Role of pleiotrophin and its receptor PTPRZ1 in hypoxia-induced endothelial cell migration

Master Diploma

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INTRODUCTION
1. Angiogenesis

1.1 Introduction

Blood vessels play a crucial role in developmental and adult physiology by transporting oxygen, nutrients, cells, and signaling molecules. Insufficient blood supply prevents tissues and organs from sustaining normal activities. The formation of new blood vessels is vital for embryonic growth and remains crucial for physiological repair processes throughout life. Vascularization is a meticulously orchestrated, multi-step process that involves various cell types and associated signaling molecules. Three distinct processes contribute to neovascularization: angiogenesis, vasculogenesis, and arteriogenesis.

Vasculogenesis is mostly noticed during early development and refers to the beginning phases of vascular expansion. During vasculogenesis, precursor cells of endothelial cells migrate to specific sites, undergo differentiation in their current position, and assemble into solid cords of endothelial cells, thus generating the primitive circulatory system (Conway et al., 2001). During angiogenesis, these initial vessels proceed, grow, expand, and remodel into a fully established vascular network (Carmeliet & Jain, 2000a). Arteriogenesis refers to the formation of new blood vessels from existing collateral arteries. This process is triggered by mechanical stresses caused by misdirected blood flow due to blockage or narrowing in bigger arteries. Under stimulation, the pre-existing arteries can rapidly expand and bypass the occluded region to facilitate perfusion upon stimulation. In contrast to vasculogenesis, which predominantly occurs during embryonic stages, both angiogenesis and arteriogenesis are frequent processes in adulthood. Angiogenesis is significant for reproductive functions and wound healing in adults (Saman et al., 2020). Inadequate vascularization can result in ischemic conditions, hindering tissue growth and survival (Folkman 1995; Carmeliet & Jain, 2000a). Hence, induction of angiogenesis proposes a prospective approach for managing cardiovascular disorders. Conversely, excessive angiogenesis is incriminated for various disorders, both malignant and non-malignant including, among others, and the pathogenesis of cancer. Hence, the inhibition of angiogenesis emerges as a promising strategy for treating diseases like cancer, whereas induction of angiogenesis may be effective in treating ischemic conditions such as myocardial infarction (Folkman 1995).
1.2 Shifting from cancer to angiogenesis

Angiogenesis is carefully regulated to match changes in tissue size and/or metabolic needs, guaranteeing an adequate oxygen supply. Some factors that stimulate angiogenesis include hypoxia, inflammation, and mechanical forces such as stretch and shear stress (Carmeliet & Jain, 2000a). The endothelial cells (ECs) are stimulated in response to these stimuli by inducing the production of growth factors or cytokines through autocrine or paracrine signaling, resulting in their release (Milkiewicz et al., 2006).

Cancer cells possess unique attributes, such as self-reliance in growth signals, which normal cells do not have. This characteristic results from the activation of specific oncogenes and the loss of particular suppressor genes. It includes the ability of cells to replicate without limit, insensitivity to signals that inhibit growth, unresponsiveness to signals that induce cell death, and the capability to invade nearby tissues and establish colonies in distant areas (Hanahan and Weinberg, 2000). These neoplastic qualities may be crucial, but alone, they might not be adequate for a cancer cell to multiply into a population of tumor cells that is observable in clinic settings, metastatic, and fatal. To achieve this, the cancer cell must initially draw and maintain its blood supply, a process known as tumor angiogenesis (Folkman 1990; Hanahan and Weinberg, 2000). Tumor growth beyond 1-2 mm in diameter relies on angiogenesis. Due to limits in oxygen diffusion, non-angiogenic tumors significantly depend on their surroundings for oxygen and nutrition supply, leading to a size limitation of fewer than 1 mm in diameter (Torres et al., 1994). The change from a non-angiogenic to an angiogenic tumor termed the "angiogenic switch," is pivotal in cancer growth (Folkman et al., 1986). As the angiogenic switch is active, pro-angiogenic proteins surpass the local concentration of anti-angiogenic proteins leading to endothelial and other cell activation. Therefore, angiogenesis is facilitated, and the tumor is allowed to expand. Well-recognized inducers and inhibitors of the angiogenesis process are given in the tables below.
Table I1: Representative inducers of the angiogenic process (Liekens et al., 2001).

<table>
<thead>
<tr>
<th>Inducer</th>
<th>Mechanism of function on ECs</th>
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<tbody>
<tr>
<td></td>
<td>Proliferation</td>
</tr>
<tr>
<td><strong>Heparin-binding growth factors</strong></td>
<td></td>
</tr>
<tr>
<td>VEGF</td>
<td>+</td>
</tr>
<tr>
<td>PIGF</td>
<td>+</td>
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<tr>
<td>FGF-1, FGF-2</td>
<td>+</td>
</tr>
<tr>
<td>PTN</td>
<td>+</td>
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<tr>
<td>PDGF</td>
<td>+</td>
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<tr>
<td><strong>Non-heparin-binding growth factors</strong></td>
<td></td>
</tr>
<tr>
<td>EGF</td>
<td>+</td>
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<tr>
<td>IGF-1</td>
<td>+</td>
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<tr>
<td>TGF-α</td>
<td>+</td>
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<tr>
<td><strong>Inflammation factors</strong></td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>Inhibition</td>
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<tr>
<td>IL-3</td>
<td>+</td>
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<td>IL-8</td>
<td>+</td>
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<tr>
<td><strong>Enzymes</strong></td>
<td></td>
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<tr>
<td>COX-2</td>
<td>-</td>
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<tr>
<td>Angiogenin</td>
<td>-</td>
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<tr>
<td>MMPs</td>
<td>+</td>
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<tr>
<td><strong>Hormones</strong></td>
<td></td>
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<tr>
<td>Estrogens</td>
<td>+</td>
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<tr>
<td><strong>Oligosaccharides</strong></td>
<td></td>
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<tr>
<td>Hyaluronic acid fragments</td>
<td>+</td>
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<tr>
<td><strong>Hematopoietic growth factors</strong></td>
<td></td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>+</td>
</tr>
<tr>
<td>G-SCF</td>
<td>+</td>
</tr>
<tr>
<td>GM-SCF</td>
<td>+</td>
</tr>
<tr>
<td><strong>Cell adhesion molecules</strong></td>
<td></td>
</tr>
<tr>
<td>VCAM-1</td>
<td>-</td>
</tr>
<tr>
<td>E-selectin</td>
<td>-</td>
</tr>
<tr>
<td>Integrins</td>
<td>+</td>
</tr>
<tr>
<td>VE-cadherin</td>
<td>-</td>
</tr>
<tr>
<td><strong>Other molecules</strong></td>
<td></td>
</tr>
<tr>
<td>NO</td>
<td>+</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>-</td>
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</tbody>
</table>
Table 12: Representative inhibitors of the angiogenic process (Liekens et al., 2001).

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Mechanism of function on ECs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein fragments</strong></td>
<td></td>
</tr>
<tr>
<td>Angiostatin (plasminogen)</td>
<td>↓ proliferation of ECs and ↑ apoptosis of ECs</td>
</tr>
<tr>
<td>Endostatin (collagen XVIII)</td>
<td>↓ proliferation of ECs and ↑ apoptosis of ECs</td>
</tr>
<tr>
<td>Prolactin</td>
<td>↓ proliferation of ECs</td>
</tr>
<tr>
<td><strong>Soluble regulators</strong></td>
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<tr>
<td>TSP-1</td>
<td>↓ proliferation of ECs and ↑ apoptosis of ECs</td>
</tr>
<tr>
<td>Troponin I</td>
<td>↓ proliferation of ECs</td>
</tr>
<tr>
<td>IFN-α</td>
<td>↓ proliferation of ECs, ↑ apoptosis of ECs, and ↓ FGF-2-induced angiogenesis</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>↓ proliferation of ECs</td>
</tr>
<tr>
<td>IL-12</td>
<td>↑ INF-γ</td>
</tr>
<tr>
<td>IL-4</td>
<td>↓ migration of ECs</td>
</tr>
<tr>
<td>IP-10</td>
<td>↓ proliferation of ECs and ↓ FGF-2 and IL-8 induced cell migration</td>
</tr>
<tr>
<td>PEDF</td>
<td>↓ migration of ECs and ↓ FGF-2-induced angiogenesis</td>
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<tr>
<td>TIMP-1, TIMP-2</td>
<td>↓ MMPs activity</td>
</tr>
<tr>
<td>PAI-1</td>
<td>↓ uPA activity</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>↓ migration of ECs</td>
</tr>
<tr>
<td>Ang-2</td>
<td>↓ vessel maturation</td>
</tr>
<tr>
<td>2-methoxyestradiole</td>
<td>↓ proliferation of ECs, ↑ migration of ECs, and ↑ apoptosis of ECs</td>
</tr>
<tr>
<td><strong>Tumor suppressor genes</strong></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>↑ TSP-1 production, ↓ VEGF production</td>
</tr>
<tr>
<td>VHL</td>
<td>↓ VEGF production</td>
</tr>
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1.3 The mechanism of angiogenesis in tumor development

Tumor angiogenesis is now thought to arise from six different primary biological pathways. These mechanisms include (1) classical sprouting angiogenesis; (2) vascular co-option, in which cancer cells grow around and take advantage of pre-existing vasculature; (3) vessel intussusception, in which pre-existing vessels are split to produce daughter vessels; (4) vasculogenic mimicry, in which cancer cells integrate into the blood vessel wall; and (5) bone marrow-derived vasculogenesis, in which endothelial progenitor cells are recruited from the bone marrow or live in vascular walls. Furthermore, (6) cancer stem-like cells contribute to vasculogenesis, in which cancer cells differentiate into endothelial cells (ECs).
and take part in the formation of mosaic vessels (Jain and Carmeliet, 2012). Sprouting angiogenesis is the mechanism that has been explored the most among these.

A monolayer of non-proliferating endothelial cells held together by junctional molecules such as VE-cadherin and claudins makes up the stable structure of blood arteries. This monolayer is covered by supporting mural tissue, such as adventitia, pericytes, and the basement membrane. A dormant vessel that senses an angiogenic signal—such as VEGF or FGF—released by a hypoxia or tumor cell causes the mural tissue to rupture, and this is the first step in the angiogenesis process. Pericytes release themselves from the basement membrane during angiogenesis by breaking loose from the vessel walls via proteolytic degradation. Endothelial cells (ECs) unhitch their connections concurrently. The endothelial cell layer's permeability rises because of VEGF-induced vessel dilatation. Plasma proteins extravasate to the surrounding tissues, which results in the creation of a transient matrix scaffold. ECs move onto this scaffold in response to integrin signaling. Several growth factors that support the angiogenic process are released because of the extracellular matrix's (ECM) breakdown. These factors provide a particular milieu that facilitates the invasion of endothelial cells. In this stage, a particular endothelial cell (tip cell) is selected, and nearby cells divide to elongate (stalk cell). The process of lumen creation is simultaneous. The new sprout bridges with another vascular branch and blood flow initiates, reestablishing the barrier. Basement membrane deposition and pericyte recruitment stabilize the newly created vessel (Carmeliet and Jain, 2011).
1.3.1 Tip/stalk model and activation of endothelial cells

The initial step in endothelial activation involves picking certain ECs inside the capillary to commence the process of angiogenic growth. As noted, the vascular sprout is led by a tip cell that goes towards an enhanced VEGF-A gradient formed by tumor cells (Carmeliet and Jain, 2011). The tip cell undergoes a metamorphosis into an invasive phenotype and travels toward the angiogenic stimulation by removing connections between cells and the ECM. Another population of ECs, referred to as stalk cells, proliferates and supports the sprout elongation (Gerhardt et al., 2005).

Notch and VEGF signaling act as the key regulators of endothelial cell fate, determining the different behaviors between tip and stalk cells. Vascular endothelial cells express Notch1, Notch4, Jagged1, Jagged2, (Delta-like 1) Dll1, and (Delta-like 4) Dll4 (Tung et al., 2012). Among these, Dll4 appears as the main Notch ligand in inducing angiogenesis.
Sprouting initiates when VEGF-A, produced by tumor cells, interacts with VEGF receptor-2 (VEGFR-2) on the endothelial cell membrane. This interaction leads to the overexpression of dll4 and consequently induces Dll4 expression. EC expressing both VEGFR-2 and Dll4 converts into a non-proliferative tip cell, defined by the breakdown of ECM. The tip cell then travels towards angiogenic factors. Dll4 on the membrane of the tip cell interacts with Notch1 receptors on surrounding endothelial cells, creating a "stalk" cell proliferative phenotype. This proliferation leads to the elongation and development of the lumen of the newly formed vessel (Jakobsson et al., 2010).

The angiogenic process can also be controlled by factors (Table I1) that mediate the expression of either VEGF and/or its receptors. Some of them that it is worth to be mentioned are Basic Fibroblast Growth Factor 2 (FGF-2) (Seghezzi et al., 1998), pleiotrophin (PTN) (Kong et al., 2012), Tumor Necrosis Factor α (TNF-α) (Ryuto et al., 1996), Platelet-derived growth factor (PDGF) (Finkenzeller et al., 1997) and interleukins (ILs) IL-1β and –7 (Cohen et al., 1996).

The relationship between integrins and VEGFR-2 is important during vascularization (Somanath et al., 2009). Integrin αvβ3 demonstrates heightened expression in ECs during tumor angiogenesis, actively working with VEGFR-2 to govern endothelial cell migration, survival, and tube formation (Soldi et al., 1999). In vivo, investigations confirm that the VEGFR-2-αvβ3 connection is necessary for tumor-induced angiogenesis (Mahabaleshwar et al., 2007).

ECM is a vital element in angiogenesis whose modification influences the bioavailability and bioactivity of growth factors, with VEGF being one of the factors affected (Lee et al., 2005). The proteolytic cleavage of PTN-VEGF inhibitory complexes caused by Matrix Metalloproteinase-2 (MMP-2) could play a regulatory role in the angiogenic process (Dean et al., 2007). mRNA splicing results in different isoforms of VEGF, identified by differences in the C-terminal region, particularly the main basic Heparin-binding Domain (HBD). This HBD is responsible for promoting the interaction between VEGF and components such as the ECM, cell surface heparan sulfate proteoglycans (HSPGs), and neuropilin-1 (Koch et al., 2011). Regulated release of VEGF by heparan sulfate (HS) enhances the development of VEGF gradients (Fleury et al., 2006). Consequently, the tip cells of sprouting blood vessels respond and therefore migrate to these gradients, which are controlled by interactions with HS proteoglycans (Ruhrberg et al., 2002). Evidence suggests that the supply of VEGF to VEGFR-2 via HS proteoglycans produced on nearby cells, such as
pericytes, leads to delayed and increased signal transduction, possibly by preventing receptor internalization (Jakobsson et al., 2006).

1.3.2 Degradation of the extracellular matrix (ECM)

The breakdown and modification of the ECM play a major regulatory role during tumor angiogenesis. This procedure is crucial to enable activated endothelial cells (ECs) to migrate and facilitate the invasion of cancer cells into the adjacent tissues (Kessenbrock et al., 2010). The ECM consists of structurally and biochemically different components, containing proteins, proteoglycans, and glycoproteins. Elastin and fibrillar collagens have a function in imparting viscoelasticity to the tissue, while other proteins including fibronectin, laminin, and nidogen serve as connectors’ proteins, contributing to the development of the matrix network (Daley et al., 2008). The remodeling of the ECM through the action of proteases plays a vital role in promoting cell migration, a crucial stage in the formation of new vasculature (Kessenbrock et al., 2010). Proteases contribute to the release of matrix-bound growth factors, either directly or through the action of angiogenic factors, thereby boosting angiogenesis through the augmentation of endothelial migration and growth. The key enzymes involved in ECM remodeling are metalloproteinases (MMPs). Among the metalloproteinases, the family of membrane-type MMPs (MT-MMPs) and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS), specialize in the destruction of the ECM. Serine proteinases, such as the plasminogen activator (PA)-plasmin system and cathepsin G, possess the ability to degrade proteins-components of ECM (Carmeliet, 2004). Additionally, cysteine proteases, notably cathepsins B and L, when released, can contribute to ECM breakdown (Green and Lund, 2005).

Matrix metalloproteinases (MMPs)

Matrix metalloproteinases (MMPs) represent a major class of calcium-dependent zinc-containing endopeptidases. Their activity is regulated by hormones, growth factors, and cytokines and they contribute to ovarian functions. MMPs are secreted by numerous connective tissue and pro-inflammatory cells. Initially, these enzymes are expressed in an inactive form known as zymogens, and their activation necessitates subsequent processing by other proteolytic enzymes, particularly serine proteases, to generate the active forms.
Endogenous tissue inhibitors of metalloproteinases (TIMPs) neutralize active MMPs (Page-McCaw et al, 2007). The latter have extensive substrate selectivity, targeting diverse ECM and other proteins. For instance, MMP-1 has a selectivity for collagen III, whereas MMP-3 and -10 focus on proteoglycans, fibronectin, and laminin. MMP-8 and -13 primarily target collagen I and II, respectively. MMP-2 and -9 dissolve denatured collagen, commonly known as gelatin (Cawston and Young 2010). MMP-3 and -10 can cleave precursors of MMP-1, -8, and -13, and by that activating them. Moreover, the membrane-type MMP family, and more specifically MT-MMP-14, -16, -24, and -25 is implicated in the activation of pro-MMP-2, and MMP-14 specifically activates pro-MMP-13 (Page-McCaw et al, 2007). On the other hand, ADAMTS enzymes are principally responsible for the proteolysis of proteoglycans. ADAMTS-1, -4, -5, -8, -9, -15, -16, and -18 are termed proteoglycanases due to their ability to breakdown aggrecan, brevican, and versican (Apte et al., 2009).

Among the members of the family described above it is worth to notice MMP-2, -9, -14, and MT1-MMP that cause tumor angiogenesis. The expression of the MT1-MMP is up-regulated in endothelial tip cells, as proteomic studies have shown. ADAMTS-1, -9, and -16 are also up-regulated, suggesting their probable participation in ECM degradation and stimulation of sprout formation (del Toro et al., 2010). As mentioned, in addition to its involvement in regulating tissue architecture through impacts on the ECM, MMP proteolysis plays a critical role in stimulating angiogenesis. More specifically, this is achieved by several mechanisms including: (1) aiding in the detachment of pericytes from vessels, (2) cracking endothelial cell-cell bindings’, (3) generating ECM component fragments that exhibit pro-migratory action, (4) exposing pro-angiogenic integrin binding sites in the ECM and (5) releasing ECM-bound angiogenic growth factors such as VEGF and FGF-2 (van Hinsbergh and Koolwijk, 2008). Conversely, MMPs can also provide signals that restrict angiogenesis. For example, the breakdown of collagen IVa3 by MMP-9 leads to the generation of tumstatin, which is acknowledged as a strong inhibitor of the angiogenesis process (Hamano et al., 2003). Moreover, MMP-3, -7, -9, -13, and -20 are implicated in the metabolism of several ECM components creating endostatin, which is known to inhibit angiogenesis (Enge et al., 2002; Heljasvaara et al., 2005). These data suggest that the impact of MMPs on angiogenesis might differ, depending on the time and the availability of substrates.
Plasminogen activator (PA)-plasmin system

Plasmin is a serine protease with a broad spectrum, capable of directly or indirectly degrading various extracellular proteins, notably fibrin (Mekkawy et al., 2014). In contrast, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) are serine proteases predominantly targeting plasminogen.

uPA plays a predominant function in tumor cell biology. It is a secreted protein with high-affinity binding to membrane-bound urokinase plasminogen activator receptor (uPAR) (Vassalli et al., 1985). tPA found in the blood arteries is implicated in the plasmin-dependent proteolysis of blood fibrin clots. Plasminogen activator inhibitor-1 and uPAR participated in a series of interactions with integrins and vitronectin. These interactions resulted in changes in the properties of endothelial cells related to proliferation, adhesion, and migration. While wound healing via angiogenesis and development looks unaffected in animals lacking uPA, uPAR, PAI-1, and plasminogen, various in vivo investigations have revealed that those biological actors are necessary for tumor angiogenesis (Soff et al., 1995).

