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Table of contents

List of Figures

List of Tables

List of Abbreviations

Akt	protein kinase B
ANG	angiogenin
ANGPT-1/2	angiopoietin 1/2
ANOVA	one-way analysis of variance
ASC(s)	adipose-derived stem cell(s)
AX	axitinib
Bcl-2	B-cell lymphoma-2
BDNF	brain-derived neurotrophic factor
BM-MSC(s)	bone marrow mesenchymal stem cell
BMP-2	bone morphogenetic protein 2
CAD	cardiac artery disease
CAM	chorioallantoïc membrane
CCL	chemokine (C-C motif) ligand
CD	cluster differentiation
CM	conditioned medium
$CoCl_2$	cobalt chloride
CSF	colony-stimulating factor
CXCL	chemokine (C-X-C motif) ligand
CXCR-1/2	C-X-C motif chemokine receptor 1
DFX	deferoxamine
DMOG	dimethyloxalglycine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DPPIV	dipeptidyl peptidase IV
DPSC(s)	dental pulp stem cell(s)
DSC(s)	dental stem cell(s)
EC(s)	endothelial cell(s)
ECM	extracellular matrix
EDN1	endothelin-1
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGM	endothelial growth medium

ELISA	enzyme-linked immunosorbent assay
ELR_+	acid-leucine-arginine positive
ENA-78	epithelial-derived neutrophil-activating peptide 78
EX	exudate
FBS	fetal bovine serum
FDA	food and drug administration
FGF	fibroblast growth factor
FGFR(s)	fibroblast growth factor receptor(s)
FITC	fluorescein isothiocyanate
FSC(s)	dental follicle stem cell(s)
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GRO	growth-regulated oncogene
HDMEC(s)	human dermal microvascular endothelial cells
hDPC(s)	human dental pulp cells
HGF	hepatocyte growth factor
HIF-(1α/β)	hypoxic inducible factor (1α/β)
HLA	human leukocyte antigen
HMEC-1	human microvascular endothelial cell-1
HUVEC(s)	human umbilical vein endothelial cell(s)
ICAM-1	intercellular adhesion molecule-1
IFN-γ	interferon-γ
IGF-1	insulin-like growth factor
IGFBP-3	insulin-like growth factor binding protein-3
IHC	immunohistochemistry
IL	interleukin
LIF	leukemia inhibitory factor
L-PRF	leukocyte- and platelet-rich fibrin
L-PRP	leukocyte- and platelet-rich plasma
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
MEM	Minimal Essential Medium
MI	myocardial infarct
MPP	metalloprotease

MSC(s)	mesenchymal stem cell(s)
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAP-2	neutrophil activating protein-2 NEDB non-enzymatic dissociation buffer
NO	nitric oxide
NT-3	neurotrophin-3
PAD	peripheral artery disease
PAI-1	plasminogen activator inhibitor-1
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PDL	periodontal ligament
PDLSC(s)	periodontal ligament stem cell(s)
PE	phycoerythrin
PECAM-1	platelet endothelial cell adhesion molecule-1
PEGF	pigment epithelium-derived factor
PFA	paraformaldehyde
PHD	prolyl hydroxylase
PI	propidium iodide
PlGF	placental growth factor
PPP	platelet-poor plasma
P-PRF	pure platelet rich fibrin
P-PRP	pure platelet rich plasma
PRFM	platelet rich fibrin matrix
PRP	platelet rich plasma
PTX-30	pentraxin-3
RANTES	regulated on activation, normal T cell expressed and secreted
RCT	randomized clinical trial
SCAP(s)	stem cells from the apical papilla
SDF-1	stromal cell derived factor-1
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SHED(s)	stem cells from human exfoliated deciduous teeth.
TBS	tris-buffered saline
TGF(-α/β)	transforming growth factor-α/β
THBS-1	thrombospondin-1

TIMP-1/4	tissue inhibitor of matrix metalloproteinase-1/4
TNF-α	tumor necrosis factor-α
uPA	urokinase-type plasminogen activator
VCAM-1	vascular cell adhesion molecule-1
VEGF	vascular endothelial growth factor
VEGFR-1/2/3	vascular endothelial growth factor receptor-1/2
WO	wash out

Chapter 1

Introduction and Aims

Based on:

Ratajczak, J., et al., The Neurovascular Properties of Dental Stem Cells and Their Importance in Dental Tissue Engineering. Stem Cells Int, 2016. 2016: p. 9762871.

Bronckaers A, Wolfs E, Ratajczak J, Hilkens P, Gervois P, Lambrichts I, et al. Dental Stem Cells: Their Potential in Neurogenesis and Angiogenesis. In: Şahin F, Doğan A, Demirci S, editors. Dental Stem cells: Springer; 2016. p. 217-42.

1.1 Angiogenesis

Within the human body, blood vessel formation occurs via one of three mechanisms: vasculogenesis, arteriogenesis or angiogenesis [1]. During the early stages of embryogenesis, blood vessels are formed through differentiation of angioblasts into endothelial cells, a process called vasculogenesis [1]. Arteriogenesis can be defined as the rapid proliferation of collateral arteries from pre-existing anastomoses [2, 3]. Within the adult human body, the predominant and most studied form of blood vessel formation is **angiogenesis.** Physiological angiogenesis plays a fundamental role during embryonic development, wound healing and the female reproductive cycle [4]. In general, angiogenesis is defined as the sprouting of new capillaries from pre-existing blood vessels in response to specific stimuli such as inflammation or hypoxia [1, 5]. Angiogenesis is a complex multi-step process consisting of vasodilatation, degradation of the basement membrane and extracellular matrix (ECM), followed by the activation of endothelial cells (ECs). Once activated, ECs can start to proliferate, migrate and form tubules (Figure 1.1). Lastly, the newly formed tubules undergo stabilization and maturation [1, 4]. This well-coordinated biological process is regulated by a broad range of proteins, which maintain a natural balance between stimulatory and inhibitory signaling pathways. Under physiological conditions, inhibitory signals outweigh the stimulatory molecules and blood vessels remain in a quiescent state. Angiogenic inhibitors include: thrombospondin, endostatin and angiostatin [6]. When angiogenic activators become more abundant than inhibitors, a transient switch towards a pro-angiogenic state occurs. Angiogenesis is initiated in response to a specific stimulus, such as hypoxia or inflammation. As a result, pericytes detach and both the ECM and basement membrane are degraded, leading to destabilization of blood vessels and an increase in vascular permeability. Degradation of the ECM is accompanied by the release of sequestered angiogenic factors and chemokines, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). These factors in turn stimulate endothelial proliferation and migration [1, 7]. After vascular sprouting and lumen formation, the resulting tubules fuse with existing capillaries. During

the final maturation step, new ECM is deposited around the newly formed tubules and pericytes are recruited to envelop the new blood vessel [7].

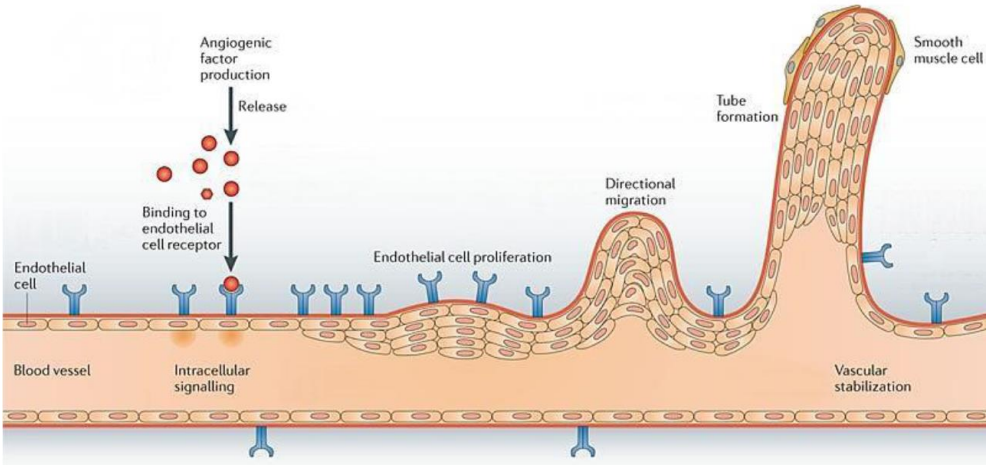


Figure 1.1 Mechanisms of action during the process of angiogenesis. Angiogenesis is a multistep process, which involves release of angiogenic stimuli, degradation of extracellular matrix, activation, proliferation and migration of endothelial cells, tube formation, and stabilization and maturation of the newly formed blood vessel. *Adapted from Zhang et al., 2012 [8].*

The equilibrium between pro-and anti-angiogenic factors strictly regulates the rate of blood vessel formation [9]. Angiogenic stimulators include: FGF-2, stromal cell derived factor-1 (SDF-1), placental growth factor (PlGF) and VEGF. VEGF has been shown to stimulate both physiological and pathological angiogenesis through VEGF receptor-2 [7, 10]. The VEGF family currently contains seven members: VEGF-A – VEGF-F and PlGF, and all members share a common VEGF homology domain [11]. So far, three VEGF tyrosine kinase receptors have been identified: VEGFR1, 2 and 3. VEGFR-2 appears to be the most important receptor for VEGF-induced mitogenesis and permeability. VEGF is also involved in EC migration and tube formation, it stimulates production of nitric oxide (NO) synthase and plasminogen activator and downregulates endothelial apoptosis [12]. Another major player in the process of angiogenesis is FGF. To date, the family is known to contain at least 20 FGF proteins and 5 receptors (FGFR1-5). FGFs play a role in development,

tissue regeneration and angiogenesis [13]. FGF-1, -2, -4 and -9 have been reported to have the highest mitogenic activity *in vitro* [14]. Furthermore, the use of recombinant FGF-1, FGF-2 and FGF-4 gene therapy has already been explored as a treatment strategy for cardiovascular pathologies [15].

1.2 Therapies to enhance angiogenesis

Angiogenesis is a tightly regulated process and imbalanced angiogenesis can result in pathological conditions such as ischemic stroke, coronary artery disease, cancer and diabetes [16, 17]. According to a report of the World Health Organization on the Global Burden of Disease (Table 1.1), treatments effectively regulating angiogenesis could eliminate up to 31% of all death globally, thereby saving 18.3 million lives each year [17]. Patients who are in the early stages of ischemia usually undergo surgical procedures in order to restore blood supply. However, treating patients who are in advanced stages of ischemia is a more challenging task, since these patients require a tissue regenerative treatment. Such regenerative treatments need to facilitate the transport of oxygen and nutrients into the damaged tissue as well as the removal of metabolites and waste materials from the tissue defects [18, 19]. Stimulating angiogenesis in a controlled manner could offer a promising strategy to treat ischemia and improve wound healing and tissue regeneration.

Table 1.1 The role of angiogenesis in global disease burden. Annual deaths worldwide are expressed in millions. *Adapted from [17]***.**

CAUSE OF DEATH	ANNUAL DEATHS WORLDWIDE	% OF ALL DEATHS	IMPROVED ANGIOGENESIS COULD
Coronary heart disease	7.20	12.2	Increase recovery of cardiac muscle
Cerebrovascular diseases	5.71	9.7	Augment functional recovery of the brain
Chronic pulmonary disease	3.02	5.1	Allow alveolar regeneration
Diabetes Mellitus	1.10	1.9	Enable wound healing
TOTAL	17.03	28.9	

1.2.1 Protein-based therapies

The process of angiogenesis is driven by the interaction between various angiogenic cytokines and growth factors and endothelial cells. Due to increasing knowledge of the role of these angiogenic signaling factors, many of them have been tested in pre-clinical and clinical settings for the purpose of revascularization [19, 20]. Using recombinant signaling molecules is associated with minimal risks of host inflammation or pathogenic infection [19]. However, different parameters should be taken under consideration: single growth factor treatment versus a cocktail of angiogenic factors or local administration versus systemic administration.

One of the cytokines that has been studied extensively for therapeutic angiogenesis is VEGF. Enhancing angiogenesis is an important treatment strategy for ischemic stroke since it is hypothesized to contribute to brain plasticity and functional recovery [21]. Overexpression of VEGF has been shown to promote angiogenesis following focal cerebral ischemia in mice [22]. Administration of VEGF has been shown to improve collateral blood supply in rabbit models of limb ischemia [23-26] and to stimulate angiogenesis in canine [27] and porcine [28, 29] models of cardiac artery disease. VEGF overexpression has been reported to improve tissue perfusion and hypoxia in mice [30] and rabbits [31] and to enhance angiogenesis in pigs [32, 33].

Moreover, the safety of VEGF treatments for myocardial [34-39] and peripheral ischemia [40] has already been demonstrated in several phase I clinical trials. A clinical phase II trial by Makinen *et al.* reported significant improvements in perfusion following VEGF gene delivery in patients suffering from peripheral vascular disease [41] whereas Kusumanto *et al.* showed that treating patients suffering from peripheral artery disease with plasmids encoding VEGF did not alter limb salvage rates [42]. However, in patients with myocardial ischemia, injection of adenoviral VEGF demonstrated significant improvements in exercise tolerance [43] and was associated with improved myocardial perfusion [44]. Nevertheless, the successes of several other phase I clinical trials, could not be reproduced in larger scale, placebo controlled phase II clinical trial [45, 46].

The lack of efficacy or reproducibility in these clinical trials could be related to the adenoviral vector used to deliver the VEGF as well as the associated inflammation, edema and excess growth of capillaries [47]. VEGF also induces angiogenesis via a nitric oxide mediated pathway, while bioavailability of NO is compromised in patients suffering from ischemic vascular disease. These patients also present with endothelial dysfunction, further limiting the VEGF response [48]. The use of adjuvant therapies focusing on improving NO bioavailability and restoring endothelial function, could substantially improve functional outcome after VEGF-based treatments.

Another pro-angiogenic cytokine that has been investigated for possible applications in therapeutic angiogenesis is FGF. Administration of FGF-2 increased revascularization in a rabbit model of hind limb ischemia [49, 50]. Furthermore, combining FGF and VEGF had a superior effect on revascularization after hind limb ischemia, compared to single growth-factor treatment [49, 51, 52]. Based on the positive results from studies using FGF to improve angiogenesis in rodent [53, 54], canine [55, 56] and porcine [54, 57-62] models of myocardial infarction, FGF-based therapies have also been evaluated in human clinical trials.

Phase I clinical trials of myocardial and peripheral ischemia have shown the safety of plasmid DNA encoding human FGF [63], adenoviral vectors expressing FGF [64] and of recombinant FGF [63, 65]. Moreover, a phase II clinical trial using plasmid FGF-1 treatment for patients with critical limb ischemia reported a 39% reduction in amputation rate [66]. The safety of intracoronary infusions with an adenovirus encoding FGF-4 was demonstrated by the phase I AGENT (Angiogenic Gene Therapy) trial [64]. The AGENT 2 study served for dose optimization while AGENT 3 was designed to investigate the efficacy of adenoviral FGF-4 treatment for therapeutic angiogenesis for myocardial ischemia. Despite an encouraging trend towards improved myocardial perfusion, no significant difference in exercise tolerance could be stated [67, 68].

The difference in pathology between cardiovascular disease and peripheral ischemia offers a first possible explanation as to why FGF showed clinical efficacy in phase II clinical trials involving peripheral artery disease (PAD) patients and not in trials with cardiac artery disease (CAD) patients [48]. Not only do pathologies differ between different clinical trials but also the delivery routes are dramatically

different, which further complicates the comparison. It should also be noted that administering FGF has been associated with hypotension, nephropathy, anemia and thrombocytopenia [69, 70] and just as is the case for VEGF, endothelial dysfunction further limits the FGF responsiveness from the patient [48].

Unfortunately, angiogenic therapies based on the delivery of cytokines or growth factors, are often associated with only limited success [48, 71]. This is probably due to the complicated nature of the angiogenic process, which requires the sequential activation of a plethora of signaling factors. Optimization of dose and even more importantly treatment duration, remains the biggest challenge for this kind of therapy [72]. Furthermore, administering high dose angiogenic factors could promote inflammation or lead to leaky and dysfunctional vessels [73]. The limited success of growth factor-based revascularization urged the need to promote angiogenesis with a more regenerative approach by means of (stem) cell-based therapies [74, 75].

1.2.2 Cell-based therapies

Cell based therapies have emerged as an alternative treatment option for ischemia related disorders [71]. Since their discovery by Friedenstein *et al.* mesenchymal stem cells (MSCs) have captured the attention of many scientists [76]. This novel cell type was characterized by their osteogenic potential, their rapid adherence to plastic, their fibroblast-like morphology and their high clonogenic expansion rates [76, 77]. Since their isolation from bone marrow, MSCs have become extensively studied cells, which can now be isolated from a wide array of tissues including: bone marrow, adipose tissue [78], umbilical cord [79] and several dental tissues [80-82]. In order to qualify as MSCs, cells need to fulfill a few minimal requirements. According to the 'International Society for Cellular Therapy' MSCs should be plastic adherent, should express specific surface antigens (CD73, CD90, CD73) and should be able to differentiate towards osteogenic, adipogenic and chondrogenic lineages [83]. Furthermore, MSCs do not express HLA class-II antigens or co-stimulatory molecules (CD40, CD80 and CD86) [84]. Next to their mesodermal differentiation capacity (bone, cartilage and fat), they have also been shown to give rise to cardiomyocytes, hepatocytes and neural cells *in vitro* [85]. MSCs are considered to establish therapeutic angiogenesis by either paracrine secretion of angiogenic growth factors or by differentiation into endothelial cells

[86-88], although the latter has only been reported under certain *in vitro* conditions.

Despite the extensive amount of research being performed with BM-MSCs, the possible therapeutic benefits of these SCs is partly offset by the invasiveness of the bone marrow isolation procedure and the associated donor site morbidity [82].

1.2.3 Dental stem cells as pro-angiogenic therapy

Recently, it has been demonstrated that different parts of the tooth contain stem cell populations. These include cells from the **periodontal ligament (PDL)** that links the tooth root with the bone, cells from the tips of developing roots (apical pads), cells from the tissue surrounding the impacted tooth (dental follicle) and from the pulp of both exfoliated and adult teeth [80, 81, 89, 90].

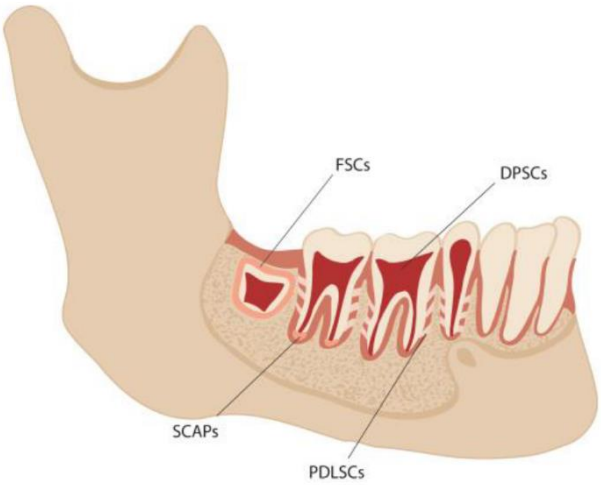


Figure 1.2 The *in vivo* location of dental stem cell populations. DPSCs = dental pulp stem cells; FSCs = dental follicle derived stem cells; PDLSCs = periodontal ligament stem cells; SCAPs = stem cells from the apical papilla. *Reprinted from Hilkens et al., 2015 [91]*.

With regard to the angiogenic properties of dental stem cells (DSCs), studies have indicated the secretion of a broad range of regulatory proteins. DPSCs, for example, have been reported to express stimulatory growth factors such as platelet-derived growth factor (PDGF), FGF-2 and VEGF, either in basal conditions or in response to noxious stimuli, e.g. injury or hypoxia [92-96]. Other angiogenesis-promoting factors that have been detected in DPSCs are: angiogenin

(ANG), angiopoietin- 1 (ANGPT1), colony-stimulating factor (CSF), dipeptidyl peptidase IV (DPPIV), endothelin-1 (EDN1), interleukin-8 (IL-8), insulin-like growth factor binding protein-3 (IGFBP3), monocyte chemoattractant protein-1 (MCP-1) and urokinase-type plasminogen activator (uPA) [82, 97]. Nevertheless, the secretome of DPSCs also comprises several inhibitory proteins, such as endostatin, pentraxin-3 (PTX-3), pigment epithelium-derived factor (PEGF), plasminogen activator inhibitor-1 (PAI-1), tissue inhibitor of matrix metalloproteinase-1/4 (TIMP-1/4) and thrombospondin-1 (THBS-1) [82, 97]. Comparable findings were also described for SCAPs and FSCs, albeit with variable expression levels between the different stem cell populations [82, 98-102]. With regard to the secretome of SHEDs and PDLSCs, literature indicates the expression of ANGPT2 [103], FGF-2 [99, 103, 104], endostatin [99], hepatocyte growth factor (HGF) [105], insulin-like growth factor-1 (IGF-1) [103] and VEGF [103-106].

Since DSCs express a wide variety of angiogenesis regulating proteins, stimulatory as well as inhibitory, it is important to determine their potential impact on the behavior of endothelial cells and angiogenesis altogether. Each well-coordinated event within the angiogenic process can be mimicked by a series of *in vitro* assays. A significant increase of both survival and proliferation of human umbilical vein endothelial cells (HUVECs) was observed after incubation with conditioned medium (CM) of a $CD31^-/CD146^-$ subpopulation of DPSCs [107]. Aranha *et al.* also reported a time-dependent increase in the proliferation of human dermal microvascular endothelial cells (HDMECs) when incubated with CM of hypoxia-preconditioned DPSCs [92]. Hilkens *et al.*, on the other hand, reported no pronounced effect of CM of DPSCs, SCAPs and FSCs on the proliferation of human microvascular endothelial cells (HMECs) [82]. To date, the potential effect of SHEDs and PDLSCs on endothelial proliferation has not been described. In order to evaluate whether endothelial cells migrate along a gradient of DSC-derived chemokines, a transwell migration assay could be performed. Using this method, DPSCs as well as SCAPs have been shown to significantly augment endothelial migration in comparison to FSCs [82, 97]. In terms of endothelial tubulogenesis, Yuan *et al.* indicated an increased formation of capillary-like structures during a direct co-culture of SCAPs and HUVECs [108]. Similar outcomes were found for DPSCs, SHEDs and PDLSCs [96, 104, 109-111]. During these direct co-cultures,

DSCs are thought to adopt a pericyte-like function as they are often found in close proximity to the endothelial cells [108, 110, 111]. Alternatively, endothelial tube formation can also be mediated by paracrine factors, as was shown by Dissanayaka *et al.* through an indirect co-culture of DPSCs and HUVECs [112]. In line with these findings, Tran-Hung *et al.* and others reported a significant increase in endothelial tubulogenesis caused by CM of DPSCs [82, 96]. With regard to the impact of PDLSCs and SHEDs on the functional behavior of endothelial cells, more research is required.

The angiogenic properties of DSCs have also been elaborately investigated *in vivo*. Yeasmin *et al.*, for example, indicated significant vascularization after subcutaneous transplantation of PDLSCs and endothelial cells. Since there was no detection of human-derived blood vessels, PDLSCs were considered to secrete paracrine mediators or to act as pericytes [104]. Mouse DPSCs were found to induce angiogenesis in a VEGF-dependent manner in a mouse matrigel plug assay [110]. DPSCs and SCAPs also caused a significant increase in angiogenesis in a chorioallantoic membrane assay [82, 97]. In terms of clinically relevant disease models of angiogenesis, Gandia *et al.* demonstrated a significant improvement of left ventricular function after injection of GFP-labeled DPSCs in a rat model of myocardial infarction. Apart from a reduction in infarct size and thickening of the anterior ventricular wall, an increase in capillary density was also detected. No differentiated DPSCs were observed within the heart tissue, suggesting that the aforementioned improvement was probably mediated through paracrine factors [113]. These findings were supported by Iohara *et al.*, who reported a high capillary density after transplantation of a $CD31^-/CD146^-$ subpopulation of DPSCs in a mouse model of hindlimb ischemia. The close location of the stem cells near the newly formed blood vessels suggests a paracrine role for DPSCs [107]. The above-mentioned subpopulation of DPSCs also promoted functional recovery in rats suffering from focal cerebral ischemia. Besides neurotrophic factors, the authors also demonstrated augmented levels of VEGF, which potentially played a role in the stimulation of vasculogenesis and neurogenesis in the ischemic rat brain [114, 115].

1.2.3.1 Preconditioning of dental stem cells to enhance their angiogenic potential

Multipotent stem cells possess many characteristics that make them suitable for clinical applications. However, a major concern in the field of regenerative medicine is the survival of these stem cells after transplantation. In order to overcome this hurdle, attempts have been made to modulate the stem cells prior to transplantation to improve cell survival and engraftment [116]. A recent paradigm shift has emerged, suggesting that that the beneficial effects of stem cell transplantation may be due to their paracrine effects rather than their differentiation potential. Therefore, a variety of different approaches has been examined, mainly focusing on increasing stem cell survival and thereby increasing the amount of trophic factors that are secreted [117].

Genetic modifications such as overexpressing anti-apoptotic genes such as Bcl-2 [118] or Akt [119, 120] offer a potential strategy to increase stem cell survival, . Another possibility is to modify the expression of a key protein of a certain illness such as dopamine for Parkinson's patients or insulin for diabetics [121]. However, genetic modification is a new and developing field and many questions remain to be resolved before clinical applications using genetically modified stem cells can be deemed possible [121].

Preconditioning stem cells by exposing them to a defined stimulus may be helpful to enhance the secretion of trophic factors (Table 1.2). In contrast to genetic modification, which usually affects a single target, preconditioning results in a more global response [117]. As mentioned above, **hypoxia** is a potent stimulus for the secretion of certain trophic factors. Not surprisingly, hypoxia preconditioning has gained a lot of attention as a method to improve the paracrine actions of a variety of stem cell sources [116]. By hypoxia preconditioning researchers aim to improve the resistance of cultured cells against hypoxic conditions by taking advantage of the known mechanism leading to survival during *in vivo* ischemia and creating *in vitro* simulations. Hypoxic preconditioning has been shown to increase stem cell survival and paracrine activity and even increased angiogenesis in an *in vivo* model of murine hind limb ischemia [122-124].

Oxygen tension in dental pulp tissues is lower compared with that in the atmosphere, since oxygen can only reach the pulpal cells via the vasculature in the narrow root. Since they naturally reside in a low oxygen environment, DPSCs are likely the best candidates to survive severe hypoxic conditions. Iida and colleagues demonstrated that hDPCs cultured under hypoxic conditions show an increased proliferation rate as well as an increased expression of STRO-1 [125, 126]. However, hypoxic preconditioning does not influence proliferation of porcine DPCs [127]. Furthermore, hypoxic preconditioning has been shown to enhance the expression of hypoxia-inducible factor-α (HIF-1α) and VEGF [92]. Hypoxia also increases the migration of DPSCs according to Kanafi *et al.* [128]. Other stem cell populations from dental origin have also been shown to react to hypoxic preconditioning. Although hypoxia does not seem to influence the proliferation rate of SCAPs, it has been reported to increase the production of VEGF as well as influence SCAPs differentiation potential [100]. Subjecting human PDL cells to hypoxia resulted in an increased secretion of VEGF and IL-6 after 24 and 48 hours of hypoxia. Moreover, after 24 or 48 hours of hypoxia, reoxygenation resulted in an even greater increase in VEGF and IL-6 production after 6 hours of normal (20%) oxygen tension [129]. Amemiya *et al.* reported increased proliferation rations of rat PDLCs and an augmented expression of VEGF mRNA [130]. SHEDs have been shown to exhibit an increased rate of cell migration under hypoxia, superior to the migration of DPSCs [128].

All these reports indicate that hypoxic preconditioning could be an effective strategy to improve stem cell survival and stimulate their pro-angiogenic and chemoattractive effects (Table 1.2). Mimicking hypoxia using **pharmacological pretreatment** could represent a more convenient alternative [116]. One particular group of chemical agents, that mimic the hypoxic response by inhibiting the activity of prolyl hydroxylase (PHD), a key enzyme of the oxygen sensing pathway, has gained a lot of interest [131]. Typical **PHD inhibitors** include: cobalt chloride ($CoCl_2$), dimethyloxalglycine (DMOG) or iron chelators such as hinokitiol, deferoxamine (DFX) or L-mimosine [132-134]. Four of these PHD inhibitors ($CoCl_2$, DMOG, DFX and L-mimosine) have been reported to increase VEGF secretion and HIF-1α expression in both dental pulp derived cells as well as cells derived from the periodontal ligament [132, 135]. Yuan *et al.* reported an upregulation of HIF-1α and VEGF secretion in $CoCl_2$ treated SCAPs. Furthermore,

co-culture of HUVEC and SCAPs under artificial hypoxic conditions ($CoCl_2$) resulted in an increased number of endothelial tubules, tubule lengths and branching points [136]. Trimmel *et al*. demonstrated that L-mimosine is able to increase the VEGF production via HIF-1α in a tooth slice organ culture model in which the dental pulp surrounded by dentine [137]. The iron chelator hinokitiol has also been demonstrated to increase HIF-1α expression and VEGF production in dental pulp cells. According to Kim *et al*. conditioned media of hinokitiol-treated pulp cells enhance angiogenesis *in vitro* and *in vivo*. Conditioned media of hinokitiol-treated pulp cells enhanced the capillary network formation of HUVEC compared to control CM, thereby demonstrating an increased angiogenic potential of hinokitiol-treated dental pulp cells. Moreover, CM of hinokitiol treated pulp cells increased neovascularization *in vivo* compared to CM of untreated pulp cells. [138]. Together these data indicate a promising future for the use of hypoxia mimicking agents and more in particular the PHD inhibitors.

Besides hypoxia mimicking agents there is a plethora of other cytokines, growth factors and chemical agents that has been investigated for their potential to augment the angiogenic profile of stem cells (Table 1.2). For example, bacterial lipopolysaccharide (LPS) has been shown to increase VEGF production in murine BM-MSCs [139], murine and human DPSCs [140, 141] and it has been reported that LPS stimulates FSC migration [142]. There are reports confirming the LPS responsiveness of PDLCs [143, 144] and SCAPs [145], however these studies do not mention the effects on VEGF secretion or the angiogenic profile of these cells. Other pretreatments such as IL-1α [106] and TNF-α [146] have been reported to increase VEGF secretion in periodontal ligament stem cells and adiponectin stimulates PDLC proliferation and wound healing [147].

Table 1.2 Dental stem cells and the effects of preconditioning.

PRIMING	(ANGIOGENIC) EFFECT	REFERENCE
Dental pulp stem cells		
Hypoxia	Increased proliferation rate Increased HIF-1α and VEGF expression/secretion Increased migration	[125, 126] [92] [128]
PHD inhibitors	Increased HIF-1α and VEGF expression/secretion	[135]
Hinokitiol	Increased HIF-1α and VEGF expression/secretion Increased hemoglobin content in mouse matrigel plug assay	[138]
FGF-2	Enhanced EC capillary network formation	[148]
Lipopolysaccharide (LPS)	Increased VEGF expression	[139, 140]
Stem cells from human exfoliated deciduous teeth		
Hypoxia	Increased migration	[128]
Periodontal ligament stem cells		
Hypoxia	Increased VEGF expression	[129]
PHD inhibitors	Increased HIF-1α and VEGF expression/secretion	[132]
IL-1α	Increased VEGF expression	[106]
TNF-α	Increased VEGF expression	[146]
Adiponectin	Increased proliferation rate Increased wound healing	[147]
Follicle stem cells		
Lipopolysaccharide (LPS)	Increased migration	[142]
Stem cells from the apical papilla		
Hypoxia	Increased VEGF expression	[100, 102]
PHD inhibitors ($CoCl_2$)	Increased HIF-1α and VEGF expression/secretion Enhanced EC capillary network formation	[136]

1.2.3.2 Clinical application of DSCs and its challenges

Despite the promising outcomes of DSC transplantation in a preclinical setting, the progression of DSCs from bench to bedside still holds some major challenges. Standardized cell isolation procedures, for example, are indispensable to safeguard the clinical safety, reproducibility and efficacy of DSC therapy [149]. However, the extraction of third molars as well as the isolation of DSCs are currently being performed using diverse isolation procedures on donors of different ages with molars at different stages of development, which not only impairs in-depth comparison of experimental outcomes but also hinders the development of a standardized treatment protocol [91, 150-152]. Next to consistent isolation procedures, the clinical implementation of DSCs also entails the upscale production of these stem cells in xeno-free culture conditions, in order to provide an adequate amount of cells without any contamination of potential infectious agents [153-155]. Nevertheless, due to the inherent batch-to-batch variety as well as the current lack in reliable study protocols regarding the use of human blood-derived products as a potential alternative, more research is required before any educated decision can be taken by both scientists and regulatory agencies [156-158]. In addition to the challenges associated with the processing and culturing of DSCs, one also needs to take into account the intrinsic behavior of the stem cells, as it can be influenced by a broad range of different donor-related factors, such as (oral) health, age and orthodontic tooth movement [91].

Although numerous studies have elaborately described the immunomodulatory effects of DSCs *in vitro*, little is known concerning the effects of allogeneic DSC transplantation *in vivo* [159-168]. Tomic *et al.*, for example, reported the formation of granulomatous tissue after xenogeneic transplantation of human DPSCs and FSCs in immunocompetent mice [169]. When transplanting rat DPSCs in mice suffering from colitis, on the other hand, a clear reduction of inflammation was observed [170]. There were also no signs of immune rejection after injection of human SHEDs in a canine model of muscular dystrophy [171]. In line with these findings, conditioned medium of SHEDs was found to alleviate autoimmune encephalomyelitis as well as to improve the cognitive function in a mouse model of Alzheimer's Disease through the induction of anti-inflammatory M2-phenotype microglia [172, 173]. Nevertheless, the outcome of allogeneic DSC transplantation

for dental tissue engineering purposes in particular remains largely unknown, as most ectopic transplantation models are performed in immunocompromised mice and most *in situ* models apply autologous DSCs [112, 174-195]. More research is thus required regarding the immunomodulatory behavior of allogeneic DSCs *in vivo* and potential graft-versus-host responses.

When making the switch from bench to bedside it is also important to include sufficient patient-centered outcomes. All too often, dental clinical trials focus on technical, clinician-centered outcomes instead of patient-centered outcomes. Developing a standardized set of core outcomes could help overcome this fixation with clinician-based outcomes and lead to more consistent study designs [196].

Despite these challenges, a few clinical studies using DSC-based therapies are currently recruiting participants (Table 1.3). In India, a clinical study is currently ongoing in which patients suffering from chronic periodontitis receive a local injection of allogeneic human DPSCs in order to improve periodontal tissue regeneration (NCT02523651). Allogeneic DPSCs are also being applied in a clinical trial in China, investigating the effect of DPSCs on osseointegration of dental implants (NCT02731586). Also in China, a second clinical trial focuses on the revitalization of young immature permanent teeth with necrotic pulps using autologous SHEDs (NCT01814436). Finally, Nagpal *et al.* announced a study protocol for evaluating safety and feasibility of autologous DPSC-based stem cell therapy in patients with chronic disability after stroke, however according to the website ClinicalTrials.gov this study is not yet recruiting participants [197].

Table 1.3: Clinical application of DSCs

CONDITION	CELL TYPE	STATUS	IDENTIFIER
Dental implants	Allogenic DPSCs	Recruiting	NCT02731586
Periodontal disease	Allogenic DPSCs	Recruiting	NCT02523651
Pulp necrosis	Autologous SHEDs	Recruiting	NCT01814436
Stroke	Autologous DPSCs	/	TBA

1.2.4 Platelet derivatives as pro-angiogenic therapy

Angiogenesis requires an interplay between numerous signaling molecules and growth factors. Using only a single growth factor to guide or enhance this intricate process is usually insufficient. In order to compensate for this oversimplified approach, the signaling molecule of choice is often administered in supraphysiological doses, which can cause adverse effects [198, 199]. Cell-based therapies are one possibility to offer a more comprehensive treatment strategy. An increasing amount of studies are focusing on other natural sources of growth factors cocktails, such as **platelet concentrates**, either as a single treatment or in combination with cell-based treatments [200]. The use of platelet concentrates is on the rise in different medical fields, not in the least due to their availability, cost-effectiveness and most importantly their autologous nature, which limits the risk of rejection [200, 201].

Despite their recent up rise, platelet concentrates were already described decades ago. Fibrin glue, considered as the precursor of platelet concentrates, was already used as a surgical additive as early as the 1970's [202]. Over the years several production techniques have been explored and every technique results in a slightly different product. Each product has different applications, growth factors secretions and a different composition with regard to leukocyte content and fibrin architecture [198]. All these differences urge the need for some clear classification and product characterization. Based on the evolution in production techniques, platelet concentrates can be divided into first and second generation concentrates [202].

1.2.4.1 First generation platelet concentrates

As mentioned earlier, the first blood derivatives used as surgical additives were the **fibrin sealants or fibrin glues.** Fibrin is the activated form of a plasma molecule called fibrinogen, which is not only present in plasma but also in platelet granules [202]. The use of autologous fibrin sealants would avoid the issue of possible cross-contamination, however, autologous fibrin sealants are generally less stable compared to commercial sealants. Furthermore, fibrin polymerization is always initiated using bovine thrombin [202]. These disadvantages contributed to the development of **platelet rich plasma (PRP)**, an autologous blood derivative which contains a fibrin matrix as well as platelets. By combining the

properties of fibrin sealants with the growth factors released from platelets, PRP offers an suitable growth factor delivery system at the site of injury [202]. Based on their leukocyte content, the PRP family can be subdivided into 'Pure-PRP (P-PRP)' with little to no leukocytes present and 'PRP with leukocytes (L-PRP)'. Both PRP subtypes can be used as a liquid solution or an activated gel form and they both have a low density fibrin network (Figure 1.3A) [203]. PRP has been widely used in maxillofacial surgery, for both soft-tissue and hard-tissue reconstruction. Despite the wide range of clinical applications, there are some drawbacks to working with PRP. First of all, the productions consists of a two-step process, which requires the use anticoagulants and the use of calcium chloride and bovine thrombin in order to initiate polymerization. Moreover, the abundance of preparation protocols, results in different end products, making it difficult to compare study outcomes. The centrifugation protocol has a critical impact on the quality and composition of the platelet derivatives, further urging the need for a consensus on preparation protocols and terminology [198, 202, 203].

