-mitogenic effect, possibly the 21 kDa PRL-like protein (Clapp *et al.* 1998). The expression of several PRL variants by different endothelial cells, together with the paradoxical effects attributed to them, adds to the known functional heterogeneity of endothelial cells thought to play a profound role in the tissue-specific regulation of angiogenesis (Lelkes *et al.* 1996).

Finally, it should be mentioned that endothelial-derived PRL may act in a paracrine manner on neighboring cell types to regulate events dependent and independent of angiogenesis. As mentioned before, 16K PRL stimulates iNOS expression and NO production by rat lung fibroblasts and type II alveolar epithelial cells (Corbacho *et al.* 2000*b*). NO is a gaseous free radical with both pro- and anti-inflammatory functions, and it plays important roles in host defense, inflammatory responses, vasodilation and inhibition of leukocyte and platelet adhesion to the blood vessel wall (Clancy *et al.* 1998). Recent data suggest that PRL may regulate leukocyte trafficking across the vascular endothelium (Montes de Oca *et al.* 2000). The treatment of peripheral blood mononuclear cells with PRL stimulates their adhesion to human umbilical vein endothelial cell monolayers, and this effect appears to involve a PRL-induced activation of integrins LFA-1 and VLA-4 in leukocytes (Montes de Oca *et al.* 2000). These observations clearly warrant studies of PRL isoforms which focus on effects not directly related to the endothelium.

Ocular prolactin

Although different lines of evidence indicate that PRL isoforms can regulate angiogenesis, it is necessary to determine their physiological contribution to the process *in vivo*. It has been hypothesized that endogenous, anti-angiogenic PRL isoforms may restrain the angiogenic process *in vivo*, helping to maintain the avascularity of certain tissues like the cornea. Consistent with this hypothesis, corneal implants containing anti-PRL-directed antibodies specifically stimulate the local outgrowth of new blood vessels (Dueñas *et al.* 1999*a*). The possibility that these antibodies unmasked anti-angiogenic PRL molecules present in the cornea was substantiated by recent findings in rats, showing immunoreactive PRL in the aqueous humor and 23 kDa and 16 kDa PRL-immunoreactive proteins in corneal homogenates (Dueñas *et al.* 1999*b*). The possible involvement of PRL in ocular angiogenesis is also suggested by the presence of PRL in the aqueous humor and subretinal fluid of patients with premature retinopathy, an ocular neovascular disease (Quiroz *et al.* 2000).

Ocular angiogenesis is a leading cause of blindness worldwide; it occurs in response to hypoxia in diseases that include diabetic retinopathy, premature retinopathy, and age-related macular degeneration. For example, in diabetes there is a reduction in blood flow through areas of the retinal microvasculature that results in ischemia. In premature babies, hyperoxic conditions resulting from the incubator environment lead to the occlusion of normal retinal blood vessels (Stone & Maslim 1997). In all cases, ischemia leads to retinal hypoxia, a major stimulus for the release of angiogenic factors that cause the outgrowth of blood vessels in the retina that extend into the vitreous. The new blood vessels recruit other cells, and the process results in the formation of fibrovascular scar tissue that causes loss of vision from vitreous hemorrhage and/or retinal detachment (Stone & Maslim 1997, Adamis *et al.* 1999).

PRL measured in the ocular fluids of patients with premature retinopathy may originate intra-ocularly from the newly formed blood vessels. PRL mRNA can be amplified by RT-PCR from the fibrovascular tissue within the vitreous compartment (Quiroz *et al.* 2000) and localized by *in situ* hybridization within endothelial cells and infiltrating leukocytes (P Montes de Oca & C Clapp, unpublished observations). This observation is consistent with the fact that actively proliferating endothelial cells from rat retinas stand out among other vascular endothelium in their ability to produce and release PRL (Ochoa *et al.* 2001). In conclusion, products from the PRL gene are produced in the eye of patients with retinal neovascularization and may affect angiogenesis. There is evidence for hyperprolactinemia in diabetes but no correlation was found between serum PRL and the occurrence of retinopathy (Cerasola *et al.* 1981, Larinkari *et al.* 1982, Mooradian *et al.* 1985). Analysis of the expression and

proteolysis of PRL within the eye of patients with diabetic retinopathy might provide important insights relevant to the pathophysiology of this disease.