1.3.3 Formation of the lumen and vessel development

For a blood vessel to become functional, it is required to be stabilized. The evolution of vessel maturation entails a sequential conversion from a growing vascular bed to a stable and functional network (Carmeliet and Jain 2011). Maturation initiates with the merging of freshly created capillaries. The tip cell fuses with its target to generate a new branch, by a mechanism that does not comprehend. In the forming mouse retina, filopodia from "tip" cells contact those of a nearby "tip" cell, creating a "bridge" that becomes the basis for a new blood vessel (Bentley et al., 2009). At this stage, the movement of "tip" cells halts, and strong adhesive connections between cells are established. As the transition from active sprouting to quiescence occurs, ECs take on a "phalanx" character, halting proliferation and aiding the stability and maturation of vasculature (Bautch et al., 2009). Except for the heightened adhesion between endothelial cells (ECs) and the deposition of matrix and basement membrane, another critical stage in the maturation process involves the recruitment of pericytes and smooth muscle cells (Cleaver et al., 2003; Xian et al., 2006). Pericytes establish direct intercellular contact with endothelial cells and compose the walls of capillaries. In contrast, the walls of adult blood vessels, particularly of the big arteries and veins, consist of numerous layers of smooth muscle cells that are separated from the endothelium by a layer of basement membrane (Karamysheva et al., 2008). It has been
proposed that the Tie2/Ang-1 signaling cascade promotes the contact between pericytes and endothelium, decreasing vascular permeability (Armulik et al., 2005). Growth hormones such as PDGF and TGF-β improve maturation by encouraging the migration of pericytes and smooth muscle cells (Hirschi et al., 1998).

1.4 Endothelial cell movement in the angiogenic process

numerous chemotactic stimuli activate the migration of ECs. Generally, this process comprises the destruction of ECM and needs the activation of signal transduction pathways that entail the remodeling of the cytoskeleton. The ongoing reorganization of the actin cytoskeleton results in the creation of structures known as filopodia, lamellipodia, and stress fibrils, all of which play a critical role in aiding cell migration. Three mechanisms are involved in the ECs migration: (a) chemotaxis refers to the guided movement of cells toward a concentration gradient of a soluble component (b) mechanotaxis is the guided movement of cells in response to mechanical stimuli (c) haptotaxis is the directed migration of cells along an adhesive gradient site (e.g., site located in the ECM) (Li et al., 2005). Growth factor stimulation promotes chemotaxis, while the interaction between integrins and ECM stimulates haptotaxis. Endothelial cell migration in the angiogenic process is the product of all the above mechanisms (Stupack et al., 2000).

2. Hypoxia

2.1 Introduction

Molecular oxygen (O₂) is essential for aerobic metabolism to uphold the cellular bioenergetics. Ensuring the equilibrium of O₂ levels is crucial for the survival of most prokaryotic and eukaryotic organisms. Any disparity between oxygen availability and its requirement within cells can lead to hypoxia. When hypoxia occurs, intricate adaptive mechanisms are activated at the cellular, tissue, and organismal levels to align oxygen supply with metabolic and bioenergetic requirements (Nakazawa, et al., 2016). Oxygenation in healthy tissues differs at different tissues, with normal ranges that vary significantly. However, the term "normoxia" is employed to denote the normal oxygen levels in tissues, typically around 20–21% oxygen (PO₂ 160 mmHg) (S R McKeown, 2014). Universally used terms for describing low oxygen levels include mild hypoxia (1-5% O₂, or PO₂ 8-38
mmHg), hypoxia (<1% O₂ or PO₂ <8 mmHg), and anoxia (<0.1% O₂ PO₂ or <0.08 mmHg) (Bhutta et al., 2022).

In the presence of hypoxia, various molecular responses, which have been conserved throughout evolution, become activated. These include pathways regulated by HIF transcriptional factors, autophagy, a wide array of O₂-dependent dioxygenases, mTORC1, ER stress responses, and other oxygen-sensitive mechanisms. These responses mediate alterations in metabolism, in the mobility of cells, in inflammation, and of course, in angiogenesis. Moreover, in hypoxic conditions, systemic changes also occur in respiration, erythropoiesis, and cardiac function. As a result, responses to oxygen and nutrient availability play a crucial role in various biological processes, including the development, and initiation of diseases such as stroke, inflammation, and cancer (Nakazawa, et al., 2016).

In particular, solid tumors create oxygen and nutrient-deprived microenvironments as cancer cells surpass the available oxygen supply, and abnormal angiogenic signaling disturbs the recruitment of normal blood vessels. Research in cancer reveals that hypoxia serves a significant role in fostering tumor angiogenesis. In the hypoxic tumor microenvironment, hypoxia-inducible factor (HIF) is expressed. Within hepatocellular carcinoma tumors, HIF-1α increases angiogenesis by stimulating the transcription of downstream target genes such as VEGFA, VEGFR1, and EphA1. Hypoxia can additionally alter the expression of different components of the ECM, encouraging tumor angiogenesis. Studies have demonstrated that hypoxia promotes the development of metalloproteinases: MMP2 and MMP9, important enzymes implicated in tumor cell invasion and thus metastasis (MacLauchlan SC et al., 2017). Moreover, the elevation of integrin β3 expression induced by hypoxia can affect endothelial cell tube formation (Befani C et al., 2017).

### 2.2 Cellular reactivity to hypoxia

Tumor and endothelial oxygen deficit promote biological processes aiding tumor development and spread. Within tumor cells, these reactions involve the beginning of specialized signal cascades, stimulating selection mechanisms for gene alterations, tumor cell death, autophagy, and new blood vessel development to sustain tumor growth (Miller et al., 2017). Tumor vascularization is necessary for tumors to develop larger than 1 mm in diameter. The explanation is that the diffusion of oxygen from the surrounding blood vessels of the host becomes restricted (Hillen et al., 2007). The subsequent decrease in local blood circulation results in hypoxia in the tumor’s microenvironment, which drives the
development of new blood vessels (by neovascularization). This phase is critical for giving sufficient oxygen to the growing tumor cells and sustaining tumor growth. The endothelial cell layer reacts to stimuli such as circulating substances and hypoxic cues (Reiterer et al., 2020). Two different responses of ECs have been reported, depending on the intensity and the duration of hypoxia. Brief durations of hypoxia (acute hypoxia) cause endothelial cells to produce chemicals that enhance the binding of neutrophils to the endothelium, a process in which P-selectin and E-selectin are implicated (Pinski, 2002). MMPs, which are also activated, stimulate the breakdown of ECM, e.g., specific activation of MMP-9 in hypoxic settings results in collagen IV degradation (Faller et al., 1999). Extended periods of hypoxia (chronic hypoxia) promote the secretion of growth factors and cytokines which foster tumor vascularization and enhance endothelial cell permeability and cell adhesion. Those modifications are crucial for tumor cell functions including intravasation and extravasation (Evans et al., 2012).

The key drivers of the earliest transcriptional reactions to hypoxia are the Hypoxia-Induced Factors: HIFs. These proteins are involved in the transcription of genes crucial for various aspects of cancer biology such as angiogenesis (Liao and Johnson, 2007), invasion and metastasis (Chan and Giaccia, 2007), the transition from epithelial to mesenchymal, genetic instability (Huang et al., 2007) and radiation resistance (Moeller et al., 2007). HIF activation results in increased production of angiogenic growth factors, such as PDGF, VEGF, bFGF, and IGF. PDGF stimulates the expression of additional growth factors, notably VEGF (Faller 1999). Nevertheless, HIF-independent mechanisms may also change gene expression under hypoxic conditions. These signaling pathways contain a vast array of receptors, ion channels, and enzymes with regulatory roles, such as different protein kinases and phosphatases (Bardos and Ashcroft, 2004; Mizukami et al., 2007; Fahling, 2009) and inducible transcription factors, including AP-1 and NF-κB.

### 2.3 Hypoxia Inducible Factors (HIFs)

For processes like oxidative phosphorylation and many other oxygen-dependent biochemical reactions, cells must maintain a balance in oxygen levels, which is a difficult but necessary effort (Raymond et al., 2006). The balance between oxidative and glycolytic metabolism to match the availability and demand of oxygen, is regulated by HIFs. Additionally, HIFs increase the delivery of oxygen by starting the transcription of genes that control angiogenesis and erythropoiesis, which increases the supply of oxygen both
locally and systemically (Semenza et al., 201, Kierans et al., 2021; Semenza et al., 2022; Watts et al., 2020; Rey et al., 2010; Rodriguez et al., 2021). The direct binding of HIFs to hypoxia response elements (HREs) found in target genes results in the production of RNA that is stimulated by hypoxia (Semenza et al., 1993). According to Wang et al. (1995), HIFs are heterodimeric proteins made up of an O2-sensitive α subunit and a continuously expressed β component, also known as ARNT. Every subunit belongs to the family bHLH-PAS (Per/Arnt/Sim). Three α-isofoms have been identified in mammals: HIF-1α, HIF-2α (also called EPAS1), and HIF-3α (Tian et al., 1997). HIF-1α and HIF-2α are mostly linked to HIF's impact on tumor growth; however, HIF-3α has occasionally been shown to have a strong negative effect on other HIF α-isofoms (Maynard et al., 2007; Heikela et al., 2011). Two PAS domains and one bHLH domain make up each α subunit. These domains are necessary for DNA binding and the heterodimerization of the α and β subunits (Jiang, 1996). The TAD-N and TAD-C domains make up HIF-1α, and they are joined by an inhibitory linker region (Ruas et al., 2002). The TAD-C domain interacts with co-activators, including p300/CBP, and is required for full transcriptional activation of HIF-1α. Conversely, the TAD-N domain is associated with the ODD domain and contributes to HIFs stabilization (Lando et al., 2003). Six HIF-1α splice variants and at least seven HIF-3α splice variants are produced in humans as a result of alternative splicing. These variations can increase or decrease other HIF complexes' activity (Monsef et al., 2010). Moreover, Hirose et al. (1996) have identified two isoforms of ARNT. HIF-1α appears to be expressed broadly in nearly all cell types, whereas HIF-2α and HIF-3α are expressed specifically in some organs, including bone marrow macrophages, liver parenchymal cells, renal interstitial cells, and vascular and lung epithelial cells (Bertout et al., 2008). Research has indicated that HIF-1α nuclear accumulation occurs quickly and early in hypoxic settings in various endothelial cells (e.g. HUVECs, HDMECs, HPAECs, HSaVECs). On the other hand, HIF-2α expression is weak or absent in the endothelial cells of arteries and veins, while it is increased in HDMECs with a short delay (Nilsson et al., 2004).

Hypoxia in the tumor microenvironment serves as a trigger for the upregulation of HIF-1α and HIF-2α protein expression. Elevated levels of either or both of these proteins, as identified through immunohistochemical analysis of the initial tumor biopsy, correlate with heightened patient mortality across various solid cancers and leukemias. While certain cancers exhibit a hypoxia-induced pattern of HIF-1α expression, characterized by maximal expression in cells farthest from blood vessels, others demonstrate a uniform elevation in HIF-1α expression throughout the tumor, as observed through immunohistochemistry. This
suggests that in some cases, mechanisms independent of oxygen levels drive increased HIF-1α expression (Aebersold et al., 2001). In certain tumors like glioblastomas one of the HIF-α subunits is linked to a dismal prognosis, indicating its primary significance in the progression of these specific tumor types (Scrideli et al., 2007; Griffiths et al., 2008). Each cancer cell typically expresses only a portion of HIF-regulated RNAs, which contribute to various crucial steps of cancer progression. These include tumor vascularization, invasion, alterations in metabolic pathways, cell motility, as well as resistance to radiation therapy and chemotherapy. (Cowman et al., 2022; Noman et al., 2019; Samanta et al., 2018; Yuen et al., 2020).

2.3.1 Oxygen levels as regulators of HIF expression

Post-translational changes like ubiquitination, acetylation, and hydroxylation strictly regulate HIF protein production. Among these, hydroxylation is the most important for HIF protein stability (Brahimi-Horn et al., 2005). In normoxic conditions, a proline residue inside HIF-1α, HIF-2α, or HIF-3α undergoes oxygenation by HIF prolyl hydroxylases (PHD1, PHD2, PHD3) while non-heme Fe2+ and ascorbate act as cofactors to the reaction (Schofield and Ratcliffe, 2004). Subsequently to this, the von Hippel-Lindau (VHL) protein specifically binds to hydroxylated HIF-α subunits, designating them for ubiquitination and destruction by the 26S proteasome (Maxwell et al., 1999; Ohh et al., 2000). Under low oxygen tension, in hypoxic conditions, prolyl-hydroxylation of HIF-α is impeded, preventing its recognition by the pVHL ubiquitin-ligase complex and thus, leading to the accumulation of non-hydroxylated HIF-α subunits (Berra et al., 2001). These subunits then form dimers with HIF-1β and bind to HREs within target genes, initiating transcriptional activation (Semenza et al., 2020). The stabilization of HIF-α is regulated equally across all three isoforms through the PHD-pVHL pathway. In cervical cancer, glioma, and neuroblastoma cells, HIF-2α protein accumulates when oxygen levels range between 2-5%, but HIF-1α stabilizes only within the 0-2% oxygen range (Nilson et al., 2005; Holmqvist-Mengelbier et al., 2006; Li et al., 2009). Furthermore, in lung cancer, HIF-1α levels rise immediately upon exposure to hypoxia but decrease after several hours. In contrast, HIF-2α protein levels stay high in cultures throughout extended hypoxia (Uchida et al., 2004; Holmquist-Mengelbier et al., 2006). This shift can be clarified by the unique role of HIF-associated factor (HAF) on HIF-1α stability (Koh et al., 2008). HAF, which declines during acute hypoxia but increases during chronic hypoxia, stimulates pVHL-independent
proteasomal degradation of HIF-1α in extended hypoxic settings, while concurrently encouraging HIF-2α transactivation (Koh et al., 2008; 2011).

HIF-α stabilization can occur independently of oxygen levels as well. Various growth factors and cytokines, such as insulin, FGF-2, EGF, PDGF, TGF, and IL-1β, are identified to stabilize HIF-α even under normoxic circumstances (Zelzer et al., 1998; Black et al., 2008; Roos et al., 2011; McMahon et al., 2006; Stiehl et al., 2002). c-Jun suppresses HIF-1α degradation by interacting with its ODD domain in a non-transcriptional fashion (Yu et al., 2009). Moreover, the tumor suppressor p53 promotes the breakdown of HIF-1α, while the oncogene c-myc supports protein stability. Both pathways influence tumor-induced angiogenesis (Chen et al., 2013). In addition to protein stabilization, the expression of HIF can be controlled at both transcription and translation levels. It is noteworthy that chronic hypoxia downregulates HIF-1α expression through two mechanisms: 1) instability of HIF-1α mRNA, and 2) rise in the amounts of a natural HIF antisense RNA (Uchida et al., 2004). Conversely, the silencing of both HIF-1α and HIF-2α leads to a drop in HIF-3α levels, showing an autoregulatory loop between the α-subunits of HIF and hypoxia (Augstein et al., 2011). Increased crosstalk between cancer cells via oncogenic signaling can drive the synthesis of HIF-α mRNA through processes not reliant on oxygen, facilitated by activated cellular kinase pathways (Lee et al., 2003).

2.3.2 Activation of HIF and its transcriptional functionality

Activation of the TAD-C domain is critical for transcriptional and angiogenic consequences mediated by HIF-1α (Tal et al., 2008). When the oxygen level is healthy, a hydroxylase domain protein (FIH) known as a factor inhibiting HIF, which is largely found in the cytoplasm, acts in the inhibitory control, specifically targeting HIF-1α, by hydroxylating Asp-803 inside the TAD-C domain. This hydroxylation impedes connections between HIF-α and the transcriptional co-activator p300/CBP and by that hinders the activation of HIFs (Metzen et al., 2003; Bracken et al., 2006; Jokilehto and Jaakkola, 2010). During hypoxic conditions, cytoplasmic HIF-α relocates to the nucleus, where it forms a heterodimer and interacts with transcriptional co-activators. This complex then binds to hypoxia response element (HRE) sequences, hence starting gene expression (Semenza and Wang, 1992; Forsythe et al., 1996; Semenza et al., 1996). Hypoxia response elements (HREs) are containing the consensus sequence 5’-RCGTG-3’. These components are positioned at varied locations and orientations throughout the coding area of multiple hypoxia-regulated
genes (Wenger et al., 2005). Interestingly, HIFs might indirectly modulate gene expression by stimulating the expression and/or activation of other transcription factors like c-jun (Laderoute et al., 2002) and c-myc (Gordan et al., 2007) in genes that HRE sequences are not functional (Forsythe et al., 1996; Semenza et al., 1996). MAPK-mediated phosphorylation, acetylation, and sumoylation are other mechanisms that mediators in DNA binding activity or the affinity of HIFs to their co-factors (Mylonis et al., 2006; Greer et al., 2012).

Studies in experiments demonstrate that the spectrum of target genes of HIF-1α differs from that of HIF-2α in angiogenic and hematopoietic pathways (Mole et al., 2009). Moreover, vascular factors produced by one of the two factors may operate competitively or be inhibitory to the function of the other transcription factor (Loboda et al., 2012). The transcriptional specificity of HIF-1 and HIF-2 is dictated by the N-terminal activation domain, resulting in different interactions with transcriptional co-factors (Keith et al., 2011). Ets1 is classified as a unique co-factor that solely participates in the formation of HIF-2-transcriptional complexes. Ninety percent of genes specific to HIF-2 possess binding sites for Ets family transcription factors, including VEGFR-2 (Elvert et al., 2003), PAI-1, and EPO (Aprelikova et al., 2006). Hypoxia can also suppress the expression of hundreds of genes in a way dependent on HIF-1. Whereas direct binding of HIF-1 to these genes is not detected, the postulated mechanism via which inhibition occurs, might include the HIF-1-mediated production of transcriptional repressors (Yun et al., 2002).

### 2.3.3 HIF-target genes that mediate angiogenesis

In keeping with the substantial involvement of hypoxia in sprouting angiogenesis, multiple genes relevant to various phases of angiogenesis display responsiveness, either directly to HIFs or hypoxia. These genes include VEGF, PDGF-B, and angiopoietins, along with their associated receptors, as well as genes implicated in matrix metabolism such as MMPs, PAIs, and PARs (Yang et al., 2013). As discussed previously, VEGF occupies a crucial function in angiogenesis. It improves vascular permeability, endothelial cell survival, proliferation, migration, and invasiveness. Endothelial gene deletion of HIF-1α in vivo decreases the hypoxia-induced increase in transcriptional level of VEGF. On the other hand, gene deletion of HIF-2α has not shown the same effect (Skulli et al., 2012). While VEGFR-1 is directly up-regulated in hypoxic settings through HREs positioned in the VEGFR-1 promoter of the gene, the up-regulation of VEGFR-2 happens through regulation at the post-transcriptional
level (Tang et al., 2004). Hypoxia-induced angiogenesis involves several additional growth factors, substances, and receptors with pro-angiogenic potential in addition to the VEGF/VEGFR axis. The signaling pathway of nitric oxide (NO) plays a vital function in modulating the response to VEGF-A signaling. Proteomic research demonstrated that the components participating in the pathway are up-regulated in experiments with hypoxia-treated bone marrow-endothelial progenitor cells (Hoffman et al., 2013). Endothelial nitric oxide synthase (eNOS) is a recognized hypoxia-inducible gene, and this enzyme is responsible for NO generation. Its transcription is induced by HIFs with two hypoxia response element (HRE) sites situated inside the eNOS promoter, found in HUVECs (Coulet et al., 2003). Interestingly, under prolonged hypoxic circumstances in pulmonary endothelial cells (Cui et al., 2011), nitric oxide NO production undergoes a considerable drop. This drop is related to the HIF-2-dependent up-regulation of arginase II, which competes with endothelial nitric oxide synthase eNOS for the substrate L-arginine (Krotova et al., 2010). HIF-1α directly binds to the promoter of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-2 (NOX2) in human microvascular ECs resulting in the production of ROS which in turn, activates HIF-1, generating a positive feedback loop that supports angiogenesis (Diebold et al., 2012). In cancer cells, HIF-2α binds HRE near the promoter of the ROS scavenger superoxide dismutase 1 (SOD-1) gene (Tuller et al., 2009). Furthermore, HIF-1α has the power to boost the expression of components inside the cytochrome c oxidase complex, such as COX4-2. Consequently, this shift can disrupt the flow of electrons along the respiratory chain, resulting in alterations in ROS generation (Fukuda et al., 2007). Hypoxia has the potential to alter vascular branching via influencing Notch signaling, wherein HIF-1α binds to the Notch intracellular domain, subsequently boosting its transcriptional activity (Gustafsson et al., 2005). In vivo, investigations have shown that Dll4 and several Notch target genes (e.g., Hey1, Hey2, and Hes1), are controlled by HIF-2α and operate as downstream effectors in endothelial cell migration (Skuli et al., 2012). Migrating ECs need to unfasten their attachment and destroy the ECM through mechanisms that increase the production of ECM proteases. In cancer cells, HIF-1α reduces E-cadherin mRNA levels (Jing et al., 2012). MMP-2 is a transcriptional target of HIF-1α, enhancing EC migration caused by hypoxia (Ben-Yosef et al., 2005). Moreover, in cancer cells, silencing of HIF-1α results in the down-regulation of MMP-2 mRNA expression (Jing et al., 2012). The receptors Tie 1 and Tie 2 are expressed in practically all ECs and are major angiogenic factors (Ziegler et al., 1993). Ang-1 is the ligand of Tie 2, and this complex is implicated in angiogenesis (Suri et al., 1996). Transgenic embryos expressing a dominant-
negative version of HIF display lower levels of the endothelium angiopoietin (Ang) receptor Tie-2. However, other endothelium indicators, such as PECAM-1, Tie-1, and VE-cadherin, remain unchanged by this modification (Licht et al., 2006). The elimination of HIF-2α primarily in endothelial cells, does not affect Tie-2 expression but leads to a decrease in Ang-2 expression. On the contrary, the selective deletion of HIF-1α in endothelial cells has negligible effects on the Ang-2 protein generated by hypoxia but dramatically reduces the transcript levels of Ang-1 in hypoxic settings. (Skuli et al., 2012).