1.2.4.2 Second generation platelet concentrates

Platelet rich fibrin (PRF) is an alternative to PRP and is considered as a second generation platelet concentrate. This category can also be divided into two subtypes: pure 'platelet rich fibrin (P-PRF)' without leukocytes and **'leukocyte and platelet rich fibrin (L-PRF)'** with leukocytes (Figure 1.3A) [204]. The production of P-PRF still requires biochemical handling of the blood sample, while L-PRF is a completely physiological material due to the lack of biochemical interference during the production protocol [201, 202, 205]. L-PRF is solid biomaterial with a strong fibrin architecture which can be produced with a single step centrifugation protocol. Thereby L-PRF offers a simplified and more cost effective alternative to PRP [205].

L-PRF is produced by collecting whole blood in 9 mL glass-coated tubes and low speed centrifugation (400g) for 12 minutes [206]. Due to the absence of anticoagulants, platelet activation and fibrin polymerization occurs due to the natural coagulation process. After centrifugation, the tube contains three different layers: red blood cells at the bottom, an acellular plasma supernatant and the L-PRF clot in the middle of the tube (Figure 1.3B)[207]. L-PRF contains over 50% of leukocytes and the majority of the platelets in the blood sample all trapped in

a strong fibrin matrix [200, 206]. L-PRF therefore only exists in its activated form and does not have an inactive, injectable suspension phase. Due to the lack of biochemical handling, the centrifugation protocol needs to start immediately after the collection of the blood sample in order to avoid diffuse polymerization [201].

L-PRF has several clinical applications, with the majority pertaining the field of maxillofacial surgery [208], but it can also be used in ear-nose-throat, orthopedic or esthetic surgery or in the treatment of diabetic ulcers [209]. Despite this wide variety of possible applications, only little is known about the intrinsic biology of L-PRF and more in-depth research could shed a light on the effects of L-PRF on the healing process and the source of interpatient variability for example.

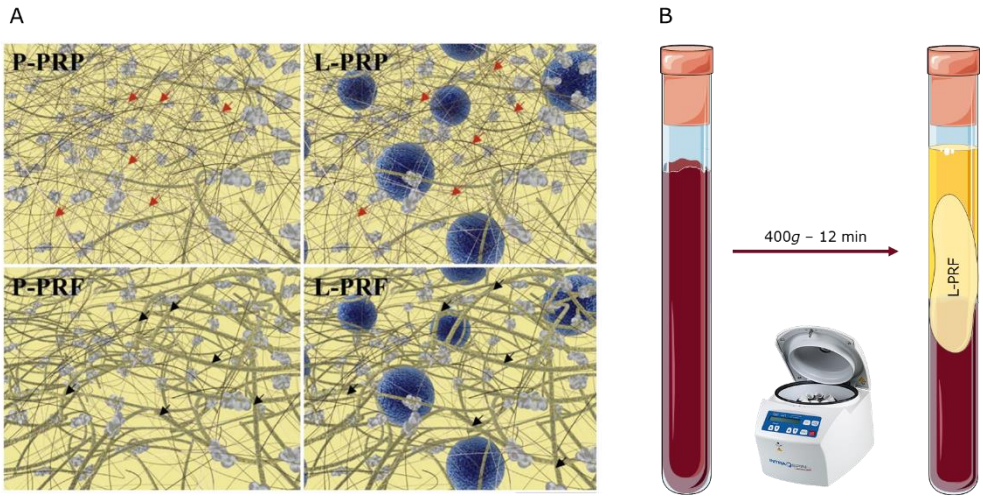


Figure 1.3 Schematic illustration of the matrix and cell architecture of the four categories of platelet concentrates and the production protocol for L-PRF. (A) Platelet concentrates can be divided into 4 distinct families, based on two key parameters: leukocyte content (blue circles) and the density of the fibrin network. PRP preparations (top panels) contain an immature fibrin network, with thin fibrin fibers (red arrows), due to simple fiber polymerization. PRF preparations however, have a resistant fibrin matrix, consisting of thick fibers (black arrows). In every subtype, platelets are assembled on the fibrin fibers. (B) Single step production protocol for L-PRF. After collection, whole blood is immediately centrifuged (400*g*) for 12 min, resulting in three different compartments within the tube: red blood cells at the base, the L-PRF clot in the middle of the tube and an acellular plasma portion on top. P-PRP = pure platelet rich plasma; L-PRP = platelet rich plasma with leukocytes; P-PRF = pure platelet rich fibrin, L-PRF = leukocyte and platelet rich fibrin. *Panel A was based on copyright from Dohan Ehrenfest et al. 2014 [203].*

1.2.4.3 The angiogenic properties of leukocyte and platelet rich fibrin

L-PRF is made up out of 3 major components: leukocytes, platelets and fibrin, all of which can influence the angiogenic process. **Leukocytes** can secrete both pro-angiogenic and anti-angiogenic signaling molecules [210-212]. Whether or not leukocytes stimulate angiogenesis, solely depends on the microenvironment [213]. Several types of leukocytes can be found in the perivascular niche, including mast cells and macrophages [214]. Mast cells can release histamine and VEGF in response to inflammatory stimuli, in order to increase vascular permeability, whereas certain macrophages can modulate vascular branching during embryogenesis [215] or are associated with wound healing and angiogenesis [216-219]. Macrophages can stimulate angiogenesis by the secretion of PDGF, FGF-2 and TNF-α [220, 221], while monocytes can even differentiate into endothelial-like cells given the right circumstances [222]. Besides the leukocytes present in the perivascular niche, leukocytes can also be recruited to site of hypoxia or injury [214]. Macrophages, eosinophils and neutrophils can promote angiogenesis by secreting factors such as VEGF and matrix metalloproteases (MMPs) [218, 223]. Neutrophils are known to produce MMP-9, which promotes angiogenesis by degrading the extracellular matrix as well as by releasing matrix-bound VEGF [210, 214]. Besides producing pro-angiogenic factors including IL-8 and hepatocyte growth factor (HGF) [211, 214], neutrophils may also stimulate angiogenesis by direct cell-to-cell interactions with endothelial cells [212].

Platelets are an important source of biomolecules which can stimulate angiogenesis or induce proliferation and activation of other cells involved in wound healing [224, 225]. Using platelet concentrates offers a promising approach to locally administer platelet-derived factors from an autologous source in order to stimulate tissue healing [224]. Platelets are anucleated cell fragments which contain three types of reservoir organelles: lysosomes, dense granules and alpha granules, with the latter being considered as the key platelet organelles [224, 226]. These alpha granules contain platelet-specific proteins, cytokines, growth factors, proteoglycans and angiogenic factors. Upon degranulation, platelets release VEGF, FGF-2 and PDGF, which are all important regulators of angiogenesis [224, 227]. Platelets have been reported to stimulate endothelial cell proliferation

and tube formation [228], however little is known about their role in blood vessel formation and wound healing *in vivo* [224].

The third, and sometimes underestimated, component of L-PRF is **the fibrin matrix.** The fibrin matrix is the result of transforming the soluble plasma fibrinogen into fibrin and the architecture of this fibrin network is an important characteristic of a platelet concentrate [229]. This matrix is not only able to capture the factors released by the platelets and leukocytes resulting in a slow progressive release over time, but it also provides a suitable scaffold for inflammatory cells or endothelial cells during wound healing [204, 230, 231].

Several preclinical studies have already reported a positive influence of platelet concentrates on angiogenesis. PRP has been described to enhance endothelial proliferation [232-234] migration, and tube formation [235]. Furthermore, PRP gels have been shown to enhance wound healing in preclinical animal models [236, 237]. Roy *et al.* reported that a platelet rich fibrin matrix was able to enhance porcine wound healing by stimulating angiogenesis [238]. Despite all these promising reports, all platelet derivatives used in the aforementioned studies were obtained by a protocol involving biochemical handling of the blood sample, urging the need for more research into the capacities of the completely biological L-PRF.

1.3 Aims of the study

As mentioned earlier, impaired angiogenesis is involved in pathological conditions such as ischemic stroke, coronary artery disease and diabetes. Stimulating angiogenesis in a controlled fashion could offer a promising approach to alleviate ischemia and improve wound healing and tissue regeneration. This dissertation aimed to enhance angiogenesis using biological approaches.

After only limited success of protein-based therapies, attention shifted towards cell-based strategies, where MSCs are used as a biological delivery system of a wide range of growth factors in order to stimulate angiogenesis. The human tooth and its surrounding tissues have been described to contain a number of different stem cell populations. Due to their ease of isolation and lack of ethical concerns, DSCs have become an attractive source for cell-based therapies to enhance angiogenesis. Whereas the angiogenic potential of DPSCs, FSCs, and SCAPs has

already been described, little is known about the angiogenic characteristics of PDLSCs [82]. Furthermore, pharmacological preconditioning of stem cells has been proposed as a promising method to augment their therapeutic efficacy. **The first aim of this thesis is to investigate the angiogenic properties of PDLSCs and enhance these properties by pharmacological pretreatment**.

Besides the use of cell-based therapies, there is another biological product that can serve as a delivery mechanism of a wide variety of growth factors, namely platelet concentrates. Due to their simple production protocol, cost effectiveness and the possibility for autologous use, platelet concentrates have captured the attention of a great number of scientists and clinicians. As a second generation platelet concentrate, L-PRF has the advantage of not requiring any biochemical handling. However, up until now, the majority of the research on platelet concentrates has focused on first generation products. **The second aim of this dissertation is to evaluate the growth factor release from L-PRF and to determine its effect on endothelial cells and angiogenesis *in ovo*.**

The field of tissue engineering aims to predictably regenerate damaged tissues and all regenerative strategies should focus on developing a vascular network in order to obtain successful clinical outcomes [239]. Wound healing is considered the natural regenerative response to tissue injury. By combining, macrophages, neutrophils, platelets; all of their released growth factors and a fibrin matrix L-PRF offers all the key players in wound healing packed into one biological tissue construct. **The third aim of this thesis is to determine the regenerative capacities of L-PRF in a murine cutaneous wound healing model.**

Chapter 2

Angiogenic Capacity of Pretreated Periodontal Ligament Stem Cells

Based on:

Ratajczak J, Hilkens P, Gervois P, Wolfs E, Jacobs R, Lambrichts I, Bronckaers A. Angiogenic Capacity of Periodontal Ligament Stem Cells Pretreated with Deferoxamine and/or Fibroblast Growth Factor-2. PLoS One. 2016;11(12).

2.1 Abstract

Periodontal ligament stem cells (PDLSCs) represent a good source of multipotent cells for cell-based therapies in regenerative medicine. The success rate of these treatments is severely dependent on the establishment of adequate vasculature in order to provide oxygen and nutrients to the transplanted cells. Pharmacological preconditioning of stem cells has been proposed as a promising method to augment their therapeutic efficacy. In this study, the aim was to improve the intrinsic angiogenic properties of PDLSCs by *in vitro* pretreatment with deferoxamine (DFX; 100µM), fibroblast growth factor-2 (FGF-2; 10 ng/mL) or both substances combined. An antibody array revealed the differential expression of several proteins, including vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). ELISA data confirmed a 1.5 to 1.8-fold increase in VEGF for all tested conditions. Moreover, 48 hours after the removal of DFX, VEGF levels remained elevated (1.8-fold) compared to control conditions. FGF-2 and combination treatment resulted in a 5.4 to 13.1-fold increase in PlGF secretion, whereas DFX treatment had no effect. Furthermore, both PDLSCs and pretreated PDLSCs induced endothelial migration. Despite the significant elevated VEGF levels of pretreated PDLSCs, the induced endothelial migration was not higher by pretreated PDLSCs. We found that the observed induced endothelial cell motility was not dependent on VEGF, since blocking the VEGFR1-3 with Axitinib (0.5 nM) did not inhibit endothelial motility towards PDLSCs. Taken together, this study provides evidence that preconditioning with DFX and/or FGF-2 significantly improves the angiogenic secretome of PDLSCs, in particular VEGF and PlGF secretion. However, our data suggest that VEGF is not the only player when it comes to influencing endothelial behavior by the PDLSCs.

2.2 Introduction

A decade ago, a mesenchymal stem cell (MSC)-like cell population was discovered in the periodontal ligament of human teeth [81]. These periodontal ligament stem cells (PDLSCs) have been identified as a good source of multipotent cells for cell-based therapies in regenerative medicine. However, a major concern is the survival of these stem cells after transplantation. Injured tissue is usually poorly perfused resulting in a lack of oxygen and nutrients for both grafted and resident cells [116]. It has only recently been demonstrated that PDLSCs possess the ability to stimulate angiogenesis [104]. Furthermore, Yeasmin *et al.* showed that PDLSCs secrete soluble pro-angiogenic factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) and induce blood vessel formation after co-transplantation with endothelial cells (EC)[104].

In light of the recent insight that the paracrine actions of MSCs are responsible for their tremendous therapeutic potential, researchers have been investigating different approaches to modulate and enhance the MSC secretome [117]. Hypoxia is a potent stimulus for the secretion of numerous trophic factors. Not surprisingly, hypoxic preconditioning has gained a lot of attention as a method to improve the paracrine actions of a variety of stem cell sources [116]. Low oxygen levels increase stem cell survival and the secretion of VEGF and FGF-2 and was also shown to increase angiogenesis in an *in vivo* model of murine hind limb ischemia [122-124, 240]. Despite the proven success of hypoxic preconditioning, mimicking hypoxia using pharmacological pretreatment could represent a more convenient alternative [116]. Deferoxamine (DFX) is such a chemical agent which simulates hypoxia by inhibiting the activity of prolyl hydroxylase (PHD), a key enzyme of the oxygen sensing pathway [131]. This drug is an FDA-approved iron chelator which is applied in the treatment of iron overload diseases and has been reported to increase VEGF secretion of both dental pulp-derived cells and cells derived from the periodontal ligament [132, 135]. Besides hypoxia-mimicking agents, a plethora of cytokines, growth factors and chemical agents have been investigated for their potential to augment the angiogenic profile of stem cells. Pretreatments such as IL-1α [106] and TNF-α [146] increase VEGF secretion in PDLSCs, while adiponectin stimulates PDLSC proliferation and wound healing [147]. In this chapter we aim to improve the angiogenic capacities of PDLSCs by *in vitro* preconditioning with DFX, FGF-2 or a combination of both substances.

2.3 Materials and Methods

2.3.1 Cell Culture

Periodontal ligaments were obtained from patients (16–27 years of age) undergoing extraction of third molars for orthodontic or therapeutic reasons at Ziekenhuis Oost Limburg, Genk, Belgium. All participants provided written informed consent, in the case of patients under the age of 18, informed consent was obtained via their guardians. This study protocol and consent procedure was approved by the medical ethical committee from Hasselt University. Periodontal ligaments were removed from extracted molars with forceps and rinsed with Minimal Essential Medium, alpha modification (α-MEM, Sigma-Aldrich, St. Louis) supplemented with 100 U/mL Penicillin (Sigma-Aldrich) and 100 µg/mL Streptomycin (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich) containing 10% fetal bovine serum (FBS; BiochromAG, Berlin, Germany), after which periodontal ligament stem cells (PDLSCs) were isolated according to the explant method as described in detail previously (15). Briefly, periodontal ligaments were mechanically minced into fragments of 1–2 mm3, which were cultured in 6-well plates in standard culture medium, to allow cellular outgrowth. When the wells reached confluence, cells were passaged using 0.05% trypsin-EDTA.

A human microvascular endothelial cell line (HMEC-1) was obtained from the Center of Disease Control and Prevention (Atlanta, GA, USA). The cells were cultured in MCDB-131 medium (Invitrogen, Carlsbad, CA) supplemented with 100 U/mL Penicillin and 100 µg/mL Streptomycin, 10 mM L-glutamine, 10% FBS, 10 ng/mL human epidermal growth factor (hEGF, Gibco, Paisley, UK) and 1 µg/mL hydrocortisone (Sigma).

2.3.2 PDLSC differentiation

Following trypsinization, PDLSCs were seeded onto 12-mm ø glass coverslips for light microscopy at a density of 21 000 cells/cm² as recommended by manufacturer (R&D systems, Minneapolis, USA). Cells were kept in normal culture medium and cultured at 37°C in a humidified atmosphere containing 5% CO2. When 90-100% confluence was reached, medium was changed to adipogenic induction medium (StemXVivoTM; R&D Systems, UK; CCM011) containing hydrocortisone, isobutylmethylxanthine and indomethacin. Medium was changed every 3-4 days and after 3 weeks, cells were washed with phosphate buffered

saline (PBS) and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Adipogenic differentiation was evaluated based on the presence of lipid droplets, which were identified using 0.3% Oil Red O staining.

For osteogenic differentiation, PDLSCs were seeded onto 12-mm ø glass coverslips at a density of 4.200 cells/cm². Cells were kept in normal culture medium at 37°C in a humidified atmosphere containing 5% CO2. When 50-70% of confluence was reached, medium was changed to osteogenic differentiation medium (StemXVivoTM; R&D Systems, UK; CM008) containing dexamethasone, ascorbatphosphate and β-glycerolphosphate. Medium was changed twice a week. After 3 weeks, cells seeded on glass coverslips were washed with PBS and fixed with 4% PFA for 20 min at room temperature. Osteogenic differentiation was evaluated by the production of extracellular matrix and calcium deposits, which were visualized using 40 mM Alizarin Red S staining.

Differentiation of PDLSCs to chondrogenic cells was initiated according to manufacturer's guidelines (R&D systems). A pellet containing 250 000 cells in a 15-mL conical tube was subjected to chondrogenic differentiation medium consisting of D-MEM/F12 containing 1% insulin – transferrin – selenious acid (ITS) supplement and 1% chondrogenic supplement (R&D systems). This supplement contained dexamethasone, ascorbate-phosphate, proline, pyruvate and transforming growth factor-β3. Every 3–4 days, differentiation medium was replaced. After 21 days of differentiation, pellets were fixed with 4% PFA for immunohistochemistry (IHC). For IHC analysis, chondrogenic pellets were embedded in paraffin and sections of 7 µm in thickness were cut. These sections were stained for aggrecan, in order to assess the presence of cartilaginous extracellular matrix.

2.3.3 Preconditioning of PDLSCs

With regard to pharmacological pretreatment, PDLSCs were treated with DFX (Calbiochem, Milipore Corp., Billerica, MA, USA), FGF-2 (Immunotools, Friesoythe, Germany) or both substances combined (Figure 2.3). DFX was solubilized in MiliQ at a final concentration of 100 mM and sterile filtered (0.2 µm). PDLSCs (passages 3-8) were seeded in a 6-well plate at a density of 10 405 cells/cm² in standard PDLSC culture medium. The next day cells were rinsed with PBS and standard culture medium was replaced by 1.4 mL standard medium containing only 0.1%

FBS supplemented with 1 µM – 1 mM DFX or with 0.1 – 100 ng/mL FGF-2. Conditioned medium (CM) containing the PDLSC secretome was harvested after 8, 24, 48 and 72 hours. To evaluate the production of vascular endothelial growth factor (VEGF) and placental growth factor (PlGF) after pharmacological removal, a wash-out (WO) experiment was performed. Therefore, cells were rinsed with PBS and priming medium was replaced with standard PDLSC culture medium containing 0.1% FBS. To evaluate how the VEGF production was affected in time after removal of the priming agent, wash-out CM was harvested at 8, 24 and 48 hours. All CM was stored at -80°C until further use.

2.3.4 Flow Cytometric Analysis

The expression of stem cell surface markers was evaluated with flow cytometry before and after priming. PDLSCs were tested for the lack of hematopoietic cell surface markers: CD34 and CD45 and the expression of MSC surface markers: CD44, CD73, CD90, CD105. Table 2.1 summarizes the used detection antibodies and matched isotype controls. Flow cytometric analysis was performed under standard culture conditions (10% FBS), under low serum conditions (0.1% FBS) and after pretreatment.

After priming, PDLSCs were harvested by trypsinization and resuspended in PBS containing 2% FBS and cells were incubated for 30 minutes at room temperature to allow re-expression of cellular markers. Next, cells were incubated with fluorescently labeled antibodies for 45 minutes. Matched isotype controls were included as a negative control for nonspecific background staining. After incubation, PDLSCs were washed three times followed by fluorescent activated flow cytometry analysis on a FACS Calibur flow cytometer equipped with CellQuest Pro software (BD Biosciences, Franklin Lakes, N.J., USA). For each condition 10,000 cells were counted and included in the analysis.

Table 2.1 Conjugated antibodies and matched isotype controls for flow cytometry

ANTIBODY	ISOTYPE	DILUTION	SUPPLIER
CD 34 PE	IgG1 PE	1:100	Immunotools
CD 45 PE	IgG1 PE	1:100	eBioscience
CD 44 PE	IgG2b PE	1:100	Immunotools
CD 73 FITC	IgG1 FITC	1:100	Biolegend
CD 90 FITC	IgG1 FITC	1:100	eBioscience
CD 105 PE	IgG1 PE	1:100	eBioscience

All isotype controls were purchased from eBioscience, Vienna, Austria. Biolegend, San Diego, CA, USA.

2.3.5 Antibody array

In order to analyze the angiogenesis-related proteins secreted by treated and untreated PDLSCs, the human angiogenesis array kit (R&D systems) was used as a general screening tool. This array was performed according to the manufacturer's instructions. Conditioned medium of treated and untreated PDLSCs was incubated with a detection antibody cocktail for one hour, prior to adding this mixture to the array membranes. Membranes were incubated overnight and after rinsing, streptavidin-HRP was added and incubated for 30 minutes, at room temperature. Immunoreaction was visualized with chemiluminescent reagents (ECL plus, GE healthcare, Little Chalfont, UK). Finally the membranes were exposed to X-ray films for different time points and the results were analyzed by ImageJ using a Dot Blot analyzer plug-in.

2.3.6 Enzyme-linked immunosorbent assay

To evaluate the concentration range of the produced angiogenic factors VEGF and PlGF, an enzyme-linked immunosorbent assay (ELISA) was performed on CM of PDLSCs after DFX, FGF-2 and combination treatment. ELISA's for VEGF (Raybiotech, USA) and PlGF (Boster, USA) were performed according to manufacturer's instructions and at least five different patient samples were tested.

2.3.7 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

In order to evaluate the effect of PDLSCs on the proliferation of endothelial cells a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–assay

was performed. HMEC-1 (passages 3-9) were seeded at 31 250 cells/cm² in a flat bottom 96-well plate. After overnight incubation, HMEC-1 were washed with PBS and incubated for 48 hours with either CM of PDLSCs, standard PDLSC culture medium containing 0.1% FBS (negative control) or standard PDLSC culture medium containing 10% FBS (positive control). An additional negative control for FGF-2 and combination treatment was added, containing the priming agents, in order to correct for the presence of FGF-2 and DFX in the CM. All conditions were performed in triplicate. After 48 hours of culturing, media were replaced with 500 µg/mL MTT in standard PDLSC culture medium containing 0.1% FBS. Four hours later, the MTT solution was removed and replaced with a DMSO (Sigma) and 0.01M Glycine (Sigma) mixture to dissolve formazan crystals. Absorbance was measured with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 570 nm. This assay was performed on a total of eight patient samples.

2.3.8 Transwell migration assay

HMEC-1 migration towards PDLSC CM of treated and untreated PDLSCs was measured with a transwell migration assay, using a 24-well plate with inserts containing a filter with an 8 µm pore size (Thincert™, Greiner Bio-One). For migration towards treated and untreated cells, PDLSCs were seeded in a 24-well plate at a density of 25 000 PDLSCs/cm² and left for culturing overnight. The next day, PDLSCs were rinsed with PBS and pretreatment was started. For the migration of HMEC-1 towards CM, CM of treated and untreated PDLSCs was added to the wells before adding the culture inserts. Inserts were seeded with 100 000 HMEC-1 in standard PDLSC culture medium with 0.1% FBS and placed over the wells. Standard PDLSC culture medium with either 0.1% FBS or 10% FBS were added to the wells underneath as negative and positive controls respectively. Furthermore, media containing 10 ng/mL FGF-2 or 10 ng/mL FGF-2 and 100 µM DFX were used as controls for FGF-2 and combination treatment. In order to eliminate the effect of VEGF on endothelial migration, VEGFR1-3 on HMEC-1 were blocked by adding 0.5 nM Axitinib (Pfizer, New York, USA) to the culture inserts. After 24 hours of transmigration, HMEC-1 cells were fixed with 4% paraformaldehyde (PFA) for 20 minutes at room temperature and stained for analysis with 0.1% crystal violet in 10% ethanol for 10 minutes at room temperature. Two 10x representative pictures were taken per insert with an

inverted phase-contrast microscope (Nikon, Eclipse TS100) equipped with a ProgRes® C3 digital microscope camera (Jenoptik AG, Jena, Germany). The degree of migration was expressed as mean area covered with cells (in percentage) and quantified with AxioVision software 4.6.3 (Carl Zeiss Vision, Aalen, Germany). Migration assays were independently performed on eight different patient samples.

2.3.9 Western Blot

Evaluation of HIF-1α expression was investigated with Western blotting. For protein expression analysis, 500 000 PDLSCs were seeded in a culture flask and after overnight incubation, cells were treated with either 100 µM DFX, or a combination of 10 ng/mL FGF-2 and 100 µM DFX. After pretreatment, PDLSCs were lysed with cell lytic buffer (Sigma-Aldrich) supplemented with 1:100 protease inhibitor (Sigma-Aldrich). Protein concentrations were determined using PierceTM BCA Protein Assay Kit according to manufacturer's guidelines (Thermo Fisher Scientific, Erembodegem, Belgium). Lysate was stored at -80 °C until blotting. Total protein (10 µg) and a Bio-rad dual color ladder control (Bio-rad, Hercules, CA, USA) were separated by 12% sodium dodecyl sulfate-polyacrylamide gelelectrophoresis and blotted onto Polyvinylidene difluoride membranes. The membranes were blocked with 5% milk in tris-buffered saline with 0.1% tween-20 (TBS-T) and incubated overnight at 4°C with primary HIF-1α antibody (Table 2.2). After incubation, blots were rinsed in TBS-T and incubated with secondary horseradish peroxidase-conjugated antibody (goat anti-mouse) for one hour at room temperature. As a loading control, blots were stained for β-actin. Therefore, blots were stripped with stripping buffer consisting of 15 g/L glycine, 1 g/L SDS, 10 mL/L tween-20 (pH 2.2) and stained with primary β-actin antibody and the aforementioned secondary antibody. Separated proteins were visualized with Pierce™ ECL Plus Western Blotting Substrate chemiluminescent kit (Thermo Fisher Scientific) and pictures were acquired and analyzed with an Image quant LAS 4000 mini camera (General Electric). Band intensities were quantified using ImageQuant TL software (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK).

Table 2.2 Antibodies used for Western Blot.

ANTIBODY	SPECIES	DILUTION	DILUTION 2nd AB	SUPPLIER
HIF-1α	Mouse	1:1000	1:1000	Novus
β-ACTIN	Mouse	1:1000	1:5000	Santa Cruz

Secondary antibody was purchased from Dako, Glostryp, Denmark. Novus Biologicals, Littleton, CO, USA. Santa Cruz Biotechnology, Dallas, TX, USA.

2.3.10 Chorioallantoic membrane assay

Angiogenic properties of treated and untreated PDLSCs were tested *in vivo* with a chorioallantoic membrane (CAM) assay. Fertilized white leghorn chicken eggs (Gallus gallus) were incubated in a humidified atmosphere at 37° C for three days. At 3 days of embryonic age (E3), albumin was removed to detach the developing CAM from the eggshell. At E9, eggs with exposed CAM were incubated with plastic discs containing 500 ng recombinant human VEGF 165 (R&D systems) or 50 000 pretreated or untreated PDLSCs dissolved in growth factor-reduced Matrigel™ droplets (BD Biosciences). Plastic discs containing pure Matrigel™ droplets were used as a negative control. Eggs were incubated for three consecutive days and at E12 the CAM was dissected to analyze angiogenesis. Images were taken with a Sony HDR-XR350VE handycam camera (Sony corporation, Tokyo, Japan) and quantified by drawing a circle (radius 4 mm) over the plastic discs on the picture and counting intersecting blood vessels. Blood vessels were counted by two different researchers in a blinded fashion.

2.3.11 Statistical Analysis

Statistical analysis was performed using Graphpad Prism software 5.03 (Graphpad Software, La Jolla, CA). Data normality was tested with D'Agostino & Pearson normality test. When Gaussian distribution was reached, experimental groups were compared using a one-way analysis of variance (ANOVA) with a Bonferroni post-test for groups ≤ 5. Non-parametric data were evaluated with a Kruskall-Wallis test combined with Dunn's post-test. ELISA data were statistically analyzed by means of a two-way ANOVA with a Bonferroni post-test for multiple comparisons. Statistical significance was reached at p-values ≤ 0.05. All data were expressed as mean ± standard error of the mean (SEM).

2.4 Results

The resulting PDLSC population displayed mesenchymal-like cell morphology comprising spindle-shaped and polygonal cell types (Figure 2.1A). The immunophenotype was evaluated by means of flow cytometry, which showed a high expression level of the stem cell markers: CD44, CD73, CD90 and CD105 (Figure 2.1E). In order to assess the multilineage differentiation potential, PDLSCs were differentiated towards adipogenic, chondrogenic and osteogenic lineages (Figure 2.1B-D). Successful adipogenic, chondrogenic and osteogenic induction was confirmed by histochemical staining of respectively lipid droplets with Oil red O, aggrecan a major structural protein of cartilage and staining of calcified matrix by means of Alizarin Red S staining. Together these data demonstrate that the PDLSC populations used in this study comply with the minimal requirements for mesenchymal stem cells [83].

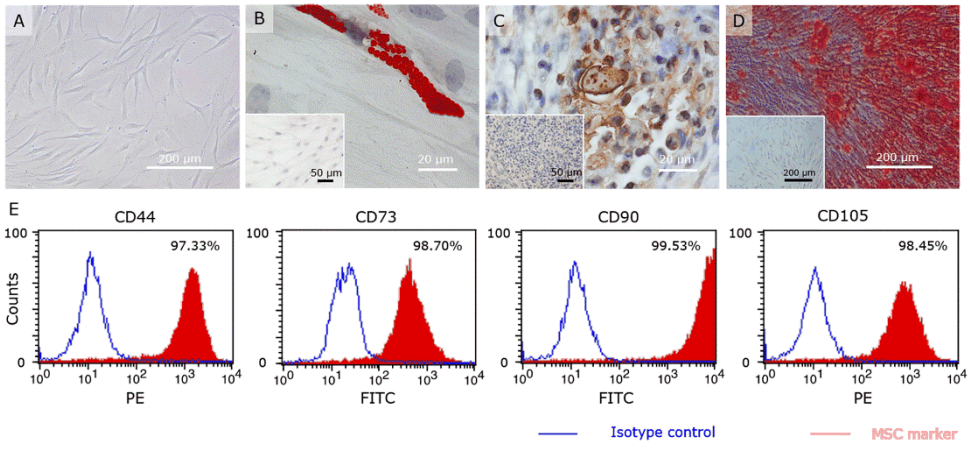


Figure 2.1 Characterization of human periodontal ligament stem cells. PDLSCs display mesenchymal-like cell morphology comprising spindle-shaped and polygonal cell types (A). Successful adipogenic, chondrogenic and osteogenic induction (n=3) is confirmed by histochemical staining of respectively lipid droplets with Oil red O (B), aggrecan (C) and the presence of calcified matrix by means of Alizarin Red S staining (D). Furthermore, flow cytometric-analysis showed the expression of MSC-markers CD44, CD73, CD90 and CD105 (E)

2.4.1 The effect of different concentrations and exposure times of DFX and FGF-2 on VEGF secretion by PDLSCs

In order to determine the optimal PDLSC pretreatment protocols, the effects of DFX and FGF-2 on VEGF secretion and cell viability were evaluated. PDLSCs were exposed to 1 µM – 1 mM DFX or to 0.1 – 100 ng/mL FGF-2 for 8, 24, 48 and 72 hours (Figure 2.2). Exposing PDLSCs to 1 µM and 10 µM of DFX did not affect VEGF secretion or cell viability. However, 100 µM of DFX, resulted in a significant increase in VEGF secretion after 48 hours. Unfortunately, this time point also corresponded with a significant decrease in cell numbers (Figure 2.2B). Pretreatment with 1 mM of DFX resulted in an increased VEGF secretion after 24 hours (Figure 2.2A), but this concentration and timepoint was also detrimental for cell viability, as demonstrated by the declining cell numbers (Figure 2.2B).

Pretreating PDLSCs with 0.1 ng/mL or 1 ng/mL FGF-2 did not affect VEGF secretion or cell viability. Exposure to 10 ng/mL FGF-2 lead to an increase in VEGF secretion, with only a minor increase in cell proliferation (Figure 2.2C-D). While adding 100 ng/mL FGF-2 to the culture medium resulted in a significant increase in VEGF after 24, 48 and 72 hours, it also caused and increase in proliferation and cells obtained a more elongated morphology (data not shown).

Based on the parameters described above, pretreating PDLSCs with 100 µM of DFX for 24 hours or with 10 ng/mL FGF-2 for 72 hours were considered optimal priming conditions and were used for further experiments (Figure 2.3).

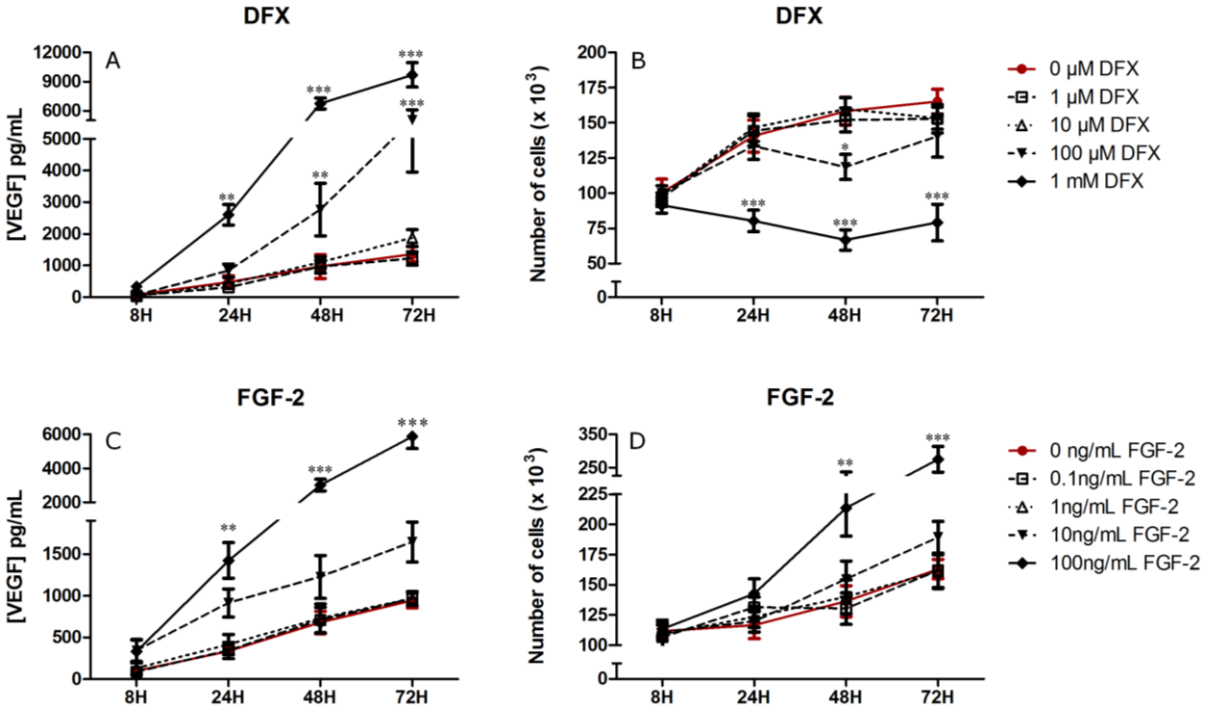


Figure 2.2 The effect of different concentrations and treatment times of DFX and FGF-2 on the VEGF production and viability of PDLSCs. (A) Concentration and time dependent responses of PDLSC VEGF secretion after DFX treatment as determined by ELISA (n=5). (B) Cell viability of PDLSCs treated with different concentrations of DFX for 8 – 72 hours (n=5). Cell proliferation was monitored during treatment by means of Trypan Blue Exclusion assay (C) Concentration and time dependent responses of PDLSC VEGF secretion after FGF-2 treatment (n=5). (D) Cell viability of PDLSCs treated with different concentrations of FGF-2 for 8 – 72 hours(n=5). Data are expressed as mean ± SEM and analyzed with Two-Way-ANOVA, ** = $p \leq 0.01$; *** = $p \leq 0.001$.

