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The Lymphangiogenic Potential of Hypoxia-Preconditioned Serum (HPS) on in vitro Lymphatic Endothelial Cells and Lymphatic Vessels

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Abstract

Lymphangiogenesis is vital for treating lymphedema and poor-healing wounds and can be enhanced by supplying lymphangiogenic growth factors. In this study, we developed a novel growth factor-enriched blood derivative, the Hypoxia-Preconditioned Serum (HPS), to explore its pro-lymphangiogenic potential and optimal concentration in comparison to Platelet-Rich Plasma (PRP). Our findings showed that HPS and PRP were both pro-lymphangiogenic, with HPS containing higher levels of VEGF-C, PDGF-BB, and bFGF, and promoting greater cell proliferation and migration at HPS-40% and stronger lymphatic tubulogenesis and vessel sprouting at HPS-10%. The thesis highlights the superior lymphangiogenic potential of HPS over PRP and reveals differentiated demands of HPS for cell proliferation and vessel development, suggesting a promising technique for addressing lymphatic dysfunction.

Zusammenfassung

Die Verbesserung der Lymphangiogenese ist ein vielversprechendes Mittel zur Behandlungsergänzung von Lymphödemen und Wundheilung und kann durch die Zufuhr von lymphangiogenen Wachstumsfaktoren erreicht werden. In dieser Studie untersuchten wir das pro-lymphangiogene Potenzial und die optimalen Konzentrationen eines neuartigen, mit Wachstumsfaktoren angereicherten Blutprodukts, dem Hypoxia-Preconditioned Serum (HPS), im Vergleich zu Platelet-rich Plasma (PRP). Unsere Ergebnisse zeigen, dass sowohl HPS als auch PRP pro-lymphangiogen sind. HPS weist höhere Konzentrationen von VEGF-C, PDGF-BB und bFGF auf und förderte bei einer 40%igen Verdünnung (HPS-40%) eine stärkere Zellproliferation und -migration sowie, bei einer 10%igen Verdünnung (HPS-10%), eine stärkere Bildung von Gefäßsprossen. Die Daten unterstreichen, dass HPS eine überlegene lymphangiogene Potenz im Vergleich zu PRP aufweist und unterschiedliche optimale Anforderungen an HPS für das Zellwachstum und die Gefäßentwicklung zeigt. Unsere Studie deutet darauf hin, dass HPS bei bestimmten Konzentrationen vielversprechende Möglichkeiten zur Behandlung von lymphatischen Dysfunktionen bietet.

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Acronyms and abbreviations

2D	Two-dimensional
3D	Three-dimensional
AKT	Protein kinase B
ANG	Angiopoietin
ANOVA	Analysis of variance
ARE	Adenylate-uridylate-rich element
AUF1	ARE-binding protein 1
BEC	Blood endothelial cell
bFGF	Basic fibroblast growth factor
$CaCl_2$	Calcium chloride
CD	Cluster of differentiation
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
CT	Computed tomography
CV	Cardinal vein
CXCR3	Chemokine receptor 3
DAPI	4′,6-diamidino-2-phenylindole
Dll4	Delta-like ligand 4
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
E	Embryonal day
EBM MV2	Endothelial cell basal medium MV2

ECM	Extracellular matrix
EGM MV2	Endothelial cell growth medium MV2
eNOS	Endothelial nitric oxide synthase
ERK	Extracellular signal regulated kinase
ES	Endostatin
ET	Endothelin
ET_BR	Endothelin B receptor
FCS	Fetal calf serum
FGFR	Fibroblast growth factor receptor
GFR	Growth factor reduced
G_i	Heterotrimeric G protein, G_i subtype
HAS	Hyperacute serum
HDLEC	Human dermal lymphatic endothelial cell
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
hPL	Human platelet lysate
HPS	Hypoxia-preconditioned serum
HRE	Hypoxia-responsive element
HRP	Horseradish peroxidase
HSP70s	70-kDa heat shock proteins
HuR	Human antigen R
IFNγ	Interferon γ
IGF	Insulin-like growth factor
IGF-1R	Insulin-like growth factor -1 receptor

IGF-2R	Insulin-like growth factor -2 receptor
IGFBP	Insulin-like growth factor-binding protein
IHC	Immunohistochemistry
IL	Interleukin
IRES	Internal ribosome entry site
ISL1	Islet 1
ISV	Interosmitic vessel
JNK	JUN N-terminal kinase
LEC	Lymphatic endothelial cell
LN	Lymph node
L-PRP	Leukocyte-rich PRP
LPS	Lipopolysaccharide
LV	Lymphatic vessel
LVD	Lymphatic vessel density
LYVE1	Lymphatic vessel endothelial hyaluronan receptor 1
MR	Magnetic resonance
MSC	Mesenchymal stromal cells
NCL	Nucleolin
NGS	Normal goat serum
NIR	Near-infrared
NO	Nitric oxide
NRP	Neuropilin
NS	Non-hypoxia-preconditioned (normal) serum
O_2	Oxygen

OD	Optical density
PBC	Peripheral blood cells
PBS	Phosphate-buffered saline
PBSTx	Phosphate-buffered saline with Triton X-100
PDFC	Platelet-derived factor concentrate
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDGFRβ	Platelet-derived growth factor receptor beta
PF4	Platelet factor 4
pH	Potential hydrogen
PITX2	Pituitary homeobox 2
P-PRP	Pure platelet-rich plasma
PRF	Platelet-rich fibrin
Prox1	Prospero-related homeobox 1
PRP	Platelet-rich plasma
PVB	Peripheral venous blood
RCF	Relative centrifugal force
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S1P	Sphingosine-1-phosphate
SEM	Standard error of the mean
sGC	Soluble guanylyl cyclase
SLO	Secondary lymphoid organs
TD	Thoracic duct
TGFβ	Transforming growth factor-beta

Th	T-helper cell
TIE2	Tunica interna endothelial cell kinase 2
TNFα	Tumor necrosis factor α
TSP1	Thrombospondin 1
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

Lymphangiogenesis is a critical component in wound repair, as it regulates interstitial pressure and inflammation, thereby influencing the healing process [1]. Despite its essential roles, lymphangiogenesis has received relatively limited attention compared to angiogenesis, which not only facilitates blood circulation, but also participates in various tissue regenerations (e.g., regenerations of muscle, nerve, and connective tissues) by delivering growth factors, nutrients, and oxygen [2]. However, angiogenesis alone has limited effects in alleviating high interstitial pressure or modulating inflammation, which conversely can be further improved by lymphangiogenesis [3]. To comprehend lymphangiogenesis, it is imperative to understand lymphatic development, signaling mechanisms, the relationship between lymphangiogenesis and angiogenesis, and its function in lymphedema and wound healing. Based on these comprehensions, diverse approaches have been employed to promote lymphangiogenesis, such as applying recombinant growth factors, injecting stem cells, or utilizing autogenous blood derivatives [4-6]. This thesis aims to evaluate the lymphangiogenic potential of various secretomes derived from blood, including the recently proposed Hypoxia-preconditioned Serum (HPS) and the conventional Platelet-rich Plasma (PRP), in comparison to the baseline normal serum (NS).

1.1 Mechanisms of lymphangiogenesis

Unlike the looped blood circulation system, lymphatic microvessels are blind-ended tubular structures constructed by thin, highly permeable endothelium with loose junctions. The junctions are enveloped by basement membranes to support large vessels like the thoracic duct and collecting vessels. This vasculature forms an intricate lymphatic vessel (LV) network which transports interstitial fluids and macromolecules into lymph and blood circulations and finally links different tissues with the bloodstream or secondary lymphoid organs (SLO) [7]. An efficient lymph transportation can prevent lymphedema by adjusting oncotic and hydrostatic pressure of the interstitium and modulate wound inflammation by conveying immune cells, antigens, and macromolecules [8, 9]. To maintain these lymphatic functions, normal lymphangiogenesis is mandatory for keeping the integrity and turnover of LVs. Until

today, the precise biological mechanisms underlying lymphangiogenesis remain elusive.

1.1.1 Development of lymphatic vessels

To date, two major mammalian lymphangiogenic mechanisms, namely the lymphatic endothelial cell (LEC) organizing theory and the vessel sprouting theory, have been proposed, followed with several new theories based on these two mechanisms [10-12]. Both mechanisms coexist in the lymphatic development, with the de novo cell organizing mechanism commencing earlier.

The cellular origin of lymphangiogenesis is still debatable and has been divided to venous versus non-venous origins, and organ-specific versus region-specific origins [10]. Venous-derived lymphatic endothelial precursors, which express the Prospero-related homeobox 1 gene (Prox1, the first known LEC marker) and then detach from the cardinal vein (CV) or interosmitic vessels (ISV) on embryonic day (E) 9.5, are the primary source of LECs [13]. These Prox1-positive LECs aggregate and bud into mesenchyme to form a primitive lymph sac, wherein the sprouting process occurs (**Figure 1. A**) [14]. In recent years, this venous-derived lymphangiogenic theory has been complemented by other theories upon non-venous sources. For instance, LV formation in the dorsal midline and lumbar regions of murine skin was found to be non-venous in origin, without expression of the vascular endothelial-specific marker TIE2 (Tunica interna endothelial cell kinase 2). LECs of non-venous origin merge to form clusters and subsequently generate LVs to communicate with venous-derived LVs on E12.5- E14.5 [15]. Additionally, new discoveries in murine embryo studies have enriched theories of non-venous sources. For example, LEC progenitors originated from dermal capillary plexus contributed to LV development in the dorsocervical area [10], pituitary homeobox 2 (PITX2)-dependent cranial mesenteric artery formed mesenteric LVs in the ventral left dorsal region [16], and Islet1 (ISL1)-positive mesodermal cells played a critical role for cardiac lymphangiogenesis in the pharyngeal region [17].

Apart from the cell organizing mechanism, sprouting from preexisting LVs can generate new microvessels in a pattern analogous to angiogenesis. LV sprouting prevails in postnatal lymphangiogenesis under various pathological or non-

pathological circumstances like ovarian maturation, pregnancy, neoplasm, inflammation, and wound healing [18-20]. The sprouting is guided by a specified LEC, (i.e., tip cell), and is elongated by proliferation and migration of ordinary LECs (i.e., stalk cells) (**Figure 1. B**) [21, 22]. The LEC growth and sprout elongation primarily rely on vascular endothelial growth factors (VEGF), particularly VEGF-C [23, 24]. Some other relevant biomolecules are introduced in the following chapter 1.1.2. Eventually, the newly sprouted vessels coalesce and anastomose to form a lymphatic network that functions as previously described in chapter 1.1.

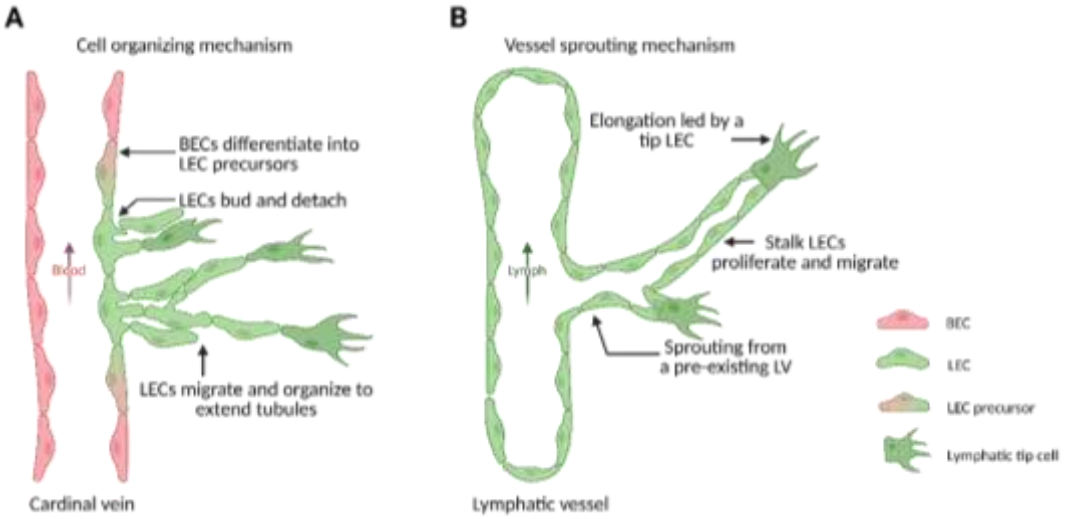

Figure 1. Mechanisms underlying the lymphatic development

Note. Two distinct lymphangiogenic mechanisms coexist in the lymphatic development. **(A)** The schematic plot shows the cell organizing mechanism of venous origin. **(B)** The plot exhibits the vessel sprouting mechanism based on parental LVs. BEC: blood endothelial cell; LEC: lymphatic endothelial cell. Created with BioRender.com.

1.1.2 Signaling in lymphangiogenesis

Numerous growth factors have been discovered to transmit lymphangiogenic signals. Although many of these signals have played parts in angiogenesis and have been recognized for decades as only angiogenic signals [25], researchers came to find that some of them were also transduced characteristically in lymphangiogenic pathways. Understanding these growth factor pathways is the cornerstone to establish pro- or anti-lymphangiogenic treatment strategies in both fundamental and clinical studies. In

this thesis study, we focused on six growth factors that play key roles in either promotion or inhibition to lymphangiogenesis. (**Figure 2**).

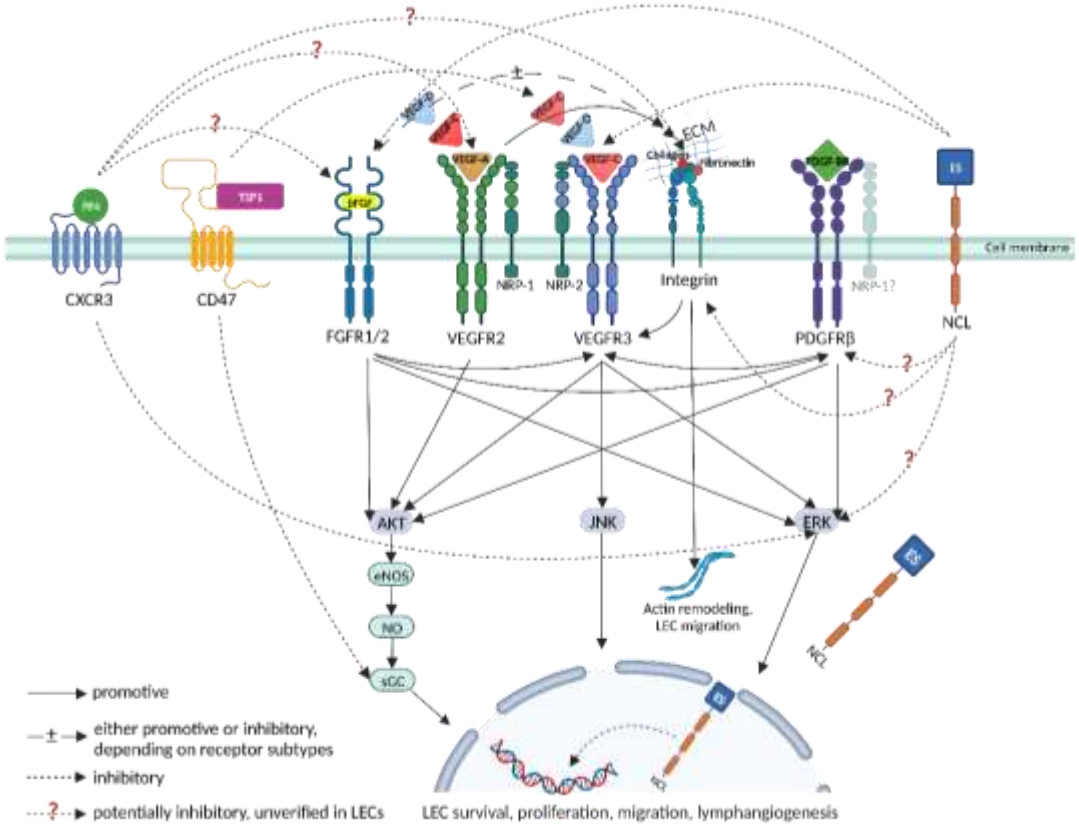

Figure 2. Lymphangiogenic signaling pathways in lymphatic endothelial cells (LEC)

Note. The schematic plot illustrates three pro-lymphangiogenic and three anti-lymphangiogenic pathways that crucially intertwine in LECs, responsible for LEC survival, proliferation, migration, and vessel formation. AKT: protein kinase B; bFGF: basic fibroblast growth factor; CD47: cluster of differentiation 47; CXCR3: chemokine receptor 3; ECM: extracellular matrix; eNOS: endothelial nitric oxide synthase; ERK: extracellular signal regulated kinase; ES: endostatin; FGFR1/2: FGF receptor-1/2; JNK: JUN N-terminal kinase; NCL: nucleolin; NO: nitric oxide; NRP-1/2: neuropilin-1/2; PDGF-BB: platelet-derived growth factor-BB; PDGFRβ: PDGF receptor beta; PF4: platelet factor-4; sGC: soluble guanylyl cyclase; TSP1: thrombospondin-1; VEGF-A/C/D: vascular endothelial growth factor-A/C/D; VEGFR2/3: VEGF receptor-2/3. Created with BioRender.com.

1.1.2.1 Pro-lymphangiogenic signaling pathways

1.1.2.1.1 VEGF-C/VEGFR3 pathway

VEGF-C is a paramount lymphangiogenic regulator for LECs, with high affinity for VEGF receptor (VEGFR) 3. Its activation requires neuropilin (NRP)-2 acting as a coreceptor. In addition to these upstream molecules, several downstream effectors have also been identified in the past two decades, such as the extracellular signal–regulated kinase (ERK), JUN N-terminal kinase (JNK), and protein kinase B (AKT) pathways, which can promote LEC proliferation, migration, and lymphatic microvessel formation [26, 27]. In AKT downstream, endothelial nitric oxide synthase (eNOS)-mediated lymphangiogenesis is intensified through activation of the NO (nitric oxide)-sGC (soluble guanylyl cyclase)-cGMP (cyclic guanosine monophosphate) axis [28].

In mouse embryos, the VEGF-C gene deletion resulted in LV aplasia and consequential edema, leading to prenatal death [29]. In transgenic mice that release soluble VEGFR3, which competed with the inherent VEGFR3, lymphangiogenesis was halted and the formed LVs degraded [30]. Additionally, since the LV formation relies on NRP2 coreceptor, the NRP2 inhibition through either blocking antibodies or gene ablation could disrupt lymphatic sprouting [31, 32]. Contrary to the inhibited lymphangiogenesis caused by deficient VEGF-C or its (co)receptors, LV regeneration in different murine models was reported be promoted through either endogenous overexpression or exogenous supplementation of VEGF-C [24, 33]. This evidence reveals that lymphangiogenesis is triggered and retained by sufficient expression of VEGF-C and engagement of VEGFR3 and NRP2.

In addition to VEGF-C, several other VEGF family members were found to contribute to lymphangiogenesis. For example, VEGF-D is pro-lymphangiogenic with the activation of VEGFR3. However, VEGF-D deletion could not compromise LV development and it was non-fatal for mouse embryos, unlike the lethal consequence caused by the VEGF-C deletion [34]. Besides, VEGF-D and VEGF-C are both compatible to VEGFR2 with the aid of coreceptor NRP-1. However, the VEGFR2 activation mostly increases the LV diameter, rather than increases LV number [35]. Both VEGFR2 and VEGFR3 enable the NO-mediated lymphangiogenesis [28]. Interestingly, another potent angiogenic VEGF, namely the VEGF-A, also exhibits remarkable pro-lymphangiogenic properties. The lymphangiogenic effects of VEGF-

A result from both LEC stimulations via VEGFR2 and monocyte recruitments via VEGFR1, the latter of which leads to release of other pro-lymphangiogenic biomolecules [36, 37].

1.1.2.1.2 PDGF-BB/PDGFRβ pathway

The platelet-derived growth factor (PDGF) family, with active forms of one heterodimer (PDGF-AB) and four homodimers (PDGF-AA, -BB, -CC, and -DD), plays a crucial role in both angiogenesis and lymphangiogenesis [38]. In lymphangiogenesis, PDGF-BB, which selectively binds to the platelet-derived growth factor receptor beta (PDGFRβ) on LECs, is the key subtype to promote lymphatic growth [39]. Although it is presently unknown whether this binding in LECs relies on a coreceptor, like the NRP-1-assisted binding of PGFG-BB and PDGFRβ in vascular smooth muscle cells [40], its resultant pro-lymphangiogenic effect is known to be achieved in two ways. First, the PDGF-BB/PDGFRβ signaling can directly activate the ERK and AKT effectors to induce LV growth [41]. Second, the PDGF-BB/PDGFRβ signaling can synergistically enhance the VEGF-C/VEGFR3 pathway, increasing the density and diameter of LVs and stabilizing these neovasculatures [42-44]. Moreover, PDGF-BB activation also leads to LEC migration and LV formation through Prox1 upregulation [39].

In contrast, blocking the combination of PDGF-BB/PDGFRβ was found to impair lymphangiogenesis or lymphatic function through various antagonistic ways, such as downregulating VEGF-C expression by paracrine feedback [44], suppressing inflammatory LV regeneration by impeding ERK phosphorylation [41], and reducing LV tonic constriction and lymph trafficking via NO-regulated smooth muscle relaxation [45]. Therefore, PDGF-BB/PDGFRβ signaling is indispensable for functional lymphangiogenesis.

1.1.2.1.3 bFGF/FGFR1/FGFR3 pathway

The basic fibroblast growth factor (bFGF, also known as FGF-2) can strongly induce LEC growth and tube formation. This pro-lymphangiogenic effect is activated by combination of bFGF with the extracellular domains of specific FGF receptors (FGFR), i.e., the FGFR1 and FGFR3 [46-48]. On the contrary, the lymphangiogenesis can be inhibited by suppressing bFGF/FGFR signals [49].

Administration of bFGF can downregulate microRNA-381, which leads to upregulation of VEGF-C [50]. For example, the VEGF-C expression was observed to be increased in rat tail lymphedema after delivering additional bFGF [51]. Meanwhile, bFGF exerted a dosage-proportional influence on VEGF-C-modulated lymphangiogenesis in corneas [52]. In summary, bFGF virtually intertwined with VEGF-C in lymphangiogenesis [53]. In practice, more complicated interrelationships have been noticed between bFGF and other biomolecules such as PDGF-BB, yet further research is still required [44, 54].

Parallel to the VEGF-C-modulated lymphangiogenic manner, bFGF per se directly leads to LEC proliferation and migration by phosphorylating the downstream ERK and AKT [47, 53]. For example, in skin melanoma, bFGF was found to increase the lymphatic vessel density (LVD) independent of VEGF-C expression, suggesting a direct pro-lymphangiogenic effect of bFGF [55].

1.1.2.2 Anti-lymphangiogenic signaling pathways

1.1.2.2.1 TSP1/CD47/CD36 pathway

The thrombospondin (TSP) family is widely recognized for its strong angiostatic effects. Among its isoforms, TSP1 has been found to be capable of repressing lymphangiogenesis with its high affinity for CD47 and CD36 [56, 57]. The distribution and anti-lymphangiogenic role of CD47 remain debatable. HH Oh's findings indicated that the TSP1/CD47 pathway was activated in tumor cells and unable to increase the LVD, whereas B Singla reported that CD47 was expressed in LECs and could inhibit lymphangiogenesis and its deletion led to a curative outcome [57, 58]. The anti-lymphangiogenic effect of TSP1/CD47 axis was considered from its negative interference on the AKT-mediated NO signaling, wherein sGC is downregulated [28, 59]. In addition to CD47, CD36 is primarily localized in inflammatory monocytes rather than LECs, making the direct anti-lymphangiogenic action of TSP1 on non-inflammatory tumor LVs less likely [60]. However, during inflammatory LV regeneration, where numerous $CD36^{+}$ macrophages are mobilized, TSP1 can exhibit its inhibitory bioactivities indirectly through macrophages probably by upregulating transforming growth factor (TGF)-β and downregulating VEGF-C [61, 62]. Vice versa, gene deletion of TSP1 and CD36 in cornea inflammation resulted in VEGF-C upregulation in macrophages and subsequent LV regeneration [56, 63]. Thus, TSP1 plays its anti-lymphangiogenic role mainly with the aid of inflammation, such as in

the circumstances of lymphedema or wound healing. Although the overexpression of TSP1 has been shown to delay wound healing via its anti-angiogenetic effect [64], the extent to which the anti-lymphangiogenic effect of TSP1 influences wound healing is questionable.

1.1.2.2.2 PF4/CXCR3 pathway

The platelet factor 4 (PF4, also known as CXCL4) is released from platelets' alpha-granules and some tumor cells and transmits lymphangio/ angiostatic signals by combining with chemokine receptor 3 (CXCR3) [65, 66]. Although the PF4/CXCR3 complex can evoke the downstream ERK phosphorylation in certain cell types (e.g., lymphocytes), it conversely inhibits ERK phosphorylation in LECs and therefore restrains LV regeneration [67, 68]. Vice versa, LV regeneration in mice was observed to be improved when knocking out the CXCR3 genes [69].

The angiostatic mechanisms for PF4 have been extensively studied for decades [70]. In summary, PF4 binds to bFGF and its heparan sulfate coreceptors on endothelial cells, blocking the bFGF-related angiogenesis pathway [71]. Meanwhile, the VEGF-A-related angiogenesis can also be interrupted by PF4 [72]. Furthermore, PF4 can restrain blood endothelial cells (BEC) from forming tubes and sprouts by competitively binding to specific integrins, such as α5β1 [73]. Integrin α5β1 has been identified as a crucial component for LV formation, as detailed in section 1.1.2.4 [74]. As a result, integrin occupation has the potential to serve as an inhibitory mechanism for PF-regulated lymphangiogenesis. However, the concrete anti-lymphangiogenic function of PF4 is presently ambiguous, particularly due to its crosstalk with bFGF, VEGF-C, or other lymphangiogenic growth factors.

1.1.2.2.3 Endostatin/NCL pathway

Endostatin (ES) is a bioactive proteolytic fragment of collagen type XVIII from the basement membrane in nearly all organs including blood vessels [75]. It can also be released from platelet degranulation that is activated by thrombin [76, 77]. ES has a potent angiostatic effect when targeting nucleolins (NCL), which subsequently translocate into the BEC nuclei, resulting in deactivation of the casein kinase 2 (CK2) and impairment of DNA transcription and protein synthesis. This inhibition process can even counteract the angiogenic effect of VEGF and bFGF [77]. Aside from its angiostatic effect on BECs, ES also shows high affinity towards NCLs in LECs and

thus prevents in vitro and in vivo LV formation. However, not all LVs can be affected by the ES/NCL complex because NCLs are specifically positioned on proliferative, nascent vessels, rather than non-proliferative, fully mature ones [78]. Furthermore, previous research has unraveled indirect anti-lymphangiogenic pathways mediated by ES. For example, the overexpression of ES was found to restrict VEGF-C production from mast cells and endothelial cells, thereby inhibiting VEGF-C-mediated lymphangiogenesis [79, 80]. Additionally, ES-bearing fragments that decomposed from collagen XVIII were reported to restrain bFGF-mediated lymphangiogenesis [81].

