Chapter

Role of the IL-6/Jak/Stat Pathway in Tumor Angiogenesis: Influence of Estrogen Status

José Manuel García-Castellano, David García-Padrón, Nerea Martínez-Aragón, Margarita Ramírez-Sánchez, Vicente Vera-Gutiérrez and Leandro Fernández-Pérez

Abstract

Solid tumors, despite being hypervascularized, are hypoxic. This is due to the imbalance that exists between the inputs of the blood vessels that supply nutrients and O_2 and that remove metabolic waste products, on one side; and the demands of the tumor cells that are part of the neoplasm that is forming, on the other. From this perspective, we briefly review the sequence of morphological events that occur during neo-angiogenesis; what chemical mediators are involved in this process; and we emphasize how the IL-6/Jak/Stat signaling pathway is involved in the control of these mediators. At the same time, we review how estrogens intervene in this control procedure, and how it opens the door to understanding the mechanism of action of these mediators. This would make it possible to propose alternative treatments, which can be added to the conventional ones, and which would exploit the findings described here in the search for new antitumor therapies.

Keywords: hypoxia, solid tumor, HIF, VEG, neovascularization, Jak/Stat, estrogens

1. Introduction

Blood vessels formation is an essential activity for the proper development of the organism. The development of new blood vessels is a well-regulated process, but it is a double-edged sword. Hence, in a physiological situation, such as embryonic development, it leads to the formation of a correct vascular network directed to provide the necessary nutrients and O_2, as well as to waste products removal. However, in a tumoral scenario, is a problem since it uncontrollably feeds the tumor and provides the ways for its spread.

Paradoxically, although solid tumors are invariably hypervascularized, they contain hypoxic regions [1], with low pO_2 levels. This is because the high rate of tumor growth is greater than the rate of new vessel formation [2] and there is no balance between supply and demand. This causes neoplastic cells to be too far from a blood vessel [1], generating a nutrient and O_2 deficient state [3]. The hypoxia generated is also due to a poor O_2

diffusion and to the fact that the cells of the neo-vessels are structurally abnormal [4]. The consequence of this tumor hypoxia leads to therapeutic radio- and chemo-resistance, as well as an increased probability of generating metastatic disease [4, 5]. The cellular change towards a state of tumoral hypoxia provokes an adaptive response that facilitates cell proliferation or angiogenesis, coordinated by the activity of HIF-1α [6]. The adaptive response to hypoxia generated by HIF-1α through angiogenesis and enhanced glucose metabolism confers a survival and growth advantage to hypoxic tumor cells [7].

2. Hypoxic tumor cells generate new capillaries

Neoangiogenesis is the process by which new capillary vessels grow out of pre-existing ones (sprouting angiogenesis). These blood vessels will provide oxygen and nutrients and will remove the metabolic waste [6], which is regulated by a variety of pro- and anti-angiogenic factors [1].

The process of sprouting angiogenesis involves several sequential steps [8] that starts with the activation of endothelial cells due to diverse angiogenic stimulus, like hypoxia or inflammation [8]. The activity of endothelial cells, normally joined by adhesion molecules such as cadherins, is mediated by growth factors released after degranulation of platelet alpha granules [9, 10]. Pericytes, surrounding endothelial cells, inhibit the proliferation of the endothelial cells, also releasing cell survival signals such as VEGF and Angiopoietin-1.

As a consequence of this activation, there is a rupture of the endothelial cells tight junctions; the pericytes detach from the wall and the basement membrane, which, together with the extracellular matrix, will be degraded by activated proteases (metalloproteinases). Loss of junctions between endothelial cells allows them to invade into the surrounding interstitial tissue and, subsequently, proliferate and migrate through the matrix. These endothelial cells afterward become motile tip cells, which are located at the growing ends of the new vessels [11, 12].

Angiogenic factors, such as VEGF, increase the vascular permeability of endothelial cells, causing extravasation of plasmatic proteins and generating an extracellular matrix (ECM). In response to integrin signaling, cells migrate within that ECM, following the tip cells.

Endothelial cells move forward following the angiogenic signal sent by the tip cell that will guide them in the specific direction [12]. Adjacent cells to the tip cell will follow them, dividing to elongate the stalk and establish the lumen. This structure thus formed is an immature vessel [13].