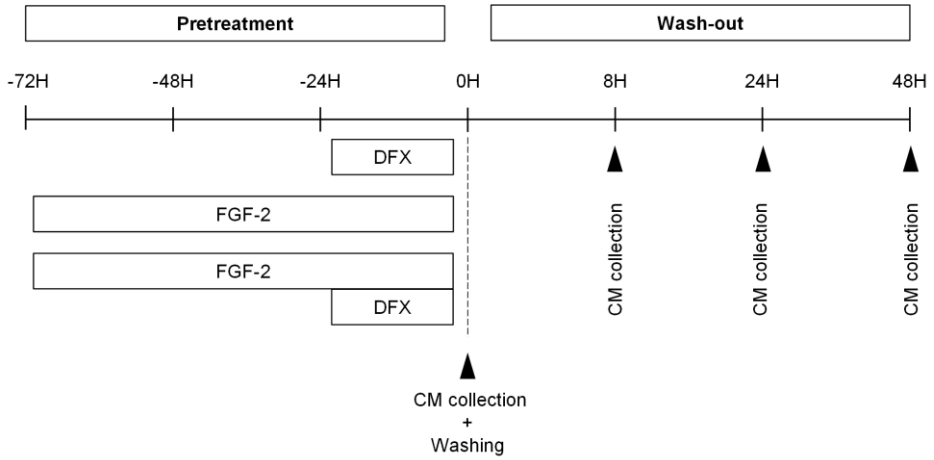
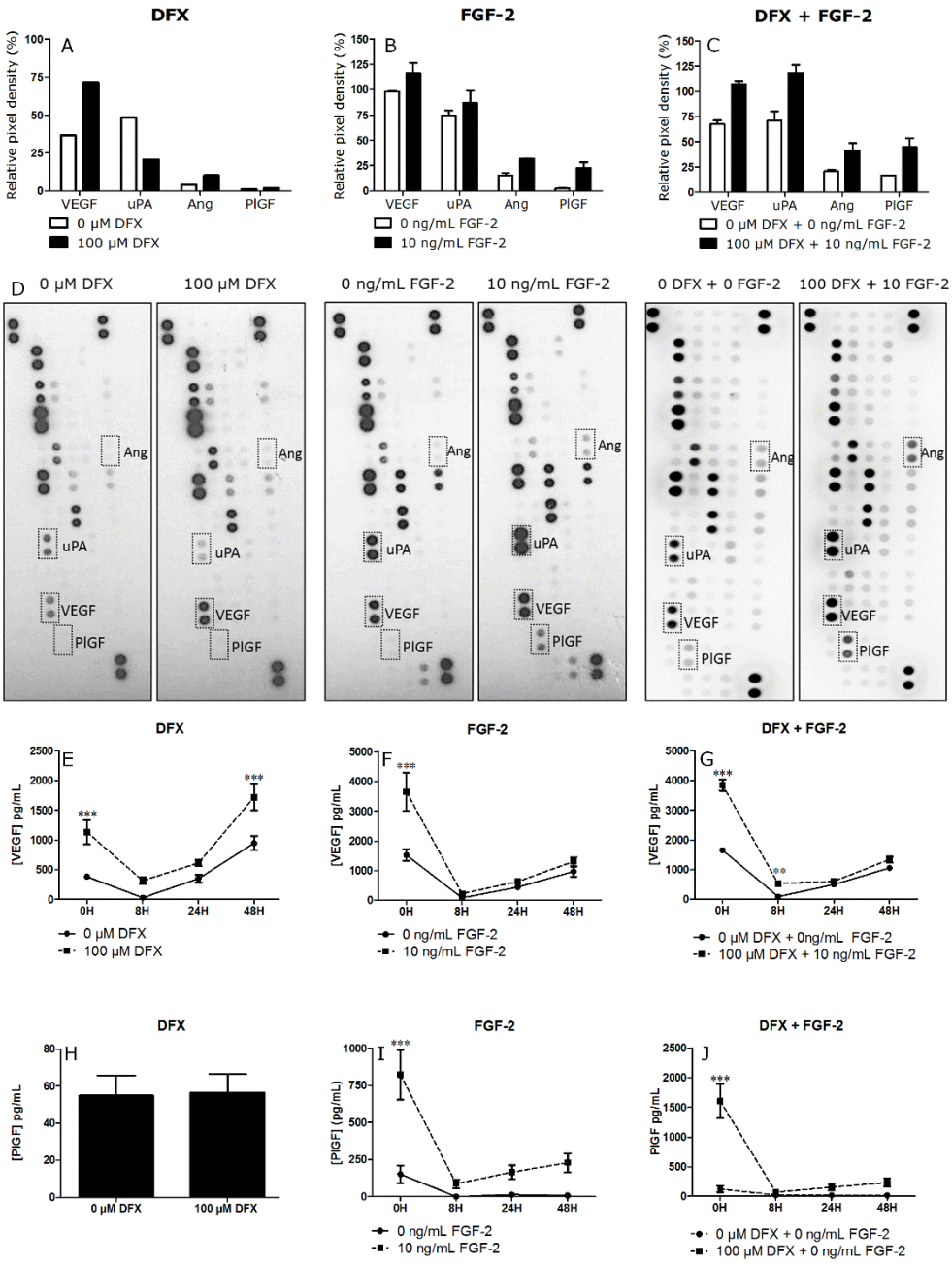


Figure 2.3 Schematic overview of PDLSC pretreatment procedures. After overnight incubation standard PDLSCs culture medium was changed to standard medium containing only 0.1% FBS, supplemented with either 100 µM DFX or 10 ng/mL FGF-2. CM of DFX-treated cells was collected after 24 hours whereas CM of FGF-2-treated cells was collected after 72 hours. In the case of combination treatment, DFX was added 48 hours after the start of FGF-2 pretreatment and CM was collected 24 hours later. Subsequently cells were washed and priming media were replaced by standard culture medium containing 0.1% FBS. This wash-out medium was collected after 8, 24 and 48 hours.

2.4.2 Effect of DFX and FGF-2 on the angiogenic profile of PDLSC

The first part of this study focused on the analysis of angiogenic factors secreted by PDLSCs before and after pretreatment. PDLSCs were either treated with 100 µM DFX for 24 hours, 10 ng/mL FGF-2 for 72 hours or with a combination of both substances. Dot blot analysis of CM of untreated and pretreated PDLSCs was performed to investigate the relative expression of a wide variety of angiogenic proteins secreted by PDLSCs (Figure 2.4A-D). VEGF secretion was increased after pretreatment as well as some other angiogenic factors such as angiogenin (Ang), urokinase (uPA), and placental growth factor (PlGF). As the difference in secretion of VEGF and PlGF was the most pronounced, the production of both factors was validated by means of ELISA (Figure 2.4E-J). Pretreating PDLSCs with 100 µM DFX for 24 hours resulted in a 1.8 fold increase in VEGF and pretreatment with 10 ng/mL FGF-2 for 72 hours resulted in an 1.5 fold increase of VEGF compared to untreated PDLSCs. When both factors were combined, VEGF secretion increased


DFX
FGF-2
DFX + FGF-2
A
B
C
Relative pixel density (%)
VEGF
uPA
Ang
PlGF
0 μM DFX
100 μM DFX
0 ng/mL FGF-2
10 ng/mL FGF-2
0 μM DFX + 0 ng/mL FGF-2
100 μM DFX + 10 ng/mL FGF-2
D
0 μM DFX
100 μM DFX
0 ng/mL FGF-2
10 ng/mL FGF-2
0 DFX + 0 FGF-2
100 DFX + 10 FGF-2
Ang
uPA
VEGF
PlGF
E
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[VEGF] pg/mL
0H
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24H
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[PlGF] pg/mL
PlGF pg/mL
0 μM DFX + 0ng/mL FGF-2
100 μM DFX + 0 ng/mL FGF-2

Figure 2.4 Treatment with DFX and/or FGF-2 increases VEGF and PlGF secretion of PDSLCs. PDLSCs were pretreated either with 100 µM DFX for 24 hours, with 10 ng/mL FGF-2 for 72 hours or with a combination of both substances. (A-D) Antibody array of the CM of PDLSCs pretreated with DFX (n=1), FGF-2 and the combination treatment (n=2). Graphs A-C show pixel density of the differentially expressed proteins. As determined by ELISA (n=5), DFX treatment resulted in a 1.8 fold increase in VEGF secretion (E), whereas FGF-2 treatment increased VEGF secretion 1.5 fold (F). Finally, a combination of both agents led to a 2.7 fold increase in VEGF secretion (G). ELISA of PlGF showed that not DFX (H) but treatment with FGF-2 (I) and FGF-2 combined with DFX (J) significantly upregulated PlGF secretion. Data are expressed as mean ± SEM and analyzed with Two-Way-ANOVA, ** = $p \leq 0.01$; *** = $p \leq 0.001$.

2.7 fold (Figure 2.4G). For combination therapy, first 10 ng/mL FGF-2 was added to the culture medium and 48 hours later, the medium was supplemented with 100 µM DFX. The CM was harvested after a total incubation of 72 hours. Furthermore, the effect of DFX persisted, even when it was removed from the culture medium (wash-out) VEGF secretion remained elevated, 1.8 fold (Figure 2.4E). When cells were treated with FGF-2 no difference in VEGF levels was present after withdrawal of the growth factor (Figure 2.4F). Following combination treatment however, the effects persisted for 8 hours, with no further difference between the VEGF secretion of pretreated and untreated PDLSCs after longer incubation times.

PlGF secretion increased significantly after FGF-2 and combination treatment, 5.4 fold and 13.1 fold respectively (Figure 2.4I-J), whereas DFX treatment did not influence PlGF secretion (Figure 2.4H). After the removal of FGF-2 or FGF-2 and DFX in the case of combination treatment, the levels of PlGF remained elevated, 25.5 fold and 14.4 fold respectively, in the treated PDLSCs compared to untreated PDLSCs, however no statistical significance could be stated (Figure 2.4I-J).

With flow cytometry the effect of the FGF-2 and DFX pretreatment on the expression of stem cells markers was analyzed. Preconditioning did not influence PDLSC surface marker expression (Figure 2.5) or proliferation rates (Figure 2.6).

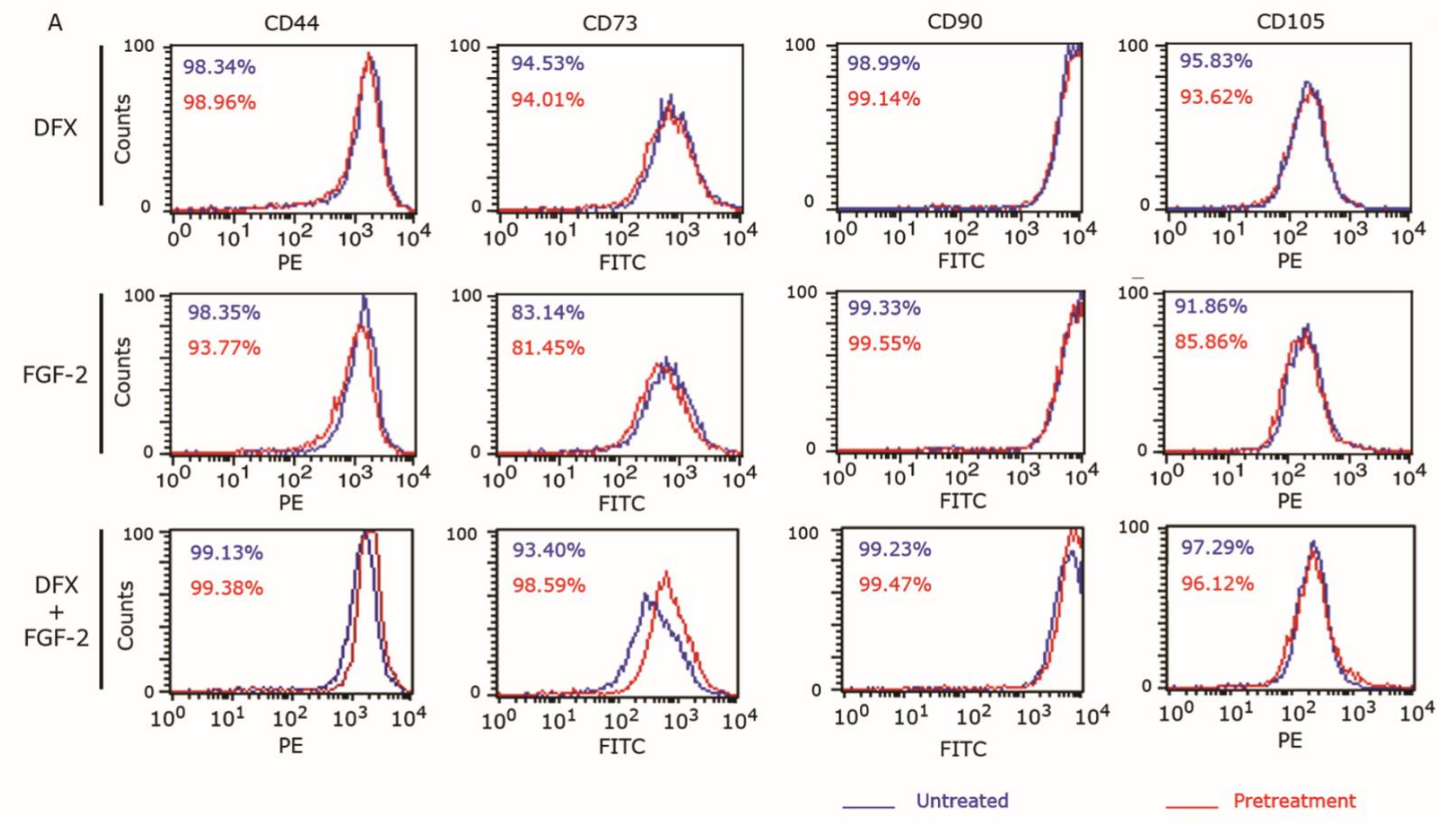

Figure 2.5 The effect of DFX, FGF-2 or combination treatment on the MSC marker expression as demonstrated by flow cytometry. Pretreatment does not influence MSC marker expression compared to untreated PDLSCs (n=6).

2.4.3 DFX upregulates the expression of HIF-1α

In order to determine whether the increased VEGF expression after DFX treatment was mediated by HIF-1α, the protein expression level of HIF-1α was assessed using western blot analysis (Figure 2.7). Untreated PDLSCs show low levels of HIF-1α expression, however, after DFX treatment there is a clear upregulation in HIF-1α. The increased expression is more pronounced after single treatment compared to combination treatment with FGF-2.

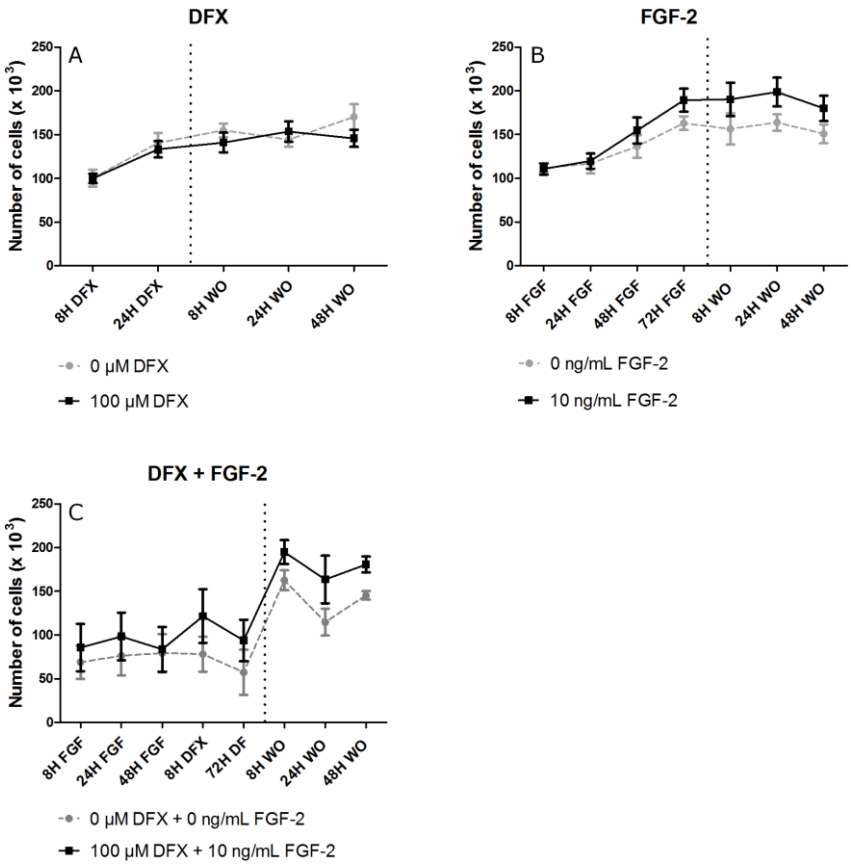

Figure 2.6 Pretreatment does not alter PDLSC proliferation. Cell proliferation was monitored during treatment by means of Trypan Blue Exclusion assay (n=5). (A) DFX pretreatment does not affect PDLSC viability. (B) Pretreating PDLSCs with FGF-2 results in a slight increase in cell numbers. (C) similar to single FGF-2 pretreatment, combination treatment with DFX and FGF-2, only caused a minor increase in cell count.

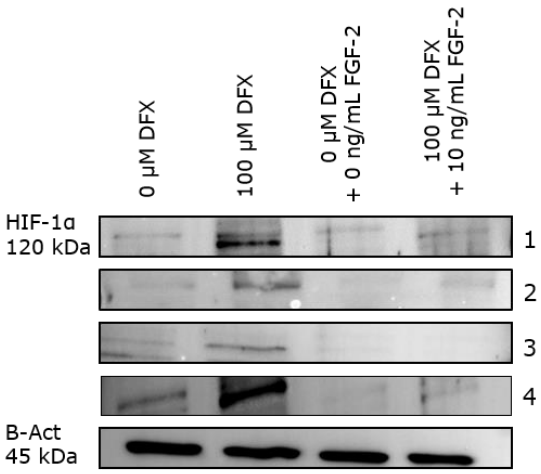

Figure 2.7 Deferoxamine treatment upregulates HIF-1α expression in PDSLCs. Western blot analysis of HIF-1α expression in protein lysates (10 µg) collected from PDLSC after DFX or combination treatment. When PDLSCs are treated with DFX or with a combination of DFX and FGF-2, HIF-1α expression increases. Each row represents the samples from one donor (n=4). DFX = Deferoxamine, FGF-2 = fibroblast growth factor-2, HIF-1α= Hypoxia inducible factor-1α, PDLSCs = periodontal ligament stem cells.

2.4.4 PDLSC induce endothelial migration *in vitro*

As angiogenesis is a complex multistep process, it is not only important to determine the factors secreted by PDLSCs but it is also crucial to assess their influence on endothelial cell behavior. Therefore, several *in vitro* assays were performed in order to mimic the different stages of angiogenesis. One of the first steps is the proliferation of endothelial cells. The capacity of PDLSCs to influence endothelial cell metabolism was investigated by means of an MTT assay (Figure 2.8A-C). HMEC-1 were cultivated in CM of either untreated or DFX or FGF-2 treated PDLSCs for 48 hours after which no difference in metabolic activity could be detected. However, CM harvested from PDLSCs receiving combination treatment seemed to significantly decrease HMEC-1 viability. This effect can be ascribed to an interaction between DFX and MTT. To further demonstrate that the decrease in viability is due to technical difficulties and not to the presence of DFX itself, cell numbers were monitored with the Trypan Blue Exclusion assay. Only

after being exposed to 100 µM DFX for 72 hours, cell numbers started to decline (Figure 2.9).

During angiogenesis, endothelial cells also respond to chemotactic stimuli, which direct them to the site in need of vascularization. Therefore, the chemotactic potential of untreated and treated PDLSCs was evaluated by means of a transwell migration assay. In order to induce transmigration, either treated or untreated PDLSCs were seeded in bottom wells, after overnight incubation, medium was changed to α-MEM 0.1% FBS. Twenty-four hours prior to the priming endpoint, HMEC-1 were added in culture inserts.

Both treated and untreated PDLSCs were capable of inducing transmigration of HMEC-1 (Figure 2.8D-G). However, no difference could be stated between treated and untreated PDLSCs. Despite the fact that combination treatment resulted in a 2.7 fold increase in VEGF secretion compared to 1.8 fold for DFX treatment and 1.5 fold for FGF-2 treatment, the effect on endothelial migration was the least pronounced in this condition (Figure 2.8G). In order to eliminate the possibility that the induced migration was due to the presence of FGF-2 or DFX in the CM, control samples were included containing equal concentrations of FGF-2 or DFX, but lacking growth factors secreted by PDLSCs.

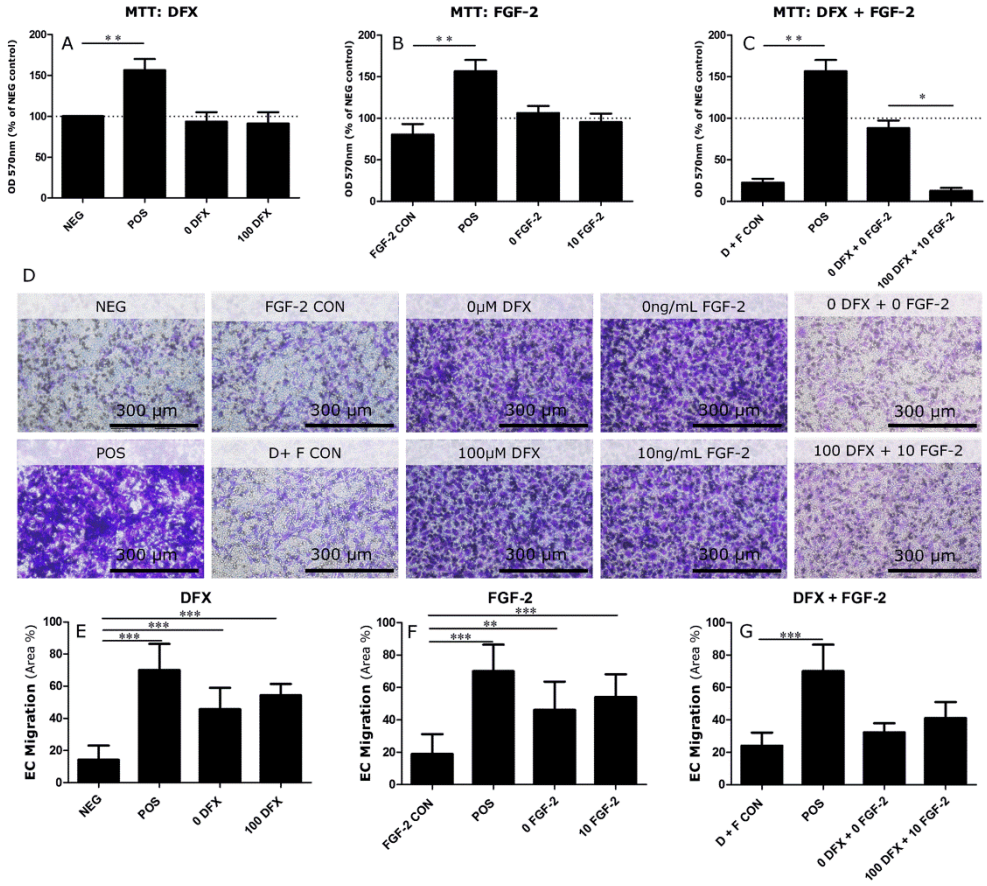

Figure 2.8 Effects of control and primed periodontal ligament stem cells on endothelial cell behavior *in vitro.* (A) CM of control PDLSCs or cells treated with DFX or FGF-2 alone does not induce HMEC-1 proliferation as determined by MTT (n=8). (B) PDLSCs induce endothelial cell migration in the transwell migration assay. Both untreated and treated PDLSCs are able to induce HMEC-1 migration, but there is no difference in chemotactic potential between control and primed PDLSCs. Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. FGF control: culture medium containing 10 ng/mL FGF-2. D+F control: culture medium containing 100 µM DFX and 10 ng/mL FGF-2. Scale bar = 300 µm. Data are expressed as mean ± SEM. ** = p-value ≤0.01; *** = p-value ≤ 0.001 compared to either FGF-2 control or D+F control.

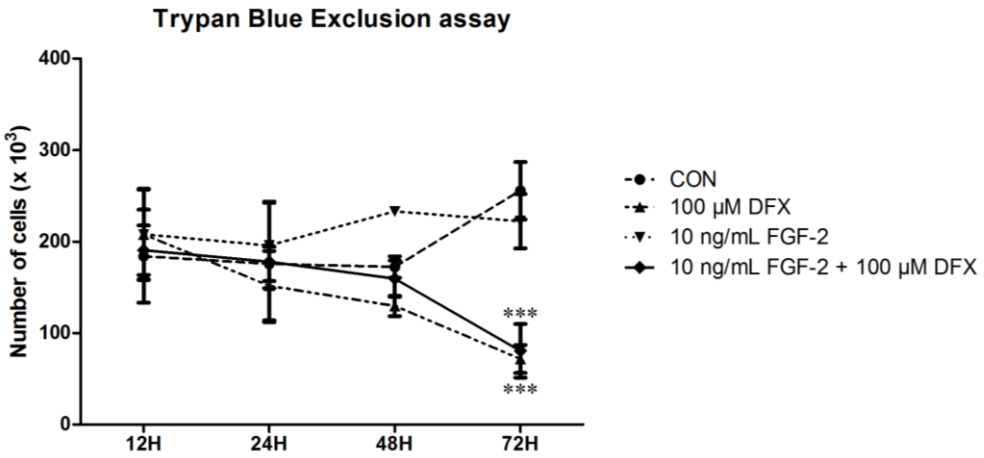

Figure 2.9 Exposure to DFX leads to decreased cell numbers of endothelial cells after 72 hours. Cell numbers were monitored via Trypan Blue Exclusion, only 72 hours of DFX treatment resulted in a decreased cell count (n=3). Control: culture medium containing 0.1% FBS. The conditions included in this experiment only contained the different priming agents and were lacking factors secreted by PDLSCs. DFX = deferoxamine; FGF-2 = fibroblast growth factor-2; PDLSC = periodontal ligament stem cells. Data are expressed as mean ± SEM and were analyzed by Two-Way ANOVA. *** = p-value ≤0.001.

2.4.5 PDLSCs induce endothelial cell migration independent of VEGF

Despite the significant increase in VEGF secretion after pretreatment, pretreated PDLSCs do not cause a greater endothelial cell migration. In order to investigate whether chemotaxis is dependent on VEGF, migration assays were performed with HMEC-1 cells exposed to Axitinib, which is a potent VEGFR1-3 inhibitor (Figure 2.10) [241]. A first indication that VEGF is not solely responsible for the induction of endothelial migration is the fact that 100 ng/mL of recombinant VEGF causes only a minimal increases in endothelial motility. This migration is inhibited by adding 0.5 nM Axitinib to the culture inserts containing HMEC-1. The presence of Axitinib has little to no impact on the endothelial migration elicited by the CM of either pretreated or untreated PDLSCs. This suggests that this migration is not dependent on VEGF.

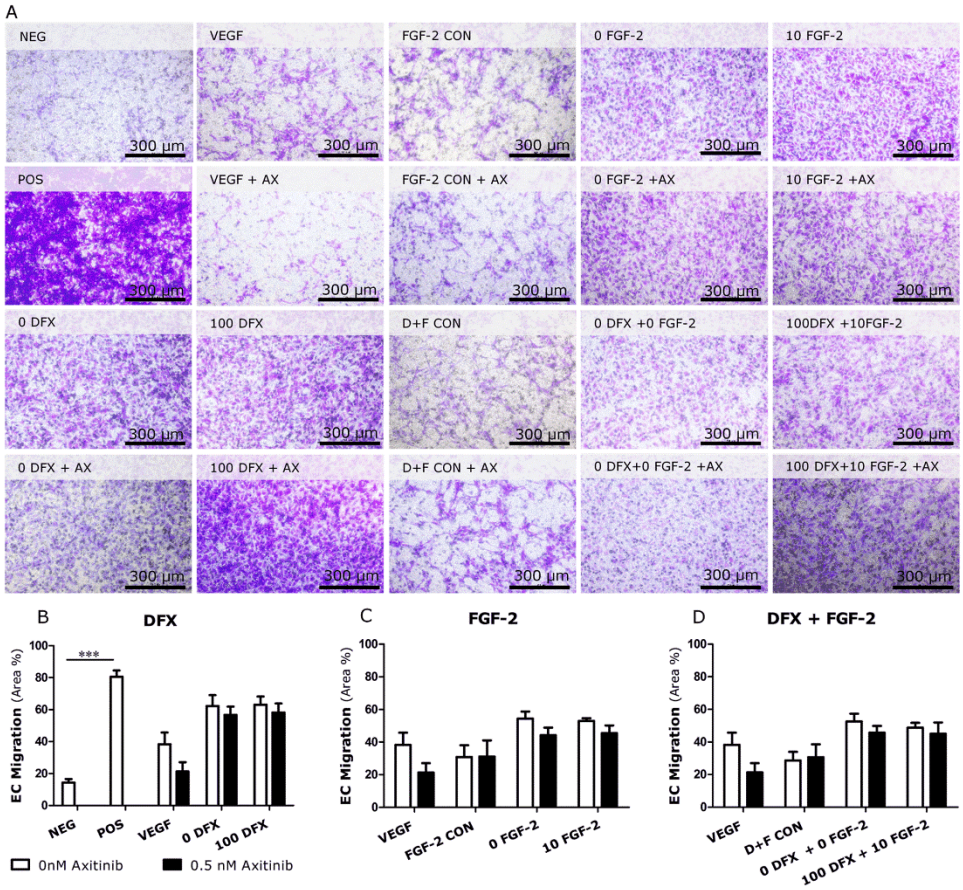

Figure 2.10 Periodontal ligament stem cells do not elicit endothelial cell migration via VEGF. Conditioned medium of untreated and treated PDLSCs induces endothelial cell migration. In the presence of 0.5 nM Axitinib (AX), a VEGFR1-3 inhibitor, endothelial cells continue to migrate towards factors secreted by PDLSCs. Negative control: culture medium containing 0.1% FBS. Positive control: culture medium containing 10% FBS. FGF control: culture medium containing 10ng/mL FGF-2. D+F control: culture medium containing 100 µM DFX and 10 ng/mL FGF-2. Scale bar = 300 µm. Data are expressed as mean ± SEM. *** = p-value ≤ 0.001.

2.4.6 PDLSCs do not induce angiogenesis *in ovo*

In order to investigate whether PDLSCs could induce angiogenesis in an *in vivo* setting, a CAM assay was performed. In this assay, treated and untreated PDLSCs were dissolved in growth factor-reduced Matrigel™ and placed onto the developing chorioallantoic membrane of a fertilized chicken egg at E9. Pure Matrigel™ droplets were used as a negative control. After three days of incubation, the CAM was removed and images were taken. In order to quantify angiogenesis, a concentric circle (radius = 4mm) was drawn and intersecting blood vessels were counted. However, neither untreated nor treated PDLSCs increased the number of blood vessels compared to control conditions (Figure 2.11). In contrast, incubation with 500 ng of human recombinant VEGF resulted in increased blood vessel formation.

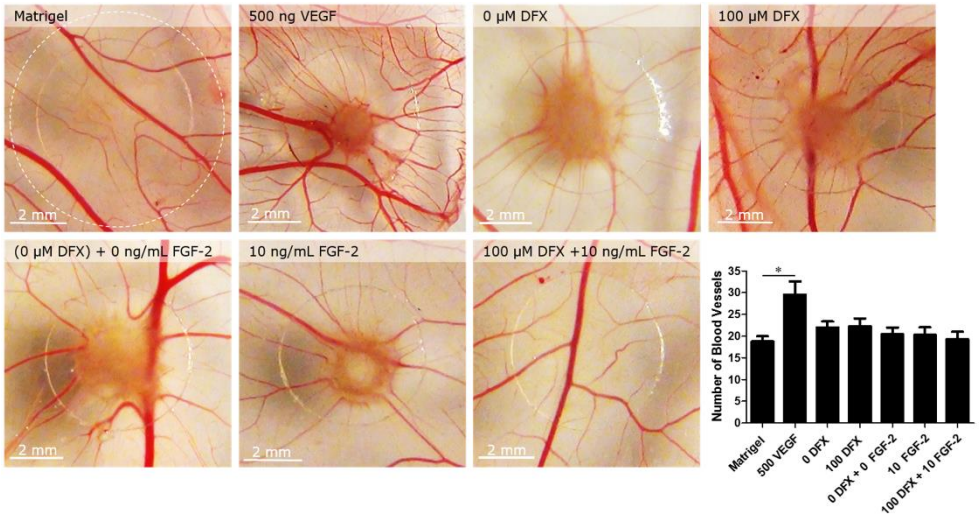

Figure 2.11 PDLSCs do not induce angiogenesis *in ovo.* Images on the left show representative vascularization of the chorioallantoic membrane following three days of incubation with growth factor reduced MatrigelTM containing either 500 ng VEGF (n=13), untreated PDLSCs cultured in low serum conditions for 24 (n=27) or 72 hours (n=26) or PDLSCs treated with 100 µM DFX (24h, n=24), 10 ng/mL FGF-2 (72h, n=24)) or a combination therapy (n=21). Neither untreated (24h control, 72h control) nor treated PDLSCs were able to increase the average number of blood vessels. Scale bar = 2mm. Data are expressed as mean ± SEM. * = p-value ≤ 0.05. PDLSCs = periodontal ligament stem cells; VEGF = vascular endothelial growth factor; DFX = deferoxamine; FGF-2 = fibroblast growth factor-2.

2.5 Discussion

Since supporting vasculature is necessary for the survival of the transplanted cells/tissues, the efficiency of regenerative medicine could be ameliorated by enhancing the angiogenic potential of stem cells [116]. In this study, we investigated the possibility of boosting the angiogenic potential of PDLSCs by means of *in vitro* preconditioning with DFX and/or FGF-2.

PDLSCs were found to express a characteristic set of MSC markers, with very little variation between different populations. High expression levels of these MSC markers together with their adherence to plastic and multilineage differentiation potential, qualify the PDLSCs used in this study as multipotent mesenchymal stromal cells according to the minimal requirements provided by the society for cellular therapy [83].

Pharmacological interventions to increase stem cell survival and biological activities, prior to transplantation, are considered one of the most promising strategies for future applications in regenerative medicine [116]. In order to enhance the angiogenic potential of PDLSCs, cells were primed with FGF-2 or with the iron chelator DFX. A number of studies have already implicated VEGF as a major player in the process of angiogenesis [242, 243]. VEGF has been described to regulate vessel permeability and to stimulate endothelial proliferation and migration [6]. This study showed that DFX and FGF-2 pretreatment significantly enhanced VEGF secretion of PDLSCs and this effect was time- and concentration dependent. DFX concentrations up to 100 µM did not show any cytotoxic effects, whereas higher concentrations of FGF-2 had a positive effect on cellular proliferation. The initial screening of the PDLSC secretome revealed a differential secretion of several angiogenesis related factors after pretreatment, including VEGF and PlGF. PlGF is a member of the VEGF family, and like VEGF, it binds to the VEGF-1 receptor [244]. Array results were validated by means of ELISA, which showed a significant upregulation of VEGF secretion after pretreatment for all three conditions. Moreover, 48 hours after removal of DFX from the culture medium, VEGF levels remained elevated in the pretreated PDLSC populations. This wash-out effect is important with regards to future *in vivo* applications, since it indicates a prolonged effect of the *in vitro* priming with DFX. Similar effects of DFX preconditioning have already been reported with regard to BM-MSCs [245] and

adipose-derived stem cells (ASCs) [246]. Moreover, Agis and colleagues already reported an increased VEGF secretion after treating periodontal ligament fibroblasts with DFX [132]. Whereas, Yanagita and colleagues demonstrated an increased VEGF secretion in FGF-2-primed mouse PDL cells. The report mentions similar results were shown using human PDL cells however no data are shown [247]. Combining FGF-2 and DFX priming is to our knowledge a unique and previously undescribed approach.

As an iron chelator, DFX is considered a hypoxia mimetic and has been described to upregulate HIF-1α in several stem cell types [132, 245, 248, 249]. DFX treated PDLSCs displayed an increased expression of HIF-1α, which is in accordance with other reports. However, after combination treatment, the upregulation of HIF-1α is much less pronounced, suggesting a protective effect of FGF-2 under hypoxic conditions. The protective effect of FGF-2 is also evidenced by the lack of the DFX-driven wash-out effect after combination therapy.

In order to assess the functional effects of PDLSCs on the different steps of angiogenesis, several *in vitro* tests were performed with HMEC-1. Despite the substantial secretion of VEGF, neither untreated nor pretreated PDLSCs had an effect on the proliferation of HMEC-1. These results are in agreement with the study by Liu *et al.* who reported no effect of DFX pretreated ASCs on HMEC-1 proliferation. Furthermore, previous reports from our lab also demonstrated no effect of dental pulp stem cells (DPSCs), stem cells from the apical papilla (SCAPs) and of the dental follicle (FSCs) on HMEC-1 proliferation [82]. Surprisingly, MTT results indicated a major decrease in HMEC-1 viability when exposed to CM of PDLSCs receiving combination treatment. This effect can probably be ascribed to an interaction between DFX and MTT, which was also reported by Agis and colleagues [132]. Furthermore, Trypan Blue Exclusion assay confirmed that exposure to 10 ng/mL FGF-2 and 100 µM DFX for 48 hours did not influence HMEC-1 proliferation.

A second important step in the process of blood vessel formation is endothelial migration [4]. Both untreated and pretreated PDLSCs induced EC motility. However, despite the increase in VEGF secretion, pretreatment of PDLSCs did not result in an increase in endothelial migration. In order to evaluate whether the HMEC-1 migration is dependent on VEGF, Axitinib was used to block VEGFR1-3.

Both untreated and pretreated PDLSCs were able to induce migration when HMEC-1 were exposed to 0.5 nM Axitinib. We showed that recombinant VEGF is able to attract HMEC-1 and this process can be reversed by adding 0.5 nM Axinitib. Together, these data indicate that the *in vitro* chemotaxis of HMEC-1 towards PDLSCs is not dependent on VEGF alone, but is possibly the result of a synergistic effect of several chemotactic factors secreted by both untreated and primed PDLSCs.

Finally, in order to assess the angiogenic potential of untreated and primed PDLSCs *in vivo* a CAM assay was performed. Unfortunately this study was unable to demonstrate an increased blood vessel formation in the presence of PDLSCs. VEGF, as used in this and numerous other studies [4, 134], as a positive control, significantly induced blood vessel formation. Thus despite the induced endothelial cell migration, no increase on *in vivo* angiogenesis was seen. Our lab previously demonstrated the ability of DSPCs and SCAPs [82] to enhance CAM angiogenesis. However, no induction of CAM angiogenesis was reported for the FSC population. This might in part explain the lack of PDLSC-induced angiogenesis, since the dental follicle eventually develops into the periodontal ligament and both populations are therefore closely related [134]. Nevertheless, also the priming by DFX and FGF-2 and thus the induced VEGF and PlGF secretion was not able to compensate the lack of angiogenic activities of PDLSCs *in vivo*.

In conclusion, this study provides evidence that pretreatment of PDLSCs with DFX and FGF-2 is able to upregulate VEGF and PlGF production, and in the case of DFX even after the removal of the priming agent. Especially the presence of a wash-out effect could have major implications for future uses of DFX primed cell transplants. Furthermore, we demonstrated that both untreated as well as pretreated PDLSCs were able to induce endothelial migration, however, this migratory response was not dependent on VEGF secretion. This VEGF independent response may therefore in part explain the lack of functional effects of PDLSCs on endothelial proliferation and blood vessel development in the CAM assay, despite their potent secretome. For future attempts to enhance angiogenic effects of stem cells, it might be important to focus on other inducers of the angiogenic cascade then the extensively studied VEGF.

Chapter 3

The Angiogenic Potential of Leukocyte and Platelet Rich Fibrin

3.1 Abstract

Leukocyte and Platelet Rich Fibrin (L-PRF) is an autologous platelet concentrate and represents an innovative tool in regenerative medicine. L-PRF consists of a fibrin matrix enriched with platelets, leukocytes and a plethora of cytokines and growth factors. Since L-PRF can be produced bedside from whole blood without the use of an anti-coagulant, it is a popular adjuvant in maxillofacial surgeries. More importantly, different types of platelet concentrates have been described to stimulate revascularization, which is essential for successful tissue regeneration. Therefore this study aimed to characterize L-PRF and explore its angiogenic potential. L-PRF growth factor release was determined by means of an antibody array and revealed an abundance of CXCR-2 ligands and EGF present in L-PRF CM. Both L-PRF CM and L-PRF exudate induced endothelial proliferation, migration and tube formation *in vitro*. Inhibition of CXCR-2 or EGF receptor (EGFR) did not affect the endothelial response to L-PRF CM. Flow cytometric analysis showed only small populations of endothelial cells expressing CXCR-2 or EGFR. Furthermore, L-PRF is able to induce blood vessel formation *in ovo* as was demonstrated by means of a chorioallantoic membrane assay. In conclusion, the results of this study demonstrated the angiogenic capacity of L-PRF both *in vitro* and *in ovo*. Since endothelial cells showed only low levels of CXCR-2 and EGFR expression and inhibition of these receptors did not alter endothelial behavior when exposed to L-PRF, no conclusive statements could be made about the involvement of these pathways in the angiogenic effects of L-PRF. However, characterization of L-PRF remains important since patient variability still represents a clinical issue for the application of L-PRF.