Beyond its anti-lymphangiogenic effect, ES exhibits other bioactive properties. Specifically, ES can disrupt the PDGF-BB/PDGFRβ signal transduction by suppressing PDGFR expression and ERK phosphorylation in human fibroblasts [82]. In human umbilical vein endothelial cells (HUVEC), ES can also inhibit the integrin-engaged tube formation [83-85]. Given that both PDGF-BB and integrins play critical roles in lymphangiogenic signaling, their inhibitions may contribute to the anti-lymphangiogenic property of ES. However, whether these analogous pathways are shared in LECs remains uncertain and further investigation is necessary.

1.1.2.3 Other lymphangiogenic/ lymphangiostatic signals

Lymphangiogenesis involves a complicated signaling network beyond the aforementioned mechanisms [25]. In this context, many old and novel biomolecules have been discovered to interact. To exemplify, the insulin-like growth factor (IGF)-1 and IGF-2 conjugate with IGF receptors (IGF-1R and IGF-2R) and IGF-binding proteins (IGFBP) to activate the cascading pathways such as ERK and AKT, consequentially promoting lymphangiogenesis regardless of VEGF-C/-D/VEGFR3 signals [86]. Sphingosine-1-phosphate (S1P) can also stimulate lymphangiogenesis through S1P1/G_i pathway, which increases calcium influx and subsequently promotes LEC growth [87]. Angiopoietin (ANG)-1 and ANG-2, with their corresponding receptors TIE1 and TIE2, can increase the VEGFR3 expression and thus enhance lymphangiogenesis [88, 89]. Lymphangiogenesis mediated by hepatocyte growth factor (HGF) or epidermal growth factor (EGF) may implicate both direct and indirect (VEGF-mediated) signaling pathways [90-92]. Endothelin (ET)-1, by binding to the endothelin B receptor (ET_BR), can increase the expression of VEGFs and hypoxia-inducible factor (HIF)-1α, the latter of which in turn stimulates more VEGF expression and amplifies lymphangiogenesis [93]. These growth factors are part of a complex

signaling system that regulates lymphangiogenesis. There are numerous other biomolecules contributing to this system as well.

For example, some inflammatory cytokines play lymphangiogenic or lymphangiostatic roles either directly on LECs or indirectly by recruiting inflammatory cells (e.g., macrophages, dendritic cells, and T lymphocytes) to further release pro- and anti-lymphangiogenic factors [94, 95]. The pro- and anti-lymphangiogenic effects of these cytokines generally comply with their known pro- and anti-inflammatory roles. To illustrate the point, the pro-inflammatory cytokines, such as interleukin (IL)-1β, IL-7, IL-33, and tumor necrosis factor (TNF) α, can enhance lymphangiogenesis [96-99]. While some anti-inflammatory cytokines, such as IL-4 and IL-13 from T-helper 2 cells (Th2), tend to do the opposite [100, 101]. However, exceptions are not rare. For instance, the pro-inflammatory cytokines like interferon (IFN) γ and IL-12 are anti-lymphangiogenic [102-104], while the anti-inflammatory cytokines like IL-6 and IL-10 are pro-lymphangiogenic [105, 106].

Interestingly, some biomolecules display dual roles. As reported, the delta-like ligand (Dll) 4 and Notch complex had a positive impact on LV regeneration [107], while in another study, it impaired LEC proliferation, migration, and LVD [108]. Similar alternating functions appeared on TGFβ and Lipopolysaccharide (LPS), which were reported to be either positive or negative in lymphangiogenesis [62, 109-112].

1.1.2.4 Integrin-mediated lymphangiogenesis

Integrins, which are a family of transmembrane receptors that links with specific ECM ligands, such as fibronectin or collagen, translate biomechanical signals from ECM to cytoskeletons to induce cell migration and further structure formation [112]. During lymphangiogenesis, integrins with β1 subunit, such as α4β1 or α5β1, facilitate LEC migration and LV formation in two ways. Firstly, β1-integrins act as an amplifier for VEGF-C/-D/VEGFR3 complex, intensifying the pro-lymphangiogenic signals. On the other hand, integrins enhance the adhesion of LECs to ECM and conduct the extracellular traction stimuli into cells, subsequently triggering intracellular actin remodeling and membrane undulation and ultimately mobilizing LECs [74]. LECs mobilize in ECM to form microvascular structures, which can be further influenced by mechanical stimuli, such as the flow of interstitial fluids [113]. Conversely,

knockdown of the β1 subunit can repress the formation of microvascular structures in an ECM milieu [112].

In addition to the β1 subunit, several α subunits, such as α1, α2, α5, and α9, are expressed on the LEC membrane to assist cell-ECM adhesion. Inhibition of these integrins can result in hypogenetic and malfunctional LVs, even leading to death of the experimental mice [114-116].

Moreover, the crosstalk between integrins and growth factors extends beyond the VEGFR3 pathway and involves various lymphangiogenic factors. For example, VEGF-A was found to increase the levels of α1β1 and α2β1, consequently promoting LV formation [36]. Addition of bFGF leads to elevation of α2β1 and α5β1 integrins while decreasing the α1β1 integrin [117]. The intricate relationships between growth factors (or their receptors) and various integrin members, as well as their specific actions on lymphangiogenesis, necessitate further research.

1.1.3 Interrelationship of lymphangiogenesis and angiogenesis

Lymphangiogenesis interplays with angiogenesis (also termed hemangiogenesis) by partly sharing some similar endothelial cell properties and signaling pathways. Their similarities allow for utilization of some angiogenic metrics in lymphangiogenesis research, such as tube formation and vessel sprouting [118]. However, lymphangiogenesis still differs from angiogenesis in terms of development (see chapter 1.1.1), signaling patterns (see chapter 1.1.2) and spatiotemporal scales (as discussed in chapter 4).

As introduced in chapter 1.1.1, veins are major origins of de novo lymphangiogenesis, wherein the blind-ended lymphatic vessels bud and are attracted by tip precursors and ultimately differentiate and detach from preexisting blood vessels (BV). In contrast, de novo angiogenesis is initiated by directly dividing from the parental vessels [119]. Research has demonstrated that the simultaneous occurrence of lymphangiogenesis and angiogenesis in the same area hinders both processes. In a murine mesenteric angio/lymphangiogenesis model, lymphangiogenesis was observed to suppress angiogenesis in the coexistence of BVs [120]. The coexistence of LVs also weakened the VEGF-C-mediated angiogenesis, leading to an angiogenetic tendency remote from the LV-occupied areas [121]. In addition, lymphangiogenesis temporally lags behind

angiogenesis, but the regenerated LVs endure even after resolution of inflammation and recession of BVs [120, 122].

The lymphangiogenic and angiogenic responses to mechanical stress also vary. For instance, the engineered LVs tend to organize LECs both longitudinally and vertically to the applied mechanical stress, whereas the engineered BVs only oriented endothelial cells vertically to the same stress [123].

Due to the differential signaling and formation patterns of lymphangiogenesis in comparison to angiogenesis, and the relatively limited research attention it has received, lymphangiogenesis warrants further investigation, particularly with regard to its indispensable role in improving lymphedema and wound healing (as described in the subsequent chapter 1.2).

1.2 Lymphangiogenesis in lymphedema and wound healing

Lymphedema is characterized by persistent interstitial fluid entrapment and recalcitrant soft tissue swelling. Despite the statistical discrepancies presented in epidemiology studies, the prevalence of lymphedema was conservatively estimated to be 250 million in the world and over 1‰ in Europe [124, 125]. The most common cause of lymphedema is acquired impairment of the lymphatic system, such as traumatic or radiologic injuries to lymph nodes (LN) or LVs [124]. Thus, re-establishment of lymphatic continuity is essential for treating edema. Treatment strategies include lymphovenous anastomoses (LVA), autologous LNs transfer, and lymphangiogenesis enhancement [8, 126]. However, the LVA operation is difficult to conduct in advanced lymphedema stages due to the scarcity of functional LVs, and LNs transfer presents a low integration rate of less than one-third [8, 127]. As a result, lymphangiogenesis has emerged as a feasible option. Research showed that enhanced lymphangiogenesis could mitigate lymphedema by increasing lymphatic density, while impaired lymphangiogenesis led to the reverse [30, 51, 52]. Regarding the exacerbating effect of lymphedema on compromised wounds, it becomes inescapable that lymphangiogenesis plays a consequential role in wound healing [1, 128].

Wound healing has been a significant global healthcare issue with expenditure over $25 billion. According to a recent analysis, the global market of advanced wound care

is anticipated to experience an average annual growth rate of 6.6% across the period spanning 2020 to 2027, wherein Europe is one of the main drivers [129]. To control the costs and growth rate on wounds and improve patients' quality of life, it is imperative to understand the underlying healing mechanism and promote the healing process. The wound healing course comprises of four sequential stages, i.e., hemostasis, inflammation, proliferation, and remodeling stage (**Figure 3**) [130]. The initial hemostasis stage is triggered by vascular disruption, leading to activation of clotting factors and subsequent platelet embolism. In this stage, platelets degranulate to release chemotactic biomolecules that also participate in the following inflammation stage. The inflammation stage is characterized by recruitment of immune cells and outburst of growth factors such as lymphangiogenic VEGFs and bFGF [130]. In this phase, the pro- and anti-inflammatory signals coordinate to progress to the third stage of proliferation, where the augmented growth factors promote tissue regeneration, including angiogenesis and lymphangiogenesis. Finally, the regenerated structures, such as LVs and ECM, undergo remodeling as the immune cells and growth factors subside [131]. As wound healing proceeds, not only platelets, but also erythrocytes, leukocytes, and the other peripheral blood cells (PBC), are engaged in the overall stages. These PBCs contribute to generating and releasing more growth factors, including lymphangiogenic growth factors like VEGFs, PDGFs, and FGFs [132].

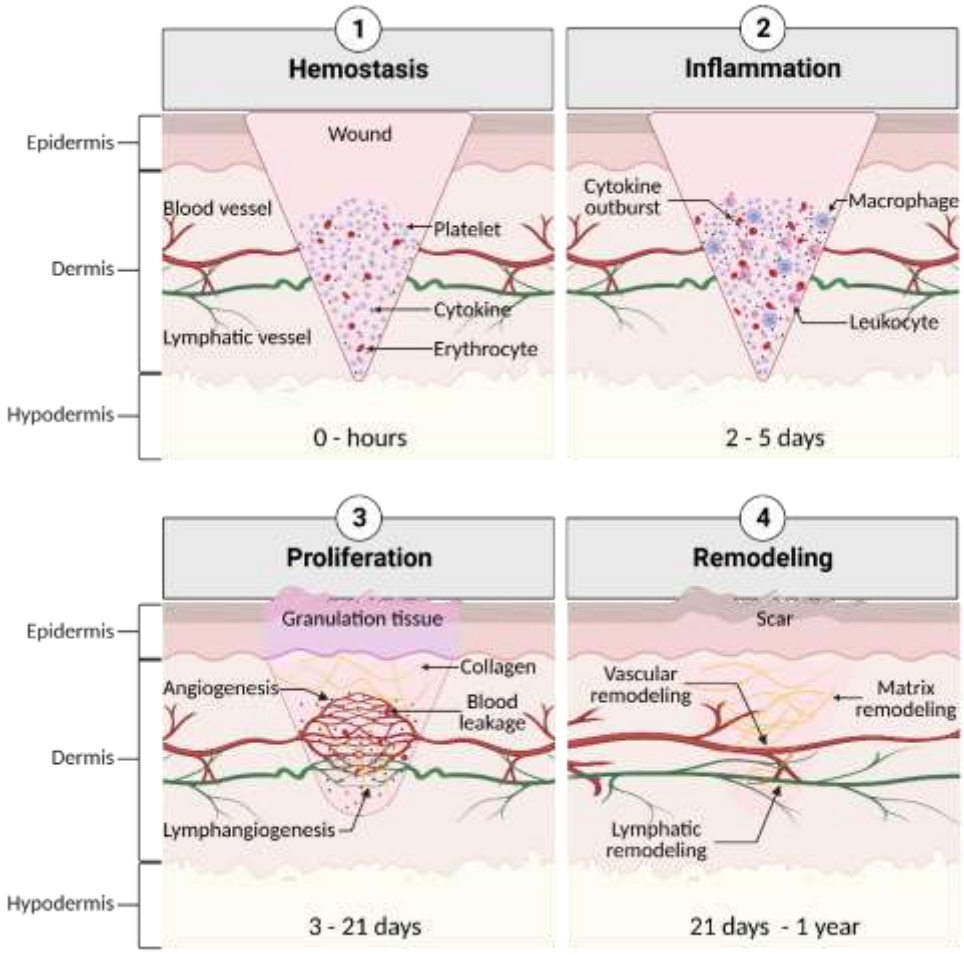

Figure 3. Wound healing process

Note. The wound healing process is subdivided into four consecutive stages. The inflammation and proliferation stages are of paramount importance for lymphangiogenesis due to the growth factor explosion. Created with BioRender.com.

Since lymphangiogenesis is critically integral to the inflammation and proliferation stages, any disruption to these stages can cause insufficient LV regeneration and lymphedema, which in turn impedes transportation of immune cells and release of growth factors and exacerbates lymphatic disorder, ultimately compromising wound healing and even proceeding to poor healing [1]. Similar to the treatment for lymphedema, management of poor-healing wounds necessitates lymphangiogenesis [131, 133]. Today, various techniques have been developed to regenerate functional LVs, such as direct injection of VEGF-C or adipose-derived stem cells (ADSCs) into wounds, artificial LV engineering, and variant modifications [1, 4, 5, 134]. However, extensive application of these techniques shows limitations, such as vascular

hyperpermeability after simple utilization of VEGF-C, cell population contamination during ADSC separation, and the repelling risk from biomaterials [127, 135, 136]. To circumvent these limitations, blood-derived products, such as PRP, have been proposed as prospective tools in the past two decades due to their autologous property, simple preparation, convenient administration, and foremost, their multiple growth factors. Several commonly used blood derivatives and their regenerative potential based on their enriched growth factors are introduced in the follow chapter.

1.3 Blood derivatives used in lymphangiogenesis

Blood circulates throughout the body, supplying nutrients and transporting inflammatory cells and biomolecules to the wound sites. The whole blood consists of 45% (v/v) of cellular portion, which includes erythrocytes, leukocytes, platelets, etc., and 55% (v/v) of liquid portion, which is presented in the form of the serum or plasma [137]. Plasma contains clotting factors, such as fibrinogen, which can be depleted from serum after blood coagulation [138]. Erythrocytes constitute the majority of cellular portion and not only serve as an inert oxygen carrier, but also as a vast depot of growth factors that is significantly larger than platelets' storage [132, 139]. Leukocytes, due to their high immunogenicity, are often removed from the end products like PRP and NS. During blood derivative preparation, the density gradients among different blood components enable blood to separate into three strata, i.e., the upper serum or plasma, the middle clot or buffy coat (rich in platelets), and the lower erythrocyte layer. Based on this component layering, various formulations of cellular or acellular blood derivatives can be extracted, providing enriched growth factors and other bioactive molecules (i.e., secretomes). Furthermore, autologous blood derivatives show their advantages of repeatable collection, excellent biocompatibility, and avoidance of disease transmission [140].

Among these blood derivatives, the platelet-derived secretomes, particularly PRP, have been widely used in tissue regeneration and are consistently evolving to concentrate more platelets or growth factors [141]. Interestingly, the enriched growth factors in NS are found to be also mainly from the activated platelets [138]. However, compared to the platelets that comprise less than 1% of the whole blood, erythrocytes constitute an overwhelming portion of cell components and thus provide more growth

factors [139]. To augment the production of growth factors and harvest them from erythrocytes, as well as from platelets and other cell components, our lab team has previously exploited a pericellular hypoxia preconditioning technique on PBCs to create a novel blood derivative, i.e., HPS, as introduced in chapter 1.3.3.

As the blood derivatives supply multiple growth factors, including lymphangiogenic factors like bFGF, PDGF-BB, and IL-7, they may have the potential to influence lymphangiogenesis [138]. One of our goals in this thesis is to investigate the pro-lymphangiogenic effect of HPS and PRP, in comparison to the baseline NS.

1.3.1 Human serum

Serum, such as fetal bovine serum (FBS) and human serum, is an essential culture supplement to support cell proliferation. In some cases, this supplement can be replaced with plasma, which is usually anticoagulated by adding sodium citrate to neutralize the endogenous calcium in blood. Despite this, the extra calcium in the culture medium may override the citrates, resulting in untoward clotting. Although this side effect can be addressed by using recalcified plasma, plasma does not deliver substantial advantages over serum in term of proteome patterns [138].

In some fundamental and clinical research settings, human serum has been utilized instead of FBS to support cell proliferation. The proliferative effect of human serum stems primarily from platelet activation elicited by blood coagulation cascade, which releases the pre-stored growth factors from α-granules [138, 142]. For example, one of the serum products, the hyperacute serum (HAS), was considered to be a byproduct of the platelet-rich fibrin (PRF) [143]. To facilitate the platelet degranulation, two technically opposing strategies are commonly used: slow and rapid preparation. The rapidly prepared products, such as HAS, are manufactured using high-speed centrifugation, similar to the single-centrifugation procedure used for PRP preparation. Compared to PRP, despite having lower levels of some growth factors (e.g., PDGF-BB and TGFβ1), HAS can promote cell proliferation [143]. On the other hand, the slow serum preparation methods, such as natural sedimentation (4℃, 24 h), provide alternatives for degranulating platelets. It has been reported that the slowly prepared serum yielded higher levels of PDGFs than rapidly prepared serum (20℃, 30 min) [144]. In summary, human serum qualifies as a cell culture supplement. A properly

prepared serum contains high amounts of growth factors, such as PDGF, VEGF, and S1P, exhibiting potent angiogenic capability [138, 142, 145]. However, its lymphangiogenic capability has not yet been explored.

In this thesis, we adopted an equivalently slow approach by shortening the incubation time while elevating temperature for spontaneous serum production. In order to distinguish this non-hypoxia stimulated serum from HPS, herein we refer to our human serum product as normal serum (NS). NS serves as a baseline reference to HPS and PRP in this lymphangiogenesis study.

1.3.2 Platelet-rich plasma (PRP)

PRP is defined as an autologous blood secretome with highly concentrated platelets and active proteins secreted from α granules, encompassing both promotive and inhibitive angio/ lymphangiogenic growth factors [141]. In regenerative medicine, at least a threefold platelet concentration (approximately 1×10^6 platelets/mL) above baseline is typically adopted [146, 147]. However, in practice, there is no standardized protocol for preparing PRP due to various preferences on centrifugation speed and time [141]. Moreover, diverse formulations derived from PRP preparation have been introduced, for instance, the pure PRP (P-PRP) that contains only concentrated platelets, PRP that contains other cells or ECM components like leukocyte-rich PRP (L-PRP) and PRF, and PRP lysates without cell components like platelet-derived factor concentrate (PDFC) and human platelet lysates (hPL) [148]. In this thesis, PRP is extracted as the easy-to-use PRP-lysate, where the pre-stored factors (e.g., VEGFs, PDGFs, bFGF, TSP1, PF4, ES) have been released into the supernatants after platelet degranulation. Platelet degranulation in wound healing normally occurs in the earliest hemostasis stage and may lack an integral proteome which can be released from the ensuing inflammation and proliferation stages.

In wound regeneration, PRP has shown its pleiotropic potency on angiogenesis, dermatogenesis, musculogenesis, osteogenesis, etc., which is intricately attributable to its productive growth factors like VEGF, PDGF, IGF-1, and bFGF [2, 149]. However, its lymphangiogenic potential has not been extensively studied. In a mouse tail circumcision model, PRP significantly improved lymph drainage, diminished wound size, and enhanced LEC viability [6]. Nevertheless, little is known about cellular

changes under PRP administration, such as LEC migration and organization. Despite being considered the gold standard among assorted blood derivatives when treating wounds, PRP still needs further research to elucidate its lymphangiogenic effect in LEC proliferation, migration, and LV sprouting.

1.3.3 Hypoxia-preconditioned serum (HPS)

Hypoxia is a potent stimulant for yielding growth factors. Different from PRP or NS that are manufactured under normoxia, hypoxia-preconditioned secretomes are generated by stimulating cells in a low-oxygen blood milieu and dissolved in serum, plasma, or specific media.

1.3.3.1 State of the art about hypoxia-preconditioning with blood

Numerous approaches have been employed in experimental settings to create hypoxic milieus for cell survival or tissue regeneration. For instance, the hypoxic incubator (33℃, 2% O_2) could be used to increase the viability and neovascularization potential of peripheral blood mononuclear cells [150]. Similarly, the blood-derived mesenchymal stromal cells (MSC) cultured in hypoxia (37℃, 1% O_2) were resistant to apoptosis and showed intensified vasculogenic ability [151]. In addition, some variants of hypoxia-preconditioning techniques have been proved practical in in vivo circumstances. Specifically, in murine subjects, exposure to hypoxia-hypobaria (60.8 kPa, 12.6% O_2) ameliorated both myocardial damage and heart function [152]. In human subjects, an intermittently induced limb ischemia and hypoxia protected vascular endothelial cells and maintained normal vasorelaxation [153]. The above approaches all aim to precondition cells or tissues in a hypoxic macro milieu in order to increase their viability and proliferation tendency. Their positive results provided evidence-based availability of using hypoxia to increase the regenerative capability of cells in blood. However, these techniques necessitate dedicated devices (e.g., hypoxia incubator, low-pressure chamber, or medical monitor) and meticulous parameter refinement, which may cause additional cost and limit their extensive application.

To simplify the hypoxia-creating procedures, our lab has previously developed an extracorporeally preconditioning technique by culturing venous blood (i.e., PBCs) in a self-adaptive hypoxic micromilieu. This hypoxic micromilieu (approximately 1% O_2) is formed through spontaneous oxygen utilization by PBCs during a four-day

incubation (37℃, 21% O_2, and 5% CO_2) [154, 155]. As the incubation proceeds, PBCs precipitate and secrete growth factors into serum, which is defined as HPS. HPS is then extracted through a filter, obviating additional necessity for spinning that is mandatory during PRP preparation [156]. HPS is formed within a routine CO_2 incubator, without the need for other special devices or parameter monitoring. It has shown strong regenerative potential attributable to hypoxic stress [156].

1.3.3.2 Mechanisms underlying the HPS-stimulated lymphangiogenesis

The hypoxic stress is known to upregulate certain lymphangio/angiogenic growth factors through both hypoxia-inducible factor (HIF)-1-related and HIF-1-unrelated pathways [157]. In the HIF-1-related pathway, HIF-1 binds to hypoxia-responsive elements (HRE) located in promoter regions of specific genes, such as PDGF and VEGF, to transduce transcriptional signals (**Figure 4. A**) [158]. This transcriptional regulation could fit PBCs that possess nuclei. However, erythrocytes and platelets are anucleate cells, instead containing mRNAs in their cytoplasm. Thus, the mRNA-based pathways (HIF-1-unrelated) may play a prominent role for synthesis of growth factors in erythrocytes and platelets at the translational level. During translation, hypoxia directly activates the internal ribosome entry sites (IRES) located in mRNAs that encode growth factors, such as VEGF-C, VEGF-A, and bFGF (**Figure 4. B**) [159-161]. In addition, at RNA modification level (HIF-1-unrelated), hypoxia contributes to stabilizing the growth factor mRNAs that contain adenylate-uridylate-rich elements (ARE), such as VEGF-A mRNA, and thence prolongs the half-life of these mRNAs by elaborate regulations from various ARE-binding proteins, such as ARE-binding protein-1 (AUF1), human antigen R (HuR), and the 70-kDa heat shock proteins (HSP70s) (**Figure 4. C**) [160]. To sum up, hypoxia serves as a strong inducer and stabilizer in multiple ways of producing growth factors, including lymphangiogenic factors. By utilizing hypoxia on the peripheral blood, we generated the novel serum secretome (i.e., HPS) from extensively distributed PBCs [154, 162-164]. PBCs circulate through the entire span of four wound healing stages, participating in more healing events (e.g., inflammation and proliferation) than most platelets that are activated in the first hemostasis stage [165]. Therefore, the PBC-derived HPS can

theoretically provide all-inclusive and biomimetic constitutes compared to the platelet-derived secretomes, like PRP and NS.

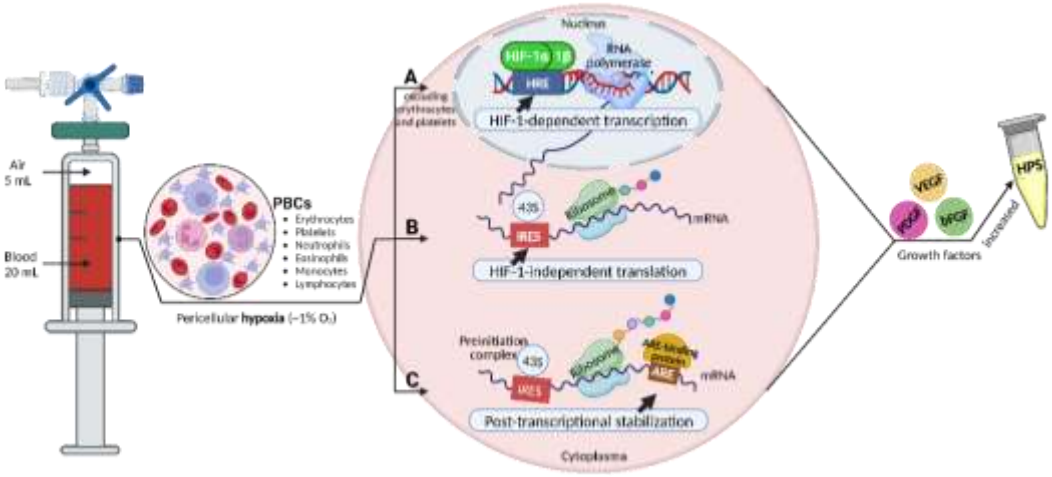

Figure 4. The effect of hypoxia on peripheral blood cells (PBC)

Note. Hypoxia increases the production of lymphangiogenic growth factors in three ways: **(A)** HIF-1(a heterodimer of 1α and 1β subunit)-related DNA transcription, **(B)** HIF-1-unrelated mRNA translation initiated by interaction of IRES and 43S preinitiation complex, and **(C)** HIF-unrelated mRNA stabilization mediated by combination of ARE and ARE-binding proteins. Notably, erythrocytes and platelets lack nuclei but have mRNAs in cytoplasm, rendering them impervious to the transcriptional regulation by HIF-1. 43S: 43S preinitiation complex; ARE: adenylate-uridylate-rich elements; HIF: hypoxia inducible factor; HRE: hypoxia-responsive elements; IRES: internal ribosome entry sites. Created with BioRender.com.