Endothelial cells then rapidly proliferate [8], form tight and adherens junctions with other endothelial cells [11, 12], and finally, the endothelial cell migration and proliferation are inhibited.

The stabilization of the immature vessels is established by the recruitment of pericytes, which will line the capillary walls and stabilize the new vessels [11, 14]. Finally, a new extracellular matrix will be generated [15].

3. Neo-angiogenesis is a well-regulated process

The process of sprouting angiogenesis is tightly controlled by positive and negative regulators whose purpose is to control in a balanced way the structured formation of new vessels through the action of growth factors and cytokines (**Table 1**).

Factor	Function	Cell	References
HIF-1α	Transcription genes: angiogenesis; erythropoiesis; cell proliferation; energy metabolism.	Tumor cell	[16, 17]
VEGF	Stimulation of endothelial cell survival, proliferation, and motility	Tumor cells, macrophages, platelets, endothelial cells	[18]
FGF	Cell differentiation, proliferation, migration, morphogenesis, survival of endothelial cells; extracellular matrix degradation	Macrophages vascular endothelial cells	[19]
PDGF	Potent mitogen	Platelets, smooth muscle cells, activated macrophages, and endothelial cells	[20, 21]
Anp	Angiogenesis Wound healing	Pericytes, vascular endothelial cells macrophages involved in angiogenesis	[22]
HGF	HGF stimulates mitogenesis, cell motility, and matrix invasion	Endothelial cells, smooth muscles cells, bone brown-derived endothelial progenitor cells.	[23–25]
Ang	Inhibit endothelial cell proliferation and migration, tube formation, neutrophil activation and migration, monocyte and macrophage migration, leukocyte recruitment, MMP expression induces endothelial cell apoptosis and the production of anti-angiogenic factors, such as thrombospondin-1	Endothelial cells, tumor cell	[26]
Endostatin	Inhibits the migration of vascular endothelial cells Inhibiting VEGF-induced phosphorylation Apoptosis in proliferating endothelial cells	Endothelial cells	[27, 28]
PF 4	Strong angiogenesis inhibitor	Platelets	[29]
TSP-1	Inhibition of migration, proliferation, and survival of endothelial cells and the formation of capillary tubes	Platelets	[30]

Table 1.
Relationship were the most common growth factors during the neo-angiogenesis process are reported, their function, and the most common cells that produce them.

We will distinguish those factors that will improve the forming action of new vessels (enhancing factors), from those that are designed to modulate and stop the appearance or development of these vessels (inhibitory factors).

3.1 Enhancing factors

3.1.1 Hypoxia-inducible factor

Hypoxia-inducible factor (HIF-1α) is a transcription factor that regulates and coordinates the cellular response to hypoxia [31, 32], by activating genes encoding pro-angiogenic factors, such as VEGF, angiopoietin or PDGF.

When tissue and cellular oxygen levels are in a normal range, HIF-1α is degraded, disrupting the signaling cascade aimed at improving vascularization by means of pro-angiogenic factors [33].

Under low pO_2 status, HIF-1α is involved in hypoxia response by binding to canonical DNA sequences (hypoxia-responsive elements or HREs) in the promoters or enhancers of target genes [34–38]. Also, HIF-1α, through the union of hypoxia-responsive elements or HREs with the promoters of target genes, coordinates a broad response to counteract the effects of hypoxia. Under hypoxic conditions, proteasomal degradation of HIF-1α ends, and it translocates to the nucleus to activate hypoxia-inducible genes [36, 39], such as VEGF, angiopoietin, PlGF, or PDGF [40].

3.1.2 VEGF

VEGF is a glycoprotein that plays an essential role in the development of new vessels. It is produced by tumor cells, macrophages, platelets, and endothelial cells, binding to the VEGF-R1/R2 receptors present on endothelial cells. This growth factor stimulates the endothelial cells survival, proliferation, and motility, initiating the growth of new capillaries by activating the RAS/MEK/ERK pathways or the PI3K/AKT/mTOR pathway. The final effect is the stimulation of endothelial cell survival, proliferation, and motility, initiating the growth of new capillaries.