Other members of the PRL/GH/PL family with effects on angiogenesis

Growth hormone

The role of GH as an angiogenic factor was initially proposed in association with the mechanisms underlying the development of diabetic retinopathy (Flyvberg 1990, Merimee 1990, Sharp 1995). The initial implication stems from the observation that retinal neovascularization in diabetic patients diminished after the ablation of the pituitary gland (Poulsen 1953, Flyvberg 1990, Merimee 1990). Although the reduction in retinal vasculature could be attributed to the elimination of other pituitary hormones, the role of GH was supported by the correlation between diabetic retinopathy and elevated GH levels in the circulation (Johansen & Hansen 1969, Hansen & Johansen 1970, Passa *et al.* 1977, Sundkvist *et al.* 1984). Likewise, in two patients given GH following hypophysectomy, retinal neovascularization continued to develop (Ray *et al.* 1968). Finally, GH deficiency in diabetic subjects is associated with reduced retinopathy when compared with diabetic controls (Merimee *et al.* 1970, Passa *et al.* 1977, Merimee 1978).

These clinical observations led investigators to postulate that GH is an angiogenic factor and were followed by *in vitro* and *in vivo* experimental approaches to confirm direct effects of GH on the promotion of angiogenesis. GH receptors were found in blood vessels of the human fetus (Werther *et al.* 1993), the human ovary (Sharara & Nieman 1994), and in the myometrium, endometrium, and ovaries of the rat (Lobie *et al.* 1990). *In vitro* studies illustrated that GH stimulates proliferation of human retinal microvascular endothelium (Rymaszewski *et al.* 1991) and bovine brain capillary endothelial cells (Struman *et al.* 1999). Moreover, GH stimulates *in vivo* angiogenesis in the late-stage CAM assay (Gould *et al.* 1995, Struman *et al.* 1999). However, as observed for PRL and PL, GH actions on angiogenesis appear to depend on conditions in the various tissues. For example, the proliferation of human umbilical vein endothelial cells is not altered by GH (Rymaszewski *et al.* 1991), and GH does not stimulate angiogenesis in the early-stage CAM (Struman *et al.* 1999). There is evidence that GH can be proteolytically cleaved in pituitary tissue (Lewis *et al.* 1980) and that putative cleavage sites around amino acids 130–140 of GH exist for the pituitary proteases, thrombin, plasmin and collagenase (Baumann 1991, Alam *et al.* 1998, Aramburo *et al.* 2001). Such enzymatic processing results in a two-chain structure linked by disulfide bonds, which upon reduction generates a 16K N-terminal GH fragment homologous to those arising from PRL and PL. Like the latter hormones, the 16K GH fragment inhibits endothelial cell proliferation, PAI-1 expression and angiogenesis in the early-stage CAM (Struman *et al.* 1999). Surprisingly, in a recent report Aramburo and colleagues (2001) showed that the N-terminal 16K fragment of chicken GH generated by thrombin cleavage between amino acids Arg^{133} and Gly^{134} stimulates the proliferation of cultured bovine endothelial cells. This finding may relate to species-specific differences with the human molecular counterpart.

In addition to the direct effects of GH on endothelial cells, this hormone also induces the secretion of insulin-like growth factor (IGF)-I, mainly by the liver (Delafontaine 1995). Particularly IGF-I, but also IGF-II, act as key mediators of GH's effects (Cohick & Clemmons 1993, Delafontaine 1995). Actually, both IGF-I and IGF-II have been implicated as direct angiogenic factors (Bar *et al.* 1988, Nakao-Hayashi *et al.* 1992, Nicosia *et al.* 1994, Kim *et al.* 1998, Beckner 1999, Dunn *et al.* 2000, Lee *et al.* 2000) and the action of GH in the promotion of retinal neovascularization appears to involve systemic or locally produced IGFs. IGF-I is elevated in the circulation and in the vitreous humor of patients with diabetic retinopathy (Merimee *et al.* 1983, Grant *et al.* 1986, Hyer *et al.* 1989, Dills *et al.* 1991, Meyer-Schwickerath *et al.* 1993), and intravitreal application of IGF-I stimulates retinal angiogenesis in rabbits (Grant *et al.* 1993). The contribution of IGF-I to neovascularization is also supported by the presence of IGF-I receptors in endothelial cells (Bar & Boes 1984, King *et al.* 1985, Boes *et al.* 1991, Spoerri *et al.* 1998) and by *in vitro* studies showing that IGF-I stimulates endothelial cell proliferation (King *et al.* 1985), migration (Grant *et al.* 1987), uPA production (Grant & Guay 1991), and angiogenesis *in vivo*. Moreover, evidence has been presented that the IGF-I gene is expressed by endothelial cells (Kern *et al.* 1989, Delafontaine *et al.* 1991).