3.2 Introduction

Within the field of tissue engineering, establishing a vascular network is a key aspect in successfully regenerating damaged tissues [239, 250]. Newly developing vasculature supports cellular function and survival by allowing exchange of nutrients, oxygen and waste products [250]. The use of biological products for wound treatment and surgical procedures has known an immense growth over the last two decades [224, 250]. In particular the use of platelet concentrates, as a source of biomolecules involved in angiogenesis and wound healing, has gained a lot of attention due to their autologous nature and their cost-effectiveness [200, 224]. The first preparation protocols involved two centrifugation steps and the use of anticoagulants, calcium chloride and bovine thrombin [239]. Much advancement has been made since, in order to develop a second-generation platelet concentrate, that can be produced without biochemical handling of the blood sample [239]. Leukocyte and platelet rich fibrin (L-PRF) can be produced with one single centrifugation step (400*g* – 12min) and without the need for biochemical handling [201, 202, 205]. L-PRF consists of three different components, all of which can influence angiogenesis and wound healing. The white blood cells present in L-PRF, including neutrophils and macrophages, secrete pro-angiogenic molecules [211, 214, 220, 221]. Platelets are known to release a plethora of growth factors (VEGF, FGF-2, PDGF) and cytokines upon degranulation [224, 227]. Last but not least, the fibrin matrix also contributes to the angiogenic potential of L-PRF. By capturing the released biomolecules, the fibrin matrix ensures a progressive release of these molecules over time [204, 230, 231]. To date, numerous studies have investigated the angiogenic and regenerative potential of platelet derivatives. PRP has been described to enhance endothelial proliferation [232-234], migration, and tube formation [235] as well as enhance wound healing in preclinical animal models [236, 237]. For PRF, so far only one report investigated the effect of PRF on angiogenesis *in vitro* [238]. Roy *et al.* reported a slow and steady release of VEGF and the induction of endothelial cell mitogenesis. However, the PRF used by Roy and colleagues was produced using trisodium citrate and calcium chloride [238]. The aim of the present study is to evaluate growth factor release of L-PRF and to determine its effect on endothelial proliferation, migration and tube formation *in vitro.* Finally, the capacity of L-PRF to induce blood vessel formation is tested in an *in vivo* setting.

3.3 Materials and Methods

3.3.1 Preparation of Leukocyte and Platelet Rich Fibrin

Blood samples were obtained from 18 healthy (11 male, 7 female) volunteers with written informed consent. This study protocol and consent procedure was approved by the medical ethical committee from Hasselt University and the Clinical Trial Center from KU Leuven (S58789 / B322201628215). Blood samples were collected in glass-coated plastic tubes (VACUETTE® 9 ml Z Serum Clot Activator Tubes, Greiner Bio-One) by means of a venipuncture, and centrifuged immediately (IntraSpin™ Centrifuge, Intra-Lock, Boca Raton, Florida, USA) for 12 minutes at 2700 rpm (400*g*). After centrifugation, L-PRF was removed from the tube using sterile tweezers and separated from the red blood cell base using a straight iris spatula (Fine Science Tools (FST), Heidelberg, Germany).

For the preparation of conditioned medium (CM), L-PRF clots were incubated in 6 mL of serum-free α-MEM, supplemented with 2 mM L-glutamine (Sigma-Aldrich), 100 U/ml Penicillin (Sigma-Aldrich) and 100 µg/ml Streptomycin (Sigma-Aldrich). After 48h, 96h or 144h the medium was collected, centrifuged for 6 minutes at 300*g* and sterile filtered (Filtropur S 0.2, Sarstedt, Nümbecht, Germany). To collect exudate (EX), L-PRF clots were transferred to a sterile box (Xpression™ Fabrication Box, Intra-Lock). The weighted press of the box transformed the L-PRF clot into a thin membrane and the exudate was released from the clot during this process. The exudate was collected and filtered.

3.3.2 Human Cytokine Antibody Array

In order to investigate growth factor release from L-PRF a Human Cytokine Antibody Array (ab133998, Abcam, Cambridge,UK) was performed. L-PRF CM and exudate of two different donors were used at a protein concentration of 10 mg/ml, previously determined by the BCA method (Pierce™ BCA Protein Assay Kit, Catalog number 23225, Thermo Scientific, Erembodegem, Belgium). The BCA kit was used following the manufacturer's instructions to determine the total protein concentration. The cytokine antibody array was performed in accordance with the manufacturer's guidelines. Briefly, membranes were blocked with 1X blocking buffer for 30 minutes at room temperature, followed by overnight incubation with L-PRF CM or exudate at 4°C. The next day, 1X biotin-conjugated anti-cytokines solution was added to the membranes for one hour at room temperature. After

repeated washing, membranes were incubated for two hours with HRP-conjugated streptavidin, before adding a mixture of detection buffers to the membranes. Immediately afterwards, images were taken (Image Quant LAS 4000 mini, GE Healthcare Life Sciences, Diegem, Belgium) with exposure times between four and ten seconds. Quantification was performed using ImageQuant™ TL software (GE Healthcare Life Sciences).

3.3.3 Enzyme-Linked Immunosorbent Assay (ELISA)

To evaluate the concentration range of the released EGF, IL-8 and VEGF, enzyme-linked immunosorbent assays (ELISA) were performed on L-PRF CM harvested after 48, 96 and 144 hours and on L-PRF exudate. ELISA's (Raybiotech, USA) were performed according to manufacturer's instructions.

3.3.4 Cell culture

Human umbilical vein endothelial cells (HUVEC) (HUVEC-2, 354151, BD) were cultured in endothelial cell growth medium (EBM-2, Lonza, Walkersville, MD, USA) supplemented with growth factors (EGM-2 SingleQuots™, CC-4176, Lonza) and 10% FBS and maintained at 37°C, 5% CO_2. HUVEC were seeded in fibronectin-coated (1µg/mL, R&D systems) culture flasks and culture medium was changed twice a week. When confluent, HUVEC were sub-cultured using 0.05% trypsin/EDTA. For all experiments, HUVEC from passage 6-10 were used.

3.3.5 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – assay

In order to evaluate the effect of L-PRF on the proliferation of endothelial cells a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)–assay was performed. HUVEC were seeded at 31 250 cells/cm² in a flat bottom 96-well plate. After overnight incubation, HUVEC were washed with PBS and incubated for 48 hours with either L-PRF CM, L-PRF EX, standard culture medium containing 0% FBS (negative control) or standard culture medium containing 10% FBS (positive control). All conditions were performed in triplicate. After 48 hours of culturing, media were replaced with 500 µg/mL MTT in standard culture medium containing 0% FBS. Four hours later, the MTT solution was removed and replaced with a DMSO (Sigma) and 0.01 M Glycine (Sigma) mixture to dissolve formazan crystals. Absorbance was measured with a Benchmark microplate reader (Bio-Rad Laboratories, Hercules, CA) at a wavelength of 570 nm.

To examine whether the effect of L-PRF on EC viability was mediated by the C-X-C chemokine receptor-2 (CXCR-2) pathway, 100 nM SB225002 (Selleckchem, Munich, Germany), a selective CXCR-2 antagonist was added to different L-PRF conditions (50% CM, 100% CM and 3% exudate). HUVEC were pre-incubated with 100 nM SB225002 for 15 minutes to ensure effective blocking, before cells were exposed to L-PRF.

3.3.6 Propidium iodide assay

Endothelial cells (HUVEC) were seeded into fibronectin coated (5 µg/mL) black 96-well plates with a clear flat bottom (Greiner Bio-One, 655096) at a density of 31 250 cells/cm². After overnight incubation, standard endothelial culture medium was replaced with different concentrations of L-PRF CM (50% and 100%) and L-PRF exudate (1%, 3%, 10%). Serum free α-MEM served as a negative control and α-MEM supplemented with 10% FBS was included as a positive control. After 48 hours of incubation, medium was replaced by 50 µL Lysis buffer A100 (ChemoMetec, Allerod, Denmark) and subsequently 50 µL of stabilization buffer B (ChemoMetec) supplemented with 1/50 Propidium iodide solution (Sigma). Cells were placed back into the incubator for 15 minutes before measuring the fluorescent signal using the Fluostar Optima plate reader (BMG Labtech, Germany) at an excitation wavelength of 540 nm and a emission wavelength of 612 nm.

To examine whether the effect of L-PRF on endothelial proliferation was mediated by EGF, 50 nM AZD8931 (Selleckchem), a reversible inhibitor of EGFR, ErbB2 and ErbB3, was added to different L-PRF conditions (50% CM, 100% CM and 3% exudate). HUVEC were pre-incubated with 50 nM AZD8931 for 15 minutes to ensure effective blocking, before cells were exposed to L-PRF conditions or to 100 ng/mL human recombinant EGF (Immunotools, Friesoythe, Germany).

3.3.7 Transwell migration assay

HUVEC migration toward L-PRF CM and exudate was evaluated by means of a transwell migration assay. Semipermeable Thincert™ tissue culture inserts (8 µm) were seeded with 100 000 HUVEC, dissolved in serum free α-MEM and placed in a 24-well plate. Serum free α-MEM or α-MEM containing 10% FBS were added to the wells underneath as negative and positive controls respectively. To determine whether L-PRF can induce HUVEC migration, different concentrations of L-PRF CM

(50%, 100%) and L-PRF exudate (1% , 3% and 10%) were added to the underlying wells. HUVEC were allowed to migrate for 24 hours before transmigrated cells were dissociated from the membrane and labeled using calceine acetoxymethyl (1,67 mM, BD) dissolved in gentle cell dissociation reagent (STEMCELL technologies, Grenoble, France). After a one hour incubation period the dissociation buffer, now containing the labeled transmigrated cells, is transferred to a black, flat bottom 96-well plate. Fluorescence was measured using the Fluostar Optima plate reader (BMG Labtech) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

In order to investigate the role of the CXCR-2 pathway and EGF in the migratory response elicited by L-PRF, inhibitors for these pathways were added to the transwell migration experiments. The CXCR-2 pathway was inhibited by adding 100nM SB225002 (Selleckchem) to the HUVEC 15 minutes prior to seeding the cells in the culture inserts. The effect of EGF were inhibited by inhibiting the EGF receptor with 50 nm of AZD8931 (Selleckchem).

3.3.8 Tube Formation

In order to examine the effect of L-PRF on endothelial tubulogenesis, a tube formation experiment was performed. The wells of an Angiogenesis µ-slide (Ibidi, Planegg/Martinsried, Germany) were coated with growth factor reduced, phenol red-free Matrigel™ Basement Membrane Matrix (Corning, Bedford, MA, USA). After the matrix had solidified, 10 000 HUVEC were seeded per well suspended in serum free α-MEM (negative control), α-MEM 10% FBS (positive control), L-PRF CM (50%, 100%) or in L-PRF exudate (1%). After 6 hours two representative images were taken at a 4x magnification level with an inverted Nikon eclipse TS100 microscope equipped with a relay lens (Nikon Microscope DXM Relay Lens MQD42070) and a Jenoptik ProgRes C3 camera. The number of nodes and total branching length were determined using the Angiogenesis Analyzer plugin in Image J.

The role of the CXCR-2 ligands and EGF, in the stimulation of endothelial tubulogenesis was investigated by adding inhibitors for these pathways. In order to inhibit the CXCR-2 pathway 100 nM of SB225002 was added, while 50 nM AZD8931 was added to inhibit the EGF receptor of endothelial cells.

3.3.9 Flow cytometry

The cell surface expression of CXCR-1, CXCR-2 and EGFR on HUVEC was analyzed with flow cytometry. HUVEC were seeded in standard endothelial culture medium (EGM-2) and left for overnight attachment. The next day, medium was replaced by α-MEM 0% FBS, α-MEM supplemented with 100 ng/mL IL-8 (R&D systems), α-MEM supplemented with 100 ng/mL EGF, 50% L-PRF conditioned medium or 10% L-PRF exudate. After 24 hours of incubation, HUVEC were harvested using a gentle cell dissociation buffer and resuspended in PBS containing 2% FBS. Cells were incubated with primary antibodies for 45 minutes at room temperature. Matched isotype controls were included as a negative control for nonspecific background staining. In the case of EGFR, cells were incubated with a PE-labeled secondary antibody for an additional 30 min before cells were analyzed with a FACS Calibur flow cytometer equipped with CellQuest Pro software (BD Biosciences, Franklin Lakes, N.J., USA).

Table 3.1 Antibodies used for flow cytometric analysis.

ANTIBODY	ISOTYPE	DILUTION	SUPPLIER
CXCR-1 FITC	IgG2b FITC	1:20	Biolegend
CXCR-2 PE	IgG1 PE	1:20	Biolegend
EGFR	IgG1	1:100	Biolegend
Goat α mouse PE	IgG1 PE	1:250	Invitrogen

3.3.10 Chorioallantoic membrane assay

In order to investigate the capacity of L-PRF to induce blood vessel formation *in vivo*, a chorioallantoic membrane (CAM) assay was performed. Fertilized white leghorn chicken eggs (Gallus gallus) were incubated in a humidified atmosphere at 37° C for three days. At 3 days of embryonic age (E3), albumin was removed to detach the developing CAM from the eggshell. At E9, eggs with exposed CAM were incubated with plastic discs containing 500 ng recombinant human FGF-2 (immunotools), L-PRF CM or L-PRF exudate dissolved in growth factor-reduced Matrigel™ droplets (BD Biosciences) in a 1:1 ratio. Plastic discs containing Matrigel™ droplets diluted 1:1 with serum-free α-MEM were used as a negative control. Eggs were incubated for three consecutive days and at E12 the CAM was dissected out of the eggs to analyze angiogenesis. Images were taken with a Sony

HDR-XR350VE handycam camera (Sony corporation, Tokyo, Japan) and quantified by drawing a circle (radius 4 mm) over the plastic discs on the picture and counting intersecting blood vessels. Blood vessels were counted by two different researchers in a blinded fashion.

In a second experiment, Matrigel™ was replaced by 18% pluronic F-127 gel (Sigma-Aldrich) as a control. Eggs were treated with plastic discs containing a fibrin gel consisting of 20 mg/mL human fibrinogen (Merck, Darmstadt, Germany), 2.5 U/mL human thrombin (Merck) and 20 mM of $CaCl_2$ (Sigma Aldrich). A third group was treated with L-PRF membranes. For this purpose, L-PRF clots form 5 different donors were pressed into membranes using the Xpression™ Fabrication Box. From these membranes, 6 mm discs were created with a biopsy punch (Stiefel, Middlesex, UK).

3.3.10 Statistical Analysis

Statistical analysis was performed using Graphpad Prism software 5.03 (Graphpad Software, La Jolla, CA). Data normality was tested with D'Agostino & Pearson normality test. When Gaussian distribution was reached, experimental groups were compared using a one-way analysis of variance (ANOVA) with a Bonferroni post-test for groups ≤ 5. Non-parametric data were evaluated with a Kruskall-Wallis test combined with Dunn's post-test. In the case of experiments involving the use of SB225002 of AZD8931, paired data were compared by a Friedman test followed by a Dunn's multiple comparison post-hoc test for non-parametric data. When Gaussian distribution was reached, results were analyzed with Repeated Measures ANOVA with Bonferroni's multiple comparison. Statistical significance was reached at p-values ≤ 0.05. All data were expressed as mean ± standard error of the mean (SEM).

3.4 Results

3.4.1 Characterization of the L-PRF secretome

The first part of this study focused on investigating the growth factor release from L-PRF. In a first step, VEGF release was evaluated by means of ELISA at different time points (Figure 3.1). VEGF levels in L-PRF exudate were significantly lower compared to VEGF levels in conditioned medium. VEGF levels increased with increasing incubation time. After incubating the L-PRF clot in medium for 96 hours, an average of 1322 pg/mL VEGF was released into the conditioned medium. Incubating the clot an extra two days, did not markedly increase VEGF levels in the conditioned medium. Therefore all further experiments were conducted using conditioned medium which was collected after 96 hours of incubation.

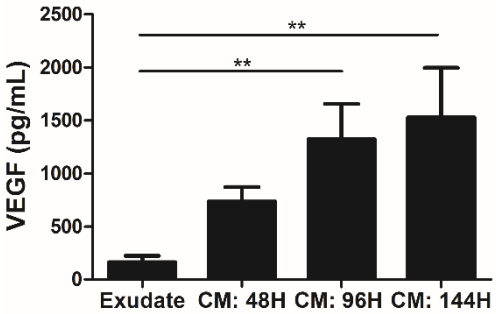

Figure 3.1 VEGF release from L-PRF over time. In order to evaluate VEGF release over time, L-PRF clots were incubated in medium for 48H (n=7), 96H (n=9) and 144 hours (n=5) before VEGF levels were measured with ELISA. L-PRF exudate (n=8) contained only low amounts of VEGF compared to L-PRF CM. VEGF contents increased with increasing time, however only a minimal increment was present between 96H and 144H. Data are represented as mean ± SEM. **= p-value < 0.01. CM = conditioned medium, L-PRF = leukocyte and platelet-rich fibrin; VEGF = vascular endothelial growth factor.

An antibody array was performed in order to obtain a more general screening of the growth factors released from L-PRF (Figure 3.2). The array was performed on exudate and CM from two different donor samples. Analysis indicated high levels of EGF present in L-PRF CM compared to L-PRF exudate. The CCL-5 protein, also known as RANTES, was highly expressed by three out of four samples.

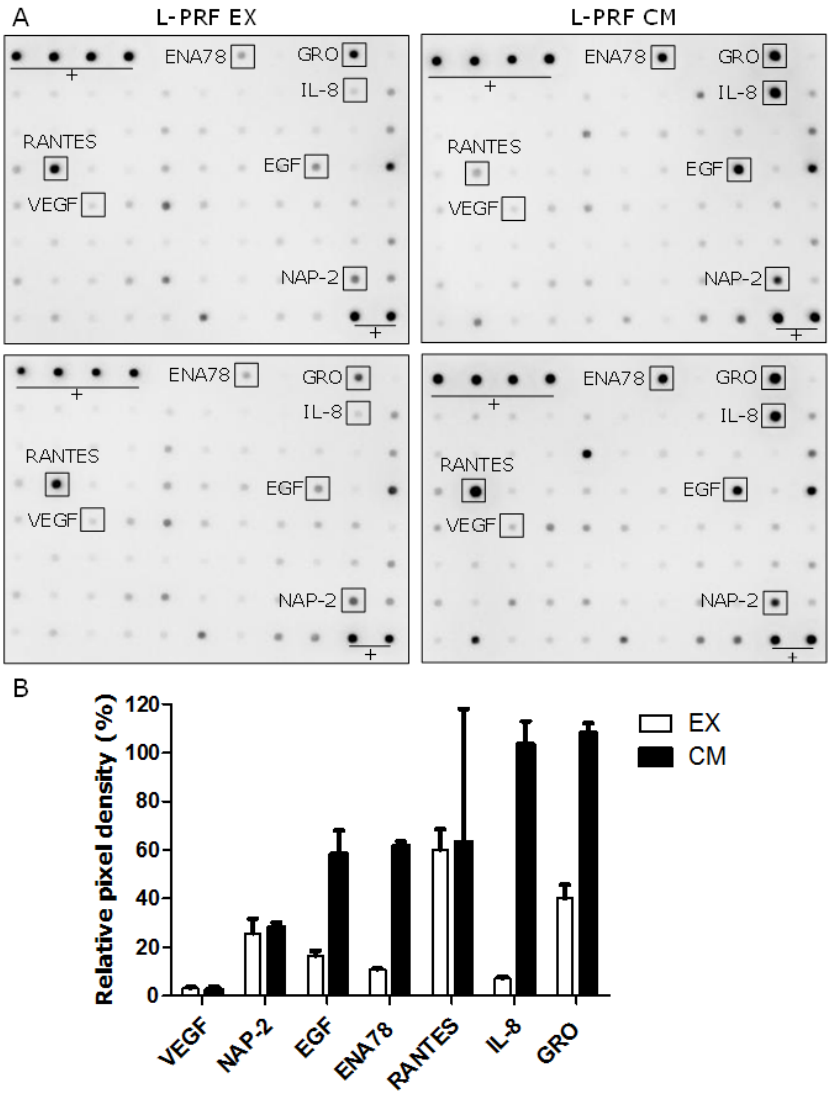

Figure 3.2 Protein release profile of L-PRF exudate (EX) and conditioned medium (CM). (A) Antibody array of the proteins released from L-PRF exudate or conditioned medium (n=2). (B) Relative pixel density was measured using ImageJ in order to compare relative protein levels between L-PRF EX and L-PRF CM. (+) positive control spots; ENA 78 = epithelial-derived neutrophil-activating peptide 78; EGF = epidermal growth factor; GRO = growth regulated oncogene; IL-8 = interleukin-8; NAP-2 = neutrophil-activating peptide-2; RANTES = regulated on activation, normal T cell expressed and secreted; VEGF = vascular endothelial growth factor. Data are expressed mean ± SEM.

Furthermore, four other proteins: epithelial-derived neutrophil-activating peptide (ENA78), growth regulated oncogene (GRO), neutrophil-activating peptide-2 (NAP-2) and interleukin-8 (IL-8) were found to be abundantly present in conditioned medium, whereas only minor levels of these proteins were detected in L-PRF exudate. All four of these proteins are considered ligands to the IL-8 receptor beta, also known as CXCR-2.

The levels of EGF and IL-8 release from L-PRF at different time points were quantified by means of ELISA (Figure 3.3). As suggested by the protein array, exudate contains over a 1000-times less IL-8 and 3-times less EGF than L-PRF CM (96h). IL-8 concentrations showed a minor increase with increasing time (Figure 3.3A). EGF levels did not increase between medium harvested after 48 hours and 96 hours of incubation with L-PRF (Figure 3.3B). Since neither VEGF (Figure 3.1), nor IL-8 or EGF concentrations markedly increased after 96 hours, this time was chosen for harvesting L-PRF CM for all following experiments. After 96 hours the medium contained on average 1322 pg/mL VEGF, 7.7 ng/mL IL-8 and 3.3 pg/mL EGF.

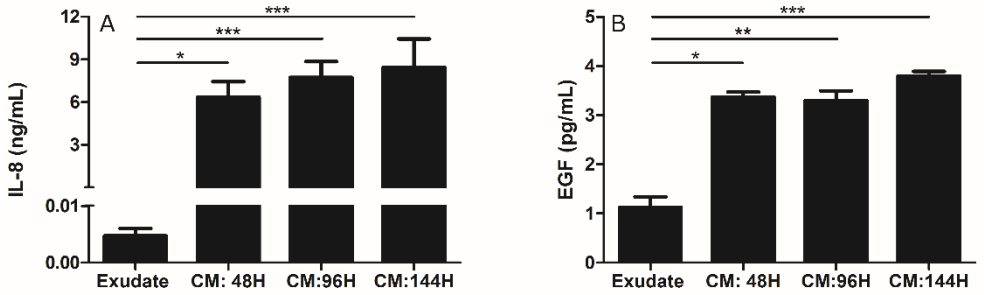

Figure 3.3 IL-8 and EGF release from L-PRF over time. IL-8 and EGF concentrations in L-PRF CM and exudate were quantified by means of ELISA. L-PRF CM was harvested after 48 (n=8), 96 (n=12) and 144 hours (n=8). (A) IL-8 levels were substantially lower in L-PRF EX (n=8) compared to L-PRF CM. IL-8 concentrations displayed minor increments with increasing incubation times of the CM. (B) L-PRF EX contained markedly lower levels of EGF compared to L-PRF CM. EGF levels in L-PRF CM remained stable over time. Data are represented as mean ± SEM. *** = p-value < 0.001, **= p-value < 0.01 and * = p-value <0.05. L-PRF = leukocyte and platelet rich fibrin, CM = conditioned medium; EGF = epidermal growth factor; IL-8 = interleukin-8; EX = exudate.

3.4.2 Functional analysis of the angiogenic potential of L-PRF *in vitro*

As angiogenesis is a tightly regulated biological process involving a myriad of regulating proteins, it is not only important to identify the factors released by L-PRF but also to investigate the effect of L-PRF on endothelial behavior. For this purpose, multiple *in vitro* assays were performed in order to mimic the different steps involved in angiogenesis.

One of the first steps in angiogenesis is endothelial proliferation. Hence, the effect of L-PRF on endothelial metabolic activity and proliferation was investigated by means of an MTT assay and propidium iodide (PI) assay (Figure 3.4). Incubating HUVEC with L-PRF CM and L-PRF EX for 48 hours significantly increased the metabolic activity of the cells compared to the negative control, which is serum free culture medium (Figure 3.4A). Increasing the concentration of L-PRF CM or L-PRF EX did not seem to correspond with an increase in metabolic activity. Culture medium supplemented with 10% FBS served as a positive control.

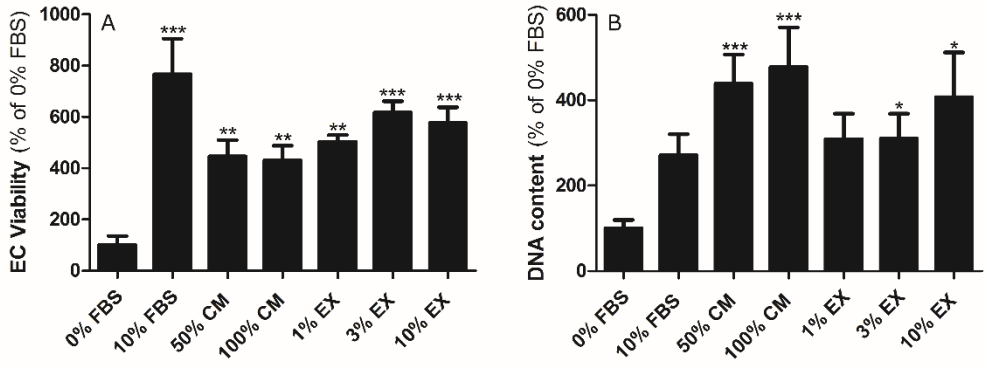

Figure 3.4 L-PRF enhances endothelial metabolic activity and proliferation. (A) L-PRF CM (n=10) and L-PRF exudate (n=8) enhance the metabolic activity of HUVEC as determined by MTT-assay.(B) Incubating HUVEC with L-PRF CM (n=9), 1% EX (n=7), 3% EX (n=9) and 10% EX (n=7) resulted in increased proliferation, based on DNA content, compared to the negative control (0% FBS, n=6). Data are represented as mean ± SEM. *** = p-value < 0.001, **= p-value < 0.01 and * = p-value <0.05 compared to 0% FBS. CM = conditioned medium; EC = endothelial cell; EX = exudate; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells.

Incubating HUVEC with L-PRF CM also resulted in an increased DNA content, and thus in the number of cells compared the negative control (Figure 3.4B). Exposure to L-PRF exudate resulted in a three- to four-fold increase in DNA content, but

only incubation with 3% and 10% exudate yielded a statistically significant increase. In contrast to the MTT assay, the effects of L-PRF CM and L-PRF EX did exceed the effect of 10% FBS with regard to DNA content (Figure 3.4B).

In response to chemotactic stimuli, endothelial cells migrate towards the site in need of vascularization. In order to determine the capacity of L-PRF to induce endothelial migration, a transwell migration assay was performed (Figure 3.5). HUVEC were seeded on top of semipermeable membranes and were allowed to migrate towards L-PRF CM, L-PRF EX or negative (0% FBS) or positive (10% FBS) control samples. Both L-PRF CM and L-PRF EX caused a significant increase in endothelial migration, compared to the negative control. Even when L-PRF CM was diluted 1:1 it elicited a similar response as pure CM and as the positive control. Migration towards L-PRF exudate showed to be dose dependent, with 10% exudate even exceeding the migratory response of the positive control containing 10% FBS.

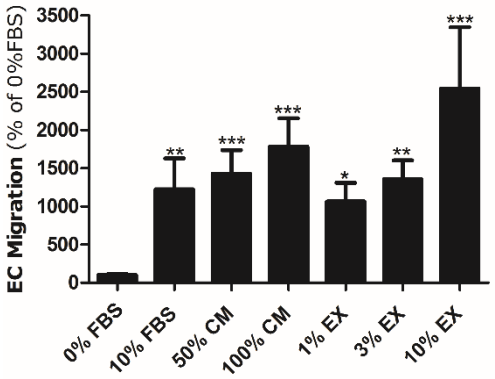

Figure 3.5 L-PRF induces endothelial migration *in vitro*. L-PRF CM (n=14) and L-PRF EX (n=8) induces endothelial migration in the transwell migration assay. Both 50% CM as well as the addition of 100% L-PRF CM results in a significant increase in endothelial migration, compared to the negative control (0% FBS, n=11). All three concentrations (1% - 3% - 10%) of exudate that were tested, were able to induce endothelial migration, in a dose dependent manner. 0% FBS = negative control; 10% FBS = positive control;. Data are represented as mean ± SEM. *** = p-value < 0.001, **= p-value < 0.01 and * = p-value <0.05 compared to 0% FBS. CM = conditioned medium; EC = endothelial cell; EX = exudate; FBS = fetal bovine serum.

Finally, the capacity of L-PRF to induce tubulogenesis was investigated using a Matrigel™ tube formation assay (Figure 3.6). Endothelial cells were incubated with L-PRF CM or L-PRF EX for 6 hours. Despite the two-fold increase in total branching length when HUVEC were exposed to LPRF-CM, only 1% L-PRF EX induces significantly more tubulogenesis compared to the negative control. Incubation with either 50% L-PRF CM or pure L-PRF CM resulted in a three-fold increase in the number of nodes whereas 1% L-PRF EX caused a four-fold increment.

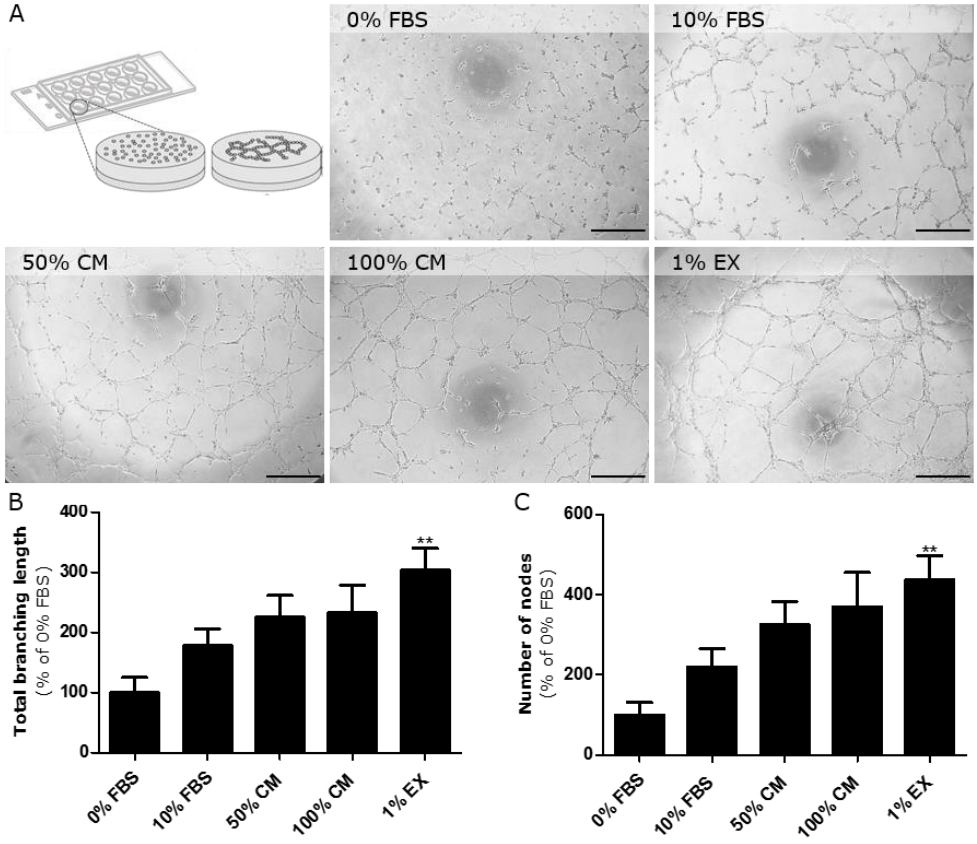

Figure 3.6 L-PRF induces endothelial tube formation *in vitro*. (A) Schematic overview of the tube formation experiment and representative images of endothelial tube formation after 6 hours of incubation with control medium (n=5), L-PRF CM (n=9) or L-PRF EX (n=7). CM Incubation with L-PRF CM and LPRF EX has a positive impact on endothelial tubulogenesis. Scale bar = 200 µM. (B) Incubating HUVEC with 1% L-PRF EX resulted in an increase in total branching length, whereas the increase in total branching length, caused by L-PRF was not significant. (C) The graph shows the average number of nodes, which was increased for all of the tested conditions except for the positive control. 0% FBS = negative control; 10% FBS = positive control;. Data are represented as mean ± SEM. **= p-value < 0.01 and * = p-value <0.05 compared to 0% FBS. CM = conditioned medium; EC = endothelial cell; EX = exudate; FBS = fetal bovine serum.

3.4.3 The role of CXCR-2 and EGFR in L-PRF induced angiogenesis

Investigation of the L-PRF secretome revealed an abundancy of EGF and IL-8, particularly in L-PRF CM. As shown previously, both L-PRF CM and L-PRF EX were able to induce endothelial proliferation, migration and tube formation. In the current section we investigated whether these pro-angiogenic effects were mediated via the CXCR-2 and EGFR pathways by using a CXCR-2 antagonist (SB225002) and an inhibitor for the EGFR (AZD8931). Incubating HUVEC with L-PRF CM for 48 hours resulted in a two-fold increase in cell viability. However, addition of 100 nM of SB225002, a selective inhibitor of CXCR-2, did not alter the L-PRF induced increase in viability, suggesting that CXCR-2 does not influence endothelial viability (Figure 3.7A). Incubating HUVEC with L-PRF CM caused a 5 to 6 fold increase in proliferation. Adding 50 nM of AZD8931, a reversible inhibitor of EGFR, ErbB2 and ErbB3, resulted in a 11.27% decrease in DNA content when added to 50% of CM and a 10.24% decrease when added to pure L-PRF CM (Figure 3.7B). Since L-PRF EX was shown to contain markedly less EGF compared to L-PRF CM, it was not surprising that adding AZD8931 to L-PRF EX did not alter the HUVEC response. Exposing HUVEC to 100 ng/mL EGF caused a four-fold increase in DNA content, which was decreased by 34.51% by adding 50 nM AZD8931.

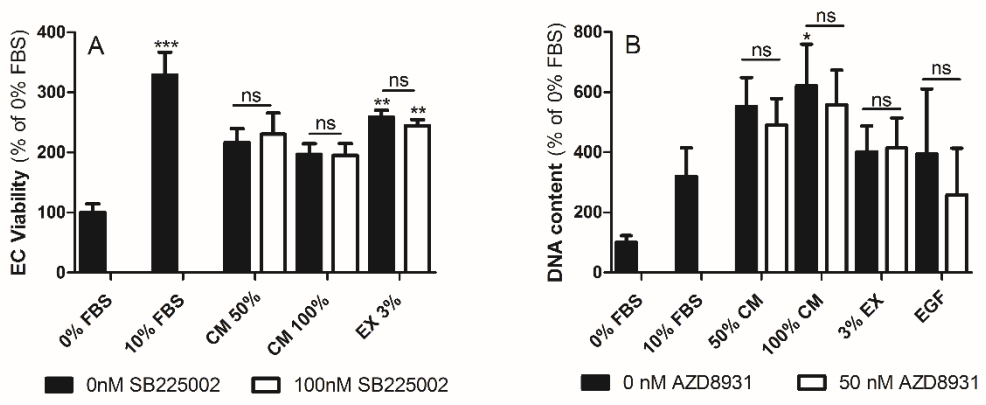

Figure 3.7 The effects of CXCR-2 antagonist SB225002 and EGFR inhibitor AZD8931 on HUVEC metabolism and proliferation. (A) Addition of 100 nM of SB225002 to L-PRF CM (n=6) or EX (n=8), did not alter the effects of L-PRF on HUVEC viability. (B) Addition of 50 nM of AZD8931 did not alter the endothelial response to L-PRF CM or L-PRF EX. However, incubating HUVEC with 100 ng/mL EGF caused a 4 fold increase in proliferation, which was reduced by 34.51% by adding AZD8931. Data are represented as mean ± SEM. *** = p-value < 0.001, ** = p-value < 0.01 and * = p-value <0.05. CM = conditioned medium; EGF = epidermal growth factor (100 ng/mL); EGFR = epidermal growth factor receptor; EX = exudate; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells; ns = non-significant.

To examine whether L-PRF induces endothelial migration via the CXCR-2 pathway or the EGFR pathway, migration experiments were performed after pre-incubating the endothelial cells either with 100 nM SB225002 to inhibit CXCR-2 (Figure 3.8A) or with 50 nM AZD8931 to inhibit the EGF receptor (Figure 3.8B). Pre-incubation with SB225002 did not alter the endothelial migration toward L-PRF CM. In contrast, when HUVEC were pre-incubated with 50 nM of AZD8931 and allowed to migrate toward 50% L-PRF CM, the migratory response was significantly lowered (Figure 3.8B). However, AZD8931 was not able to decrease endothelial migration towards 100% L-PRF CM. Data indicate that despite the minor role on endothelial proliferation, the EGFR pathway is involved in endothelial migration.