3.1.3 FGF

FGFs are a family of proteins, mostly with angiogenic effects. The best known are FGF-1 and FGF-2. They are essentially secreted by macrophages and vascular endothelial cells. They are involved in numerous processes, including the induction of endothelial cell differentiation, proliferation, migration, morphogenesis, and survival of endothelial cells; and extracellular matrix degradation by stimulating the secretion of proteases [19, 41]. FGF-1 is necessary for the differentiation and proliferation of all the cell types necessary for creating the vessel wall; while FGF-2 signaling is related to the preservation of vascular endothelial cell junctions and vessel permeability [19].

3.1.4 PDGF

Platelet-derived growth factor is a dimeric glycoprotein synthesized, stored (in the alpha granules of platelets), and released by platelets upon activation, it is also produced by other cells including smooth muscle cells, activated macrophages, and endothelial cells. PDGF is a potent mitogen for cells of mesenchymal origin.

3.1.5 Angiopoietins

Family of proteins involved in vascular repair. Ang-1 and Ang-2 are the best known. Its function is carried out by coupling an angiopoietin to its corresponding receptor (Tie-1 and Tie-2). These receptors are expressed specifically on vascular endothelial cells and on a certain type of macrophages involved in angiogenesis.

3.1.6 Hepatocyte growth factor

HGF is a factor secreted by mesenchymal cells in a paracrine manner that exerts its function through its c-Met receptor. This receptor is expressed in several cell types,

such as endothelial cells, smooth muscles cells, and bone brown-derived endothelial progenitor cells. HGF stimulates mitogenesis, cell motility, and matrix invasion.

3.2 Inhibitory factors

3.2.1 Angiostatin

Angiostatin is a protein produced by autoproteolytic cleavage of certain proteins, like plasminogen. Its function is to inhibit endothelial cell proliferation and migration, tube formation, and tumor cell invasion. In addition, it decreases VEGF expression and induces endothelial cell-mediated apoptosis by thrombospondin-1.

3.2.2 Endostatin

Endostatin is a C-terminal type XVIII collagen fragment, cleaved by the proteolytic activity of MMP-7. It has anti-angiogenic activity by inhibiting FGF-2 and VEGF [1]. It also has an anti-migratory effect by binding to the α5-αv-integrins. It has the ability to directly combine to VEGFR2, inhibiting the VEGF-induced phosphorylation and consequently down-regulating receiver, as well.

3.2.3 Platelet Factor 4

It is a small protein belonging to the CXC chemokine family, usually associated with complexes with proteoglycans and released from alpha-granules of activated platelets during platelets aggregation. It is a potent inhibitor of angiogenesis, especially when acting in conjunction with the receptors of FGF2 and VEGF, leading to downstream effects on endothelial cell migration and proliferation.

3.2.4 Thrombospondin-1

TSP-1 is a glycoprotein that mediates intercellular interactions or with the ECM. This protein can bind to elements of this ECM (to fibrinogen, fibronectin, laminin, collagen types V and VII, and integrins alpha -V/beta-1), and exerts an inhibitory effect on the migration, proliferation, and survival of endothelial cells and the formation of capillary tubes.

4. Role of the IL-6/Jak/Stat pathway on the neoangiogenesis process

After a tissue injury, a cascade of events is set in motion aimed at repairing the damage. The products generated by tissue destruction stimulate the cells of the immune system. In response to this damaging process, immune cells in the tumor environment secrete multiple cytokines, such as histamine, serotonin, prostaglandins, leukotrienes; and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and various chemokines. Many of them belong to the IL-6 family [42]. These substances help to repair healthy tissues but nevertheless have deleterious effects on tumors.

4.1 IL-6/Jak/Stat pathway

Janus kinase (Jak), the signal activation transducer (Stat) pathway, is recognized as an evolutionarily conserved signaling pathway (**Figure 1**). After binding the