Smith and coworkers (1997) have explored the role of the somatostatin/GH/IGF-I axis in retinal neovascularization. Transgenic mice expressing a GH antagonist (dwarf phenotype) or normal mice treated with a somatostatin analog to inhibit GH secretion were subjected to ischemia in order to induce retinal neovascularization. Retinal blood vessel growth was reduced in both types of mice when compared with controls, suggesting that normal GH levels promote the growth of new blood vessels under ischemic conditions. Consistent with this finding, neovascularization was partially or completely restored when GH or IGF-I was co-injected with somatostatin, an inhibitor of GH secretion. Accordingly, these data suggested that GH can stimulate ischemia-induced neovascularization, probably by acting through IGF-I (Smith *et al.* 1997). Most studies now agree that hypoxia-inducible VEGF is the principal factor mediating ischemia-associated ocular neovascularization (Stone & Maslim 1997). However, mice with decreased GH and IGF-I serum levels are

resistant to hypoxia-induced retinopathy, even when retinal expression of VEGF remains high (Smith *et al.* 1997). A later study showed that an IGF-I receptor antagonist suppresses retinal neovacularization *in vivo*, and that IGF-I interaction with the IGF-I receptor is necessary to induce maximal neovascularization by VEGF (Smith *et al.* 1999). On the other hand, it should be mentioned that in addition to reducing GH levels, somatostatin can act directly on endothelial cells to inhibit angiogenesis (Danesi *et al.* 1997, Woltering *et al.* 1997, Albini *et al.* 1999), a finding that adds to the debated function of somatostatin and its analogs in the control of tumor growth (Albini *et al.* 1999).

Although the data discussed above implicate GH and IGF-I in the promotion of retinal neovascularization, abnormally increased GH levels do not appear to exacerbate ocular angiogenesis. Transgenic mice expressing a GH agonist (giant phenotype) showed no increase in retinal neovascularization compared with controls, and IGF-I alone did not increase neovascularization over control levels (Smith *et al.* 1997).

Another example that illustrates the stimulatory effect of GH and IGF-I on neovascularization comes from the study of aging and cerebral cortical vasculature. Both GH and IGF-I plasma levels decrease with age, and this correlates with a decrease in cerebral cortical vasculature (Sonntag *et al.* 1997). Interestingly, in contrast to the effect observed in the retina (Smith *et al.* 1997), injection of GH stimulated the growth of blood vessels in the cerebral cortex of aging rats when compared with non-treated animals (Sonntag *et al.* 1997). Taken together these results suggest that the effects of GH and IGF-I on angiogenesis may be determined by their local or circulating levels, the specific tissues, and the ontogenic period.

In contrast with the newly discovered angiogenic factors that are currently entering clinical trials, GH treatment has been used for decades to treat GH deficiency. Thus, the effects of GH deficiency or its therapeutic administration on angiogenesis can already be evaluated in human subjects. Elevated levels of GH (as occur in acromegaly) show little correlation with retinopathy. Moreover, long-term GH replacement therapy does not appear to increase the risk of retinopathy in children or adults (Hellström *et al.* 1999, Blank *et al.* 2000, Radetti *et al.* 2000) and is rarely associated with retinal neovascularization (Koller *et al.* 1998, 2000). Nevertheless, concerns are being raised regarding GH and IGF-I treatment of diabetic children and adolescents due to its potential to exacerbate retinopathy, particularly since isolated cases of retinopathy have been identified that are associated with exogenous GH therapy in GH-deficient non-diabetic patients (Koller *et al.* 1998). In one of these patients, discontinuation of GH treatment was followed by full remission of the retinopathy in the absence of additional treatment (Hansen *et al.* 2000).

