The effects of EPO on cutaneous wound healing, dermal stem cell marker activation, and gene expression patterns

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Meimanat Fathi, Karshenasi arshad (Master), Islamic Azad University

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Referent	:	Prof. Dr. rer. nat. Thomas Scheper
Korreferent	:	Univ.-Prof. Dr. med. Augustinus Bader
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ZUSAMMENFASSUNG

Narben wirken sich oft negativ auf die individuelle Selbstwahrnehmung von Schönheit, Lebensqualität und Selbstvertrauen aus. Um diese negativen Auswirkungen zu verringern, versuchen Forscher die biologischen Mechanismen, die zur Narbenbildung führen, besser zu verstehen, damit wirksamere klinische Behandlungen entwickelt werden können. Langfristige Ziele sind die Hautregeneration zu beschleunigen und die narbenlose Heilung von Wundverletzungen. In früheren Studien haben Forscher gezeigt, dass niedrig dosiertes Erythropoietin (EPO) regenerative und zytoprotektive Wirkungen in präklinischen Tiermodellen und sogar bei klinischen Patienten mit Verbrennungen oder diabetischen Wunden hat. Allerdings sind die molekularen Mechanismen, durch die EPO die Narbenbildung und/oder Wundheilung im menschlichen Hautgewebe beeinflusst, unklar. Daher werden in diesem Forschungsprojekt die Wirkung von EPO auf die Wundheilung von Hauttransplantaten beim menschlichen Patienten mit immunhistochemischen Methoden untersucht, und dann zusätzliche In-vitro-Experimente durchgeführt, um zu prüfen, wie die Wirkung von EPO durch das Vorhandensein anderer kleiner Moleküle, sogenannter proinflammatorischer Zytokine, beeinflusst wird. Im Einzelnen wurde in einer Studie (i) die Wirkung einer systemischen Anwendung von EPO auf immunhistochemische Indikatoren der Wundheilung anhand von Hautproben aus einer klinischen Studie untersucht, bei der EPO-behandelte Gruppen mit Placebo-behandelten Gruppen verglichen wurden. In einer zweiten In-vitro Studie wurde: (ii) die Wirkung der EPO-Verabreichung (allein und in Kombination mit verschiedenen Zytokinen, z. B. IL-6, TNF-a) auf die Expressionsniveaus von Genen, die mit der Wundheilung in Verbindung stehen, getestet. In der ersten Studie ergaben die immunhistochemischen Analysen keine eindeutigen Muster, die die Behandlungs- und Kontrollgruppen unterschieden, mit Ausnahme des von-Willebrand-Faktor, der Anzeichen einer verringerten Expression in der der EPO-behandelten Gruppe zeigte. In der zweiten Studie führte die Verabreichung von EPO und die gleichzeitige Verabreichung von EPO mit den Zytokinen TNF-a und IL-6 zu einigen Veränderungen der Genexpression in den Mesenchymalen Stammzellen die aus der Vorhaut gewonnen wurden. Die Behandlung mit EPO allein und die gleichzeitige Verabreichung von EPO mit TNF-a und IL-6 führte jedoch nicht zu statistisch signifikanten Unterschieden in GO- oder KEGG-Signalwegen.

Schlagwörter: Erythropoetin, EPO, Wundheilung, Hautregeneration, Proinflammatorische Zytokine, Genexpression

ABSTRACT

Scars often have a negative impact on an individual's self-perception of beauty, quality of life, and self-confidence. In order to reduce these negative impacts, researchers are attempting to better understand the biological mechanisms that lead to scar formation, so that more effective clinical treatments can be developed. A long-term goal is to accelerate skin regeneration and facilitate scarless healing of wound injuries. In previous studies, researchers have shown that low dose erythropoietin (EPO) has regenerative and cytoprotective effects in preclinical animal models, and even in clinical patients presenting with burns or diabetic wounds. However, the molecular mechanisms through which EPO affects scar formation and/or wound healing in human skin tissue remains unclear. Therefore, in this research project, the effect of EPO on skin-graft donor site wound healing in human patients is evaluated using immunohistochemical methods, and then additional *in vitro* experiments are conducted to test how the effects of EPO may be impacted by the presence of other small molecules called pro-inflammatory cytokines. Specifically, in one study: (i) the effect of systemic application of EPO on immunohistochemical indicators of wound healing is tested using skin samples from a clinical trial that compared EPO-treated to placebo-treated groups. In a second *in vitro* study: (ii) the effect of EPO administration (alone and in combination with different cytokines, e.g., IL-6, TNF-α) on expression levels of genes linked to wound healing was tested. In the first study, immunohistochemical analyses failed to uncover any strong patterns that differentiated treatment and control groups, with the exception of von Willebrand factor, which showed signs of decreased expression in the EPO-treated group. In the second study, EPO administration, and co-administration of EPO with TNF-α and IL-6 cytokines, led to some changes in gene expression levels in foreskin-derived mesenchymal stem cells. However, treatment with EPO alone, and co-administration of EPO with TNF-α and IL-6, did not lead to statistically significant differences in GO or KEGG signaling pathways.

