

## Pigment epithelium-derived factor: clinical significance in estrogen-dependent tissues and its potential in cancer therapy

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### ABSTRACT

Pigment epithelium-derived factor (PEDF) is a glycoprotein that belongs to the family of non-inhibitory serpins. The broad spectrum of PEDF biological activity is evident when considering its effects in promoting cell survival and proliferation, as well as its antiangiogenic, antitumor, and anti-metastatic properties. Although the structural domains of the PEDF gene that mediate such diverse effects and their mechanisms of action have not been completely elucidated, there is a large body of evidence describing their diverse range of activities; this evidence combined with the regulation of PEDF expression by sex steroids and their receptors have led to the idea that PEDF is not only a diagnostic and prognostic marker for certain diseases such as cancer, but is also a potential therapeutic target. In this manner, this paper aims to generally review the regulation of PEDF expression and PEDF interactions, as well as the findings that relate PEDF to the role of estrogens and estrogen receptors. In addition, this manuscript will review major advances toward potential therapeutic applications of PEDF.

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## Introduction

Pigment epithelium-derived factor (PEDF) was initially described as a neurotrophic and antiangiogenic factor secreted by the human fetal retinal pigment epithelium (1). Expression of PEDF has been reported in a variety of organs and tissues, such as the brain (2), spinal cord (3), eyes (1), lung, heart (4), liver (5), uterus (6–8), ovary (9), prostate, pancreas (10), bone (11), and plasma (12). The multiple biological actions of PEDF have been identified not only in healthy conditions but also in several diseases, such as cancer (12–15), endometriosis (16–19), polycystic ovarian syndrome (20, 21), insulin resistance (22, 23), metabolic syndrome, type II diabetes (24, 25), diabetic retinopathy (26, 27), and cardiovascular disease (28, 29), among others.

The available evidence indicates that the multifunctionality of PEDF is due to the large number of signaling pathways it activates and to the various microenvironments in which PEDF can interact with multiple molecules and undergo various post-translational modifications (30).

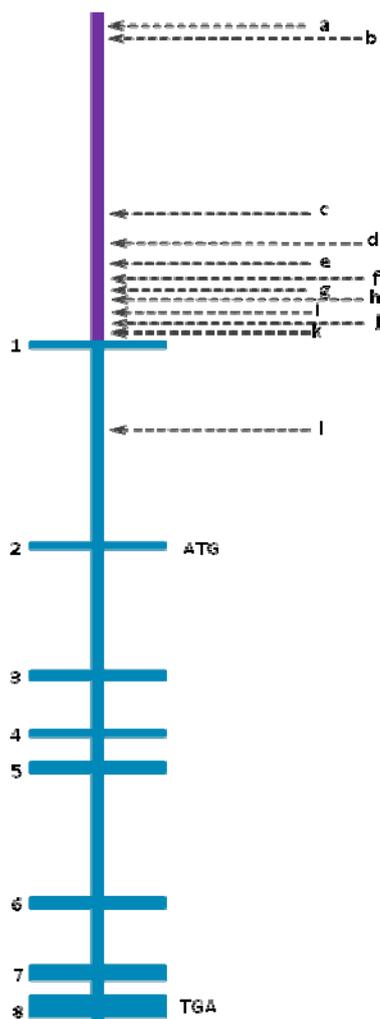
The high levels of PEDF expression in normal tissues, in contrast to the low levels found in cancer

tissues, have been attributed to tumor suppressive activity (31, 32). In addition, PEDF has become a central focus of research in the past decade due to the discovery that in normal cells such as neurons, PEDF induces cell survival and differentiation (1, 33), whereas in animal and human models of malignant tumors, PEDF has potent antiangiogenic and anti-metastatic activity (7, 34–37).

### PEDF: the gene and protein

The gene encoding PEDF, also known as SERPINF1, is located on chromosome 17p13.3 and contains eight exons and seven introns and spans approximately 16 kb (30) (Figure 1) while the encoded glycoprotein consists of 418 amino acids and has a molecular weight of 50 kDa. Similarly to other serpins, the tertiary structure of PEDF is composed of three  $\beta$  sheets and ten  $\alpha$ -helices plus a typical reactive center loop (RCL) near the C-terminus. However, compared with the serpins that inhibit serine/cysteine proteases, the RCL region of PEDF contains substitutions such as the replacement of four consecutive alanines (aa 360-364) with one

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**Figure 1.** Pigment epithelium-derived factor (PEDF) gene structure containing its promoter region and its eight exons and seven introns

- Promoter is represented in purple
- **a**, **b** and **c** represent three different putative response elements to p63 y p73, which are located at -6362, -5996, and -1366
- **d**, **f** and **h** are binding sites for transcription factors, located at ALU sequences (-682, -480, and -150). The most striking and unique feature of this region is the dense cluster of Alu elements which comprises 70% of the most proximal 1 kb upstream region. A sequence identical to Alu subclass (consensus sequence GGTCA(n)<sub>3</sub> TGGTC(n)<sub>9</sub> TGACC), which can function as an estrogen receptor-dependent transcriptional enhancer, is present in the PEDF upstream Alu repeat and a sequence differing by only one nucleotide is present in the proximal Alu repeat, i.e., within 200 bps of the translational start site<sup>141</sup>.
- **e** is a sequence that is recognized by the C/EBP (*CAAT-enhancer binding protein*) family of transcription factors
- **g** represents a sequence contained within TREp (*palindromic thyroid hormone-responsive element*). It is similar to the developmentally-regulated RAR (*retinoic acid receptor*) (-204).
- **i** is the sequence for PEA3 (*polyomavirus enhancer activator-3*). It is present in tandem at -122,-129 and again at -141
- **j** is a binding element for transcription factors such as the Oct (*octamer-binding factor*) family (-113)
- **k** represents binding sites for CAAT-enhancer-binding proteins, C/EBPs or CHOP (-40), HNF4 (*hepatocyte nuclear factor 4*) (-60) and USF (*upstream stimulatory factor*)
- **l** represents the MITF binding site (intron 1)
- Boxes represent exons (1-8)

phenylalanine and one glutamic acid in the last two positions, which explains why PEDF is unable to bind to the target serine/cysteine proteases (38-40).

In positions 63-70, PEDF contains an unconventional nuclear localization signal (NLS) peptide (Figure 2); arginines 67 and 69 in this sequence interact with lysines 48 and 53, from which they are separated by 12 residues, so that a bipartite NLS signal is generated from its own YxxYRVRS sequence and a sequence similar to the consensus NLS sequence KKRRK (41, 42). This signal mediates the transport of PEDF by TRN-SR2 (transportin 3) to the nucleus, where it has specific functions such as participating in cell cycle regulation (43). In addition to the nuclear localization signal, PEDF contains other evolutionarily conserved regions, such as one signal peptide for the secretion of the protein, one C-terminal glycosylation site, and four peptide regions: two near the N-terminus (aa 40-67 and aa 78-95) and two near the C-terminus (aa 277-301 and aa 384-415) (44) (Figure 2). The high degree of homology between the N-terminal amino acids 78-95 and the C-terminal residues 384-415 with all other serpins has led researchers to suggest that these regions are responsible for the shared functions of PEDF and other serpins. Additionally, the finding that the N-terminal residues 40-67 and the C-terminal amino acids 277-301 are unique to PEDF, suggests that they are related to unique functions of the protein (39).

The presence of a secretion signal peptide (Figure 2) located in the RCL C-terminus indicates that PEDF can also be transported outside of the cell, where it acts on target cells by binding to various receptor types.