In summary, evaluation of GH effects on angiogenesis is limited by the complex endocrine status of disease states. Although a large body of evidence indicates an angiogenic role for GH and IGF-I, their actions appear to be influenced by systemic and local factors. Understanding these interactions may open new therapeutic avenues for the treatment and prevention of vascular diseases, such as diabetic retinopathy.

Placental lactogen

Angiogenesis is essential during the development of the placenta, when remodeling of the maternal uterine vasculature and growth of fetal vessels into the placenta takes place. Placental hormones of the PRL family may regulate reorganization and growth of maternal and fetal blood vessels. PL binds with high affinity to the PRL receptor, mimicking the action of PRL (Kelly *et al.* 1976). Accordingly, PL could promote angiogenesis under the same conditions in which PRL is active. Consistent with this idea, both PL and PRL stimulate new capillary blood vessel formation *in vivo* in the late-stage CAM bioassay, but they do not affect the proliferation of bovine brain capillary endothelial cells (BBCEC) *in vitro* (Struman *et al.* 1999). As in the case of the intact hormones, the N-terminal 16K fragments of PRL and PL have similar actions. An homologous N-terminal 16K PL fragment produced by recombinant DNA displays inhibitory actions on angiogenesis equivalent to those of 16K PRL both *in vivo* and *in vitro*. 16K PL inhibits BBCEC proliferation and PAI-1 expression, as well as the outgrowth of new blood vessels in the early-stage CAM, but not in late-stage CAM quiescent capillaries (Struman *et al.* 1999). Although there is no evidence yet that 16K PL occurs naturally *in vivo*, PL and 16K PL bear a striking resemblance to PRL and 16K PRL in their opposing actions on angiogenesis *in vitro*. Taking into consideration all these data, it is likely that PL isoforms exert opposite actions on angiogenesis via the PRL and 16K PRL receptors.

Proliferin and proliferin-related protein

While the angiogenic and anti-angiogenic actions of PRL, GH, or PL reside within a single molecule, two non-classical members of the mouse placental PRL family, proliferin and proliferin-related protein, work as independent molecules to modulate angiogenesis (Linzer & Fisher 1999). Proliferin and proliferin-related protein act as pro- and anti-angiogenic factors respectively (Jackson *et al.* 1994). These two proteins share structural features (location and type of the intron/exon splice sites, chromosome location, nucleic acid and amino acid similarity, etc.) with PRL and PL (Soares *et al.* 1998).

Proliferin (PLF), also known as mitogen-regulated protein (MRP) (Nilsen-Hamilton *et al.* 1980), was originally detected in 3T3 mouse fibroblasts *in vitro* (Nielsen-Hamilton *et al.* 1980, Linzer & Nathans 1984) and shortly thereafter its expression was also demonstrated in the mouse placenta (Linzer *et al.* 1985, Lee *et al.* 1988).

Similarly, proliferin-related protein is expressed in the placenta of mice (Linzer & Nathans 1985, Colosi *et al.* 1988) and rats (Toft & Linzer 2000). Both hormones are present in the circulation of the pregnant mouse and whereas proliferin reaches peak levels by midgestation (Lee *et al.* 1988), proliferin-related protein levels are increased in the second half of pregnancy (Lopez *et al.* 1993).

The function of proliferin and proliferin-related protein remained unknown until the discovery that they could efficiently compete with 16K PRL for binding to endothelial cells (Clapp & Weiner 1992), data that suggested direct actions of these hormones on the angiogenic process. Thus, proliferin and proliferin-related protein were tested in both *in vitro* and *in vivo* assays for angiogenesis. Proliferin was found to stimulate the migration of endothelial cells *in vitro* and the growth of blood vessels in the rat cornea assay, while proliferin-related protein exerted inhibitory effects in both assays (Jackson *et al.* 1994). Consistent with the temporal pattern of circulating levels during mouse pregnancy, proliferin was found to be a major component of the angiogenic activity present in the placenta during midgestation, whereas proliferin-related protein was shown to contribute to the anti-angiogenic activity detected in late-pregnancy placental tissues (Jackson *et al.* 1994). Indeed, proliferin and proliferin-related protein are synthesized specifically in the placental trophoblast giant cells (Linzer & Nathans 1984, Linzer *et al.* 1985, Lee *et al.* 1988, Carney *et al.* 1993) and could act as paracrine factors regulating the local growth of blood vessels.