Keywords: Erythropoetin, EPO, wound healing, skin regeneration, pro-inflammatory cytokines, gene expression

Dedicated to my mother, Fatemeh Shojaian, my father, Ebrahim Fathi,
my brother, Mohammed Ali Fathi, and my partner Cody Ross.

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ACRONYMS

ABL2 Abelson-related gene 2

ACE 3-amino-9-ethyl-carbazol substrate

ADAMTS18 ADAM metallopeptidase with thrombospondin type 1 motif 18

ADNP Activity dependent neuroprotector homeobox

AGER Advanced glycosylation end-product specific receptor

AGT Angiotensinogen

AHNAK Neuroblast differentiation-associated protein

AKT Protein kinase B (PKB)

ALOX15 Arachidonate 15-lipoxygenase

ALOX15 Arachidonate 15-lipoxygenase

AMPK AMP-activated protein kinase

AQP-3 Aquaporin-3

ARHGAP24 Rho GTPase activating protein 24

αSMA Alpha smooth muscle actin

ATG3 Autophagy related 3

ATP Adenosine triphosphate

Bcl-2 B-cell lymphoma 2

Bcl-XL B-cell lymphoma-extra large

BLK B lymphoid tyrosine kinase

BP Biological processes

CAM1 Calmodulin-1

CC Cellular component

CCR2 C-C motif chemokine receptor 2

CD Cluster of differentiation

CD10 Cluster of differentiation 10

CD105 Cluster of differentiation 105 or Endoglin

CD106 Cluster of differentiation 106

CD11b Cluster of differentiation 11b

CD129 Cluster of differentiation 129

CD13 Cluster of differentiation 13

CD14 Cluster of differentiation 14

CD146 Cluster of differentiation 146

CD19 Cluster of differentiation 19

CD26 Cluster of differentiation 26

CD271 Cluster of differentiation 271 or low-affinity nerve growth factor receptor (LNGFR)

CD31 Cluster of differentiation 31 or platelet endothelial cell adhesion molecule (PECAM-1)