1.3.3.3 Pros and cons of HPS and its pro-lymphangiogenic hypothesis

HPS is a promising tool for regenerative medicine due to its simple manufacture, autologous origin, and its broad-spectrum growth factors. Our preceding studies have revealed elevations of several angiogenic growth factors in HPS, including VEGF, bFGF, IL-8, etc., when compared to serum that was not hypoxically stimulated [155, 156, 162, 165, 166]. These results accord with our hypothesis that HPS can provide more growth factors due to its source of the entire PBC spectrum and additional stimulation from hypoxia. With the enriched growth factors, HPS has shown robust proliferative effects on several cell types, including HUVECs, fibroblasts, and osteoblasts [156, 167]. Aside from cell proliferation, HPS showed significant promotion for tissue regeneration in wounds, accompanied with both blood and lymphatic vessel formation [166]. However, the underlying pro-lymphangiogenic

mechanism of HPS and its lymphangiogenic potential versus the gold standard PRP or baseline NS remain elusive. These knowledge gaps need to be elucidated by comparatively investigating specific protein levels of these secretomes and their stimulative effects on LECs. To this end, we designed this project to quantify six key lymphangiogenesis-related growth factors (VEGF-C, PDGF-BB, bFGF, TSP1, PF4, ES) in HPS, PRP and NS, and evaluate the feasibility of these secretomes on LEC proliferation, migration, two-dimensional (2D) tubulogenesis, and three-dimensional (3D) microvessel sprouting.

Despite the forementioned advantages of HPS, it has some limitations, such as the long incubation time (i.e., 4 days) and concurrent elevations of some inhibitory growth factors, which may partially offset the positive regenerative effect of HPS [165]. To mitigate the shortcoming of elevated inhibitory factors, we made serial dilutions of HPS to rebalance its pro- and anti-lymphangiogenic growth factors and anticipated to identify the optimal dilution(s) for LEC proliferation and LV sprouting.

1.4 Summary of the thesis

1.4.1 Hypothesis of the thesis

Lymphangiogenesis is a requisite in treating lymphedema and poor-healing wounds, yet it has been overshadowed by angiogenesis and received less attention. The neglected lymphangiogenic disorders can lead to persistent edema and hinder wound repair [168]. Therefore, effective lymphedema or wound management requires techniques that can promote lymphangiogenesis. So far, several experimental and practical strategies have been introduced. Traditional pro-lymphangiogenic strategies, such as topical administration of a single growth factor (e.g., VEGF-C) can cause concomitant LV and BV expansion, distortion, and leakage, and hence exacerbate edema [127, 135]. Other cutting-edge techniques, like tissue engineering [123, 169], stem cell injection [5] and gene therapy [170], are still distant from achieving clinical safety. Conversely, as a safe tool derived from autologous blood, PRP has been clinically applied to reduce wound size and lymphedema [171]. However, there is no “standard strategy” due to variant growth factor properties, lymphedema causes, wound bed conditions, and patient’s immunities. Moreover, upon literature reviews,

there is little research into the behaviors of LECs or patterns of LV neovascularization when stimulated by blood secretomes and there is a lack of comparative data about HPS, PRP, and NS. To resolve this knowledge gap and improve lymphangiogenic effect of blood-derived secretomes, herein we proposed investigating the lymphangiogenic potential of HPS, which is stimulated by pericellular hypoxia, a condition that commonly exists in poor-healing wounds.

Another intriguing part of this thesis is that HPS, which has shown its effectiveness in promoting BEC proliferation and BV formation, would be also highly possible to promote LEC proliferation and LV formation if we could harmonize the pro- and anti-lymphangiogenic growth factors in it. Furthermore, HPS treatment in fundamental science has the potential for an easy clinical translation since the manufacture of HPS is uncomplicated and avoids using specific equipment. This approach is likely to connect basic research on lymphangiogenesis and practical therapy for lymphedema and is promising for expediting wound healing and improving patients' quality of life.

1.4.2 Scope of the thesis

This thesis study centered on exploring the pro-lymphangiogenic potential of HPS. To this end, we manufactured individual secretomes of HPS, PRP, and NS from 9 blood donors for protein analysis, as well as pooled secretomes from 10 donors for the subsequent in vitro experiments on LECs from 3 human donors and LVs from 3 murine donors, expecting to achieve the following deliverables:

(a) identification of the ideal concentration(s) of HPS for lymphangiogenesis while ensuring an appropriate equilibrium between pro- and anti-lymphangiogenic effects,

(b) characterization of LEC proliferation and migration under HPS stimulation,

(c) characterization of LV regeneration (including LEC tube formation and LV sprouting) under HPS stimulation,

and (d) comparison of the lymphangiogenic potential of HPS with that of PRP and NS.

1.4.3 Aims of the thesis

This project was conducted aiming to:

1) determine six key pro- and anti-lymphangiogenic factors in HPS in comparison to PRP and NS.

   The focus is to discern the combined effect of these growth factors, which can help us understand the general regulation picture of lymphangiogenesis.

2) investigate the in vitro behaviors of LECs in response to HPS, PRP, and NS stimulation.

   The focus is to evaluate cell proliferation, migration, and tube formation (LEC organization), the latter of which includes the metrics of tubule number, length, branching points, and area percentage.

3) assess the lymphangiogenic potential of HPS, PRP, and NS in an ex vivo 3D culture model of mouse thoracic duct.

   The focus is to quantify the number and length of lymphatic sprouts originating from preexisting vessels, which is the keystone for verifying the pro-lymphangiogenic potential of HPS.

4) determine the ideal concentration(s) of HPS for lymphangiogenesis by performing graduated dilutions of HPS in comparison to NS and PRP.

2 Materials and methods

2.1 Ethical approval

The investigation received approval from the ethics committee at the Technical University of Munich, Germany, with a file number of 497/16S and a corresponding amendment granted on 11 November 2016. All implemented methodologies adhered to the ethical principles outlined in the World Medical Association Declaration of Helsinki (2013).

2.2 Materials

2.2.1 Cells and animals

- Human dermal lymphatic endothelial cells (PromoCell®, C-12217, PromoCell GmbH, Heidelberg, Germany)
- Crl:CD1 mice (Charles River Laboratories, Wilmington, DE, USA)

2.2.2 Reagents

- Antibiotic/antimycotic solution 100× (AAS-B, Capricorn Scientific GmbH, Ebsdorfergrund, Germany)
- Antigenfix (P0014, Diapath S.p.A, Martinengo, Italy)
- Calcein AM (invitrogen™, C3099, Life Technologies corp., Eugene, OR, USA)
- Collagen type I, rat tail (Millipore®, 08–115, EMD Millipore Corp., Temecula, CA, USA)
- DAPI (invitrogen™, D3571, Molecular Probes, Inc., Eugene, OR, USA)
- DMEM (PAN™ Biotech, P04-04500, PAN Biotech GmbH, Aidenbach, Germany)
- DPBS 10× w: Ca^{2+} and Mg^{2+} (Sigma®, D1283, Sigma-Aldrich Chemie GmbH, Steinheim, Germany)
- DPBS w/o: Ca^{2+} and Mg^{2+} (PAN™ Biotech, P04-36500, PAN Biotech GmbH, Aidenbach, Germany)

- Endothelial cell basal medium MV2 (PromoCell®, C-22221, PromoCell GmbH, Heidelberg, Germany)
- Goat anti-rabbit IgG (H&L) antibody (Alexa Fluor® 488, ab150077, Abcam plc, Berlin, Germany)
- Human bFGF ELISA kit (DuoSet®, DY233, R&D Systems, Inc., Minneapolis, MA, USA)
- Human endostatin ELISA kit (DuoSet®, DY1098, R&D Systems, Inc., Minneapolis, MA, USA)
- Human PDGF-BB ELISA kit (DuoSet®, DY220, R&D Systems, Inc., Minneapolis, MA, USA)
- Human PF4 ELISA kit (DuoSet®, DY795, R&D Systems, Inc., Minneapolis, MA, USA)
- Human TSP1 ELISA kit (DuoSet®, DY3074, R&D Systems, Inc., Minneapolis, MA, USA)
- Human VEGF-C ELISA kit (DuoSet®, DY752B, R&D Systems, Inc., Minneapolis, MA, USA)
- Matrigel®, growth factor reduced, phenol red free (Corning®, 356231, Corning Inc., MA, USA)
- Normal goat serum (ab7481, Abcam plc, Cambridge, UK)
- Rabbit anti-mouse LYVE1 antibody (ab33682, Abcam plc, Berlin, Germany)
- Resazurin sodium salt (Sigma®, R7017, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany)
- SupplementMix (PromoCell®, C-39226, PromoCell GmbH, Heidelberg, Germany)
- SupplementPack (PromoCell®, C-39221, PromoCell GmbH, Heidelberg, Germany)
- Thrombin (TISSEEL, Baxter Deutschland GmbH, Unterschleißheim, Germany)
- Triton X-100 (Triton™, 9036-19-5, Sigma-Aldrich, Saint Louis, MO, USA)

2.2.3 Equipment

- 0.2 µm-filter (Minisart®, 16534K, Sartorius GmbH, Goettingen, Germany)

- 24-well plate (CELLSTAR®, 662162, Greiner Bio-One GmbH, Frickenhausen, Germany)
- 30 mL-syringe (Omnifix®, 4617304F, B Braun AG, Melsungen, Germany)
- 96-well plate (CELLSTAR®, 655180, Greiner Bio-One GmbH, Frickenhausen, Germany)
- µ-Slides (ibidi®, 81506, ibidi GmbH, Martinsried, Germany)
- C-Chip hemocytometer (DHC-N01, NanoEnTek Inc., Gyeonggi-do, Korea)
- Cell culture flask (CELLSTAR®, Red filter screw cap, Greiner Bio-One GmbH, Frickenhausen, Germany)
- Centrifuge conical tube (Falcon®, 352070, Corning Science, Tamaulipas, Mexico)
- Culture-insert 2 well (ibidi®, 80209, ibidi GmbH, Martinsried, Germany)
- Evacuated blood collection system (BD Vacutainer®, 366575, Becton, Dickinson and Company, Plymouth, UK)
- Inverted fluorescence microscope platform (Axio Observer Z1, Carl Zeiss, Jena, Germany).
- Inverted phase contrast light microscope system (Axio Vert.A1, Carl Zeiss, Jena, Germany)
- Mini centrifuge (Ministar C1413, VWR international GmbH, Darmstadt, Germany)
- Multimode microplate reader (Mithras LB 940, Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany)
- Surgical microscope (OPMI® pico S100, Carl Zeiss Meditec AG, Jena, Germany)
- Three-way stopcock (Discofix®, 16496C, B Braun AG, Melsungen, Germany)

2.2.4 Softwares

- GraphPad Prism (Version 9.3.1, GraphPad Software LLC, San Diego, CA, USA)
- ImageJ platform (Version 1.52v, National Institutes of Health, Bethesda, MD, USA)
- MikroWin 2000 (Version 5.17, Mikrotek Laborsysteme GmbH, Overath, Germany)

- NeuronJ plugin (Version 1.4.3, Erick Meijering)
- WimScratch and WimTube tools (Wimasis, Onimagin Technologies SCA, Córdoba, Spain)

2.3 Production of human blood derived secretomes

All blood donors offered informed consent. Participants were excluded with cigarette smoking, pregnancy, active illnesses, or medication within the preceding six weeks, which may cause fluctuations of abundant growth factors [172, 173]. For pooled secretome preparation, ten healthy male and female donors (7:3, n=10) between 20 and 34 years of age were included. While separate secretomes for growth factor measurements were derived from nine donors (n=9), as one female donor was unable to participate due to illness. Blood collection and subsequent processing were conducted under aseptic conditions. The resultant blood-derived products were cryopreserved at -80℃ for up to three months, avoiding repeated freeze-thaw cycles, to ensure the effectiveness of secretomes [165].

2.3.1 Platelet-rich plasma (PRP) preparation

We utilized a multi-centrifugation procedure at high centrifugal forces (RCF) to increase the platelet concentration in PRP [174]. Venous blood was drawn using an evacuated blood collection system and transferred to a conical tube after thoroughly mixed with trisodium citrate. The anticoagulated blood was centrifuged under 1,300 RCF for 20 min, resulting in three distinctive layers (from top to bottom: the platelet-poor plasma, buffy coat comprised of platelets and leukocytes, erythrocytes). The first and second layers, including the superior 5 mm of the bottom layer, were harvested to encompass as many platelets as possible and were centrifuged again under 1,800 RCF for 15 min to split into platelet sediments and plasma supernatant. The supernatant was then removed, leaving the concentrated platelets to be mixed with 1 IU/mL thrombin that had dissolved in basal medium and further activated at 37℃ for 30 min. The activated PRP was subsequently centrifuged under 2,500 RCF for 20 min to isolate the supernatant, which was technically the PRP-lysate (approximately 0.5 mL PRP out of 6 mL blood) (**Figure 5. A**). In the following chapters, the term “PRP” was used to

refer to PRP-lysate to obviate conceptual confusion. This PRP product was cryopreserved at -80 ℃ in either pooled or separate aliquots, depending on experimental requirements.

Since the optimal protocol for preparing PRP remains a subject of debate, we guaranteed our protocol by counting the platelet number of non-activated PRP versus the whole blood counterpart from three randomly selected donors (n=3). The PRP and whole blood were diluted in PBS respectively by factors of 400 and 200 and pipetted to C-Chip hemocytometers (10 μL/chamber). The hemocytometers were observed under an inverted phase contrast microscope with a 40× objective. Platelet numbers in five small squares (center and corners) were counted and concentrations were computed according to the product manual.

2.3.2 Normal serum (NS) preparation

The baseline NS was obtained through natural sedimentation under normoxic conditions. Briefly, 20 mL of blood was drawn from the antecubital vein using an anticoagulant-free 30 mL-syringe. The syringe was capped with a 0.2 μm-filter attaching to an opened three-way stopcock, and the entire apparatus was kept upright and undisturbed at room temperature (20 ℃) for 4 h. The resultant yellowish supernatant comprised NS (approximately 5 mL NS out of 20 mL blood) and was strained into a new syringe. NS was immediately cryopreserved at -80℃ in either pooled or separate aliquots, depending on experimental requirements (**Figure 5. B**).

2.3.3 Hypoxia-preconditioned serum (HPS) preparation

The HPS was produced by PBC culture in a hypoxia micromilieu within a regular normoxic incubator, which had been reported in detail by our prior laboratory team [156, 165]. In brief, 20 mL of peripheral blood was drawn from the antecubital vein into an anticoagulant-free 30 mL-syringe. The syringe was kept upright and sealed after drawing in 5 mL of air via a 0.2 μm-filter connected with a three-way stopcock. During a four-day incubation (5% CO_2, 37 ℃), the pericellular oxygen level spontaneously declined to approximately 1% owing to oxygen utilization in PBCs. At the end of incubation, the blood separated to three layers, with HPS on top, followed

by leukocytes and clotted platelets in the middle, and erythrocytes at the bottom. The lower two layers were filtered out, leaving HPS in a newly attached syringe (minimally 5 mL HPS out of 20 mL blood). HPS was then collected as either pooled or separate aliquots, depending on experimental requirements, and immediately cryopreserved at -80℃ (**Figure 5. C**).

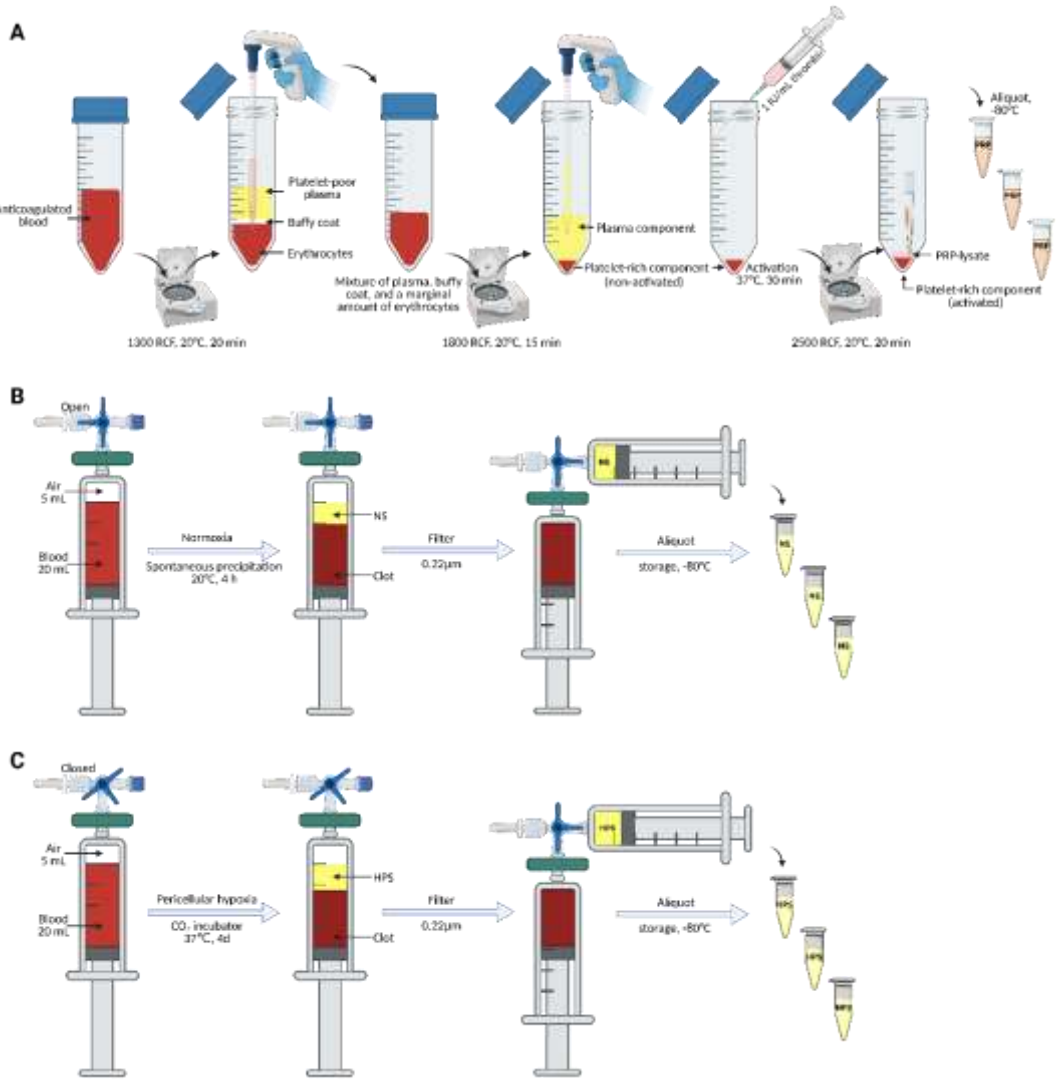

Figure 5. Schematic flow charts for manufacturing blood-derived secretomes

Note. Three representative blood-derivatives were produced in this thesis study. **(A)** PRP preparation with the multi-centrifugation protocol. **(B)** NS preparation by natural precipitation. **(C)** HPS preparation, a novel technique rendering pericellular hypoxia in a regular incubator. Created with BioRender.com.

2.4 Cell culture

The human dermal lymphatic endothelial cells (LEC) were cultured in flasks using the endothelial cell growth medium MV2 (EGM MV2). This growth medium was composed of endothelial cell basal medium MV2 (EBM MV2) and its matching SupplementMix, providing a combination of 0.05 mL/mL fetal calf serum (5% FCS), 5 ng/mL EGF, 10 ng/mL bFGF, 20 ng/mL IGF-1 (long R3), 0.5 ng/mL VEGF-165, 0.2 µg/mL hydrocortisone, and 1 µg/mL ascorbic acid. Since the formulation of EGM MV2 has been commercially optimized for LEC culture, it was used as our positive control. The negative control, which also serves as our basal medium to dilute the blood-derived secretomes, was made up by adding 1% FCS, 0.2 µg/mL hydrocortisone, and 1 µg/mL ascorbic acid to EBM MV2. The sample media in this study included pure HPS and NS and their graduated dilutions in basal medium (0.1/1/10/40%, v/v), and pure PRP without any dilution due to its highly concentrated nature by PRP definition. LECs were cultured at 37℃, 5% CO_2 and subcultured between passage 3 and 5 for the following cell experiments, each of which was repeated in triplicate with LECs from three donors (n=3).

2.5 Protein quantification for lymphangiogenic growth factors

To determine the levels of selected promotive (VEGF-C, PDGF-BB, bFGF) and inhibitory lymphangiogenic growth factors (TSP1, PF4, ES) in blood-derived secretomes, we implemented enzyme-linked immunosorbent assays (ELISA) on separate samples of HPS, NS and PRP (n=9) with respective reagent kits. As per the kits' instructions, we sandwiched the secretome samples with specific capture and detection antibodies, and then initiated an oxidation reaction between streptavidin-HRP (horseradish peroxidase) and substrate to generate fluorescent signals. The signals were instantly detected at 450 nm by a microplate reader and converted to growth factor concentrations by the MikroWin 2000 software.

2.6 Cell proliferation assay

The LEC proliferation was assessed by detecting viable cells through mitochondrial reduction of non-fluorescent resazurin, the primary constituent of alamarBlue, to

fluorescent resorufin, which can be quantified as optical density (OD). In our preliminary tests, we have established the optimal plating density and incubation time for LECs. Specifically, 3,100 cells in 150 μL basal medium were seeded per well in 96-well plates, followed by an overnight starvation. After starvation, the basal medium was substituted with sample media (HPS- and NS-0.1/1/10/40/100%, and PRP), positive and negative control media (150 μL/well), and LECs were cultured at 37℃ and 5% CO_2 for 96 h without changing media. At the end of culture, the spent media for all groups were changed to EGM MV2 mixed with 1/10 volume of the pre-dissolved 10× resazurin solution. Plates with the renewed resazurin medium were incubated at 37℃ for 4 h. Subsequently, 100 μL of the supernatants was collected per well for OD measurements at 560 nm in the microplate reader. LEC proliferation in each group was expressed in OD percentage relative to the negative control.

2.7 Cell migration assay

The migration of LECs is integral to LV formation and elongation [21]. To investigate the migration ability, we seeded cells in double-chambered culture-inserts with a gap of 500 μm in width, allowing for cell mobilization. In brief, LECs were suspended in basal medium and seeded in inserts that had been pre-attached on 24-well plates at a density of 40,000/cm^2 (70 μL/chamber). An additional volume of basal medium (400 μL/well) was supplemented around the insert. LECs were cultured at 37℃, 5% CO_2 for 18 h to allow for adhesion before removal of the inserts. Subsequently, cells were washed with PBS and cultured with sample media: HPS and NS (0.1/1/10/40/100%), PRP, positive and negative controls (1 mL/well). The incubation proceeded without changing media until the fastest migration group completely closed the gaps (24 h). Gap closures were recorded under an inverted phase contrast microscope at 0, 12, and 24 h, and were analyzed as acellular area percentage by WimScratch tool.

2.8 Endothelial cell tube formation assay

The tube-like structures formed from LEC organization on basement membrane were observed to evaluate the lymphangiogenic effect of different blood-derived secretomes. The optimal plating density and incubation time for this assay had been determined in

preliminary tests. Briefly, after the overnight starvation in basal medium, LECs were suspended in sample media (HPS- and NS-0.1/1/10/40/100%, and PRP), positive and negative controls. Cell suspensions were then evenly loaded into μ-Slides (8,000 cells in 50 μL/well), which had been prefilled with growth factor reduced Matrigel (10 μL/well) and had polymerized at 37℃ for 30 min. LECs on Matrigel were incubated at 37℃, 5% CO_2 for 8 h to allow lymphatic tubules to develop. Tubules from four random fields per well were then captured under an inverted phase contrast microscope. To demarcate living cells, tubules were immediately stained with 1 μg/mL Calcein AM diluted in DPBS (1×, with Ca^{2+} and Mg^{2+}) for 5 min and observed under a fluorescent microscope at 498 nm. The tube-forming capability of various secretomes was analyzed using the WimTube service in terms of total tube number, cumulative tube length, total branching points, and cell-covered area percentage per field.

2.9 Lymphatic sprouting assay

In order to replicate the multidimensional interactions between LECs and extracellular matrix (ECM) and mimic the vascular budding from parental LVs, we applied the sprouting assay by culturing thoracic duct rings in rat tail collagen type I to evaluate the lymphangiogenic potential of different secretomes [175]. This ex vivo sprouting assay simulated the real LV regeneration in a 3D structure, only involving primary LECs and excluding any immune cell interference. Based on the preceding results obtained from LEC proliferation, migration, and tube formation, we selected HPS and NS of 10% and 40% and deselected groups with concentrations of 0.1, 1, and 100% that were unlikely to benefit LEC growth.

2.9.1 Animal dissection and 3D culture

Animal dissections were performed with sterile microsurgical instruments under a surgical microscope (12.5× eyepieces, f = 250 mm, magnification changer γ factor = 0.6) in the animal operation theatre. Thoracic ducts of the freshly euthanized Crl:CD1 mice (aged between 8 to 12 weeks) were exposed and carefully harvested posteromedially to the descending aorta (**Figure 6. A**). The ducts were soaked in DMEM premixed with 1× antibiotic/ antimycotic solution and subsequently separated

from fibroadipose sheaths and uniformly cut into segments (~25 segments/duct, thickness < 500 μm) (**Figure 6. B**). The segments (i.e., lymphatic rings) were then kept in an incubator (37℃, 5% CO_2) for a maximum of 3 h.