In addition to its actions on the placenta, proliferin may have specific developmental functions. Proliferin, but not proliferin-related protein, can be transported from the placenta through the extraembryonic membranes of the fetus (yolk sac) to the amniotic fluid, where it is in direct contact with the developing fetus (Lee *et al.* 1988). Remarkably, in the fetus proliferin binds to the developing heart, the blood vessels around the dorsal artery, and the endothelial cells of the growing ribs (Jackson & Linzer 1997). Although the physiological effects of proliferin in the fetus are not yet known, it has been proposed that it may stimulate endothelial cell migration promoting angiogenesis during the development of fetal tissues.

Furthermore, recent findings suggest that proliferin may participate in angiogenesis in instances other than during pregnancy and development. In a model of progressive fibrosarcoma in mice, the expression of proliferin increased in association with the progression of the tumor from mildly noninvasive to aggressively invasive stage of tumor development, a stage at which the tumor becomes highly angiogenic. The expression of proliferin in the fibrosarcoma was associated with angiogenic activity in *in vitro* and *in vivo* models of angiogenesis (Toft *et al.* 2001), indicating that proliferin may be secreted by tumoral cells and act as a pro-angiogenic factor to induce tumor angiogenesis.

Proliferin comprises a group of homologous proteins (PLF1, PLF2, MRP3, MRP4) encoded by four distinct genes (Linzer & Nathans 1984, Wilder & Linzer 1986, Nilsen-Hamilton *et al.* 1987, Jackson-Grusby *et al.* 1988, Connor *et al.* 1989, Fassett *et al.* 2000). All forms of proliferin are very similar in their amino acid sequences and are glycosylated proteins; however, they can differ in their degree of glycosylation (Fassett *et al.* 2000), a characteristic that may determine differences in the functional interactions with the proliferin receptors *in vivo* (Jackson *et al.* 1994, Nelson *et al.* 1995, Jackson & Linzer 1997, Fassett *et al.* 2000). Although the proliferin originally found in 3T3 mouse fibroblasts (Nielsen-Hamilton *et al.* 1980, Linzer & Nathans 1984) and in the mouse placenta (Linzer *et al.* 1985) corresponds to PLF1, all forms of proliferin have been detected in the mouse placenta, MRP3 being the most abundant (Linzer *et al.* 1985, Wilder & Linzer 1986, Lee *et al.* 1988, Fang *et al.* 1999, Fassett *et al.* 2000). Also, the proliferin detected in skin fibrosarcomas corresponds to PLF1 (Toft *et al.* 2001).

Recently, the expression of proliferin was investigated in the adult mice and appeared to be limited to tail and ear skin (Fassett *et al.* 2000), hair follicles (MRP3, MRP4), small intestine (PLF1, MRP3 and MRP4) (Fassett *et al.* 2000, Fassett & Nilsen-Hamilton 2001) and skin keratinocytes during wound healing processes *in vivo* (MRP3) (Fassett & Nilsen-Hamilton 2001). It is remarkable that proliferin is expressed in wound healing processes and in developing hair follicles (Fassett *et al.* 2000, Fassett & Nilsen-Hamilton 2001), events that represent two of the few processes accompanied by angiogenesis in the adult. Although it has not been directly proven, the very specific site of proliferin expression suggests its participation in normal angiogenic processes in the adult.