CD34 Cluster of differentiation 34

CD44 Cluster of differentiation 44

CD45 Cluster of differentiation 45

CD54 Cluster of differentiation 54

CD71 Cluster of differentiation 71

CD73 Cluster of differentiation 73

CD79a Cluster of differentiation 79a

CD90 Cluster of differentiation 90 or Thy-1

CFUe Colony forming unit erythroid

CHMP1B Charged multivesicular body protein 1B

CHMP6 Charged multivesicular body protein 6

CK1 Cytokeratin 1

CK10 Cytokeratin 10

CK14 Cytokeratin 14

CNR1 Cannabinoid receptor 1

CO_2 Carbon dioxide

Cont Control

COX2 Cyclooxygenase 2

CTSG Cathepsin G

CX3CR1 C-X3-C motif chemokine receptor 1

DAMPs Damage-associated molecular patterns

ddH2O Double-distilled water

DFFB DNA-fragmentation factor subunit beta

DGKA Diacylglycerol kinase alpha

DHODH Dihydroorotate dehydrogenase

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

ECM Extracellular matrix

EDTA Ethylenediaminetetraacetic acid

EGF Epidermal growth factor

EN1 Engrailed homeobox 1

eNOS Endothelial nitric oxide synthase

EPHA7 Ephrin type-A receptor 7

EPO Erythropoietin

EPOR Erythropoietin receptor

FBS Fetal bovine serum

FCS Fetal calf serum

FGF Fibroblast growth factor

FGG Fibrinogen gamma chain

FMR1 Fragile-X messenger ribonucleoprotein 1

FMSCs Human foreskin-derived mesenchymal stem cells

FOXO3a Forkhead box O3

g Gravitational force

$\gamma\,\delta$ T cells Gamma delta T cells

GNAS Guanine nucleotide binding protein, alpha stimulating activity polypeptide

GO Gene ontology

GP5 Glycoprotein V platelet

HE Hematoxylin and eosin stain

HGFAC Hepatocyte growth factor activator

HLA-DR Human leukocyte antigen – DR isotype

HPS4 Hermansky-Pudlak syndrome 4 protein

IF Intermediate filament

IgG1 Immunoglobulin G 1

IHC Immunohistology

IL-1 Interlukin 1

IL-10 Interlukin 10

IL-33 Interleukin 33

IL-6 Interlukin 6

IL-8 Interleukin 8

IL6ST Interleukin 6 cytokine family signal transducer

iNOS Inducible nitric oxide synthase

IU International unit

JAK2 Janus kinase-2

JNK c-Jun N-terminal kinase

KANK1 KN motif and ankyrin repeat domains 1

KDa Kilo dalton

KEGG Kyoto encyclopedia of genes and genomes

KGF Keratinocyte growth factor

KO Knock out

LNGFR Low affinity nerve growth factor receptor

LSP1 Lymphocyte specific protein 1

LTK Leukocyte receptor tyrosine kinase

M1 Macrophage 1

M2 Macrophage 2

MADD MAP kinase activating death domain

MAPK Mitogen-activated protein kinase

MAPK8IP3 Mitogen-activated protein kinase 8 interacting protein 3

MCP1 Monocyte chemoattractant protein 1

MEF2A Myocyte enhancer factor 2A

MF Molecular function

MIA3 Melanoma inhibitory activity protein 3

MIL-6Rα Membrane interleukin-6 receptor alpha

min Minute

μL Microlitre

μm Micrometer

MMP11 Matrix metallopeptidase 11

mRNA Messenger RNA

MSC Mesenchymal stem cells

MUC16 Mucin 16

N_2 Nitrogen

Neg cont Negative control

NF-κB Nuclear factor kappa B

Ng Nanogram

NK Natural killer cells

NO Nitric oxide

NQO1 NAD(P)H quinone dehydrogenase 1

NR4A1 Nuclear receptor subfamily 4 group A member 1

O_2 Oxygen

P P-value

P75NTR P75 neurotrophin receptor

PAX2 Paired box 2

PBS Phosphate-buffered saline

PDGF Platelet-derived growth factor

PDGFB Platelet-derived growth factor subunit B

PDGFRA Platelet-derived growth factor receptor alpha

PDZK1 PDZ domain containing 1

Pen/Strep Penicillin and streptomycin solution

PI3K Phosphoinositide 3-kinase

PIK3CG Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma

PRDM11 PR-domain containing protein 11

RAP2B Ras-related protein Rap-2b

RELT Receptor expressed in lymphoid tissues

rHuEPO Recombinant human EPO

RLD Repetitive-low-dose

RLT RNeasy lysis buffer

RNA Ribonucleic acid

ROI Region of interest

RPE Buffer for washing membrane-bound RNA

RPM Revolutions per minute

RW1 Buffer for washing membrane-bound RNA

SDC1 Syndecan 1

SDW Second degree burn wounds

SERPINC1 Serpin family C member 1

SGDS Skin graft donor site

SHH Sonic hedgehog signaling molecule

SIL-6Rα Soluble interleukin-6 receptor alpha

SLC12A2 Solute carrier family 12 member 2

Smad Small worm phenotype mothers against decapentaplegic

SNAI Snail family transcriptional repressor 1

SPDEF SAM pointed domain containing ETS transcription factor

SSCs Spermatogonial stem cells

SSEA-4 Stage-specific embryonic antigen-4

STAT Signal transducer and activator of transcription

STAT5a/b Signal transducer and activator of transcription 5a/b

STNF Soluble TNF

STRO-1 Stromal cell surface marker-1

TBXA2R Thromboxane A2 receptor

TDW Third degree burn wounds

TGF-β Transforming growth factor beta

TGF-βR Transforming growth factor beta receptor

TH T-helper

Thy1 Thymocyte differentiation antigen 1

TLRs Toll-like receptors

TNF-α Tumor necrosis factor alpha

TNFR1 Tumor necrosis factor receptor 1

TNFR2 Tumor necrosis factor receptor 2

TNFRSF8 TNF receptor superfamily member 8

TNFSF13 TNF superfamily member 13

TPR Tissue protective receptor

Treg T-regulatory cells

TRPC5 Transient receptor potential cation channel subfamily C member 5

UV Ultraviolet

VCAM1 Vascular cell adhesion protein 1

VEGF-A]Vascular endothelial growth factor-A

VEGF Vascular endothelial growth factor

VEGFα Vascular endothelial growth factor alpha

VEGFB Vascular endothelial growth factor B

VIL1 Villin 1

VWF Von-Willebrand factor

WNT Wingless/Integrated

XKR5 X-Kell blood-group precursor-related family, member 5

ZMYND11 Zinc finger MYND-type containing 11

1 EPO AND WOUND HEALING

1.1 INTRODUCTION

In many cases, damage or trauma to human skin can be repaired through natural regenerative mechanisms. However, in some cases, an abnormal and unwelcome healing process may occur, resulting in the formation of excessive scar tissue. The formation of scar tissue is a complex process, made up of different cellular and extracellular mechanisms. A variety of biochemical regulatory processes have been shown to impact scaring, including the synthesis of extracellular matrix (ECM) [11].

For many years, little progress was made in identifying treatments for wound injuries that can eliminate scaring. However, over many years, promising techniques have been developed in the field of regenerative medicine. These techniques include: (i) using skin tissue transplanted from other donor sites (e.g., foot or forearm) to close and repair wound injuries, (ii) using laboratory-created skin cells derived from stem cell cultures to mitigate the need for donor tissue, and (iii) using topical application of bioactive molecules to trigger the regenerative effects of endogenous stem cells and decrease the period of wound healing [124, 162].