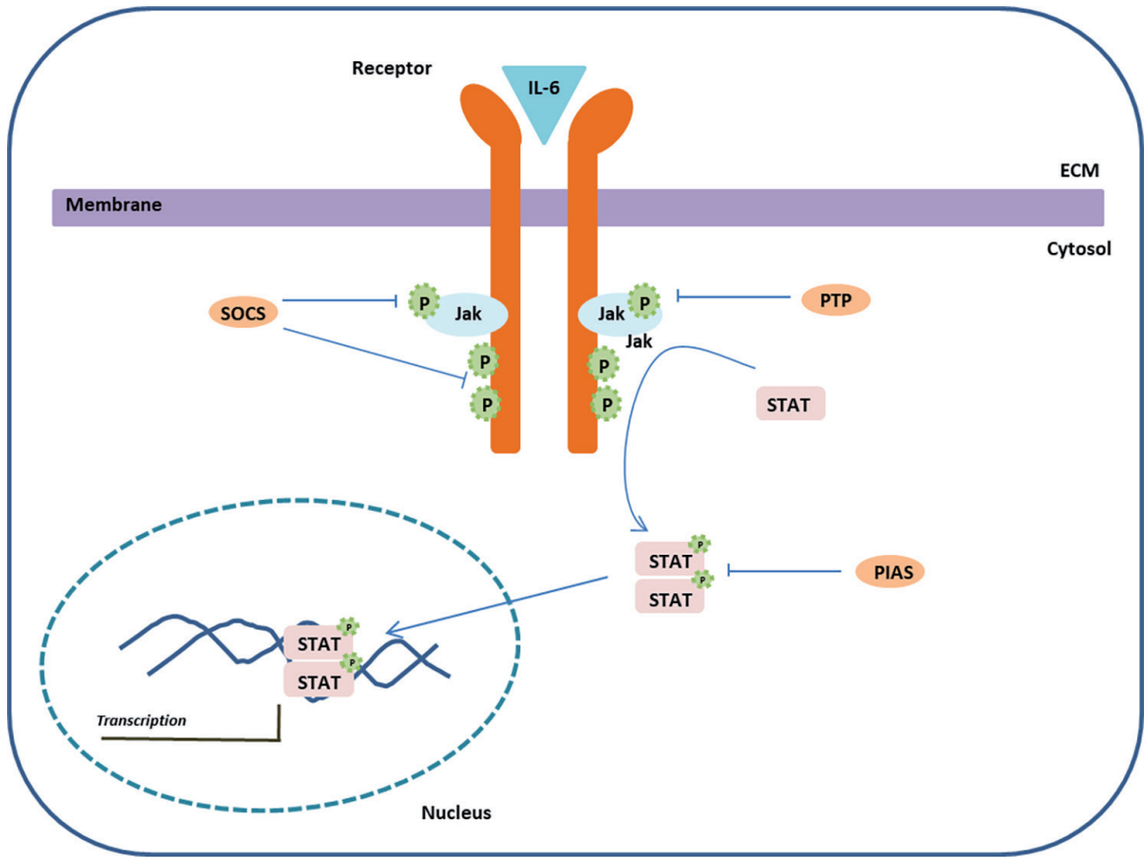

Figure 1.
IL-6/Jak/Stat pathway. After binding the cytokine to the receptor, Jak is activated by the specific tyrosine residues phosphorylation. Phosphorylated Jak, in turn, induces the phosphorylation of Stat, which, after dimerization, translocate into the nucleus where it regulates the transcription of numerous genes.

cytokine to the receptor, Jak is activated by the specific tyrosine residues phosphorylation. Phosphorylated Jak in turn induces the phosphorylation of Stat, which, after dimerization, translocate into the nucleus where it regulates the transcription of numerous genes [43].

The IL-6/Jak/Stat pathway is overexpressed in various tumors, causing continuous transcription of cell growth factors that promote tumor progression. However, this pathway not only regulates aspects such as tumor proliferation, survival, and invasion, but also contributes significantly to tumor neo-angiogenesis [44, 45], enhancing endothelial cells survival, infiltration of the ECM by immune cells followed by activation of mesenchymal cells, and finally the generation of metastases [46].

Jak/Stat is activated upon stimulation by IL-6, among several effectors, promoting endothelial cell migration and tumor angiogenesis. This function is suppressed when Jak inhibitors are administered, ending the observed endothelial cell migration *in vitro* [47].

Regulation of tumor angiogenesis is dependent on VEGF and HIF-1α transcription by endothelial cells [48–50]. This action is induced by tumor IL-6 and mediated by Stat3 [51]. These results are validated by the fact that the aberrant expression of Stat3 causes an increase in the expression level of HIF- 1α and VEGF, as well as of the metalloproteinases MMP-9 and MMP-7, enhancing tumor progression and aggressiveness [52]. This pathway is reciprocally enhanced by the action of IL-6 secreted by endothelial cells on tumor cells [53]. This boost signal is also produced by other pathways, such as that promoted by EGFR, HER2, Ras, and Rho, which lead to Stat3 activation [46].