The expression of proliferins appears to be tissue-specifically regulated by different growth factors, including bFGF, epidermal growth factor (EGF), keratinocyte growth factor (KGF) and transforming growth factor β (TGFβ) (Nilsen-Hamilton *et al.* 1980, Chiang & Nilsen-Hamilton 1986, Fassett & Nilsen-Hamilton 2001). In addition, their actions on endothelial cells appear to be mediated by the IGF-II/mannose 6-phosphate receptor, and the glycosylated state of proliferin appears to be essential for the binding to this receptor (Lee & Nathans 1988, Volpert *et al.* 1996). Although the signaling pathway associated with the IGF-II/mannose-6-phosphate receptor is poorly understood, binding of either proliferin or IGF-II activates a G protein that leads to MAPK activation (Groskopf *et al.* 1997). Finally, proliferin can bind to a specific, high affinity receptor present in the uterus that is distinct from the IGF-II/mannose-6-phosphate receptor and mediates cell proliferation (Nelson *et al.* 1995). However, the identity of this specific receptor remains unknown. Therefore, different receptors may mediate different actions of proliferin. Binding of proliferin in the fetus occurs through the IGF-II/mannose-6-phosphate

Table 4 Structural characteristics, receptors and signaling mechanisms of members of the PRL/GH/PL family with effects on angiogenesis

	MW (kDa)	Posttranslational and posttranscriptional modifications	Receptor	Intracellular signaling pathway implicated in angiogenesis	Effect on angiogenesis
PRL	23	Native form	PRL receptor	JAK/STAT pathway?	None or stimulatory
	16	N-terminal fragment	Unknown	Inhibition of Ras/Raf/MEK/MAPK Stimulation of PAI-1 expression Activation of caspases Inhibition of Bcl-X	Inhibitory
	14	N-terminal fragment	Unknown	Unknown	Inhibitory
	21	Lacking third exon sequence	Unknown	Unknown	Stimulatory
PL	22	Native form	PRL receptor	JAK/STAT pathway?	None or stimulatory
	16	N-terminal fragment	Unknown	Unknown	Inhibitory
GH	22	Native form	GH receptor	JAK/STAT pathway?	Stimulatory
	16	N-terminal fragment	Unknown	Unknown	Inhibitory
Proliferin	27–38	N-glycosylated forms	IGF-II/mannose 6-phosphate receptor and other	G protein and MAPK activation	Stimulatory
Proliferin-related protein	34–45	N-glycosylated form	Unknown	Inhibition of arachidonic acid release	Inhibitory

MW, molecular weight.

receptor (Jackson & Linzer 1997), whereas the transport of proliferin through the yolk sac appears to be independent of the IGF-II/mannose-6-phosphate receptor (Jackson & Linzer 1997) and may be mediated by the specific receptor identified in the uterus.

Contrasting with the pro-angiogenic effects of proliferin, the anti-angiogenic properties of proliferin-related protein make it an endogenous factor that has the potential to impede angiogenesis-dependent pathologies, such as tumor growth. To test the anti-angiogenic potential of proliferin-related protein to block tumor growth, two tumor cell lines, SVT2 fibroblasts (SV40-transformed BALB/c 3T3 mice fibroblasts) and C6 glioma cells, were engineered to secrete proliferin-related protein (Bengtson & Linzer 2000). When injected into mice, tumor cells secreting proliferin-related protein generated tumors that were significantly smaller and showed a marked reduction in vascular density, in comparison with the tumors produced by the control cancer cells (Bengtson & Linzer 2000). Consistent with proliferin-related protein's anti-angiogenic properties *in vitro*, these results demonstrate that proliferin-related protein can restrict tumor growth, most likely by acting directly on endothelial cells and restricting tumor angiogenesis. Although a protein homologous to proliferin-related protein has not been identified in humans, proliferin-related protein can inhibit human, rat, mice, and bovine endothelial cells, indicating that the receptor and cell responses are conserved among mammals (Bengtson & Linzer 2000). Finally, the receptor for proliferin-related protein has not been identified, but the signaling pathway appears to involve the inhibition of arachidonic acid release (Bengtson & Linzer 2000).

Conclusions and future directions

Numerous studies have sought to identify molecules that regulate blood vessel growth (for reviews see Browder *et al.* 2000, Carmeliet 2000, Hagedorn & Bikfalvi 2000). The complexity of the angiogenic cascade and the concept of an angiogenesis or anti-angiogenesis-based therapy have attracted scientists with widely ranging interests in basic and clinical science. Among them, endocrinologists are analyzing the effect of classical hormones on the angiogenesis process (Clapp *et al.* 1993, Jackson *et al.* 1994, Ponce *et al.* 1997, Franck-Lissbrant *et al.* 1998, Struman *et al.* 1999).