In preclinical studies, Günter et al. [45] were able to show that a number of cytokines and growth factors play a role in stem cell activation at wound sites. It is even suggested that erythropoietin (EPO) is one of the key active agents in skin regeneration [45]. EPO plays a key role in skin regeneration, especially in treating acute and chronic tissue injuries [6, 38]. Studies have shown that adding EPO to mesenchymal stem cell (MSC) cultures under hypoxic conditions increases the proliferation rate of MSCs [45]. EPO stimulates angiogenesis, mitosis, vascularization, and cell-cycle activation [45]. EPO is expressed in hematopoetic and several non-hematopoietic tissues, where it plays a role in improving cell survival and delaying inflammation processes caused by hypoxia, toxicity, or injury [45]. It has even been suggested that EPO might have a potential to restore impaired wound healing and stimulate angiogenesis and cell-cycle in burn-damaged tissues [5].

Understanding the molecular and cellular mechanisms responsible for wound healing may provide clues that enable us to develop faster—and possibly scar-less—wound healing treatments. The development of such alternative, rapid, efficient, and cost-saving therapies to treat wounds is a goal of some medical research. Herein, we focus on reviewing the molecular and cellular basis of regeneration and skin

tissue repair, with special attention to dermal mesenchymal stem cells (MSCs). Differences in the speed of healing in various regenerative models can provide key insights for the development of therapeutic strategies that may alleviate some of the morbidity associated with scarring after traumatic injuries.

1.2 AIMS OF THE PROJECT

1.2.1 *Motivations*

The Department of Cell Techniques and Applied Stem Cell Biology at the Centre for Biotechnology and Biomedicine at the University of Leipzig has been engaged in the field of skin regeneration for several years. Their research interests have focused especially on the acceleration of wound healing and skin regeneration in traumatic ulcers and severe burn or scald injuries. In order to investigate the mechanisms responsible for wound healing, *in vitro* cell cultures of mesenchymal fibroblasts (FMSCs) were isolated from human skin donors, cultured, and maintained under normal and trauma conditions with the addition of certain proinflammatory cytokines, like IL-6 and TNF-α. The extent of cell proliferation with and without EPO co-administration was measured and compared. Although the stimulation of FMSCs with EPO alone had an inhibitory effect on stem cell growth *in vitro*, a significant increase in fibroblast growth was observed after 7 days of EPO-cytokine co-administration. Similarly, the administration of IL-1β, IL-6, and/or TNF-α with EPO helped to encourage cell replication. Furthermore, expression of the EPO receptor at the mRNA level was improved [6, 38].

In animal experiments carried out in cooperation with the Department of Plastic and Hand Surgery, University of Lübeck, Lübeck, Germany, recombinant human EPO (rHuEPO) was applied to mice with deep second-degree scald injuries by infusion pump (continuous systemic application). Vascularization was remarkably higher in the rHuEPO treatment group than in the control group [38]. In another study, local topical application of rHuEPO gel was given in deep second-degree scald injury sites in rats [38]. Histological analysis showed that the rate of epithelialization was significantly higher in the rHuEPO gel-treated group than in the control group. Additionally, immunohistochemical studies have revealed that rHuEPO gel-treated groups show higher expression of key cell-surface markers (such as CD31, CD90, CD71, and nestin) than control groups [6, 38]. Here, we aim to study how such results generalize to treatments in clinical settings.

1.2.2 *Methodological outline*

This dissertation focuses on the role that recombinant human erythropoietin (rHuEPO) plays in wound healing and gene expression. Two analyses are presented here. The first is a subanalysis from the "EPO in Burns" project [45–48] that investigates the effect of EPO on vasscularization, re-epithelialization, and dermal stem cell activation at skin-graft donor sites. The second study is a novel *in vitro* test of how gene expression is impacted by co-application of EPO and pro-inflammatory cytokines. Before presenting these empirical studies, a review of the literatures on skin structure, wound healing, scar formation, and the biology of proinflammatory cytokines and EPO is presented.

In the first study, skin samples from the donor sites of skin grafts were used to test if systemic application of EPO can accelerate wound healing. Specifically, in both treatment and placebo groups, we quantified the expression of cell surface markers over a duration of 16 days. To conduct this quantification of cell surface marker expression, histology, immunohistochemistry, and image analysis were used.

In the second study, foreskin mesenchymal stem cell cultures were used to study how the expression of genes involved in wound healing is affected by *in vitro* administration of EPO and trauma cytokines. To conduct this analysis, foreskin mesenchymal stem cells were isolated from human skin samples and were then cultured with and without EPO and trauma cytokines. RNA from the samples was extracted and used for microarray analyses.

The data from both studies were analysed using the R software package [114]. In the second study, data from various bioinformatics databases (e.g., the Gene Ontology Consortium and the Kyoto Encyclopaedia of Genes and Genomes) were integrated into the study, in order to investigate whether genes linked to specific functions (e.g., wound healing, skin fibrosis, or apoptosis) are up-regulated or down-regulated by treatment with EPO.