Other major factors generated in hypoxic conditions, and especially regulated by HIF-1α, include TGF-α and TGF-β (Pertovaara et al., 1994), as well as PDGF-B that plays a pivotal role in stabilizing newly formed vessels (Kelly et al., 2003; Nilsson et al., 2004). The overexpression of HIF-1α induces the levels of PDGF-Bin pulmonary endothelial cells (Kelly et al., 2003), as well as in the vascular network of tumors under low oxygen levels (Yoshida et al., 2005). Studies have revealed that hypoxia drives reconfiguration of the pulmonary vascular network through HIF-1α-mediated suppression of tyrosine phosphorylation (ten Freyhaus et al., 2011).

There are methods outside of HIFs that increase the expression of genes associated with angiogenesis in a hypoxic environment (Arany et al., 2008). These pathways involve the participation of transcription factors such as AP-1, Ets1, NF-κB, Sp1, c-myc, and Nrf-2 (Webster et al., 1993, Royds et al., 1998; Faller, 1999; Xu et al., 2000; Alfranca et al., 2002; Gordan et al., 2007; Salnikow et al., 2008; Kim et al., 2010). AP-1 consists of homo- and heterodimers from the oncogene families: c-fos and c-jun, controlling the transcription of numerous factors, including PTN (Polytarchou et al., 2005). It is directly activated under hypoxic conditions, both in vivo and in vitro (Laderoute et al., 2002; Rupec et al., 1995).

### 2.3.4 HIFs and hypoxia orchestrating angiogenesis

Mice lacking HIF-1α display defects in cephalic vascularization, resulting in embryo mortality (Ryan et al., 1998). Nevertheless, targeted deletion of HIF-1α in endothelial cells does not affect normal vascular development (Tang et al., 2004) and embryos lacking HIF-2α do not consistently demonstrate vascular defects (Tian et al., 1998; Peng et al., 2000; Compernolle et al., 2002; Scortegagna et al., 2003). This shows that HIFs are important in non-endothelial cells for normal vascular formation, and the deficit of HIF-1 function can be partly compensated by HIF-2, and inversely. Moreover, targeted ablation of HIF-1α in endothelial cells affects angiogenesis and tumor formation in adult mice, while deletion of
HIF-2α in endothelial cells leads to tumors of lesser size but with a similar number of vessels (Skuli et al., 2009; 2012). This research indicates that HIF-1α and HIF-2α may supplement each other's roles in the tumor endothelium, where HIF-1α is important for vessel growth and HIF-2α promotes vascular maturation. In the mouse retina model, the lack of VHL that results in constitutive activation of HIF-1α and HIF-2α leads to persisting vasculature and severe retinal degeneration (Kurihara et al., 2010). The question of whether hypoxia produces independent endothelium proliferation remains a source of discussion. Endothelial cell proliferation produced by hypoxia has been connected with the target of rapamycin (mTOR) signaling pathway (Li et al., 2007). The impact of hypoxia on endothelial cell proliferation appears to be regulated by the degree of hypoxia and the type of cells. For instance, HUVEC development was improved in 5% hypoxia compared to HUVECs grown in air oxygen or 1% hypoxia. However, no difference in cell proliferation was detected for human endothelial progenitor cells across the various oxygen concentrations specified (Abaci et al., 2010). On the contrary, some research has claimed that chronic hypoxia affects the endothelial cell cycle, leading to cell cycle arrest, thus inhibiting cell development and ending in apoptosis (Heidi et al., 2003). Conversely, endothelial cells missing HIF-2α isolated from mouse lungs demonstrated heightened levels of tube formation and migration, while demonstrating lower adherence to ECM proteins (Skuli et al., 2012). HIF-1β is also engaged in endothelial cell migration and vessel outgrowth and it is hypothesized to have a vital role in EC viability and proliferation when mild hypoxia occurs (Han et al., 2012b). Elevated expression of HIF-1α increases chemotaxis and adhesion and modulates the differentiation of BMSCs into ECs. Moreover, HIF-1α stimulates tube formation by ECs (Ben-Shoshan et al., 2008).

Cancer stem cells create differentiated cancer cells under HIF control inside both primary and secondary tumors, and they play key roles in tumor angiogenesis (Mimeault and Batra, 2013). HIF-1α, recognized for regulating the glycolytic properties of tumor-associated macrophages (TAMs), promotes the motility, invasion, and clustering of TAMs at inflammatory locations (Cramer et al., 2003). Conversely, the lack of HIF-2α in TAMs resulted in decreased macrophage-driven angiogenesis and tumorigenesis in vivo (Imtiyaz et al., 2010).
3. **Pleiotrophin (PTN)**

3.1 Introduction

Pleiotrophin (PTN), a secreted growth factor, is essential for several biological processes, such as angiogenesis and development (Papadimitriou et al., 2021). The 136 amino acid cytokine PTN, is highly conserved and has a variety of roles in the body, such as controlling the differentiation of glial progenitor cells, neurite outgrowth, and angiogenesis. The PTN gene is upregulated in cells exhibiting an early differentiation phenotype during wound repair, and it is expressed during early differentiation stages in various developmental contexts, highlighting its significant contribution to organ development, particularly in the nervous and hematopoietic systems. Notably, PTN is acknowledged as a proto-oncogene and is expressed strongly in a variety of human tumor types.

PTN is a member of the structurally related family of heparin-binding growth factors that includes midkine. Comprehensive analysis of a wide range of human tumor cell lines and tumor samples from different sources has shown that PTN is widely expressed in many types of cancer. PTN has become recognized in the field of cancer biology as a complex component that possesses the ability to both induce and promote tumor growth. PTN has been implicated in cellular quiescence, suggesting that it plays a function in preserving equilibrium within the tumor microenvironment, even as it directly affects tumor cells, promoting their growth and development (Mikelis et al., 2007).

In vivo investigations have revealed that PTN overexpression promotes angiogenesis, a mechanism necessary for tumor growth and metastasis, and speeds up tumor growth (Deuel et al. 2002). This suggests the importance of PTN in carcinogenesis. Moreover, PTN's control over endothelial cells implies a critical role in angiogenesis as well. The role of PTN in mammary gland development is further explained by research which shows that PTN keeps epithelial cells in a progenitor state, postponing their maturity and differentiation (Rosenfield et al. 2012). Given that PTN expression is significantly elevated in a variety of cancers, contributing to their mitogenic, pro-angiogenic, and metastatic characteristics, this pathway may have consequences for tumor biology. Additionally, PTN serum levels may be high in non-malignant circumstances, such as acute inflammation, acute vascular disease, and acute trauma (Zugmaier et al. 2018). PTN promotes a pro-metastatic niche in the tumor microenvironment, which facilitates tumor cell escape and secondary site colonization (Ganguly et al. 2022).
3.2 *Ptn* gene and regulation of its expression

PTN's significance in numerous physiological and pathological processes, such as tumor angiogenesis, is reflected in the complex network of transcriptional, post-transcriptional, and epigenetic mechanisms that regulate its expression. Tumor angiogenesis is known to be positively regulated by the *Ptn* gene, a feature that is especially evident in primary tumor tissues and cell lines related to breast cancer. At least two promoters regulate *Ptn* expression: one is species-conserved, and the other is human-specific; the latter is created when a human endogenous retrovirus-like element (HERV) is inserted into the *Ptn* gene. Together with the ordinary *Ptn* transcripts, this HERV insertion causes the transcription of HERV-PTN fusion transcripts, which are present in a variety of human tissues such as primary human breast tumor specimens, breast cancer cell lines, and even certain normal breast tissues (Zhang et al., 2022).

The expression of these fusion transcripts is significantly regulated by the HERV-PTN promoter element, which has been shown to have several regulatory factors that affect its function. PTN expression is further modulated by transcription factors that bind to these regulatory sites, suggesting a complex degree of transcriptional regulation (Wellstein, 2012). Furthermore, it has been discovered that the transcription factor YY1 represses the HERV-PTN fusion transcript in breast cancer cell lines, indicating that *Ptn* expression is also regulated by transcriptional repression (Kurisaki et al., 2012; Schulte et al., 2000).

PTN's function is also controlled at the signal transduction level, as evidenced by the activation of a cascade of second messengers in a variety of cell lines through its interaction with its receptor, anaplastic lymphoma kinase (ALK), which goes beyond transcriptional regulation. The relationship between PTN and ALK may be particularly significant for angiogenesis and tumor formation, as PTN expression in mammary cells triggers ALK activation in stromal cells, which promotes tumor growth (Perez-Pinera et al., 2007).

The oviduct, where estrogen stimulates PTN mRNA and protein expression, has been the focus of research on PTN expression in chickens. Post-transcriptional mechanisms, such as miR-499 and miR-1709, which target the 3'-UTR of PTN mRNA, further influence this regulation, indicating a common mechanism of PTN regulation across species (Lee et al., 2012). Furthermore, the CpG methylation status and the PTN gene expression pattern in malignant and normal ovaries suggest that epigenetic alterations also regulate PTN expression; demethylation in diseased cells enhances PTN expression (Zhang et al., 2015).
3.3 PTN protein structure

PTN protein interaction with different receptors and its role in angiogenesis, tissue regeneration, and neuronal development are all dependent on its shape. PTN has broad fundamental surfaces in both of its domains that are organized. While not involved in heparin binding, PTN's C-terminal tail is necessary for stable interactions with chondroitin sulfate A (CSA), which is often present on its receptor protein tyrosine phosphatase ζ (PTPRZ). This structural characteristic highlights the tail's function in mediating PTN's biological activities by explaining why PTN variants without the C-terminal tail are unable to communicate through PTPRZ (Ryan et al., 2016).

Fujikawa et al. (2019) provided additional insight into the PTN-PTPRZ interaction by revealing the structural underpinnings of ligand-induced PTPRZ receptor deactivation. According to their research, the PTPRZ intracellular region adopts a "head-to-toe" dimer configuration, with the first domain's catalytic site being hidden by the second phosphatase domain (D2). PTN binding causes this dimerization and deactivation of the phosphatase activity of PTPRZ1. This is important for the myelination of the central nervous system and the differentiation of oligodendrocyte precursor cells. This discovery offers important new understandings of the molecular processes by which PTN regulates PTPRZ1 activity and its function in cellular signaling cascades.

Furthermore, the dynamic conformational landscape of PTN's structure contains as-yet undiscovered subtleties that may control its varied functions in a range of biological settings. New methods in structural biology, in conjunction with advanced computer modeling tools, could help to clarify the mysterious details of PTN architecture and how it relates to cellular physiology and pathology. By gaining a thorough grasp of the structural details of PTN, scientists hope to open new therapeutic options that will use PTN regulation to fight a variety of crippling illnesses, from cancer to neurological problems and beyond. The story of PTN's molecular complexities is expected to enthrall the scientific world as the structural exploration drama develops, opening the door for revolutionary advances in biological research and treatment.

3.4 Biological functions of PTN

As a versatile cytokine, PTN is involved in many distinct biological processes, such as tissue regeneration, cell differentiation, and proliferation. Its functions encompass important
functions like immunological and defensive responses, inflammatory responses, and the control of cell division and death. Bioinformatics analysis revealed PTN's participation in more than 100 pathways, including metabolic, cellular, and regulatory activities (Choi et al. 2015). This indicates the importance of PTN in a range of physiological and pathological settings.

Choi et al. (2015) investigated the impact of PTN on melanogenesis and discovered that PTN expression in human skin melanocytes and fibroblasts could reduce melanogenesis by degrading MITF via Erk1/2 activation. This adds another aspect to PTN's many biological roles by indicating that it regulates skin pigmentation. Furthermore, PTN's role in the development of tumors and angiogenesis has been extensively studied. Its biological actions are mediated by its interactions with multiple receptors, including N-syndecan (Yao et al., 2017), anaplastic lymphoma kinase (ALK) (Bowden et al., 2002), and protein tyrosine phosphatase receptor zeta 1 (PTPRZ1) (Papadimitriou et al., 2016). PTN's ability to activate migratory responses in endothelial cells has been demonstrated to depend on the creation of a functional complex consisting of integrin αvβ3 and RPTPβ/ζ, underscoring its potential significance in tumor metastasis and angiogenesis (Koutsoumpa et al., 2015).

Research around the intracellular signaling pathways and receptors of PTN and its homolog, midkine (MK), as well as their structural features could potentially reveal key characteristics of PTN’s functional role in cancer. Given their high expression in a variety of malignancies and their roles in mitogenicity, inflammation, angiogenesis, oncogenesis, and stem cell self-renewal, PTN and MK are important biomarkers and targets for anticancer treatments. Their potential for treating degenerative disorders is further enhanced by their involvement in tissue regeneration, encompassing the heart, cartilage, neurons, muscle, and bone (Xu et al. 2014).

PTN's significance in supporting neuronal survival, axonal development, and synaptic plasticity has also drawn attention due to research on its neurological functions. It has been discovered to alter the expression of neural cell adhesion molecule (NCAM) and to trigger intracellular signaling pathways that are essential for the growth and regeneration of neurons, including the PI3K/Akt and MAPK pathways (Mi et al., 2007; Sanchez-Alegria, 2018).

PTN has been linked to heart development, angiogenesis, and cardioprotection in the context of cardiovascular biology (Papadimitriou et al., 2022). According to Zhang et al. (2015), there is evidence that PTN can protect against ischemia injury, promote the
migration and tube formation of endothelial cells, and increase the proliferation of cardiomyocytes through a variety of mechanisms, including the activation of Akt and ERK signaling pathways. These results demonstrate the therapeutic potential of PTN in the treatment of cardiovascular disorders and heart regeneration.

Furthermore, it is becoming more widely acknowledged that PTN plays a role in regulating immunological responses. PTN may be used as a therapeutic target in inflammatory conditions, immune-mediated pathologies, and autoimmune diseases due to its immunomodulatory qualities. It has been demonstrated to control the proliferation, differentiation, cytokine generation, and migration of immune cells, including T cells, B cells, macrophages, and dendritic cells (Shen et al., 2017). Numerous studies have highlighted PTN's role in angiogenesis, inflammation, and immunological regulation, indicating that it may be a promising target for the treatment of diseases such as multiple sclerosis, atherosclerosis, and rheumatoid arthritis (RA). Patients with RA have markedly elevated levels of PTN, a factor that is normally expressed during development, especially in synoviocytes and endothelial cells. PTN may be a potential paracrine angiogenesis and growth factor in RA, as indicated by this increase, which correlates with the severity of the disease (Pufe et al., 2003). Furthermore, via activation of JAK and STAT, IFN-γ induces PTN expression in macrophages (Li et al., 2010). FGF-2 expression is correlated with demyelination, whereas PTN and midkine expression is elevated throughout Experimental Autoimmune Encephalomyelitis (Liu et al., 1998).

### 3.5 The role of PTN in angiogenesis and cancer progression

Especially in the case of breast cancer, PTN is essential for angiogenesis and the advancement of malignancy. Many breast malignancies produce the secretory cytokine PTN, and substantial modification of the tumor microenvironment and the rapid progression of scirrhous carcinoma have been associated with its improper expression. This includes enhanced angiogenesis of tumors and marked up-regulation of several procollagens and elastin, as seen in mouse models where PTN was induced via the mouse mammary tumor virus (MMTV) promoter in conjunction with the middle T antigen of the polyomavirus (PyMT). According to these results, PTN may modify the microenvironment to facilitate tumor metastasis and progression in addition to stimulating tumor development (Chang et al., 2007).
Furthermore, PTN is also raised in various types of cancer. Tissue samples from lung cancer patients were used in immunohistochemical methods to detect PTN expression, and findings showed that PTN expression is significantly higher in lung cancer tissues compared to controls, with small cell lung cancer exhibiting the highest expression among the different cancer types (Wang & Wang 2015). These results suggest that PTN expression could be a novel diagnostic and prognostic marker for lung cancer, particularly small-cell lung cancer, where PTN expression is primarily observed in the cytoplasm and cell membrane. Apart from the previously reported results, another study on levels of PTN in the serum and plasma of lung cancer patients showed that lung cancer patients had higher serum levels of PTN (Jager et al. 2002). The study compared patients with small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) to healthy control people. The tumor patient group had considerably greater mean serum concentrations of PTN than the control group. PTN serum levels also showed an adverse relationship with therapy response and a positive correlation with the illness stage. On the other hand, VEGF levels in plasma were higher in a smaller percentage of lung cancer cases and did not appear to be associated with the disease stage. PTN may help track the effectiveness of treatment and act as an early warning of lung cancer, according to the study; however, larger studies are needed to confirm these findings. Additionally, research has been done on PTN and N-syndecan expression in pancreatic cancer and their relationships to tumor growth and perineural invasion. While N-syndecan expression strongly correlates with tumor size, it does not affect survival time. Elevated PTN expression correlates with big bloody ascites, liver metastases, and lower survival time (Yao et al., 2017). Due to its limited expression in normal tissue, PTN has also been found to be a crucial growth factor in colorectal cancer (CRC), underlining its potential as a prognostic diagnostic and therapeutic target in CRC therapy (Kong et al., 2012). A correlation between PTN levels has been found in gliomas and survival outcomes in both human patients and animal models, highlighting its potential as a therapeutic target for halting the progression of gliomas (Zhang et al. 2015). PTN as a neurotrophic cytokine, is also strongly linked to metastasis and exhibits higher expression levels in aggressive breast tumors. Metastatic cancer cells also adapt to new settings in secondary organs, boosting their survival and proliferation. Furthermore, a significant correlation between elevated blood levels of PTN and metastasis is as well as lower survival rates in patients with breast cancer (Ganguly et al. 2023). Investigations are ongoing to determine the mechanism by which PTN encourages metastasis. PTN causes cytokine
production to alter in cancer cells via activating NF-κB (Ganguly et al. 2023). Consequently, neutrophil recruitment and the creation of an immune-suppressive microenvironment are followed. By pharmacologically or genetically inhibiting PTN, tumor-associated neutrophil accumulation decreases, and local immune suppression is reversed. This reversal inhibits metastasis and increases T-cell activation. Additionally, immune checkpoint inhibition and chemotherapy are much more successful in lowering the metastatic burden in mice when PTN is blocked.