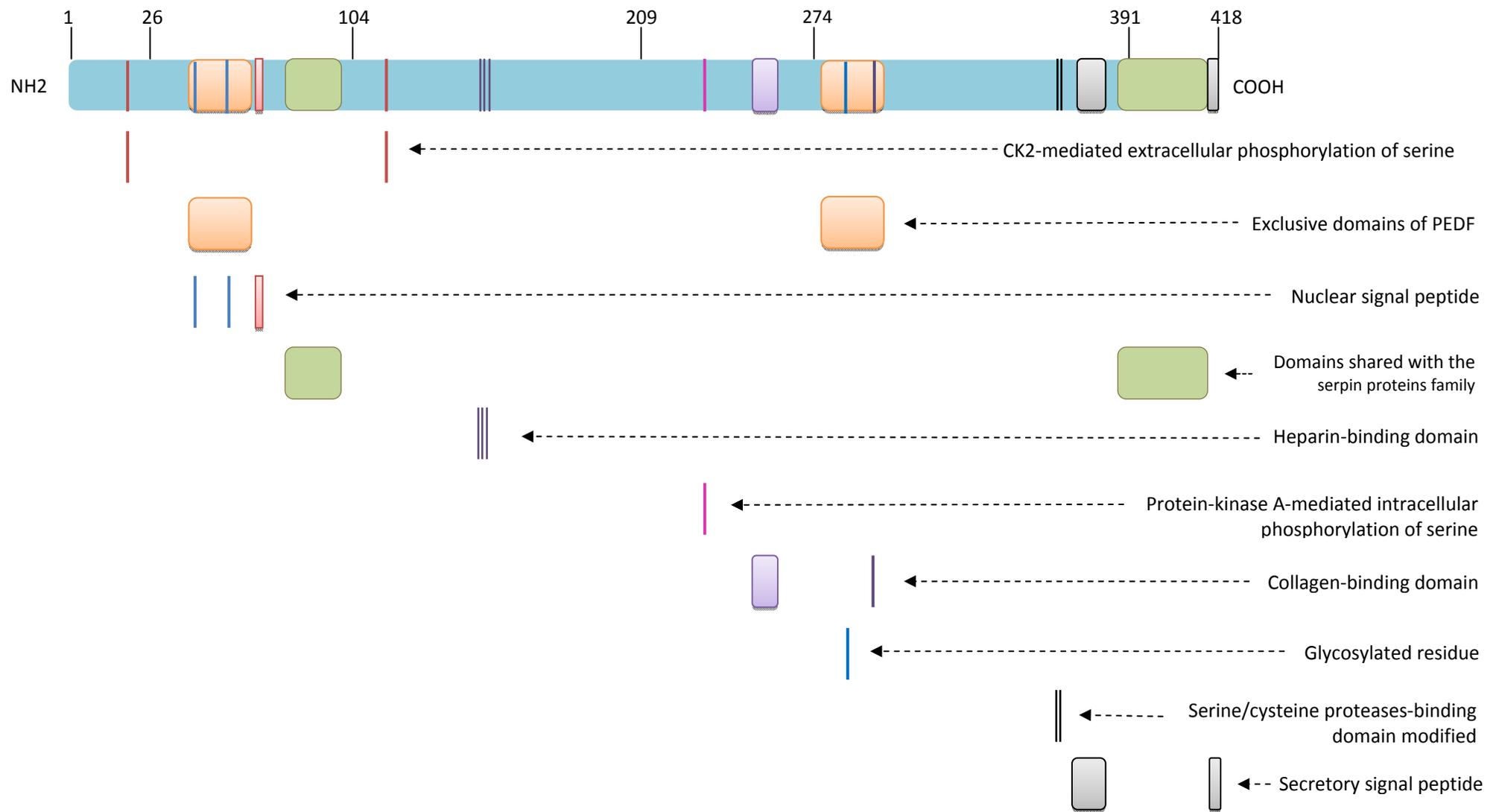
### Cellular localization and functions

PEDF may localize to either the nucleus or the cytoplasm (44-46) or may be secreted, demonstrating that the various functions of the protein can be performed both inside and outside of the cell that produces the protein.

Little is known about the specific activity of PEDF in the nuclear and cytoplasmic compartments, although other members of the same family, such as the avian serpin MENT, have been associated with an inhibition of gene expression by epigenetic mechanisms and the nuclear inhibition of cysteine proteases similar to papain thus promoting the accumulation of the target proteins of papain, for example, Rb (47) and the expression of certain genes by epigenetic mechanisms. These findings coupled with the presence of the nuclear localization peptide and the strong immunolocalization of PEDF in the nucleus of many mammalian cell types indicate the relevance of the PEDF protein in the nuclear compartment.

### Cell cycle regulation

In the nucleus, PEDF interacts with transcription factors that regulate the cell cycle. It has been established that the promoter region involved in this



**Figure 2.** Pigment epithelium-derived factor (PEDF) protein has binding domains for heparin (residues 145, 146 and 148), collagen (regions 255–257, 299) and a modified binding domain for serine/cysteine proteases. It further contains a bipartite nuclear localization peptide (NLS) (regions 48, 53 and 67–69), and a secretory peptide (regions 373–380 and 415–418). It has exclusive domains (regions 40–67 and 277–301) and also domains shared with other serpins (regions 78–95 and 384–415). The protein is target of various post-translational modifications, such as extracellular phosphorylation at serines 24 and 114 by CK2 as well as intracellular phosphorylation of serine 277 by PKA and glycosylation at residue 285

process is located at least 1759 bp upstream of the transcriptional start site (48). In the 5 kb adjacent to the 5' end of the gene, there is an accumulation of Alu repeats that are involved in the regulation of gene expression (49) through their binding motifs for transcription factors involved in cell cycle regulation, such as ER $\alpha$ , YY1 and Sp1 (48).

There is also evidence that the PEDF gene promoter contains response elements for the nuclear proteins p63 and p73 (-1366 to -1330), which belong to the p53 family (Figure 1). The amino acid sequence and molecular structure of both proteins have a significant level of homology with p53, which suggests that p63 and p73 share similar functions with p53. In this regard, Sasaki *et al* 2005 (50) noted that although p63 and p73 can induce PEDF expression and promote cell differentiation, the physiological role of these proteins as potential inducers of PEDF expression has not yet been clarified.

#### Induction of p53

The indirect effect by which PEDF induces p53 expression (51) by activation of the PEDF receptor (PEDFR) is particularly important. Certain fatty acids such as arachidonic acid metabolites that are released upon activation of this receptor and oxysterol derivatives of the cholesterol biosynthetic pathway in turn activate PPAR $\gamma$  (*peroxisome proliferator-activated receptor gamma*) transcription factors. The same effect can also be observed when cPLA(2)- $\alpha$  (*cytosolic calcium-dependent phospholipase A(2)-alpha*) and p38 MAPK (*mitogen-activated protein kinase*) are sequentially activated (52). It is worth mentioning that PPAR $\gamma$  also can trigger anti-neoplastic processes, including the inhibition of cell proliferation and the induction of apoptosis, due to the affinity of these proteins for the p53 promoter region (52–54). Evidence indicates that PEDF inhibits the cell cycle by inducing p53 expression, suggesting that this effect does not correspond to an immediate cell response but rather to a long-term response.