In the following chapters, the literature reviews, materials and methods, and results and discussion for each of the studies are described in detailed.

2

STUDY 1: EPO AND CLINICAL WOUND HEALING

2.1 STUDY 1: LITERATURE REVIEW

In this section, the structure and function of human skin is first described. Then, a review of the literature on the general biology of cutaneous wound healing is presented. Finally, the literature on wound healing at skin graft donor sites is discussed.

2.1.1 *Skin structure and function*

The skin is the largest organ, and the most important protective layer of the human body. It varies in thickness from less than a millimeter (on the lower eyelid) to several millimeters (on the soles of the feet) [30, 77]. The skin is a defensive barrier against external microbes, and protects the body against mechanical, chemical, osmotic, thermal, and ultraviolet (UV) damage. It has self-healing and renewable properties. The skin is also a sensory organ that allows individuals to sense the environment, react to important stimuli, and avoid potentially dangerous circumstances—e.g., touching toxic plants or hot surfaces. Additionally, the skin stores lipids (fats) and water for the body. Sweat glands, blood vessels, and adipose tissue are involved in the regulation of body temperature, metabolism, and excretion of various substances [30, 77]. The details are reviewed below.

2.1.1.1 *Skin Structure*

The skin is a complex organ. An average of one square inch of skin contains 650 sweat glands, 20 blood vessels, and more than 1,000 nerve receptors [17, 30, 77]. Although the skin is only a few millimeters thick, it makes up about one-seventh of our body weight. The skin also makes up part of the body's immune system. The skin has different thicknesses in different areas of the body. For example, the skin is thinnest under the eyes and thickest on the back, and on the soles of the feet and palms [22, 77].

The dermal structure consists of three main layers: epidermis, dermis, and hypodermis [22, 77].

EPIDERMIS:
The outermost layer of the skin is known as the epidermis. It has an ectodermal origin and consists of epithelial tissue. The epidermis is responsible for regenerating new skin cells, producing pigmentation,

and creating a protective barrier between the body and the external environment. The main function of the epidermis is to build new skin cells at the boundary of the dermal and epidermal layers. It takes about a month for these cells to reach the top layer of the epidermis, where old cells are replaced with new cells. Epidermal cells produce protein that strengthens the skin and protects the skin from drying out [22, 77, 158].

There are different cells located in the epidermis—e.g., keratinocytes, melanocytes, langerhans cells, and merkel cells. Keratinocytes are the most common cell type within the epidermis. Langerhans cells in the epidermal layer of the skin are part of the immune system and protect the body against pathogens. Melanocytes are cells that are present in the epidermal layer and control skin pigmentation. They produce a pigment called melanin, which provides extra protection from the sun's UV rays. The epidermis has no blood vessels, and so epidermal cells must exchange materials with dermal blood vessels. Between the epidermis and the dermis is a thin sheet of fibers called the basement membrane, which attaches the epidermis and dermis together [158].

The epidermis is divided into five layers; from outer to inner, these layers are known as the stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum germinativum [158]. The outermost layer of the epidermis is called the stratum corneum, and consists of 20–30 layers of dead keratinocytes. These dead cells are constantly shed and replaced with new cells that are made in the inner layers of the epidermis. Within a period of about 4 weeks, new skin cells emerge, pass towards the outer surface of the skin, harden, and are finally shed. Keratinocytes in this layer release defensins, and so this layer is the first part of body's immune defense [158].

The stratum lucidum is composed of two to three cell layers of dead keratinocytes, which are located under the stratum corneum [158].

The stratum granulosum is located under the stratum lucidum, and consists of three to five cell layers. Cells in this region have a diamond shape and contain keratohyalin granules and lamellar granules [158].

The stratum spinosum is composed of 8–10 layers of cells. In this region, desmosome structures attach keratinocytes together. These structures help to maintain the integrity of the skin. Also, dendritic cells might appear in the stratum spinosum [8, 158].

The stratum germinativum, or stratum basale, is the deepest layer of the epidermis. It is attached to the basement membrane (basal lamina) by hemidesmosomes, which form the boundary between the epidermis and the dermis. This region consists of different cell types, including stem cells, keratinocytes, and melanocytes [158].

DERMIS:

The dermis is the middle layer of skin, and is also the largest. Approximately 90% of the weight of the skin is located beneath the epidermis

and above the hypodermis [105]. The dermis has a mesodermal origin and consists of connective tissue.

The dermal–epidermal junction of human skin occurs between the basal epidermis and the papillary dermis, and consists of networks of finger-like protrusions [22]. This thick connective membrane is composed of an interconnected network of blood and lymph capillaries, nerves, sensory fibers and terminals, collagen, and elastin proteins that support nerve fibers [145].