Angiogenesis is a crucial process in the growth and spread of tumors. It is controlled by several angiogenic factors and entails the creation of new blood vessels from pre-existing ones. PTN is one of these variables, playing a part in the intricate interaction that promotes the proliferation of endothelial cells and enhanced vascularity. PTN, by numerous molecular pathways, plays a crucial role in controlling angiogenesis. Recent research has demonstrated the direct angiogenic qualities of PTN, which cause an angiogenic switch to occur in a variety of cancer types in vivo. PTN regulates angiogenesis by several different mechanisms, such as remodeling the stromal microenvironment, inducing monocyte transdifferentiation into endothelial cells, modulating signaling pathways involving basic fibroblast growth factor and vascular endothelial growth factor, and directly interacting with endothelial cells to promote proliferation, migration, and tube formation. Furthermore, peptides that can block PTN signaling have also been found in recent research, along with specific PTN domains that support angiogenesis (Perez-Pinera et al., 2008; Rountree et al., 2021; Elahouel et al., 2015; Fang et al., 1992). Since increased angiogenesis is associated with lower survival rates in the setting of breast cancer, it is imperative to comprehend angiogenic mechanisms, particularly PTN’s function, to create effective treatments. Potential therapeutic approaches for slowing the course of cancer and enhancing patient outcomes include targeting angiogenic pathways, such as those involving PTN (Madu et al., 2020).

PTN has an impact on the phenotype of tumor-associated macrophages in the tumor microenvironment, which fosters a pro-malignant state that aids in the development of malignancy, metastasis, and treatment resistance. Patients with breast cancer who have PTN-correlated macrophages in their tumors are likely to have a worse prognosis. PTN contributes to the development of a pro-tumorigenic milieu that is favorable to the advancement of cancer through its interactions with its receptors, including ALK, and its capacity to activate macrophages (Shi et al., 2017). PTN exhibits variable effects in different
tumor types, stimulating angiogenesis, metastasis, and cancer progression via unique molecular pathways. For example, PTN activates the RPTPβ/ζ pathway to promote tumor development and angiogenesis in breast cancer, and through ALK-dependent and RPTPβ/ζ-dependent processes, it promotes migration and proliferation in glioblastoma (Lu et al., 2005). It's interesting to note that different PTN isoforms have distinct effects on the behavior of glioblastomas, which emphasizes how intricate PTN-mediated signaling is in the development of cancer. Furthermore, preclinical research has demonstrated encouraging outcomes when PTN expression or activity is inhibited, indicating that PTN may be a prospective therapeutic target for the treatment of cancer. PTN is unquestionably important as a predictive biomarker and therapeutic target in a variety of malignancies, offering important insights into the molecular mechanisms behind cancer metastasis and progression (Zhou et al., 2018).

### 3.6 Molecules and receptors that interact with PTN

PTN is involved in several physiological and pathological processes, such as the development of the nervous system, tissue healing, and the advancement of cancer. Its many biological activities are facilitated by interactions with other molecules, such as growth factors, proteoglycans, and receptors, which work together to control an intricate web of signaling pathways.

PTN interacts with chondroitin sulfate (CS) proteoglycans, such as the long isoform of PTPRZ1. The ECM and cell surfaces are rich with CS proteoglycans, which control cell adhesion, migration, and proliferation. PTN's biological actions are enhanced by its binding to CS proteoglycans, which contributes to its localization and concentration in particular tissue microenvironments. In their investigation of PTN's interactions with artificial versions of CS tetrasaccharides, García-Jiménez et al. (2022) found that PTN binds to these glycosaminoglycans, but less strongly than its homolog midkine (MK). This interaction emphasizes the significance of the ECM components in regulating PTN's actions and is critical for PTN's function in brain development and stem cell control.

Protein tyrosine phosphatase zeta 1 (PTPRZ1) is another important PTN target that was found in the nervous system. PTPRZ1 is a receptor protein tyrosine phosphatase that interacts with PTN through its chondroitin sulfate chains and core protein. PTN binding boosts PTPRZ1's downstream signaling pathways, including the activation of different
kinases and integrins, which are crucial in multiple cellular processes (Papadimitriou et al., 2009; 2016).

ALK, a receptor associated with some cancers, is a significant component in the PTN signaling network. PTN binds to ALK, and when the two bind together, signaling pathways downstream that are critical in cell migration, survival, and proliferation are triggered. This PTN-ALK axis is especially significant when considering carcinogenesis because enhanced PTN expression and concomitant ALK signaling activation have been reported to boost the formation and spread of cancer cells (Perez-Pinera et al., 2007; King et al., 2015; Kalamatianos et al., 2018).

PTN also interacts with transmembrane receptors known as integrins, which mediate interactions between cells and the ECM. The binding of PTN to αvβ3 integrin on endothelial cells is connected to enhanced cell migration and angiogenesis (Mikelis et al., 2009) while the binding of PTN to αMβ2 on leukocytes is linked to inflammatory responses (Shen et al., 2017).

SDC3, also known as syndecan-3, was the first functional PTN receptor in brain neurons, necessary for PTN's interaction and control of synaptic plasticity. This interaction includes the heparan sulfate chains of SDC3 and needs the coordinated activity of both TSR-1 domains of PTN. Moreover, two other members of the family also bind to PTN, SDC1 and SDC4. However, their functions in PTN-mediated activities remain unclear. For SDC1, both heparan and chondroitin sulfate chains, as well as the core protein, contribute to its interaction with PTN (Papadimitriou et al., 2009; 2016).

NCL (Nucleolin) is indicated as a low-affinity PTN receptor on the cell surface, particularly on activated endothelium and cancer cells. It may interact with PTN indirectly through other molecules such as αvβ3 integrin and PTPRZ1, altering PTN signaling and nuclear translocation (Koutsoumpa & Papadimitriou, 2014). Another PTN receptor is Neuropilin-1 which binds PTN via its TSR-1 domains, leading to the activation of ERK1/2 kinase and increasing endothelial cell motility. This receptor plays a key role in the development of the neurological and circulatory systems (Elahouel et al., 2015). Lastly, VEGFR2 interacts preferentially with PTN in endothelial cells, where PTN suppresses VEGFA-induced phosphorylation of VEGFR2. This interaction alludes to the regulatory role of PTN in the VEGF signaling pathway (Lamprou et al., 2020).
4. Protein tyrosine phosphatase receptor zeta 1 (PTPRZ1)

Tyrosine residues on target proteins are dephosphorylated by protein tyrosine phosphatases (PTP), which can be found intracellularly or on the cell membrane. PTPRZ1 is a receptor-type PTP, which can interact with different ligands, among which PTN (Papadimitriou & Kanellopoulou, 2023). PTPRZ-A and PTPRZ-B are isoforms of PTPRZ1 that have tandem PTP domains (D1 and D2), with only the D1 domain having catalytic activity. The inhibition of PTPRZ1’s phosphatase activity is caused by PTN binding to its extracellular region. This has important ramifications for cellular processes like the differentiation of oligodendrocyte precursor cells (OPCs) and the myelination in the central nervous system (CNS). The processes underlying the ligand-induced inactivation of PTPRZ1 comprise a "head-to-toe" dimerization of its intracellular region, which obstructs the D1 domain's catalytic site and hence prevents it from functioning. The physiological roles of PTPRZ1, specifically in the regulation of OPC development and myelination, depend on this dimerization and subsequent inactivation (Fujikawa et al., 2019).

The involvement of PTPRZ1 is not limited to the CNS; it is also linked to some pathological disorders, such as cancer. When the receptor interacts with its ligands, like PTN, it can modify cellular signaling pathways that are important in cell survival, migration, and proliferation, which in turn can affect the formation and spread of tumors. The significance of PTPRZ1 in cellular signaling networks is further highlighted by its identification as a regulator of receptor tyrosine kinases (RTKs) phosphorylation (Lee & Bennett, 2015; Papadimitriou & Kanellopoulou, 2023).

4.1 PTPRZ1 expression and functions in cancer development

PTPRZ1 is involved in several biological processes, such as adhesion, migration, and the transition from epithelial to mesenchymal tissue. It is a significant molecule in the initiation and spread of cancer because of its involvement in the control of cancer stem cells and treatment resistance. To perform its functions within the tumor microenvironment, PTPRZ1 interacts with PTN, midkine, interleukin-34, β-catenin, VEGFA, NF-κB, HIF-2, PSD-95, MAGI-3, contactin, and ErbB4 (Wang et al., 2022; Fujiwara et al., 2015; Gonzalez-Sanches et al., 2022; Ma et al., 2022; Kastana et al., 2023; Koutsioumpa et al., 2015; Huang et al., 2018; Wang et al., 2010; Xia et al., 2019).
It has been established that PTPRZ1 is expressed in a variety of tumor tissues and that its function in survival signaling helps to predict the prognosis of some cancers (Xia et al., 2019; Papadimitriou & Kanellopoulou, 2023). Because PTN and other ligands of PTPRZ1 together promote carcinogenic pathways, the interaction between these molecules is of great interest. As an illustration, PTN binding to PTPRZ1 may initiate downstream signaling pathways that promote tumor development and metastasis. In the case of glioblastoma, where PTN and PTPRZ1 are frequently overexpressed and add to the tumor's aggressiveness, this interaction is essential (Xia et al., 2019). Furthermore, PTPRZ1's involvement in cancer goes beyond how it interacts with growth factors. Additionally, it has a role in controlling intracellular signaling pathways that are crucial for cell survival and proliferation, like those mediated by NF-κB and β-catenin. PTPRZ1's potential as a therapeutic target is highlighted by its involvement in these pathways, as modifying its activity may affect the tumor's growth and resistance to treatment (Papadimitriou & Kanellopoulou, 2023).

Its significance is further highlighted by the prognostic significance of PTPRZ1 expression in cancer. Elevated PTPRZ1 has been linked to a bad prognosis in several malignancies, indicating that it may function as a biomarker for aggressive illnesses and may direct treatment choices (Chen et al., 2015). Research is currently being conducted to develop therapeutic drugs that specifically target PTPRZ1, to reduce its carcinogenic properties and enhance patient outcomes.

Human neuroendocrine tumor (NET) tissues express PTPRZ1, and PTPRZ1 protein has been shown to have a critical oncogenic function in the advancement of small cell lung cancer (SCLC) in mouse xenograft models (Makinoshima et al., 2012). Furthermore, the same study reveals that PTPRZ1 controls the tyrosine phosphorylation of calmodulin (CaM) in response to PTN stimulation in SCLC cells, indicating a crucial connection between PTPRZ1 activity and CaM phosphorylation. These data suggest that PTPRZ1 may be a useful target for therapy in both NETs and SCLC.

The blood-brain barrier (BBB) makes it difficult for drugs to be delivered to patients with glioblastoma (GBM), a highly aggressive brain tumor with a poor prognosis. However, there is a promising therapeutic approach based on the PTN-PTPRZ1 signaling axis that uses an inventive nanocarrier to penetrate the BBB, precisely target the tumor microenvironment, and work in concert with traditional GBM chemotherapeutic agents (Yang et al., 2023). Furthermore, PTPRZ1-MET and MET exon 14 skipping have been
highlighted as possible therapeutic targets, in addition to the significance of thorough DNA and RNA sequencing in brain metastases (Chai et al., 2022).

Fu et al. (2016) investigated the expression of PTPRZ1 and PTN in 325 cases of breast cancer, including luminal A, luminal B, Her-2-enriched, TNBC, and relapsed TNBC (RTNBC), using a retrospective immunohistochemical analysis. The RTNBC group had considerably higher expression levels of PTPRZ1 and PTN in comparison to other subtypes, as per the findings. Furthermore, PTPRZ1 expression varied significantly across the TNBC, breast cancer (BC), and control groups, whereas PTN expression varied between the BC and RTNBC groups. Notably, there was no discernible relationship between the expression of CD24 and CD44, tumor size, menopausal status, molecular subtypes, and PTPRZ1/PTN expression. All things considered, elevated PTPRZ1 expression could function as a stand-alone risk factor for TNBC metastasis and recurrence.

In a study by Kastana et al. (2023) simulating lung adenocarcinoma (LUAD) development in smokers using an animal model, it was revealed that lower expression of the protein tyrosine phosphatase receptor type Z1 (PTPRZ1) corresponds with increased tumor growth and angiogenesis, as well as poorer survival in animals. These findings are confirmed by TCGA data which also demonstrate that decreased PTPRZ1 expression is related to poorer patient survival in LUAD patients (Xia et al., 2019). Immunohistochemistry and data from the Human Protein Atlas suggest that PTPRZ1 is largely expressed in club and respiratory cells in the adult lung, which are crucial in the development of LUAD following chemical exposure (Spella et al., 2019). Additionally, mutations or epigenetic alterations such as hypermethylation of the Ptprz1 gene promoter, similar to those reported in colorectal malignancies, can suggest a poorer prognosis for LUAD (Laczmanska et al., 2013).

Studies have identified a contradictory expression pattern of PTPRZ1 in osteosarcoma and colorectal cancer. PTPRZ1 is variably expressed in osteosarcoma patients, with roughly 73% of patient samples exhibiting overexpression and 27% showing under-expression, with no obvious association with patient outcomes or clinicopathological characteristics (Toledo et al., 2010). Intriguingly, mouse model research revealed that deletion of the Ptprz1 gene can increase osteosarcoma growth through improved tyrosine phosphorylation and cell proliferation, putting PTPRZ1 possibly as a tumor suppressor in this kind of cancer (Baldauf et al., 2015). On the other hand, colorectal malignancies often display a drop in PTPRZ1 mRNA levels relative to neighboring normal mucosa, with additional research employing bioinformatics and promoter methylation analysis confirming the idea of PTPRZ1 being a
downregulated gene in these tumors (Yamakawa et al., 1998; Laczmanska et al., 2013; Xia et al., 2019). This downregulation has been related to lower overall and disease-free survival, making PTPRZ1 a potential for future research as a biomarker and a target for innovative treatment techniques in the management of colorectal cancer.

4.2 PTPRZ1 interactions related to activation of tumorigenic cascades

PTPRZ1 interacts with a variety of ligands and participates in several signaling pathways, which are important for the activation of tumorigenic cascades. The regulation of cellular activities like survival, migration, and proliferation—many of which are dysregulated in cancer—depends on these connections.

PTN is one of the main partners of PTPRZ1. The phosphatase activity of PTPRZ1 is inactivated when PTN binds to it, which causes downstream signaling molecules including β-catenin and the mitogen-activated protein kinase (MAPK) pathway to become phosphorylated and activated. In the case of GBM, where PTN and PTPRZ1 are frequently overexpressed and add to the tumor's aggressiveness, this interaction is important (Xia et al., 2019). MK also interacts with PTPRZ1 and stimulates tumor-promoting signaling pathways, including the PI3K/Akt pathway, which controls cell survival and proliferation (Baldauf et al., 2015).

The PTPRZ1-interleukin-34 (IL-34) interaction also plays a role in the carcinogenic process. IL-34 interacts with PTPRZ1 and syndecan-1, in addition to acting as a ligand for the CSF-1R receptor. Its functions include innate immunity, inflammation, and cancer, albeit it is yet unclear how specifically it contributes to cancer. Whereas IL-34 expression did not correspond with a positive prognosis in individuals with basal breast cancer, it did in those with luminal and HER2 breast cancer subtypes (Zins et al. 2018). It's interesting to note that myeloid cell infiltration was strongly correlated with the expression of the CSF-1 and CSF-1R genes; however, there was little to no link found between PTPRZ1, syndecan-1, IL-34, and myeloid cells. Cytokine IL-34 binds to PTPRZ1 and increases cancer cell survival and proliferation (Zwicker et al., 2016). This relationship is especially important when it comes to breast cancer, as expression of PTPRZ1 and IL-34 is linked to a worse prognosis and a higher risk of metastasis (Kajihara et al., 2020).

Moreover, PTPRZ1 controls the epithelial-to-mesenchymal transition (EMT), a crucial step in the spread of cancer. By interacting with molecules like NF-κB and β-catenin, PTPRZ1
can activate signaling pathways that support EMT and increase the invasiveness of cancer cells. The function that PTPRZ1 plays in the progression and spread of cancer is highlighted by its modulation of EMT. A further component of PTPRZ1’s role in carcinogenesis is its participation in treatment resistance.

PTPRZ1 is implicated in numerous pathways linked to cell adhesion and migration via interacting with other angiogenic molecules, such as VEGFA (Koutsioumpa et al., 2015). VEGFA seems to bind to the protein core of PTPRZ1 (Choleva et al., 2023) and PTPRZ1 is required for its stimulatory effect on endothelial cell migration. FGF-2 (fibroblast growth factor 2) also binds to the protein core of PTPRZ1, but the functional significance of this interaction remains unknown (Papadimitriou and Kanellopoulou, 2023).

4.3 Therapeutic agents that target PTPRZ1

Given PTPRZ1's important involvement in carcinogenesis, the discovery of therapeutic drugs targeting PTPRZ1 is a growing topic of interest in cancer therapy.

Targeting the PTN-PTPRZ1 connection is one strategy, as it is essential for the activation of tumorigenic pathways. This connection can be disrupted by inhibitors or neutralizing antibodies, which may reduce the oncogenic signaling mediated by PTPRZ1. It was found that an antibody against the extracellular PTPRZ1 domain in GBM stem cells inhibits PTN binding and hence decreases GBM development in mice, resulting in longer survival (Shi et al., 2017). Moreover, in research examining targeted therapeutics for ovarian cancer, siRNA-mediated suppression of PTN and PTPRZ1 in epithelial ovarian cancer cell lines elicited considerable apoptotic cell death, suggesting their potential as therapeutic targets (Sethi et al., 2015).

The most common intraocular malignancy in adults, uveal melanoma (UM), presents considerable treatment problems because it tends to metastasize, is aggressive, and has frequent recurrence. Li et al. (2022) have examined the potential therapeutic benefits of targeting UM Formation-Transcript 1 (OUM1) and its downstream target gene PTPRZ1 in UM tissues and cells. They clarified the regulatory process via which OUM1 influences the course of UM by modifying the activity of the tyrosine phosphatase of PTPRZ1. By specifically knocking down OUM1 and PTPRZ1, targeting the OUM1/PTPRZ1 pathway with siRNAs decreases PTP activity, tampers with protein tyrosine phosphorylation, inhibits UM proliferation and metastasis, and increases susceptibility to cisplatin.
The maintenance of cancer stem cell features has been linked to elevated levels of PTPRZ-B, an isoform of PTPRZ, which was seen in sphere-forming cells produced from GBM lines. Reduced PTPRZ expression was shown in knockdown studies to impact key stem cell transcription factors and decrease the capacity of these cells to form spheres, underscoring PTPRZ's critical involvement in maintaining cancer stem cell characteristics (Fujikawa et al., 2017). As dual-targeting drugs inhibit two or more tumorigenesis-related targets at once, they present a promising option for cancer therapy. With PTPRZ1's participation in numerous signaling pathways and interactions with ligands, designing medications that target PTPRZ1 and other important molecules in cancer pathways may improve treatment outcomes and get around resistance mechanisms (Singh et al., 2019).
AIM OF STUDY
Angiogenesis refers to a complex, dynamic process through which the formation of most new blood vessels is achieved. The angiogenic process involves the participation of endothelial cells through critical processes that contribute to their proliferation, migration, and maturation (Eelen, 2018). Angiogenesis is also directly related to cancer. In particular, the growth of tumors requires the formation of new blood vessels through which the supply of oxygen and nutrients is achieved (Maishi, 2019). As a tumor grows, the lack of oxygen in its inner mass due to insufficient or dysfunctional vasculature reduces oxygen availability and consumption, creating a hypoxic environment (Jain, 2005), which activates pro-angiogenic signaling pathways that have an important regulatory role in cancer development, angiogenesis, and progression (Liu, 2018; Du, 2019).