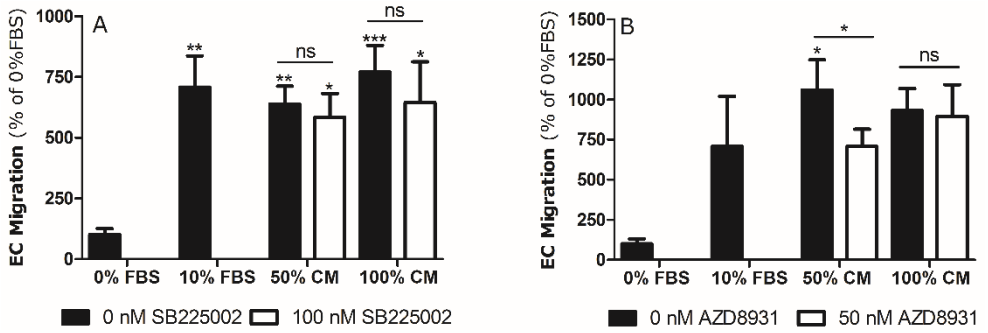

Figure 3.8 Endothelial migration towards L-PRF is partially mediated by the EGFR. (A) L-PRF CM induces endothelial migration with our without the addition the CXCR-2 antagonist SB225002 (n=8). (B) The migratory response of HUVEC towards 50% L-PRF CM (n=6) can be diminished by adding 50 nM of AZD8931 to inhibit the EGFR. However, adding 50 nM of AZD8931 to 100% L-PRF CM did not reduce HUVEC migration. Data are represented as mean ± SEM. *** = p-value < 0.001, ** = p-value < 0.01 and * = p-value <0.05. CM = conditioned medium; EC = endothelial cell; FBS = fetal bovine serum; HUVEC = human umbilical vein endothelial cells; L-PRF = leukocyte and platelet rich plasma; ns = non-significant.

In order to examine the role of CXCR-2 and EGFR in the induction of endothelial tubulogenesis, HUVEC were pre-incubated with the CXCR-2 antagonist SB225002 (n=7) or EGFR inhibitor AZD8931 (n=3), before seeding in L-PRF CM. No decrease in tube formation could be demonstrated following pre-incubation with 100 nM SB225002. Moreover, incubating HUVEC prior to exposure to 50% L-PRF CM even caused an increase in tube formation compared to untreated HUVEC which were incubated with 50% L-PRF CM (Figure 3.9A,B). Pre-incubating HUVEC with 50 nM AZD8931 had similar results. Adding the EGFR inhibitor did not alter the endothelial response to 100% L-PRF CM, but did increase the response to 50% L-PRF CM (Figure 3.9C,D).

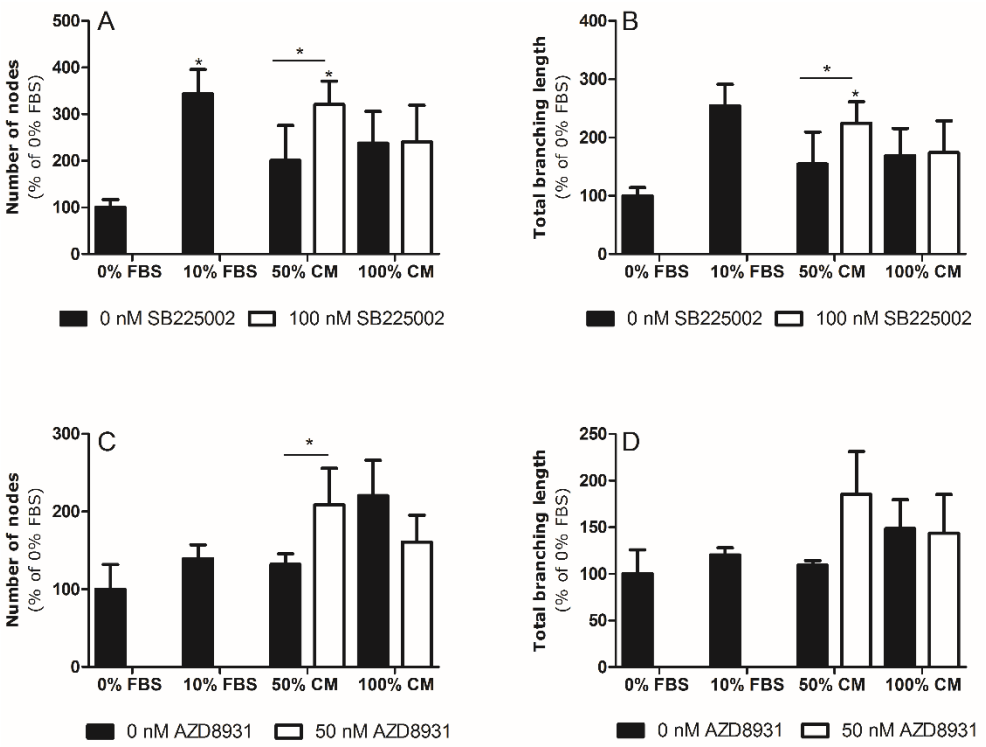

Figure 3.9 Endothelial tube formation is not mediated via CXCR-2 or EGFR. Surprisingly, pre-incubation of HUVEC with 100 nM SB225002 (A,B) or 50 nM AZD8931 (C,D) before exposure to 50% L-PRF CM seemed to increase the endothelial tubulogenesis. However, incubation with 100 nM SB225002 (n=7) or 50 nM AZD8931 (n=3) prior to cultivating HUVEC in 100% L-PRF CM did not alter tubulogenesis. Data are represented as mean ± SEM. * = p-value <0.05. CM = conditioned medium; FBS = fetal bovine serum

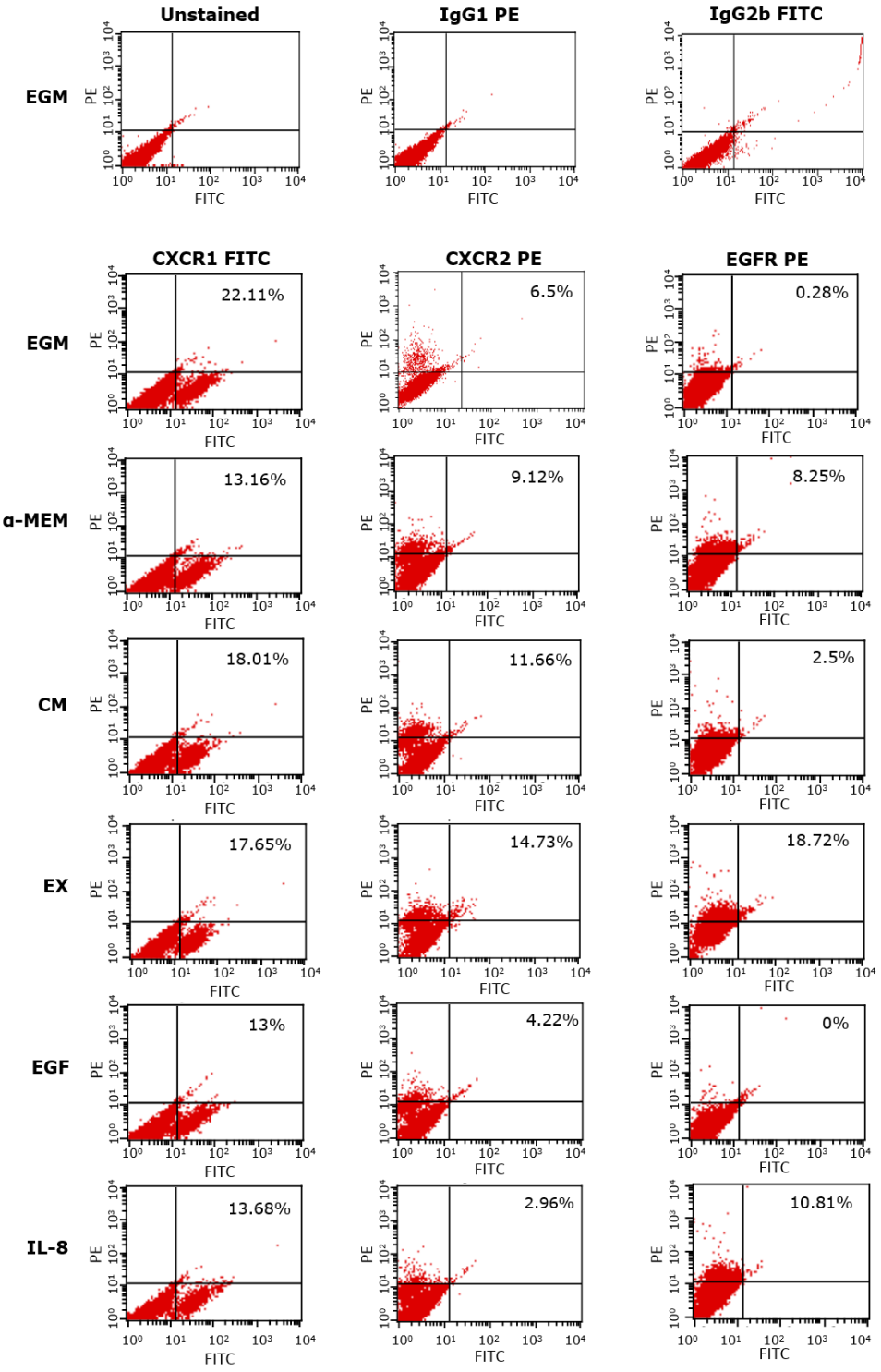
Unstained
IgG1 PE
IgG2b FITC
CXCR1 FITC
CXCR2 PE
EGFR PE
EGM
α-MEM
CM
EX
EGF
IL-8
22.11%
6.5%
0.28%
13.16%
9.12%
8.25%
18.01%
11.66%
2.5%
17.65%
14.73%
18.72%
13%
4.22%
0%
13.68%
2.96%
10.81%
PE
FITC

Figure 3.10 Flow cytometric analysis of HUVEC surface expression of CXCR-1, CXCR-2 and EGFR under different culture conditions. Exposure to EGF results in a down-regulation of the EGF receptor. Incubation with L-PRF CM and L-PRF EX upregulates the expression of CXCR-1 and CXCR-2 compared to α-MEM. α-MEM = Minimal Essential Medium, alpha modification; CM = conditioned medium; EGF = epidermal growth factor; EGFR = epidermal growth factor receptor; EGM = standard endothelial growth medium; EX = exudate; FITC = Fluorescein isothiocyanate; Ig = Immunoglobulin; IL-8 = Interleukin-8; PE = phycoerythrin.

In order to evaluate the expression levels of CXCR-1, CXCR-2 and EGFR on HUVEC, flow cytometric analysis was performed under standard culture conditions and after exposure to L-PRF CM or L-PRF EX (Figure 3.10). When HUVEC are kept in standard endothelial growth medium (EGM) supplemented with 10% FBS, only a minority of cells express CXCR-2 (6.5%) or EGFR (0.28%). Under these conditions 22.11% of cells express CXCR-1, also known as IL-8 receptor alpha. Replacing standard EGM medium with serum-free α-MEM led to a 8.9% decrease in cells expressing CXCR-1. However, when HUVEC were exposed to 50% L-PRF CM or 10% L-PRF EX for 24 hours, the number of cells expressing CXCR-1 only decreased by 4.1% and 4.46% respectively. Exposing HUVEC to 100 ng/mL EGF or 100 ng/mL IL-8 did not alter CXCR-1 expression levels compared to serum-free α-MEM. Under standard culture conditions only 6.5% of cells stained positive for CXCR-2, switching to serum-free α-MEM resulted in a 2.62% increase. However exposure to L-PRF CM and L-PRF EX further increased CXCR-2 expression levels to 11.66% and 14.73%. Supplementing α-MEM with either EGF or IL-8 lowered expression levels even further compared to standard culture conditions. In the presence of EGF, the expression of EGFR seems to be downregulated as is evidenced by the fact that EGF-stimulated HUVEC show no expression of EGFR and neither do HUVEC under standard culture conditions, since EGM is also supplemented with EGF. It is therefore also not surprising that only 2.5% of HUVEC stain positive for EGFR after exposure to L-PRF CM compared to 18.72% after incubation with L-PRF EX, which has been shown to contain markedly less EGF. The low expression levels of CXCR-2 and EGFR, when HUVEC are exposed to L-PRF CM, could explain the limited effects of SB225002 and AZD8931.

3.4.4 L-PRF induces blood vessel formation *in ovo*

L-PRF has been shown to improve endothelial proliferation, migration and tube formation *in vitro.* However, these are only small interdependent steps of the angiogenic process. To investigate whether L-PRF is also capable of inducing blood vessel formation *in vivo,* a CAM assay was performed. Following three days of incubation, a characteristic spooks wheel pattern of blood vessels was seen in every condition. Both L-PRF CM and L-PRF EX significantly increased blood vessel formation compared to the control condition. Exudate and conditioned medium were dissolved in Matrigel™ in a 1:1 ratio. For this reason, the Matrigel™ droplets serving as negative controls, were also diluted in the same ratio and in the same culture medium that was used for the production of L-PRF CM.

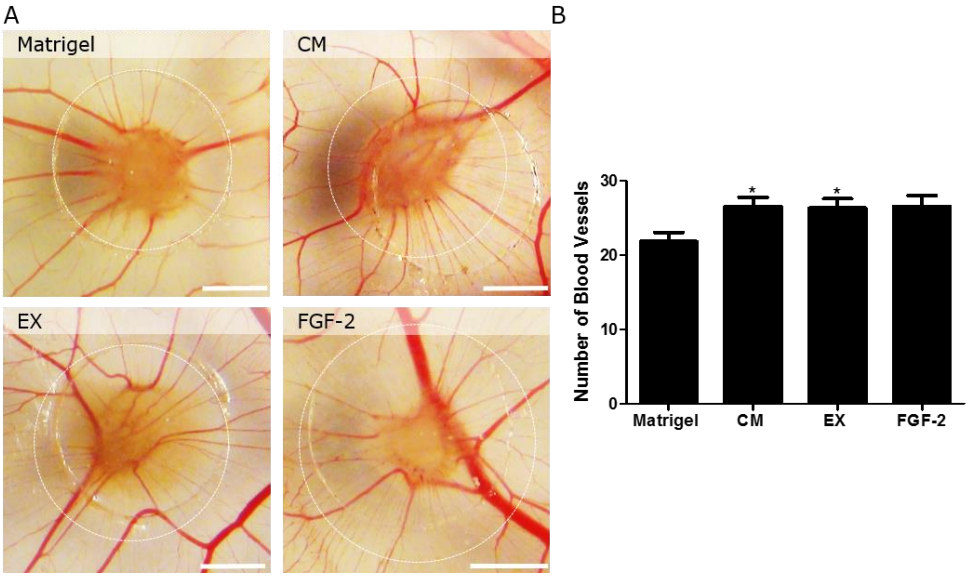

Figure 3.11 L-PRF induces blood vessel formation *in ovo*. (A) Representative images of chorioallantoic membranes after 3 days of incubation with growth factor-reduced Matrigel™ (n=46) or with Matrigel™ containing L-PRF CM (n=57), L-PRF EX (n=49) or 500 ng FGF-2 (n=28) as a positive control. Scale bar = 2 mm. (B) Average number of blood vessels intersecting with the circle. L-PRF CM and L-PRF EX increased the number of capillaries. Data are represented as mean ± SEM. * = p-value <0.05. CM = conditioned medium; EX = exudate; FGF-2 = fibroblast growth factor-2.

Due to the fact that even growth-factor reduced Matrigel™ still contains growth factors, the number of blood vessels in the negative control of the previous experiment was rather high. Furthermore, the exact mixture and concentration of the growth factors present in Matrigel™ is unknown and can show batch-to-batch differences. For these reasons the next experiment was carried out with a growth factor-free gel, namely pluronic F-127. Compared to untreated eggs, incubation with pluronic F-127 did not induce blood vessel formation. Fibrin is one of the major components of L-PRF which is responsible for the slow release of growth factors. But fibrin itself has also been shown to induce blood vessel formation or wound healing. In order to determine the role of the fibrin matrix in the angiogenic capacities of L-PRF, eggs were treated with a fibrin gel, based on human fibrinogen and thrombin. Since the previous CAM experiment (Figure 3.11) used Matrigel™ as a vehicle for the delivery of L-PRF CM and L-PRF exudate, the experimental setup did not allow the evaluation of the role of the fibrin matrix in enhancing blood vessel formation. Therefore, the eggs were incubated with 6 mm discs made from L-PRF membranes (Figure 3.12). By using the L-PRF membranes, the CAMs are not only exposed to the factors which are released from the L-PRF but also to the fibrin network. Incubation with L-PRF membranes resulted in a significant increase in the number of blood vessels, compared to untreated eggs and eggs treated with pluronic F-127 gel.

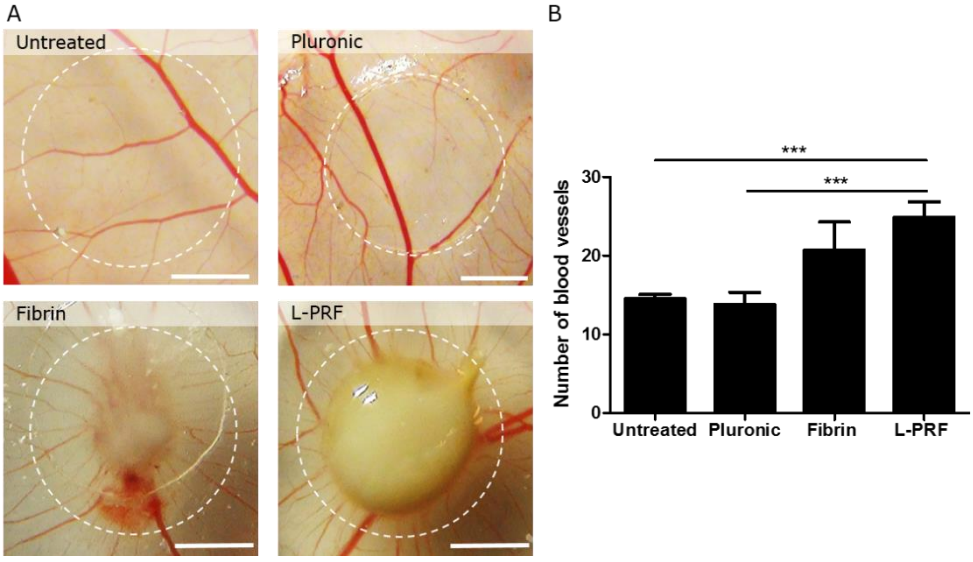

Figure 3.12 L-PRF membrane punches induce blood vessel formation *in ovo.* (A) Representative images of chorioallantoic membranes at E12. Untreated membranes (n=21) were left completely untreated while others were incubated for 3 days with pluronic F-127 gel (n=13), fibrin gel (n=10) or with L-PRF membrane discs (6mm; n=24). Scale bar=2 mm. (B) Average number of blood vessels intersecting with the circle. Eggs incubated with L-PRF membranes showed significantly more blood vessels compared to control CAMs or CAMs treated with pluronic F-127 gel. Data are represented as mean ± SEM. *** = p-value <0.001. CAMs = chorioallantoic membranes L-PRF = leukocyte and platelet rich fibrin; E12 = embryonic day 12.

3.5 Discussion

Angiogenesis is indispensable for wound healing and tissue regeneration. However, impaired or deregulated angiogenesis can also contribute to the pathogenesis of cancer, ischemic diseases or chronic wounds [1, 233]. Platelet-derived growth factors play an important role in tissue regeneration and revascularization [238].

This study investigated the angiogenic potential of L-PRF and its growth factor release. Since the main goal of the this study entailed the evaluation of the angiogenic potential of L-PRF, the first growth factor that was investigated was VEGF. The levels of VEGF release were determined at different time points. Data showed 45 times more VEGF in L-PRF conditioned medium harvested after 96 hours compared to L-PRF exudate. This rather large discrepancy, can probably be ascribed to growth factor entrapment in the fibrin matrix. During centrifugation, polymerization of the L-PRF clot occurs slowly, creating a flexible fibrin network which supports cytokine enmeshment, resulting in a slow and gradual growth factor release [251-253]. Apart from cytokine entrapment, the production of VEGF by leukocytes could also partly account for the difference in VEGF levels between L-PRF CM and L-PRF EX [206]. Moreover, incubation of the L-PRF clot for 96 hours resulted in a two-fold increase in VEGF, compared to the CM harvested after 48 hours. However, incubating L-PRF for an additional 48 hours, did not cause a further increase in VEGF levels. This is in contrast with other studies reporting a sustained VEGF release up to seven days [206, 254]. However, in the study by Ehrenfest *et al.* VEGF release from compressed L-PRF membranes was evaluated, in contrast to the uncompressed L-PRF clots used in this study. Compression of the fibrin matrix could affect it architecture and therefore influence cytokine entrapment and release kinetics [255]. Furthermore, in these studies the medium was repeatedly renewed which further stimulates cytokine secretion since every medium change creates a cytokine-poor environment [254]. Unfortunately, *in vitro* studies cannot account for the influence of the physiological environment on the behavior of the platelet concentrate with regard to cellular crosstalk and growth factor release [206]. However, *in vitro* characterization of these platelet concentrates remains an important step towards better understanding their effects *in vivo*.

To further investigate the plethora of factors released from L-PRF, a cytokine antibody array was performed, allowing the simultaneous detection of 80 different proteins. This array was performed on L-PRF EX and L-PRF CM harvested after 96 hours, since ELISA data showed no increase in VEGF release past this time point. The protein array revealed an abundancy of ENA-78 (CXCL5), GRO (CXCL1), IL-8 (CXCL8) and NAP-2 (CXCL7) in L-PRF CM. All these factors bind to CXCR-2, also known as IL-8 receptor beta, with high affinity [256-258]. These cytokines belong to the glutamic acid-leucine-arginine positive (ELR_+) subfamily of CXC chemokines. These ELR_+ cytokines have been reported to have pro-angiogenic effects which are mediated via CXCR-2 [259, 260]. Since a cytokine array only indicates relative expression levels, a more precise quantification of IL-8 release was performed. L-PRF exudate contained on average 4.7 pg/mL IL-8, which is in range with previous reports of IL-8 serum levels [261-263]. However, IL-8 concentrations were substantially higher in L-PRF CM (7.7 ng/mL). The high levels of IL-8 in L-PRF CM are probably due to the induction of IL-8 production by fibrin. Fibrin has been described to induce IL-8 secretion in oral squamous cell carcinoma cells [264], HUVEC [265] and neutrophils [266]. Besides the ELR_+ cytokines, the protein array also revealed the presence of relatively high amounts of EGF. Quantification by means of ELISA demonstrated a 5 times higher concentration of EGF in L-PRF CM compared to L-PRF EX. However our results demonstrated only an average of 3.3 pg/mL EGF in L-PRF CM after 96h, which is substantially less than the reported serum levels ranging from 200 pg/mL [267] to ~1 ng/mL [268] and even higher [269]. Bertrand-Duchesne *et al.* reported high levels of EGF (513 pg/mL) in human platelet rich plasma (PRP)[232]. The low levels detected in this study are probably due to the short half-life and the rapid cell diffusion of EGF [270]. Since PRP supernatant was stored at -85°C immediately after production this would not have been an issue in the study of Bertrand-Duchesne and colleagues and could therefore account for the discrepancy between their study and the results reported here.

Besides investigating the protein release of L-PRF, this study also examined the angiogenic potential of L-PRF *in vitro.* Different *in vitro* experiments were performed in order to evaluate the effect of L-PRF on endothelial proliferation, migration and tube formation, which represent the main phases of the angiogenic process. Results from the current study demonstrated the ability of L-PRF CM and

L-PRF EX to increase endothelial viability and proliferation. These results are in line with a other reports showing that both human [232, 234, 271] and murine [233] platelet derivatives induce endothelial proliferation. The next crucial step in the angiogenic process is endothelial migration. The migratory response of HUVEC towards L-PRF CM or L-PRF EX was investigated by means of a transwell migration assay. This study confirmed the ability of L-PRF to induce endothelial migration in a dose dependent matter as previously demonstrated by Schär *et al.* [206]. Furthermore, L-PRF was also shown to induce endothelial tube formation. Although PRP has already been reported to induce endothelial tubulogenesis, to our knowledge this is the first study to report similar effects of L-PRF CM and L-PRF CM [235, 271, 272].

In order to investigate whether the pro-angiogenic effects of L-PRF were mediated by the CXCR-2 or EGFR pathway, *in vitro* experiments were repeated with the addition of SB225002, a selective CXCR-2 antagonist or AZD8931, a reversible inhibitor of EGFR. Addition of SB225002 did not lower HUVEC viability. While incubating endothelial cells with AZD8931, decreased their response to EGF by 34.51% it did not cause a considerable decrease in their response to L-PRF CM or L-PRF EX. Furthermore, the addition of SB225002 to HUVEC did not influence endothelial migration towards L-PRF CM. However, addition of AZD8931 did cause a significant decrease in the migratory response to 50% L-PRF CM but not to 100% L-PRF CM. Possibly the used concentration of AZD8931 was not sufficient to inhibit EGF in the 100% CM condition.

Unexpectedly, both SB225002 or AZD8931 increased endothelial tubulogenesis, when cells were incubated with 50% L-PRF CM. Except for a decreased number of nodes after incubation with AZD8931, none of the inhibitors influenced the response to 100% L-PRF CM.

A possible explanation to our data is that inhibition of the EGFR or the CXCR-2 receptors induces compensatory expressions of other growth factor receptors and thus increases tube formation. The numerous growth factors could have a synergistic effect to induce endothelial behavior. Using a combination of SB225002 and AZD8931 or a combination of AZD8931 and an inhibitor of VEGF, could help to further elucidate the pathways involved.

The large variety of growth factors released by L-PRF could explain the inability to reduce the endothelial response to L-PRF by inhibiting just one receptor. However, by inhibiting CXCR-2 we aimed to eliminate the effect of four abundant proteins (ENA-78, GRO, IL-8 and NAP-2) at once. Another possibility is of course that HUVEC do not express CXCR-2 or that the abundance of ligands downregulates CXCR-2. While there are some reports stating that IL-8 induces HUVEC proliferation [273, 274], migration and tube formation [273-275], according to Petzelbauer *et al.* IL-8 does not directly increase HUVEC proliferation and migration [276]. Furthermore, expression of IL-8 receptors may be dependent on culture conditions. Results from the flow cytometric analysis performed in this study actually support this idea, since different culture conditions led to differences in receptor expression. Under standard culture conditions only 6.5% of HUVEC stained positive for CXCR-2 and expression of CXCR-2 actually increased when cells were exposed to L-PRF CM or L-PRF EX. This increase in CXCR-2 is not necessarily mediated by the presence of IL-8 since stimulating HUVEC with IL-8 caused a further decrease in the amount of cells expressing CXCR-2. Since IL-8 is known to bind to CXCR-1 the expression of this receptor was also evaluated. Under standard culture conditions 22.1% of HUVEC expressed CXCR-1. As is the case for CXCR-2, data on CXCR-1 expression of HUVEC are also controversial. Our data are in line with reports by Salcedo *et al*. who showed that only a small portion of HUVEC express low levels of CXCR-1 [277]. In contrast, other studies reported higher levels of CXCR-1 expression in HUVEC [274, 278]. Evaluation of EGFR expression revealed almost no endothelial cells expressing EGFR under standard culture conditions and EGFR was completely absent after stimulation with EGF for 24 hours. This is in line with extensive literature reporting rapid internalization and degradation of EGFR after exposure to EGF, possibly to adjust the cell's subsequent sensitivity to EGF [279-282]. Since L-PRF CM contained higher levels of EGF compared to L-PRF EX, it is therefore not surprising that incubation with CM leads to lower expression levels compared to L-PRF EX. However, flow cytometric analysis was only performed on one HUVEC population as a pilot experiment and should be repeated before drawing any conclusions.

In order to evaluate the ability of L-PRF to induce blood vessel formation *in vivo* a CAM assay was performed. Despite the significant increase in the number of

blood vessels, the vehicle treated membranes also showed a substantial amount of blood vessels. For this reason a second experiment was performed, in which Matrigel™ was replaced by pluronic F-127. Pluronic F-127 is a non-toxic thermoreversible hydrogel, which is completely free of growth factors and has already been used for drug delivery [283]. Treating eggs with pluronic F-127 did not increase the number of blood vessels compared to untreated membranes. Furthermore, there are distinctly less blood vessels in pluronic-treated eggs as there are in Matrigel™ treated membranes, indicating that pluronic F-127 is a more inert vehicle compared to Matrigel™. Up until now this study has mainly been focusing on the angiogenic potential of the L-PRF secretome. However, growth factors are only one aspect of L-PRF that can be beneficial for blood vessel formation. Fibrin matrix has been demonstrated to induce angiogenesis and guide the coverage of damaged tissues by influencing epithelial cells and fibroblasts [284]. Therefore, a second CAM assay included a fibrin gel derived from human fibrinogen and thrombin and intact L-PRF membranes, in order to combine the fibrin matrix with the platelet derived growth factors and to include the leukocytes. The human-derived fibrin gel was able to induce blood vessel formation *in ovo* which is in accordance with a report by Smith *et al.* [285]. Incubating eggs with L-PRF membranes increased the number of blood vessels to a comparable level as L-PRF CM and L-PRF EX did in the previous experiment. The increased angiogenesis cannot be ascribed to an inflammatory response as a reaction to the xenogeneic origin of L-PRF since the chick embryo lacks a mature immune system at this point in the development [286].

In conclusion, this study demonstrates a strong pro-angiogenic effect of L-PRF *in vitro* and *in ovo*. This study aimed to identify the growth factors released from L-PRF and the key mediators of the pro-angiogenic effects of L-PRF. Despite the high levels of CXCR-2 ligands and EGF, these pathways did not seem to be indispensable for stimulating endothelial proliferation, migration or tube formation. Due to the variety in preparation protocols of platelet derivatives it is very difficult to compare biomolecule composition and angiogenic potential and thus obtaining a detailed characterization remains a challenge. Despite these challenges our data suggest a promising role for platelet concentrates in the clinical setting of wound healing and tissue regeneration.

Chapter 4

In vivo Wound Healing Capacities of Leukocyte and Platelet Rich Fibrin

4.1 Abstract

Leukocyte and platelet rich fibrin (L-PRF) unites all components (i.e. platelets, leukocytes, fibrin) of a blood sample, favorable for blood vessel formation and healing, into one biological product. As described in the previous chapter L-PRF contains numerous growth factors able to promote angiogenesis. The objective of this study was to investigate the ability of Matrigel™ impregnated with human-derived L-PRF conditioned medium (CM) and L-PRF exudate (EX) to enhance wound healing. A full thickness wound was created on the dorsum of BALB/cJRj mice and splinted in order to counteract wound contraction. Evaluation of wound sizes over time revealed that Matrigel™ impregnated with L-PRF CM or L-PRF EX did not enhance wound closure compared to vehicle treated animals. In order to explore the role of the supporting fibrin matrix in the wound healing capacities of L-PRF, follow-up studies were performed in which wounds were treated with L-PRF membranes or a fibrin gel. While L-PRF conditions or the fibrin gel did not have any detrimental effects on murine wound healing, they were also not able to transcend the natural wound healing capacities of control mice treated with a growth factor-free pluronic F-127 gel. Histological analysis revealed almost complete re-epithelization of wounds in control animals as well as more infiltrating cells and less collagen deposition in fibrin and L-PRF treated animals. In conclusion, human-derived L-PRF has no detrimental effects on murine wound healing but it does also not offer an advantage over natural wound healing in healthy mice.

4.2 Introduction

The primary function of the skin is to act as a protective shield against the environment [287, 288]. The skin protects against water loss, penetration of harmful UV radiation and serves as a first line of immunological defense against intruding pathogens [288, 289]. Due to the constant exposure to potential injury, an optimized wound healing process is indispensable for the survival of all higher organisms [289]. Wound healing is a dynamic and intricate process involving crosstalk between blood vessels, epidermis, dermis, the nervous system and subcutaneous tissue layers [290]. Wound healing is a conserved evolutionary process which can be divided into three major stages: the inflammatory phase followed by the proliferation phase and finally the remodeling phase [289, 290]. However, these three phases can overlap both spatially and temporally [287, 289].

All these processes are regulated by a multitude of growth factors and cytokines [291]. Identifying key growth factors could offer new strategies for the application of exogenous growth factors to improve clinical outcomes of non-healing wounds [291, 292]. These factors are responsible for regulating the immune response as well as promoting angiogenesis. Angiogenesis is vital for wound repair as it allows the removal of debris and provides nutrients, oxygen and immune cells to the wound bed [293]. Several growth factors have already been investigated in clinical trials including: platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2) and granulocyte-macrophage colony stimulating factor (GM-CSF) [291]. Despite the high expectations, delivery of exogenous growth factors has had only limited success with regard to improved clinical outcomes [294]. Due to the involvement of a multitude of growth factors, it is not surprising that treatments based on the delivery of one single growth factor have only had minimal success. The lack of improved clinical outcomes is probably due to the short half-life of the delivered growth factors and the hostile environment, all contributing to the quick degradation of the treatment [292].

Successful wound treatment not only aims for rapid wound closure but also aims for a functional and aesthetically satisfying scar [287]. Unfortunately, the number of patients suffering from impaired wound healing (e.g., diabetes) is reaching

epidemic proportions [291, 293, 295, 296]. Clinical research often struggles with demonstrating the efficacy of treatments in large-scale clinical trials due to the complex and heterogeneous patient population [295]. Furthermore, preclinical research is hampered by the search for suitable animal models which correctly mimic human conditions [295]. A wide range of animal species have been used for the study of cutaneous wound repair, but the laboratory mouse remains by far the most used animal model for biomedical research [296]. Despite the fact that murine skin consists of the same three layers (epidermis, dermis and hypodermis) as human skin, a few differences between mouse skin and human skin need to be taken under consideration (Figure 4.1). Apocrine sweat glands and rete ridges/ dermal papillae are absent in murine skin but are found in human skin. A unique characteristic of mouse skin is de the presence of a *panniculus carnosus*, a thin muscle layer underneath the dermis, which causes rapid wound contraction after injury. Human skin, in contrast, heals via re-epithelialization and the formation of granulation tissue.

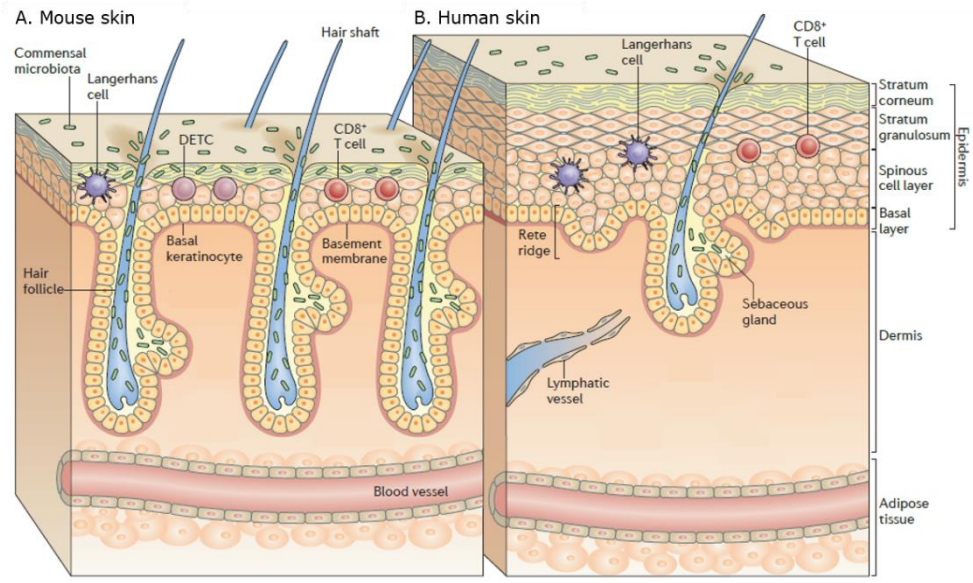

Figure 4.1 Schematic comparison between human skin and murine skin. Human skin has a thicker dermis with protruding epidermal rete ridges and a thicker epidermis with more cell layers compared to mouse skin. Adapted from Pasparakis *et al*. [288].

A commonly used mouse models to study wound repair is the excisional wound model. In order to mimic the circumstances during human wound repair as closely as possible, the created wounds extend through the *panniculus carnosus* and a silicone splint is sutured around the wound thereby minimizing wound healing via contraction. This relatively simple animal model allows for a large sample size, easy access for the application of topical agents and the possibility of histological examinations of the wounds after harvest [296].

Growth factors derived from platelets play a pivotal role in wound repair and neovascularization [238]. Autologous platelet derivatives such as leukocyte and platelet rich fibrin (L-PRF), combine the growth factors released from platelets with a fibrin matrix and may serve a resorbable membrane [297]. The effects of PRF on soft tissue healing and angiogenesis have been investigated using different animal models [239]. Suzuki and colleagues showed that PRF accelerated wound healing and angiogenesis in rats [298]. While Roy *et al.* found that PRF significantly improved angiogenesis after 14 days in a porcine model of ischemic excisional wounds [238]. PRF was also shown to improve skin graft take in pigs [299]. Together these studies demonstrate the improved soft tissue healing under the influence of PRF, probably mediated by enhanced angiogenesis at the site of injury [239]. Furthermore, a first wave of clinical research has provided evidence that PRF seems to promote soft tissue healing, however results need to be interpreted with caution due to missing controls and the lack of larger randomized controlled trials [239]. Moreover, to date little is known about the effects of fibrin architecture and leukocyte content on tissue regeneration [239]. Future studies should therefore focus on identifying the impact of the fibrin matrix and leukocyte content of L-PRF on tissue regeneration and clinical studies should be designed with the inclusion of appropriate controls [239].

In the current study full thickness wounds were created on the dorsum of BALB/cJRj mice and a silicone splint was used to counteract wound contraction. The objective of this study was to investigate the ability of L-PRF conditioned medium and L-PRF exudate to enhance wound healing. Mice were also treated with L-PRF membranes or a fibrin gel in order to evaluate the wound healing capacities of fibrin.

4.3 Materials and Methods

4.3.1 Production of Leukocyte and Platelet Rich Fibrin

Blood samples were obtained from 3 healthy (2 male and 1 female) volunteers with written informed consent. This study protocol and consent procedure was approved by the medical ethical committee from Hasselt University and the Clinical Trial Center from KU Leuven (S58789 / B322201628215). Blood samples were collected by means of a venipuncture, and centrifuged immediately for 12 minutes at 2700 rpm as described in chapter 3 (3.3.1).

For the preparation of conditioned medium (CM), L-PRF clots were incubated in 6 mL of serum-free Minimal Essential Medium, alpha modification (α-MEM) for 96 hours as described in chapter 3 (3.3.1). To collect exudate, L-PRF clots were transferred to a sterile box and transformed into thin membranes thereby releasing the exudate. The exudate was collected and sterile filtered as described in chapter 3 (3.3.1). By means of a biopsy punch (Stiefel, Middlesex, UK) 8 mm disks were created out of the L-PRF membranes.

4.3.2 Full thickness excisional wound model

All experimental procedures were approved by the ethical committee for animal experiments from Hasselt University and were performed according to the guidelines described in Directive 2010/63/EU on the protection of animals used for scientific purposes. Male BALB/cJRj mice (n=48) (Janvier, Le Genest-Saint-Isle, France) were housed individually in a temperature-controlled room (20 ± 3 °C) on a 12 h light-dark schedule and with food and water ad libitum.