The dermis consists of different cell types, glands, vessels, and other materials (e.g., extracellular matrix or ECM). The cells in the dermis include fibroblasts, langerhans cells, melanocytes, endothelial cells, smooth muscle cells, neurons, and tissue-resident immune cells. Fibroblasts are the main cells located in the dermis, and they are responsible for producing ECM, elastin, collagen, growth factors, and cytokines. The dermis is mostly made up of a collagen-protein layer linked in strong networks of collagen and elastin protein fibers, which provide elasticity, resilience, and strength to the skin tissue [145].

ECM components, blood vessels, and lymphatic vessel components are also located in the dermis [87]. Hair follicles, sebaceous glands, and sweat glands can also be found in this layer. The dermis is rich in proteoglycans and glycosaminoglycans that protect the body from fluid waste and act as a water storage source. These same chemicals also help to protect the skin from mechanical damage [87, 145]. The main task of the dermis is to maintain the firmness and elasticity of the skin [145].

The dermis also plays an important role as a sensory receptor and internal regulator [145]. The nerves in dermis contain receptors that transmit signals relating to pain, pressure, touch, itching, and temperature to the brain. It is a place for production of sweat and fat. The sweat glands help cool the body, and the sebaceous glands produce soft, moist fats for the skin. The hair follicles in the dermis cause hair growth on the body. Hair also helps to control body temperature and protect the body against damage. Blood vessels in the dermis nourish the skin and help control body temperature. These vessels nourish the epidermis. When the skin gets too hot, blood vessels dilate to release heat from the surface of the skin. On the other hand, when the skin gets too cold, blood vessels constrict to keep the body warm. Lymphatic vessels, which are an important part of the immune system, are located in the dermis. They protect the body against infection [145].

SUBCUTANEOUS TISSUE OR HYPODERMIS:

The deepest innermost layer of the skin is called the subcutaneous tissue or the hypodermis. This layer is not structurally part of the skin; however, it helps to connect the skin to the underlying bones and

muscles. The hypodermis is mostly made up of fat, such as panniculus and adiposus. It is also composed of loose connective tissue and elastin protein. Elastin fibers help tissues return to their normal positions after stretching. Fat layers can protect the body against heat and cold, and are especially suited at preventing excessive heat loss. This layer is very elastic due to the abundance of fat cells, and acts as a shock absorber as well, protecting bones and muscles from the shocks associated with falls. Neurons and blood vessels are also located in subcutaneous tissues [152].

2.1.1.2 *Wound healing*

Cutaneous wound healing is a complex of biological and physiology processes in the body that involves multiple cell populations, interaction between epidermal and dermal cells, chemotaxis, mitosis, angiogenesis, ECM synthesis, and the action of soluble mediators, such as growth factors and cytokines [144].

The skin constitutes the most important barrier between the body and outside environment. The epithelium is the first, and most critical layer in the body's defensive barrier. When skin becomes injured or cut, pathogens can enter the body and cause infection. It is, therefore, essential that the skin can quickly repair itself via re-epithelialization during wound healing [122].

The repair process in the skin occurs in four overlapping phases, consisting of coagulation and haemostasis, inflammation, proliferation, and tissue remodeling. The aim of tissue repair is generally to achieve tissue haemostasis, and a return to elasticity and integrity. However, sometimes excessive or pathological scar formation may occur, especially when the trauma to the skin is severe [36].

Tissue injuries cause mechanical stress in the tissue, can change ECM components, and create inflammation in the environment surrounding the damaged area. One of the key factors that may lead to excessive scar formation is prolonged and acute inflammation. Studies comparing scar-free embryonic wound healing to the formation of scar tissue in adults, show that the undeveloped immune system present in the embryo may be responsible for reduced scar formation [45]. Also, inflammatory cascades in the embryo are not advanced, and the immune reaction is greatly reduced and shorter in duration, compared to wound healing in adults. The embryonic wound healing environment is rich in platelet-derived growth factor (PDGF) and transforming growth factors (e.g., TGF-β3). It has been suggested that adding PDGF and TGF-β3, as well as neutralizing TGF-β1 and 2, can promote wound healing in adults [e.g., 45], but more research is needed.

At wound sites, fibroblasts activate, proliferate, release ECM, and differentiate into a contractile phenotype, called myofibroblasts. Fibroblasts and myofibroblast express contractile microfilament bundles,

α-SMA, and ECM at the site of injuries [56, 125]. Myofibroblasts migrate to wound sites, proliferate, and contract the wound. Wound closure is essential for the haemostasis needed to restore damaged tissue. However, accumulation, persistence, and excessive activity of myofibroblasts during tissue repair and healing can cause fibrotic scarring. In addition, excessive production of ECM and rarification of the microvasculature can transform granulation tissue into a hypertrophic scar [56, 125].

In the following subsections, the phases of wound healing are discussed in more detail.

THE HAEMOSTASIS PHASE:

When tissue injury occurs, blood vessels may be damaged and blood may flow into the wound area. The body responds by constricting blood vessels and thus restricting blood flow. Platelets can then aggregate together and create clots in the wall of the injured vessels, in order to stop the bleeding. Fibrin clots are composed of platelets, thrombin, fibronectin, and collagen [37]. Afterwards, the dilation of blood vessels allows the influx of white blood cells and thrombocytes to the wound site.