Key regulators of response to the hypoxic conditions are the Hypoxia-Inducible Factors, HIFs. HIFs are heterodimers composed of two proteins: an oxygen-sensitive α-subunit whose stabilization is regulated by oxygen levels and a constitutively expressed β-subunit (Semenza, 2003). In mammals, three isoforms of HIFα-subunits have been identified. HIF1α and HIF2α are structurally similar and are well-studied, while less is known about HIF3α, whose action seems to inhibit HIF1α and HIF2α. In hypoxic conditions, the α subunit is stabilized and translocated to the nucleus, forming a heterodimeric complex with the β subunit. This complex constitutes the active transcription factor HIF, which binds to hypoxia response elements upstream of their target genes. HIFs induce the transcription of pro-angiogenic factors, such as Vascular Endothelial Growth Factor A (VEGFA), basic fibroblast growth factor (bFGF or FGF2), angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Platelet-Derived Growth Factor B (PDGFB), Transforming growth factor beta (TGFβ), thus highlighting the association of hypoxia with angiogenesis (Skuli, 2009; Oh H, 1999). HIF1α and HIF2α might regulate angiogenesis by activating distinct sets of target genes. HIF1α mediates the expression of genes involved in vascular endothelial growth, ECM reorganization, and endothelial cell migration, while HIF2α mediates vascular endothelial remodeling and cell-cell adhesion. (Fengyan2019).

Pleiotrophin (PTN) is a secreted growth factor of molecular weight 18 kDa. It belongs to a family of proteins with a high binding affinity to heparin. PTN is overexpressed in various human cancers and promotes cancer angiogenesis in vitro and in vivo, as well as expansion, and metastasis of tumor cells. PTN exerts its biological action by binding to various receptors that include Protein Tyrosine Phosphatase Receptor zeta 1 (PTPRZ1), Anaplastic Lymphoma Kinase (ALK), αβ3 integrinand nucleolin (Papadimitriou 2016;
PTN expression has been reported to increase in response to hypoxia in rat hepatic stellate cells (Antoine et al., 2005). Previous studies of our research group have shown that chemically-induced hypoxia, achieved by the administration of dimethylxallylglycine (DMOG or cadmium chloride, increases the expression and secretion of PTN in human endothelial cells (Droggiti, MSc thesis, 2015; Poimenidi, Ph.D. thesis, 2016). Acute hypoxia also increased PTN mRNA and protein levels in human endothelial cells in a HIF-dependent manner, although the ptn promoter does not contain any of the known HIF response elements (Poimenidi, Ph.D. thesis, 2016). The functional significance of the hypoxia-induced up-regulated PTN expression in endothelial cells is unknown.

PTPRZ1 is a transmembrane tyrosine phosphatase and appears in three forms, a full-length (chondroitin sulfate proteoglycan) transmembrane isoform, a short transmembrane isoform, and a secreted isoform that corresponds to the extracellular domain of the full-length transmembrane isoform (Papadimitriou and Kanellopoulou, 2023). The involvement of PTPRZ1 in endothelial cell migration stimulated by PTN (Polykratis et al., 2005), nitric oxide (NO) (Polytarchou et al., 2009), aprotinin (Koutsioumpa et al., 2009) and VEGFA (Koutsioumpa et al., 2015) suggests a role of the receptor in angiogenesis. By binding to PTPRZ1, PTN leads to the activation of signaling pathways, such as c-Src, FAK, and PBK kinases (Polykratis et al., 2005). PTPRZ1 and αvβ3 integrin interact to form an active complex on the membrane of endothelial cells that mediates PTN-induced β3 Tyr773 phosphorylation, through which c-Src is activated (Mikelis et al., 2009). The same pathway seems to be also activated by VEGFA (Koutsioumpa et al., 2015). Following chemically-induced hypoxia, protein levels of PTPRZ1 are increased in endothelial cells (Droggiti, MSc thesis, 2015). PTPRZ1 is preferentially up-regulated by HIF2α (Wang, 2005), at least partly due to the cooperative binding of HIF2α and the E26 transformation-specific (Ets) factor ELK1 to nearby sites on the PTPRZ1 promoter region. (Wang et al., 2010). Similarly to PTN, the functional significance of the hypoxia (HIF)-induced up-regulated PTPRZ1 expression in endothelial cells is not known.

Based on the above, the present MSc thesis aimed to study the role of PTN and PTPRZ1 in hypoxia-induced endothelial cell migration by primarily using endothelial cells isolated from mice which are knock-out for the ptn gene (PTNko) and their corresponding wild-type mice (PTNwt), as well as endothelial cells derived from mice knock-out for the ptprz1 gene (PTPRZ1ko) and their corresponding wild type mice (PTPRZ1wt). Human umbilical vein
endothelial cells (HUVEC) and a selective pharmacological inhibitor of the tyrosine phosphatase activity of PTPRZ1 have been also used to investigate the potential role of the tyrosine phosphatase activity of PTPRZ1.
Materials and Methods
6.1 Cell cultures

Human umbilical vein endothelial cells (HUVECs), endothelial cells derived from mice lungs lacking ptprz1 gene (knock-out) or expressing ptprz1 gene (wild type) and lacking ptn gene or expressing ptn gene, were grown in cultures that were used in this study. Therefore, the full culture medium that was used was intended exclusively for endothelial cells grown. All cell cultures were kept at 37°C, with 5% CO₂, and maintained at 100% humidity.

Materials and Solutions

Culture medium M199 or Dulbecco’s modified Eagle medium (DME)

Penicillin-Streptomycin 100 U/ml
Gentamicin 50 µg/ml
Amphotericin 2.5 µg/ml
L-glutamine 2 mM

In culture mediums added, under sterile conditions, the antibiotics listed above. Penicillin-streptomycin and Gentamicin prevent bacterial growth, while amphotericin prevents fungus growth. Hereafter, when culture medium is mentioned, it will correspond to this medium, unless otherwise clarified.

Full culture medium for endothelial cells:

M199 85% v/v or DMEM 90% v/v
Fetal Bovine Serum (FBS) 15% v/v
Endothelial Cell Growth Supplement (ECGS) 150 µg/ml
Heparin 5 U/ml

ECGS is enriched in fibroblast growth factor (FGF) and is mandatory for efficient EC growth. Heparin is used for the activation of ECGS. The dissolution of ECGS demands incubation of the medium in a water bath at 37°C for 30 minutes with a light shaking every 10 minutes. The final step involves the filtration of the medium through a sterilization filter (0.2 μm pore diameter) using a syringe. The medium is stored at 4°C.
6.2 Cell recultivation

Materials and Solutions

PBS (1X) pH 7.2-7.4:

Na₂HPO₄ 10 mM
KH₂PO₄ 2 mM
NaCl 137 mM
KCl 2.7 mM

Before using PBS 1X on the cells, it is sterilized using an autoclave (121°C for 20 minutes) and then stored at room temperature.

Full culture medium for endothelial cells

As described in paragraph 1

Trypsin EDTA

0,02-0,05% v/v in PBS Ca²⁺ free

Hemocytometer Neubauer

Pipette Pasteur

Always used sterile.

Gelatin solution

Gelatin solution 2% w/v which is diluted before use, to a final concentration of 1% with sterile PBS 1X. Stored at 4°C.

Experimental procedure

For the culture of endothelial cells, it is necessary to coat the plate to be used with a 1% gelatin solution. After coating, the plates are transferred to the incubation chamber and remain there for at least 20 minutes. During this time, the substrate: gelatin, binds to the bottom of the plates so that the cells can then adhere to the bottom. The incubation time follows a quick wash with PBS 1X solution to remove excess gelatin and the process of recultivation begins.

1. Initially, the cells are observed under the microscope, in order to determine the percentage of coverage of the plate. The reculturing process is carried out when the cells have covered 80-90% of the surface of the plate. Cell morphology was also studied, as it is indicative of their condition. It is therefore assessed whether the cells are healthy and can be studied in upcoming experiments.
2. The plate is then transferred to a nematic fluid flow chamber, as all cell handling procedures must be carried out under sterile conditions. The culture medium is aspirated under vacuum with a Pasteur pipette.

3. The cells are then washed two or three times with PBS 1X solution to remove the remains of FBS, which contains trypsin inhibitors and, if not removed, will affect the cell detachment process.

4. Apply 1 ml of trypsin per 100 mm plate diameter or 0.5 ml of trypsin per 60 mm plate diameter and then incubate the cells in the incubation chamber for 3 minutes for trypsin to maximize its action. Trypsin is a proteolytic enzyme that breaks down the connections between cells, or cell-substrate junctions. In case of non-detachment of the cells, the incubation time with trypsin can reach up to 10 minutes. After this time, trypsin can cause toxicity.

5. The cells are observed under the microscope, and in case of successful detachment, they are distinguished as spherical structures.

6. The plate is transferred to the nematic flow chamber where 1 mL of full culture medium containing 10% FBS is added. In this step, the medium is added to inhibit trypsin activity.

7. Cells are collected in a sterile 15 mL falcon and the cell solution is centrifuged for 5 minutes at 500 g at room temperature.

8. The supernatant is aspirated with a pipette. The cell pellet is then resuspended using a full culture medium twice the volume of the added trypsin (step 4). The handling of the cells at this point must be done in such a way as to ensure as much as possible a uniform distribution of the cells throughout the volume of the suspension.

9. From this solution and using a pipette we take 10 µL which we place in the special inlet of the hemocytometer. From the same point and in the same way, a second volume of 10 µL is taken, which is placed in a second special slot in the hemocytometer. Count the number of cells under the microscope at 10X magnification.

10. After counting the cells, calculations are performed to determine the number of cells that must be transferred from the suspension to the plate for reculturing. For each reculture of endothelial cells, 800,000 cells/mL and 300,000 cells/mL of full culture medium are obtained for 100mm and 60mm plates, respectively.

*Note 1: Endothelial cells used in the present study were recultured up to the P4 generation.*

*Note 2: The number of cells mentioned above and/or the medium to be used are varied in cases where cells are used in specific experiments.*
6.3 Isolation of human umbilical ECs

Endothelial cells in this study were isolated from human umbilical cords which provided by the Department of Obstetrics of the University Hospital of Patras. The umbilical cord was embathed with sterile PBS1X in order to prevent infections until the isolation of endothelial cells occurs in the laboratory.

Materials and Solutions

**PBS (1X) pH 7.2-7.4:**

- Na₂HPO₄ 10 mM
- KH₂PO₄ 2 mM
- NaCl 137 mM
- KCl 2.7 mM

Before using PBS 1X on the cells, it is sterilized using an autoclave and then stored at room temperature.

**Collagenase 1% w/v**

100 mg type 1A Collagenase in 10 ml serum-free M199 or DMEM medium

The initial collagenase solution is divided into smaller portions ( aliquots ) and preserved at a temperature of -20°C.

**Culture medium M199 or DMEM**

As described in paragraph 1

**Full culture medium for endothelial cells**

As described in paragraph 1

**1% Gelatin solution**

As described in paragraph 2

Experimental procedure

1. A portion of the collagenase stock solution is diluted 100 times in serum serum-free medium containing Ca²⁺ ions, and therefore collagenase final concentration is 0.01%. Ca²⁺ ions act as activators for enzymatic activity, and fetal bovine serum (FBS) is excluded due to its collagenase-inhibitory properties.

2. The umbilical cord is comprised of two arteries and one vein, with the umbilical vein being readily identifiable due to its larger diameter. To eliminate excess blood and clots, a
20ml syringe is introduced at one end of the vein. Subsequently, the vein is flushed with PBS 1x until no traces of blood are observable in the eluate. Maintaining the integrity of the vein walls is essential to advance to the subsequent step.

3. The free end of the umbilical cord is clamped using hemostatic forceps.

4. The collagenase solution is meticulously injected into the other end of the vein using a syringe, taking care to prevent the formation of air bubbles.

5. Once the vein is fully infused with the collagenase solution, the same end of the umbilical cord is clamped with the hemostatic forceps.

6. The umbilical cord is then carefully positioned in a sterile jar and subjected to incubation for 20-25 minutes at 37°C.

7. After the incubation follows the removal of the hemostatic forceps from one end of the umbilical cord, collecting the eluate in a sterile 50 ml falcon tube.

8. Remove the second pair of hemostatic forceps. Flush the vein with PBS 1x, collecting the eluate in the same tube as before.

9. Centrifuging of the eluate at 500 g for 4 minutes at room temperature is then followed. The supernatant is then discarded and the cells are resuspended in 10ml of full culture medium.

10. Cells are plated in a 60mm culture plate and incubated overnight at 37°C, 5% CO2, and 100% humidity.

11. The next day, the cells are washed twice with PBS 1X, and fresh full culture medium is added. This step removes erythrocytes and non-adherent cells from the culture.

12. Cells are observed under an optical microscope. Endothelial cells at this point have a very characteristic form and more specifically at the bottom of the plate form clusters.

13. Incubation of the cells until reaching 80-90% confluency. Full culture medium must renewed every three days.

Note: Before adding the cell suspension, the plates were incubated with 1% gelatin solution, as described in paragraph 2.

### 6.4 Counting cells with a hemocytometer Neubauer

Counting cells with a hemocytometer is the simplest and most direct way of cell measurement. A hemocytometer is a slide whose two surfaces are smooth and properly treated. In each of them and with the use of a microscope we distinguish, as can be seen in Figure 1, a square grid. This grid consists of 9 individual squares, with a side length of 1 mm (square area 1 mm²). Each of these squares is defined by three parallel lines which are at a distance of 2.5 mm from each other. These lines determine the position of the cells.
inside or outside the grid. The level of the mesh is 0.1 mm lower than the two ridges on which the cover lays. Between the points on which the coverslip rests and the outer side of each squared surface, with the help of a pipette, the cell suspension is placed, which spreads over each squared surface due to capillary effects. The volume of cell suspension covering each of the nine squares is 0.1 mm³ (1mm² x 0.1mm) or 1 x 10⁻⁴ ml. So the concentration of cells in the suspension, expressed as a number of cells/mL is:

Number of cells in one of the central squares X 10000.

Figure 1: Hemocytometer Neubauer. The method of measuring cells is presented schematically.

6.5 Cell freezing

Long-term preservation of animal cells preserved in liquid nitrogen tanks, maintaining temperatures ranging from -195°C to -210°C. For a shorter period of time, the cells can be stored at -80°C. Before freezing, cells must be in a healthy state and achieve 80-90% confluency. It is also important that cooling is achieved gradually. For this reason, a special device containing isopropanol is used and gradually reduces the temperature by 1°C/h until a critical temperature point of -20°C. To prevent ice crystal formation and water loss during cell freezing, dimethyl-sulfoxide (DMSO) is used as a cryoprotectant.

Materials and Solutions

Dimethyl- sulfoxide (DMSO)

FBS

Cryovials
Special collector for cooling cells with isopropanol

Dewar container with special inlets for storing cryovials

Liquid N\textsubscript{2} tanker

**Experimental procedure**

1. Cells are trypsinized, as described in paragraph 2

2. Cell resuspension is centrifuged at 500 g for 5 minutes at room temperature. The supernatant is aspirated. The pellet was resuspended using 950 µL FBS.

3. Cell solution is transferred in cryovials which are noted the cell type, cell generation, and the date that the procedure took place.

4. 50 µL of DMSO is placed in the cell solution, in the cryovials. The addition of DMSO includes quick movements. More specifically, DMSO when added is piped up and down and then follows an immediate transfer of the cryovials in an isopropanol chamber due to the toxicity of concentrated DMSO in the cells. The concentration of DMSO should not exceed 20% in the final solution.

5. Placing the chamber at -80° C.

The following steps are for cases where we want to achieve cell storage for long periods of time. If these cells are to be used in future experiments (a few months), the freezing process can be stopped at this step.

6. Placing the collector at -80° C for 24h. In this way, the temperature reduction is achieved gradually (1°C/h).

7. Finally, the cryovial is placed in special inlets in liquid nitrogen for as long as required.

*Note: In the present study only ECs were cultured and therefore the above procedure applies exclusively to endothelial cells.*
6.6 Cell thawing

The thawing process is carried out to reuse the cells that had been stored in nitrogen. Thawing is performed under sterile conditions and rapidly to avoid remaining the cells in DMSO.

Materials and Solutions

Culture medium M199 or DMEM
As described in paragraph 1

Full culture medium for endothelial cells
As described in paragraph 1

Pipette Pasteur
Autoclaved

Experimental procedure

1. 9 mL of serum-free culture medium is added to 15 ml falcon. Remove the cells to be thawed from the liquid nitrogen and transfer them, under sterile conditions, to the 15 ml falcon. This step is performed to dilute the content of DMSO in the medium where the cells are.

2. The cell solution is then centrifuged at 500 g for 5 minutes at room temperature.

3. The supernatant is aspirated, and the pellet is resuspended using 1 mL of full culture medium.

4. The suspension is then transferred to a 100 mm culture plate in which a full culture medium was previously placed. The cells, after being observed under the microscope, are placed in the incubation chamber and allowed to grow.

5. Until the cell culture covers 80-90% of the surface of the plate, the full culture medium is changed every three days.

Note: In the present study endothelial cells were used and therefore before adding the culture medium, the plate was previously coated using 1% gelatin solution as described in paragraph 2.

6.7 Stimulation of cells with chemical reagents for determination of EC proliferation

The incubation of the cells with chemical reagents aims to study the effect of each added factor on the angiogenic functions of the cell. The functions studied in this project were the proliferation and migration (described below in the ‘‘Transfilter assay’’ chapter) of
endothelial cells. The added chemical reagents cause, by a different mechanism, the stabilization of HIFs whose signaling cascade mediates the effects of hypoxia. Before the effect with chemical reagents, the cells are cultured and they are allowed to grow until they have covered 70-80% of the surface of the plate (confluency).

Before effecting the cells are incubated for 16-18 hours in a serum-free medium. FBS and other components contained in serum are known to stimulate protein expression and induce specific cell functions. Therefore this step ensures that the observed effect is due to the presence of the agent under study, and in our case, as a result of hypoxic conditions. In addition, the incubation in a serum-free medium ensures that at the time of the effect, the cells will be in the same stage of the cell cycle.

**Materials and Solutions**

**PBS (1X) pH 7.2-7.4:**

\[ \text{Na}_2\text{HPO}_4 \text{ 10 mM} \]

\[ \text{KH}_2\text{PO}_4 \text{ 2 mM} \]

\[ \text{NaCl} \text{ 137 mM} \]

\[ \text{KCl} \text{ 2.7 mM} \]

Before using PBS 1X on the cells, it is sterilized using an autoclave and then stored at room temperature.

**Culture medium M199 or DMEM**

Supplemented with 0.25% BSA

**Full culture medium for endothelial cells**

**Chemical reagents**

Desferrioxamine mesylate (dfx):

The stock solution, diluted in distilled water, 20 mM concentration is prepared. The solution is divided into smaller portions (aliquots) and preserved at a temperature of -20° C.

Dimethyloxaglycine (dmog):

The stock solution, diluted in DMSO, 100 mM concentration is prepared. The solution is divided into smaller portions (aliquots) and preserved at a temperature of -20° C.

**Experimental procedure**

1. Full culture medium is aspirated when cells have reached 70-80% confluency, and cells are incubated overnight with culture medium supplemented with 0.25% BSA.

2. The culture medium is removed, and the cells undergo two washes with PBS 1X.
3. The cells are exposed to chemical reagents for a duration of 24 hours.

4. The culture medium is removed and the cells' recultivation steps are repeated until the step in which we determine by direct counting the cell number in the suspension of the cells, using a hematocytometer Neubauer.

6.8 Protein extraction from endothelial cell cultures

Protein extraction procedure from frozen and non-frozen eukaryotic cell cultures is used prior to techniques that demand specific amounts of protein concentration in the samples (e.g. protein analysis by Western)

Materials and Solutions

PBS (1X) pH 7.2-7.4:

- Na$_2$HPO$_4$ 10 mM
- KH$_2$PO$_4$ 2 mM
- NaCl 137 mM
- KCl 2.7 mM

Before using PBS 1X on the cells, it is sterilized using an autoclave (121°C for 20 minutes) and then stored at room temperature.