#### Extracellular localization and interactions

Outside of the cell, PEDF is found in body fluids, in which it reaches significant concentrations, and in the extracellular matrix, where it establishes interactions with collagen and glycosaminoglycans such as hyaluronan, heparan sulfate, chondroitin sulfate, chondroitin sulfate-C, dermatan sulfate, and dextran sulfate (4, 55); each of these glycoproteins interacts with a different binding domain of PEDF. Thus, whereas the heparan sulfate-binding domain includes a positively charged region, the collagen-binding region requires Asp(255), Asp(257), and Asp(299), and the heparin-binding domain contains three acidic residues: Arg(145), Lys(146), and Arg(148) (56).

The PEDF protein has affinity for various receptor types, such as PEDFR, the laminin receptor (LR), F1-

ATPase synthase, LRP6 (*low-density lipoprotein receptor-related protein 6*), ATGL (*adipose triglyceride lipase*), LRP5 (*low-density lipoprotein receptor-related protein 5*), and PLA2 (*phospholipase A2*), and specific receptor interactions determine the broad pleiotropy of PEDF biological activity (39, 55, 57–59). For example, anti-apoptotic activity of PEDF has been demonstrated in retinal cells as a result of binding between PEDF and the PEDFR transmembrane receptor (60). In this case, PEDF-PEDFR binding triggers the activity of phospholipase A2 (61) and, therefore, the release of membrane fatty acids or bioactive lipids capable of inducing apoptosis (62). In cerebellar granule neurons, the PEDF-PEDFR complex activates NF $\kappa$ B (*nuclear factor kappa beta*) target genes with anti-apoptotic and neuroprotective functions against glutamate cytotoxicity. NF $\kappa$ B signaling is released via IP3 and RAS downstream of PEDF-PEDFR complex formation (63). In contrast, PEDF binding to the LR induces pro-apoptotic signaling in endothelial cells (64–66), whereas PLA binding triggers a similar signal in neurons and cardiomyocytes (67). In cultured cells and in animal models, the activation of the LR by PEDF has antiangiogenic, anti-thrombogenic, and anti-inflammatory effects (59, 66). It has further been reported that PEDF binding to the ATGL receptor triggers antiangiogenic and proinflammatory activity (68, 69).

In neurons, PEDF induces the expression of genes that promote survival, such as c-IAP1, c-IAP2, FLIPs, A1/Bfl-1, and Mn-SOD. In murine models, PEDF secretion in the nervous system has been demonstrated in certain regions of neurogenesis, such as in the hippocampus and subventricular zone; here, PEDF may specifically regulate the induction of self-renewal in neural stem cells (70).

In addition to the above mentioned cell types, other cells such as neonatal pericytes and astrocytes are also PEDF targets. PEDF stimulates the synthesis of PDGF-B (*platelet-derived growth factor-B*), a factor necessary for survival and microvascular homeostasis (71) in pericytes, whereas neonatal astrocytes, in turn, begin to express early and proinflammatory genes as a result of NF $\kappa$ B or CREB (*cyclin AMP-responsive element binding protein*) activation (72). In recent studies, it has been reported that adipocytes secrete PEDF, which has been associated with insulin resistance and inflammatory processes in adipocytes and muscle cells (42).

#### The PEDF is the most potent inhibitor of angiogenesis

Although PEDF lacks the serine/cysteine protease inhibitory activity characteristic of many serpins (39), PEDF does share antiangiogenic and antitumor properties with some serpin family members, such as antithrombin, angiotensinogen, and maspin (73), presumably due to the presence of a common structural determinant. However, among all

natural angiogenesis inhibitors known thus far, PEDF is the most effective, with greater potency than angiostatin, endostatin, and thrombospondin 1 (74). This antiangiogenic activity is the result of various PEDF functions, including the induction of endothelial cell apoptosis, the inhibition of cell migration and new vessel formation, and the inhibition of the response to VEGF (vascular endothelial cell growth factor), the most potent proangiogenic factor, as well as the response to bFGF (*basic fibroblast growth factor*) (74).

PEDF has been shown to inhibit the neovascularization promoted by VEGF through different pathways (Figure 3):

- The PEDF-PEDFR complex can act as an upstream inducer of NF $\kappa$ B, a factor that, in turn, downregulates the anti-apoptotic factor FLIP 1 (*FLICE inhibitory protein*) which means that PEDF-PEDFR complex interferes with caspase 8 activation and increases cell resistance to the pro-apoptotic effects of FasL (75).
- PEDF can promote  $\gamma$ -secretase-dependent proteolysis of the transmembrane portion of VEGFR1 and VEGFR2 (76, 77) and also inhibit VEGF-induced VEGFR1 phosphorylation, or promote the dephosphorylation of this receptor, by inducing the upregulation of presenilin-1 and the subsequent aggregation of vascular endothelial protein-tyrosine phosphatase and VEGFR1 (78).
- PEDF inhibits motility and thus migratory behavior both in endothelial and cancer cells, and this effect is mediated by the PEDF-LR complex and subsequent activation of the p38 MAPK pathway (79).
- PEDF directly induces apoptosis in endothelial cells (74) by upregulating the expression of the pro-apoptotic gene p53 in human umbilical vein and mouse cornea endothelial cells *in vitro* through the sequential activation of p38 MAPK, cPLA(2)- $\alpha$ , and PPAR $\gamma$  (80). In a more recent study, it was shown that PEDF induces the expression of TRAIL (*TNF-related apoptosis inducing ligand*) also through cPLA(2)- $\alpha$  and PPAR $\gamma$  to generate a pro-apoptotic effect mediated by macrophages in tumor cells *in vitro* (52) and also suggested that macrophages may be involved in the antiangiogenic mechanism of PEDF.

There is evidence that the inhibition and induction of angiogenesis depend on the differential secretion of regulatory factors by the fibroblasts present in the tissues. Thus, when fibroblasts are quiescent, they secrete PEDF (43), whereas cells in the proliferative state secrete VEGF to arrest or induce mitosis in the endothelial cells of adjacent blood vessels depending on the situation (81). Accordingly, it has been proposed that the regulation of angiogenesis depends on changes in the balance between PEDF and VEGF. In this vein, both the increase in PEDF expression and the decrease in

VEGF have a negative effect on angiogenesis, whereas the increase in VEGF promotes blood vessel formation (82). Similarly, studies conducted in mice (83) showed that bone marrow neovascularization depends on the ratio of VEGF expression and PEDF produced by stromal cells. In any case, the mechanisms underlying the antiangiogenic property of PEDF are not fully elucidated, underscoring the need for further studies.