In the meantime, the type I collagen was prepared on ice by thoroughly mixing with 10× DMEM, 1× DMEM, and ultrapure water (1:5:4, v/v), to reach a concentration of 1.5 mg/mL. The pH was adjusted to 7. Bubbles were removed by a mini centrifuge (< 2,000 RCF, 10 s). Next, 50 μL of the liquid collagen was dispensed per well onto a 96-well plate using chilled pipettes within 15 min and polymerized at 37℃ for 20 min. On surface of the polymerized collagen, the lymphatic segments were loaded and overlain with another 50 μL of the liquid collagen. This collagen I-embedded lymphatic ring complex was incubated again (37℃, 30min) for polymerization and then fed sample media (HPS- and NS-10/40%, PRP) and control media (150 μL/well) (**Figure 6**. C). The spent media were renewed the other day and then every three days. On day 7, 9, and 11, sprouting was observed before degradation and the ring-sprout entities were photographed using an inverted phase contrast microscopy. By tuning the focal depth, 2-3 images were obtained per well to include all sprouts (**Figure 6**. D). The sprouts were traced using the semi-automatic plugin of NeuronJ in ImageJ platform. The lymphatic sprouting assay was repeated in triplicate with different murine donors (n=3).

2.9.2 Immunostaining

In order to authenticate the lymphatic lineage of both the rings and their sprouting microvessels, we employed the whole-mount immunostaining with lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) antibodies before and after the 3D culture, respectively [176]. To facilitate the antibody penetration through collagen, we removed the upper collagen layer without compromising sprouts prior to the immunostaining procedures.

The tissue segments (before the 3D culture) and collagen-embedded tissue segments (after the 3D culture) were rinsed three times with DPBS before a 2 h-fixation in 4% paraformaldehyde (Antigenfix, 200 μL/well) at room temperature, ensued with thorough washings and a 1.5 h-blocking with 20% normal goat serum (NGS) in 0.2%

TritonX-100 in DPBS (20% NGS/ 0.2% PBSTx, 150 μL/well). The blocked tissue segments were incubated overnight at 4℃ with primary antibodies (1:300 LYVE1 antibodies in 5% NGS/ 0.2% PBSTx, 150 μL/well), then were rinsed with 0.2% PBSTx and conjugated with secondary antibodies (1:400 Alexa Fluor® 488 in 5% NGS/ 0.2 PBSTx, 150 μL/well) at room temperature in the dark for 2 h. Tissue segments were further washed and stained with the DNA-binding DAPI solution (1:20,000) for 1 h, followed by immediate fluorescence detection at excitation wavelengths of 498 nm and 359 nm.

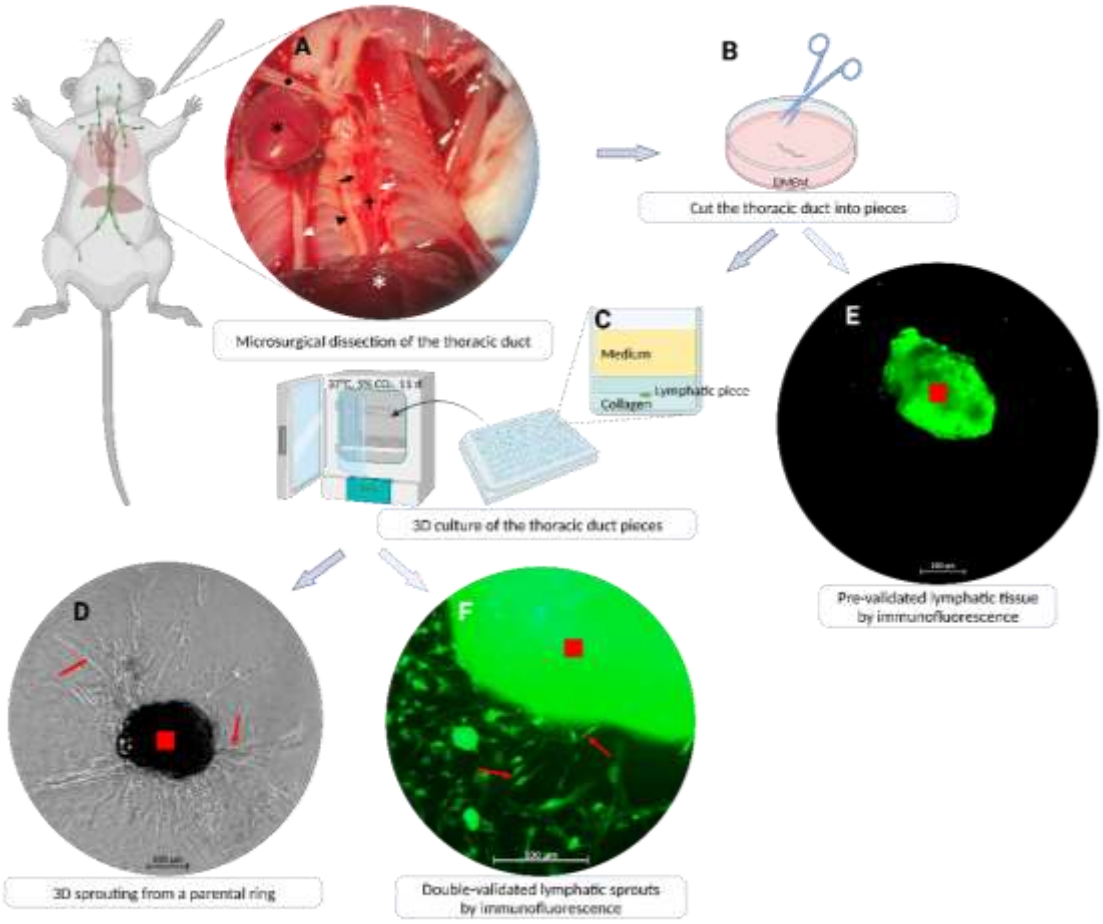

Figure 6. Sprouting assay of thoracic duct rings

Note. Segments of the mouse thoracic duct were cultured in type I collagen matrix to develop 3D microvessels. **(A)** The translucent thoracic duct was dissected from the thoracic cavity with a 5.1 × magnification. Caution was exercised to prevent mechanical damage to the delicate structure and to keep it moisturized with sterile DPBS. Black arrow: thoracic duct; black arrowhead: fibroadipose sheath wrapping the thoracic duct; white arrow: azygos vein; white arrowhead: inferior vena cava; black star: descending aorta; black asterisk: heart; white asterisk: liver covered by diaphragm superiorly; black dot: esophagus (cut and upturned). **(B)** The lymphatic strip was immersed in DMEM and soon cut into thin segments. **(C)** The lymphatic segment was mounted in a collagen "sandwich". **(D)** 3D sprouts radiated from the central lymphatic ring into the collagen matrix. Captured on the 11th day. **(E)** The harvested tissue was pre-validated by LYVE1 antibodies specific to lymphatic tissues. **(F)** The lymphatic origin of sprouts was double-validated with LYVE-1 antibodies. Red square: thoracic duct segment; red arrow: lymphatic sprout. Scale bar = 100 μm. **(F)** is adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI. Created with BioRender.com.

2.10 Statistical analysis

All data were presented as mean ± SEM (standard error of the mean). One-way ANOVAs with Tukey test, using GraphPad Prism (version 9.3.1 for Windows), were performed for multiple comparisons. For paired comparisons between two groups, paired t tests were conducted. Statistical significance was defined as $p < .05$ (* $p < .05$, ** $p < .01$, *** $p < .001$, and **** $p < .0001$).

3 Results

3.1 VEGF-C, PDGF-BB, and bFGF increased in HPS

The present study investigated the levels of representative lymphangiogenic growth factors in different secretomes. The levels of pro-lymphangiogenic VEGF-C, PDGF-BB and bFGF were shown to be significantly higher in HPS versus in PRP and NS ($p = .0439$ and $p < .0001$, respectively) (**Figure 7**). Compared with NS, PRP had higher levels of VEGF-C (12.24 ± 1.28 vs 7.25 ± 0.60 ng/mL, $p = .0024$), similar levels of PDGF-BB (2.34 ± 0.21 vs 2.25 ± 0.32 ng/mL, $p = .9715$), and lower levels of bFGF (18.60 ± 8.50 vs 66.07 ± 23.97 pg/mL, $p = .0400$). To ensure the quality of PRP used in this study, platelet number before the activation procedure was counted to be 4.9 times more than that in whole blood (1,213.33 ± 126.66 × 10^9 vs 246.66 ± 38.44 × 10^9/L, $p = .0083$) (**Figure 8**).

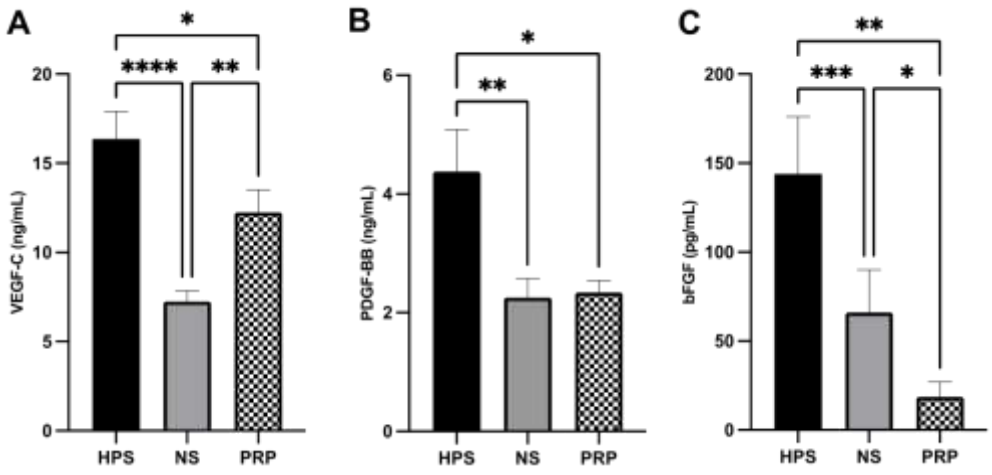

Figure 7. Concentrations of pro-lymphangiogenic growth factors

Note. HPS exhibited a significantly increase in the levels of VEGF-C, PDGF-BB, and bFGF in comparison to PRP and NS **(A-C)**. One-way repeated measures ANOVAs with Tukey test were used to compare means of multiple groups. N (blood donor) = 9, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

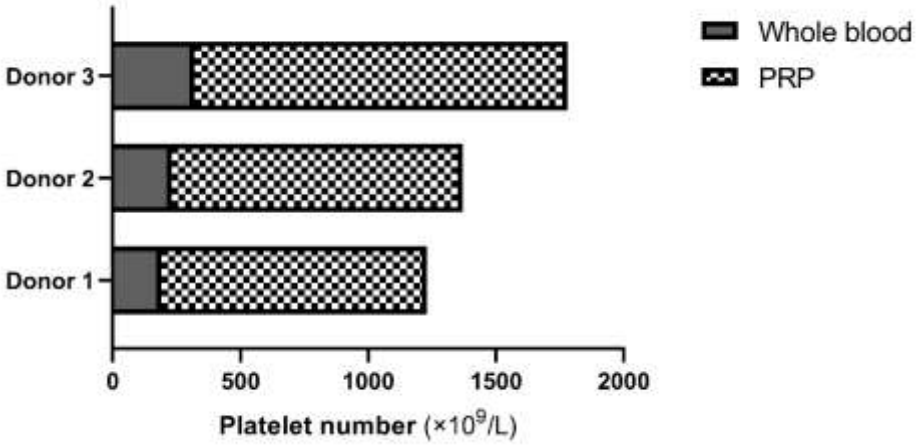

Figure 8. Platelet count

Note. The mean platelet number in non-activated PRP was 4.9-fold as many as in whole blood ($p = .0083$). Paired t test was performed with a two-tailed 95% confidence interval. N (blood donor) = 3.

With regard to anti-lymphangiogenic factors, the levels of TSP1 increased comparably in HPS and PRP (14.51 ± 1.20 vs 18.35 ± 2.23 μg/mL, $p = .1705$), and both were higher than those in NS (8.87 ± 0.77 μg/mL, $p = .0006$ and $p = .0029$, respectively). PRP comprised threefold as much PF4 as HPS (8.73 ± 0.68 vs 2.96 ± 0.32 μg/mL, $p < .0001$), while HPS contained more PF4 than NS (2.15 ± 0.22 μg/mL, $p = .0076$). ES levels were higher in HPS compared to both NS and PRP (24.07 ± 0.75 vs 16.63 ± 0.63 and 3.22 ± 0.56 ng/mL, respectively, $p < .0001$) (**Figure 9**).

Overall, the above findings suggest that HPS has a unique pro-lymphangiogenic and anti-lymphangiogenic factor profile, which may have positive implications for LEC proliferation and LV formation.

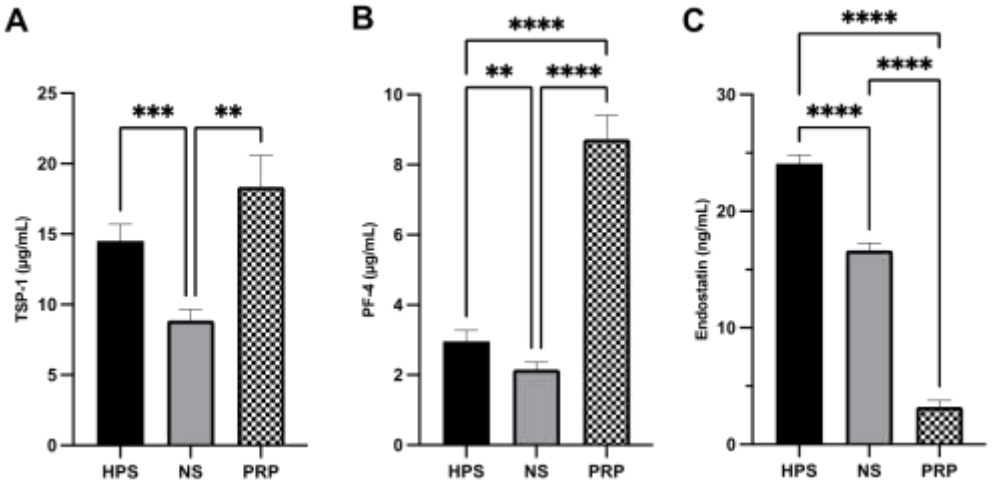

Figure 9. Concentrations of anti-lymphangiogenic growth factors

Note. The levels of TSP1, PF4, and ES increased significantly in HPS versus the baseline NS **(A-C)**. PRP showed higher levels of TSP1 than NS **(A)**, the highest levels of PF4 **(B)**, and the lowest levels of ES **(C)**. One-way repeated measures ANOVAs with Tukey test were used to compare means of multiple groups. N (blood donor) = 9, * $p < .05$, ** $p < .01$, *** $p < .001$, **** $p < .0001$. Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

3.2 HPS-40% maximized LEC proliferation

We investigated the proliferation effect of different secretomes on LECs by Alamar blue assay. LEC proliferation was assessed at 96 h by calculating the OD percentage relative to the negative control. The results demonstrated a significantly higher OD percentage in HPS-40% (872.39 ± 12.33%) compared to all other groups (**Figure 10**). At either lower (HPS-10/1/0.1%) or higher concentrations (HPS-100%), the relative OD value decreased, with HPS-10% and HPS-1% still displaying superior results in comparison to PRP ($p < .0001$ and $p = .0004$, respectively). PRP treatment showed a higher OD percentage than the negative control (195.48 ± 3.01 vs 100.00 ± 20.78%, $p = .0432$), whereas comparable to pure HPS and NS ($p = .9996$ and $p = .3486$). When compared with NS head-to-head, except for HPS-40%, the other HPS secretomes showed no statistical differences. These findings suggest that HPS-40% has the most

proliferative effect on LECs, while PRP has a moderate effect compared to the negative control.

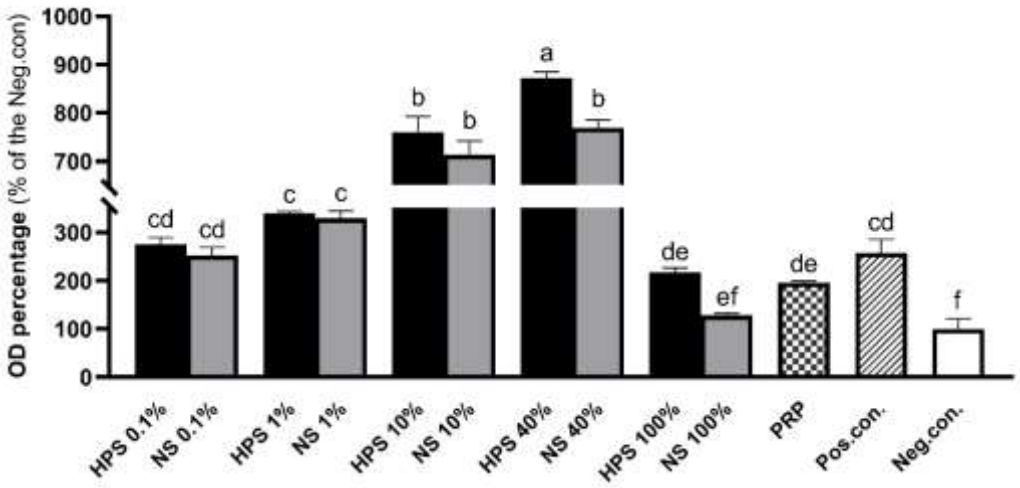

Figure 10. Influence of different blood secretomes on LEC proliferation

Note. The LEC proliferation was assessed as relative cell viability in terms of % OD of the negative control, using resazurin reduction assay. LECs treated with HPS-40% showed the greatest proliferation with statistical significance. One-way repeated measures ANOVA with Tukey test was used to compare means of multiple groups. N (cell donor) = 3. Bars with different lowercase letters on top are statistically different ($p < .05$). Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

3.3 HPS-40% reduced migration gap more than PRP

In addition to proliferation, LEC migration is essential for LV elongation, and was evaluated by calculating the acellular area percentage per field. The results showed that HPS-40% had a greater potential on reducing the gap area compared to both PRP (1.32 ± 0.60 vs $10.00 \pm 0.23\%$, $p = .0311$) and NS-40% ($20.23 \pm 3.90\%$, $p < .0001$), and was comparable to the positive control ($0.27 \pm 0.17\%$, $p > .9999$) (**Figure 11**). Despite no statistical gap area difference between conditions of HPS-40% and HPS-10%, the mean value of the former was approximately 1/3 of the latter (1.32 ± 0.60 vs $3.85 \pm 0.72\%$, $p = .9939$). As opposed to the negative control, PRP and all the HPS conditions, except HPS-0.1%, significantly reduced the gap area. The area percentage

in PRP was nearly equivalent to that in HPS-100% (10.00 ± 0.23 vs 11.24 ± 1.64%, $p > .9999$), and much less than in NS-100% (43.05 ± 3.30%, $p < .0001$). Additionally, in all pairwise comparisons with NS, HPS revealed strong gap-reducing capability, with HPS-40% significantly outranked. These findings indicate that both the HPS and PRP can enhance LEC movement, with HPS-40% more potent than PRP.

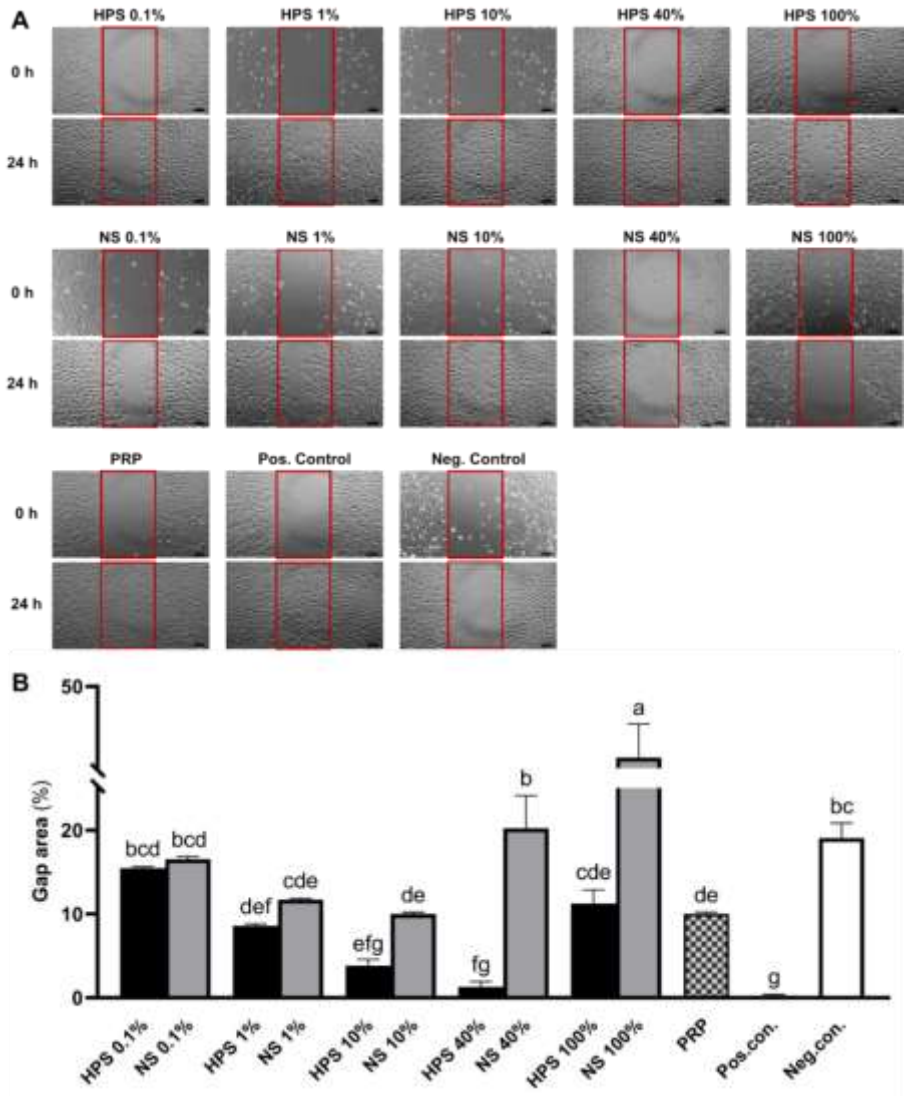

Figure 11. Influence of blood secretomes on LEC migration

Note. LEC migration was evaluated by measuring the acellular gap area (% of the total area per field). A smaller gap area percentage represented a strengthened cell motility, and thus a stronger pro-migration effect of the blood secretomes. The blood secretomes (HPS, NS, and PRP) were pooled from 10 donors. **(A)** Micrographs showed the initial cell distribution at 0 h and cell migration status at 24 h. The red rectangles illustrate the normalized acellular gap created by ibidi® culture-inserts. Scale bar = 100 μm. **(B)** The chart showed gap closure at 24 h under different conditions, among which HPS-40% treatment remained significantly smaller gaps than treatments from PRP, NS, and the other concentrations of HPS. One-way repeated measures ANOVA with Tukey test was used to compare means of multiple groups. N (cell donor) = 3. Bars with different lowercase letters on top are statistically different ($p < .05$). Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

3.4 HPS-10% triggered superior tubulogenesis versus HPS-40% and PRP

The formation of tube-like structures from LECs exposed in different secretomes demonstrates the cell organization ability that is based on proliferation and migration. This tubulogenic potential can be quantitively assessed by computing the total tube number, cumulative tube length, number of branching points, and area percentage occupied by cells, as presented in the live cell staining images (**Figure 12. A**).

The results revealed that treatment with HPS-10% yielded a significantly larger total tube number (10.07 ± 0.32/field) than all groups except the positive control (9.43 ± 0.30/field, p = .8900). The tube number decreased at both lower (HPS-10/1/0.1%) and higher concentrations (HPS-40/100%). Even, the higher concentrations of HPS (40 and 100%) induced nearly the same fewer tubes than the negative control (2.83 ± 0.09 and 2.87 ± 0.23 vs 4.37 ± 0.07/field, p = .0198 and p = .0243, respectively). In comparison to the negative control, PRP stimulated a larger number of tubes (6.20 ± 0.21/field, p = .0029) (**Figure 12. B**).

Similarly, the cumulative tube length in HPS-10% was significantly longer than in other media, except for the positive control and NS-10% (7.35 ± 0.03 vs 7.02 ± 0.06 and 6.45 ± 0.09 mm/field, p = .9963 and p = .2162, respectively). The lengths decreased bidirectionally toward lower (HPS-1/0.1%) and higher HPS concentrations (HPS-40/100%). The mean length in PRP was 0.77 mm longer than in the negative control, despite lacking statistical significance (p = .4258) (**Figure 12. C**).

The number of branching points displayed a similar trend to that of tube number and length, where the HPS-10% treatment, nearly as potent as the positive control (36.53 ± 0.79 vs 37.93 ± 0.54, p = .8513), generated significantly greater number of branching points versus other treatment conditions. Secondary to HPS-10%, PRP induced significantly more branching points (29.83 ± 0.44) than other HPS media (0.1/1/40/100%) and the negative control (**Figure 12. D**).

The percentage of cell-covered area per field, which was proportional to the area covered by tube-like structures, was significantly larger in HPS-10% group (56.27 ± 1.27%) when compared to PRP and HPS-0.1/1/40%. Different from the downtrends past summits in HPS-10% for tube number, length, and branching points, the area

percentage in HPS-100% conversely increased by 36.67% as compared to HPS-40%, despite lacking statistical significance ($p = .5253$). Although the mean value of area percentage in PRP was larger than that in negative control, it was nonsignificant (36.09 ± 3.94 vs $30.18 \pm 1.17\%$, $p = .9895$) (**Figure 12. E**).

In summary, both HPS and PRP were able to trigger tube formation from LECs. However, HPS-10%-treated LECs developed into a significantly higher number of tube-like structures with greater length, branching points, and cell-covered area when compared to PRP. Furthermore, among these parameters, HPS-10% significantly outperformed the other HPS concentrations except for the area percentage in HPS-100%.