RIPA buffer (pH 7.4)

- PBS 1X
- Sodium dodecyl sulfate (SDS) 0.1%
- Triton X-100 1%
- PMSF 1 mM
- EDTA 5 mM
- Aprotinin 1μg/ml
- Na$_3$VO$_4$ 20 nM

Pipette Pasteur

Experimental procedure

1. The culture medium from adherent cells is meticulously aspirated using a pipette Pasteur.

2. Cells undergo three washes with cold PBS 1X.
3. Addition of either 600 μl or 300 μl of cold RIPA buffer to a 100 mm or 60 mm culture plate respectively. The plate is placed on ice for 10 minutes, with occasional swirling to ensure even distribution of the RIPA buffer.

4. The lysate is collected using a cell scraper and then transferred to a tube.

5. Centrifugation of the samples at 20,000 g for 30 minutes at 4°C is then followed.

6. The supernatants (where proteins are) transferred to new collecting tubes.

7. Determination of the protein amount using the Bradford assay.

6.9 Bradford protein assay

The Bradford protein assay is an easy and highly reliable spectroscopic analytical method employed for determining the protein concentration in a solution. This procedure relies on the interaction between Coomassie G-250 dye and the basic amino acids present in proteins, leading to an absorbance shift. The observed alteration in optical density is directly correlated to the protein concentration in the solution. Standard curves are generated by graphing the absorbance values of known concentrations of bovine serum albumin (BSA) samples.

Materials and Solutions

Bradford solution (1x)

Coomasie G-250 0.1 mM
95% ethanol 5% v/v
85% H₃PO₄ 10% v/v

The solution is filtered three times using filter paper.

Spectrophotometer

Hitachi U-1100

Experimental procedure

1. Firstly, the spectrophotometer is powered on at least 30 minutes before the measurement takes place and calibrated to 595 nm.

2. 8μl of the protein solution is mixed with 1992 μl of Bradford solution. A solution without proteins serves as the blank.

3. Samples are roughly vortexed and subsequently incubated for five minutes at room temperature.
4. The spectrophotometer is blanked using the solution without proteins (blank), and the absorbance is read at 595 nm.

5. The protein concentrations of the unknown samples are determined based on the extinction coefficient derived from the standard curve.

6.10 Protein analysis by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE)

The basic principle on which the technique is based is the movement of charged molecules, such as proteins, in an electric field in order to separate them. The movement of the molecules depends on three factors: the strength of the electric field (E), the net charge of the protein (z), and the coefficient of friction (f). The gel acts as a molecular sieve and is a three-dimensional network of long aliphatic polyacrylamide chains. Smaller molecules move more easily and quickly through the pores of the gel, while larger molecules move more slowly.

**Materials and Solutions**

**Acrylamide 30% w/v**

Acrylamide 30 g

Methylenebisacrylamide 1 g

Volume is adjusted to 100ml with distilled water

**Tris-HCl buffer 1.5M, pH 8.8**

Tris-base 18.165 g

Distilled water 80 ml

pH is adjusted to 8.8 and the volume is filled to 100 ml with distilled water

**Tris-HCl buffer 0.5M, pH 6.8**

Tris-base 6.055 g

Distilled water 80ml

pH is adjusted to 6.8 and the volume is filled to 100ml with distilled water

**Sodium Dodecyl Sulfate solution (SDS) 10% w/v**

SDS 10 g

Distilled water 90ml

The solution is heated at 68° C and the volume is adjusted to 100 ml with distilled water
Ammonium persulfate (AMPS) 20% w/v
Dissolved in distilled water

Laemmli sample buffer (2X)
Tris-HCl pH 6.8 0.05 M
SDS 0.03 M
Glycerol 10% v/v
Bromophenol blue 2% v/v
Prior to use β-mercaptoethanol 10% v/v. is added

Running buffer (5X)
Glycine 2M
Tris-base 0.25M
SDS 0.02M
Prior to use the buffer is diluted 5 times with distilled water

Experimental procedure

1. First, the device- gel cassette- in which the gel will be placed is assembled.

2. The protein separation gel is prepared (separating gel). The recipe to be carried out for its preparation is determined by the concentration of acrylamide which is determined based on the molecular weight of the protein under study. For low molecular weight proteins, a high acrylamide concentration gel is chosen (Table M1).

3. Then the separation gel is placed in the device, and more specifically, it is poured into the intermediate area between the two glass plates. Then 1-2 mL of distilled water is added on the top of the gel. Handling in this step must be carried out softly. Adding distilled water prior to polymerization facilitates the formation of a straight level in the gel and removes air bubbles that might have formed. Wait until the polymerization of the gel is complete.

| Polyacrylamide gel | 17.5% | 15% | 7.5% | 6.2% | 5% |
4. The protein stacking gel is then prepared. Its concentration depends on the concentration of the separating gel (Table M2). Generally, TEMED acts as a catalyst for the polymerization initiated by AMPS. So, these two components are added after we discard the water from the surface of the separation gel. Once the separation gel has been polymerized, we remove excess water by absorption, using filter paper. After we finish the preparation of stacking gel, we also add it to the glass plates from a certain point, at a constant rate. When its level reaches the edge of the device, we place a component: a well-forming comb, which creates the sample addition positions.

5. Stacking gel is left to polymerize for 10-20 minutes.

6. The samples to be analyzed are prepared. They are first heated at 90°C for 5 minutes and then centrifuged at 13,000 g for 1 minute at room temperature.

7. After polymerization of the stacking gel, the well-forming comb is gently removed and the wells are carefully rinsed with distilled water in order to remove pieces of acrylamide. Then, the glass plates are placed in the electrophoresis apparatus in which we add electrophoresis buffer 1X.

8. Loading a specific amount of samples, depends on the size of the plate (where the cells are grown) from which the extract solution was carried out. In this step, the loading in the well is performed by using a Hamilton syringe.
9. A constant voltage of 100 Volt, is applied in the electrophoresis tank, to create an electric field that allows protein to migrate toward the positive charged electrode (anode). Proteins move from the negative to the positive pole, due to their negative charge. The distance each protein will travel in the gel is inversely proportional to its molecular mass.

10. After the completion of electrophoresis, western analysis follows.

**6.11 Protein Analysis by Western**

Western analysis is a technique used to detect proteins in a sample. This technique is performed after gel electrophoresis to separate the denatured proteins and involves transferring the proteins to a nitrocellulose or PVDF membrane. The proteins are then detected using specific antibodies.

**Materials and Solutions**

**Blotting buffer**
- Glycine 0.04 M
- Tris-base 0.05 M
- SDS 1 mM
- Methanol (absolute) 20% v/v

**TBS buffer pH 7.6 (10X)**
- Tris-base 0.2 M
- NaCl 1.36 M
- Distilled water

The buffer's pH is tuned to 7.6, and before utilization, the buffer undergoes a tenfold dilution with distilled water.
TBS-Tween 20 buffer, pH 7.6 (TBS-T)

TBS buffer (1X)

Tween-20 0,05% v/v

PVDF (polyvinylidene fluoride) membrane

Amersham Hybond P 0,45 μm PVDF

Nonfat dry milk (Regilait)

Bovine Serum Albumin (BSA)

Chemiluscent detection system

The integrated chemiluminescence system: SuperSignal West Pico PLUS was used

Primary antibodies

Primary antibodies and the conditions applied for Western blot analysis are detailed in Table M3 and M4 respectively

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Monoclonal antibody, Cell signaling</td>
</tr>
<tr>
<td>eNOS</td>
<td>Polyclonal antibody, Santa Cruz</td>
</tr>
<tr>
<td>β-actin</td>
<td>Monoclonal antibody, Santa Cruz</td>
</tr>
<tr>
<td>Vinculin</td>
<td>Polyclonal antibody, Santa Cruz</td>
</tr>
</tbody>
</table>

Table M3: The list of primary antibodies used for Western blot analysis is organized with columns indicating the protein-antigen and the manufacturer, reading from left to right.

Secondary antibodies

Secondary antibodies used in this project are outlined below. The conditions utilized for Western blot analysis are presented in Table M4

Anti-mouse IgG peroxidase-conjugated (Cell Signalling)

Anti-rabbit IgG peroxidase linked (Santa Cruz)

Anti-rabbit IgG peroxidase linked (Cell Signalling)
**Table M4:** The conditions employed for Western blot analysis of the protein of interest are presented in columns from left to right, indicating the protein-antigen, the reagents used for blocking, and the incubation conditions with both primary and secondary antibodies.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Blocking reagent</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>5% nonfat dry milk in TBS-T 0,05%</td>
<td>1:1000 in TBS-T 0,05% containing 5% w/v BSA</td>
<td>a-rabbit 1:2000 in TBS-T 0,05%</td>
</tr>
<tr>
<td>eNOS</td>
<td>5% nonfat dry milk in TBS-T 0,05%</td>
<td>1:1000 in TBS-T 0,05% containing 5% nonfat dry milk</td>
<td>a-rabbit 1:2500 in TBS-T 0,05%</td>
</tr>
<tr>
<td>β-actin</td>
<td>5% nonfat dry milk in TBS-T 0,05%</td>
<td>1:1000 in TBS-T 0,05%</td>
<td>a-mouse 1:2000 in TBS-T 0,05%</td>
</tr>
<tr>
<td>vinculin</td>
<td>5% nonfat dry milk in TBS-T 0,05%</td>
<td>1:1000 in TBS-T 0,05%</td>
<td>anti-rabbit 1:5000 in TBS-T 0,05%</td>
</tr>
</tbody>
</table>

**Experimental procedure**

1. After completion of electrophoresis (Paragraph 10) the separation gel and PVDF membrane are immersed in transfer buffer and allowed to incubate for 10 min. PVDF membrane must first be activated. Therefore, a quick wash (30 seconds) using methanol is performed before the incubation.

2. Separation gel is then placed in contact with the PVDF membrane, and this system (sandwich) is placed in a device to which a constant current of 0.1 A is applied for 30 min so that the negatively charged proteins are transferred from the gel to the membrane. The transfer time is proportional to the molecular size of the proteins to be transferred. The higher the molecular weight of a protein, the longer it takes for the proteins to be transported.

3. After the transfer is completed, the membrane is immersed in TBT-Tween buffer solution where three successive washes of 5 minutes are performed under shaking at room temperature.

4. Then saturation of the non-specific binding sites of the antibodies (blocking) by incubating the membrane in a buffer solution at room temperature under shaking. This buffer solution is prepared using either 0.05% TBS-Tween or 0.1-3% BSA or 5% nonfat dry milk. The choice of buffer is based on the antibody chosen to be used next. The incubation time ranges from 1-3 h, depending on the protein under study. In cases where a higher level of non-specific binding is detected, the process can be blocked for 16 hours under shaking at 4°C.

5. Incubation of the membrane with the appropriate first antibody follows. First antibody binds specifically to the protein under study. The first antibody is diluted in TBS-Tween buffer and the incubation time is 16 hours at 4°C with shaking.
6. After incubation the first antibody solution is returned and stored at -20°C where it can be reused. The membrane is washed three times for 5 minutes with 0.05% TBT-Tween, under shaking at room temperature.

7. Incubate with an appropriate second antibody that recognizes and binds to specific epitopes of the first antibody. Signal amplification is achieved. The secondary antibody is diluted appropriately with TBS-Tween. Incubation is done at room temperature for one hour, with shaking.

8. Shedding of the second antibody. Five-minute washes are carried out on the membrane under shaking. The first three washes were performed with TBS-Tween solution and the last two with 1X TBS. Those steps were carried out in order to remove tween.

9. For the immunolabeling of the protein under study, the chemiluminescence (ECL) system is used. The immunoprint of the membrane protein is shown on a film in a darkroom. The ECL contains the horseradish peroxidase enzyme substrate which is conjugated to the second antibodies. In the chemiluminescence reaction, peroxidase catalyzes the oxidation of luminal, a reagent that emits light and therefore "burns" the film and gives a signal which corresponds to the protein under study.

6.12 Antibody release process from PVDF membrane (stripping)

The specific procedure completely achieves the release of both the first and the second antibody from the membrane. In this way, the membrane can be reused to detect and study other proteins. This process leaves the existing proteins unaffected, which remain bound to the membrane through hydrogen bonds.

Materials and Solutions

Stripping buffer
20 mL SDS 10%
12,5 ml 0,5M Tris-HCL pH 6.8
67,5 ml absolute water
0.8 mL β-mercaptoethanol is added to the solution by using the hood

TBS-Tween 20 buffer, pH 7.6 (TBS-T)

TBS (1X)
Tween-20 0,05% v/v

Experimental procedure
1. After the completion of the exposure of the membrane to the film, three incubations of 5 minutes are carried out in TBS-Tween solution under shaking.

2. The membrane is then incubated in an antibody release solution- stripping buffer- for 30 minutes at 50-60°C in a water bath in which the membrane is constantly under shaking.

3. Then the membrane is washed six consecutive times, for 5 minutes each, in TBS-Tween solution under shaking. By this step, the removal of β-mercaptoethanol is crucial because its leftovers may damage the antibodies to be used.

4. The membrane is re-incubated in a blocking solution.

5. The protein analysis steps by Western (described in paragraph 10) are repeated.

### 6.13 Quantification of immunoreactive bands

For the quantification of each immunoreactive band, the films are first digitized with the help of a scanning machine. The images are then processed using the ImageJ system.

This software calculates the area and intensity of each band, and the data is displayed in a spreadsheet. The product of the above two parameters is proportional to the amount of protein, corresponding to each band, and is used in further analyses and to make inferences regarding changes in protein levels.

### 6.14 Transfilter assay

The method of studying cell chemotaxis is an in vitro technique that assesses the ability of cells to migrate across a specific membrane. Specifically, over a micro-well in which we placed serum-free medium and the studying reagent, we applied an insert the bottom of which consisted of a microporous membrane with a pore size (8μm) in order to allow the pass of the cells (Figure M2). The cell suspension is placed on top of the insert. The cells under the influence of a specific reagent move through the membrane and after the membrane is fixed, the migrated cells can be quantified. In the present work, we study the hypoxia-induced migratory effect in endothelial cells. In order to exclude that the observed result is due to hypoxia and not because of the chemotaxis that the reagents may caused, reagents were placed in both microwell and transwell.

### Materials

**Culture medium for migration experiments**

Serum-free culture medium supplemented with 0,25% BSA, glutamine, and antibiotics. BSA was added last and after its dissolution, the solution was filtered through a PVDF bacteriostatic filter.
Transfilter assay

Transwell Costar transfilters with 8km membrane pore size (Costar, Avon, France).

Toluidine blue solution

0,33% v/v toluidine blue in PBS 1X

Carson’s solution

Formaldehyde 37%, 10% v/v

NaH₂PO₄·H₂O 0.1 M

NaOH 0.1 M

Distilled water

Figure M2: Illustrative depiction of a Transwell Costar. (a) The cell suspension is positioned on the top of the porous membrane and the substances causing hypoxia are placed in both the well below and also in the top of the porous membrane. (b) While incubating at 37°C, cells migrate through the pores to the lower section of the membrane. The pore size permits only active cell passage. (c) Cells that have not migrate remove. (d) Fixing and staining of the remaining cells. (e) At higher magnification of the preceding image, membrane pores are indicated by small arrows, and the stained, fixed cells are discernible with big arrows. (Polytarchou et. al, 2007).
Experimental procedure

Cells were incubated for 16 hours in a serum-free medium supplemented with 0.25% BSA (migration culture medium). The cells should have covered 80% of the plate’s area surface to perform the experiment and then the cell’s recultivation steps are followed as described in paragraph 2. Subsequently:

1. In every microwell (bottom well) 600 μl of migration culture medium with or without (control) the hypoxia-cause reagents are added.

2. In the transwell (specific insert with the porous membrane) 100 μl of cell suspension is placed (10⁴ cells in 0.1 ml migration culture medium) with the hypoxia-cause reagents.

3. Microplate is transferred to the incubator for 4 hours.

4. Transwells are then embathed in Carson’s solution for 20 minutes at room temperature. In this step, we achieve the fixation of the cells that migrated to the bottom of the membrane.

5. Very carefully, using a cotton swab, we remove the cells on the upper side of the membrane. Always check while doing this step in order to make sure that removing non-migrated cells is effective.

6. With great care, by using a scalpel, the membrane is cut thus recovering the largest possible surface area of the membrane.

7. Membranes are then placed on a microscope slide with the surface of the migrated cells on the top.

8. Toluidine blue is added for at least 20 minutes at room temperature in order to stain the migrated cells.

9. Membranes are then washed with distilled water and transferred to a new microscope slide with the side of the migrated cells facing down.

10. A few drops of distilled water are then added to prevent the dryness of the membrane and coverslips are placed on the top.

11. Counting of stained cells in the whole area of the membrane using a microscope. If it is necessary, we carry out the counting by using a special grid.
6.15 Immunofluorescence

In the present work, the cellular localization of HIF-1α was detected by immunofluorescence technique.

Materials and Solutions

Small coverslips

The coverslips are sterilized using an autoclave (121°C) for 20 minutes

Gelatin solution

As described in paragraph 2

Thin-tipped tongs

Insulating gelatin-type Parafilm

PBS (1X) pH 7.2-7.4:

Na2HPO4 10 mM

KH2PO4 2 mM

NaCl 137 mM

KCl 2.7 mM

It is kept sterile and stored at 4°C

PBS-Triton X-100 buffer

Buffer solution PBS 1X

Triton X-100 0.5% v/v

4% PFA solution

Formaldehyde 37% v/v

Buffer solution PBS 1X

Bovine Serum Albumin, BSA

FBS
Draq5

Used in 1:1000 final concentration diluted in PBS 1X

Mowiol 4-88

Primary antibody staining

Antibodies and the conditions under which they are used in immunofluorescence studies are listed in Table M5.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Manufacturer</th>
<th>Blocking Reagent</th>
<th>Primary Antibody</th>
<th>Secondary Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Cell Signaling</td>
<td>3,3% BSA, 10% FBS in PBS</td>
<td>1:400 in 3,3% BSA/PBS</td>
<td>a- rabbit 1:500 in 3,3% BSA/PBS</td>
</tr>
</tbody>
</table>

Table M5: The conditions employed for the immunofluorescence technique of the protein of interest are presented in columns from left to right, indicating the protein-antigen, the manufacturer, the reagents used for blocking, and the incubation conditions with both primary and secondary antibodies.

Secondary antibody staining

The secondary antibody, whose conditions are shown in the Table M5 above, is as follows:

Alexa Fluor® 488 chicken anti-rabbit IgG (H+L)

Experimental procedure

Endothelial cells were used in this procedure, and the steps are as follows:

1. Deposit a sterile coverslip into each well of a 6-well plate.

2. Coating plates (including the coverslip) with 1% gelatin solution. This step demands special care in order to cover the entire surface of the coverslip.

3. Incubation for 20 minutes.

4. A brief wash with PBS 1X at room temperature is followed.

5. Cell recultivation steps are followed, as described in paragraph 2, and the appropriate number of cells is plated in the coverslip.

6. Observation of cells under the microscope. The cells should have covered 70-80% of the coverslip’s surface to perform the experiment.
7. A quick wash with cold PBS 1X (stored at 4°C) is performed.

8. Cell fixation with 4% PFA for 10 minutes at room temperature.

9. The above step is followed by three washes of 5 minutes each with PBS-Triton under gentle stirring to achieve the opening of pores in the cell membrane.

10. Blocking using 3,3% BSA/10% FBS in PBS 1X for 1 hour under shaking at room temperature.

11. Transfer the coverslips to 60mm plates on top of which wettex-type material has been placed and moistened, in order to maintain humidity, and gelatin-type Parafilm on top. In this way, diffusion of the antibody is prevented.

12. This is followed by incubation of the first antibody in the conditions listed in Table M5, for 16 hours at 4°C.

13. Three washes of 5 minutes each with PBS 1X under shaking at room temperature are followed.

14. Incubation of the secondary antibody in the conditions listed in Table M5 for 1 hour at room temperature. From this step onwards, the process is carried out in low light conditions.

15. Three washes of 5 minutes each with PBS 1X under shaking at room temperature are followed.

16. Nuclei staining using Draq5 for 15 minutes in 37°C.

17. Coverslips are then lightly touched in an absorbent paper to remove an excessive amount of Draq5.

18. Deposition of 8 μl Mowiol 4-88 on a slide.

19. Transfer the coverslips with the surface of the cells facing the platted Mowiol.

20. Incubation for 1 hour at room temperature.

21. Storage the coverslips at 4°C until observation of the cells in a confocal scanning fluorescence microscope Leica SP5.