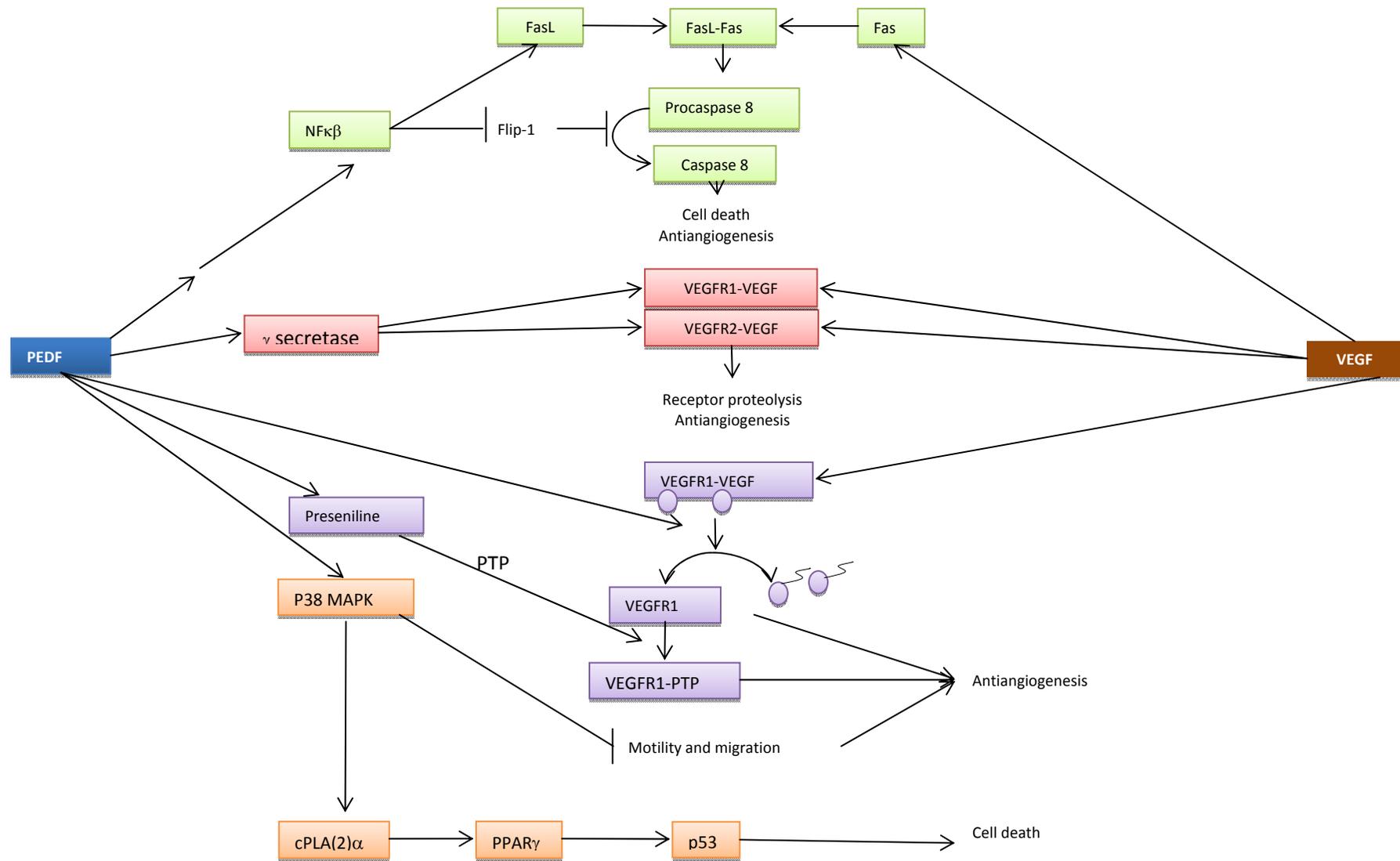
### The role of the PEDF in senescence and aging

PEDF expression declines not only during tumor progression but also during senescence and aging. Senescence is defined as the state of irreversible cell cycle arrest reached by cells that have exhausted their capacity for proliferation, either as part of the natural aging process or as a result of mechanisms such as the activation of certain oncogenes, the inactivation of tumor suppressors, oxidative stress, and genotoxic damage, among others (84, 85). Aging is the result of a gradual decrease in the ability to repair and regenerate tissues in complex organisms (86).

Similarly to mitotic cells, PEDF is not expressed in senescent cells (43). However, when cells have recently become senescent or, in the case of fibroblasts, when cells are cultured under serum deprivation, PEDF levels are elevated, in contrast to the low levels found in long-term senescent cells (43). In cells that have recently become senescent, these elevated levels of PEDF begin to decline until they become undetectable, which has led to the suggestion that PEDF may induce senescence in mitotically active cells, after which senescence would be maintained by other genes (43, 87). However, it has been reported that in certain cells, such as senescent melanocytes, PEDF expression is upregulated by MITF (*microphthalmia-associated transcription factor*), which activates gene transcription by directly binding to the first intron (88). Thus, the elevated PEDF expression is likely associated with a recent acquisition of the senescent state in these cells, which is in agreement with the findings reported by Pignolo *et al* 1993 (87).

In cultured human mesenchymal cells, Cao *et al* 2013 (89) established that PEDF reduces oxidative stress and thus indirectly delays the onset of senescence and preserves the proliferative and differentiation potentials of the cells. Considering that oxidative stress precedes p53 activation (90), it is reasonable that when stress is reduced, the expression of genes associated with senescence, such as p53 and p16, also decreases, as determined by Cao *et al* 2008 (89).

In contrast to this finding, Steinle *et al* 2008 (91) showed that during normal aging in rats, the retinal pigment choroid-epithelium complex shows a decrease in PEDF and VEGF expression levels. The decrease in PEDF may be due to its antiangiogenic



**Figure 3.** Signaling transduction pathways involved in the antiangiogenic activity of Pigment epithelium-derived factor(PEDF). The antiangiogenic response depends on the balance between PEDF and vascular endothelial cell growth factor (VEGF) and on the regulatory factors induced by them

properties. In this vein, the lack of PEDF may be the result of a compensatory effect to offset the decline in microvessel density, a common event during this stage of life. Furthermore, the loss of PEDF may generate an imbalance in angiogenic modulators, contributing to the onset of diseases characterized by abnormal vasculogenesis (92). The decreased PEDF expression associated with aging could also be ascribed to the activity of transcription factors whose activity decreases with age, such as NF $\kappa$ B and AP-1, which can potentially bind to specific binding motifs located at multiple Alu repeats present in the PEDF promoter (48).

### **Antitumor activity of the PEDF**

Several studies have shown that PEDF exerts strong antitumor activity and is downregulated in solid tumors and cancer cell lines. Assays aimed at restoring gene expression have allowed researchers to assess the impact of PEDF loss on tumor progression. The restoration of PEDF expression in prostate, osteosarcoma, ovary, pancreas, malignant glioma, and neuroblastoma cells reduces cell growth rate and microvessel density of the tumor mass (32, 35, 93–96), indicating that low PEDF levels are significantly associated with metastatic potential, tumor grade, and poor prognosis (51).

Because the expression pattern of PEDF is strongly linked to angiogenesis, disease progression is dependent on the balance between PEDF and VEGF. Increased PEDF or decreased VEGF expression inhibits angiogenesis, whereas increased VEGF promotes angiogenesis (82). In invasive ductal breast tumors, decreased PEDF expression is correlated with an increase in microvessel density; for this reason, PEDF is considered to predict poor prognosis (26). Moreover, a decrease in PEDF in this type of tumor and in others, such as pancreatic ductal adenocarcinoma and melanoma, has been associated not only with increased tumor aggressiveness but also with increased metastatic potential and shorter survival time (97). Thus, PEDF may serve as a therapeutic target for certain cancers, including breast, liver, lung, colorectal, and Wilms tumor (97–100) and may also provide prognostic value where high PEDF expression is associated with better total and disease-free survival.

Although the PEDF mechanism of action in tumors has not yet been revealed, it has been established in malignant melanoma that PEDF expression is upregulated by MITF, which activates gene transcription in a manner similar to what occurs in senescent melanocytes (88).

### **The PEDF in normal and pathological estrogen-dependent tissues**

Estrogens are steroid hormones that act on a number of tissues to promote growth and differentiation. Estrogens possess a wide range of

influence, which allows them to participate in a myriad of physiological processes. For this reason, it is not surprising that estrogens are considered to be important players in the onset and progression of certain cancers (breast, ovarian, prostate, endometrial, and colorectal) and other diseases such as diabetic retinopathy, osteoporosis, endometriosis and obesity as well as various types of neuropathies, systemic lupus erythematosus, and cardiovascular disease (101–109).