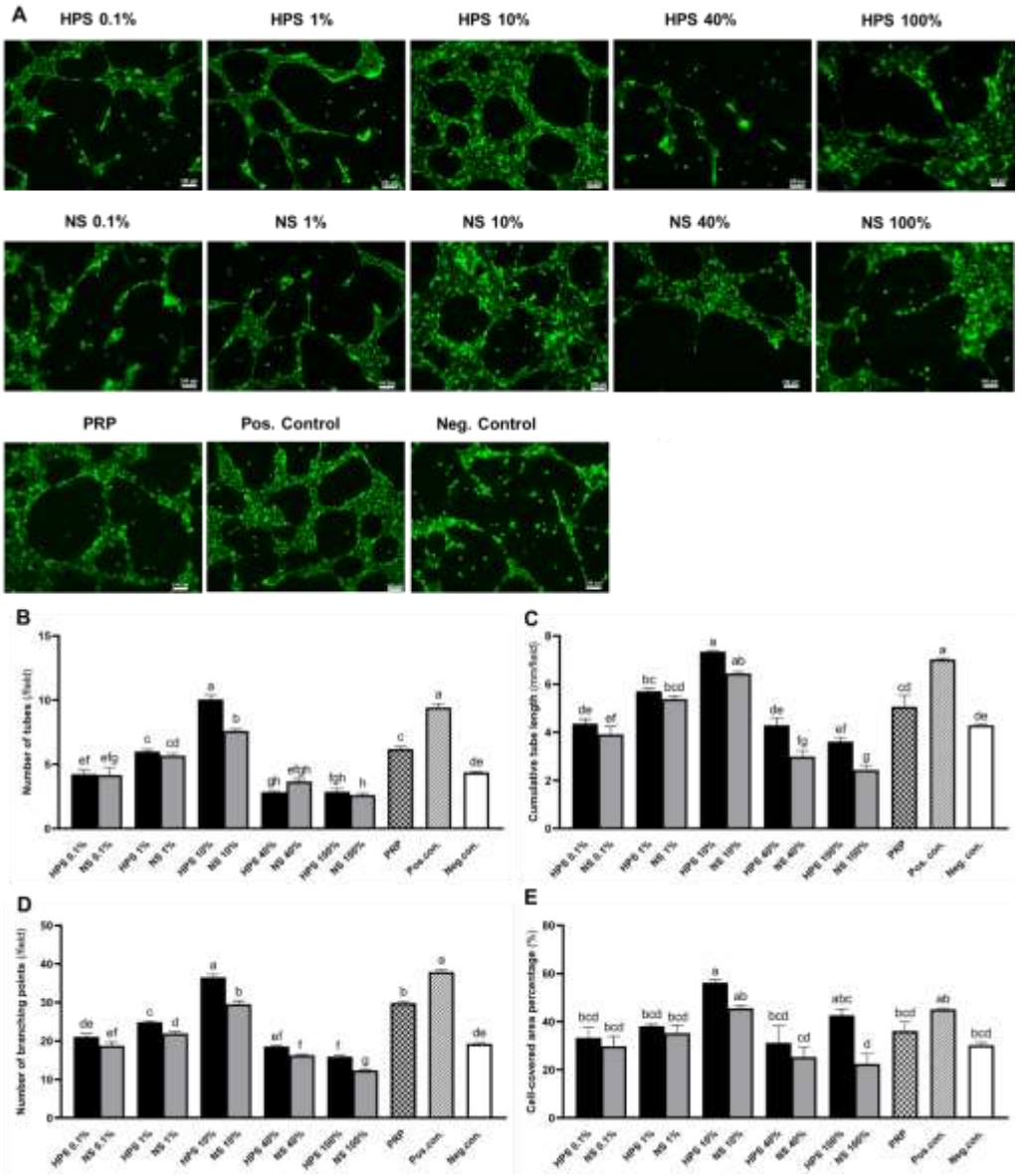

Figure 12. Influence of blood secretomes on LEC tube formation

Note. The tubulogenic capability of HPS, NS, and PRP (pooled from 10 blood donors) on LECs were evaluated after 8 h-treatments. **(A)** Live LECs were labeled with Calcein AM and observed via GFP channel. Scale bar = 100 μm. The tubulogenesis results were quantified in terms of total number of tubes per field **(B)**, cumulative tube length per field **(C)**, total number of branching points per field **(D)**, and % area occupied by cells **(E)**. One-way repeated measures ANOVAs with Tukey test were used to compare means of multiple groups. N (cell donor) = 3. Bars with different lowercase letters on top are statistically different ($p < .05$). Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

3.5 HPS-10% enhanced lymphatic sprouting greater than HPS-40% and PRP

The thoracic duct ring sprouting assay was used to investigate the pro-lymphangiogenic potential of different secretomes by inducing LV generation from preexisting vessels. Microvessel sprouting is an alternative and primary LV formation route aside from cell organization, resulting in more mature LVs than LEC tube formation due to its extended incubation and 3D structure support. To confirm the lymphatic origin of the ring and its budding sprouts in the matrix, the whole-mount immunostaining was conducted using LYVE1 antibodies before and after the 3D culture (**Figure 6. E and F**). Referring to the previously determined secretome concentrations ideal for either LEC proliferation and migration (40%) or tube formation (10%), we selected HPS of 10% and 40%, as well as corresponsive baseline NS dilutions and the gold standard pure PRP, for this sprouting assay. Sprouting micrographs on day 11 were captured for analysis in terms of total sprouting number and cumulative sprouting length per thoracic duct ring (**Figure 13. A**).

The results showed that HPS-10% treatment significantly promoted the most and longest sprouts among all groups. Regarding the sprouting number, the HPS-40% treatment produced precipitously fewer sprouts than HPS-10% (26.33 ± 4.10 vs 57.33 ± 7.84/ring, $p = .0010$), but a comparable number to PRP condition (29.33 ±3.38/ ring, $p = .9975$). PRP yielded more sprouts than the negative control (29.33 ± 3.38 vs 9.00 ± 0.58/ring, $p = .0305$) (**Figure 13. B**).

In terms of sprouting length, both PRP and HPS-40% elongated the sprouts greater than the negative control (4.35 ± 0.40 and 3.13 ± 0.57 vs 0.72 ± 0.22 mm/ring, $p = .0015$ and $p = .0388$, respectively). However, they exhibited a significantly weaker elongation effect than HPS-10% (8.79 ± 0.10 mm/ring, $p = .0002$ and $p < .0001$, respectively) (**Figure 13. C**).

To summarize, HPS-10% treatment on the ex vivo thoracic ducts led to significantly more sprouts with greater lengths than the other groups, including the PRP treatment, which although demonstrated similar sprouting number and length to the positive control ($p = .9653$ and $p = .8541$, respectively) and were greater than the negative

control. HPS-40% treatment, on the other hand, exhibited much fewer sprouts and shorter lengths than HPS-10%, comparable to PRP.

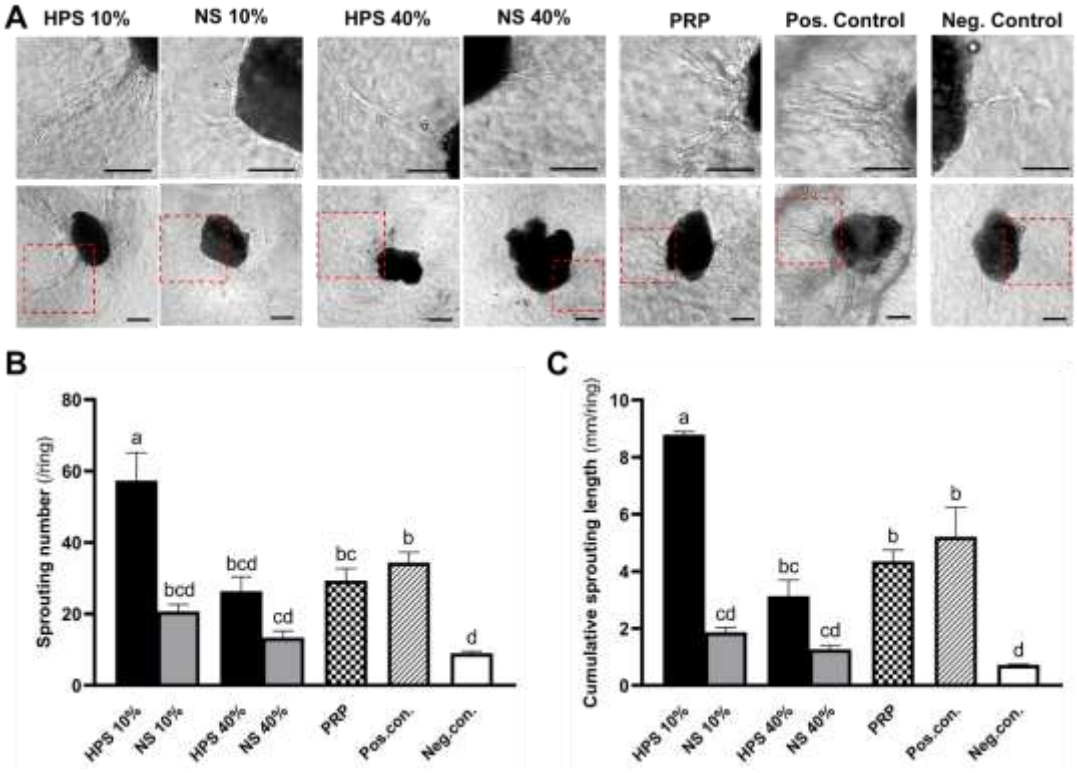

Figure 13. Influence of blood secretomes on thoracic duct sprouting

Note. The 3D culture of lymphatic rings in collagen type I with HPS (10/40%), NS (10/40%), and PRP (pooled from 10 blood donors). **(A)** Sprouting contours on the 11th day. Dashed red rectangles delineated the magnified area in the upper row. Scale bar = 100 μm. Sprouts were traced and analyzed in terms of: **(D)** total sprouting number and **(E)** cumulative length per ring. One-way repeated measures ANOVAs with Tukey test were used to compare means of multiple groups. N (mouse donor) = 3. Bars with different lowercase letters on top are statistically different ($p < .05$). Adapted with permission from "In Vitro Comparison of Lymphangiogenic Potential of Hypoxia Preconditioned Serum (HPS) and Platelet-Rich Plasma (PRP)", by J Jiang and X Cong, et al. 2023, *Int J Mol Sci*, 24, p.1961. Open access by MDPI.

4 Discussion

The hypoxia-preconditioning strategy has been widely employed in tissue regeneration and has evolved into various forms, such as anaerobic incubation, ischemia, intermittent hypoxia, and combined hypobaria-hypoxia [151, 152, 177]. In addition to these artificially created hypoxic milieus, spontaneous pericellular hypoxia can be formed in inflammatory zones as a result of restricted oxygen transportation, which is caused by disrupted vasculatures and increased oxygen consumption from recruited immune cells and is typical during wound healing [178].

Wound healing is spatiotemporally a biological event intertwined with regeneration of different tissues, especially epidermis and BVs. Thus, re-epithelialization and neovascularization have been emphasized in wound repair [179]. Current repairing strategies focus on replenishing growth factors indispensable for dermatogenesis or angiogenesis, either by exogenous delivery or by endogenous synthesis [180, 181]. These strategies have been reported effective, yet still exposed limitations such as vascular leakage when frequently using a single growth factor, like VEGF [135]. Moreover, wound healing involves more than dermatogenesis and angiogenesis, and includes lymphangiogenesis as well. However, lymphangiogenesis has received less attention than angiogenesis. Complementary to angiogenesis, lymphangiogenesis is integral for wound healing, particularly in the proliferation stage [131]. Insufficient lymphangiogenesis leads to entrapment of interstitial fluids, inflammatory cells, macromolecules, and ultimately lymphedema [1]. As the lymphedema proceeds, inflammation exacerbates and oxygen diffusion distance increases, which cause hypoxia and promote poor-healing wounds [182].

In this thesis, we were inspired by the pericellular hypoxia that typically exists in wounds and then replicated such a spontaneous hypoxia micromilieu to produce HPS and examined its pro-lymphangiogenic potential. HPS is derived from the autologous blood and contains an abundance of growth factors secreted from the entire spectrum of PBCs. The PBCs, including platelets and the other cell types, are natural depots of growth factors and circulate through all stages of wound healing [139]. Compared to platelets that are mainly activated in the early stage, the PBCs, as a larger depot throughout both early and later stages, release more growth factors, especially when

stimulated by hypoxia (i.e., HPS formation) [139, 165]. In this sense, the PBC-derived HPS is theoretically more advantageous for tissue regeneration than the platelet-derived PRP or NS, particularly for lymphangiogenesis that commences later than angiogenesis and thus may benefit more from the constant growth factor release at later stages [120, 138, 183].

This thesis validated the hypothesized lymphangiogenic superiority of HPS by revealing that HPS significantly increased LEC proliferation, migration, tube formation, and vessel sprouting, outperforming both PRP and NS in head-to-head comparisons. The results showed that HPS, PRP, and NS, all include pro- and anti-lymphangiogenic growth factors, with HPS containing more VEGF-C, PDGF-BB, and bFGF and consequently providing stronger lymphatic regeneration. This work examined lymphangiogenesis in both cell organization (LEC tube formation with cell proliferation and migration) and vessel sprouting (LV sprouting) patterns in relation to the three secretomes.

Another knowledge gap was about the equilibrium of pro- and anti-lymphangiogenic effects in HPS. Although HPS has been shown to be effective in speeding wound healing and causing formation of both LVs and BVs in a previous study [166], it was unknown at which concentrations the pro-lymphangiogenic factors would maximally compensate the anti-lymphangiogenic ones. We were able to address this gap by serially diluting HPS and determining the appropriate concentrations through in vitro experiments on LEC proliferation and LV formation.

4.1 Coordination of pro- and anti-lymphangiogenic factors in secretomes

Lymphangiogenesis is a complex process regulated by a vast array of pro- and anti-lymphangiogenic growth factors, among which VEGF-C is of particular importance [184, 185]. VEGF-C acts as a mediator for either positive, such as PDGF-BB, bFGF, IGF, and HGF [44, 51, 86, 90], or negative lymphangiogenic signals, such as TSP1, PF4, and IL-12 [56, 80, 186]. These growth factors are released into blood-derived secretomes like HPS, NS, and PRP, allowing us to evaluate the lymphangiogenic potential of each secretome in the first step through measuring the growth factor levels. In this thesis, six key promotive (VEGF-C, PDGF-BB, bFGF) and inhibitory growth

factors (TSP1, PF4, ES) were selected to evaluate the comprehensive lymphangiogenic effect of HPS, in comparison to PRP and NS.

4.1.1 Levels of pro-lymphangiogenic factors

The levels of VEGF-C, PDGF-BB, and bFGF in secretomes were assessed individually (**Figure 7**). Our findings indicated that HPS could have a greater lymphangiogenic potential probably due to its higher amounts of these growth factors. Explicitly, HPS demonstrated a 2.3-fold increase in VEGF-C versus NS and 1.4-fold increase versus PRP, essentially supporting its stronger pro-lymphangiogenic effect because VEGF-C is a pivotal lymphangiogenic mediator. Interestingly, the PDGF-BB levels in our PRP, which had been validated to have 4.9 times more platelets than whole blood, were found to be comparable to those in NS. This consists with a preclinical study involving both human serum and platelet lysates, in which human serum exhibited similar PDGF-BB levels to the latter [187]. Moreover, PDGF-BB levels in centrifuged human serum were even higher than the centrifuged and activated plasma that is equivalent to platelet lysates [138]. The concentrations of bFGF in HPS were 7.7-fold higher than in PRP and 2.2-fold higher than in NS, which still contained 3.5-fold more bFGF than PRP. The relatively lower bFGF levels in platelet-derived secretomes (PRP and NS) conform to a previous study showing that erythrocytes, which were also a cell source during our HPS preparation, were a significant growth factor depot and released considerably more bFGF than platelets and leukocytes [139]. Additionally, the amounts of VEGF-C, PDGF-BB, and bFGF in HPS were higher than those in NS, proving that our proposed hypoxia-preconditioning technique has a significant promotion on producing these pro-lymphangiogenic growth factors.

4.1.2 Levels of anti-lymphangiogenic factors

Regarding the TSP1, PF4, and ES, it is difficult to predict their counteractions on lymphangiogenesis since their levels in HPS, NS, and PRP were disproportionate to the alterations of pro-lymphangiogenic factors (**Figure 9**). Nevertheless, an increasing trend of inhibitory growth factors in HPS and PRP above baseline is anticipated because they are concomitantly released as a result of prolonged incubation or biomechanical stresses (i.e., hypoxia, centrifugation and thrombin activation) [148, 165]. Our findings revealed that the TSP1 levels in HPS and PRP were higher than

those in NS, with a 1.6-fold increase in HPS and a 2.1-fold increase in PRP. However, the PF4 levels were significantly higher in PRP than in HPS or NS, which may be attributable to a greater α-granule degranulation during PRP preparation as a result of thrombin and calcium activation [148, 188]. Meanwhile, the PF4 levels in HPS were elevated by 1.4-fold compared to NS, which could be due to its longer incubation at a higher temperature (4 d, 37℃ for HPS vs 4 h, 20℃ for NS), rather than the hypoxic stress, because hypoxia had little impact on PF4 release [189]. Levels of ES in HPS were 1.4-fold higher than in NS and 7.5-fold higher than in PRP, implying that hypoxia may boost ES production or release. This hypoxia-driven increase in ES matches the in vivo data from both hypobaria-hypoxia-treated mice and COVID-19-induced hypoxic state in humans [190, 191].

4.1.3 Overview of lymphangiogenic growth factors in HPS and PRP

Despite the attempt to better understand lymphangiogenic signaling processes by quantifying these crucial growth factors, a comprehensive interpretation remains elusive due to their signaling complexity. Firstly, the discovery of either novel or existing biomolecules related to lymphangiogenesis can exert unpredictable influences over the coordinated outcome [192, 193], indicating that the mechanism cannot be accurately reflected by measuring only a few growth factors. Secondly, these growth factors are interactive, with some suppressing or increasing the expression of others (e.g., PF4 suppresses bFGF expression, and bFGF intensifies VEGF-C expression), while some playing dual roles between pro- and anti-lymphangiogenesis (e.g., TGFβ maintains dynamic homeostasis of lymphangiogenesis) [53, 71, 109, 110]. Thirdly, the lymphangiogenic efficacies of these growth factors vary apparently, with some contributing strongly while others weakly in different concentration ranges. For example, our prior study showed that TSP1 and PF4 of higher levels in HPS were unable to significantly suppress the lymphatic sprouting when compared to the lower levels of these inhibitory factors in hypoxia-preconditioned plasma, suggesting their limited anti-lymphangiogenic efficacies at high concentrations [176].

In summary, the increased levels of VEGF-C, PDGF-BB, and bFGF in HPS can support its lymphangiogenic superiority over PRP and NS. However, determining the overall lymphangiogenic effect within a framework of several key growth factors is

difficult, especially when they have different efficacies and some of them are elevated disproportionally. However, the disproportionate efficacies and concentrations of pro- and anti-lymphangiogenic factors can regain balance (i.e., rebalance) as HPS becomes diluted. In order to ascertain an ideal dilution with appropriately rebalanced growth factors and comprehensively evaluate the lymphangiogenic potential of HPS, we analyzed experimental results from LECs and thoracic ducts, as discussed in the following chapters.

4.2 HPS maximizes LEC proliferation and migration at a concentration of 40%

Post-natal lymphatic regeneration is dominated by sprouting from preexisting vessels, rather than cell organization from LEC clusters. However, LEC proliferation and migration remain a prerequisite for LV growth. In wound healings, LV neovascularization by means of cell organization has been observed and can be enhanced through exogenous LEC injection, probably mediated by VEGF signaling [11, 194, 195]. Furthermore, the addition of extra VEGF-C to LECs in a biomaterial was found to facilitate LEC organization to form LVs and ameliorate lymphedema [169]. In this thesis, HPS serves as a lymphangiogenic stimulant by providing growth factors more than VEGFs. In addition, we reset the balance of these growth factors by serially diluting HPS from 100% to 40%, 10%, 1%, and 0.1%, to determine an appropriate concentration in practical settings. As a baseline comparison, NS was parallelly diluted. On the contrary, PRP was used as concentrated platelet lysates without further dilutions, as a diluted PRP secretome would resemble NS, which has the characteristic feature of unconcentrated platelet lysates.

4.2.1 The role of HPS in LEC proliferation

HPS has been used at various concentrations to promote proliferation of different cells, including fibroblasts, osteoblasts, and HUVECs [156, 167]. Here, for LEC proliferation, HPS manifested maximal effectiveness with a 40% dilution according to the cell viability results (**Figure 10**). HPS-40% exhibited a 4.5-fold increase in proliferative capability compared to PRP and a 3.4-fold increase compared to the positive control. As the concentration of HPS rose to 100%, LEC proliferation dramatically decreased by 75% versus that in HPS-40%, presenting a unimodal HPS

curve higher than the proliferation curve observed in NS treatment. We speculate that HPS-40%, despite containing fewer growth factors than pure HPS, achieves an optimum rebalance of pro- and anti-lymphangiogenic interactions for cell proliferation. Besides, HPS-10% also manifested a substantial promotion on LEC proliferation, which was 4.3-fold higher than PRP and 2.6-fold higher than the positive control. This outcome consists with our prior studies showing that both HPS-10% and HPS-40% were effective in promoting proliferation of other cell types, such as fibroblasts and osteoblasts [156, 167]. However, for LEC proliferation, HPS-40% treatment was more effective than HPS-10%.

4.2.2 The role of HPS in LEC migration

LEC migration is driven by growth factor gradient (i.e., from high concentration to low concentration) and extracellular mechanical stimuli (e.g., interstitial flow), even prior to the onset of LEC proliferation [194]. Similar to our findings on LEC proliferation, HPS-40% showed greater capability of promoting LEC migration (as illustrated by smaller gap area percentages in **Figure 11**) compared to all except the positive control and HPS-10% treatment, indicating a proper balance of pro- and anti-lymphangiogenic factors in HPS-40%. Although the migration results between HPS-40% and HPS-10% treated LECs were not statistically different, HPS-40% exhibited its effectiveness of 7.6-fold higher than PRP, while HPS-10% exhibited 2.6-fold higher than PRP. However, the migration results in extremely low (0.1%) and high (100%) concentrations of HPS were both comparable to those in the negative control, indicating the less effective balances of growth factors in them, where the anti-lymphangiogenic factors could largely offset cell migration caused by pro-lymphangiogenic ones.

In addition to the proper balance of growth factors in HPS-40%, its greater potential for LEC migration may be related to its relatively high viscosity. A relatively higher extracellular fluid viscosity can suppress cell membrane undulation, increase cell adhesion, and promote actin remodeling, thus facilitating membrane protrusion and cell movement [196, 197]. However, cell movement can be conversely impeded in overly high viscosity and resistance [198]. Although the specific viscosities of HPS at different dilutions were not measured in this study, increases in viscosity from HPS-

0.1% to HPS-100% are anticipated because serum viscosities were found to be proportionate to the increased concentrations [199]. Herein, HPS with a concentration of 40% appears to have the preferential viscosity for LEC migration.

4.2.3 Overview of LEC proliferation and migration in secretomes

The superior promotive effects of HPS on LEC proliferation and migration are likely due to the higher levels of lymphangiogenic growth factors released from hypoxia preconditioned PBCs when compared to PRP. Although PRP demonstrated greater promotion than the negative control, it was significantly outperformed by HPS-40%, which showed better performance on both LEC proliferation and migration. This superior performance in diluted HPS probably results from an ideal rebalance of the pro- and anti-lymphangiogenic factors that improves LEC proliferation, as well as a relatively high viscosity that induces LEC migration.

While the dilution of HPS rebalanced pro- and anti-lymphangiogenic effects, highly diluted HPS (e.g., 0.1%) exerted weaker effects on LEC proliferation and migration than HPS-40% and was comparable to PRP. The decreased lymphangiogenic performance is more likely caused by a deficiency of the overall growth factors. In general, deficient growth factors in low-serum media can halt cell growth [200, 201]. On the other side, cell growth can also be restricted at overly high serum concentrations due to deprivation of the low molecular weight nutrients from medium, such as salts, vitamins, and amino acids [202]. Nutrient deprivation is present in pure HPS and NS because no additional medium is added. In contrast, pure PRP used in our study has no such nutrient-deprivation issue since it is technically prepared as concentrated platelet lysates that are dissolved in basal medium.

This study has a limitation concerning the asymmetrical comparisons between diluted HPS and undiluted PRP. However, pure PRP remains a gold standard control by its definition about the highly enriched platelets (or lysates) [146]. In addition, to avoid side effects caused by nutrient deprivation in pure plasma, we dissolved PRP lysates in basal medium. This allows us to compare the diluted HPS to undiluted PRP, without concerning the nutrient paucity.

In conclusion, our findings demonstrate that HPS-40% promoted LEC proliferation and migration greater than PRP. In this context, further lymphedema or wound healing

models may benefit from co-application of LECs and HPS-40%. However, the following lymphatic neovascularization results presented a different concentration preference for LEC tube formation and LV sprouting, as discussed in chapter 4.3.

4.3 HPS enhances lymphatic vessel formation at a concentration of 10%

LV regeneration is the basis for treating lymphedema caused by trauma, radiotherapy, or surgery [8]. Mature LVs can transport lymph, stabilize interstitial fluid pressure, and subsequently mitigate lymphedema [128, 131]. Therefore, it is imperative to study the lymphangiogenic potential of HPS in terms of vessel formation.

Our previous studies have observed that HPS could enhance LV formation from preexisting vessels. However, details about LEC organization behavior, the optimal HPS concentration, and comparisons with PRP, all remain to be investigated [166, 176]. In this study, we utilized the tube formation (in vitro cell organization, 2D) and lymphatic sprouting assay (ex vivo microvessel sprouting, 3D) to investigate the pro-lymphangiogenic capability of HPS by comparison with PRP. The results showed that LV formation requires a relatively lower HPS concentration, with the optimum being 10%. This differs from the ideal HPS concentration of 40% for LEC proliferation and migration, indicating a lower growth factor demand during LV regeneration.

4.3.1 The role of HPS in 2D tube formation

The tubulogenic results addressed the knowledge gap regarding the de novo LV formation under secretome stimulations in cell organization pattern. The organized tube-like structures on an ECM substrate were optimally developed by treating LECs with HPS-10%, as indicated in parameters like total number of tubes, total tube length, number of branching points, and area percentage covered by cells (**Figure 12**). This tubulogenic superiority of HPS-10% surpassed not only PRP but unexpectedly also HPS-40%, which had previously shown its excellent promotion on both LEC proliferation and migration. Similar to the proliferation curve, the tubulogenic capability of HPS tapered at both higher (40%, 100%) and lower (1%, 0.1%) concentrations. This modest concentration of HPS (10%) for LEC tube formation resembles a HUVEC tube formation study using hPL, which demonstrated a better

result at 5-10% of hPL when compared to 2.5% and 1% [203], implying that a relatively low concentration of secretomes is demanded for an optimal endothelial cell tube formation.

In addition to the tube number, tube length, and branching point number, we observed significantly larger cell-covered areas in HPS-10% than in HPS-40% and PRP, suggesting a larger cell number in HPS-10%. Since the cell number can dictate diameters of the emerging tube lumen, which consequentially predicts a functional role of LVs [204, 205], HPS-10% is likely to have greater potential to promote the formation of functional tubes.