Wounds were created at the age of 11 weeks and mice were anesthetized with 2% isoflurane (IsofFlo, Abbot Animal Health, Belgium). The day before surgery, dorsal hair was shaved, followed by a depilatory cream (Veet, Reckitt Benckiser Group, Slough, UK) in order to remove the remaining short hairs. For the induction of full thickness wounds, the dorsum was disinfected using an iodine solution and the pattern of the wound was traced using a biopsy punch (Stiefel) with a diameter of 6 mm. Full thickness wounds protruding through the *panniculus carnosus* were made using curved Vannas spring scissors (Fine Science Tools, Heidelberg, Germany). A doughnut-shaped silicone splint (Thermo Fisher Scientific, Erembodegem, Belgium) with an external diameter of 8 mm was placed over the wound and fixed using Histoacryl tissue glue (B. Braun, Diegem, Belgium). Next,

the splint was sutured into place using 6/0 polyamide sutures (Ethilon, Johnson & Johnson Medical, New Brunswick, NJ, USA). The wound was covered with a semi-occlusive dressing (Opsite Flexifix, Smith&Nephew, London, UK) and dressed with a self-adhesive bandage (Peha-haft, Hartmann, Heidenheim an der Brenz, Germany).

Depending on the experiment, L-PRF CM or L-PRF exudate was diluted in either growth factor-reduced Matrigel™ (BD Biosciences) or in a 18% pluronic F-127 gel (Sigma-Aldrich). Fifty microliters of gel was applied to the wound every two or three days, depending on the experiment, starting from the day of wound induction. Animals treated with fibrin gel received a gel consisting of 20 mg/mL human fibrinogen (Merck, Darmstadt, Germany), 2.5 U/mL human thrombin (Merck) and 20 mM of $CaCl_2$ (Sigma-Aldrich) dissolved in PBS. For the animals treated with an L-PRF membrane, 8 mm circles were punched out of the L-PRF membranes as described above, using an 8 mm biopsy punch (Stiefel). L-PRF punches were placed into the wound and the edges were secured to the dermis using Histoacryl tissue glue. When animals lost the silicone splint during the course of the experiment, they were excluded from the analysis. Without the silicone splint, wound healing can occur through wound contraction instead of the formation of granulation tissue.

4.3.3 Macroscopic analysis of wounds

Dressings were removed every other day, unless stated otherwise. Wounds were photographed under isoflurane anesthesia using a stereomicroscope (Leica, Wetzlar, Germany). All treatments except for the L-PRF membranes were reapplied when the wounds were photographed. Images were used to macroscopically analyze wound size using Image J software (National Institutes of Health, Bethesda, MD, USA). This analysis was performed starting from the day of surgery until day 14 or 15.

4.3.4 Histological stainings

Skin samples were fixed in 4% paraformaldehyde for 24 hours after which the samples were dehydrated in graded alcohol and embedded in paraffin. Tissues were cut into 7 µm sections and a Masson's trichrome staining was performed. Tissue sections were scanned with a Mirax digital slide scanner (Carl Zeiss Vision, Aalen, Germany). Re-epithelization was quantified in a blinded fashion by two

researchers by means of Case Viewer software (3DHISTECH Ltd., Budapest, Hungary). The amount of cellular infiltration and collagen deposition was quantified based on Masson's trichrome staining of skin biopsies using the AxioVision software 4.6.3 (Carl Zeiss Vision, Aalen, Germany). Counterstaining with Hematoxylin allowed the quantification of cellular infiltrates based on the area (pixels²) of the total wound bed, consisting of nuclei. Collagen deposition was quantified based on the Masson's trichrome staining, by calculating the percentage of collagen content in the wound bed, normalized to the wound area.

4.3.5 Statistical analysis

Statistical analysis was performed using Graphpad Prism software 5.03 (Graphpad Software, La Jolla, CA). All data were expressed as mean ± standard error of the mean (SEM). Data of wound area quantification were to be analyzed by means of a two-way ANOVA with a Bonferroni post-test for multiple comparisons. However, excluding animals due to splint loss, resulted in missing data points, making it impossible to perform a two-way ANOVA.

4.4 Results

4.4.1 Human-derived L-PRF does not enhance wound healing compared to vehicle treated animals

In order to evaluate the wound healing capacities of L-PRF, a pilot study was performed to compare wound healing between control mice treated with growth factor-reduced Matrigel™ (n=6) and mice treated with growth factor-reduced Matrigel™ enriched with either L-PRF CM (n=6) or LPRF-EX (n=6). Wounds were photographed every other day and both control and treatment gels were reapplied. Quantitative analysis of the wound area confirmed a gradual decreasing wound size over time (Figure 4.2). More importantly, images show a gradual wound healing in all three groups without any signs of infection over a period of 14 days (Figure 4.3). Unfortunately, L-PRF treatments did not seem to accelerate wound closure. After 14 days none of the groups showed complete wound closure. In the control group the average wound area after 14 days of healing was reduced by 79.1 % while L-PRF CM treatment reduced wound size by 66.8% and L-PRF EX was responsible for a 48.7% reduction. Furthermore, after 14 days every group was reduced to three animals, because animals were excluded from the analysis when they lost the silicone splint (Table 4.1).

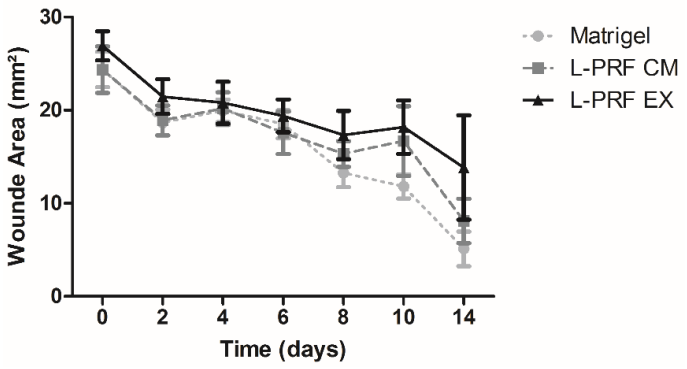

Figure 4.2 Quantitative evaluation of wound area over time. Quantification of wound area shows a gradual decrease of wound size over time in all three groups. However, none of the groups reached complete wound closure at day 14. Data are represented as mean±SEM. L-PRF = leukocyte and platelet rich fibrin; CM = conditioned medium; EX = exudate.

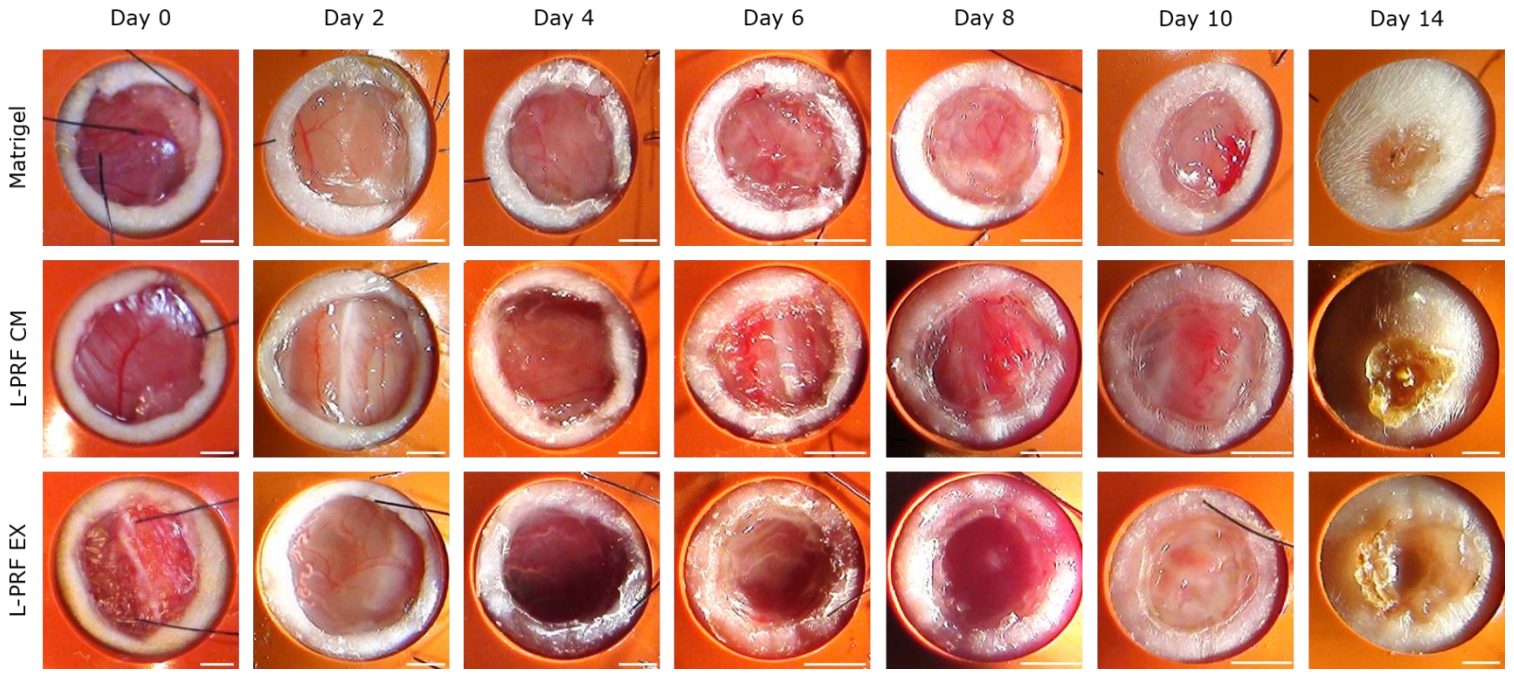

Figure 4.3 Representative images of the time course of macroscopic wound healing in mice treated with L-PRF CM or L-PRF EX. Wound size steadily decreases in all three groups, however none of the groups reach complete wound closure after 14 days. Scale bar = 2 mm. L-PRF = leukocyte and platelet rich fibrin; CM = conditioned medium; EX = exudate.

Table 4.1 Wound area over time and the number of animals included in the analysis.

	MATRIGEL		L-PRF CM		L-PRF EX	
	Wound Area	Mice (n)	Wound Area	Mice (n)	Wound Area	Mice (n)
Day 0	24.4 ± 1.9	6	24.4 ± 2.5	6	26.9 ± 1.6	6
Day 2	18.7 ± 1.4	6	18.9 ± 1.6	5	21.5 ± 1.8	6
Day 4	19.9 ± 1.2	6	20.2 ± 1.7	5	20.8 ± 2.2	6
Day 6	18.5 ± 1.5	5	17.6 ± 2.3	4	19.4 ± 1.8	5
Day 8	13.3 ± 1.5	5	15.3 ± 1.4	5	17.3 ± 2.6	5
Day 10	11.8 ± 1.3	4	16.7 ± 3.7	4	18.2 ± 2.9	4
Day 14	5.1 ± 1.9	3	8.1 ± 2.4	3	13.8 ± 5.6	3

Wound area (mm^2) is expressed as mean ± SEM. L-PRF = leukocyte and platelet rich fibrin; CM = conditioned medium; EX = exudate.

4.4.2 Wound healing capacities of L-PRF membranes

In a second set of *in vivo* experiment, growth factor-reduced Matrigel™ was replaced by pluronic F-127 gel, which is completely free from growth factors. In this study animals were treated with L-PRF membranes, in order to investigate the influence of the fibrin matrix of L-PRF on wound healing. A fibrin gel, composed of human-derived fibrinogen and thrombin was used as a control group. In order to secure the L-PRF membrane punches, small drops of Histoacryl tissue glue were placed at the edge of the wound. Since L-PRF membranes do not need to be replaced and fibrin gel has a slower resorption rate compared to Matrigel™, wounds were only photographed at 3-day intervals, at which point pluronic gel and fibrin gel was reapplied but L-PRF membranes were left untouched.

Quantitative analysis showed a steady decrease in wound size, with an almost complete wound healing (92%) in control animals treated with pluronic F-127 gel (Figure 4.4). Treatment with a human-derived fibrin gel resulted in an average wound reduction of 75.4% while treatment with L-PRF membranes only reduced wound size with 55.3% compared to the initial wound size at the day of surgery. In this study only one animal per group was excluded from the analysis due to the loss of the silicon splint (Table 4.2).

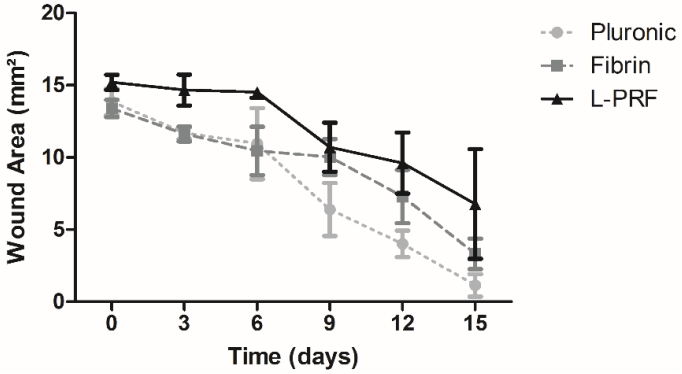

Figure 4.4 Quantitative analysis of wound area over a 15-day period. Quantification of wound area shows a gradual decrease of wound size over time in all three groups. However, none of the groups reached complete wound closure at day 15. Data are represented as mean±SEM. L-PRF = leukocyte and platelet rich fibrin.

Despite the presence of human leukocytes in the L-PRF membranes, macroscopic images of the wounds show no external signs of inflammation or swelling. At day 6, granulation tissue was present in pluronic and fibrin groups (Figure 4.5). In wounds treated with L-PRF membrane, it was difficult to evaluate the presence of granulation tissue macroscopically.

Table 4.2 Wound area over time and number of animals included in the analysis.

	PLURONIC		**FIBRIN**		**L-PRF**	
	Wound Area	Mice (n)	Wound Area	Mice (n)	Wound Area	Mice (n)
Day 0	13.8 ± 0.9	4	13.4 ± 0.6	4	15.2 ± 0.5	4
Day 3	11.7 ± 0.4	4	11.6± 0.5	4	14.7 ± 1.1	4
Day 6	10.9 ± 2.5	4	10.5 ± 1.7	4	14.5 ± 0.4	4
Day 9	6.4 ± 1.8	3	10.0 ± 1.2	4	10.7 ± 1.7	3
Day 12	4.0 ± 0.9	3	7.3 ± 1.8	4	9.6 ± 2.1	2
Day 15	1.1 ± 0.8	3	3.3 ± 1.0	3	6.8 ± 3.8	3

Wound area (mm²) is expressed as mean ± SEM. L-PRF = leukocyte and platelet rich fibrin

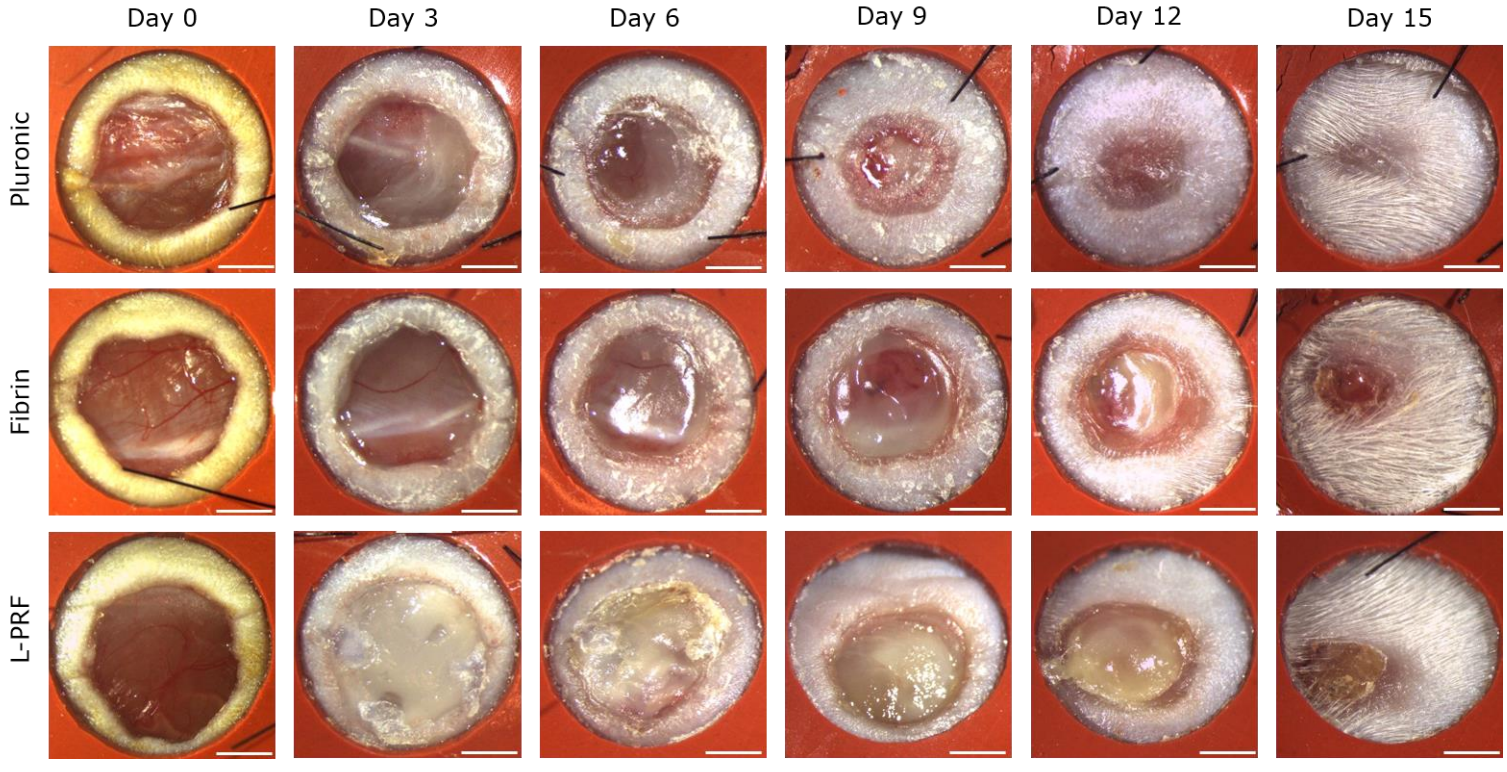

Figure 4.5 Representative images of the wound healing time course in mice treated with fibrin gel or L-PRF membranes. Wound size steadily decreases in all three groups. Control animals treated with pluronic F-127 gel achieve almost complete wound healing after 15 days. From day 6, granulation tissue is visible in pluronic and fibrin treated wounds. Scale bar = 2 mm. L-PRF = leukocyte and platelet rich fibrin.

4.4.3 L-PRF or fibrin treatment do not exceed the natural healing capacities of healthy mice

We further investigated the wound healing capacities of L-PRF exudate, in a setting without the interference of the unknown growth factors. Unlike the experiment in section 4.4.1, L-PRF EX was dissolved in growth factor-free pluronic F-127 and not in Matrigel™. A human-derived fibrin gel was included as a control condition to independently evaluate the influence of the fibrin component of L-PRF on wound healing. Quantitative analysis of the wound area showed a continued decrease in wound size over a two-week period (Figure 4.). At day 10, pluronic and L-PRF EX were responsible for a 49.3% and 50.6% reduction in wound area, while animals treated with fibrin showed only a 31.6% reduction in wound size. However, after 14 days of healing, quantification of wound sizes yielded no difference between control animals and animals treated with fibrin gel of with L-PRF EX. Control wounds showed a reduction in wound size of 61.2%, whereas fibrin treatment caused a 67.2% reduction and L-PRF EX reduced wound size with 65.2%.

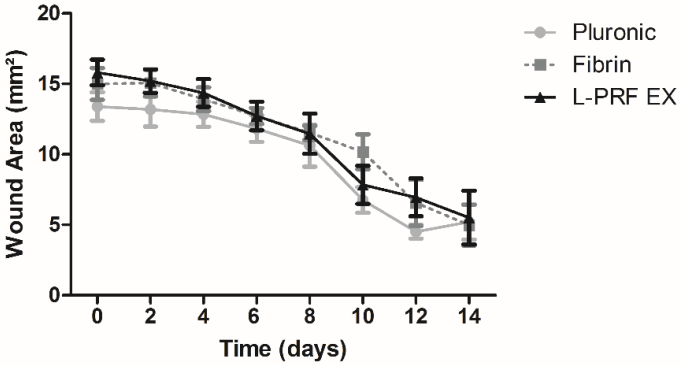

Figure 4. Quantitative evaluation of wound area over time. Quantification of wound area shows a gradual decrease of wound size over time in all three groups. However, none of the groups reached complete wound closure at day 14. Data are represented as mean ± SEM. L-PRF = leukocyte and platelet rich fibrin; EX = exudate.

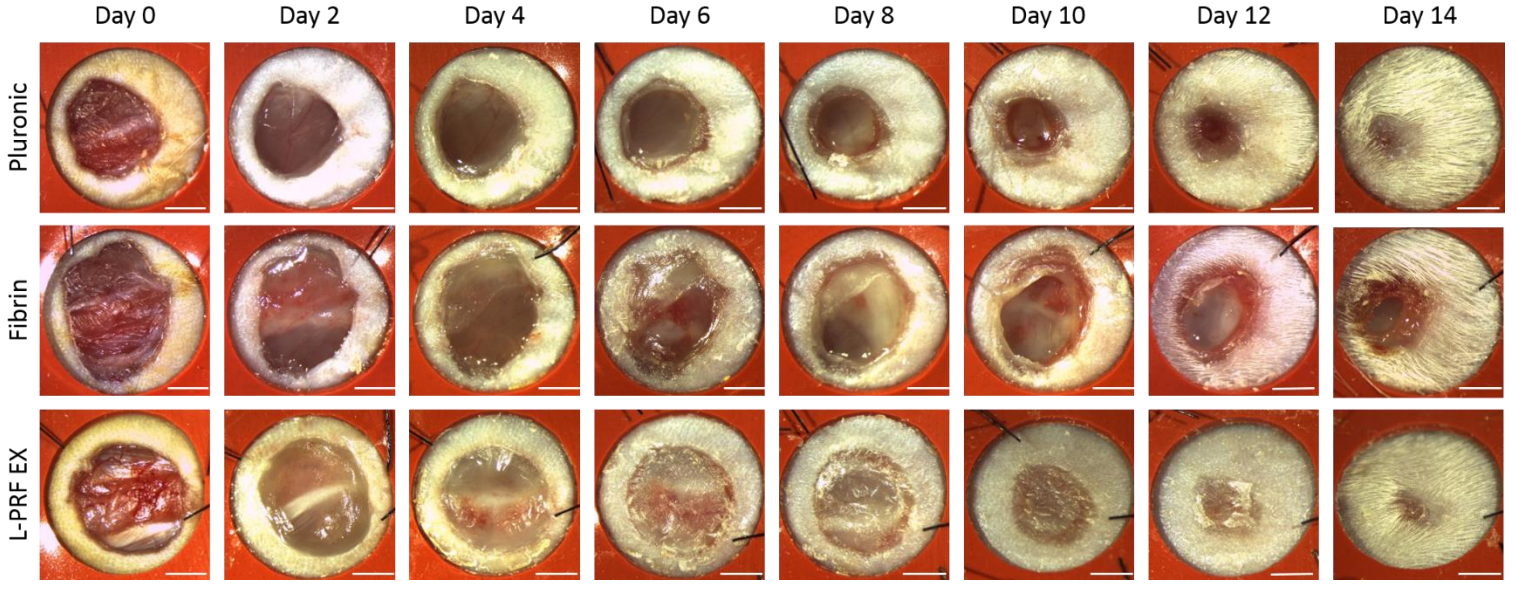

Figure 4.6 Representative images of the wound healing time course in mice treated with fibrin gel or L-PRF EX dissolved in pluronic F-127 gel. Wound size steadily decreases in all three groups. Control animals treated with pluronic F-127 gel achieve almost complete wound healing after two weeks. At day 6, all groups show granulation tissue at the wound edge, which increases over time. Scale bar = 2 mm. L-PRF = leukocyte and platelet rich fibrin; EX = exudate .

Macroscopic images show no external signs of inflammation (Figure 4.6). Similar to the previous experiment, granulation tissue was first seen at day 6 in all three groups and increased with time. At day 10 wound healing in the fibrin group seemed to lag behind, however at day 14 all three groups showed very similar levels of wound reduction. Until day 10 all animals could be included in the analysis, which was the best inclusion rate so far. Unfortunately, at day 14 only the pluronic group was still completely intact, while in both the fibrin group and the L-PRF EX group 2 animals had to be excluded from the analysis due to loss of the splint (Table 4.3).

Table 4.3 Wound area over time and the number of animals included in the analysis.

	PLURONIC		**FIBRIN**		**L-PRF EX**	
	Wound Area	Mice (n)	Wound Area	Mice (n)	Wound Area	Mice (n)
Day 0	13.4 ± 1.0	6	14.9 ± 1.1	6	15.8 ± 0.9	6
Day 2	13.2 ± 1.2	5	15.1 ± 0.9	5	15.2 ± 0.8	6
Day 4	12.8 ± 0.9	6	13.9 ± 0.8	6	14.4 ± 1.0	6
Day 6	11.8 ± 0.9	6	12.7 ± 0.6	6	12.7 ± 1.0	6
Day 8	10.6 ± 1.5	6	11.5 ± 0.5	5	11.5 ± 1.4	6
Day 10	6.8 ± 0.9	6	10.2 ± 1.2	6	7.8 ± 1.3	6
Day 12	4.5 ±0.5	6	6.6 ± 1.6	5	6.9 ± 1.3	4
Day 14	5.2 ±1.2	6	4.9 ± 1.4	4	5.5 ± 1.9	4

Wound area (mm^2) is expressed as mean ± SEM. L-PRF = leukocyte and platelet rich fibrin; EX = exudate.

At day 14, all remaining animals were sacrificed and tissues were collected for histological analysis. A Masson's trichrome staining was performed on the tissue sections in order to evaluate re-epithelization, cellular infiltration and collagen deposition (Figure 4.7). The degree of re-epithelization varied substantially within fibrin and L-PRF EX groups, while four out of five tissues of control animals showed 90–100% re-epithelization (Figure 4.9) The results for other groups were rather inconclusive, due to the low number of tissues sections included in the analysis for fibrin treated animals (n=2) and L-PRF EX (n=3) treatment. In all three groups the newly formed epithelial layer was thicker compared to the epithelium covering the adjacent healthy skin (Figure 4.7D-F). All three groups showed strongly

vascularized wound beds with smaller as well as larger vessels (Figure 4.7G-I). Compared to control samples, fibrin and L-PRF EX treatment led to more infiltrating cells and less deposition of collagen (Figure 4.9B,C). Fibrin treatment resulted the highest amount of infiltrating cells, based on nuclei count, and the lowest amount of collagen deposition, suggesting an inverse relationship between both parameters.

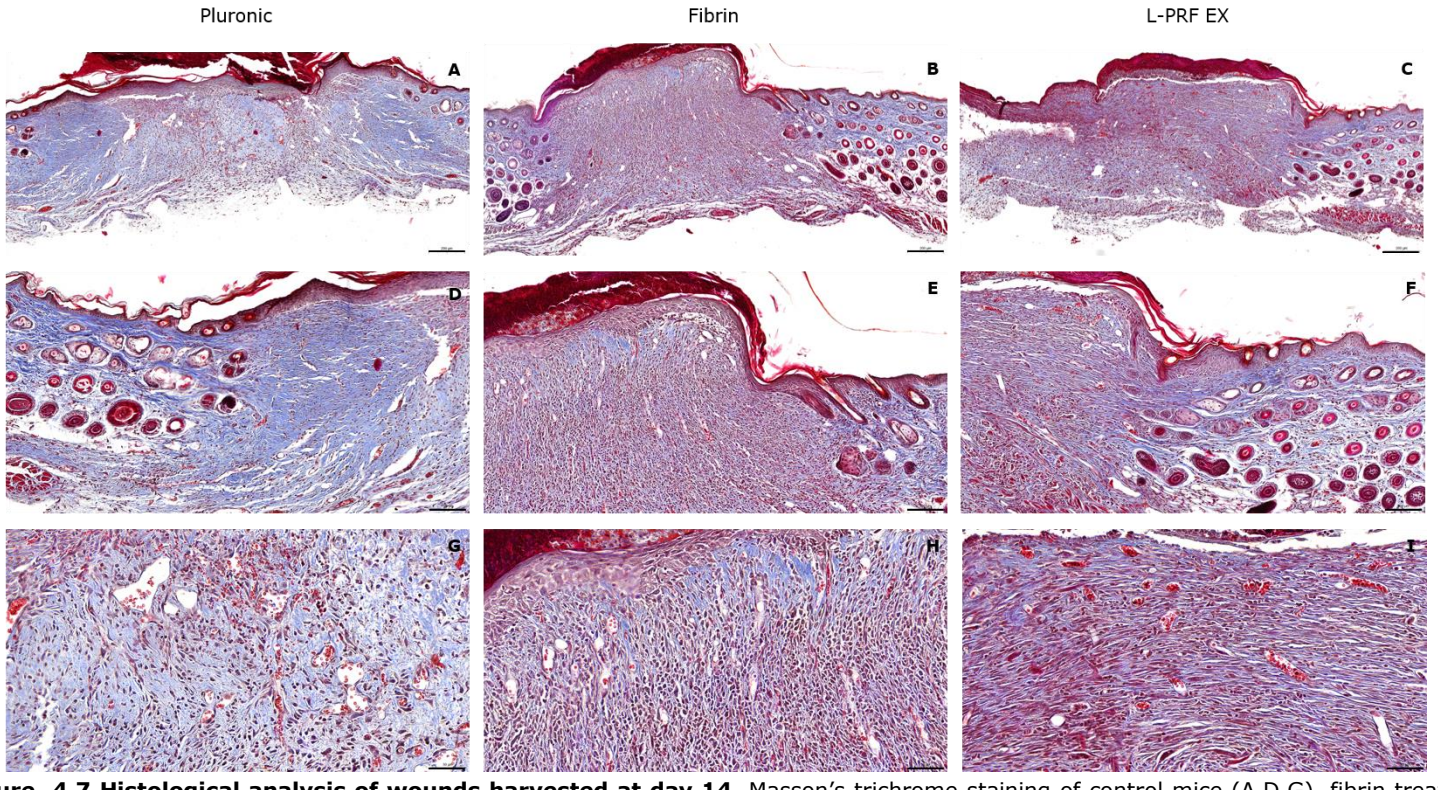

Figure 4.7 Histological analysis of wounds harvested at day 14. Masson's trichrome staining of control mice (A,D,G), fibrin treated mice (B,E,H) and mice treated with L-PRF EX (C,F,I). In all groups the newly formed epithelial layer is thicker compared to the epithelium of the adjoining healthy skin (D, E, F). All groups show highly vascularized wound beds (G, H, I) but wounds in fibrin (H) and L-PRF EX (I) groups show a higher cellular density compared to control mice, which show more collagen fibers in the wound bed (G). Scale bars (A-C) = 200 µm, scale bars (D-F) = 100 µm, scale bars (G-I) = 50 µm.

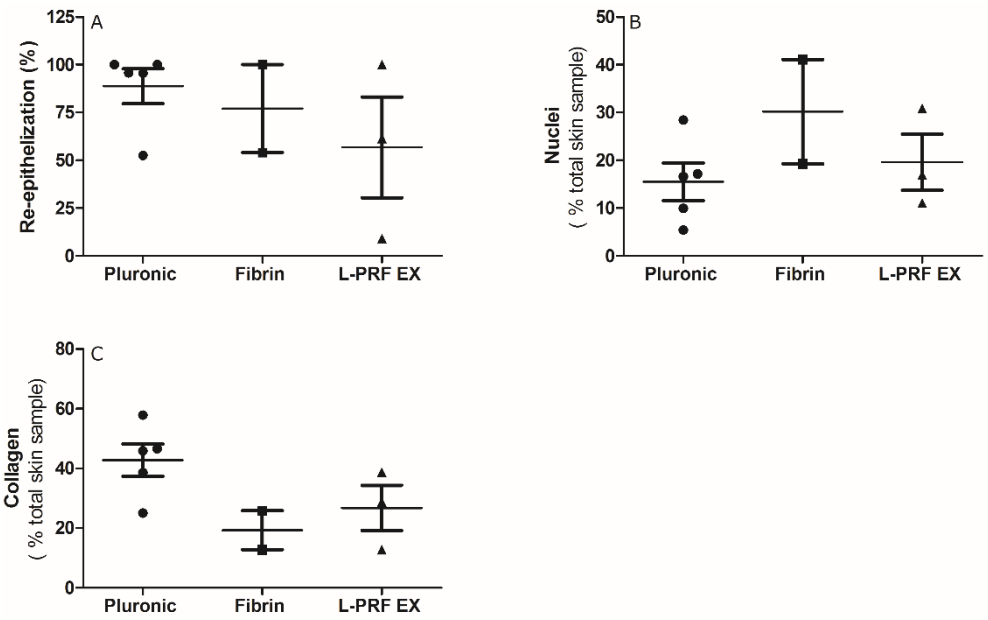

Figure 4.8 Histomorphometric analysis of re-epithelization, cellular infiltration and collagen deposition after 14 days of healing. Histomorphometric analysis of Masson's trichrome stained skin biopsies, harvested 14 days after injury. (A) Four out of five tissue samples in the pluronic group showed 90-100% re-epithelization. The fibrin and L-PRF EX groups each contained one sample which showed complete re-epithelization. (B) Pluronic treatment led to fewer infiltrating cells compared to fibrin and L-PRF EX treatment based on nulcei count. (C) Quantification of mean collagen fraction revealed higher levels of collagen in control animals. Data are represented as mean ± SEM. L-PRF = Leukocyte and platelet rich fibrin; EX = exudate.

4.5 Discussion

Platelet concentrates including platelet rich plasma (PRP) and L-PRF are being used in a variety of medical fields mainly because they represent a safe, reliable and cost-effective means to enhance tissue repair [239]. Because L-PRF represents a second generation platelet concentrate, the majority of studies to date have focused on the use of PRP to enhance wound healing. Unfortunately the importance of the fibrin architecture and leukocyte content of L-PRF, with regard to the wound healing process, is often neglected. This urges the need for more research into the contribution of these indispensable components [239].

While human skin heals via re-epithelialization and the formation of granulation tissue, the natural healing mechanism in mice is wound contraction. In contrast to human skin, mouse skin contains a thin muscular layer called the *panniculus carnosus*, which is responsible for the rapid contraction of cutaneous wounds in mice [296]. Under natural conditions, mice have been reported to achieve complete wound closure of 6 mm skin defects after only 7.8 days [300]. In order to better simulate the human condition, this study used a splinted mouse model for excisional wound healing. In this model full thickness wound are created extending through the *panniculus carnosus* and a silicone splint is sutured around the wound in order to counteract wound contraction [296, 301]. The use of wound splints has been shown to prolong the time needed to achieve complete wound closure with almost five days [300]. In this study, macroscopic images show the presence of granulation tissue, indicating a successful implementation of the splinted mouse model. Furthermore, histological analysis of the harvested tissues after 14 days of healing, showed partial to complete re-epithelialization and the absence of the *panniculus carnosus* at the site of wound creation.

In order to investigate the effects of the growth factors present in L-PRF CM and L-PRF EX on murine wound healing, a first *in vivo* experiment was performed, using growth factor-reduced Matrigel™ as a vehicle. L-PRF CM and L-PRF EX did not result in improved wound closure, compared to control animals. Matrigel™ is used for a variety of *in vitro* and *in vivo* assays focusing on angiogenesis [302, 303] and was therefore considered a suitable vehicle. However, one of the major drawbacks of the use of Matrigel™ is the fact that it is not chemically defined, leading to batch-to-batch differences and possible unknown confounding factors

[302]. Furthermore, Matrigel™ is derived from mouse sarcoma cells and could therefore, despite the usage of the growth factor-reduced variant, still contain murine growth factors and thus influence wound healing. Gorelik and colleagues found Matrigel™ to increase the rate of epithelialization of split-thickness wounds in rats [304]. Together, this suggests a positive effect of Matrigel™ , which could explain the inability of L-PRF CM or L-PRF EX to exceed vehicle induced wound healing.

A second *in vivo* study was performed in which Matrigel™ was replaced by a 18% pluronic F-127 gel. Pluronic gel has the advantage of being a nontoxic, thermoreversible hydrogel completely free of growth factors [283]. At a moderate concentration, an F-127 solution is soluble at refrigerated temperatures, but solidifies at body temperature [283]. It has been evaluated for a variety of biomedical applications ranging from drug and gene delivery to covering burn wounds [283, 305]. Furthermore, pluronic F-127 has already been used as a vehicle for murine wound healing experiments [306, 307]. By using L-PRF CM and L-PRF EX, the first experiment focused on the wound healing capacities of the growth factors released from L-PRF. However, growth factors are only one aspect of L-PRF that can be beneficial for wound healing. Fibrin matrix has been demonstrated to induce angiogenesis and guide the coverage of damaged tissues by influencing epithelial cells and fibroblasts [284]. Therefore, a fibrin gel derived from human fibrinogen and thrombin was included in a second *in vivo* experiment. For this particular experiment, intact L-PRF membranes were used, in order to combine the fibrin matrix with the platelet-derived growth factors and to include the leukocytes. This approach, although clinically relevant, was not without risk, since immunocompetent mice were treated with human-derived L-PRF, containing human leukocytes. This was not a concern in the first experiment because cells were removed form L-PRF CM and exudate by sterile filtration. Comparable to our data , Anitua *et al.* also treated immunocompetent mice with human-derived PRP [234] without complications. According to Suzuki *et al.* treating skin defects in rats with canine PRP did also not induce a severe inflammatory response [298]. These reports suggest the feasibility of using xenogeneic platelet derivatives in immunocompetent animals. Treating wounds with human-derived fibrin gels, did not improve wound closure compared animals treated with pluronic gel. These results are in line with a report by Mehanna *et al.* who showed no difference

between untreated and fibrin (human origin) treated wounds in rats [308]. Application of L-PRF membranes did not cause a fulminant inflammation since macroscopic evaluation did not show signs of swelling or pus formation. However, L-PRF membranes were not incorporated into the surrounding tissues, as would be expected when using autologous L-PRF. Due to small blood volumes and low blood pressure of mice, using autologous L-PRF would not be technically feasible.