In short, platelet activation leads to production of a scaffold of fibrin clots and granulation tissue. This tissue then facilitates repair of the vascular system of the injured tissue [37].

THE INFLAMMATORY PHASE:

An inflammatory response happens when haemostasis is achieved and chemotaxis is initiated. Platelet-derived growth factor (PDGF) stimulates chemotaxis and proliferation, activates cells with mesenchymal origin, attracts fibroblasts, and enhances fibroblast mitosis [110]. Damaged tissue releases damage-associated molecular patterns (DAMPs), including ATP, DNA fragments, and interlukines like IL-33 and IL-1α. DAMPs can stimulate inflammatory effects and immune cells [71].

At wound sites, immune cells recognize pathogens and DAMPs through toll-like receptors (TLRs) [40]. TLRs are located on different immune and non-immune cells, such as dendritic cells, macrophages, natural killer cells (NK), T cells, B cells, epithelial cells, endothelial cells, cardiomyocytes, astrocytes, and fibroblasts [26, 157].

Spontaneously, leukocytes, such as neutrophils and macrophages, infiltrate and clean the wound environment of cell debris, damaged cells, and pathogens [110]. Monocytes and tissue-resident macrophages differentiate. They synthesize pro-inflammatory cytokines, such as IL-1α, IL-1β, IL-6, IL-17, TNF-α, and IFN-γ, which increase inflammation. In this phase, the M1 macrophage phenotype is most common [71].

Thrombocytes and white blood cells release mediators, like serotonin, histamine, and cytokines to accelerate the inflammation process. Cells involved in healing and repair migrate to the wound site. At

the site of injury, damaged cells release some cytokines, such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), and interleukin 8 (IL-8/CXCL-8) that create an inflammatory response. In fact, damaged cells and fibrin clots recruit immune cells, such as neutrophil cells and macrophages, by sending chemical signals to the body. The key goal of the inflammation process is the prevention of infection and the cleaning of the wound environment [71, 110].

During the inflammatory phase, the wound site is generally swollen, due to the presence of growth factors, enzymes, and increased blood flow, which increases the heat, pain, and redness of the wound site [110]. The duration of this stage normally depends on the size of the wound. It can be problematic when this phase takes an excessive period of time, as prolonged inflammation can have negative consequences. For example, a long inflammatory period may lead to hypertrophic scar formation [71].

THE PROLIFERATIVE PHASE:

Around 2-10 days after an injury, the proliferation phase typically starts. The important events that occur in this phase consist of vascularization, ECM deposition, granulation tissue formation, and re-epithelialization [71].

Proliferation begins with the infiltration of fibroblasts and the release TGF-β to the wound area. Fibroblasts then deposit a collagen-I scaffold [71]. Additionally, some cytokines—such as IL-6— trigger a change from the inflammatory phase to the proliferative phase. IL-6, as a profibrotic cytokine, induces differentiation, activation, and proliferation of leukocytes, endothelial cells, keratinocytes, and fibroblasts in the wound site [71]. These changes also cause the M1 macrophage phenotype to shift to the M2 phenotype. This new phenotype drives the profibrotic signalling necessary for scaring and wound repair [71].

Keratinocytes in the epidermis migrate to the damage site, and—in addition—angiogenesis begins. Angiogenesis results from the stimulation of bone marrow and endothelial progenitor cells at normal concentrations of oxygen. It is important that during the proliferation phase, new blood vessel networks rebuild. Then, the granulation tissue can receive nutrients, exchange O_2/CO_2, and dispose of waste materials via the blood stream. Fibroblasts, macrophages, and capillary sprouts grow and replace the granulation tissue. Fibroblasts are modified to the myofibroblast phenotype, which has a role in wound closure during the proliferative phase. Both fibroblasts and myofibroblasts synthesize ECM proteins and deposit collagen into damaged areas [65].

During wound healing, growth factors also bind with ECM proteins in the wound microenvironment and modulate vascularization [65].

Fibroblasts also secrete glycosaminoglycans, which together with collagen, create a scaffold in the wound bed and stabilize the wound area. In this stage, myofibroblasts, collagen, and ECM contract the wound, and new tissue is rebuilt. Collagen fibers in the wound create thick and disorganized networks. Although wound closure is very critical to wound healing, an undesirable complication of these components can create excessive contractual scarring. Therefore, it is important to maintain a balance between deposition and degradation of ECM proteins to avoid the risk of abnormal scar formation at the healing site [21].