The tube formation experiment embodies the LEC organization mechanism and reveals an ideal HPS concentration of 10% as compared to PRP in 2D lymphatic neovascularization. However, this 2D model has limitations such as lacking natural LV structures and complete LEC-ECM interactions [206]. To overcome these limitations, we employed a sprouting model to mimic the genuine LV formation in a 3D extracellular milieu. This model double validated the lymphangiogenic potential of HPS. Furthermore, the sprouting pattern observed in this 3D model closely correlates with the predominant vessel sprouting mechanism during lymphangiogenesis [11].

4.3.2 The role of HPS in 3D lymphatic sprouting

The ex vivo culture of thoracic duct simulates the 3D cell-ECM interactions and reflects vessel sprouting mechanism, different from tube formation that implicates cell organization mechanism and 2D cell-ECM interactions. Since collagen I was reported to be more suitable for LV sprouting, we selected it as ECM instead of Matrigel to provide reliable LV regeneration results [207]. The results showed that thoracic ducts treated with HPS-10% exhibited the greatest values in both sprouting number and length, surpassing those observed in HPS-40%, NS-10%, and PRP. Notably, PRP demonstrated a comparable effect to the positive control (**Figure 13. B** and **C**). The greater LV sprouting effect of HPS-10% was in accordance with the maximal stimulation by the same HPS concentration on BV formation in our previous aortic ring sprouting experiments, indicating superior potential of HPS-10% on promoting microvessel formation [207].

This superior promotive effect of HPS-10% could be partly attributed to its appropriate rebalance of lymphangiogenic growth factors that are preferable for LV sprouting, which was different from the preferred balance of HPS-40% for LEC proliferation and migration but coincided with the ideal concentration of 10% in tube formation. Secondly, the involvement of ECM components can lead to coordination of integrins and VEGFR3, which subsequently enhances the VEGF-C/VEGFR3 signaling [74]. This additional signal enhancement may reduce the dependency of lymphangiogenesis on growth factor levels, and hence lower the requirement for high concentrations of HPS. Thirdly, the high bFGF level in HPS may enable LECs to upregulate the expressions of certain β1 integrins (e.g., α2β1 and α5β1) [117], thereby may reinforce the already enhanced β1 integrin-VEGFR3 signals and then form positive feedback. In these contexts, HPS at a slightly lower concentration of 10% manifested the greatest ability for promoting LV sprouting in ECM structures.

During the analysis of lymphatic sprouts, despite the presence of background noise interference from collagen and the question regarding tissue origin, we managed to accurately count sprouts. This was achieved by fine-tuning microscope focus at different depths and employing immunohistochemical staining to identify the lymphatic origin of both the parental ring and its sprouts before and after the culture.

4.3.3 Overview of lymphatic microvessel formation

The formation of lymphatic microvessels is a multifaceted activity that involves various contributors, including growth factors, ECM structure, cell proliferation, cell movement, etc. In this study, we examined the effects of different concentrations of HPS and pure PRP on LEC tube formation and LV sprouting. Our findings reveal that both HPS-10% and PRP promote the formation of lymphatic tubules and sprouts, with HPS-10% exhibiting a stronger promotion.

To the best of our knowledge, this was the first work to determine the appropriate concentration of HPS and investigate the role of PRP in lymphatic microvessel formation. PRP, which has been widely used in tissue regeneration, is highlighted by its autologous origin and rich growth factors from platelets. However, platelets are not the only source of growth factors in blood, wherein other PBCs like erythrocytes can provide large amounts of growth factors as well, including lymphangiogenic factors

[139]. Additionally, with the hypoxia stimulation, we obtained more lymphangiogenic growth factors in the PBC-derived HPS (**Figure 7**). HPS displayed stronger promotive effects on LV regeneration than platelet derived PRP.

The relatively weaker lymphangiogenic potential of PRP may also result from its high levels of anti-lymphangiogenic growth factors, particularly the platelet-originated PF4, which interferes with integrin signal that is essential for cell-ECM adhesion and consequentially inhibits vessel formation [73, 208]. Our prior research on BV formation has shown that angiogenesis was enhanced at low PF4 levels but inhibited at high levels, even in the presence of abundant VEGF [165, 166, 209]. In this LV formation study, PRP had 3-fold higher PF4 levels than HPS, thus might exert relatively more negative impact. Given the fact that HPS also contains elevated anti-lymphangiogenic factors of TSP1, PF4 and ES, determining a properly rebalanced dilution formula becomes a requisite. As a result, HPS-10% was proved beneficial to both 2D (cell organizing) and 3D (vessel sprouting) microvessel development, as compared to either PRP or the corresponsive NS-10%.

Apart from the increased number and length of microvessels, an increased lumen diameter (or vessel width) facilitates rapid lymph transportation [210]. However, measuring the average sprout width is impractical due to the overlapping vessels and inconsistent widths along the length. Nonetheless, we estimated the widths of these sprouts to be similar by subjective visual inspection of the micrographs. This was different from the LEC tube formation with apparently larger cell-covered areas in HPS-10%, which might predict a broader lumen formation as the tubules grow [204]. Since the tube formation represents an alternative cell organization route for microvessel regeneration and is independent of sprouting mechanism, our findings provide a likelihood for regenerating large-diameter LVs by offering exogenous LECs treated with HPS-10%. This extrinsic LEC introduction may serve as a promising supplement to the intrinsic sprouting pattern, potentially aiding lymph flow and ameliorating lymphedema.

4.4 Demands for HPS vary between LEC proliferation/migration and LV formation

It is unexpected to find that the ideal HPS concentrations for LEC proliferation/ migration and LEC tube formation/ LV sprouting were different, where 40% and 10% respectively emerged as the most effective levels. This discrepancy suggests that different growth factor formulations are demanded for cell recruitment and vessel formation, which are actually two events temporospatially intertwined in the wound healing process.

In temporal terms, LECs mobilize during the inflammation and early proliferation stages along the interstitial fluid flow and require strong stimuli of high levels of lymphangiogenic factors [11]. These growth factors, such as VEGF-C, bFGF, and PDGF-BB, can be supplied in HPS-40%. When LVs develop and extend during the sequential late proliferation stage, the levels of these growth factors subside. Within the context of this thesis, the growth factor subsidence corresponds with the decline of HPS concentration from 40% to 10%. This hypothesis about dynamic alterations of growth factors aligns with other studies, which discovered an up-and-down switch of VEGF and a spectrum shift of cytokines throughout wound healing stages [211, 212]. At the final remodeling stage, it could be anticipated that highly diluted HPS of less than 10% with diminutive growth factors would even fit the lymphatic network remodeling.

From a spatial perspective, LV regeneration is driven by the flowing lymph and interstitial fluid in a distal-to-proximal direction, in which the levels of lymphangiogenic growth factors decline [11, 194]. In another lymphangiogenesis study, high levels of VEGF-A and VEGF-C were observed distal to the wound due to fluid stasis, while low levels were found in proximal tissues due to fluid flow [184]. The reduced growth factor levels make the less concentrated HPS (i.e., 10% within the context) a reasonable choice for LV formation. However, in oversized wounds, growth factors may fall excessively in the proximal region and become insufficient to maintain LV regeneration. In such a case, delivering HPS-10% to the proximal region to maintain relatively low levels of growth factors may offer an improved treatment strategy.

In addition to the above spatiotemporal possibilities, the lower HPS demand for LV formation could be also attributable to the additionally amplified VEGFR3 signaling, which is caused by ECM involvement and β1-integrin activation [74]. This amplified pro-lymphangiogenic signal may decrease the requirement for growth factors during LV formation when compared to the ECM-free LEC proliferation and migration, where the lymphangiogenic signals remain unamplified. In the meantime, the high bFGF levels in HPS may elevate integrin synthesis and thus reinforce the integrin-VEGFR3 signaling [117]. Therefore, the signal threshold for lymphangiogenesis could be lowered in ECM-dependent tube formation and sprouting, whereby the HPS concentration of 10% was proved sufficient for a superior microvessel formation in comparison to HPS-40%.

In summary, the discrepancy in HPS demand between cell proliferation and vessel formation unveils the dynamic regulation of growth factors during lymphangiogenesis. To promote lymphangiogenesis effectively, fixed concentrations of HPS need to be readjusted in real time according to the regeneration phases, thereby forming new levels and balances of pro- and anti-lymphangiogenic factors. Based on my thesis findings, we present that HPS-40% is optimal for LEC proliferation and migration, while HPS-10% is preferred for LV formation.

5 Conclusion and outlook

5.1 Conclusion

The current study elucidated the lymphangiogenic potential of two blood-derived secretomes, HPS and PRP. Both demonstrated promotive effects, with HPS exhibiting superior potential to PRP. The lymphangiogenic superiority of HPS is probably attributed to its higher levels of growth factors released from hypoxia stimulated PBCs. While in PRP, growth factors are purely derived from platelets under normoxia. Although PRP has been widely used in regenerative medicine due to its multiple growth factors, it may be overrated because of its concomitantly elevated inhibitive factors, such as the anti-lymphangiogenic PF4.

Since lymphangiogenesis is coordinately regulated by both promotive (e.g., VEGF-C, PDGF-BB, bFGF) and inhibitive growth factors (e.g., TSP1, PF4, and ES), it is imperative to decide a proper balance among them. By serially diluting HPS to reset the balance and comparing HPS with PRP and NS, our study unexpectedly discovered that HPS-40% demonstrated superior performance in LEC proliferation and migration, while HPS-10% showed better results in LV formation. These results indicate the differentiated demands for growth factors in LEC recruitment and microvessel formation and embody the superiority of HPS in both cell organization and vessel sprouting patterns during the lymphatic development.

5.2 Outlook

Our findings provide in vitro evidence for the superior pro-lymphangiogenic potential of HPS over the gold standard PRP, not only through vessel sprouting but also cell organization. Apart from this thesis research, further research in several directions is warranted to advance our understanding of the pro-lymphangiogenic role of HPS. One promising avenue is to examine the expression of lymphangiogenic growth factors in LECs and their feedback regulations in response to HPS treatment, using techniques such as reverse transcription quantitative polymerase chain reaction (RT-qPCR). Additionally, gene-deleted animal models may help to elucidate the influence of HPS on specific signaling pathways in LECs, such as VEGF-C/VEGFR3, bFGF/FGFR1 or FGFR3, PDGF-BB/PDGFRβ, or their related downstream signals.

Another investigative direction lies in in vitro culture of LECs or lymphatic ducts by dynamically altering HPS concentrations. Unlike the conventional cell culture with a fixed medium concentration, a sequential shift of HPS from 40% to 10% could be implemented according to real-time stages of cell proliferation and vessel formation. This dynamic concentration shift would examine our theory that differentiated demands for HPS (i.e., differentiated growth factor demands) are necessary for optimum LEC proliferation and LV formation.

Besides morphological aspects (e.g., vessel number and length), the functionality assessments (e.g., lymph flow and vessel contractility) on LVs under HPS and PRP stimulations need to be focused on in future in vivo studies. A variety of tools could be applied, such as lymphangiography with contrast-enhanced computed tomography (CT) or magnetic resonance (MR) and percutaneous detection of LV contractility with near-infrared (NIR) dyes and devices. These techniques could evaluate the trafficking function of mature LVs and distinguish them from immature, dysfunctional LVs.

From a microvessel formation perspective, in order to intensify the role of cell organization and combine it with vessel sprouting, we propose an in vivo injection of LEC suspensions in HPS-10%, using the model of murine skin wound or tail lymphedema. This strategy, unlike our previous approaches that only applied HPS on wounds, may double enhance the cell-organized tubulogenesis and lumen formation in addition to LV sprouting. It could also be utilized on proper biocompatible materials in lymphatic engineering.

Furthermore, since LNs and LVs are both responsible for lymph drainage, the regeneration of LNs is equally crucial to that of LVs. To alleviate lymphedema, which can result from damaged LNs due to trauma, radiotherapy, or surgical removal, HPS could be a helpful treatment tool. Appropriate concentrations of HPS can be administered to in vitro LN-mimicking organoids or in vivo lymphedema models. This allows for studying their histological and functional outcomes and exploring the effectiveness of HPS for LN regeneration.

In addition, future studies should consider other contributors, such as interstitial pressure and flowing shear stress, which are relevant to lymphatic regeneration. Addressing all the aforementioned research gaps will pave the way for further preclinical and clinical applications of HPS in treating lymphedema.

6 Patents

Device-based methods for localized delivery of cell-free carriers with stress-induced cellular factors. (AU2013214187 (B2); 9 February 2017): Arndt F Schilling, Ektoras Hadjipanayi, Hans-Günther Machens.

Bibliography

1. Yoshida, S.; Koshima, I.; Hamada, Y.; Sasaki, A.; Fujioka, Y.; Nagamatsu, S.; Yokota, K.; Harima, M.; Yamashita, S., Lymphovenous Anastomosis Aids Wound Healing in Lymphedema: Relationship Between Lymphedema and Delayed Wound Healing from a View of Immune Mechanisms. *Adv Wound Care (New Rochelle)* **2019,** 8, (6), 263-269.
2. Oneto, P.; Etulain, J., PRP in wound healing applications. *Platelets* **2021,** 32, (2), 189-199.
3. Renò, F.; Sabbatini, M., Breaking a Vicious Circle: Lymphangiogenesis as a New Therapeutic Target in Wound Healing. *Biomedicines* **2023,** 11, (3), 656.
4. Saaristo, A.; Tammela, T.; Farkkila, A.; Karkkainen, M.; Suominen, E.; Yla-Herttuala, S.; Alitalo, K., Vascular endothelial growth factor-C accelerates diabetic wound healing. *Am J Pathol* **2006,** 169, (3), 1080-7.
5. Zhou, J.; Wei, T.; He, Z., ADSCs enhance VEGFR3-mediated lymphangiogenesis via METTL3-mediated VEGF-C m(6)A modification to improve wound healing of diabetic foot ulcers. *Mol Med* **2021,** 27, (1), 146.
6. Ackermann, M.; Wettstein, R.; Senaldi, C.; Kalbermatten, D. F.; Konerding, M. A.; Raffoul, W.; Erba, P., Impact of platelet rich plasma and adipose stem cells on lymphangiogenesis in a murine tail lymphedema model. *Microvasc Res* **2015,** 102, 78-85.
7. Hancock, D. G.; Potezny, T. M.; White, P. M., Immune regulation by the peripheral lymphatics and its implications for wound healing and infection control in lymphoedema. *Wound Practice & Research: Journal of the Australian Wound Management Association* **2016,** 24, (2), 76-83.
8. Stritt, S.; Koltowska, K.; Makinen, T., Homeostatic maintenance of the lymphatic vasculature. *Trends Mol Med* **2021,** 27, (10), 955-970.
9. Kastenmuller, W.; Torabi-Parizi, P.; Subramanian, N.; Lammermann, T.; Germain, R. N., A spatially-organized multicellular innate immune response in lymph nodes limits systemic pathogen spread. *Cell* **2012,** 150, (6), 1235-48.
10. Pichol-Thievend, C.; Betterman, K. L.; Liu, X.; Ma, W.; Skoczylas, R.; Lesieur, E.; Bos, F. L.; Schulte, D.; Schulte-Merker, S.; Hogan, B. M.; Oliver, G.; Harvey, N. L.; Francois, M., A blood capillary plexus-derived population of progenitor cells contributes to genesis of the dermal lymphatic vasculature during embryonic development. *Development* **2018,** 145, (10).
11. Rutkowski, J. M.; Boardman, K. C.; Swartz, M. A., Characterization of lymphangiogenesis in a model of adult skin regeneration. *Am J Physiol-Heart C* **2006,** 291, (3), H1402-H1410.
12. Hagerling, R.; Pollmann, C.; Andreas, M.; Schmidt, C.; Nurmi, H.; Adams, R. H.; Alitalo, K.; Andresen, V.; Schulte-Merker, S.; Kiefer, F., A novel multistep mechanism for initial lymphangiogenesis in mouse embryos based on ultramicroscopy. *EMBO J* **2013,** 32, (5), 629-44.
13. Yang, Y.; Garcia-Verdugo, J. M.; Soriano-Navarro, M.; Srinivasan, R. S.; Scallan, J. P.; Singh, M. K.; Epstein, J. A.; Oliver, G., Lymphatic endothelial progenitors bud from the cardinal vein and intersomitic vessels in mammalian embryos. *Blood* **2012,** 120, (11), 2340-8.
14. Francois, M.; Short, K.; Secker, G. A.; Combes, A.; Schwarz, Q.; Davidson, T. L.; Smyth, I.; Hong, Y. K.; Harvey, N. L.; Koopman, P., Segmental territories along the cardinal veins generate lymph sacs via a ballooning mechanism during embryonic lymphangiogenesis in mice. *Dev Biol* **2012,** 364, (2), 89-98.

15. Martinez-Corral, I.; Ulvmar, M. H.; Stanczuk, L.; Tatin, F.; Kizhatil, K.; John, S. W.; Alitalo, K.; Ortega, S.; Makinen, T., Nonvenous origin of dermal lymphatic vasculature. *Circ Res* **2015,** 116, (10), 1649-54.
16. Mahadevan, A.; Welsh, I. C.; Sivakumar, A.; Gludish, D. W.; Shilvock, A. R.; Noden, D. M.; Huss, D.; Lansford, R.; Kurpios, N. A., The left-right Pitx2 pathway drives organ-specific arterial and lymphatic development in the intestine. *Dev Cell* **2014,** 31, (6), 690-706.
17. Maruyama, K.; Miyagawa-Tomita, S.; Mizukami, K.; Matsuzaki, F.; Kurihara, H., Isl1-expressing non-venous cell lineage contributes to cardiac lymphatic vessel development. *Dev Biol* **2019,** 452, (2), 134-143.
18. Rutkowski, J. M.; Ihm, J. E.; Lee, S. T.; Kilarski, W. W.; Greenwood, V. I.; Pasquier, M. C.; Quazzola, A.; Trono, D.; Hubbell, J. A.; Swartz, M. A., VEGFR-3 neutralization inhibits ovarian lymphangiogenesis, follicle maturation, and murine pregnancy. *Am J Pathol* **2013,** 183, (5), 1596-1607.
19. He, Y.; Rajantie, I.; Ilmonen, M.; Makinen, T.; Karkkainen, M. J.; Haiko, P.; Salven, P.; Alitalo, K., Preexisting lymphatic endothelium but not endothelial progenitor cells are essential for tumor lymphangiogenesis and lymphatic metastasis. *Cancer Res* **2004,** 64, (11), 3737-40.
20. Martinez-Corral, I.; Olmeda, D.; Dieguez-Hurtado, R.; Tammela, T.; Alitalo, K.; Ortega, S., In vivo imaging of lymphatic vessels in development, wound healing, inflammation, and tumor metastasis. *Proc Natl Acad Sci U S A* **2012,** 109, (16), 6223-8.
21. Qutub, A. A.; Popel, A. S., Elongation, proliferation & migration differentiate endothelial cell phenotypes and determine capillary sprouting. *BMC Syst Biol* **2009,** 3, 13.
22. Zheng, W.; Tammela, T.; Yamamoto, M.; Anisimov, A.; Holopainen, T.; Kaijalainen, S.; Karpanen, T.; Lehti, K.; Yla-Herttuala, S.; Alitalo, K., Notch restricts lymphatic vessel sprouting induced by vascular endothelial growth factor. *Blood* **2011,** 118, (4), 1154-62.
23. Joukov, V.; Pajusola, K.; Kaipainen, A.; Chilov, D.; Lahtinen, I.; Kukk, E.; Saksela, O.; Kalkkinen, N.; Alitalo, K., A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* **1996,** 15, (7), 1751.
24. Sweat, R. S.; Sloas, D. C.; Murfee, W. L., VEGF-C induces lymphangiogenesis and angiogenesis in the rat mesentery culture model. *Microcirculation* **2014,** 21, (6), 532-40.
25. Coso, S.; Bovay, E.; Petrova, T. V., Pressing the right buttons: signaling in lymphangiogenesis. *Blood* **2014,** 123, (17), 2614-24.
26. Shin, M.; Male, I.; Beane, T. J.; Villefranc, J. A.; Kok, F. O.; Zhu, L. H. J.; Lawson, N. D., Vegfc acts through ERK to induce sprouting and differentiation of trunk lymphatic progenitors. *Development* **2016,** 143, (20), 3785-3795.
27. Salameh, A.; Galvagni, F.; Bardelli, M.; Bussolino, F.; Oliviero, S., Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. *Blood* **2005,** 106, (10), 3423-31.
28. Lahdenranta, J.; Hagendoorn, J.; Padera, T. P.; Hoshida, T.; Nelson, G.; Kashiwagi, S.; Jain, R. K.; Fukumura, D., Endothelial Nitric Oxide Synthase Mediates Lymphangiogenesis and Lymphatic Metastasis. *Cancer Research* **2009,** 69, (7), 2801-2808.
29. Karkkainen, M. J.; Haiko, P.; Sainio, K.; Partanen, J.; Taipale, J.; Petrova, T. V.; Jeltsch, M.; Jackson, D. G.; Talikka, M.; Rauvala, H.; Betsholtz, C.; Alitalo, K.,

Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* **2004,** 5, (1), 74-80.
30. Makinen, T.; Jussila, L.; Veikkola, T.; Karpanen, T.; Kettunen, M. I.; Pulkkanen, K. J.; Kauppinen, R.; Jackson, D. G.; Kubo, H.; Nishikawa, S.; Yla-Herttuala, S.; Alitalo, K., Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* **2001,** 7, (2), 199-205.
31. Yuan, L.; Moyon, D.; Pardanaud, L.; Breant, C.; Karkkainen, M. J.; Alitalo, K.; Eichmann, A., Abnormal lymphatic vessel development in neuropilin 2 mutant mice. *Development* **2002,** 129, (20), 4797-806.
32. Xu, Y.; Yuan, L.; Mak, J.; Pardanaud, L.; Caunt, M.; Kasman, I.; Larrivee, B.; Del Toro, R.; Suchting, S.; Medvinsky, A.; Silva, J.; Yang, J.; Thomas, J. L.; Koch, A. W.; Alitalo, K.; Eichmann, A.; Bagri, A., Neuropilin-2 mediates VEGF-C-induced lymphatic sprouting together with VEGFR3. *J Cell Biol* **2010,** 188, (1), 115-30.
33. Huggenberger, R.; Ullmann, S.; Proulx, S. T.; Pytowski, B.; Alitalo, K.; Detmar, M., Stimulation of lymphangiogenesis via VEGFR-3 inhibits chronic skin inflammation. *J Exp Med* **2010,** 207, (10), 2255-69.
34. Baldwin, M. E.; Halford, M. A.; Roufail, S.; Williams, R. A.; Hibbs, M. L.; Grail, D.; Kubo, H.; Stacker, S. A.; Achen, M. G., Vascular endothelial growth factor d is dispensable for development of the lymphatic system. *Mol Cell Biol* **2005,** 25, (6), 2441-2449.
35. Wirzenius, M.; Tammela, T.; Uutela, M.; He, Y.; Odorisio, T.; Zambruno, G.; Nagy, J. A.; Dvorak, H. F.; Yla-Herttuala, S.; Shibuya, M.; Alitalo, K., Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J Exp Med* **2007,** 204, (6), 1431-40.
36. Hong, Y. K.; Lange-Asschenfeldt, B.; Velasco, P.; Hirakawa, S.; Kunstfeld, R.; Brown, L. F.; Bohlen, P.; Senger, D. R.; Detmar, M., VEGF-A promotes tissue repair-associated lymphatic vessel formation via VEGFR-2 and the alpha1beta1 and alpha2beta1 integrins. *FASEB J* **2004,** 18, (10), 1111-3.
37. Cursiefen, C.; Chen, L.; Borges, L. P.; Jackson, D.; Cao, J.; Radziejewski, C.; D'Amore, P. A.; Dana, M. R.; Wiegand, S. J.; Streilein, J. W., VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* **2004,** 113, (7), 1040-50.
38. Chen, P. H.; Chen, X. Y.; He, X. L., Platelet-derived growth factors and their receptors: Structural and functional perspectives. *Bba-Proteins Proteom* **2013,** 1834, (10), 2176-2186.
39. Cao, R.; Bjorndahl, M. A.; Religa, P.; Clasper, S.; Garvin, S.; Galter, D.; Meister, B.; Ikomi, F.; Tritsaris, K.; Dissing, S.; Ohhashi, T.; Jackson, D. G.; Cao, Y., PDGF-BB induces intratumoral lymphangiogenesis and promotes lymphatic metastasis. *Cancer Cell* **2004,** 6, (4), 333-45.
40. Pellet-Many, C.; Frankel, P.; Evans, I. M.; Herzog, B.; Junemann-Ramirez, M.; Zachary, I. C., Neuropilin-1 mediates PDGF stimulation of vascular smooth muscle cell migration and signalling via p130Cas. *Biochem J* **2011,** 435, (3), 609-18.
41. Miyazaki, H.; Yoshimatsu, Y.; Akatsu, Y.; Mishima, K.; Fukayama, M.; Watabe, T.; Miyazono, K., Expression of platelet-derived growth factor receptor beta is maintained by Prox1 in lymphatic endothelial cells and is required for tumor lymphangiogenesis. *Cancer Sci* **2014,** 105, (9), 1116-23.
42. Liu, J. N.; Liu, C. Y.; Qiu, L. Y.; Li, J.; Zhang, P.; Sun, Y. P., Overexpression of both platelet-derived growth factor-BB and vascular endothelial growth factor-C and its association with lymphangiogenesis in primary human non-small cell lung cancer. *Diagn Pathol* **2014,** 9.