The findings accumulated over the last decade indicate that members of the PRL/PL/GH family are potential endogenous regulators of physiological and pathological angiogenesis (Table 4). These proteins can act as circulating hormones and/or as paracrine and autocrine factors, in various stages of the formation and remodeling of new blood vessels, including endothelial cell proliferation, protease production, and apoptosis. Furthermore, the receptors for these hormones are members of the class 1 cytokine

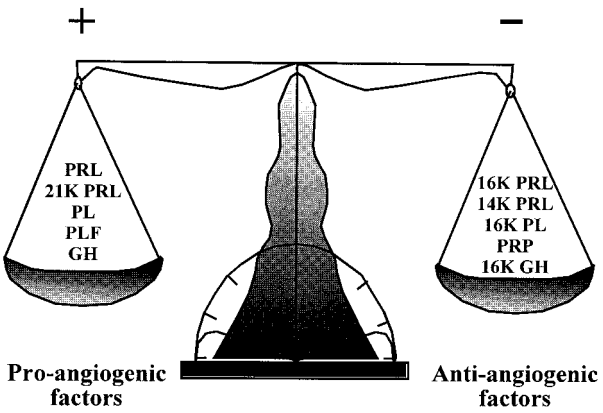

Figure 3 Members of the PRL/GH/PL family with pro-angiogenic or anti-angiogenic actions. PLF, proliferin; PRP, proliferin-related-protein.

receptor superfamily, which also includes receptors for cytokines with recently discovered angiogenesis-related effects, such as interleukin-2 (Sakkoula *et al.* 1997, Johansson *et al.* 2000), interleukin-15 (Angiolillo *et al.* 1997), and erythropoietin (Yasuda *et al.* 1998).

In contrast to previously identified regulators of angiogenesis, the PRL/PL/GH family comprises homologous molecules that can either stimulate or inhibit the process (Fig. 3, Table 4). It is particularly interesting that, in some cases, the potential to exert opposing effects resides within the same molecule. The inhibitory activity can remain quiescent in a stimulatory molecule (PRL, PL, GH) until the parental protein is proteolytically cleaved, giving rise to an anti-angiogenic fragment (16K PRL, 16K PL, 16K GH). This appears to be a very efficient and low cost mechanism to simultaneously down-regulate a stimulatory factor and up-regulate an inhibitory factor.

It is important to mention that several other endogenous inhibitors of angiogenesis result from the proteolytic cleavage of proteins with functions distinct from angiogenesis, for example, angiostatin (from plasminogen), endostatin (from collagen XVIII), endostatin XV (from collagen XV), and vasostatin (from calreticulin) (for review see Folkman 1997, Hagedorn & Bikfalvi 2000). Together, these findings suggest that proteolytic cleavage may be a general mechanism underlying the production of inhibitors of angiogenesis at a local site. In view of the unique properties of the fragments versus the full-length molecules, the regulation of the protease activity responsible for the specific protein cleavage would critically influence the angiogenic process.

Along with the generation of angiogenic or anti-angiogenic factors, an important element to be considered is the expression of specific receptors. The opposing actions of members of the PRL/GH/PL family appear to be mediated by different receptors. However, the nature of the receptor for 16K PRL or for the other 16K hormones remains unknown. Because the 16K PRL receptor is different from the PRL receptor, the absence of an angiogenesis phenotype in PRL receptor-deficient mice (Bole-Feysot *et al.* 1998) may reflect the lack of interference with 16K PRL inhibition of angiogenesis, but also the compensatory actions of angiogenic molecules other than PRL. The multiple levels of redundancy built into the mammalian systems may also compensate for the deficiencies in PRL isoforms in the PRL knockout mice, where no apparent angiogenesis-related alteration is observed. However, in these mice disruption of the PRL gene was not complete, leaving an 11K N-terminal PRL molecule (Horseman *et al.* 1997) that could still activate the 16K PRL receptor. Furthermore, in mice lacking the gene for collagen XV, a protein that is proteolytically cleaved to generate the potent anti-angiogenic peptide endostatin XV, no increase in the number of blood vessels is seen (Eklund *et al.* 2001), suggesting that under normal physiological conditions the absence of a single anti-angiogenic agent can be compensated for by other mechanisms. Therefore, besides the regulation of hormonal cleavage, the selective expression of specific receptors and their associated signal transduction pathways must play a decisive role in the outcome of hormonal effects on the angiogenic process.