On the other hand, IL-6-induced activation of Stat3 in tumor and stromal cells protects neoplastic cells from the immune surveillance system. This pathway promotes immune evasion [54], by modulating the secretion of various inflammatory factors such as IL-6 and TNF-α [55] and reducing natural killer cell activity [56, 57]. This favors tumor expansion by avoiding immunological control.

Furthermore, the metastatic process is regulated by Stat3, by controlling the capacity for cell migration and invasion of tissues. On one side, Stat3 acts directly on the promoter of MMP genes [58, 59], increasing their expression and thus the ability of cancer cells to degrade the basement membrane/extracellular matrix. Tumor cells then invade the surrounding ECM by migrating due to the action of RhoA on the cytoskeleton [60] after activation of the Stat3/ROCK-myosin pathway. The cells then spread through the circulatory or lymphatic system, forming metastatic foci in lymph nodes and distant organs.

5. Effect of the IL-6/Jak/Stat pathway on neo-angiogenesis mediators

Several authors show that the Jak/Stat pathway plays an important role in neo-angiogenesis through these growth factors.

5.1 HIF-1α

In a hypoxic environment, the HIF-1α protein is stabilized and its proteasomal degradation rate is reduced by slowing down the protein ubiquitination of HIF-1α and thereby achieving enhanced HIF-1α protein levels [61]. This increases its half-life and the cellular concentration of HIF-1α. The IL-6/Jak/Stat3 pathway mediates in the regulation of this process; in such a way that Stat3 interacts with HIF-1α and with VEGF in order to generate greater tumor vascularization (**Figure 2**).

Similar results are obtained after sustained administration of the constitutively active form of Stat3, which causes an increase in HIF-1α transcription, with the consequent increase in HIF-1α protein levels. Changes in HIF-1α levels are also due to the interaction between this molecule and PIAS [62], a negative regulator of the Jak/Stat pathway. Hypoxia causes the interaction between molecules, promoting the stabilization of HIF-1α and prolonging its half-life.

5.2 VEGF

It is well-known that HIF-1α stimulates vascularization and metastasis upon activation of VEGF expression [63]. But there are evidences that show that Stat3 plays a central role in this response. Thus, Xu et al. [64] shows that in various types of human cancer cell lines Stat3 activation induces HIF-1α and up-regulates VEGF expression, promoting tumor angiogenesis [64]. The inhibition of Stat-1/Stat-3 phosphorylation was accompanied by a decrease in VEGF transcription and secretion due to the direct transcriptional action of the VEGF gene by Stat3 (**Figure 2**). On the other hand, Stat3 cooperates with HIF-1α, binding both simultaneously to the promoter region of the VEGF gene, leading to its maximum transcriptional activation and angiogenesis [65].

This action of Stat3 on the VEGF pathway also affects its VEGF receptor. Thus, it has been seen that indirubin suppressed severely the VEGFR-mediated Jak/Stat3 signaling pathway in prostate tumor cells, affecting angiogenesis and tumor growth [66].

Similarly, in pancreatic cancer cell lines, suppression of VEGFR-2 phosphorylation and Stat3-dependent expression of HIF-1α reduced the expression of the Rho-GTPases RhoC, which is downstream of VEGF signaling. This effect plays a vital role in tumor angiogenesis and metastasis [67] because RhoC plays an essential role in transmitting the VEGF signals downstream to angiogenesis and invasiveness [51].

In addition, inhibition of Stat-1/Stat-3 down-regulates other pro-angiogenic factors, such as eNOS, iNOS, MMP-2, and FGF-2 in HUVEC, associated with reduced capillary sprouting and tumor angiogenesis [68, 69]. These molecular findings, taken to clinical practice, translate into a reduction in cell viability, proliferation, adhesion, migration, and tube formation.

Lymphangiogenesis is carried out in a similar way, observing activation of the IL-6-Jak-Stat3-VEGF-C signaling pathway in the growth and invasion process [70, 71].