43. Donnem, T.; Al-Saad, S.; Al-Shibli, K.; Busund, L. T.; Bremnes, R. M., Co-expression of PDGF-B and VEGFR-3 strongly correlates with lymph node metastasis and poor survival in non-small-cell lung cancer. *Ann Oncol* **2010,** 21, (2), 223-231.
44. Onimaru, M.; Yonemitsu, Y.; Fujii, T.; Tanii, M.; Nakano, T.; Nakagawa, K.; Kohno, R.; Hasegawa, M.; Nishikawa, S.; Sueishi, K., VEGF-C regulates lymphangiogenesis and capillary stability by regulation of PDGF-B. *Am J Physiol Heart Circ Physiol* **2009,** 297, (5), H1685-96.
45. Maejima, D.; Kawai, Y.; Ajima, K.; Ohhashi, T., Platelet-derived growth factor (PDGF)-BB produces NO-mediated relaxation and PDGF receptor beta-dependent tonic contraction in murine iliac lymph vessels. *Microcirculation* **2011,** 18, (6), 474-86.
46. Kazenwadel, J.; Secker, G. A.; Betterman, K. L.; Harvey, N. L., In vitro assays using primary embryonic mouse lymphatic endothelial cells uncover key roles for FGFR1 signalling in lymphangiogenesis. *PLoS One* **2012,** 7, (7), e40497.
47. Shin, J. W.; Min, M.; Larrieu-Lahargue, F.; Canron, X.; Kunstfeld, R.; Nguyen, L.; Henderson, J. E.; Bikfalvi, A.; Detmar, M.; Hong, Y. K., Prox1 promotes lineage-specific expression of fibroblast growth factor (FGF) receptor-3 in lymphatic endothelium: A role for FGF signaling in lymphangiogenesis. *Mol Biol Cell* **2006,** 17, (2), 576-584.
48. Perrault, D. P.; Lee, G. K.; Park, S. Y.; Lee, S.; Choi, D.; Jung, E.; Seong, Y. J.; Park, E. K.; Sung, C.; Yu, R.; Bouz, A.; Pourmoussa, A.; Kim, S. J.; Hong, Y. K.; Wong, A. K., Small Peptide Modulation of Fibroblast Growth Factor Receptor 3-Dependent Postnatal Lymphangiogenesis. *Lymphat Res Biol* **2019,** 17, (1), 19-29.
49. Larrieu-Lahargue, F.; Welm, A. L.; Bouchecareilh, M.; Alitalo, K.; Li, D. Y.; Bikfalvi, A.; Auguste, P., Blocking Fibroblast Growth Factor receptor signaling inhibits tumor growth, lymphangiogenesis, and metastasis. *PLoS One* **2012,** 7, (6), e39540.
50. Tzeng, H. E.; Chang, A. C.; Tsai, C. H.; Wang, S. W.; Tang, C. H., Basic fibroblast growth factor promotes VEGF-C-dependent lymphangiogenesis via inhibition of miR-381 in human chondrosarcoma cells. *Oncotarget* **2016,** 7, (25), 38566-38578.
51. Onishi, T.; Nishizuka, T.; Kurahashi, T.; Arai, T.; Iwatsuki, K.; Yamamoto, M.; Hirata, H., Topical bFGF Improves Secondary Lymphedema through Lymphangiogenesis in a Rat Tail Model. *Prs-Glob Open* **2014,** 2, (8).
52. Chang, L. K.; Garcia-Cardena, G.; Farnebo, F.; Fannon, M.; Chen, E. J.; Butterfield, C.; Moses, M. A.; Mulligan, R. C.; Folkman, J.; Kaipainen, A., Dose-dependent response of FGF-2 for lymphangiogenesis. *Proc Natl Acad Sci U S A* **2004,** 101, (32), 11658-63.
53. Cao, R. H.; Ji, H.; Feng, N. H.; Zhang, Y.; Yang, X. J.; Andersson, P.; Sun, Y. P.; Tritsaris, K.; Hansen, A. J.; Dissing, S.; Cao, Y. H., Collaborative interplay between FGF-2 and VEGF-C promotes lymphangiogenesis and metastasis. *P Natl Acad Sci USA* **2012,** 109, (39), 15894-15899.
54. Platonova, N.; Miquel, G.; Regenfuss, B.; Taouji, S.; Cursiefen, C.; Chevet, E.; Bikfalvi, A., Evidence for the interaction of fibroblast growth factor-2 with the lymphatic endothelial cell marker LYVE-1. *Blood* **2013,** 121, (7), 1229-37.
55. Straume, O.; Jackson, D. G.; Akslen, L. A., Independent prognostic impact of lymphatic vessel density and presence of low-grade lymphangiogenesis in cutaneous melanoma. *Clin Cancer Res* **2003,** 9, (1), 250-6.
56. Cursiefen, C.; Maruyama, K.; Bock, F.; Saban, D.; Sadrai, Z.; Lawler, J.; Dana, R.; Masli, S., Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes. *J Exp Med* **2011,** 208, (5), 1083-92.
57. Singla, B.; Aithabathula, R. V.; Ahn, W.; Csanyi, G., Deletion of CD47 Specifically in Lymphatic Endothelium Augments Arterial Lymphangiogenesis and Attenuates Atherosclerosis. *Circulation* **2022,** 146.

58. Oh, H. H.; Park, Y. L.; Park, S. Y.; Myung, E.; Im, C. M.; Yu, H. J.; Han, B.; Seo, Y. J.; Kim, K. H.; Myung, D. S.; Cho, S. B.; Lee, W. S.; Park, D.; Joo, Y. E., CD47 mediates the progression of colorectal cancer by inducing tumor cell apoptosis and angiogenesis. *Pathol Res Pract* **2022,** 240, 154220.
59. Isenberg, J. S.; Ridnour, L. A.; Dimitry, J.; Frazier, W. A.; Wink, D. A.; Roberts, D. D., CD47 is necessary for inhibition of nitric oxide-stimulated vascular cell responses by thrombospondin-1. *Journal of Biological Chemistry* **2006,** 281, (36), 26069-26080.
60. Hawighorst, T.; Oura, H.; Streit, M.; Janes, L.; Nguyen, L.; Brown, L. F.; Oliver, G.; Jackson, D. G.; Detmar, M., Thrombospondin-1 selectively inhibits early-stage carcinogenesis and angiogenesis but not tumor lymphangiogenesis and lymphatic metastasis in transgenic mice. *Oncogene* **2002,** 21, (52), 7945-56.
61. Crawford, S. E.; Stellmach, V.; Murphy-Ullrich, J. E.; Ribeiro, S. M. F.; Lawler, J.; Hynes, R. O.; Boivin, G. P.; Bouck, N., Thrombospondin-1 is a major activator of TGF-beta 1 in vivo. *Cell* **1998,** 93, (7), 1159-1170.
62. Oka, M.; Iwata, C.; Suzuki, H. I.; Kiyono, K.; Morishita, Y.; Watabe, T.; Komuro, A.; Kano, M. R.; Miyazono, K., Inhibition of endogenous TGF-beta signaling enhances lymphangiogenesis. *Blood* **2008,** 111, (9), 4571-9.
63. Maruyama, K.; Ii, M.; Cursiefen, C.; Jackson, D. G.; Keino, H.; Tomita, M.; Van Rooijen, N.; Takenaka, H.; D'Amore, P. A.; Stein-Streilein, J.; Losordo, D. W.; Streilein, J. W., Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* **2005,** 115, (9), 2363-72.
64. Streit, M.; Velasco, P.; Riccardi, L.; Spencer, L.; Brown, L. F.; Janes, L.; Lange-Asschenfeldt, B.; Yano, K.; Hawighorst, T.; Iruela-Arispe, L.; Detmar, M., Thrombospondin-1 suppresses wound healing and granulation tissue formation in the skin of transgenic mice. *EMBO J* **2000,** 19, (13), 3272-82.
65. Briquet-Laugier, V.; Lavenu-Bombled, C.; Schmitt, A.; Leboeuf, M.; Uzan, G.; Dubart-Kupperschmitt, A.; Rosa, J. P., Probing platelet factor 4 alpha-granule targeting. *J Thromb Haemost* **2004,** 2, (12), 2231-40.
66. Prats, A. C.; Van den Berghe, L.; Rayssac, A.; Ainaoui, N.; Morfoisse, F.; Pujol, F.; Legonidec, S.; Bikfalvi, A.; Prats, H.; Pyronnet, S.; Garmy-Susini, B., CXCL4L1-fibstatin cooperation inhibits tumor angiogenesis, lymphangiogenesis and metastasis. *Microvasc Res* **2013,** 89, 25-33.
67. Korniejewska, A.; McKnight, A. J.; Johnson, Z.; Watson, M. L.; Ward, S. G., Expression and agonist responsiveness of CXCR3 variants in human T lymphocytes. *Immunology* **2011,** 132, (4), 503-15.
68. Van Raemdonck, K.; Gouwy, M.; Lepers, S. A.; Van Damme, J.; Struyf, S., CXCL4L1 and CXCL4 signaling in human lymphatic and microvascular endothelial cells and activated lymphocytes: involvement of mitogen-activated protein (MAP) kinases, Src and p70S6 kinase. *Angiogenesis* **2014,** 17, (3), 631-40.
69. Li, S.; Shi, S.; Xia, F.; Luo, B.; Ha, Y.; Luisi, J.; Gupta, P. K.; Merkley, K. H.; Motamedi, M.; Liu, H.; Zhang, W., CXCR3 deletion aggravates corneal neovascularization in a corneal alkali-burn model. *Exp Eye Res* **2022,** 225, 109265.
70. Maione, T. E.; Gray, G. S.; Petro, J.; Hunt, A. J.; Donner, A. L.; Bauer, S. I.; Carson, H. F.; Sharpe, R. J., Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* **1990,** 247, (4938), 77-9.
71. Chadderton, N. S.; Stringer, S. E., Interaction of platelet factor 4 with fibroblast growth factor 2 is stabilised by heparan sulphate. *Int J Biochem Cell B* **2003,** 35, (7), 1052-1055.
72. Gengrinovitch, S.; Greenberg, S. M.; Cohen, T.; Gitay-Goren, H.; Rockwell, P.; Maione, T. E.; Levi, B. Z.; Neufeld, G., Platelet factor-4 inhibits the mitogenic activity

of VEGF121 and VEGF165 using several concurrent mechanisms. *J Biol Chem* **1995,** 270, (25), 15059-65.
73. Aidoudi, S.; Bujakowska, K.; Kieffer, N.; Bikfalvi, A., The CXC-chemokine CXCL4 interacts with integrins implicated in angiogenesis. *PLoS One* **2008,** 3, (7), e2657.
74. Vaahtomeri, K.; Karaman, S.; Makinen, T.; Alitalo, K., Lymphangiogenesis guidance by paracrine and pericellular factors. *Genes Dev* **2017,** 31, (16), 1615-1634.
75. Miosge, N.; Simniok, T.; Sprysch, P.; Herken, R., The collagen type XVIII endostatin domain is co-localized with perlecan in basement membranes in vivo. *J Histochem Cytochem* **2003,** 51, (3), 285-96.
76. Ma, L.; Hollenberg, M. D.; Wallace, J. L., Thrombin-induced platelet endostatin release is blocked by a proteinase activated receptor-4 (PAR4) antagonist. *Br J Pharmacol* **2001,** 134, (4), 701-4.
77. Shi, H. B.; Huang, Y. J.; Zhou, H.; Song, X. M.; Yuan, S. P.; Fu, Y.; Luo, Y. Z., Nucleolin is a receptor that mediates antiangiogenic and antitumor activity of endostatin. *Blood* **2007,** 110, (8), 2899-2906.
78. Zhuo, W.; Luo, C.; Wang, X.; Song, X.; Fu, Y.; Luo, Y., Endostatin inhibits tumour lymphangiogenesis and lymphatic metastasis via cell surface nucleolin on lymphangiogenic endothelial cells. *J Pathol* **2010,** 222, (3), 249-60.
79. Brideau, G.; Makinen, M. J.; Elamaa, H.; Tu, H.; Nilsson, G.; Alitalo, K.; Pihlajaniemi, T.; Heljasvaara, R., Endostatin overexpression inhibits lymphangiogenesis and lymph node metastasis in mice. *Cancer Res* **2007,** 67, (24), 11528-35.
80. Fukumoto, S.; Morifuji, M.; Katakura, Y.; Ohishi, M.; Nakamura, S., Endostatin inhibits lymph node metastasis by a down-regulation of the vascular endothelial growth factor C expression in tumor cells. *Clin Exp Metastasis* **2005,** 22, (1), 31-8.
81. Kojima, T.; Azar, D. T.; Chang, J. H., Neostatin-7 regulates bFGF-induced corneal lymphangiogenesis. *FEBS Lett* **2008,** 582, (17), 2515-20.
82. Li, Y.; Ren, H. T., Endostatin inhibits fibrosis by modulating the PDGFR/ERK signal pathway: an in vitro study. *J Zhejiang Univ Sci B* **2017,** 18, (11), 994-1001.
83. Rehn, M.; Veikkola, T.; Kukk-Valdre, E.; Nakamura, H.; Ilmonen, M.; Lombardo, C.; Pihlajaniemi, T.; Alitalo, K.; Vuori, K., Interaction of endostatin with integrins implicated in angiogenesis. *Proc Natl Acad Sci U S A* **2001,** 98, (3), 1024-9.
84. Wickstrom, S. A.; Alitalo, K.; Keski-Oja, J., Endostatin associates with integrin alpha5beta1 and caveolin-1, and activates Src via a tyrosyl phosphatase-dependent pathway in human endothelial cells. *Cancer Res* **2002,** 62, (19), 5580-9.
85. Nguyen, T. M.; Subramanian, I. V.; Xiao, X.; Ghosh, G.; Nguyen, P.; Kelekar, A.; Ramakrishnan, S., Endostatin induces autophagy in endothelial cells by modulating Beclin 1 and beta-catenin levels. *J Cell Mol Med* **2009,** 13, (9B), 3687-98.
86. Bjorndahl, M.; Cao, R.; Nissen, L. J.; Clasper, S.; Johnson, L. A.; Xue, Y.; Zhou, Z.; Jackson, D.; Hansen, A. J.; Cao, Y., Insulin-like growth factors 1 and 2 induce lymphangiogenesis in vivo. *Proc Natl Acad Sci U S A* **2005,** 102, (43), 15593-8.
87. Yoon, C. M.; Hong, B. S.; Moon, H. G.; Lim, S.; Suh, P. G.; Kim, Y. K.; Chae, C. B.; Gho, Y. S., Sphingosine-1-phosphate promotes lymphangiogenesis by stimulating S1P1/Gi/PLC/Ca2+ signaling pathways. *Blood* **2008,** 112, (4), 1129-38.
88. Korhonen, E. A.; Murtomaki, A.; Jha, S. K.; Anisimov, A.; Pink, A.; Zhang, Y.; Stritt, S.; Liaqat, I.; Stanczuk, L.; Alderfer, L.; Sun, Z.; Kapiainen, E.; Singh, A.; Sultan, I.; Lantta, A.; Leppanen, V. M.; Eklund, L.; He, Y.; Augustin, H. G.; Vaahtomeri, K.; Saharinen, P.; Makinen, T.; Alitalo, K., Lymphangiogenesis requires Ang2/Tie/PI3K signaling for VEGFR3 cell-surface expression. *J Clin Invest* **2022,** 132, (15).
89. Tammela, T.; Saaristo, A.; Lohela, M.; Morisada, T.; Tornberg, J.; Norrmen, C.; Oike, Y.; Pajusola, K.; Thurston, G.; Suda, T.; Yla-Herttuala, S.; Alitalo, K., Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. *Blood* **2005,** 105, (12), 4642-8.

90. Cao, R.; Bjorndahl, M. A.; Gallego, M. I.; Chen, S.; Religa, P.; Hansen, A. J.; Cao, Y., Hepatocyte growth factor is a lymphangiogenic factor with an indirect mechanism of action. *Blood* **2006,** 107, (9), 3531-6.
91. Kajiya, K.; Hirakawa, S.; Ma, B.; Drinnenberg, I.; Detmar, M., Hepatocyte growth factor promotes lymphatic vessel formation and function. *EMBO J* **2005,** 24, (16), 2885-95.
92. Marino, D.; Angehrn, Y.; Klein, S.; Riccardi, S.; Baenziger-Tobler, N.; Otto, V. I.; Pittelkow, M.; Detmar, M., Activation of the epidermal growth factor receptor promotes lymphangiogenesis in the skin. *J Dermatol Sci* **2013,** 71, (3), 184-94.
93. Spinella, F.; Garrafa, E.; Di Castro, V.; Rosano, L.; Nicotra, M. R.; Caruso, A.; Natali, P. G.; Bagnato, A., Endothelin-1 stimulates lymphatic endothelial cells and lymphatic vessels to grow and invade. *Cancer Res* **2009,** 69, (6), 2669-76.
94. Liao, S.; von der Weid, P. Y., Inflammation-induced lymphangiogenesis and lymphatic dysfunction. *Angiogenesis* **2014,** 17, (2), 325-34.
95. Kim, H.; Kataru, R. P.; Koh, G. Y., Inflammation-associated lymphangiogenesis: a double-edged sword? *J Clin Invest* **2014,** 124, (3), 936-42.
96. Baluk, P.; Hogmalm, A.; Bry, M.; Alitalo, K.; Bry, K.; McDonald, D. M., Transgenic overexpression of interleukin-1beta induces persistent lymphangiogenesis but not angiogenesis in mouse airways. *Am J Pathol* **2013,** 182, (4), 1434-47.
97. Al-Rawi, M. A.; Watkins, G.; Mansel, R. E.; Jiang, W. G., The effects of interleukin-7 on the lymphangiogenic properties of human endothelial cells. *Int J Oncol* **2005,** 27, (3), 721-30.
98. Han, L.; Zhang, M.; Liang, X.; Jia, X.; Jia, J.; Zhao, M.; Fan, Y., Interleukin-33 promotes inflammation-induced lymphangiogenesis via ST2/TRAF6-mediated Akt/eNOS/NO signalling pathway. *Sci Rep* **2017,** 7, (1), 10602.
99. Baluk, P.; Yao, L. C.; Feng, J.; Romano, T.; Jung, S. S.; Schreiter, J. L.; Yan, L.; Shealy, D. J.; McDonald, D. M., TNF-alpha drives remodeling of blood vessels and lymphatics in sustained airway inflammation in mice. *J Clin Invest* **2009,** 119, (10), 2954-64.
100. Savetsky, I. L.; Ghanta, S.; Gardenier, J. C.; Torrisi, J. S.; Nores, G. D. G.; Hespe, G. E.; Nitti, M. D.; Kataru, R. P.; Mehrara, B. J., Th2 Cytokines Inhibit Lymphangiogenesis. *Plos One* **2015,** 10, (6).
101. Shin, K.; Kataru, R. P.; Park, H. J.; Kwon, B. I.; Kim, T. W.; Hong, Y. K.; Lee, S. H., T(H)2 cells and their cytokines regulate formation and function of lymphatic vessels. *Nat Commun* **2015,** 6.
102. Shao, X.; Liu, C., Influence of IFN- alpha and IFN- gamma on lymphangiogenesis. *J Interferon Cytokine Res* **2006,** 26, (8), 568-74.
103. Kataru, R. P.; Kim, H.; Jang, C.; Choi, D. K.; Koh, B. I.; Kim, M.; Gollamudi, S.; Kim, Y. K.; Lee, S. H.; Koh, G. Y., T lymphocytes negatively regulate lymph node lymphatic vessel formation. *Immunity* **2011,** 34, (1), 96-107.
104. Sorensen, E. W.; Gerber, S. A.; Frelinger, J. G.; Lord, E. M., IL-12 suppresses vascular endothelial growth factor receptor 3 expression on tumor vessels by two distinct IFN-gamma-dependent mechanisms. *J Immunol* **2010,** 184, (4), 1858-66.
105. Zhao, G.; Zhu, G.; Huang, Y.; Zheng, W.; Hua, J.; Yang, S.; Zhuang, J.; Ye, J., IL-6 mediates the signal pathway of JAK-STAT3-VEGF-C promoting growth, invasion and lymphangiogenesis in gastric cancer. *Oncol Rep* **2016,** 35, (3), 1787-95.
106. Hos, D.; Bucher, F.; Regenfuss, B.; Dreisow, M. L.; Bock, F.; Heindl, L. M.; Eming, S. A.; Cursiefen, C., IL-10 Indirectly Regulates Corneal Lymphangiogenesis and Resolution of Inflammation via Macrophages. *Am J Pathol* **2016,** 186, (1), 159-71.

107. Niessen, K.; Zhang, G.; Ridgway, J. B.; Chen, H.; Kolumam, G.; Siebel, C. W.; Yan, M., The Notch1-Dll4 signaling pathway regulates mouse postnatal lymphatic development. *Blood* **2011,** 118, (7), 1989-97.
108. Muley, A.; Kim Uh, M.; Salazar-De Simone, G.; Swaminathan, B.; James, J. M.; Murtomaki, A.; Youn, S. W.; McCarron, J. D.; Kitajewski, C.; Gnarra Buethe, M.; Riitano, G.; Mukouyama, Y. S.; Kitajewski, J.; Shawber, C. J., Unique functions for Notch4 in murine embryonic lymphangiogenesis. *Angiogenesis* **2022,** 25, (2), 205-224.
109. Suzuki, Y.; Ito, Y.; Mizuno, M.; Kinashi, H.; Sawai, A.; Noda, Y.; Mizuno, T.; Shimizu, H.; Fujita, Y.; Matsui, K.; Maruyama, S.; Imai, E.; Matsuo, S.; Takei, Y., Transforming growth factor-beta induces vascular endothelial growth factor-C expression leading to lymphangiogenesis in rat unilateral ureteral obstruction. *Kidney Int* **2012,** 81, (9), 865-79.
110. Clavin, N. W.; Avraham, T.; Fernandez, J.; Daluvoy, S. V.; Soares, M. A.; Chaudhry, A.; Mehrara, B. J., TGF-beta(1) is a negative regulator of lymphatic regeneration during wound repair. *Am J Physiol-Heart C* **2008,** 295, (5), H2113-H2127.
111. Kang, S.; Lee, S. P.; Kim, K. E.; Kim, H. Z.; Memet, S.; Koh, G. Y., Toll-like receptor 4 in lymphatic endothelial cells contributes to LPS-induced lymphangiogenesis by chemotactic recruitment of macrophages. *Blood* **2009,** 113, (11), 2605-13.
112. Kumaravel, S.; Abbey, C. A.; Bayless, K. J.; Chakraborty, S., The beta(1)-integrin plays a key role in LEC invasion in an optimized 3-D collagen matrix model. *Am J Physiol Cell Physiol* **2020,** 319, (6), C1045-C1058.
113. Wiig, H.; Keskin, D.; Kalluri, R., Interaction between the extracellular matrix and lymphatics: Consequences for lymphangiogenesis and lymphatic function. *Matrix Biol* **2010,** 29, (8), 645-656.
114. Norgall, S.; Papoutsi, M.; Rossler, J.; Schweigerer, L.; Wilting, J.; Weich, H. A., Elevated expression of VEGFR-3 in lymphatic endothelial cells from lymphangiomas. *BMC Cancer* **2007,** 7, 105.
115. Huang, X. Z.; Wu, J. F.; Ferrando, R.; Lee, J. H.; Wang, Y. L.; Farese, R. V., Jr.; Sheppard, D., Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. *Mol Cell Biol* **2000,** 20, (14), 5208-15.
116. Dietrich, T.; Onderka, J.; Bock, F.; Kruse, F. E.; Vossmeyer, D.; Stragies, R.; Zahn, G.; Cursiefen, C., Inhibition of inflammatory lymphangiogenesis by integrin alpha5 blockade. *Am J Pathol* **2007,** 171, (1), 361-72.
117. Klein, S.; Giancotti, F. G.; Presta, M.; Albelda, S. M.; Buck, C. A.; Rifkin, D. B., Basic Fibroblast Growth-Factor Modulates Integrin Expression in Microvascular Endothelial-Cells. *Mol Biol Cell* **1993,** 4, (10), 973-982.
118. Kriehuber, E.; Breiteneder-Geleff, S.; Groeger, M.; Soleiman, A.; Schoppmann, S. F.; Stingl, G.; Kerjaschki, D.; Maurer, D., Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *Journal of Experimental Medicine* **2001,** 194, (6), 797-808.
119. Parsons-Wingerter, P.; McKay, T. L.; Leontiev, D.; Vickerman, M. B.; Condrich, T. K.; Dicorleto, P. E., Lymphangiogenesis by blind-ended vessel sprouting is concurrent with hemangiogenesis by vascular splitting. *Anat Rec Part A* **2006,** 288a, (3), 233-247.
120. Sweat, R. S.; Stapor, P. C.; Murfee, W. L., Relationships Between Lymphangiogenesis and Angiogenesis During Inflammation in Rat Mesentery Microvascular Networks. *Lymphatic Research and Biology* **2012,** 10, (4), 198-207.
121. Benest, A. V.; Harper, S. J.; Herttuala, S. Y.; Alitalo, K.; Bates, D. O., VEGF-C induced angiogenesis preferentially occurs at a distance from lymphangiogenesis. *Cardiovasc Res* **2008,** 78, (2), 315-323.