In order to investigate the wound healing capacities of the growth factors present in L-PRF exudate, a third *in vivo* experiment was performed. By using pluronic F-127 as a vehicle instead of Matrigel™ the unknown growth factors present in Matrigel™ were no longer a possible confounding factor. Macroscopic images of wound evolution show the presence of granulation tissue starting at day 6, which is similar to reports by Suzuki *et al.* who demonstrated the presence of granulation tissue at day 7 in rats treated with gelatin gels [298]. Quantification of wound sizes showed a 61.2% decrease in wound size in control animals compared to a 67.2% reduction in fibrin treated animals and 65.2% reduction in L-PRF treated animals. Suzuki *et al.* reported a 70% repair after treating rats with growth factor-free gelatin gels for 14 days compared to a 80% repair of scar tissue in animals receiving gelatin gels impregnated with canine PRF extract [298]. Since Suzuki et al demonstrated only a 40% recovery of untreated wounds, the high repair ratios in vehicle treated wounds are probably due to the moistening effect of the hydrogels [309]. In contrast to these reports, Notodihardjo and colleagues demonstrated improved wound healing in mice treated with platelet rich plasma (PRP) compared to control mice, already at day 7 post surgery [236]. However, Notodihardjo *et al*. treated the animals with murine PRP, suggesting that the xenogeneic origin of the human-derived L-PRF used in the current study, may be a limiting factor for improved wound healing. Furthermore, the small sample size in the current study is also a limiting factor to make any well-founded conclusions, whereas the sample size was considerably larger (n=45) in the study performed by Notodihardjo *et al.* Despite the induction of the same sized wounds, control animals in Notodihardjo's study displayed inferior wound healing at day 14 compared to the control animals in the current study [236]. The difference in mouse strain, C57bl6/J mice compared to Balb/CRj mice in this study, could account for the difference in intrinsic wound healing capacities [301].

Studies reporting positive effects of platelet derivatives, mainly investigate wound healing in animal models of impaired healing. PRP has been described to induce faster wound healing [310] and improved re-epithelialization in diabetic rodents [311]. Both studies used donor animals to produce PRP for wound treatments. Proteins derived from human platelets were also reported to improve angiogenesis and wound healing in mice with impaired wound healing capacities due to cyclophosphamide treatment [312]. Together these reports suggest that the lack of improved healing in the current study is probably due the fact that wound healing was investigated in healthy mice using xenogeneic L-PRF as a treatment. In the future, this hypothesis could be validated by performing similar experiments using an animal model of impaired wound healing, such as diabetes.

Chapter 5

General Discussion and Summary

Establishing a supporting vascular network is not only instrumental in the survival of transplanted cells and tissues, but could also ameliorate ischemic diseases such as stroke and myocardial infarction. The process of angiogenesis is tightly regulated by a delicate balance between stimulating and inhibiting proteins. Imbalanced angiogenesis can result in pathological conditions [16, 17]. Treatments aiming to enhance angiogenesis are often based on the delivery of pro-angiogenic factors [19, 20]. Unfortunately, in clinical trials these kinds of therapies have only shown limited success [48, 71] thereby urging the need for more efficient and regenerative treatment protocols [313, 314] and shifting attention towards (stem) cell-based therapies [74, 75]. Bone marrow-derived mesenchymal stem cells (BM-MSCs) have been extensively studied and were demonstrated to establish therapeutic angiogenesis [86-88]. However, isolation of BM-MSC is associated with high donor site morbidity due to the invasive harvesting procedure [82]. This led the search for alternative sources of adult MSCs. Besides bone marrow, MSCs have also been isolated from adipose tissue [78], umbilical cord [79] and several dental tissues [80-82]. The latter include cells from the periodontal ligament which have been identified as suitable candidates for cell-based therapies and more recently have been reported to stimulate angiogenesis [104]. The current paradigm states that the beneficial effects of MSCs are mainly mediated via their paracrine actions instead of cellular differentiation, which is why researchers have been focusing on further enhancing the MSC-secretome [117]. Hypoxia is a potent stimulator for the secretion of beneficial trophic factors and has therefore been intensively studied as a possible approach to boost the MSC-secretome [116]. Besides hypoxia there is also a vast array of cytokines and growth factors which could possibly augment the angiogenic profile of stem cells [106, 146-148].

This dissertation aimed to elucidate the effect of pharmacological pretreatment on the angiogenic properties of periodontal ligament stem cells (PDLSCs) *in vitro* and *in ovo.* First of all the effect of pretreatment on VEGF secretion was evaluated, after which the angiogenic properties of untreated and pretreated PDLSCs were characterized by means of protein expression profiling, functional *in vitro* angiogenesis assays and a chorioallantoic membrane (CAM) assay.

Cell-based therapies are not the only strategy that could supply a wide range of growth factors to the site of injury and even fine-tune its actions based on input from the environment. Using platelet concentrates as a supply for biomolecules involved in angiogenesis and wound healing has gained a lot of attention due to their autologous nature and cost-effectiveness [200, 204]. The preparation of the first platelet concentrates required two centrifugation steps and the use of anticoagulants, calcium chloride and bovine thrombin [239]. Much advancements have been made since then, resulting in a second generation of platelet concentrates which can be made without the need for biochemical handling [239]. Leukocyte and platelet rich fibrin (L-PRF) is such a second generation platelet concentrate which combines the growth factors released from activated platelets and those secreted by leukocytes with a strong fibrin matrix [297]. Platelet-derived growth factors play a pivotal role in wound repair and neovascularization [238]. The effects of different types of platelet concentrates on angiogenesis and soft tissue healing have already been evaluated in pre-clinical animal experiments as well as in a first wave of clinical studies [238, 239, 299, 315]. Despite its clinical applications, little is known about the mechanisms of action and biological features of this platelet concentrate. More in-depth research could shed some light on the contribution of each of the three components to L-PRF's wound healing capacities [239].

Therefore, this dissertation aimed to elucidate the protein release profile of L-PRF and to investigate the angiogenic potential of L-PRF by means of functional angiogenesis assays *in vitro* and *in ovo* using of a CAM assay. Finally, the pro-angiogenic effects and wound healing capacities of L-PRF were tested in an *in vivo* model of cutaneous wound healing. In the following paragraphs the results obtained in this dissertation are summarized and discussed.

Does pretreatment of periodontal ligament stem cells enhance their angiogenic properties *in vitro* and *in ovo*?

Periodontal ligament stem cells (PDLSCs) represent a suitable source of adult stem cells for cell-based therapies in regenerative medicine and have been shown to stimulate angiogenesis [104]. Since establishing adequate vasculature is absolutely crucial for the survival transplanted cells, pharmacological preconditioning has been explored as possible strategy to further enhance the

therapeutic efficacy of cell-based therapies [116]. Deferoxamine (DFX) is an FDA-approved iron chelator which mimics hypoxia [131]. Besides hypoxia mimicking agents there are also a variety of growth factors which have been described to augment the angiogenic capacities of stem cells [106, 146, 148]. In **chapter 2**, we investigated the possibility of boosting the angiogenic potential of PDLSCs by means of *in vitro* preconditioning with DFX, fibroblast growth factor-2 (FGF-2) or both substances combined.

Pretreating PDLSCs resulted in an increased secretion of VEGF and placental growth factors (PlGF) and induced the expression of hypoxia-inducible factor 1-alpha (HIF-1α). Moreover, 48 hours after the removal of DFX, VEGF levels remained elevated, which would be beneficial for future *in vivo* uses. Increased VEGF secretion after DFX preconditioning has already been reported for BM-MSCs [245], adipose-derived stem cells (ASCs) [246] and periodontal ligament fibroblasts [131], whereas FGF-2 treatment has been shown to increase VEGF secretion in mouse PDL cells [247]. As a hypoxia mimetic it is not surprising that DFX has been found to upregulate HIF-1α in a variety of other stem cell types as well [132, 245, 248, 249]. However, to our knowledge, this study is the first to combine DFX and FGF-2 treatment. Combination treatment also resulted in increased VEGF and PlGF levels, however the DFX-induced HIF-1α was offset by the presence of FGF-2, suggesting a protective effect of FGF-2 under hypoxic conditions.

Although PDLSCs did not affect endothelial viability, both untreated and pretreated PDLSCs induced endothelial migration. Other reports also demonstrated no effect of other dental stem cell populations [82] or even DFX treated ASCs [248] on HMEC-1 viability. Despite the higher levels of VEGF secretion after pretreatment, pretreated PDLSCs did not induce more endothelial migration. Yeasmin *et al.* also reported high levels of VEGF secretion by untreated PDLSC, but in contrast to the current study, the PDLSCs investigated by Yeasmin *et al.* were able to initiate angiogenesis *in vitro* and *in vivo* when co-cultured with endothelial cells [104]. One could argue that the difference in isolation method, enzymatic digestion in Yeasmin's study versus outgrowth method in our study, could account for the discrepancy in the results. However, Tran *et al.* compared both isolation methods and reported similar proliferation rates and MSC marker

expression in both types of PDLSC, but they did find less mineralized nodules and lipid droplets in PDLSCs isolated via the outgrowth method [316]. According to Hilkens *et al.* both isolation methods result in a similar population of dental stem cells with no differences in MSC marker expression and multilineage differentiation [150]. However, these studies only investigated the characteristics these stem cells need in order to comply with the minimal requirements to be categorized as MSCs. It would be interesting to see whether isolation method affects cross-lineage differentiation or other more functional effects of dental stem cells.

Yeasmin *et al.* suggest that the angiogenic capacities of PDLSCs reported in their study are most likely due to the production of VEGF, though their study was not designed to address the role of VEGF. In the current study, addition of Axitinib, which is a VEGFR1-3 inhibitor, revealed that endothelial motility is not solely dependent on VEGF, since it was not able to reduce endothelial migration and since recombinant VEGF only caused a 2.7 fold increase EC migration whereas PDLSCs elicited on average an 3.8 fold increase in migration. These data are in line with a study by Shao *et al*., reporting an 1.5 fold increase in HMEC-1 migration under the influence of VEGF [317]. There is substantial evidence supporting that VEGFR-2 is the major mediator of the angiogenic response, including endothelial mitogenesis, survival, microvascular permeability [74] and tube formation [318]. Shoa and colleagues reported the complete absence of VEGFR-2 expression in HMEC-1 [317]. Whereas a study by Imoukhuede *et al.* did report VEGFR-2 expression in human dermal microvascular endothelial cells [319]. In contrast to VEGFR-2, VEGFR-1 does not mediate EC mitogenesis and can even have an inhibitory effect by binding VEGF and thereby preventing its interaction with VEGFR-2 [74, 319]. However, VEGFR-1 also exhibits pro-angiogenic effects under pathological conditions [320]. Moreover, short-term exposure to VEGF has been shown to cause internalization of surface VEGFR-1 and VEGFR-2 [321-324] and 24 hours incubation with VEGF led to upregulation of VEGFR-1 and downregulation of VEGFR-2 [319]. This only adds to the complicated nature of how ligand-receptor binding eventually leads to angiogenesis. Furthermore, pretreatment also resulted in an upregulation of PlGF, which is also a ligand for VEGFR-1, making the matter at hand even more complex. In order to circumvent the difficulties and uncertainties surrounding receptor expression on endothelial cells, the use of

VEGF neutralizing antibodies would perhaps have been a more suitable option in this study.

Unfortunately, PDLSCs did not induce blood vessel formation *in ovo*. Based on the high levels of VEGF secreted by pretreated PDLSC, we expected to see an increase in blood vessel formation, since VEGF has been shown to induce blood vessel growth *in ovo* [4, 134]. Furthermore, stem cells derived from the dental pulp or apical papilla did accomplish blood vessel formation *in ovo* [82]. However, this study reported no induction of angiogenesis when eggs were incubated with stem cells originating from the dental follicle. Since the dental follicle is the precursor for the PDL, both stem cell populations are closely related [134].

Despite the promising results of other studies using dental stem cells (DSCs) for regenerative approaches and regardless of the exact type of stem cell one wants to use, some major challenges remain, before DSCs, or any cell-based therapy for that matter, can progress to the clinic. The extraction of third molars as well as the isolation of DSCs are currently being performed using a variety of isolation protocols on donors of different ages and with molars at different developmental stages. This does not only impair in-depth comparison of experimental outcome but also hampers the establishment of a standardized treatment protocol [91, 150-152]. Therefore it is crucial to set a standardized isolation procedure in order to safeguard the clinical safety, reproducibility and efficacy of DSC therapy [149]. Clinical implementation also requires the upscale production of stem cells in xeno-free culture conditions [153-155]. Recently it has been reported that PDLSCs cultivated in serum-free medium retained their MSC characteristics including their capacity of hard tissue formation *in vivo,* but serum-free culture conditions did expose PDLSCs to a variety of extrinsic cytotoxic stimuli [325, 326]. Moreover, to provide sufficient cells for clinical applications, current stem cell expansion technique are in need of some 'up-scaling'. Treatment for one patient can easily require 1 to 5 billion cells [153] which cannot be achieved with conventional cell culture flasks. This makes the use of large scale bioreactors an inevitable step in the progression from bench to bedside. However, a few engineering challenges still need to be addressed before bioreactors can be implemented for large-scale commercial cell expansion. While the use of 3D scaffolds would result in a better surface to volume ratio, the safe retrieval of cells from these culture surfaces, still

poses a problem. The high costs associated with the large volumes of specialized culture media, limits the research on large-scale cell culture techniques to laboratories/corporations with sufficient financial resources. However, quality control and production monitoring using integrated sensors remains the biggest challenge. Bioreactors are currently equipped with oxygen, pH and temperature sensors, but this is not sufficient. Novel sensors need to be development which allow real-time monitoring of the cellular characteristics at any given time during the production process [327].

Besides challenges associated with the isolation and expansion of stem cells, one also needs to consider the possible influence of donor-related factors such as (oral) health, orthodontic tooth movement and age [91]. A small study including only 14 donors divided over 2 age groups reported that age influences the proliferation and differentiation potential of human PDLSCs [328]. A larger study which included 90 donors, ranging from 16 to 75 years of age, spread over 4 age groups reported a decline in PDLSC numbers and proliferation rate with increasing age. While adipogenic and osteogenic differentiation potential of PDLSCs was demonstrated regardless of donor age, advanced donor age did reduce the differentiation potential and the ability to form periodontal tissues [329]. Zhang *et al.* therefore suggest to harvest PDLSCs from younger patients for the use in tissue engineering and cell-based therapies. Which brings us to another issue, the use of allogeneic stem cells and the possibility for long term storage via cryopreservation and bio-banking initiatives.

For allogeneic purposes, cryopreservation allows the transport of cellular products and provides a time window to screen the cells prior to transplantation. In the case of autologous use, cells can simply be stored for future clinical applications [330]. Several DMSO-based cryopreservation protocols have been tested for the preservation of DPSCs [151, 331-334], SCAPs [160] and PDLSCs [335]. PDLSCs have been shown to retain their regenerative capacity [335, 336], their immunosuppressive characteristics and even their ability to regenerate periodontal tissues [162] after cryopreservation. The possibility of storing tissue samples for longer periods of time without significant damage to the inherent stem cell population offers great perspectives for regenerative medicine and stem cell banking. Besides al the scientific considerations that need to be taken into account

for the implementation of a public stem cell banking system, it also needs to be cost-effective and requires the careful evaluation from a legislative point of view. Despite these challenges, a few clinical studies using DSC-based therapies are currently recruiting participants and are thereby taking the first steps to introducing DSC-based therapies into the clinic.

Does leukocyte and platelet rich fibrin enhance angiogenesis *in vitro* and *in ovo?*

Platelet-derived growth factors play a pivotal role in tissue regeneration and revascularization [238]. Using platelet concentrates, it is possible to apply a concentrate of autologous growth factors at the site of injury [206, 233]. Despite their increasing popularity in several medical fields, the need to characterize the underlying mechanisms and the key players for enhanced tissue healing remains [238]. In **chapter 3** we investigated the angiogenic potential of L-PRF and characterized its growth factor release.

The L-PRF growth factor release was determined with an antibody assay and revealed an abundance of CXCR-2 ligands (IL-8, ENA-78, GRO and NAP-2) and EGF present in L-PRF conditioned medium (CM) but not in L-PRF exudate (EX). The discrepancy between L-PRF CM and L-PRF EX can be explained by the cytokine production by leukocytes [206] and by growth factor entrapment in the fibrin matrix [251-253]. Furthermore, fibrin has been reported to induce IL-8 secretion in oral squamous cell carcinoma cells [264], HUVEC [265] and neutrophils [266]. Concerning growth factor entrapment, Martino *et al.* reported the fibrin binding abilities of VEGF, PDGF, TGFβ, BMP-2, NT-3, BDNF and several members of the FGF family [337]. Fibroblast growth factor-2 (FGF-2) and VEGF have been shown to bind to fibrinogen while retaining their ability to potentiate endothelial proliferation [338-342]. When IL-1β binds to fibrin(ogen) it displaces bound FGF-2 and displays enhanced stimulatory activity of endothelial cells [343]. Furthermore, IGF-I is concentrated at the site of injury by binding to fibrin-immobilized IGFBP-3 to stimulate stromal cell function and proliferation [344]. Fibrin(ogen) has therefore been considered a suitable delivery system for long term, low-dose, sustained growth factor release [345, 346]. Both fibrin [347-349] and growth factors [346, 350, 351] have been modified to further facilitate fibrin binding because the binding of growth factors to fibrin is important for wound

healing. Platelets and macrophages within a fibrin clot represent a continuous source of growth factors necessary for angiogenesis and wound healing. By capturing these growth factors, fibrin not only acts as a growth factor reservoir but also creates a biochemical gradient in order to attract cells to the site of injury [337].

An antibody array is a fast and relatively inexpensive method to screen for dozens of proteins at once. However, such an array only yields a semi-quantitative result and thus always requires validation via a quantitative method such as an ELISA. Recent advances in technology have illustrated that mass spectroscopy-based protein quantification is becoming an indispensable tool for cellular biology. With the latest technical developments, mass spectroscopy is able to identify and quantify thousands of proteins [352, 353]. However, this is a very time-consuming and complex technique. Besides proteomics, metabolomics has also emerged as a powerful tool for future scientific research and clinical practice [354]. Metabolites such as lactate have been shown to play a role in endothelial metabolism, and angiogenesis [355]. Future research would benefit from implementing both proteomics and metabolomics techniques to further characterize the L-PRF secretome.

Both L-PRF CM and L-PRF EX were shown to induce endothelial proliferation, migration and tube formation *in vitro*. This is in line with other reports which have also demonstrated the ability of human platelet concentrates to improve endothelial proliferation [232, 234, 271] migration [206] and tubulogenesis [235, 271, 272]. In order to investigate whether the pro-angiogenic effects of L-PRF were mediated by the CXCR-2 or EGFR pathway, *in vitro* experiments were repeated with the addition of SB225002, a selective CXCR-2 antagonist or AZD8931, a reversible inhibitor of EGFR. Flow cytometric analysis of CXCR-1, CXCR-2 and EGFR expression demonstrated only small populations of endothelial cells expressing these receptors. This might explain why inhibition of CXCR-2 and EGFR did not alter the endothelial response to L-PRF CM in the functional angiogenesis assays *in vitro*. Except for the migration towards 50% L-PRF CM which was significantly lowered by the addition of AZD8931. The non-peptide CXCR-2 inhibitor, SB225002 has been used in a variety of studies and has been reported to block IL-8 induced neutrophil migration [356] and to possess

antitumor activity [357, 358]. Furthermore, Devapatla *et al.* showed a reduction in HUVEC survival, migration and tube formation when cells were exposed to 1µM SB225002. However, SB225002 was more effective in combination with sorafenib, a VEGFR inhibitor [359]. AZD8931 has been shown to possess pro-apoptotic effects and inhibit xenograft growth in a range of cancer models [360]. Despite all these reports stating the efficacy of the inhibitors used in this study, these results could not be replicated in the current study. In the future, using neutralizing antibodies for the growth factors of interest would be a promising approach especially due to the low and variable expression levels of these receptors on endothelial cells. Bertrand-Duchesne *et al.* reported a 40% decrease in PRP-induced HUVEC proliferation after adding EGF neutralizing antibodies [232]. In addition, a study by Li *et al.* reported a decrease in HUVEC migration and tube formation after addition of either anti-IL-8 or anti-CXCR-2 antibodies. Whereas a CAM assay only showed inhibited neovascularization after incubation with anti-IL-8 antibodies but not after addition of anti-CXCR-2 antibodies [361]. Despite these reports indicating the efficacy of growth factor neutralizing antibodies, we hypothesized that especially in the case of CXCR-2 it is more effective to target the receptor rather than inhibiting every ELR^+ chemokine independently. As IL-8 also binds to CXCR-1, the competitive inhibition of CXCR-2 by SB225002 might induce the availability of IL-8 for the CXCR-1 receptor thereby inducing compensatory angiogenic effects. Inhibiting both CXCR-1 and CXCR-2 in order to inhibit L-PRF induced EC migration would therefore be an interesting option. Possibly some of the identified growth factors work synergistically and inhibition of two or more angiogenic factors is a therefore an interesting approach. It might also be worthwhile to investigate the involvement of more central downstream cellular signaling molecules such as ERK ½ or AKT in L-PRF induced angiogenesis [362].

Furthermore, L-PRF was also shown to induce blood vessel formation *in ovo*. Initially L-PRF CM and L-PRF EX were dissolved in growth factor-reduced Matrigel™. Although L-PRF led to more blood vessel formation compared to vehicle treated eggs, these control membranes also contained a considerable number of blood vessels. For this reason, pluronic F-127, a non-toxic, thermoreversible, growth factor-free hydrogel was investigated as a possible vehicle [283]. Pluronic F-127 showed to be more inert compared to Matrigel™, since vehicle treated eggs

displayed a similar number of blood vessels as untreated membranes did. In order to evaluate the role of the fibrin component of L-PRF in the induction of angiogenesis, CAMs were incubated with a human-derived fibrin gel or with L-PRF membranes, both of which induced blood vessel formation. Human-derived fibrin has previously been shown to induce blood vessel formation *in ovo* [285]. Since the embryo is lacking a functional immune system at this developmental stage, the xenogeneic origin of the tested substances is not a concern or a possible inducer of angiogenesis [286].

Taken together, the results from this study demonstrate a profound pro-angiogenic effect of L-PRF both *in vitro* and *in ovo*. We identified several important angiogenic molecules in L-PRF CM and EX such as IL-8 and EGF. Based on inhibition studies, these factors, and other not yet identified factors, could be acting synergistically to induce angiogenesis. Studies using combinations of EGFR, CXCR-1, CXCR-2 and possibly VEGFR inhibitors, are needed to identify the driving force behind the tremendous angiogenic potential of L-PRF. In addition, pinpointing the key players could aid in the search for biomarkers predicting L-PRF quality and possibly help with identifying patients benefitting the most from L-PRF treatment. The variety of preparation protocols and *in vitro* setups makes it difficult to compare results from different studies. This warrants standardization of preparation protocols in order further characterize these platelet concentrates, which will hopefully lead to a better understanding of their *in vivo* effects.

Does human leukocyte and platelet rich fibrin enhance murine wound healing?

Wound healing is a dynamic and complicated process involving crosstalk between blood vessels epidermis, dermis and even the nervous system and subcutaneous tissue layers [290]. The crosstalk between all these different tissues is predominantly mediated by growth factors and cytokines [291]. L-PRF not only contains numerous growth factors able to promote angiogenesis and wound healing, but the supporting fibrin matrix also serves as a natural guide for angiogenesis and wound healing. In **chapter 4** the ability of L-PRF to enhance *in vivo* wound healing was investigated.

Full thickness wounds were created and splinted in order to counteract wound contraction. Quantification of wound sizes over time revealed that L-PRF impregnated Matrigel™ was not able to enhance wound closure compared to vehicle treated animals. As Matrigel™ is used for a variety of *in vitro* and *in vivo* assays focusing on angiogenesis [302, 303] it was considered a suitable vehicle. However, Matrigel™ is not chemically defined, meaning that even the growth factor-reduced type, which was used in this study, still contains an unknown amount of murine growth factors, which according to Gorelik *et al.* increases the rate of wound epithelialization [302, 304]. This led to the search for another suitable vehicle, preferably free of growth factors. Pluronic F-127 is a nontoxic, growth factor-free thermoreversible hydrogel, which has been evaluated for a variety of biomedical applications, ranging from drug delivery to covering burn wounds [283, 305] and as a vehicle in murine wound healing experiments [306, 307]. Results from the CAM assay performed in chapter 3 showed pluronic F-127 to be more inert compared to Matrigel™, therefore the following *in vivo* experiments were carried out using pluronic F-127 as a vehicle.

In order to explore the wound healing capacities of L-PRF's supporting fibrin matrix, animals were treated with L-PRF membranes or a human-derived fibrin gel. Using human-derived L-PRF and fibrin in immunocompetent mice poses the risk of inducing a fulminant inflammatory response. However, previous studies have reported the xenogeneic use of platelet concentrates [234, 298] and fibrin [337, 363] without complications. Moreover, since the immune system plays a pivotal role in the process of wound healing, it would not be clinically relevant to investigate wound healing in immune compromised animals. For these reasons we decided to use human derived L-PRF and fibrin. Furthermore in chapter 3 the ability of these human derived biomaterials to induce cross species blood vessel formation (*in ovo*) were already demonstrated. However no immunological response had to be taken into account in these experiments since chicken embryo's had not yet developed a functional immune system. While no major signs of inflammation were seen during the course of the *in vivo* experiments, neither the fibrin gel nor the L-PRF membranes enhanced murine wound healing compared to animals treated with pluronic F-127. While there are reports on the angiogenic and wound healing capacities of fibrin [284], this study is not the first to report no improved wound healing after fibrin treatment [308, 363, 364]. The

latter reports stated that while fibrin might me a suitable vehicle for wound healing applications, it does not promote healing on its own. These contradictory results may be attributed to a different composition of the fibrin matrix, which is known to considerably influence its effects on wound healing [365]. Additionally, the efficacy of fibrin is also largely dictated by the exact surgical circumstances in which fibrin is applied [366].

In order to investigate the wound healing capacities of the growth factors present in L-PRF exudate, a third *in vivo* experiment was performed using pluronic F-127 as a vehicle. After 14 days of healing, fibrin and L-PRF treatment resulted in only minor increases in wound reduction (4-6%) compared to vehicle treated animals. While Suzuki *et al.* reported only a 40% recovery of untreated wounds, vehicle treated animals in this study displayed a 61% decrease in wound size. This discrepancy can probably be attributed to the moistening effect of the hydrogels [309]. Moreover, most studies investigating treatment strategies for improved wound healing use an animal model mimicking chronic/impaired healing, such as diabetic (*db/db*) rodents [337]. Both allogeneic [310, 311] and xenogeneic [312] platelet derivatives have been shown to improve wound healing in animal models of impaired healing. Using L-PRF CM, LPRF-EX or L-PRF membranes in animal models mimicking impaired wound healing would be an interesting option for future experiments. Furthermore, it would be interesting to compare L-PRF from diabetic patients with that of healthy patients. Since diabetes is associated with impaired healing, this might be reflected in the L-PRF-secretome and in its wound healing capacities.

The number of patients suffering from impaired wound healing is unfortunately reaching epidemic proportions [291, 293, 295, 296]. The standard treatment involves wound debridement, controlling infection, stimulating revascularization and avoiding inappropriate pressure on the wound [198]. PRF been explored as an alternative treatment for chronic wounds such as diabetic foot ulcers, venous leg ulcers and chronic leg ulcers, especially for patients not responding to standard care [367-371]. Moreover, platelet concentrates including PRP and (L-) PRF have been used for regenerative approaches in various fields of medicine including dentistry [372], maxillofacial surgery [373], dermatology, aesthetic surgery [374], orthopedics and sports medicine [375] and even neurology [376]. The

effects of PRF on soft tissue healing have been investigated in at least 31 clinical trials [239]. In dentistry, PRF is mostly utilized for the treatment of extraction sockets [377-380], gingival recession [381-383] and palatal wound closure [384-386]. Clinical research often struggles with demonstrating the efficacy of treatments in large scale clinical trials due to the complex and heterogeneous patient population [295]. Despite the fact that 27 out of 31 studies reported beneficial effects of PRF for soft tissue wound healing and angiogenesis, only 8 studies were randomized clinical trials (RCT) and more than half did not include the appropriate controls [239]. Therefore the results from these studies should be interpreted carefully and larger standardized randomized controls studies are necessary to provide sufficient evidence for the efficacy of PRF in wound healing applications.

To date, research predominantly focused on the role of platelets, since this is the common denominator between all the different types of platelet concentrates. However fibrin architecture and leukocyte content are also two important parameters, which should not be neglected. Fibrin itself has a strong influence on the healing process [251, 366]. Especially from a tissue engineering standpoint, the mechanical properties of L-PRF could be very interesting since different applications might require different tensile strengths in order to achieve optimal results. To date very little research has been performed to characterize the strength and stiffness of different platelet concentrates [387-389]. Furthermore, fibrin architecture can also influence the cells within the matrix, especially the leukocytes [221]. Leukocytes can greatly impact the wound healing process by secreting growth factors and facilitating the antibacterial immune defense [390, 391]. Future research should therefore focus on identifying the role of these cell in the wound healing process. By better understanding the role of the fibrin matrix and the leukocytes, preparation protocols could be altered in order to specifically tailor the matrix architecture or the leukocyte content to the situation at hand.

Overall platelet concentrates are a promising treatment options for enhancing angiogenesis and wound healing and so far no major health problems have arisen from the therapeutic use of platelet concentrates. However, there might be some limiting factors to the use platelet derivatives. High hematocrit levels, low platelet count or the parallel use of anti-platelet drugs such as aspirin could limit the

efficacy. Furthermore, platelets also secrete prothrombotic factors and one should therefore refrain from using these products in the vicinity of major blood vessels in patients with thrombotic risk factors [392]. Since platelet derivatives are blood derived substances, they are not classified as drugs, despite containing substantial amounts of growth factors [393]. Therefore the correct ethical approvals that need to be in place are not well defined. Since platelet derivatives are easily produced and have aesthetic applications (e.g., skin rejuvenation), this might lead to unprofessional use outside a clinical or scientific setting.

Despite some shortcomings in study design and lacking standardization, platelet concentrates have been shown to be a safe, reliable and cost-effective means to accelerate soft tissue healing [239].

In conclusion, this dissertation evaluated two completely different biological approaches to enhance angiogenesis for applications in regenerative medicine. While pharmacological pretreatment enhanced the PDLSC-secretome, stem cells only affected endothelial migration and did not induce blood vessel formation *in ovo*. L-PRF on the other hand, enhanced endothelial migration, proliferation and tube formation and was shown induce blood vessel formation *in ovo*. While the key angiogenic molecules involved remain to be elucidated, these data indicate a promising future for the use of L-PRF in the field of regenerative medicine.

Chapter 6

Samenvatting

Het snel ontwikkelen van een functionele bloedtoevoer is cruciaal voor het overleven van getransplanteerde cellen en weefsels. Verbetering van het bloedvatennetwerk is een belangrijke strategie voor de genezing van talrijke aandoeningen zoals hersen- of hartinfarcten. Het delicate evenwicht tussen stimulerende en inhiberende eiwitten bepaalt of er al dan niet nieuwe bloedvaten worden gevormd. Wanneer deze balans ontregeld is, kan dat leiden tot pathologische aandoeningen. In eerste instantie heeft men dan ook geprobeerd om de bloedvatvorming te stimuleren door het toedienen van dergelijke bloedvatvorming stimulerende eiwitten. Spijtig genoeg hadden deze studies niet het gewenste effect en worden er nu andere alternatieven verkend. Het gebruik van stamcellen en meer bepaald het gebruik van mesenchymale stamcellen afkomstig van het beenmerg zou een interessante optie kunnen zijn en werd daarom reeds intensief onderzocht. Ondanks het feit dat deze beenmergstamcellen in staat zijn om bloedvatvorming oftewel angiogenese te bevorderen, heeft het gebruik van deze stamcellen één groot nadeel: de isolatie procedure is namelijk erg invasief. Dit was de drijvende kracht achter de zoektocht naar alternatieve stamcelbronnen. Het menselijke lichaam bevat nog heel wat andere weefsels waaruit deze mesenchymale stamcellen geïsoleerd kunnen worden, zoals vetweefsel, navelstreng en zelfs tanden. De tanden bevatten verschillende stamcelpopulaties waaronder de stamcellen van het parodontaal ligament (PDLSCs). In de literatuur werd reeds beschreven dat deze stamcellen geschikte kandidaten zijn voor het gebruik in cel-gebaseerde therapieën en dat ze in staat zijn om angiogenese te bevorderen. Het heersende paradigma stelt dat de stamcellen angiogenese bevorderen door de productie van stimulerende eiwitten. Bovendien kan de productie van deze eiwitten nog verhoogd worden door de stamcellen bloot te stellen aan hypoxie of aan bepaalde groeifactoren of cytokines.

Deze doctoraatsthesis had als doel om het effect van dergelijke voorbehandelingen op de angiogene eigenschappen van PDLSCs te onderzoeken. Allereerst werd het effect van verschillende voorbehandelingen nagegaan op de productie van VEGF, één van de belangrijkste eiwitten voor de stimulatie van bloedvatvorming. De productie van dit eiwit doorbehandelde en onbehandelde stamcellen werd vergeleken, alsook het effect van de PDLSCs op endotheelcellen. Tenslotte werd er ook gekeken of deze stamcellen in staat zijn om

bloedvatvorming te induceren in onderzoeksmodel dat gebruik maakt van bevruchtte kippeneieren, namelijk het chorioallantois membraan (CAM) experiment.

Stamcellen zijn niet de enige mogelijkheid om een brede waaier aan groeifactoren toe te dienen. Ook bloedplaatjes fungeren als een bron van biomoleculen die bloedvatvorming en wondheling bevorderen. Leukocyt-en-plaatjes-rijk fibrine (L-PRF) is een plaatjesconcentraat dat bestaat uit bloedplaatjes, witte bloedcellen of leukocyten en een fibrine matrix. Eiwitten afkomstig van bloedplaatjes spelen een centrale rol in wondherstel en neovascularisatie. Zowel in dierstudies als in klinische studies werd de capaciteit van verschillende soorten plaatjesconcentraten om angiogenese en wondheling te stimuleren reeds onderzocht. Veel van deze klinische studies zijn echter kleine studies of missen de gepaste controlegroepen. Ondanks alle mogelijke klinische toepassingen is tot op heden nog maar weinig geweten over de onderliggende mechanismen en de biologische kenmerken van deze plaatjesconcentraten.

Het doel van deze doctoraatsthesis was om te onderzoeken welke eiwitten er worden vrijgezet door L-PRF en of L-PRF in staat is om bloedvatvorming te stimuleren zowel *in vitro* als *in vivo*. Tenslotte werden ook de wondheling capaciteiten van L-PRF onder de loep genomen door gebruik te maken van een muismodel voor wondheling van de huid. De resultaten van deze thesis zullen in de volgende paragrafen besproken worden.

Zorgt farmacologische behandeling van parodontaal ligament stamcellen ervoor dat hun angiogene eigenschappen verbeteren?

Farmacologische voorbehandeling werd onderzocht als een mogelijke strategie om de therapeutische efficiëntie van stamcellen te verhogen. In **hoofdstuk 2** werden PDLSCs voorbehandeld met deferoxamine (DFX), een ijzer chelator die hypoxie nabootst en met een groeifactor genaamd fibroblast groeifactor-2 (FGF-2).

Behandeling van PDLSCs leidde tot een verhoogde secretie van VEGF en PlGF alsook een verhoogde expressie van HIF-1α. Dit komt overeen met andere studies die reeds beschreven dat DFX of FGF-2 zorgde voor een verhoogde VEGF en HIF-1α expressie in tal van andere stamcel types. De PDLSCs hadden echter geen invloed op de proliferatie van endotheelcellen. Endotheelcelmigratie werd

daarentegen wel bevorderd, dit zowel door onbehandelde als door behandelde PDLSCs. Ondanks de verhoogde secretie van VEGF na behandeling, induceren de behandelde stamcellen dus niet meer migratie. Om te onderzoeken of VEGF een bepalende factor is in het stimuleren van endotheelcelmigratie, werd het experiment herhaald maar ditmaal met toevoeging van een inhibitor voor de VEGF receptor. Toevoeging van deze inhibitor kan de endotheelcellen echter niet verhinderen om naar de stamcellen te migreren. Bovendien kon recombinant VEGF wel endotheelcel migratie induceren, hetzij in mindere mate dan de stamcellen. Dit geeft aan dat VEGF wel een rol speelt in endotheelcelmigratie maar het is niet de enige speler. Bovendien zijn er in de literatuur veel tegenstrijdige data met betrekking tot de expressie van VEGF receptoren op endotheelcellen. Wanneer slechts een kleine subpopulatie van de endotheelcellen de receptor tot expressie brengt, is het vanzelfsprekend dat het inhiberen van de receptor geen grootschalig effect zal hebben. Dit probleem zou vermeden kunnen worden door VEGF direct te inhiberen met behulp van neutraliserende antilichamen. Tenslotte werd de capaciteit van de stamcellen om bloedvaten te induceren ook onderzocht in een CAM experiment. Noch de behandelde, noch de onbehandelde stamcellen bleken in staat om bloedvatvorming te induceren *in ovo.* Dit komt overeen met de resultaten uit de literatuur waar ook andere stamcelpopulaties afkomstig uit de tand, namelijk de folliculaire stamcellen, niet in staat waren om bloedvatvorming te induceren. Voor andere stamcelpopulaties afkomstig van de tand werd dit wel reeds beschreven.

Ondanks de veelbelovende resultaten die werden gerapporteerd door tal van andere studies die gebruik maakten van dentale stamcellen, zijn er toch nog enkele obstakels die overwonnen moeten worden vooraleer stamceltherapie kan worden toegepast in een klinische setting. Momenteel is er nog te veel variatie in het isoleren en opkweken van de stamcellen. Vooral de expansie zal ook op grotere schaal moeten plaatsvinden, wil men effectief patiënten gaan behandelen.

Het opkweken van stamcellen op grote schaal kan gebeuren met behulp van bioreactoren, maar dat is momenteel nog een zeer kostelijke procedure. Door de hoge kosten geassocieerd met deze techniek, beschikken slechts een klein aantal instellingen over voldoende financiële middelen om zulk onderzoek te financieren. Bovendien moet de kwaliteit van de cellen nauwgezet opgevolgd kunnen worden

doorheen het productieproces door middel van geautomatiseerde sensoren, iets wat momenteel nog niet op punt staat. Bovendien worden de stamcellen momenteel geëxpandeerd met behulp van dierlijk serum wat een risico inhoudt voor het overbrengen van bepaalde ziektes. Een eerste stap is dus het standaardiseren van de isolatieprocedure, het opgroeien van de stamcellen in grote hoeveelheden en dat allemaal zonder de toevoeging van dierlijke producten. Bovendien mogen deze aanpassingen de karakteristieke eigenschappen van de stamcellen niet beïnvloeden. Een gestandaardiseerd protocol zal het overigens ook veel eenvoudiger maken om studies met elkaar te vergelijken, iets wat momenteel een probleem is door de grote variatie.