To date, three estrogen receptor (ER) subtypes have been described: ER $\alpha$  and ER $\beta$ , which are present in the cytoplasm, nucleus and mitochondria, and GPER (*G protein-coupled estrogen receptor*), which is localized in the plasma membrane.

### **ER $\alpha$ and ER $\beta$**

Despite differences in tissue distribution and downstream signaling, ER $\alpha$  and ER $\beta$  share structural similarities. Their DNA-binding domains share approximately 95% homology, whereas the ligand binding domains are approximately 55% identical at the amino acid level and the N-terminal domains share only 15% homology (110).

At the genomic level, ER $\alpha$  and ER $\beta$  act as modulators of estrogen physiological function because they regulate gene expression as transcription factors (107, 111, 112). These ERs are primarily located in the cytoplasm, where they form complexes with the heat shock proteins (HSP) 50, 70, and 90. Once activated by ligand binding, ERs dissociate from HSPs so that the DNA-binding domain is released. ERs form  $\alpha\alpha$  or  $\beta\beta$  homodimers or  $\alpha\beta$  heterodimers. In the dimerized form, the receptors can interact with specific DNA sequences in the promoter region of target genes, known as *E2 response elements* (EREs). When not bound to DNA, the ERs interact with other factors such as SP1, NF $\kappa$ B, and AP1 (*activator protein 1*), thus affecting the transcription of target genes of these factors (113, 114). After binding to DNA, ERs recruit coregulatory molecules of the P160 family such as SRCs (*steroid receptor coactivators*) (115). The finding that ER $\alpha$  preferentially binds to EREs whereas ER $\beta$  binds to AP1 recognition sites established that DNA-binding domains are less relevant for the induction of ER $\beta$  transcriptional activity than for that of ER $\alpha$  (102).

A wide variety of rapid non-genomic effects have also been described resulting from the activation of ER $\alpha$  and ER $\beta$ . In the presence of estrogens, these ERs interact with various molecules associated with membrane receptors, as tyrosine kinase, striatin, G proteins and non-receptor tyrosine kinases and with caveolin-1, allowing them to form complexes that activate different signaling pathways, as MAPK/ERK and PI3K/AKT (*phosphoinositide 3-kinase*) pathways (103, 116); they also increase Ca<sup>2+</sup> and nitric oxide levels (117), among others.

Both the ER $\alpha$  and ER $\beta$  receptors are expressed in a wide range of cells and tissues and are involved in

regulating the functions of the reproductive, immune, skeletal, cardiovascular, and central nervous systems, on which they exert distinct and non-redundant effects (118, 119) despite their structural similarities.

Due to its effects on cell proliferation, ER $\alpha$  has been implicated not only in the development of the reproductive tract and the mammary gland but also in estrogen-dependent carcinogenesis (120). The antiproliferative effect of ER $\beta$ , meanwhile, is evident due to its antitumor (121) and anti-inflammatory activities (122). However, when both ERs are present at the promoter region of genes involved in proliferation, they exert opposing actions, so that the result of E2 stimulation corresponds to the balance of ER $\alpha$  and ER $\beta$  signaling (123).

#### GPER

The expression of GPER has been described in various organ systems, such as the reproductive, urinary, endocrine, nervous, immune, musculo-skeletal, and cardiovascular systems (119, 124). In these tissues, GPER participates in rapid signal transduction events triggered by estrogens and, to a lesser extent, in genomic signaling (125). It has been shown that in the cardiovascular and immune systems, estrogen-mediated rapid signaling responses occur as a result of GPER activation of multiple signaling pathways, an effect that occurs independently of the signal generated by steroid nuclear receptors (119, 126). This indicates that GPER increases the complexity of the physiological responses to estrogens (125).

#### PEDF is an estrogen target gene

The PEDF gene promoter contains estrogen response elements, which may explain why the protein levels change in tissues, both normal and pathological, whose growth is subject to hormonal regulation. The finding of a putative response element to 17 $\beta$ -estradiol (E2) in the region near the 5' end of the gene (127) and the fact that E2 modulates PEDF expression indicate that estrogens are involved in the transcriptional regulation of PEDF and that the ERs can influence its biological functions.

#### Estrogen-ER-PEDF signaling Endothelium

Using ginsenoside-Rb1, a steroidal saponin with a structure similar to those of the estrogens, Leung *et al* 2007 (128) demonstrated that more so than ER $\alpha$  activation, ER $\beta$  activation caused an increase in the expression and secretion of PEDF in human umbilical vein endothelial cells *in vitro*, resulting in the inhibition of endothelial tube network formation. Consequently, these authors related the antiangiogenic role of ER $\beta$  to the findings of Garvin *et al* 2006 (129), who found that E2 decreases VEGF expression in breast

cells transfected with ER $\beta$ , and those of Li *et al* 2006 (130), who reported an increased expression of PEDF mRNA in retinal Müller cells, which contain ER $\beta$ .

#### Ovary

Many studies have reported differential expression patterns of ER $\alpha$  and ER $\beta$  in the different cell types present in normal ovaries. Thus ER $\beta$  is confined to follicle granulosa cells, whereas ER $\alpha$  is only expressed in theca or ovary stromal cells (131, 132). This localization was described by Emmen *et al* 2005 (133), who noted that ER $\beta$  plays a key role in the differentiation and function of granulosa cells and that ER $\alpha$  is responsible for regulating the negative feedback of pituitary gonadotropins. However, Lenie and Smitz 2008 (134) showed that the two receptor types were expressed *in vitro* in the granulosa cells of mouse follicles, although with a different subcellular localization, and that ER $\alpha$  was expressed in theca cells. The results of this study indicated that ER $\beta$  is exclusively present in the nucleus during follicular development, whereas ER $\alpha$  is initially expressed in the cytoplasm and subsequently undergoes translocation to the nucleus, an event that also occurs in theca cells, showing that expression of the two receptors is subject to the hormonal environment.

In the human ovarian surface epithelium (OSE), ER activation is an important regulatory event upstream of PEDF expression as Cheung *et al* 2006 (32) demonstrated. In normal OSE cells, E2 directly repressed the PEDF promoter, which resulted in a rapid decrease in PEDF mRNA and protein levels in a dose-dependent and time-dependent manner that was independent of *de novo* synthesis of an intermediate protein or a change in mRNA stability. It is worth noting that in this tissue, ER $\alpha$  is the main estrogen receptor (135), which may be related to the fact that approximately 90% of ovarian tumors arise from this tissue. It also explains how E2 may exert a dual effect, suppressing or inducing PEDF according to the cell type and the activated receptor. These authors also reported that PEDF not only induces apoptosis but is also a potent inhibitor of estrogen-induced proliferation in ovarian epithelial cells, an effect that was shared by cancer cell lines of the same estrogen-responsive tissue. According to these researchers, the elevated PEDF levels that occur prior to ovulation induce apoptosis of ovarian epithelial cells adjacent to the preovulatory follicle, favoring its subsequent rupture and the release of the secondary oocyte. During ovulation, the reduction observed in the amount of PEDF is consistent with the preovulatory estrogen secretion peak, which promotes the survival and proliferation of cells around the rupture site (32).