122. Baluk, P.; Tammela, T.; Ator, E.; Lyubynska, N.; Achen, M. G.; Hicklin, D. J.; Jeltsch, M.; Petrova, T. V.; Pytowski, B.; Stacker, S. A.; Yla-Herttuala, S.; Jackson, D. G.; Alitalo, K.; McDonald, D. M., Pathogenesis of persistent lymphatic vessel hyperplasia in chronic airway inflammation. *J Clin Invest* **2005,** 115, (2), 247-57.
123. Landau, S.; Newman, A.; Edri, S.; Michael, I.; Ben-Shaul, S.; Shandalov, Y.; Ben-Arye, T.; Kaur, P.; Zheng, M. H.; Levenberg, S., Investigating lymphangiogenesis in vitro and in vivo using engineered human lymphatic vessel networks. *Proc Natl Acad Sci U S A* **2021,** 118, (31).
124. Schulze, H.; Nacke, M.; Gutenbrunner, C.; Hadamitzky, C., Worldwide assessment of healthcare personnel dealing with lymphoedema. *Health Econ Rev* **2018,** 8, (1), 10.
125. Rockson, S. G.; Rivera, K. K., Estimating the population burden of lymphedema. *Ann N Y Acad Sci* **2008,** 1131, 147-54.
126. Pereira, N.; Lee, Y. H.; Suh, Y.; Choi, D. H.; Suh, H. S.; Jeon, J. Y.; Hong, J. P. J., Cumulative Experience in Lymphovenous Anastomosis for Lymphedema Treatment: The Learning Curve Effect on the Overall Outcome. *J Reconstr Microsurg* **2018,** 34, (9), 735-741.
127. Lahteenvuo, M.; Honkonen, K.; Tervala, T.; Tammela, T.; Suominen, E.; Lahteenvuo, J.; Kholova, I.; Alitalo, K.; Yla-Herttuala, S.; Saaristo, A., Growth factor therapy and autologous lymph node transfer in lymphedema. *Circulation* **2011,** 123, (6), 613-20.
128. Asai, J.; Takenaka, H.; Hirakawa, S.; Sakabe, J.; Hagura, A.; Kishimoto, S.; Maruyama, K.; Kajiya, K.; Kinoshita, S.; Tokura, Y.; Katoh, N., Topical simvastatin accelerates wound healing in diabetes by enhancing angiogenesis and lymphangiogenesis. *Am J Pathol* **2012,** 181, (6), 2217-24.
129. Sen, C. K., Human Wound and Its Burden: Updated 2020 Compendium of Estimates. *Adv Wound Care (New Rochelle)* **2021,** 10, (5), 281-292.
130. Reinke, J. M.; Sorg, H., Wound Repair and Regeneration. *Eur Surg Res* **2012,** 49, (1), 35-43.
131. Bianchi, A.; Painter, K. J.; Sherratt, J. A., Spatio-temporal Models of Lymphangiogenesis in Wound Healing. *Bulletin of Mathematical Biology* **2016,** 78, (9), 1904-1941.
132. Parrish, W. R., Physiology of Blood Components in Wound Healing: an Appreciation of Cellular Co-Operativity in Platelet Rich Plasma Action. *Journal of Exercise Sports & Orthopedics* **2017,** 4, (2), 1-14.
133. Tammela, T.; Alitalo, K., Lymphangiogenesis: Molecular mechanisms and future promise. *Cell* **2010,** 140, (4), 460-76.
134. Huethorst, E.; Krebber, M. M.; Fledderus, J. O.; Gremmels, H.; Xu, Y. J.; Pei, J.; Verhaar, M. C.; Cheng, C., Lymphatic Vascular Regeneration: The Next Step in Tissue Engineering. *Tissue Eng Part B Rev* **2016,** 22, (1), 1-14.
135. Saaristo, A.; Veikkola, T.; Enholm, B.; Hytonen, M.; Arola, J.; Pajusola, K.; Turunen, P.; Jeltsch, M.; Karkkainen, M. J.; Kerjaschki, D.; Bueler, H.; Yla-Herttuala, S.; Alitalo, K., Adenoviral VEGF-C overexpression induces blood vessel enlargement, tortuosity, and leakiness but no sprouting angiogenesis in the skin or mucous membranes. *FASEB J* **2002,** 16, (9), 1041-9.
136. Mazini, L.; Ezzoubi, M.; Malka, G., Overview of current adipose-derived stem cell (ADSCs) processing involved in therapeutic advancements: flow chart and regulation updates before and after COVID-19. *Stem Cell Res Ther* **2021,** 12, (1), 1.
137. George-Gay, B.; Parker, K., Understanding the complete blood count with differential. *J Perianesth Nurs* **2003,** 18, (2), 96-114; quiz 115-7.
138. Ayache, S.; Panelli, M. C.; Byrne, K. M.; Slezak, S.; Leitman, S. F.; Marincola, F. M.; Stroncek, D. F., Comparison of proteomic profiles of serum, plasma, and modified media supplements used for cell culture and expansion. *J Transl Med* **2006,** 4.

139. Karsten, E.; Breen, E.; Herbert, B. R., Red blood cells are dynamic reservoirs of cytokines. *Sci Rep* **2018,** 8, (1), 3101.
140. Yogev, Y., A humoral solution: Autologous blood products and tissue repair. *Cell Immunol* **2020,** 356, 104178.
141. Amable, P. R.; Carias, R. B.; Teixeira, M. V.; da Cruz Pacheco, I.; Correa do Amaral, R. J.; Granjeiro, J. M.; Borojevic, R., Platelet-rich plasma preparation for regenerative medicine: optimization and quantification of cytokines and growth factors. *Stem Cell Res Ther* **2013,** 4, (3), 67.
142. Ross, R.; Glomset, J.; Kariya, B.; Harker, L., A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. *Proc Natl Acad Sci U S A* **1974,** 71, (4), 1207-10.
143. Kardos, D.; Simon, M.; Vacz, G.; Hinsenkamp, A.; Holczer, T.; Cseh, D.; Sarkozi, A.; Szenthe, K.; Banati, F.; Szathmary, S.; Nehrer, S.; Kuten, O.; Masteling, M.; Lacza, Z.; Hornyak, I., The Composition of Hyperacute Serum and Platelet-Rich Plasma Is Markedly Different despite the Similar Production Method. *Int J Mol Sci* **2019,** 20, (3).
144. Mizuno, M.; Katano, H.; Otabe, K.; Komori, K.; Matsumoto, Y.; Fujii, S.; Ozeki, N.; Tsuji, K.; Koga, H.; Muneta, T.; Matsuyama, A.; Sekiya, I., Platelet-derived growth factor (PDGF)-AA/AB in human serum are potential indicators of the proliferative capacity of human synovial mesenchymal stem cells. *Stem Cell Res Ther* **2015,** 6, 243.
145. English, D.; Welch, Z.; Kovala, A. T.; Harvey, K.; Volpert, O. V.; Brindley, D. N.; Garcia, J. G. N., Sphingosine 1-phosphate released from platelets during clotting accounts for the potent endothelial cell chemotactic activity of blood serum and provides a novel link between hemostasis and angiogenesis. *Faseb Journal* **2000,** 14, (14), 2255-2265.
146. Mehta, S.; Watson, J. T., Platelet rich concentrate: Basic science and current clinical applications. *J Orthop Trauma* **2008,** 22, (6), 433-438.
147. Hakimi, M.; Grassmann, J. P.; Betsch, M.; Schneppendahl, J.; Gehrmann, S.; Hakimi, A. R.; Kropil, P.; Sager, M.; Herten, M.; Wild, M.; Windolf, J.; Jungbluth, P., The composite of bone marrow concentrate and PRP as an alternative to autologous bone grafting. *PLoS One* **2014,** 9, (6), e100143.
148. Everts, P.; Onishi, K.; Jayaram, P.; Lana, J. F.; Mautner, K., Platelet-Rich Plasma: New Performance Understandings and Therapeutic Considerations in 2020. *Int J Mol Sci* **2020,** 21, (20).
149. Xu, P.; Wu, Y.; Zhou, L.; Yang, Z.; Zhang, X.; Hu, X.; Yang, J.; Wang, M.; Wang, B.; Luo, G.; He, W.; Cheng, B., Platelet-rich plasma accelerates skin wound healing by promoting re-epithelialization. *Burns Trauma* **2020,** 8, tkaa028.
150. Kubo, M.; Li, T. S.; Suzuki, R.; Shirasawa, B.; Morikage, N.; Ohshima, M.; Qin, S. L.; Hamano, K., Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance. *Am J Physiol Heart Circ Physiol* **2008,** 294, (2), H590-5.
151. Bader, A. M.; Klose, K.; Bieback, K.; Korinth, D.; Schneider, M.; Seifert, M.; Choi, Y. H.; Kurtz, A.; Falk, V.; Stamm, C., Hypoxic Preconditioning Increases Survival and Pro-Angiogenic Capacity of Human Cord Blood Mesenchymal Stromal Cells In Vitro. *PLoS One* **2015,** 10, (9), e0138477.
152. Terada, H.; Hirata, N.; Sawashita, Y.; Ohno, S.; Yoshikawa, Y.; Yamakage, M., Acute Hypobaric and Hypoxic Preconditioning Reduces Myocardial Ischemia-Reperfusion Injury in Rats. *Cardiol Res Pract* **2021,** 2021, 6617374.

153. Jarrard, C. P.; Nagel, M. J.; Stray-Gundersen, S.; Tanaka, H.; Lalande, S., Hypoxic preconditioning attenuates ischemia-reperfusion injury in young healthy adults. *J Appl Physiol (1985)* **2021,** 130, (3), 846-852.
154. Hadjipanayi, E. B., S.; Moog, P, Extracorporeal Wound Simulation as a Foundation for Tissue Repair and Regeneration Therapies. *International Journal of Transplantation & Plastic Surgery* **2018,** 2.
155. Hadjipanayi, E.; Schilling, A. F., Regeneration through autologous hypoxia preconditioned plasma. *Organogenesis* **2014,** 10, (2), 164-9.
156. Hadjipanayi, E.; Moog, P.; Bekeran, S.; Kirchhoff, K.; Berezhnoi, A.; Aguirre, J.; Bauer, A. T.; Kukrek, H.; Schmauss, D.; Hopfner, U.; Isenburg, S.; Ntziachristos, V.; Ninkovic, M.; Machens, H. G.; Schilling, A. F.; Dornseifer, U., In Vitro Characterization of Hypoxia Preconditioned Serum (HPS)-Fibrin Hydrogels: Basis for an Injectable Biomimetic Tissue Regeneration Therapy. *J Funct Biomater* **2019,** 10, (2).
157. Morfoisse, F.; Renaud, E.; Hantelys, F.; Prats, A. C.; Garmy-Susini, B., Role of hypoxia and vascular endothelial growth factors in lymphangiogenesis. *Mol Cell Oncol* **2015,** 2, (4), e1024821.
158. Liu, Y. Y.; Li, W. J.; Ma, X. Y.; He, J. B.; Lin, Y.; Lin, D. S., Rivastigmine Regulates the HIF-1 alpha/VEGF Signaling Pathway to Induce Angiogenesis and Improves the Survival of Random Flaps in Rats. *Front Pharmacol* **2022,** 12.
159. Morfoisse, F.; Kuchnio, A.; Frainay, C.; Gomez-Brouchet, A.; Delisle, M. B.; Marzi, S.; Helfer, A. C.; Hantelys, F.; Pujol, F.; Guillermet-Guibert, J.; Bousquet, C.; Dewerchin, M.; Pyronnet, S.; Prats, A. C.; Carmeliet, P.; Garmy-Susini, B., Hypoxia induces VEGF-C expression in metastatic tumor cells via a HIF-1alpha-independent translation-mediated mechanism. *Cell Rep* **2014,** 6, (1), 155-67.
160. Arcondeguy, T.; Lacazette, E.; Millevoi, S.; Prats, H.; Touriol, C., VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level. *Nucleic Acids Res* **2013,** 41, (17), 7997-8010.
161. Creancier, L.; Morello, D.; Mercier, P.; Prats, A. C., Fibroblast growth factor 2 internal ribosome entry site (IRES) activity ex vivo and in transgenic mice reveals a stringent tissue-specific regulation. *J Cell Biol* **2000,** 150, (1), 275-81.
162. Hadjipanayi, E.; Bauer, A. T.; Moog, P.; Salgin, B.; Kuekrek, H.; Fersch, B.; Hopfner, U.; Meissner, T.; Schluter, A.; Ninkovic, M.; Machens, H. G.; Schilling, A. F., Cell-free carrier system for localized delivery of peripheral blood cell-derived engineered factor signaling: towards development of a one-step device for autologous angiogenic therapy. *J Control Release* **2013,** 169, (1-2), 91-102.
163. Hadjipanayi, E.; Cheema, U.; Mudera, V.; Deng, D.; Liu, W.; Brown, R. A., First implantable device for hypoxia-mediated angiogenic induction. *J Control Release* **2011,** 153, (3), 217-24.
164. Hadjipanayi, E.; Cheema, U.; Hopfner, U.; Bauer, A.; Machens, H. G.; Schilling, A. F., Injectable system for spatio-temporally controlled delivery of hypoxia-induced angiogenic signalling. *J Control Release* **2012,** 161, (3), 852-60.
165. Moog, P.; Kirchhoff, K.; Bekeran, S.; Bauer, A. T.; von Isenburg, S.; Dornseifer, U.; Machens, H. G.; Schilling, A. F.; Hadjipanayi, E., Comparative Evaluation of the Angiogenic Potential of Hypoxia Preconditioned Blood-Derived Secretomes and Platelet-Rich Plasma: An In Vitro Analysis. *Biomedicines* **2020,** 8, (1).
166. Jiang, J.; Kraneburg, U.; Dornseifer, U.; Schilling, A. F.; Hadjipanayi, E.; Machens, H. G.; Moog, P., Hypoxia Preconditioned Serum (HPS)-Hydrogel Can Accelerate Dermal Wound Healing in Mice-An In Vivo Pilot Study. *Biomedicines* **2022,** 10, (1).

167. Jiang, J.; Röper, L.; Alageel, S.; Dornseifer, U.; Schilling, A. F.; Hadjipanayi, E.; Machens, H.-G.; Moog, P., Hypoxia Preconditioned Serum (HPS) Promotes Osteoblast Proliferation, Migration and Matrix Deposition. *Biomedicines* **2022,** 10, (7).
168. Maruyama, K.; Asai, J.; Ii, M.; Thorne, T.; Losordo, D. W.; D'Amore, P. A., Decreased macrophage number and activation lead to reduced lymphatic vessel formation and contribute to impaired diabetic wound healing. *Am J Pathol* **2007,** 170, (4), 1178-91.
169. Hadamitzky, C.; Zaitseva, T. S.; Bazalova-Carter, M.; Paukshto, M. V.; Hou, L.; Strassberg, Z.; Ferguson, J.; Matsuura, Y.; Dash, R.; Yang, P. C.; Kretchetov, S.; Vogt, P. M.; Rockson, S. G.; Cooke, J. P.; Huang, N. F., Aligned nanofibrillar collagen scaffolds - Guiding lymphangiogenesis for treatment of acquired lymphedema. *Biomaterials* **2016,** 102, 259-267.
170. Rissanen, T. T.; Markkanen, J. E.; Gruchala, M.; Heikura, T.; Puranen, A.; Kettunen, M. I.; Kholova, I.; Kauppinen, R. A.; Achen, M. G.; Stacker, S. A.; Alitalo, K.; Yla-Herttuala, S., VEGF-D is the strongest angiogenic and lymphangiogenic effector among VEGFs delivered into skeletal muscle via adenoviruses. *Circulation Research* **2003,** 92, (10), 1098-1106.
171. Akgül, A.; Cirak, M.; Birinci, T., Applications of Platelet-Rich Plasma in Lymphedema. *Lymphat Res Biol* **2016,** 14, (4), 206-209.
172. Jarmund, A. H.; Giskeodegard, G. F.; Ryssdal, M.; Steinkjer, B.; Stokkeland, L. M. T.; Madssen, T. S.; Stafne, S. N.; Stridsklev, S.; Moholdt, T.; Heimstad, R.; Vanky, E.; Iversen, A. C., Cytokine Patterns in Maternal Serum From First Trimester to Term and Beyond. *Front Immunol* **2021,** 12, 752660.
173. Zhang, W.; Lin, H.; Zou, M.; Yuan, Q.; Huang, Z.; Pan, X.; Zhang, W., Nicotine in Inflammatory Diseases: Anti-Inflammatory and Pro-Inflammatory Effects. *Front Immunol* **2022,** 13, 826889.
174. Kececi, Y.; Ozsu, S.; Bilgir, O., A cost-effective method for obtaining standard platelet-rich plasma. *Wounds* **2014,** 26, (8), 232-8.
175. Bruyère, F.; Melen-Lamalle, L.; Blacher, S.; Roland, G.; Thiry, M.; Moons, L.; Frankenne, F.; Carmeliet, P.; Alitalo, K.; Libert, C.; Sleeman, J. P.; Foidart, J. M.; Noël, A., Modeling lymphangiogenesis in a three-dimensional culture system. *Nat Methods* **2008,** 5, (5), 431-7.
176. Moog, P.; Schams, R.; Schneidinger, A.; Schilling, A. F.; Machens, H. G.; Hadjipanayi, E.; Dornseifer, U., Effect of Hypoxia Preconditioned Secretomes on Lymphangiogenic and Angiogenic Sprouting: An in Vitro Analysis. *Biomedicines* **2020,** 8, (9).
177. Dizon, M.; Szele, F.; Kessler, J. A., Hypoxia-ischemia induces an endogenous reparative response by local neural progenitors in the postnatal mouse telencephalon. *Dev Neurosci* **2010,** 32, (3), 173-83.
178. Hong, W. X.; Hu, M. S.; Esquivel, M.; Liang, G. Y.; Rennert, R. C.; McArdle, A.; Paik, K. J.; Duscher, D.; Gurtner, G. C.; Lorenz, H. P.; Longaker, M. T., The Role of Hypoxia-Inducible Factor in Wound Healing. *Adv Wound Care (New Rochelle)* **2014,** 3, (5), 390-399.
179. Rodrigues, M.; Kosaric, N.; Bonham, C. A.; Gurtner, G. C., Wound Healing: A Cellular Perspective. *Physiol Rev* **2019,** 99, (1), 665-706.
180. Ferrer-Tasies, L.; Santana, H.; Cabrera-Puig, I.; Gonzalez-Mira, E.; Ballell-Hosa, L.; Castellar-Alvarez, C.; Cordoba, A.; Merlo-Mas, J.; Geronimo, H.; Chinea, G.; Falcon, V.; Moreno-Calvo, E.; Pedersen, J. S.; Romero, J.; Navarro-Requena, C.; Valdes, C.; Limonta, M.; Berlanga, J.; Sala, S.; Martinez, E.; Veciana, J.; Ventosa, N., Recombinant Human Epidermal Growth Factor/Quatsome Nanoconjugates: A

Robust Topical Delivery System for Complex Wound Healing. *Adv Ther-Germany* **2021,** 4, (6).
181. Shams, F.; Moravvej, H.; Hosseinzadeh, S.; Mostafavi, E.; Bayat, H.; Kazemi, B.; Bandehpour, M.; Rostami, E.; Rahimpour, A.; Moosavian, H., Overexpression of VEGF in dermal fibroblast cells accelerates the angiogenesis and wound healing function: in vitro and in vivo studies. *Sci Rep* **2022,** 12, (1), 18529.
182. Jiang, X.; Tian, W.; Kim, D.; McQuiston, A. S.; Vinh, R.; Rockson, S. G.; Semenza, G. L.; Nicolls, M. R., Hypoxia and Hypoxia-Inducible Factors in Lymphedema. *Front Pharmacol* **2022,** 13, 851057.
183. Paavonen, K.; Puolakkainen, P.; Jussila, L.; Jahkola, T.; Alitalo, K., Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol* **2000,** 156, (5), 1499-504.
184. Zampell, J. C.; Avraham, T.; Yoder, N.; Fort, N.; Yan, A.; Weitman, E. S.; Mehrara, B. J., Lymphatic function is regulated by a coordinated expression of lymphangiogenic and anti-lymphangiogenic cytokines. *Am J Physiol-Cell Ph* **2012,** 302, (2), C392-C404.
185. Anisimov, A.; Alitalo, A.; Korpisalo, P.; Soronen, J.; Kaijalainen, S.; Leppanen, V. M.; Jeltsch, M.; Yla-Herttuala, S.; Alitalo, K., Activated forms of VEGF-C and VEGF-D provide improved vascular function in skeletal muscle. *Circ Res* **2009,** 104, (11), 1302-12.
186. Airoldi, I.; Ribatti, D., Regulation of angiostatic chemokines driven by IL-12 and IL-27 in human tumors. *J Leukoc Biol* **2011,** 90, (5), 875-82.
187. Philippe, V.; Laurent, A.; Abdel-Sayed, P.; Hirt-Burri, N.; Applegate, L. A.; Martin, R., Human Platelet Lysate as an Alternative to Autologous Serum for Human Chondrocyte Clinical Use. *Cartilage* **2021,** 13, (1_Suppl), 509s-518s.
188. Niewiarowski, S.; Lowery, C. T.; Hawiger, J.; Millman, M.; Timmons, S., Immunoassay of human platelet factor 4(PF4, antiheparin factor) by radial immunodiffusion. *J Lab Clin Med* **1976,** 87, (4), 720-33.
189. Kiers, D.; Tunjungputri, R. N.; Borkus, R.; Scheffer, G. J.; de Groot, P. G.; Urbanus, R. T.; van der Ven, A. J.; Pickkers, P.; de Mast, Q.; Kox, M., The influence of hypoxia on platelet function and plasmatic coagulation during systemic inflammation in humans in vivo. *Platelets* **2019,** 30, (7), 927-930.
190. Paddenberg, R.; Faulhammer, P.; Goldenberg, A.; Kummer, W., Hypoxia-induced increase of endostatin in murine aorta and lung. *Histochem Cell Biol* **2006,** 125, (5), 497-508.
191. Asif, S.; Ruge, T.; Larsson, A.; Anderberg, S. B.; Lipcsey, M.; Frithiof, R.; Hultstrom, M., Plasma endostatin correlates with hypoxia and mortality in COVID-19-associated acute respiratory failure. *Biomark Med* **2021,** 15, (16), 1509-1517.
192. Hatami, N.; Buttner, C.; Bock, F.; Simfors, S.; Musial, G.; Reis, A.; Cursiefen, C.; Clahsen, T., Cystathionine beta-synthase as novel endogenous regulator of lymphangiogenesis via modulating VEGF receptor 2 and 3. *Commun Biol* **2022,** 5, (1).
193. Tai, H. C.; Lee, T. H.; Tang, C. H.; Chen, L. P.; Chen, W. C.; Lee, M. S.; Chen, P. C.; Lin, C. Y.; Chi, C. W.; Chen, Y. J.; Lai, C. T.; Chen, S. S.; Liao, K. W.; Lee, C. H.; Wang, S. W., Phomaketide A Inhibits Lymphangiogenesis in Human Lymphatic Endothelial Cells. *Mar Drugs* **2019,** 17, (4).
194. Boardman, K. C.; Swartz, M. A., Interstitial flow as a guide for lymphangiogenesis. *Circulation Research* **2003,** 92, (7), 801-808.
195. Lee, S. J.; Park, C.; Lee, J. Y.; Kim, S.; Kwon, P. J.; Kim, W.; Jeon, Y. H.; Lee, E.; Yoon, Y. S., Generation of pure lymphatic endothelial cells from human pluripotent stem cells and their therapeutic effects on wound repair. *Sci Rep-Uk* **2015,** 5.

196. Pittman, M.; Iu, E.; Li, K.; Chen, J. J.; Potnikov, S.; Chen, Y., Membrane ruffling is a mechanosensor of extracellular fluid viscosity. *Biophys J* **2022,** 121, (3), 267a-267a.
197. Bera, K.; Kiepas, A.; Godet, I.; Li, Y. Z.; Mehta, P.; Ifemembi, B.; Paul, C. D.; Sen, A.; Serra, S. A.; Stoletov, K.; Tao, J. X.; Shatkin, G.; Lee, S. J.; Zhang, Y. Q.; Boen, A.; Mistriotis, P.; Gilkes, D. M.; Lewis, J. D.; Fan, C. M.; Feinberg, A. P.; Valverde, M. A.; Sun, S. X.; Konstantopoulos, K., Extracellular fluid viscosity enhances cell migration and cancer dissemination. *Nature* **2022,** 611, (7935), 365-+.
198. Park, S.; Jung, W. H.; Pittman, M.; Chen, J.; Chen, Y., The Effects of Stiffness, Fluid Viscosity, and Geometry of Microenvironment in Homeostasis, Aging, and Diseases: A Brief Review. *J Biomech Eng* **2020,** 142, (10).
199. Poon, C., Measuring the density and viscosity of culture media for optimized computational fluid dynamics analysis of in vitro devices. *J Mech Behav Biomed* **2022,** 126.
200. Novoa-Herran, S.; Umana-Perez, A.; Canals, F.; Sanchez-Gomez, M., Serum depletion induces changes in protein expression in the trophoblast-derived cell line HTR-8/SVneo. *Cell Mol Biol Lett* **2016,** 21, 22.
201. Antoniades, H. N.; Owen, A. J., Growth factors and regulation of cell growth. *Annu Rev Med* **1982,** 33, 445-63.
202. Jayme, D.; Watanabe, T.; Shimada, T., Basal medium development for serum-free culture: a historical perspective. *Cytotechnology* **1997,** 23, (1-3), 95-101.
203. Peters, K.; Helmert, T.; Gebhard, S.; Mailander, V.; Unger, R. E.; Nezi-Cahn, S.; Hasenburg, A.; Heller, M.; Schwab, R.; Brenner, W., Standardized Human Platelet Lysates as Adequate Substitute to Fetal Calf Serum in Endothelial Cell Culture for Tissue Engineering. *Biomed Res Int* **2022,** 2022.
204. Iruela-Arispe, M. L.; Beitel, G. J., Tubulogenesis. *Development* **2013,** 140, (14), 2851-5.
205. Davis, G. E.; Stratman, A. N.; Sacharidou, A.; Koh, W., Molecular basis for endothelial lumen formation and tubulogenesis during vasculogenesis and angiogenic sprouting. *Int Rev Cell Mol Biol* **2011,** 288, 101-65.
206. Kapalczynska, M.; Kolenda, T.; Przybyla, W.; Zajaczkowska, M.; Teresiak, A.; Filas, V.; Ibbs, M.; Blizniak, R.; Luczewski, L.; Lamperska, K., 2D and 3D cell cultures - a comparison of different types of cancer cell cultures. *Arch Med Sci* **2018,** 14, (4), 910-919.
207. Detry, B.; Erpicum, C.; Paupert, J.; Blacher, S.; Maillard, C.; Bruyere, F.; Pendeville, H.; Remacle, T.; Lambert, V.; Balsat, C.; Ormenese, S.; Lamaye, F.; Janssens, E.; Moons, L.; Cataldo, D.; Kridelka, F.; Carmeliet, P.; Thiry, M.; Foidart, J. M.; Struman, I.; Noel, A., Matrix metalloproteinase-2 governs lymphatic vessel formation as an interstitial collagenase. *Blood* **2012,** 119, (21), 5048-5056.
208. Hashiguchi, S.; Tanaka, T.; Mano, R.; Kondo, S.; Kodama, S., CCN2-induced lymphangiogenesis is mediated by the integrin alpha v beta 5-ERK pathway and regulated by DUSP6. *Sci Rep-Uk* **2022,** 12, (1).
209. Hadjipanayi, E.; Kuhn, P. H.; Moog, P.; Bauer, A. T.; Kuekrek, H.; Mirzoyan, L.; Hummel, A.; Kirchhoff, K.; Salgin, B.; Isenburg, S.; Dornseifer, U.; Ninkovic, M.; Machens, H. G.; Schilling, A. F., The Fibrin Matrix Regulates Angiogenic Responses within the Hemostatic Microenvironment through Biochemical Control. *PLoS One* **2015,** 10, (8), e0135618.
210. Eichten, A.; Hyun, W. C.; Coussens, L. M., Distinctive features of angiogenesis and lymphangiogenesis determine their functionality during de novo tumor development. *Cancer Res* **2007,** 67, (11), 5211-20.

211. Bao, P.; Kodra, A.; Tomic-Canic, M.; Golinko, M. S.; Ehrlich, H. P.; Brem, H., The role of vascular endothelial growth factor in wound healing. *J Surg Res* **2009,** 153, (2), 347-58.
212. Landen, N. X.; Li, D.; Stahle, M., Transition from inflammation to proliferation: a critical step during wound healing. *Cell Mol Life Sci* **2016,** 73, (20), 3861-85.