6.16 Statistical analysis

Statistical analysis was performed by using an unpaired t-test or ANOVA test, depending on the number of comparing groups as described in the results that follow in the next chapter.
6.17 Reagents

The reagents used in this study are listed below. The reported concentrations refer to the final concentrations used.

**Desferrioxamine mesylate** (dfx): 200 μM. Iron chelator (Merck, Germany).

**Dimethyloxaglycine** (dmog): 0.5 mM (Santa Cruz).

**MY10**: 10 μM. Eclectic inhibitor of PTPRZI’s domain by which the receptor gains its phosphatase action. (Created by the research team of Gonzalo Herradon).

**L-NG-Nitro arginine methyl ester** (L-NAME): 100 μM. A non-selective inhibitor of nitric oxide synthase (eNOS). (Sigma).
RESULTS
In the present study, the following reagents were used for the induction of hypoxia:

1. Dimethyloxaglycine (dmog), known to inhibit prolyl hydroxylases, leads to stabilization and accumulation of HIF1α in the nucleus and therefore, induces the transcriptional activity of HIFs. A previous study of our research group has shown that the stabilization of HIF1α in HUVEC occurs at concentrations greater and/or equal to 0.5 mM (Droggiti, MSc thesis, 2015). Therefore, the concentration of 0.5 mM was used in all the experiments of the present thesis.

2. Deferoxamine mesylate (dfx), which is an iron-chelating agent that binds iron and as a result, lowers the concentration of oxygen. A previous study of our research group has shown that dfx stabilizes HIF1α in HUVEC at a concentration of 200 μM (Droggiti, MSc thesis, 2015). This concentration was used in all the experiments of the present thesis.

7.1 Effect of chemically induced hypoxia on HUVEC proliferation

At first, we wanted to test the effect of chemically induced hypoxia on the proliferation of HUVEC. These results would constitute a positive control for the subsequent experiments since hypoxia is known to induce cell proliferation. Cells were treated with the tested agents for 24h, as described in Methods. As can be seen in Figure R1, hypoxia induced by both dmog and dfx significantly enhances HUVEC proliferation.

![Figure R1: Effect of chemically induced hypoxia on HUVEC proliferation. Cells were stimulated for 24h with 0.5 mM dmog or 200 μM dfx and cell numbers were determined by direct counting. Results are expressed as the mean ± standard deviation (n=3) of the number of cells relative to the group of unstimulated cells (ctl). Asterisks denote statistical significance from ctl. ****P<0.0001.](image-url)
7.2 Effect of chemically induced hypoxia on HIF1α accumulation in HUVEC

The stabilization of HIF1α by dmog and dfx was also used as a positive control marker. HUVEC were stimulated with dmog or dfx as described in Methods, followed by cell lysis, SDS-PAGE electrophoresis, and Western analysis using an antibody specific for HIF1α. Figures R2 and R3 show that dmog and dfx increase HIF1α protein levels at the used concentrations in HUVEC, as expected.

**Figure R2**: Dmog and dfx increase HIF1α protein levels in HUVEC. Cells were stimulated for 4h with 0.5 mM dmog or 200 µM dfx. A representative image from a Western blot analysis of total protein extracts of HUVEC using an antibody selective for HIF1 α is shown. β-actin was used as a loading control.

**Figure R3**: Representative images of immunofluorescence for HIF1α in HUVEC. Cells were incubated for 4h as described in Methods and then fixed and stained using a HIF1 α selective antibody (green). Nuclei were labeled using Draq5 (blue).
7.3 Effect of chemically induced hypoxia on the proliferation and migration of PTPRZ1<sup>wt</sup> and PTPRZ1<sup>ko</sup> LMVEC

Previous studies from our and other research groups have shown that hypoxia induces the expression of PTPRZ1, as discussed in the aim of the study. In this thesis, the role of PTPRZ1 in the stimulatory effects of hypoxia in endothelial cells was studied using LMVEC derived from mice lacking PTPRZ1 (PTPRZ1<sup>ko</sup>) compared to their corresponding wild-type LMVEC (PTPRZ1<sup>wt</sup>). Cells were incubated with dmog or dfx at the same concentrations that were used in HUVEC, and 24 h later, the number of cells was determined by direct counting of cells. As shown in Figure R4 and Table R1, both dmog and dfx significantly enhanced PTPRZ1<sup>wt</sup> LMVEC proliferation. The proliferation of PTPRZ1<sup>ko</sup> LMVEC was increased compared to PTPRZ1<sup>wt</sup>LMVEC, as previously described (Kastana et al., 2023). In these cells, dmog and dfx enhanced their proliferation, like their effect on PTPRZ1<sup>wt</sup>LMVEC.

**Figure R4:** Effect of PTPRZ1 expression on the hypoxia-induced endothelial cell proliferation. PTPRZ1<sup>ko</sup> and PTPRZ1<sup>wt</sup>LMVEC were incubated for 24h with 0.5 mM dmog or 200 µM dfx and cell numbers were determined by direct counting. Results are expressed as the mean ± standard deviation (n=3) of the number of cells. Asterisks denote statistical significance compared with the corresponding untreated (ctl) LMVEC. ****P<0.0001.
Table R1: Numbers of cells 24 h following treatment with dfx or dmog. Results are expressed as mean ± standard deviation (n=3).

<table>
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<tr>
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<th>PTPRZ1&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>PTPRZ1&lt;sup&gt;ko&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>untreated</td>
<td>17.75 ± 2.10 (100%)</td>
<td>28.25 ± 2.77 (100%)</td>
</tr>
<tr>
<td>dfx</td>
<td>28.75 ± 4.57 (62%)</td>
<td>45.17 ± 3.63 (60%)</td>
</tr>
<tr>
<td>dmog</td>
<td>29.63 ± 6.47 (67%)</td>
<td>46.54 ± 4.95 (65%)</td>
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</table>

Chemically induced hypoxia by either dmog or dfx significantly enhanced the migration of PTPRZ1<sup>wt</sup>LMVEC. Migration of PTPRZ1<sup>ko</sup>LMVEC was increased compared to PTPRZ1<sup>wt</sup>LMVEC, as previously described (Kastana et al., 2023), and in these cells, dmog and dfx also enhanced migration, although to a lesser extent compared to their effect on PTPRZ1<sup>wt</sup>LMVEC (Figure R5 and Table R2).

Figure R5: Effect of PTPRZ1 expression on the hypoxia-induced endothelial cell migration. PTPRZ1<sup>ko</sup> and PTPRZ1<sup>wt</sup>LMVEC were incubated for 4h with 0.5 mM dmog or 200 µM dfx and migration was studied using the transwell assay. Results are expressed as the mean ± standard deviation (n=3) of the number of cells that migrated through the filter. Asterisks denote statistical significance compared with the corresponding untreated (ctl) LMVEC. **P<0.01.
Table R2: Numbers of cells 24 h following treatment with dfx or dmog. Results are expressed as mean ± standard deviation (n=3).

<table>
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<tr>
<th></th>
<th>PTPRZ1&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>PTPRZ1&lt;sup&gt;ko&lt;/sup&gt;</th>
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<tr>
<td>untreated</td>
<td>88.4 ± 37.12 (100%)</td>
<td>195.2 ± 41.90 (100%)</td>
</tr>
<tr>
<td>dfx</td>
<td>257.0 ± 57.63 (291%)</td>
<td>293.8 ± 11.90 (150%)</td>
</tr>
<tr>
<td>dmog</td>
<td>326.0 ± 74.09 (369%)</td>
<td>360.0 ± 22.45 (185%)</td>
</tr>
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7.4 HIF1α stabilization in PTPRZ1<sup>ko</sup> LMVEC

The above results, mainly those of cell migration, raised the question of a potential stabilization and therefore activation of HIF1α in PTPRZ1<sup>ko</sup> cells. Immunofluorescence experiments were performed using an antibody selective for HIF1α, as described in Methods. As shown in Figure R6, and as expected, HIF1α was increased following cell stimulation by dfx or dmog, in both PTPRZ1<sup>ko</sup> and PTPRZ1<sup>wt</sup> LMVEC. It is also increased in untreated PTPRZ1<sup>ko</sup> cells compared to untreated PTPRZ1<sup>wt</sup> cells, suggesting that PTPRZ1 deletion leads to HIF1α upregulation.

Figure R6: Representative images of immunofluorescence using an antibody selective for HIF1α in PTPRZ1<sup>ko</sup> and PTPRZ1<sup>wt</sup> LMVEC, in the presence or absence of dfx or dmog. Cells were incubated for 4h with the tested agents, as described in Methods, and were then fixed.
and stained with an antibody selective for HIF1α (green) or Draq5 to stain nuclei (blue). The scale bar corresponds to 10 μm.

### 7.5 HIF1α stabilization in HUVEC following inhibition of PTPRZ1 tyrosine phosphatase activity

Previous data from our research group have shown that a selective PTPRZ1 tyrosine phosphatase activity inhibitor, MY10, enhances endothelial cell migration, mimicking the effect of PTPRZ1 deletion in endothelial cells (Kastana et al., 2023). In this thesis, the effect of MY10 was studied in the levels of HIF1α in HUVEC. Cells were incubated with dmog or MY10 for 4 h, as described in Methods, and the levels of HIF1α were estimated by either Western blot or immunofluorescence. MY10 did not affect HIF1α protein levels when studied by Western blot of LMVEC total protein extracts (Figure R7A). However, when studied by IF, it seems that it causes a small increase in HIF1α protein levels in HUVEC. This increase was smaller than the effect of dmog and did not affect the effect of dmog (Figure R7). The slightly increased HIF1α IF caused by MY10 was also observed only in the cell cytoplasm and not in the cell nucleus (Figure R7B).
Figure R7: Inhibition of PTPRZ1 tyrosine phosphatase activity increases HIF1α protein levels in HUVEC. Cells were stimulated for 4h with 0.5 mM dmog or 10 µM MY10 or their combination. (A) A representative image from a western blot analysis of total protein extracts of HUVEC using an antibody selective for HIF1α is shown. Beta-actin was used as a loading control. (B) Representative images of immunofluorescence using an antibody selective for HIF1α in PTPRZ1ko and PTPRZ1wtLMVEC, in the presence or absence of dfx or dmog. Cells were incubated for 4h with the tested agents, as described in Methods, and were then fixed and stained with an antibody selective for HIF1α (green) or Draq5 to stain nuclei (blue). The scale bar corresponds to 50 µm.

7.6. Effect of chemically induced hypoxia on the proliferation and migration of PTNwt and PTNko LMVEC

Previous studies of our research group show that chemically induced hypoxia stimulates the production and secretion of PTN in HUVEC. In this thesis, the functional significance of this effect was studied by using LMVEC derived from mice lacking PTN (PTNko) or expressing PTN (PTNwt). Cells were stimulated with dmog or dfx, as described in Methods, and the cell numbers were determined by direct counting. Figure R8 and Table R3 show that dfx and dmog induce PTNko and PTNwt LMVEC proliferation. The proliferation of PTNko LMVEC is smaller compared to PTNwt LMVEC, in agreement with previous studies (Kastna Pinelopi, Ph.D. thesis, 2023; Kanellopoulou Vassia, MSc Thesis, 2023) and the effect of chemical hypoxia is higher in PTNko compared to PTNwt LMVEC.
**Figure R8**: Effect of PTN expression on the hypoxia-induced endothelial cell proliferation. PTN<sup>ko</sup> and PTN<sup>wt</sup> LMVEC were incubated for 24h with 0.5 mM dmog or 200 µM dfx and cell numbers were determined by direct counting. Results are expressed as the mean ± standard deviation (n=3) of the number of cells. Asterisks denote statistical significance compared with the corresponding untreated (ctl) LMVEC. ****P<0.0001.

**Table R3**: Numbers of cells 24 h following treatment with dfx or dmog. Results are expressed as mean standard deviation (n=3).

<table>
<thead>
<tr>
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<th>PTN&lt;sup&gt;wt&lt;/sup&gt;</th>
<th>PTN&lt;sup&gt;ko&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>untreated</td>
<td>22.83 ± 3.21 (100%)</td>
<td>17.25 ± 1.13 (100%)</td>
</tr>
<tr>
<td>dfx</td>
<td>36.67 ± 3.44 (161%)</td>
<td>34.33 ± 3.52 (199%)</td>
</tr>
<tr>
<td>dmog</td>
<td>37.92 ± 3.89 (166%)</td>
<td>41.58 ± 3.08 (241%)</td>
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Like proliferation, the migration of PTN<sup>ko</sup> LMVEC is smaller compared with PTN<sup>wt</sup> LMVEC, and both dfx and dmog have a greater effect on the migration of PTN<sup>ko</sup> compared with PTN<sup>wt</sup> LMVEC (Figure R9 and Table R4).
Figure R9: Effect of PTN expression on the hypoxia-induced endothelial cell migration. PTN\textsuperscript{ko} and PTN\textsuperscript{wt} LMVEC were incubated for 4h with 0.5 mM dmog or 200 μM dfx and migration was studied using the transwell assay. Results are expressed as the mean ± standard deviation (n≥3) of the number of cells that migrated through the filter. Asterisks denote statistical significance compared with the corresponding untreated (ctl) LMVEC. **P<0.01.

Table R4: Numbers of cells that migrated through the transwell filters 4 h following treatment with dfx or dmog. Results are expressed as mean ± standard deviation (n=3).

<table>
<thead>
<tr>
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<th>PTN\textsuperscript{wt}</th>
<th>PTN\textsuperscript{ko}</th>
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<tbody>
<tr>
<td>untreated</td>
<td>108.1 ± 10.33 (100%)</td>
<td>80.9 ± 5.70 (100%)</td>
</tr>
<tr>
<td>dfx</td>
<td>199.7 ± 11.15 (185%)</td>
<td>235.3 ± 18.58 (291%)</td>
</tr>
<tr>
<td>dmog</td>
<td>223.0 ± 24.76 (206%)</td>
<td>241.7 ± 36.17 (299%)</td>
</tr>
</tbody>
</table>

Immunofluorescence experiments for HIF1\textalpha were performed using an antibody selective for HIF1\textalpha, as described in Methods. As shown in Figure R10, HIF1\textalpha was not detected in either PTN\textsuperscript{ko} or PTN\textsuperscript{wt} LMVEC and was increased following cell stimulation by dfx or dmog.
Figure R10: Representative images of immunofluorescence using an antibody selective for HIF1α in PTN\textsuperscript{ko} and PTN\textsuperscript{wt} LMVEC, in the presence or absence of dfx or dmog. Cells were incubated for 4h with the tested agents, as described in Methods, and were then fixed and stained with an antibody selective for HIF1α (green) or Draq5 to stain nuclei (blue). The scale bars correspond to 10 μm.

7.7 Expression of eNOS and role of NO in the regulation of proliferation and migration of PTPRZ1\textsuperscript{ko} cells

In the current thesis, it hasn’t been possible to detect HIF1α by Western blot in mouse LMVEC lysates. Before establishing HIF1α detection by immunofluorescence, we thought of detecting a gene that is up-regulated downstream of HIF1α, such as eNOS (Coulet et al., 2003), to verify HIF1α activation by dfx or dmog in these cells. For this purpose, total protein extracts of PTPRZ1\textsuperscript{ko} and PTPRZ1\textsuperscript{wt} LMVEC were analyzed by Western blot using an antibody specific for eNOS, as described in Methods. In contrast to what was expected, eNOS protein levels are significantly lower in PTPRZ1\textsuperscript{ko} compared with PTPRZ1\textsuperscript{wt} LMVEC (Figure R12).
Figure R12: The protein levels of eNOS are decreased in comparison with PTPRZ1^wt^LMVEC. A representative photo of a Western blot analysis using a specific anti-eNOS antibody is shown. Vinculin was used as an equal loading control. The bands were quantified by image analysis software and the results are expressed as mean ± standard deviation (n=3) of the % eNOS/vinculin ratio relative to the PTPRZ1^wt^ group considered as 100%.

To investigate whether the tyrosine phosphatase activity of PTPRZ1 is involved in the regulation of eNOS levels, we used the selective PTPRZ1 tyrosine phosphatase activity inhibitor MY10 and studied its effect in the eNOS protein levels in HUVEC. As a positive control, dmog was also used. HUVEC were incubated with MY10 and/or dmog or dfx for 4 h, followed by cell lysis, SDS-PAGE electrophoresis, and Western blot analysis using an antibody specific for eNOS. Dmog and dfx increased eNOS protein levels. MY10 also increased eNOS protein levels (Figure R13).
**Figure R13**: The protein levels of eNOS are increased by chemically induced hypoxia or by inhibition of the PTPRZ1 tyrosine phosphatase activity. A representative photo of a Western blot analysis using a specific anti-eNOS antibody is shown (n=2). Vinculin was used as a loading control. The bands were quantified by image analysis software and the results are expressed as mean ± standard deviation (n=4) of the % eNOS/vinculin ratio relative to the vehicle-treated (ctl) group considered as 100%.

The involvement of eNOS in the basal and hypoxia-stimulated proliferation and migration of the PTPRZ1ko and PTPRZ1wtLMVEC was then studied by using the eNOS inhibitor L-NAME. As shown in Figure R14, inhibition of eNOS induced cell proliferation in PTPRZ1wtLMVEC, like DMOG. The combination of the two agents caused a further increase in the number of cells. In PTPRZ1koLMVEC, on the other hand, L-NAME inhibited both basal and dmog-stimulated cell proliferation.
Figure R14: Effect of the pharmacological inhibition of eNOS under normoxic or hypoxic conditions on the PTPRZ1\textsuperscript{ko} and PTPRZ1\textsuperscript{wt}LMVEC proliferation. Cells were first stimulated for 10min with L-NAME (0.6 μM) and then with 0.5 mM dmog for 24h. The number of cells was determined by direct counting. Results are expressed as the mean ± standard deviation (n≥2 in tetraplicates) of the number of cells. Asterisks denote statistical significance compared with the corresponding untreated LMVEC. ****P<0.0001.

Inhibition of eNOS did not affect PTPRZ1\textsuperscript{wt} or PTPRZ1\textsuperscript{ko}LMVEC migration, while it abolished the stimulatory effect of dmog (Figure R15).
Figure R15: Effect of the pharmacological inhibition of eNOS under normoxic or hypoxic conditions on the PTPRZ1<sup>ko</sup> and PTPRZ1<sup>wt</sup> LMVEC migration. Cells were first stimulated for 10 min with L-NAME (0.6 μM) and then with 0.5 mM dmog for 24 h. The number of cells was determined by direct counting. Results are expressed as mean ± standard deviation (n≥2) of the number of cells. Asterisks denote statistical significance compared with the corresponding untreated LMVEC. ***P<0.001.
Discussion
Hypoxia is essential in tumor angiogenesis, contributing to a more aggressive tumor phenotype, which is characterized by heightened invasiveness (Semenza et al., 2012), enhanced potential for metastasis (Bayer et al., 2012), and increased resistance to chemotherapy. Hypoxia is a well-studied element of angiogenesis, crucial for facilitating the growth of cancer cells and ensuring sufficient oxygen supply to rapidly proliferating tumors. In line with this function, hypoxia regulates the expression of genes related to angiogenesis, including growth factors like VEGFA (Tafani et al., 2013), Ang-2 (Simon et al., 2008, Yamakawa et al., 2003), PDGF (Nilsson et al., 2004), and FGF-2 (Luo et al., 2011, Lee et al., 2009), along with their respective receptors such as VEGFR1 and VEGFR2 (Krock et al., 2011). Additionally, hypoxia regulates the enzymatic elements responsible for generating ROS (Diebold et al., 2012) and NO (Kourembanas et al., 1994; Coulet et al., 2003), as well as genes involved in matrix metabolism (Yang et al., 2013). The cornerstone regulators of initial reactions to hypoxia are the HIF transcription factors. HIFs operate by directly engaging with HRE sequences instigating the expression of specific genes (Semenza and Wang, 1992).