The presence of PEDF has also been established in the ovarian follicular fluid of patients undergoing

*in vitro* fertilization procedures (136, 137). The circulation, the oocyte, and the surrounding granulosa cells appear to be the source of the factor (9), which decreases follicular angiogenesis, preventing vascularization of the granular compartment of the follicles before ovulation and luteinization occur (138). Based on the finding that high doses of E2 and hCG (*human chorionic gonadotropin*) induced a gradual decrease in PEDF expression in human granulosa cells and rodent follicles, Chuderland *et al* 2013b (9) postulated that the regulation of PEDF was dependent on the hormonal environment predominant in each phase of the ovarian cycle, with expression increased during the early proliferative phase when E2 levels are low and decreased as the ovulation cycle progresses towards the secretory phase, when levels of E2 and progesterone increase (Figure 4). Consistent with this hypothesis, the fact that ER $\beta$ , which is the predominant estrogen receptor in the granulosa cells, maintains its nuclear localization during follicular development suggests that this receptor induces not only the differentiation of these cells but also PEDF expression. Strikingly, ER $\alpha$  translocation to the nucleus occurs only during the late follicular phase in a manner that appears to parallel the decrease in the secretion of PEDF and E2 that occurs a few hours before ovulation and the decline in the levels of pituitary gonadotropins, hormones whose downregulation is controlled by ER $\alpha$  (133).

According to Kampfer *et al* 2014 (138), in the avascular granulosa cell compartment, PEDF is responsible for generating reactive oxygen species and hence oxidative stress, which in turn leads to apoptosis of the granulosa cells. This may be involved in the genesis of fertility disorders, such as polycystic ovary syndrome (PCOS). Despite its antiangiogenic effect, PEDF has also been found in the corpus luteum, a structure characterized by its large blood supply, as well as in cultured granulosa-lutein cells.

In addition to PCOS, other fertility disorders such as OHSS (*ovarian hyper stimulation syndrome*) have been associated with alterations in ovarian angiogenesis in a manner that involves VEGF and PEDF. PCOS appears to be common in women of reproductive age with risk factors such as insulin resistance, hyperinsulinemia, and genetic predisposition. After the appearance of seemingly contradictory reports on the finding of serum levels of PEDF—both decreases and increases have been reported—in patients with PCOS (20, 139, 140), it was recently demonstrated that in the ovaries of PCOS-induced mice, low levels of PEDF and elevated levels of VEGF coexist (141). OHSS has been described as a complication from the use of gonadotropins during *in vitro* fertilization treatments and is more common in women with PCOS (142). PEDF and VEGF also participate in the pathogenesis of OHSS according to

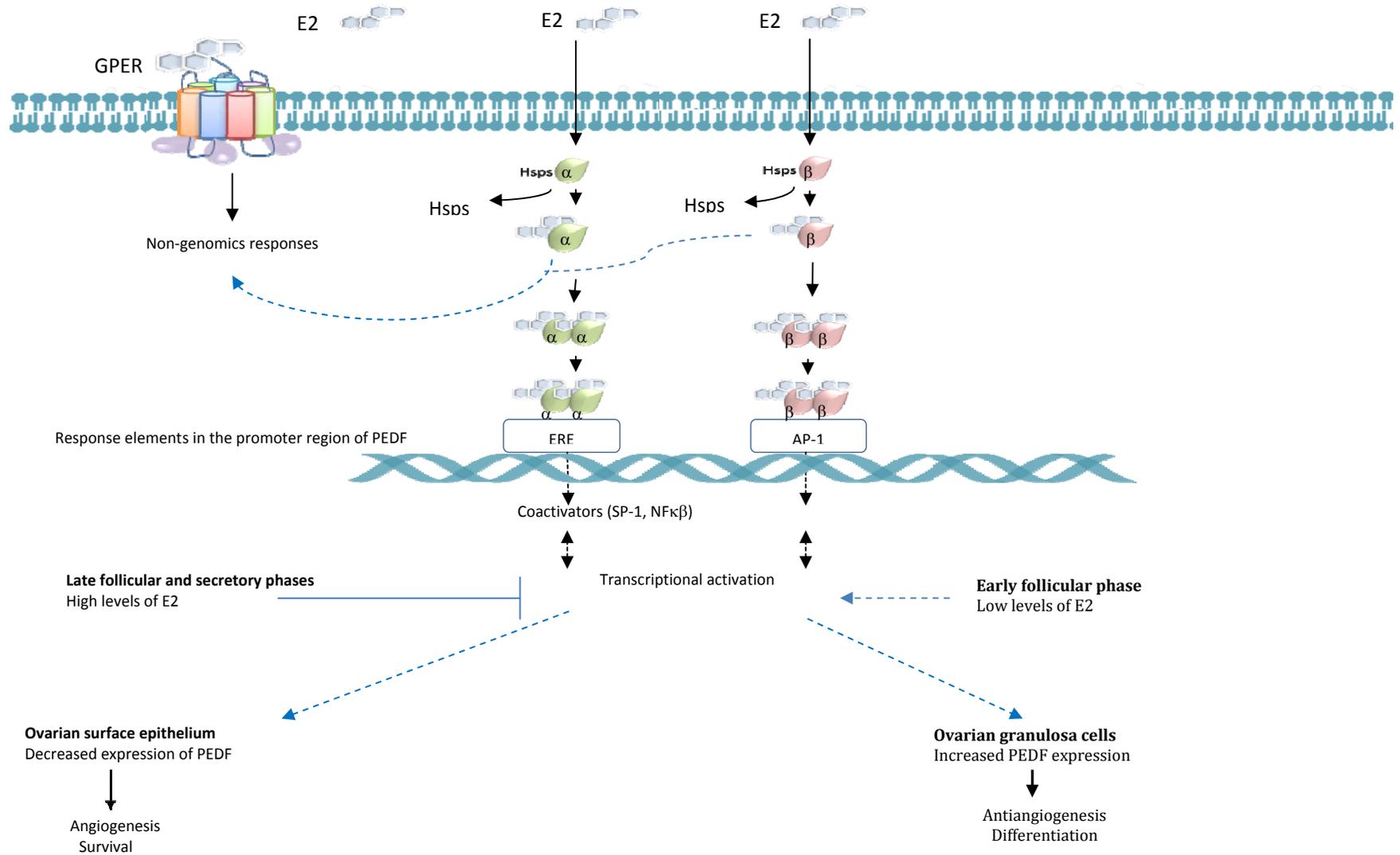
reports that describe an increase in vascular permeability due to the action of VEGF (143) and a decrease in PEDF levels in the ovaries of OHSS-induced mice (144) as well.

Because ovarian carcinoma is one of the most lethal malignancies, the presence of low PEDF expression levels in various ovarian tumor types is of particular interest (145). One of the major risk factors for ovarian cancer is the presence of elevated estrogen serum levels; thus, it is understandable that under these circumstances, PEDF levels tend to decrease, which is in line with the results of Cheung *et al* 2006 (32). Because it is generally thought that ER $\beta$  expression in ovarian tumors decreases and that of ER $\alpha$  increases, the reduction in PEDF expression may also be related to the low levels of ER $\beta$  expression reported in ovarian tumors (146–148). Similarly, the low expression of ER $\beta$  suggests that the pro-proliferative effect of ER $\alpha$  is not counterbalanced. Chan *et al* 2008 (147) demonstrated that ER $\beta$  expression is significantly higher in normal tissue compared with malignant ovarian tissues and that the expression level is inversely correlated with disease stage. However, Halon *et al* 2011 (148) determined that although ER $\beta$  expression is decreased in ovarian tumors, ER $\beta$  expression did not significantly correlate with histopathological parameters (histological type and grade) or tumor stage. This study further established that increased ER $\beta$  expression is associated with increased both disease-free survival and overall survival. Furthermore, Fujimoto *et al* 2000 (149) demonstrated that when the ER $\alpha$ /ER $\beta$  ratio is close to one, prognosis improves compared with very high or very low ratios, which are associated with poor prognosis.