5.3 PDGF

On the other hand, other aspects must be taken into account. Thus, in the angiogenesis process, it is necessary to increase the cell population, either proliferating new cells or the chemo-attraction of others (**Figure 2**). To do this, PDGF, a growth factor that stands out for being a potent mitogen and chemoattractant for VSMC, stimulates the phosphorylation of Jak-2 and Stat3 in VSMCs [10, 72, 73] and contributes to PDGF-BB-induced mitogenesis [73] and VSMC motility [72]. In addition, PDGF helps regulate the IL-6/Jak-2/Stat pathway through phosphorylation of SOCS, a natural regulator of Jak, by platelet-derived growth factor receptor tyrosine kinase [64].

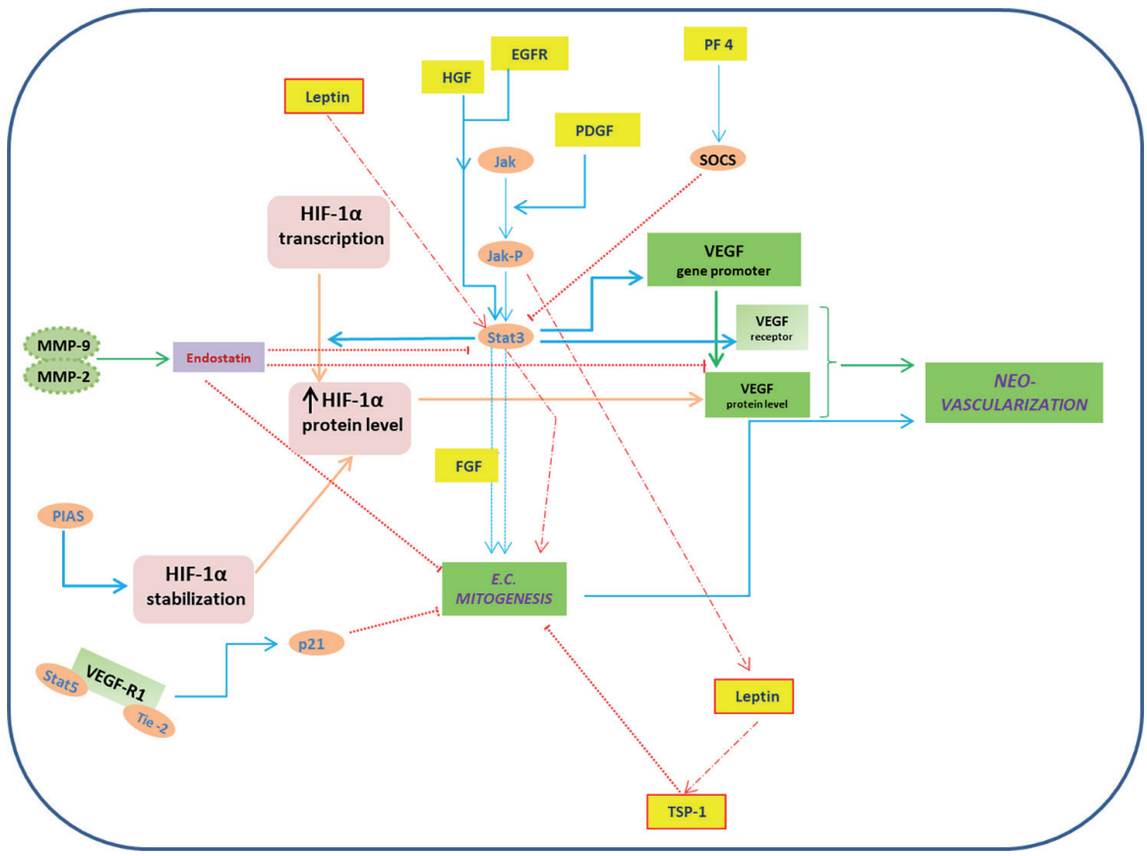

Figure 2.
Diagram that summarizes the neo-angiotizing action of certain cytokines and growth factors that influence neo-angiogenesis and how the mediators of the Jak/Stat pathway act on them.

5.4 FGF

New vessel formation is also regulated by growth factors such as FGF, another downstream effector to IL-6 that induces angiogenic activity in basal cell carcinoma cell lines [74], dependent on the activation of Jak/Stat3. Thus, IL-6 overexpression increases FGF-2 levels (**Figure 2**), tube formation by HUVEC cells, and consequently neoangiogenesis [74].