Understanding the mechanisms that regulate the interplay of PRL/PL/GH isoforms will be essential for establishing their contribution to angiogenesis-related pathologies. In this respect, ongoing studies aim to elucidate the role of GH and PRL in neovascular eye diseases, such as diabetic retinopathy and premature retinopathy. Likewise, the action of PRL/PL/GH molecules on other pathologies characterized by neovascularization, such as tumor angiogenesis and rheumatoid arthritis, has begun to be explored, and some evidence already suggests a possible contribution of this family of molecules. Surprisingly, recent work by Turner and coworkers (2000*a*) showed that pituitary tumors are less vascular than the normal pituitary gland, and that pituitary adenomas show different levels of angiogenesis. Macroprolactinomas are significantly more vascular than microprolactinomas (Turner *et al.* 2000*a*), and their blood vessel density was directly correlated with PRL levels in the circulation (Turner *et al.* 2000*b*). Conversely, no such correlation was found for macroadenomas and microadenomas secreting GH (Turner *et al.* 2000*a*,*b*), although tumors producing both GH and PRL were less vascular than tumors producing GH alone (Turner *et al.* 2000*b*). It remains to be determined whether the reduced vascular density of PRL-producing tumors is accompanied by the local or systemic proteolysis of PRL into 16K PRL. In this regard, some studies suggest the association of cathepsin D (the protease implicated in PRL cleavage and 16K PRL generation) with a tumorigenic and invasive phenotype in breast cancer cells (Rochefort 1990), a result that allows speculation about the possible production of 16K PRL in a

tumor environment. The synthesis of anti-angiogenic factors by tumor cells has been demonstrated previously (O'Reilly *et al.* 1994, 1997). Actively growing primary tumors can secrete anti-angiogenic factors into the circulation, as is the case of angiostatin and endostatin, which can maintain tumors in a dormant state (O'Reilly *et al.* 1994, 1997, Cao 1998).

Exciting new work has shown that the anti-angiogenic properties of 16K PRL (Bentzien *et al.* 2001) and proliferin-related protein (Bengston & Linzer 2000) make them promising candidates for limiting tumor growth. In addition, the potential of PRL and other members of the PRL/PL/GH family on angiogenesis in rheumatoid arthritis promises to be rewarding. Clinical and basic science observations have already implicated circulating and locally produced PRL in the pathophysiology of rheumatoid arthritis (for a review see Neidhart *et al.* 1999). The already developed GH and PRL receptor antagonists (Goffin *et al.* 1996*a*, Okada & Kopchick 2001), together with the availability of hormones (GH, PRL and IGF-I), offer hope for potential therapeutic approaches in the treatment of angiogenesis-related diseases. More recently, dopamine, the major inhibitor of pituitary PRL, was shown to act on D2 receptors present on endothelial cells to inhibit VEGF-induced angiogenesis (Basu *et al.* 2001). This finding represents a new avenue to be explored as dopaminergic inhibition of endothelial-derived PRLs may represent a new mechanism mediating dopamine anti-angiogenic properties (Basu *et al.* 2001) and inhibitory actions on tumor growth (Basu & Dasgupta 2000).

In summary, members of the PRL/GH/PL family constitute novel stimulatory and inhibitory regulators of angiogenesis. The implication of their actions for the development of therapeutic strategies against angiogenesis-dependent disorders has begun to be investigated. It is clear that much further work will be necessary before the relative importance of these hormones on physiological and pathological angiogenesis can be understood. Nevertheless, regardless of the diverse settings in which angiogenesis is encountered and the great redundancy of mediator systems that participate in the process, the characterization of the mechanisms that control the production and action of angiogenic and anti-angiogenic members of the PRL/GH/PL family will undoubtedly prove to be a fruitful area of investigation that ultimately will improve the treatment of patients who suffer from angiogenesis-dependent diseases.

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