However, based on the observation that gene expression was only lower in malignant ovarian tissues where there was also low VEGF expression, Tsuchiya *et al* 2009 (145) proposed that the malignancy of ovarian tumors with low VEGF expression was strongly dependent on PEDF expression. Thus, decreased PEDF expression could be not only an informative diagnostic marker but also a good prognostic factor for patients with ovarian cancer.

#### Endometrium

The endometrial tissue is subject to differential hormonal regulation by estrogen and progesterone. Morphological and functional changes in the human endometrium during the menstrual cycle are closely linked to PEDF expression, as demonstrated by Chuderland *et al* 2014a (8), who stated that PEDF levels varied according to a dynamic pattern, with low levels during the proliferative and early secretory cycle phase—when estrogen levels are high—and higher levels in the late secretory phase—when



**Figure 4.** Effects of estrogen signaling on Pigment epithelium-derived factor (PEDF) expression and of the PEDF transcriptional activation in ovarian granulosa cells and in epithelial cells along the ovarian cycle. The response to estrogen shows differences according to the phase of the cycle and the target ovarian cell

estrogen levels decrease. This finding suggested the existence of an association between PEDF expression and the variation of steroid hormone dominance during each phase of the cycle. E2 pro-angiogenic activity appears to be mediated by ER $\alpha$  (150), whereas the effect of progesterone is less well understood. Although the mechanisms involved are not fully elucidated, these authors suggested that PEDF counterbalances the pro-angiogenic factor VEGF in a manner similar to what occurs in other tissues, with elevated levels of VEGF during the proliferative and early secretory cycle phases and decreased levels during the late secretory phase, indicating that endometrial angiogenesis is controlled by the balance between VEGF and PEDF. They also argued that the regulation exerted by PEDF in the endometrium is autocrine-paracrine taking into account that endometrial glandular cells not only express and secrete PEDF but also respond to it via the PEDFR (8). The VEGF-dependent antiangiogenic response mediated by PEDFR combines with the VEGF-independent antiangiogenic response mediated by the LR in endothelial cells, which was previously described by Bernard *et al* 2009 (66).

Angiogenesis defects have been associated with the development of certain endometrial diseases such as endometriosis (151), endometrial cancer (152), and repetitive implantation failure (153). In endometriosis, the formation of ectopic foci of functional endometrium is necessarily preceded by a process of neovascularization. This led Chen *et al* 2011 and 2012 (16, 17) to hypothesize about the possible role of PEDF in the pathogenesis of this disease. Indeed, these authors demonstrated that PEDF expression levels are reduced in serum, peritoneal fluid, and endometriotic lesions of women with endometriosis and established that the expression level is correlated with disease severity.

#### Mammary gland

ER $\beta$  and ER $\alpha$  are expressed in the normal adult breast tissue of humans and rodents. ER $\alpha$  is expressed in approximately 10% and ER $\beta$  in 70%–80% of epithelial cells. ER $\beta$  is also expressed in stromal and immune cells present in glandular tissue (154, 155). ER $\alpha$  is necessary for growth and branching of the mammary ducts, whereas ER $\beta$  promotes the terminal differentiation of ductal epithelial cells and maintains their differentiated status (156). In contrast to normal breast tissue, the concentration of ER $\beta$  declines and that of ER $\alpha$  increases in tumor tissue (157).

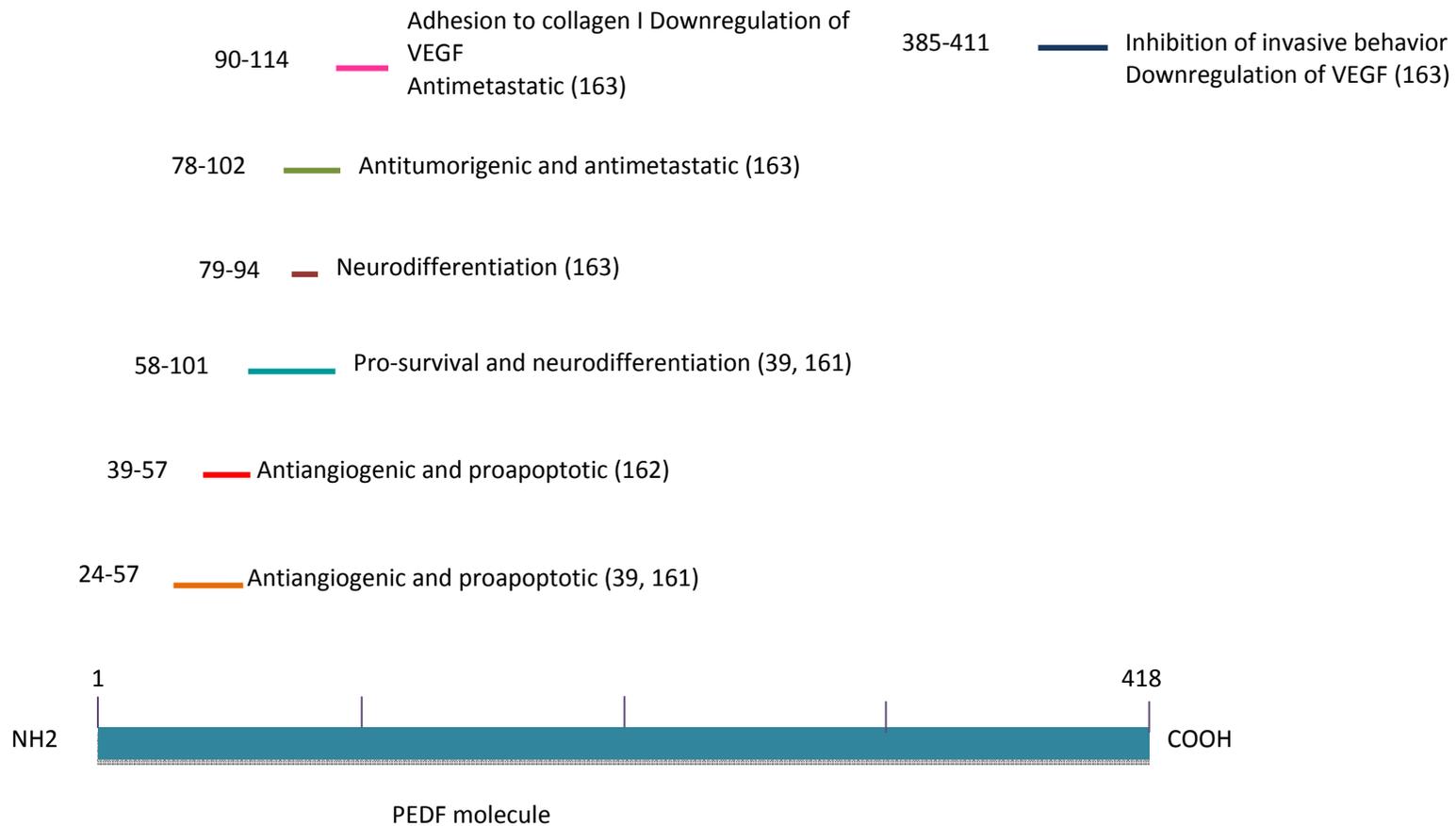
As tumor mass increases, hypoxic regions appear, which, together with other factors, induce angiogenesis. Hypoxic regions are associated with decreased PEDF expression and thus with a poor prognosis and tumor cell metastasis. Therefore, the

presence of PEDF in breast tumors has been regarded as an important biomarker because the level of PEDF is regulated by the degree of hypoxia and by VEGF, as demonstrated by Zhou *et al* 2010 (26). In breast cancer metastatic foci in the brain, it has been observed that PEDF expression is inversely related to patient survival (158). Assays restoring PEDF expression in mice with brain metastasis have shown a reduction in the size of the tumor mass without affecting microvessel development, a finding that is the first evidence that PEDF protects neurons from damage induced by cancer (158).