5.5 Angiopoietins

These molecules are also involved in relevant functions during neo-angiogenesis, such as vascular repair after binding with the endothelial cell-surface receptor tyrosine kinase, Tie2. It also highlights the regulatory activity of Stat on the cell survival, migration, and proliferation [75, 76] by Ang1/Ang2-Tie2 receptor activated. Thus, after Stat5, VEGFR-1 the Tie-2 receptor co-expressed, an increased expression of the cell cycle inhibitor p21 is induced [76], which will arrest cell proliferation (**Figure 2**). On the other hand, angiopoietin-like 4 stimulates Stat 3-mediated iNOS expression and enhances angiogenesis [77].

5.6 HGF

The IL-6/Jak/Stat signaling pathway is regulated by HGF, mediated by SOCS1 [78]. In the case of SOCS3 [79], it counteracted Stat3-dependent keratinocyte migration after being stimulated by HGF (**Figure 2**). In the case of EGFR, SOCS3 is involved in the regulation of IL-6/Jak/Stat signaling, attenuating the EGF signal [78, 80].

5.7 Endostatin

Endostatin activation in the extra cellular environment is enhanced by means of MMP-2/MMP-9 activation, which is accompanied by decreased tumor vascularization [81]. The administration of IL-35 to fibroblast-like synoviocytes produces an inhibition of vascularization due to an increase in the expression of endostatin and a decrease in the expression of VEGF, FGF-2, TNF-α, and IL-6, by means of Stat1 [82]. Synergism between endostatin and Stat3 suppression by a Stat3-siRNA has been observed. In the hepatocarcinoma model, each of both treatments had a potent antitumor effect; but, the combination had a superior effect. It was observed a decreased VEGF expression, decreased cell proliferation, induced cell apoptosis, and inhibited angiogenesis [83] (**Figure 2**).

5.8 PF4

PF4 may contribute to suppress tumor growth in the melanoma murine model, decreasing IL-17, IL-6, and p-Stat3 pathway (**Figure 2**) via up-regulation of SOCS3 expression [84].

5.9 Leptin

Leptin secreted by adipose tissue has a well-known paracrine effect on endothelial, stromal, and tumor cells, enhancing the aggressive tumor behavior. On

adipose-derived stromal cells, VEGFA, MMP-2, MMP-9, IGF-1, and b-FGF genes expression are up-regulated and angiogenesis is stimulated by the Jak/Stat3 pathway [85]. In addition, leptin increases the migration and proliferation of VSMC [86, 87], by inducing the phosphorylation of the tyrosine residue of Jak and the activation of its effectors Stat3 and MAPK [88, 89] (**Figure 2**). Jak, on the other hand, produces leptin-dependent up-regulation of TSP-1 [90].

6. Effect of the estrogens on neoangiogenesis mediators

Sex steroids cooperate with the pro- and anti-angiogenic factors involved in the tumor neo-vascularization process. The connection between the inflammatory pathway represented by the IL-6/Jak-Stat pathway, and the tumor estrogenic pathway is very close and is involved in the pathogenic processes of these diseases [43].

Recently, evidence has emerged showing that cytokines generated during the inflammatory process interact with estrogen signaling pathways [43]. On one side, there is a very close relationship between ER α protein levels and Stat 1 activity (**Figure 3**). Thus, if Stat1 levels are insufficient or its function is blocked, a decrease in ERα levels and cell proliferation is observed. This occurs through the direct action of Stat1 on the promoter region of ERα, regulating the transcription of mRNA levels [91].

On the other hand, estrogenic activity has been found in adipose tissue and tumoral stroma. Thus, immunohistochemical studies have found the expression of cytochrome P450 aromatase, responsible for the aromatization of adrenal and testicular androgens into estrogens (**Figure 3**). It is also known that the IL-6/Jak/Stat pathway stimulates the cytochrome P450 aromatase expression, transforming tissue androgens into estrogens that will act in a paracrine manner on the tumor, causing tumor growth and development [92].