Helaas is er niet alleen een grote variatie in de verschillende isolatie- en expansieprotocollen maar ook in de leeftijd en algemene gezondheid van de donoren. Er werd namelijk aangetoond dat de toenemende leeftijd van een donor zorgt voor een afnemend aantal stamcellen met een verminderde celdelingscapaciteit en verminderde differentiatiecapaciteit. Omwille van deze redenen stelt Zhang *et al.* voor om enkel stamcellen afkomstig van jongere donoren te gebruiken voor weefselherstel. Dit brengt ons echter bij een volgende kwestie, namelijk de langdurige opslag van deze stamcellen door middel van cryopreservatie en de opstart van bio-bank initiatieven. Cryopreservatie bij allogeen gebruik, heeft als functie dat de cellen getransporteerd kunnen worden en dat ook het tijdvenster tussen isolatie bij de donor en later de toediening bij de recipiënt kan vergroot kan worden. Bij autoloog stamcelgebruik is cryopreservatie van belang om stamcellen gedurende een lange tijd te bewaren voor toekomstig gebruik. Er werden reeds verschillende cryopreservatieprotocollen uitgetest op dentale stamcellen en deze tonen aan dat het invriesproces geen gevolgen heeft voor hun karakteristieke stamceleigenschappen. De mogelijkheid tot langdurige opslag van stamcellen opent perspectieven voor de regeneratieve geneeskunde. Ondanks de vele bovenvermelde uitdagingen blijken stamceltherapieën een veelbelovende behandelingsoptie voor een brede waaier aan pathologische aandoeningen.

Zorgt leukocyt-en-plaatjes-rijk-fibrine voor verhoogde angiogenese *in vitro* en *in ovo*?

Groeifactoren afkomstig van bloedplaatjes spelen een cruciale rol in het proces van wondheling. Door gebruik te maken van plaatjesconcentraten kan men lokaal een verhoogde concentratie van autologe groeifactoren toedienen. Ondanks het feit dat de toepassing van deze plaatjesconcentraten zich steeds verder verspreid in de medische gemeenschap, moeten de onderliggende mechanismen die verantwoordelijk zijn voor de verbeterde wondheling nog verder gekarakteriseerd worden. In **hoofdstuk 3** werd onderzocht welke eiwitten worden vrijgezet door L-PRF en werd het angiogeen potentieel van L-PRF onder de loep genomen.

Analyse toonde aan dat geconditioneerd medium (CM) van L-PRF een overdaad aan CXCR-2 liganden en aan EGF bevat. Dit was niet het geval voor L-PRF exudaat (EX). Het grote verschil tussen EX en CM is waarschijnlijk te wijten aan de eiwitproductie van de leukocyten. Bovendien werd reeds aangetoond dat fibrine de productie van IL-8 kan bevorderen in verschillende celtypes. Ook het feit dat de fibrine matrix de groeifactoren vasthoudt, kan een verklaring zijn voor de opmerkelijk lagere concentraties in het L-PRF EX.

In hoofdstuk 3 werd aangetoond dat zowel L-PRF CM als L-PRF EX in staat zijn om endotheelcelproliferatie,-migratie en -tubulogenese te bevorderen. Om na te gaan of de CXCR-2 liganden of EGF hierin een bepalende rol spelen, werden er inhibitoren voor CXCR-2 en de EGF receptor (EGFR) toegevoegd. Deze inhibitoren hadden echter geen effect op het gedrag van de endotheelcellen wanneer ze werden blootgesteld aan L-PRF CM. Behalve wanneer het medium verdund werd, zorgde de inhibitie van de EGF receptor voor een verminderde endotheelcelmigratie. Aan de hand van flowcytometrie werd vastgesteld dat slechts een kleine subpopulatie van de endotheelcellen CXCR-2 en EGFR tot expressie brachten. Er werd echter gekozen om CXCR-2 te inhiberen en niet rechtstreeks de liganden omdat men verwachtte om op deze manier door toevoeging van één inhibitor meteen vier liganden tegelijk te kunnen uitschakelen.

Tenslotte werd ook aangetoond dat L-PRF in staat is om bloedvatvorming te induceren in een CAM experiment. Om L-PRF CM en EX te kunnen aanbrengen op de CAM werd er gebruik gemaakt van Matrigel. Hoewel behandeling met L-PRF

resulteerde in meer bloedvaten in vergelijking met de controle waarin enkel Matrigel werd toegediend, bevatte ook deze controles een aanzienlijke hoeveelheid bloedvaten. Ondanks het wijdverspreide gebruik van Matrigel in het angiogenese-onderzoek, zijn er toch ook enkele nadelen aan verbonden. Zo is de exacte chemische samenstelling van deze gel niet gekend en bevat het altijd nog resterende groeifactoren. Daarom werd er in een volgend experiment gebruik gemaakt van een hydrogel genaamd pluronic F-127 die overigens volledig vrij is van groeifactoren. Deze pluronic gel bleek inderdaad meer inert dan de Matrigel. Bovendien werd in dit tweede experiment ook onderzocht of fibrine angiogenese kan induceren, aangezien dit ook een belangrijke component is van L-PRF. Dit experiment toonde aan dat zowel synthetische fibrine gel als L-PRF membranen in staat zijn om bloedvatvorming te stimuleren.

Ondanks dat er enkele eiwitten werden geïdentificeerd die talrijk aanwezig zijn in L-PRF CM, kon er geen concreet besluit worden getrokken over hun exacte rol in de stimulatie van angiogenese. De resultaten toonden wel aan dat L-PRF zowel *in vitro* als *in ovo* bloedvatvorming ingrijpend kan bevorderen.

Zorgt humaan leukocyt-en-plaatjes-rijk-fibrine voor verbeterde wondheling in een muismodel?

Wondheling is een complex proces dat hoofdzakelijk gereguleerd wordt door groeifactoren en cytokines. L-PRF bevat niet enkel talrijke groeifactoren die een rol spelen in wondheling en angiogenese, maar ook de fibrine matrix kan beide processen ondersteunen. In **hoofdstuk 4** werd onderzocht of L-PRF in staat is om de wondheling te bevorderen in een muismodel voor cutane wondheling.

Wanneer L-PRF CM of L-PRF EX opgelost werden in Matrigel zorgde het niet voor verbeterde wondheling in vergelijking met dieren die enkel met Matrigel behandeld werden zonder de toevoeging van L-PRF. Aangezien in hoofdstuk 3 was gebleken dat pluronic gel meer inert is dan Matrigel en er bovendien studies zijn waarbij men pluronic gel heeft gebruikt in combinatie met diermodellen voor wondheling, werden de verdere dierexperimenten uitgevoerd met deze gel als oplosmiddel. In een tweede dierexperiment werden de wondhelingscapaciteiten van een synthetische fibrinegel en van L-PRF- membranen geëvalueerd. Het gebruik van humaan materiaal in dieren met een functioneel immuunsysteem is

echter niet zonder risico. Aangezien het immuunsysteem een belangrijke rol speelt in het gehele wondhelingsproces zou het echter niet relevant zijn om dit soort onderzoek te verrichten met immuundeficiënte dieren. Bovendien hebben voorgaande studies reeds aangetoond dat het mogelijk is om plaatjesconcentraten en fibrine van xenogene oorsprong te gebruiken. Ook in deze studie werden er geen macroscopische tekenen van infectie waargenomen ten gevolge van de behandeling met humaan L-PRF of fibrine. Bovendien werd in hoofdstuk 3 reeds aangetoond dat humaan L-PRF bloedvatvorming kan induceren in een andere diersoort. Toch zorgde noch de fibrinegel, noch de L-PRF membranen voor versnelde wondheling in vergelijking met dieren die behandeld werden met de pluronic gel. In de literatuur zijn tegenstrijdige rapporten te vinden over de capaciteit van fibrine om wondheling te bevorderen. Dit zou onder meer te wijten kunnen zijn aan de samenstelling van de fibrinegel alsook de medische omstandigheden waarin fibrine gel gebruikt wordt. Tenslotte werden de wondhelingscapaciteiten van L-PRF EX nogmaals onderzocht, ditmaal opgelost in pluronic gel. Na 14 dagen deden fibrinegel en L-PRF EX het slechts 4-6% beter wat de reductie van de wondgrootte betreft, ten opzichte van de controle dieren. Aangezien deze experimenten werden uitgevoerd met gezonde dieren zou het kunnen dat L-PRF de intrinsieke helingscapaciteiten niet kan overtreffen. De meeste studies die onderzoek doen naar de wondhelingscapaciteiten van plaatjesconcentraten gebruiken modellen van chronische wondheling zoals dieren die leiden aan diabetes.

Aangezien het aantal patiënten dat te maken krijgt met chronische wonden steeds toeneemt, is het niet verwonderlijk dat een groot aantal onderzoekers zich focust op dit soort ziektebeelden. Maar er is ook interesse in plaatjesconcentraten in tal van andere vakgebieden zoals tandheelkunde, dermatologie, orthopedie en zelfs neurologie. Er zijn ondertussen 31 klinische studies uitgevoerd waarin het effect van plaatjes-rijk-fibrine op weefsel heling werd onderzocht. Van deze 31 studies rapporteerden er 27 een positief effect van de plaatjesconcentraten. Hoewel, slechts 8 van deze studies waren gerandomiseerde klinische studies en in meer dan de helft ontbraken de juiste controlegroepen. Omwille van deze redenen moet men toch voorzichtig zijn bij het interpreteren van deze resultaten.

Tot op vandaag concentreren de meeste onderzoekers zich vooral op het aandeel van de bloedplaatjes en de factoren die ze vrijzetten in de verbeterde wondgenezing. Maar ook het fibrinenetwerk zelf en de witte bloedcellen in L-PRF kunnen een belangrijke bijdrage leveren. Momenteel is er nog niet veel bekend over de mechanische eigenschappen van L-PRF of over de exacte rol van de leukocyten. Door ook deze twee componenten beter te karakteriseren zou het productieproces van L-PRF in de toekomst bijvoorbeeld aangepast kunnen worden om de leukocyt inhoud of fibrine architectuur lichtjes te wijzen. Op deze manier zou L-PRF op maat gemaakt kunnen worden, afhankelijk van de exacte toepassing. Ondanks het feit dat er dus nog enkele obstakels overwonnen moeten worden, werd er tot nu toe toch aangetoond dat plaatjesconcentraten een veelbelovende therapie zijn voor het stimuleren van angiogenese en wondheling. Bovendien blijkt het een veilige, betrouwbare en een economische manier om weefselherstel te bevorderen.

Tot slot, geven de resultaten van dit doctoraatsproefschrift aan dat er verschillende biologische materialen gebruikt kunnen worden in de regeneratieve geneeskunde. Hoewel farmacologische behandeling van PDLSC zorgde voor een verhoogde productie van stimulerende eiwitten, bleken deze cellen enkel in staat om endotheelcellen aan te trekken, maar slaagden ze er niet in om bloedvatvorming te stimuleren *in ovo*. L-PRF daarentegen stimuleerde endotheelcelproliferatie,- migratie en -tubulogenese alsook de bloedvatvorming *in ovo*. Hoewel het exacte werkingsmechanisme nog ontrafeld moet worden, voorspellen de eerste resultaten toch een veelbelovende toekomst voor het gebruik van L-PRF in de regeneratieve geneeskunde.

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Curriculum vitae

Jessica Ratajczak werd geboren op 2 februari 1991 in Waterschei. In 2008 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de afstudeerrichting Latijn-Wetenschappen aan het Sint-Pauluscollege te Houthalen. In hetzelfde jaar startte ze haar universitaire opleiding aan de Universiteit Hasselt, waar ze in 2011 haar bachelor diploma in de Biomedische Wetenschappen behaalde. Na het afronden van haar eindwerk behaalde ze in 2013 haar master diploma in de Biomedische Wetenschappen met grote onderscheiding. Haar eindwerk getiteld: 'Evaluation of iron oxide labeling as a tool for long term cell tracking and its effects on human dental pulp stem cell differentiation', werd uitgevoerd in het Biomedisch Onderzoeksinstituut (BIOMED) van de Universiteit Hasselt onder supervisie van Prof. dr. Ivo Lambrichts. Vervolgens startte ze in oktober 2013 haar doctoraat in de groep Morfologie. Tijdens deze periode maakte ze actief deel uit van het onderwijsteam van verschillende vakken in de opleidingen Biomedische Wetenschappen en Geneeskunde. Tijdens haar doctoraat volgde ze verschillende cursussen in het kader van de Doctoral School for Medicine and Life Sciencens zoals: project management, biosafety, scientific English, statistical thinking and smart experimental design en leadership skills. Bovendien heeft ze actief deelgenomen aan de organisatie twee wetenschappelijke symposia. De resultaten van dit doctoraat werden deels gepubliceerd in internationale tijdschriften en werden tevens gepresenteerd op verschillende (inter)nationale meetings. Tijdens het symposium 'Belgian Symposium on Tissue Engineering' ontving ze hiervoor in 2016 de prijs voor de beste poster presentatie.

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Dankwoord

Foei Foei, je moest je schamen! Ik durf ervoor te wedden dat je slechts vluchtig doorheen de rest van de thesis hebt gebladerd vooraleer je hier bent aanbeland. Het dankwoord blijft traditiegetrouw het meest begeerde hoofdstuk van een doctoraatsthesis. Nu je hier dan toch bent, zou ik dan toch graag even de resultaten van mijn thesis samenvatten, vooraleer ik overga tot mijn lofzang over collega's, vrienden en familie die mij hebben bijgestaan tijdens mijn doctoraat. Dus, wat heb je zoal gemist? Awel, in hoofdstuk 1… nee hoor grapje, laat de zoektocht naar je eigen naam beginnen: 3…2..1. Start!

Eerst en vooral wil ik natuurlijk mijn promotor, prof. dr. Ivo Lambrichts, bedanken. Ik weet nog goed dat je mij 's avonds belde, na mijn sollicitatie, om mij te vertellen dat ik officieel het nieuwste lid was van 'Team Ivo'. Toeval wil, dat ik net op dat moment op bezoek was bij de buren, waar ze met twee hun wijsheidstanden hadden laten trekken die dag. Ik kan me geen meer toepasselijke start van mijn doctoraat voorstellen. Ik wil je dan ook hartelijk bedanken voor de steun, het vertrouwen en het creëren van zo'n aangename werkomgeving. Natuurlijk wil ik je ook bedanken voor alle kansen die ik de afgelopen jaren heb gekregen, zoals de opstart van het L-PRF project en de presentatie op het eerste L-PRF congres maar natuurlijk ook het congres in Japan! Bedankt!

Behalve een promotor, kreeg ik niet één maar zelfs twee co-promotoren. Wendy, hartelijk bedankt voor alle steun tijdens de beginfase van mijn doctoraat. Voor je kritische blik en nieuwe ideeën voor de opstart van nieuwe experimenten en voor de bemoedigende woorden, wanneer ik het even niet meer zag zitten. Ik ben ervan overtuigd dat je leerlingen aan je lippen hangen tijdens je lessen biologie en dat ze van geluk mogen spreken met zo'n leerkracht als jij!

Annelies, mijn co-promotor, mijn steun en toeverlaat. Net terug uit zwangerschapsverlof na de geboorte van de olijke tweeling, en hopla, een tweede doctoraatsstudente op je dak. Gelukkig schreef ik net als Petra met een latje, iets waar je blijkbaar zo trots op was, dat je op BSTE verkondigde dat je vanaf nu enkel nog mensen zou aannemen die met een latje schrijven. Ik kon altijd bij jou terecht voor mijn (vaak moeilijke) vragen. Vergaderen, brainstormen of troubleshooten gebeurde standaard aan het koffietoestel, de verhuis naar Bioville zorgde zelfs voor een upgrade naar Nespresso, what else? Jouw nooit aflatend

enthousiasme vormde de perfecte balans voor mijn zwartkijken, mijn voorliefde voor schema's en planningen was dan weer een tegengewicht voor de chaos in jouw hoofd. Kortom een perfect team! Ik hoop dat het niet te warm is vandaag, want Veronique zal er wel weer op toezien dat je 'panties' draagt onder je toga, zeker nu je een gerespecteerd professor bent! ☺

Graag wil ook mijn juryleden bedanken voor het nalezen en kritisch beoordelen van mijn thesis. Bedankt prof. dr. Bert Brône, prof. dr. Virginie Bito, prof. dr. Renilde Jacobs, prof. dr. Nadia Zakaria en prof. dr. Ribatti. Prof. dr. Domenico Ribatti, thank you for evaluating my thesis and for making the trip to Belgium.

Prof. dr. Marc Quirynen en prof. dr. Joke Duyck, bedankt voor het introduceren van L-PRF in ons labo en om samen met ons te zoeken naar de meest efficiënte manier om onze verschillende expertises samen te brengen, om zo het mysterie van L-PRF te ontrafelen. Ik zou héél het L-PRF team van de KULeuven willen bedanken voor jullie enthousiasme, jullie suggesties en voor de warme ontvangst van 'Team Ivo' op vergaderingen en symposia. Nastaran, Mostafa en Geertje samen ontdekte we een klein stukje Japan, gingen we op zoek naar westerse toiletten, werkende geldautomaten of een verdwenen oorbel en trotseerde we de Japanse keuken. Nooit was een groep Belgen zo blij met friet-biefstuk als wij op de laatste avond van het PBRG symposium. Hartelijk bedankt om van dit congres een onvergetelijke belevenis te maken!

Yanick, bedankt voor de leuke samenwerking, de leuke babbels en natuurlijk de heerlijke pralines achteraf! Ik vind het nog steeds spijtig dat ik niet aanwezig kon zijn bij jouw verdediging. Nog veel succes bij Beta-cell en natuurlijk ook in je verdere carrière.

A word of thanks also goes out to dr. Ilayaraja Muthuramu and Ruhul Amin for teaching us the murine wound healing model. Without your help chapter 4 of this thesis would not have been possible. Thank you for making the trip to Hasselt, we greatly appreciated your help!

Dan nu, la moment suprême: de BIOMED collega's. Sommige onder jullie zag ik dagelijks, soms zelfs meer dan mijn huisgenoten, anderen kwam ik vooral tegen op de gang of aan het koffieapparaat. Want we weten allemaal: wie een wetenschapper zoekt, richt zich best tot het dichtstbijzijnde koffieapparaat. Zo

heeft ieder op zijn of haar eigen manier wel een steentje bijgedragen aan mijn doctoraat en gezorgd voor een aangename werkomgeving.

Mijn bureaugenootjes (the originals): Petra, Stefanie en Pascal. Dag in dag uit op elkaars lip en dat in een afgesloten sardineblik, want veel groter was de C130 niet. Pascal, jij moet nogal gevloekt hebben toen daar nog een extra vrouw op de proppen kwam, maar nog veel harder toen ineens (op 1 april) de enige vrije muur van ons blik vol hing met afbeeldingen van mannen in bloot bovenlijf. Tot onze grote verbazing mochten ze toch enkele weken blijven logeren. Jij moet (met momenten) nogal hebben afgezien met al die vrouwen, maar zelfs ten midden van een ware oestrogeen storm, bleef je nog je koele zelve. Merci voor je goede raad, je gezelschap bij Baar Gerard, het openen van de pintjes (aan de kapstok) van ons feestmaal in Stockholm en natuurlijk het uitlenen van je 'bankkaske' elke keer ik toch nog snel een Zalando bestelling moest plaatsen! Ik wens je nog véél succes en ik ben ervan overtuigd dat er nog stapels en stapels artikels zullen volgen!

Stefanie, wat had ik even geluk dat je mij al had zien rondlopen tijdens mijn seniorstage, want verandering is voor jou eerder een noodzakelijk kwaad. Gelukkig konden we het al snel heel goed met elkaar vinden en mocht ik dan toch tegenover jou blijven zitten. Blijkbaar praat ik luidop tegen mezelf, maar dat overkomt jou ook wel eens…Guacamole! Man wat heb ik een bewondering voor jouw doorzettingsvermogen! Waar jij soms de moed blijft halen om ondanks alle tegenslagen er telkens toch weer voor de volle 100% voor te gaan, gaat mijn petje te boven! Chapeau! Steen en been hebben we geklaagd over van alles en nog wat, maar we hebben ook vooral gelachen, gedronken, gedanst en gewinkeld. Want als we dan toch in het weekend moeten komen werken, kunnen we even goed het nuttige aan het aangename koppelen en nog even langs de winkels lopen (en ze leegkopen). Ondanks je muzikale beperkingen en onze zeer uiteenlopende muzieksmaken, zijn we allebei in zwijm gevallen voor onze gemeenschappelijke held, de Fonzy Fons ☺! Ik ben ervan overtuigd dat je een geweldig post-doc zal zijn voor de andere leden van 'Team Sven'. Wonder Woman (of Mega Mindy) met ballen aan haar lijf dat ben jij!

And last, but certainly not least: Petra. Ik had het dan toch bij het rechte eind in die eerste week van mijn doctoraat. Door de rariteiten van de ander hebben we

onszelf de afgelopen jaren toch vaak minder raar gevoeld. Een voorliefde voor to-do lijstjes, schema's met kleurencodes en een hekel aan te laat komen of niet gerespecteerde deadlines leken plots de meest normale zaken ter wereld. Net zoals pipetpuntjes in een strikte volgorde uit het doosje nemen (analoog met de plaat-layout natuurlijk!) of het schrijven met een latje (ja, zelfs op ruitjespapier). Trein- en busreizen worden immer weken van tevoren gepland en owhee als de voorziene ticketverkoopster niet op de verwachte plaats zit! Bedankt voor alle brainstorm sessies, de bemoedigende woorden en natuurlijk het nalezen van mijn thesis. Maar we hebben doorheen de jaren ook heel wat concerten en festivals afgeschuimd en redelijk wat kilometers gezwommen. Kortom: met of zonder latje, wij zitten altijd op dezelfde lijn ☺. Zelfs nu beginnen we gelijktijdig aan een nieuw hoofdstuk! Ik kijk er dan ook al reikhalzend naar uit om Pixel te ontmoeten. Jij en Brecht gaan dat geweldig doen!

Petra werd dr. Hilkens dus: 'Out with the old, in with the new'. Petra's plaatsje op onze bureau werd vanaf nu ingenomen door Yörg en ik was niet langer het jongste lid van de Morpho-Mountain (snif, snif). Eindelijk wat versterking voor Pascal, of dat dacht hij toch. Het duurde echter niet lang voordat ook jij een (weliswaar zeer stoer) My Little Pony personage (Sheriff Silverstar) kreeg toegewezen, deelnam aan de ladies night en je mening gaf over mogelijke Zalando aankopen ☺. Door je bureau te delen met 3 oude rotten, heeft die naïeve eerstejaars fase niet lang geduurd. Zeker naarmate onze verdedigingen dichterbij kwamen, heb je heel wat gezaag moeten aanhoren, sorry daarvoor! Bedankt voor alle keren dat je de mirax weer aan de praat kreeg en voor me weer met mijn voetjes op de grond te zetten als ik weer eens de Drama Queen uithing. Merci voor de companie tijdens onze levensreddende bloeddonaties, voor het delen van mijn Daredevil/Marvel obsessie, voor alle flauwe mopkes, onnozeliteiten en alle overige shenanigans. Ik wens je nog massa's data, minstens evenveel publicaties als Pascal en vooral véél sterkte met al die vrullie op uwe bureau binnenkort!

Ook de overige leden van de Morpho-Mountain mogen natuurlijk niet ontbreken: Esther, Tim, Ronald en natuurlijk onze newbies: Greet, Melissa en Hannelore. Esther, bedankt voor de vrolijke noot, je optimisme en voor de introductie tot de rode wijn. Tim, bedankt voor de leuke samenwerking rond de L-PRF. Ik wens je véél succes met jouw thesis en natuurlijk in je verdere carrière. Ronald, jij bent

volgens mij geboren om achter een microscoop te zitten want elke labmeeting kom je weer op de proppen met de meest fantastische foto's (en iets minder geweldige tekeningen) van tandjes. Greet, hartelijk bedankt voor alle hulp tijdens mijn dierproeven. Zonder jou had ik in het begin zeker hele dagen in het animalium moeten doorbrengen. Ik wens je het allerbeste, een doctoraat boordevol succesvolle transwells en met een hele stapel artikels! Melissa, onze Italiaanse furie en spring int veld. Na het voorval van de verdwenen pipetten had je waarschijnlijk nooit verwacht dat we het zo goed met elkaar zouden vinden. Maar onze passie voor wetenschap, wijn en winkelen maakte van ons al snel de beste collegaatjes! Ik zal natuurlijk nooit die legendarische zoektocht naar de auto met jou op mijn rug vergeten, na een avondje dansen! Met jouw overvloed aan enthousiasme, kan het gewoon niet anders dan dat je je doctoraat tot een goed einde gaat brengen, daar twijfel ik niet aan! Hannelore, eigenlijk pas officieel het nieuwste morpho-member vanaf september, maar toch nu al 'part of the gang'. Je bent al lid van de morfo-zwemclub, mede-oprichtster van de BIOMESSI supportersclub en vaste klant bij Baar Gérard. Kortom: al helemaal ingeburgerd. Véél succes!

Dan zijn er natuurlijk ook nog de leden van 'Team Sven': Daniela, Selien en Céline, beter bekend als onze buurvrouwen in Bioville. Bedankt voor de gezellige babbels als we onze bureaus moesten ontruimen voor de poetsvrouwen of het geklaag over (de zoveelste) verhuis. Ik ben ervan overtuigd dat al jullie harde werk uiteindelijk beloond zal worden met een mooi doctoraat. Véél succes ermee! Ik mag ook zeker Leen niet vergeten. Bedankt om alles in goede banen te leiden in de celkweek en voor alle mycoplasmatests, zodat we weer even gerustgesteld konden zijn.

Myriam, Nathalie, Evi, Derv, Lisa, Kathleen en Tom, jullie maakten tijdens mijn doctoraat ook allemaal deel uit van 'Team Morpho' en droegen op die manier allemaal jullie steentje bij aan mijn doctoraat.

Dan zijn er natuurlijk nog de collega's van gebouw D: Liliane, Dennis, Davy, Marc, Jeanine, Kathleen, Katrien, Marjan, Linda en Koos-Jaap. Bedankt voor de leuke babbels tijdens lunchpauzes, examentoezichten, kerstfeestjes, verjaardag traktaties of spontane acties van gezelligheid. Marc en Jeanine hartelijk bedankt voor jullie hulp bij het verwerken van de stalen van mijn dierstudies en alle tips &

tricks in verband met kleuringen. Dennis, merci voor alle keren dat ik de camera kwam lenen, voor elke vrolijke begroeting in de koffiekamer (inclusief bewondering voor mijn garderobe ☺) en natuurlijk de onvergetelijke rondleiding in de snijzaal! Liliane, bedankt voor het inbinden van alle kladversies van mijn thesis. Natuurlijk mag ik ook ons Marie-Jeanne niet vergeten! Merci voor alle witte wijntjes en alle versnaperingen op vrijdagavond, maar vooral bedankt voor goedgeluimde ontvangst! Zo gingen wij keer op keer met een glimlach op ons gezicht het weekend tegenmoet!

Behalve de leden van de Morpho-Mountain zijn er natuurlijk nog tal van andere collega's op BIOMED. Met de collega's van fysiologie is de fun ook altijd verzekerd. Silke en Dorien, jullie leerde ik tijdens mijn eerste jaar al wat beter kennen door de organisatie van het PhD symposium. Dorien, bij deze nogmaals mijn excuses voor de angstaanjagende stalker sms'en. Bedankt voor de leuke babbels op de gang of in het animalium en natuurlijk voor de occasionele vleeskeuring op Baouzza. Petra, Maxim, Sophie en Sofie bedankt voor het gezellige tijdverdrijf en natuurlijk de Cuba libres op een mooie lentedag!

Dan zijn er natuurlijk ook nog de laboranten: Katrien, Igna, Christel en Kim. Bedankt voor alle introducties, voor jullie hulp met de microscoop, cryostaat, FACS, met ethische matrixen en voor het aanleggen van geheime voorraden van ladders ☺. Bedankt Regine om je poetsplanning aan te passen elke keer als ik je voor de voeten liep door mijn celkweek experimenten in de vroege uurtjes. Joke, Paul en Yennick bedankt voor alle hulp in het animalium.

Behalve labowerk, komt er bij een doctoraat natuurlijk ook heel wat papierwerk kijken. Gelukkig kunnen we daarvoor altijd rekenen op Rani, Veronique, Stefanie en Jessica. Rani, ik heb nooit geweten dat Excel zoveel mogelijkheden had totdat ik een keer bij jou passeerde om iets te regelen voor het PhD symposium. Veronique, wat zou BIOMED toch zijn zonder jou? Ontelbare keren heb ik aan jouw deur gestaan om je te verwittigen dat ik weer een lading kippeneieren verwachtte of kwam ik weer aan je mouw trekken om voor de zoveelste keer bloed af te nemen. Multitasken kan jij als geen ander! Ik zie je nog zo de voetbaltruitjes strijken voor de match van het jaar, terwijl je bezoek ontvangt en nog wat telefoontjes pleegt en e-mails verstuurt. Veronique, hartelijk bedankt om heel de aflegprocedure zo mooi te stroomlijnen en om ervoor te zorgen dat

uiteindelijk alles mooi in de plooi viel! Stefanie, bedankt voor de begeleiding tijdens de organisatie van het PhD symposium en voor alle administratie in verband met de doctoral school. Jessica, bedankt om na elk telefoontje toch nog de bestelbon op te maken voor de zoveelste dringende bestelling. De collega's in Bioville verdienen hier ook een plaatsje: Michele, Katrien, Jelle en Stefanie, bedankt voor de goede ontvangst!

Doorheen de jaren zijn er ook wat studenten de revue gepasseerd die ook elk hun steentje hebben bijgedragen aan mijn doctoraat en dus ook zeker een plaatsje verdienen in het dankwoord. Ik was amper 3 maanden bezig toen Eline aan haar stage begon. Toeval wil dat ik net op dat moment ziek was en geen stem had. Ik heb je de eerste dag dus alles letterlijk met hand en tand moeten uitleggen, mijn excuses daarvoor. Dat was zowat de enige keer dat ik ziek ben geweest de afgelopen jaren. Bedankt voor alle hulp met de optimalisatie van het priming protocol en veel succes met je verdere carrière! Na een jaartje was ik klaar om de volgende stagiair te ontvangen, deze keer voor een periode van 8 maanden zodat ik extra veel werk verzet zou krijgen. Wim, in het begin liep het zeker niet van een leien dakje, de chaos in jouw hoofd en mijn voorliefde voor schema's, structuur en strikte deadlines leek niet de meest geschikte combinatie. Maar na wat acclimatisatie konden we het toch uitermate goed met elkaar vinden. Halverwege je stage stonden we zelfs in het labo te kibbelen als een oud getrouwd koppel en kon je aan mijn gezicht zien wanneer je me beter geen vragen kwam stellen. Tegen alle verwachtingen in, werden we dan ook de beste vriendjes. Ik wil je dan ook heel erg bedanken voor je inzet tijdens je stage en voor die keer dat je zelfs tussen twee vluchten door mij nog kwam helpen in het labo!! Er volgende dan ook nog heel wat leuke momenten: terrasjes, sushidates, cocktailavonden, Temptation verslagen en Pukkelpop. Ook al zie ik je nu wat minder nu je de wereld rondvliegt, ik hoop van harte dat we nog jaren blijven verder chateren! Kirsten, ook jij hebt zeker en vast bijgedragen aan deze thesis. Jij hebt de opstart van het L-PRF project meegemaakt, je had waarschijnlijk nooit gedacht dat iemand zo vrolijk kon worden van een geslaagde tube formation als ik. Hartelijk bedankt voor al je harde werk en veel nog veel succes verder!

Dan zijn er natuurlijk ook nog mijn klasgenootjes, die samen met mij het hele traject hebben afgelegd en waarvan sommigen zelfs collega's zijn geworden. Sam,

Chloë, Manolo, Elien, Hannelore en Martijn bedankt voor de leuke studententijd en de steun tijdens mijn doctoraat. Sam, bedankt voor de leuke momenten tijdens onze jaarlijkse reünie op Genk on Stage en natuurlijk ons tripje naar Lissabon (waar we toch ook één dag de nerd hebben uitgehangen in de plaatselijke technopolis). Chloë, elk symposium in Leuven was een goed excuus om nog eens bij te kletsen, sushi te gaan eten of een terraske te doen. Ne dikke merci voor de leuke babbels en het geklaag en gezaag! Bitches for life! Manolo, onze Mexicaanse vriend, ik zal nooit je 'drie-melkentaart' of regenboogtaart vergeten of de legendarische 'mole'! Ik weet ondertussen dat met een beetje pikant en citroen alles beter smaakt! Ik hoop dat ik je kan komen bezoeken in Mexico en wens je nog het allerbeste met je doctoraat! Hopelijk tot snel! Elien, ook jij werd verbannen naar Bioville, wat uiteindelijk toch beter meeviel dan verwacht. Bedankt voor de paaseitjes, de kerstkaartjes en de babbels op de gang. Ik ben ervan overtuigd dat ook voor jou alles goed gaat komen, ook al lijkt dat niet misschien altijd zo! Véél succes! Hannelore en Martijn, ons power couple, jullie gaan het nog ver schoppen, daar ben ik zeker van! Bedankt voor de 'liquid courage' in tijden van nood ;-)

Dan zijn er natuurlijk ook nog de vriendinnetjes uit het middelbaar: An-Katrien, Dorine, Esther, Inge en Karolien. Dames, bedankt voor alle leuke momenten doorheen de jaren! Ik ben ontzettend blij dat we na het middelbaar elkaar nog steeds op regelmatige basis blijven zien. Of we nu gezellig iets gaan eten, een filmpje meepikken, een festivalleke doen, gaan kerstschaatsen, het nieuwe jaar inzetten, een citytripke doen, in de cocktails vliegen of ons dansschoentjes bovenhalen, het is altijd even leuk. Binnenkort staat de eerste vrijgezellen op het programma en daar kijken we allemaal natuurlijk reikhalzend naar uit. Karolien, hopelijk wordt het een dag om nooit meer te vergeten (en de trouw natuurlijk ook hé)! Esther, jij hebt waarschijnlijk nog het meeste meegekregen van alle thesisperikelen, bedankt om te delen in mijn frustratie, verontwaardiging of teleurstellingen. Merci voor de (vele) peptalks! Dat er nog vele Marvel-films mogen volgen ☺. Sjoeke, van Beklim-kamp naar Amsterdam tot in Boedapest, wij hebben de afgelopen jaren toch heel wat avonturen beleefd. Ondanks alle drukte weet ik dat ik altijd bij jou terecht kan, hetzij voor een goede babbel hetzij voor een goed glas wijn (of allebei natuurlijk).

Isabel, het begon allemaal meer dan 15 jaar geleden op een camping in Nieuwpoort. Als wij samen op uitstap gaan, is dat altijd een kleine vakantie, een beetje de toerist uithangen in eigen land. Zulke uitstapjes worden dan ook vaak gekenmerkt door 'du Vin, du Pain et du Boursin' want picknicken doen we echt overal: op het Noordzeestrand, nabij de Maastrichtse stadswallen, op een Luiks stadsbankje, onder een Genkse of Brugse kerktoren of op de Gentse Graslei. De Gentse Feesten en Aalst carnaval staan ook al enkele jaren met stip op het programma en ook je vrijgezellen en trouwfeest zal ik niet snel vergeten net zoals het foute kerstfeestje van afgelopen jaar! Hartelijk bedankt voor al deze leuke momenten waardoor ik even kon ontsnappen aan de dagdagelijkse beslommeringen! Ik kijk er dan ook enorm naar uit om binnenkort jullie kleine spruit te mogen ontmoeten.

Diëm, 'my brother from another mother', na een dikke twintig jaar kan ik eindelijk je naam onthouden ☺! Ook al zien we elkaar tegenwoordig veel te weinig, toch is het elke keer een beetje thuiskomen. People watching is één van onze favoriete bezigheden en dat kunnen we ondertussen al bijna volledig telepathisch. Dat is vooral handig voor later, als we oud, versleten en doof zijn, kunnen we toch nog stiekem roddelen terwijl we van ons zjatje kaffee slurpen.

Liefste zusiewusie, eerst en vooral bedankt om mij telefonisch bij te staan toen ik als een clochard in een bank in Gent stond met al mijn carnaval gerief (belofte maakt schuld)! Zoveel als ik op ons mama lijk, zoveel verschillen wij. Maar ondanks het feit dat we meestal kat en hond zijn, kunnen we toch bij elkaar terecht als het echt nodig is. Veel succes met je masterthesis volgend jaar, ik bereid me alvast voor op minstens evenveel geklaag en gezaag, maar ook bij jou zal het uiteindelijk wel allemaal in orde komen!

Mama en papa, wat waren jullie stiekem toch een beetje opgelucht toen ik besloot om geschiedenis in te ruilen voor biomedische. Als ik jullie mag geloven, heb ik in de eerste week van de unief meer met mijn neus tussen mijn boeken gezeten dan in die 6 jaar middelbaar samen. Ik moet toegeven, ik heb ook absoluut geen spijt gehad van mijn keuze. Bedankt voor alle steun doorheen de jaren! Mama, bedankt voor alle zjatjes kaffee en lekkernijen die je mij tijdens de blok hebt gebracht. Mama, gelukkig kan jij door het werk wel heel wat verdragen en kon ik zelfs tijdens het eten over mijn avonturen in de snijzaal of over mijn muizen vertellen

zonder je eetlust te bederven. Jullie hebben je waarschijnlijk maar al te vaak afgevraagd waar ik mij toch zo druk over maakte. Jullie waren er dan ook altijd (in mijn plaats) van overtuigd dat alles wel goed zou komen.

Tenslotte wil ik ook mijn Moeke en Bompa bedanken uit de grond van mijn hart! Vanaf dag 1 stonden jullie altijd voor mij klaar en ik heb dan ook zo ontzettend veel aan jullie te danken. Bedankt voor alle liefde en warmte, voor de mooie vakanties en voor alle kaarsjes en weesgegroetjes tijdens de examens. Moeke, in het middelbaar hielp je mij al door het ondervragen van Latijnse woordjes en ook tijdens de unief kwam je tijdens de blok altijd eens horen wat ik nu weer aan het studeren was. Je was dan ook bij al mijn proclamaties steeds op post en ik zou dit boekje dan ook graag opdragen aan jou! Merci Grote Voi en Mini Moi!

"You do not really understand something,

until you can explain it to your grandmother"

- Albert Einstein