Prior investigations conducted by our research team revealed increased levels of secreted PTN and its receptor RPTPZ1, in hypoxic conditions in both cancer and endothelial cells (Evangelia Poimenidi, PhD thesis, 2026; Eirini Droggiti, MSc thesis, 2015), leading to the hypothesis that PTN or PTPRZ1 may have a functional role in the effects of hypoxia. This hypothesis was studied in the present master thesis using endothelial cells derived from PTPRZ1^ko, PTN^ko, and their corresponding wild-type LMVEC. Hypoxic conditions were achieved by using DMOG and deferoxamine mesylate which both stabilize HIFs but through a different mechanism.

Chemically induced hypoxia enhanced both PTPRZ1^wt and PTPRZ1^ko LMVEC proliferation. The stimulatory effect of hypoxia in cell proliferation was quantitatively similar in both genotypes. On the other hand, although chemical hypoxia stimulated the migration of both PTPRZ1^wt and PTPRZ1^ko LMVEC, the effect was quantitatively smaller in PTPRZ1^ko LMVEC due to the increased migration of the unstimulated PTPRZ1^ko LMVEC. These results redirected our research interest towards studying the potential stabilization and thus activation of HIF1α in PTPRZ1^ko LMVEC. Indeed, as observed, HIF1α appears to be activated in PTPRZ1 compared to PTPRZ1^wt unstimulated cells. This is in line with the observation that in PTPRZ1^ko LMVEC, the Akt/mTOR signaling pathway is activated (Kastana et al., 2023; Eleni Mourkogianni, PhD thesis, 2023) and it is well
known that HIF1α protein expression strongly depends on the PI3K/Akt/mTOR signaling pathway and that the mTOR inhibitors can prevent HIF1α expression in 70% of human cancer cell lines (Rashid et al., 2021). However, it is unknown how the increased HIF1α expression in PTPRZ1<sup>ko</sup> LMVEC affects their phenotype. For example, it has been shown that the protein levels of both VEGFA and VEGFR2 are increased in PTPRZ1<sup>ko</sup> compared to PTPRZ1<sup>wt</sup> LMVEC, but this seems to be the case at the protein and not the transcriptional level (Kastana et al., 2023). Moreover, the selective PTPRZ1 tyrosine phosphatase inhibitor MY10 does not seem to significantly affect the HIF1α protein levels in HUVEC, suggesting that inhibition of the tyrosine phosphatase activity of the PTPRZ1 receptor is not sufficient for full HIF1α activation.

Interestingly, <i>Ptprz1</i> is upregulated preferentially by HIF2 (Wang et al., 2005) and this preferential activation of PTPRZ1 by HIF2 seems to result from the cooperative binding of HIF2 and Elk1 transcription factors to nearby sites on the PTPRZ1 promoter region (Wang et al., 2010). In the present thesis it is shown that PTPRZ1 may negatively regulate the activation of HIF1 in endothelial cells. This leads to the hypothesis that when activated, HIF2 may negatively regulate the activation of HIF1-dependent genes through PTPRZ1 expression. Previous data have shown a negative effect of HIF2-dependent genes in endothelial cell sprouting (Nauta et al., 2016). Based on these and data on HIF2α<sup>ko</sup> endothelial cells, it seems that HIF2α restricts endothelial cell migration and sprouting (Nauta et al., 2014). Using PTPRZ1<sup>ko</sup> endothelial cells, our data suggest that PTPRZ1 also restricts endothelial cell activation (Kastana et al., 2023 and present study). It is tempting, therefore, to propose that PTPRZ1 upregulated by HIF2, e.g. in chronic hypoxia, contributes to the restriction of endothelial cell activation.

In contrast to what has been observed in PTPRZ1<sup>ko</sup> LMVEC, HIF1α does not seem to be activated in untreated PTN<sup>wt</sup> or PTN<sup>ko</sup> LMVEC, and its protein levels are significantly upregulated in PTN<sup>ko</sup> and PTN<sup>wt</sup> LMVEC following chemical hypoxia. It is interesting to note, however, that both proliferation and migration of PTN<sup>ko</sup> LMVEC induced by hypoxia are higher compared to PTN<sup>wt</sup> LMVEC, suggesting that PTN may act as an endogenous brake of the stimulatory effect of hypoxia in endothelial cell activation. This agrees with what has been observed in human prostate cancer cells (Evangelia Poimenidi, PhD thesis, 2016). It is also like the interplay between VEGFA<sub>165</sub> and PTN; VEGFA<sub>165</sub> stimulates PTN expression and secretion (Poimenidi et al., 2016), and PTN acts as an endogenous break in
the stimulatory effects of VEGFA_{165} in endothelial cells (Koutsiompa et al., 2015; Poimenidi et al., 2016; Vasiliki Kanellopoulou, MSc thesis, 2023).

Prior to the immunofluorescence technique, our first attempt at HIF1α detection in mouse LMVEC lysates was by Western blot analysis. With no success, we focused on looking at a gene directly regulated by HIF1α, and we chose eNOS, which has been shown to be upregulated by hypoxia (Coulet et al., 2003) and to be dependent on HIF1α (Liang et al., 2010). Surprisingly and contrary to what was expected, eNOS protein levels were extremely lower in PTPRZ1^{ko} cells, while MY10 treatment of HUVEC enhanced eNOS levels in normoxia. These data cannot lead to a clear conclusion and add to the discrepancies that already exist in the literature related to eNOS activation and expression under hypoxic conditions. These differences have been attributed to the different types of hypoxias and of course, to the type of cells under study (Martinive et al., 2009). The regulation of eNOS expression by PTPRZ1 also needs to be further studied.

Towards this latter direction, the effect of the eNOS inhibitor L-NAME was studied in the proliferation and migration of PTPRZ1^{wt} and PTPRZ1^{ko} LMVEC. It is interesting to note that L-NAME enhanced PTPRZ1^{wt}LMVEC proliferation in both unstimulated and DMOG-stimulated cells. L-NAME has also been shown to enhance HUVEC proliferation (Vasiliki Kanellopoulou, MSc thesis, 2023). In contrast, it had no effect on the proliferation of unstimulated PTPRZ1^{ko}LMVEC and completely abolished the stimulatory effect of DMOG in these cells. The data in PTPRZ1^{wt}LMVEC are in line with a previous observation that pretreatment of cells with L-NAME followed by hypoxia induction stimulates HIF1α accumulation at higher levels compared to hypoxic conditions alone and results in increased survival of endothelial cells (Martinive et al., 2009). In PTPRZ1^{ko}LMVEC that have decreased eNOS levels, L-NAME seems to antagonize the effect of DMOG on cell proliferation, suggesting a different regulation that needs to be further explored. The differential effect of L-NAME in LMVEC expressing or not PTPRZ1 may also help explain discrepancies in the literature related to the effect of L-NAME on endothelial cell proliferation (this study, Vasiliki Kanellopoulou, MSc thesis, 2023, and unpublished data) and migration (this study and Bu et al., 2022) and warrants extra research.

In summary, in the present master thesis, it has been shown that:

1. Deletion of PTPRZ1 expression leads to increased HIF1α expression in endothelial cells, in line with the enhanced proliferation and migration of these cells.
2. PTN seems to act as an endogenous brake in the stimulatory effects of hypoxia on endothelial cell proliferation and migration.

3. Expression of eNOS is differentially affected by genetic or pharmacological inhibition of PTPRZ1.

4. Pharmacological inhibition of eNOS has a different effect on the proliferation of endothelial cells expressing or not PTPRZ1.
ABBREVIATIONS
ADAMTS: A Disintegrin and Metalloproteinase with thrombospondin motifs
ALK: Anaplastic lymphoma kinase
Ang-1: Angiopoietin-1
Ang-2: Angiopoietin 2
AP-1: Activator Protein-1
ARNT: Aryl Hydrocarbon Receptor Nuclear Translocator

c-Jun: cellular homolog of the Avian myelocytomatosis virus oncogene
c-myc: cellular homolog of Avian myelocytomatosis virus oncogene
COX-2: Cycloxygenase-2
CREB: cAMP-response element binding protein
CS: Chondroitin Sulfate

dFX: Desferrioxamine
Dll-1: Delta-like ligand-1
Dll-4: Delta-like ligand-4

ECM: Extracellular matrix
ECs: Endothelial cells
EGF: Epidermal Growth Factor
eNOS: endothelial nitric oxide synthase
EPO: Erythropoietin
ERK1/2: Extracellular signal-regulated kinases 1,2
Ets 1: E-twenty-six 1 transcription factor

FAK: Focal Adhesion Kinase
FGF: Fibroblast Growth Factor
FIH: Factor Inhibiting HIF
Fyn: proto-oncogene tyrosine-protein kinase of the Src family

G
G-CSF: Granulocyte Colony-Stimulating Factor
GM-CSF: Granulocyte Macrophages Colony-Stimulating Factor

H
HAF: Hypoxia Inducible Factor-Associated Factor
HIF: Hypoxia Inducible Factor
HRE: Hypoxia Response Element
HS: Heparan sulfate
HUVECs: Human Umbilical Vein Endothelial Cells

I
IFN: Interferon
IGF: Insulin Growth Factor
IkBα: Inhibitor of NF-κBα
IL: Interleukin
IP-10: Interferon γ-induced Protein 10

J
JNKs/SAPKs: c-Jun N-terminal kinases/stress-activated kinases

M
MAPK: Mitogen-Activated Protein Kinase
MMP: Matrix Metalloproteinase
MT-MMP: Membrane-type MMP
mTOR: Mammalian target of rapamycin

N
NCL: Nucleolin
NF-kB: Nuclear Factor kB
NO: Nitric oxide
NOX-2: Nicotinamide Adenine Dinucleotide Phosphate Oxidase-2
Nrf-2: Nuclear factor erythroid 2-related factor 2
NSCLC: Non-Small Cell Lung Carcinoma
P
p300/CBP: E1A binding protein p300/CREB-binding protein co-activator complex
PAI: Plasminogen Activator Inhibitor
PAS: Periodic circadian protein-Aryl hydrocarbon receptor translocator-Single minded protein
PDGF: Platelet-derived Growth Factor
PDGFB: Platelet-derived Growth Factor subunit B
PECAM-1: Platelet Endothelial Cell Adhesion Molecule-1
PEDF: Pigment Epithelium Derived Factor
PHD1: Prolyl-Hydroxylase Domain-containing enzyme 1
PHD2: Prolyl-Hydroxylase Domain-containing enzyme 2
PHD3: Prolyl-Hydroxylase Domain-containing enzyme 3
PI3K: Phosphoinositide 3-kinase
PIGF: Placenta Growth Factor
PTN: Pleiotrophin
R
ROS: Reactive Oxygen Species
RPTPZ1: Receptor protein tyrosine phosphatase zeta 1
S
Sp1: Specificity protein 1 transcription factor
Src: proto-oncogene tyrosine-kinase
T
TAD-C: C-terminal transactivation domain
TAD-N: N-terminal transactivation domain
TAMs: Tumor-associated macrophages
TGF: Transforming Growth Factor
Tie-1: Tyrosine kinase with Ig and EGF homology domains-1
Tie-2: Tyrosine kinase with Ig and EGF homology domains-2
TIMP: Tissue Inhibitor of Metalloproteinase
TNF: Tumor Necrosis Factor
tPA: tissue-type Plasminogen Activator
U
uPA: urokinase-type Plasminogen Activator
uPAR: urokinase-type Plasminogen Activator Receptor
V
VCAM-1: Vascular Cell Adhesion Molecule-1
VE-cadherin: Vascular Endothelial cadherin
VEGF: Vascular Endothelial Growth Factor
VEGFR-1: Vascular Endothelial Growth Factor Receptor-1
VEGFR-2: Vascular Endothelial Growth Factor Receptor-2
VHL: Von Hippel-Lindau tumor suppressor
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ABSTRACTS
Angiogenesis is a critical step in cancer progression and is activated by low oxygen levels in the tumor mass. The main mediators of hypoxia are the transcription factors hypoxia-induced factors (HIFs). The recruitment of HIFs under hypoxic conditions regulates the expression of genes that activate the angiogenic switch, such as those of VEGFA, Ang-2, PDGF, and FGF-2, along with their respective receptors, such as VEGFR1 and VEGFR2.

PTN is a secreted growth factor that regulates angiogenesis mainly through its receptor tyrosine phosphatase PTPRZ1. Previous studies of our research group showed increased levels of PTN and its receptor PTPRZ1 after hypoxia in endothelial and cancer cells, suggesting a functional role of these molecules in the actions of hypoxia. Based on the above, in the present study endothelial cells isolated from the lungs of mice knocked out for PTPRZ1 (PTPRZ1\(^{ko}\)), as well as for PTN (PTN\(^{ko}\)), and the corresponding wild type (PTPRZ1\(^{wt}\), PTN\(^{wt}\)) cells were used, while regarding in hypoxic conditions a model of hypoxia was studied using the agents DMOG and deferoxamine (dfx). Proliferation and migration of PTPRZ1\(^{ko}\) and PTPRZ1\(^{wt}\) cells were found to be induced under hypoxic conditions. However, the induction of migration in PTPRZ1\(^{ko}\) cells was quantitatively less, due to the increased migratory capacity exhibited by PTPRZ1\(^{ko}\) under normoxia conditions. HIF1\(\alpha\) was activated in unstimulated PTPRZ1\(^{ko}\) compared to unstimulated PTPRZ1\(^{wt}\) endothelial cells. This observation agrees with recent findings of our research group in which it has been shown that in PTPRZ1\(^{ko}\) cells the Akt/mTOR pathway is activated, which is also the main signaling pathway of HIF1\(\alpha\) expression. These results indicate that PTPRZ1 negatively regulates HIF1\(\alpha\) activation in endothelial cells. It is known that eNOS is directly regulated by HIF1\(\alpha\), but contrary to expectations, in PTPRZ1\(^{ko}\) cells, eNOS levels were significantly lower than in PTPRZ1\(^{wt}\) cells. In contrast, in HUVEC cells and under normoxia conditions, administration of the pharmacological inhibitor MY10 led to increased levels of eNOS but not HIF1\(\alpha\). This observation suggests a difference in response after genetic silencing or pharmacological inhibition of PTPRZ1 tyrosine phosphatase, and further studies are needed. The observation that the NOS inhibitor, L-NAME, led to increased cell proliferation of PTPRZ1\(^{wt}\) endothelial cells in both normoxia and hypoxic conditions, but did not affect the proliferation of unstimulated PTPRZ1\(^{ko}\) cells, leads to the same conclusion, in which it inhibited the inducing action of DMOG. The differential effect of the inhibitor L-NAME on PTPRZ1\(^{ko}\) and PTPRZ1\(^{wt}\) endothelial cells may account for the discrepancies in the observations regarding the effect of L-NAME on endothelial cell proliferation, depending on PTPRZ1 expression. Finally, both proliferation and migration in hypoxic conditions were greater in PTN\(^{ko}\) compared to PTN\(^{wt}\) endothelial cells. This
observation suggests that PTN may act as a negative regulator of endothelial cell activation under hypoxic conditions.
Ο ρόλος της πλειοτροπίνης και του υποδοχέα της PTPRZ1 στην επαγόμενη από υποξία μετανάστευση ενδοθηλιακών κυττάρων

Η αγγειογένεση αποτελεί κρίσιμο βήμα στην εξέλιξη του καρκίνου και ενεργοποιείται από τα χαμηλά επίπεδα οξυγόνου στη μάζα του όγκου. Οι κύριοι μεσολαβητές της υποξίας είναι οι μεταγραφικοί παράγοντες hypoxia-induced factors (HIFs). Η επιστράτευση των HIFs σε υποξικές συνθήκες ρυθμίζει την έκφραση γονίδιων που ενεργοποιούν τον αγγειογενετικό διακόπτη, όπως αυτά των γονίδιων των VEGFA, Ang-2, PDGF και FGF-2, μαζί με τους αντίστοιχους υποδοχείς τους, όπως ο VEGFR1 και ο VEGFR2. Η PTN αποτελεί έναν ακριβώς διακόπτη παράγοντα που ρυθμίζει την αγγειογένεση κυρίως μέσω της αλληλεπίδρασης της με τον υποδοχέα με δράση φωσφατάσης τυροσίνης PTPRZ1.

Προηγούμενες μελέτες της ερευνητικής μας ομάδας έδειξαν αυξημένα επίπεδα PTN και του υποδοχέα PTPRZ1 μετά από την εφαρμογή υποξίας σε ενδοθηλιακά και καρκινικά κύτταρα, υποδηλώνοντας έναν πιθανό λειτουργικό ρόλο των μορίων αυτών στις δράσεις της υποξίας. Με βάση τα παραπάνω, στην παρούσα μελέτη χρησιμοποιήθηκαν ενδοθηλιακά κύτταρα που απομονώθηκαν από πνεύμονα ποντικών knockout (PTPRZ1ko), καθώς και για την PTN (PTNko), και τα αντίστοιχα wild type (PTPRZ1wt, PTNwt) κύτταρα, ενώ σε ότι αφορά στις υποξικές συνθήκες μελετήθηκαν ένα μοντέλο πρόκλησης υποξίας με χρήση των παραγόντων DMOG και δεφεροξαμίνη (dfx). Βρέθηκε ότι σε συνθήκες υποξίας επάγεται ο πολλαπλασιασμός και η μετανάστευση των PTPRZ1ko και PTPRZ1wt κυττάρων. Ωστόσο, η επαγωγή της μετανάστευσης στα κύτταρα PTPRZ1ko ήταν ποσοτικά μικρότερη, λόγω της αυξημένης μεταναστευτικής ικανότητας που παρουσίαζαν τα PTPRZ1ko σε σύγκριση με τα μη διεγερμένα PTPRZ1wt ενδοθηλιακά κύτταρα. Η παρατήρηση αυτήν έρχεται σε συμφωνία με πρόσφατα ευρήματα της ερευνητικής μας ομάδας στα οποία έχει δειχθεί ότι στα PTPRZ1ko κύτταρα είναι ενεργοποιημένη η δοκιμαστική eNOS, αλλά αντίθετα σε ότι αναμένονταν στα PTPRZ1ko κύτταρα τα επίπεδα της eNOS ήταν σημαντικά χαμηλότερα από τα κύτταρα PTPRZ1wt. Αντίθετα, σε κύτταρα HUVEC και σε συνθήκες υποξίας, η χορήγηση του φαρμακολογικού αναστολέα MY10 οδήγησε σε αυξημένα επίπεδα eNOS αλλά όχι HIF1α.
Η παρατήρηση αυτή υποδηλώνει ότι υπάρχει διαφορά στην ανταπόκριση μετά από γενετική αποσιώπηση ή φαρμακολογική αναστολή της φωσφατάσης τυροσίνης του PTPRZ1, και χρειάζονται περαιτέρω μελέτες. Στην ίδια διαπίστωση οδηγεί και η παρατήρηση ότι ο αναστολέας της NOS, L-NAME, οδήγησε σε αυξημένο κυτταρικό πολλαπλασιασμό των ενδοθηλιακών κυττάρων PTPRZ1wt, τόσο σε συνθήκες νορμοξίας όσο και σε συνθήκες υποξίας, αλλά δεν είχε καμία επίδραση στον πολλαπλασιασμό των μη διεγερμένων PTPRZ1ko κυττάρων, στα οποία ανέστειλε την επαγωγική δράση του DMOG. Η διαφορική επίδραση του αναστολέα L-NAME στα PTPRZ1ko και PTPRZ1wt ενδοθηλιακά κύτταρα, ενδεχομένως να αιτιολογεί και τις αποκλίσεις που υπάρχουν στις παρατηρήσεις σχετικά με τη δράση του L-NAME στον πολλαπλασιασμό των ενδοθηλιακών κυττάρων, ανάλογα με την έκφραση του PTPRZ1. Τέλος, στα PTNko ενδοθηλιακά κύτταρα, τόσο ο πολλαπλασιασμός όσο και η μετανάστευση σε υποξικές συνθήκες ήταν μεγαλύτερη σε σύγκριση με τα PTNwt. Η παρατήρηση αυτή υποδηλώνει πως η PTN μπορεί να λειτουργήσει ως αρνητικός ρυθμιστής της ενεργοποίησης των ενδοθηλιακών κυττάρων σε συνθήκες υποξίας.