This connection between IL-6/Jak/Stat and estrogens is regulated, in such a way that there is negative regulation of Jak2 with respect to ERα [43] because Jak2 induces

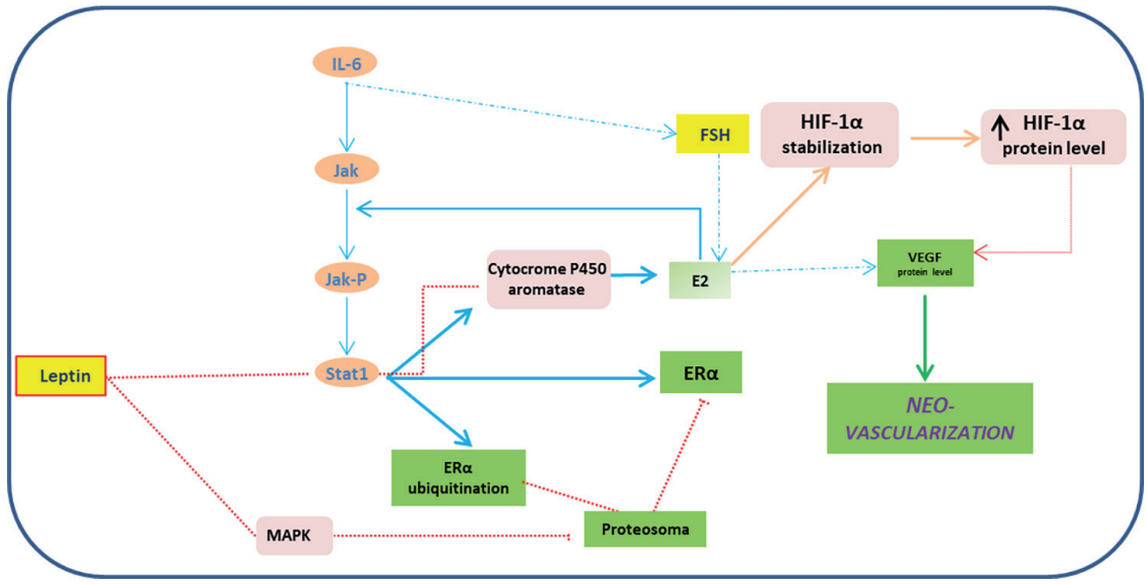

Figure 3.
Diagram summarizing the action of how estrogens and mediators of the Jak/Stat pathway interact during the process of neo-angiogenesis.

the ubiquitination of ERα for being degraded in the proteasome (**Figure 3**). On the other hand, sustained treatment with E2 induces Jak-2 expression, thus controlling the formation and destruction of these molecules.

These observations are integrated by the adipose tissue cytokine leptin function. It activates the phosphorylation of the tyrosine residue of the receptor and causes the activation of its effectors Stat3 and MAPK (**Figure 3**). In this way, the estrogenic pathway is enhanced at the tissue level, since Stat3 induces the generation of estrogens by aromatization of androgens, and MAPK stops the proteasomal degradation of ERα [93], enhancing the estrogenic status [43].

Estrogens, in addition to synergizing with the IL-6/Jak/Stat pathway, regulate the action of mediators involved in the neo-angiogenesis process (**Figure 3**). Thus, regarding HIF-1α, estrogens stabilize the protein in normoxia by regulating its expression through the Akt pathway [63, 94].

In addition, IL-6 induces the expression of VEGF in granulosa cells through FSH mediation, favoring the expression of HIF-1α and COX2, thanks to the activation of the Jak/Stat3 pathway (**Figure 3**). Other evidence indicates that ovarian steroids increase the production of HGF by peritoneal macrophages, promoting the proliferation of endothelial cells and the organization of capillaries.

The angiopoietins, promote the formation of endothelial cells through the mediation of estrogens. Thus, the up-regulation of brain Ang-1 mRNA caused an increase in the capillary density. Besides, E2 acting through ERβ up-regulates Ang-2, increased Tie-2 phosphorylation, and promoted angiogenesis [95].

In ER-positive breast cancer tumor cells, estrogens control the production of TSP-1, which is under the direct control of estrogens, performing regulatory functions favorable to tumor growth [96].

It is also the case that a growth factor is influenced by both pathways. In the case of FGF, while estrogens potentiate its release, it signaling pathway was mediated by activated Stat1 [97].

All these coordinated measures between both systems are aimed at enhancing vascular neo-formation and thus potential metastatic dissemination.

Conflict of interest

"The authors declare no conflict of interest."

Authorship

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- Participate in drafting or revising the work: DGP, NMA, MRS, VVG
- Approve the final version of the work to be published: JMGC, LFP, DGP, NMA, MRS, VVG

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