#### Clinical implications and perspectives

Because the role of PEDF in modifying the tumor microenvironment has been amply demonstrated, research on the use of this molecule for therapeutic purposes against cancer is highly important (51, 159). The design of PEDF-derived synthetic peptides forecasts a promising future therapy for cancer. Due to their high specificity for and easy penetration of target tissues and the lack of genotoxic or genotype-specific effects, PEDF synthetic peptides are currently a spearhead in the therapy to restore PEDF expression in cancer tissues (159, 160).

Some studies using peptides synthesized from functional epitopes located in the PEDF N-terminal region have ascribed properties to the peptides similar to those of the whole protein (Figure 5). For example, a 34-mer peptide (residues 24–57) with antiangiogenic and pro-apoptotic activities and a 44-mer peptide (residues 58–101) with neurotrophic activity (161) showed significant antitumor activity when introduced into prostate cancer cells (39). Within the 44-mer peptide sequence, a shorter ERT fragment (residues 79–94) was identified that causes neuroendocrine differentiation in prostate cancer cells (39). From the 34-mer peptide, Mirochnik *et al* 2009 (162) obtained a shorter 18-mer peptide (residues 39–57) that retains the ability to inhibit angiogenesis and tumor growth in prostate tumors in mice. Based on known PEDF functional epitopes, Ek *et al* 2007b (163) designed four synthetic peptides and used them against cells of the human osteosarcoma cell line SaOS2. In this study, the StVOrth-2 peptide (residues 78 to 102) exhibited an inhibitory effect on tumor cell proliferation, whereas StVOrth-3 (residues 90–114) promoted adhesion to collagen I. StVOrth-4 (residues 387–411) was the most potent inhibitor of invasion, and StVOrth-1 (residues 40–64), -2, and -3 induced osteoblast differentiation. Although none of the peptides inhibited the formation of tubular networks in the angiogenesis assay conducted in this study, StVOrth-3 and -4 downregulated VEGF expression. In addition, the StVOrth-2 and StVOrth-3 peptides inhibited *in vivo* primary tumor growth and the development of pulmonary metastases in an



**Figure 5.** Some synthetic peptides derived from Pigment epithelium-derived factor (PEDF) as summarized here, have shown remarkable effects in various experimental models, especially as it relates to counteract the malignant progression

orthotopic disease model (35). In another orthotopic murine model of tibial osteosarcoma with spontaneous metastasis, Broadhead *et al* 2012 (159) evaluated treatment with the StVOrth-2 and StVOrth-3 peptides, which were supplied continuously and systemically by micro-osmotic pumps. Their results showed that the peptides had little effect on the extent of local tumor invasion or on the induction of apoptosis or necrosis when administered together. However, when administered individually, StVOrth-2 reduced the growth of primary tumors, whereas StVOrth-3 limited the development of pulmonary metastases. Furthermore, there was no evidence of immunogenic effects or treatment-related toxicity.

Given that the size of PEDF can be a limiting factor for its use, it is important to use smaller peptides derived from the protein that can easily access systems such as the central nervous system, where features such as the permeability of the blood-brain barrier restrict the passage of a large number of pharmacological agents, proteins, and peptides. In the nervous tissue, the use of PEDF-derived peptides that retain their neuroprotective activity may be feasible to protect neurons from damage caused by metastatic processes (164).

Using a somewhat different approach to investigate the effect of recombinant PEDF (rPEDF) on neovascularization and the growth of cervical carcinoma, Yang *et al* 2010 (165) injected the protein intraperitoneally into xenografted mice. The results indicated that PEDF significantly reduced tumor growth, possibly due to its antiangiogenic effect, and microvessel density, due to its pro-apoptotic and antiproliferative activities in endothelial cells. In combination with hyperthermia, Wu *et al* 2014 (166) evaluated the therapeutic effect of an adeno-associated viral vector expressing PEDF (rAVV-PEDF) in solid tumors both *in vitro* and *in vivo*. Thus, in Meth-A cells, it was possible to identify PEDF expression and bioactivity, whereas in a mouse model of subcutaneous fibrosarcoma, therapy suppressed tumor growth and increased survival. This same type of vector and low doses of the chemotherapeutic agent cisplatin were used by He *et al* 2014 (167) in a mouse model of Lewis lung cancer. Therapy was effective in suppressing tumor angiogenesis and inducing the death of malignant cells, which resulted in inhibition of tumor growth and increased survival.

rPEDF has been proposed as a potential therapeutic strategy against OHSS based on the finding that it restored VEGF expression in a mouse model to physiological levels without affecting normal functions such as ovulation, implantation, or pregnancy (141). In addition, the possible exogenous application of rPEDF for the treatment of PCOS is also feasible, given that in theory, any change in the

physiological balance of PEDF-VEGF can be reversed by rPEDF (141).

Regarding the treatment of endometriosis, Sun *et al* 2012 (18) determined that *in vivo* -with prior use of gene therapy- PEDF induces regression and atrophy of human endometriotic cells transplanted in a mouse model by inducing apoptosis and antiangiogenesis. These authors also described similar effects of PEDF on ovarian endometriotic stromal cells *in vitro*. In addition, Chuderland *et al* 2013d (168) were able to eradicate endometrial lesions and cause a significant decrease in VEGF levels using rPEDF.

PEDF has also been used in combination with radiotherapy: In a heterogenic human xenograft model of nasopharyngeal carcinoma (NPC) in nude mice, the effectiveness of PEDF treatment on NPC was evaluated. The results showed that the combination therapy is much more effective than the single administration of PEDF or radiotherapy in reducing VEGF levels and microvessel density (169).

Given that one of the major difficulties for therapies against deep tumors, such as osteosarcoma, is inefficient drug delivery, Dass *et al* 2007 (170) reported that the application of chitosan biopolymer microparticles containing plasmid-expressed PEDF (pPEDF) decreased invasiveness and promoted adhesion in osteosarcoma (SaOS2) cells, whereas in an orthotopic model of the same disease, pPEDF decreased primary tumor growth and also decreased bone resorption and pulmonary metastasis. In an *in situ* assay, Ta *et al* 2009 (171) combined pPEDF treatment with chemotherapy and found greater suppression of tumor growth with no side effects.

## Conclusion

The multimodality of PEDF potential under normal and pathological conditions has made PEDF a strong candidate for a therapeutic agent to successfully replace the combined cancer therapies in use today. However, before proceeding to clinical trials in humans, it is necessary to elucidate completely the clues that determine its activity, i.e. gene expression, post-translational modifications and half-lives of PEDF mRNA and protein as well as the signal transduction associated with each of the activities of the protein in both normal and pathological tissues. It is also desirable to identify functional epitopes that play a key role in tumor suppression and cell protection and possess the ability to recognize receptors on cells and tissues of interest.

Integrating the findings of the genomics, proteomics, transcriptomics, and epigenomics of PEDF will result in the development of therapies against diseases caused by defects in its expression and function.

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