THE REMODELING OR MATURATION PHASE:
The last phase of wound healing is the remodeling or maturation phase. Depending on the size of the damaged area, this phase can last several months or years. In this stage, the wound is fully closed with long-lasting tissue. ECM production is considerably reduced, but not stopped. Fibroblasts degrade and remodel collagen type III (which is a major component of granulation tissue) to type I (which is the main structural component of the dermis). Afterwards, collagen fibers are arranged in orderly cross-linked networks, and the fibers are closer together, which can improve scar flexibility and lead to the return of tissue function. Vessel networks are shaped by the aggregation of smaller vascular tissues [25, 71]. At this stage, the wound area has more strength and flexibility than it had in previous stages. However, the tissue cannot typically return completely to normal physiology [71]—e.g., especially when the area affected was large, or the trauma severe.

2.1.2 *Clinically significant injury types and the effect of EPO on wound healing*

Although the skin is able to heal itself, even after severe trauma, wound areas may be impacted by severe scarring. As such, researchers have begun to study how different chemicals or treatments might improve clinical outcomes for patients affected by skin injuries. This subsection will focus specifically on reviewing the literature on the biology of EPO and its receptors. Special attention is paid to the role of EPO in wound healing. Interventions that may be able to improve clinical outcomes and change/accelerate the body's natural wound healing responses are overviewed.

2.1.2.1 *Erythropoietin (EPO)*

EPO is recognized as an evolutionarily conserved hormone, growth factor, and type 1 cytokine. EPO is a 30.4 kDa glycoprotein and is composed of 165 amino acids that form four α helices [32]. EPO as a hormone is involved in the regulation of erythrocyte proliferation

and differentiation, and the maintenance of a physiological level of circulating erythrocyte mass. The EPO gene is expressed in interstitial fibroblast cells located in the kidneys, a primary site of EPO production, and in the liver, a secondary site of EPO production in adults [28, 119]. In fetal life, however, the liver produces the majority of body's EPO [119].

When the level of erythrocytes decreases in circulating blood, oxygen delivery to specialized cells in the kidneys is decreased. Renal tubular interstitial fibroblast cells secrete EPO, which circulates in the blood, and then binds to the erythropoietin receptor (EPOR) on erythroid progenitor (CFUe) cells—which are located in the bone marrow—and therefore promotes erythropoiesis. As such, EPO expression and secretion is increased. As the mass of red blood cells increases, EPO expression and secretion then becomes down-regulated [16, 18].

In addition, studies have shown that EPO gene expression depends on hypoxic conditions in two hepatoma cell lines (HepG2 and Hep3B). These cells are sensitive to oxygen (O_2) tension variation [31].

Moreover, EPO is expressed in several non-hematopoietic tissues. The nonhematopoietic effects of EPO in skin regeneration and repair has been the center of attention for many years. Specifically, the tissue protective role of EPO in inflammation has been a target of many research projects [18, 89]. During skin injury, many tissues are under hypoxic, traumatic, and inflamed conditions, and release EPO, which might then play an important role in tissue regeneration and protection [18, 89]. For example, in animal models, EPO has been shown to play a role in protecting tissues from apoptosis and inflammation due to hypoxia, toxicity, or injury conditions [18, 89].

2.1.2.2 *EPO receptors*

EPO can bind to two different receptors, the homodimeric EPO receptor (EPOR) and the tissue protective receptor (TPR). EPORs are widely expressed in non-erythroid cells or tissues, such as those found in the cardiovascular system (cardiac myocytes, endothelial cells, smooth muscle cells), the brain (asterocytes, neural progenitor cells, neurons, oligodendrocytes), the uterus (cervix, endometrerium, ovary, oviduct), and the endocrine system (insulin-producing cells of the pancreatic islets) [18, 89]. It has also been shown that EPOR is expressed on fibroblasts, keratinocytes, endothelial cells, macrophages, mast cells, melanocytes, and hair follicles [12, 57, 66, 126].

EPO promotes erythropoeisis by binding to EPOR, but when EPO binds to TPR it can act differently—for example, by suppressing pro-inflammotory cytokines, promoting wound healing, protecting cells from apoptosis, and modulating the activation, differentiation, and function of immune cells. Both EPOR and TPR are expressed on different innate and adaptive immune cells, and EPO can directly reg-

ulate the immune system through TPR. However, the affinity of EPO towards EPOR is higher than towards TPR. The healing, immunoregulatory, and tissue protective effects of EPO at injury sites depend on high concentrations of EPO and elevated expression of the TPR receptor, so that EPO can bind to TPR effectively. In the absence of TPR and EPO-TPR, the healing and protective effects of EPO is diminished [156]. When EPO binds to TPR, it activates signalling pathways in different cells that suppress pro-inflammatory cytokines, alter the inflammatory response, and regulate the immune system. As a result, the immunoregulatory effects of EPO can down-regulate apoptosis, promote tissue survival, and accelerate wound healing [108].

2.1.2.3 *EPO signaling pathways*

When EPO binds to EPOR, EPOR undergoes a conformational change that leads to dimerization and Janus Kinase-2 (JAK2) phosphorilation. As a result of JAK2 activation, three main downstream effects are triggered. The first cascade is called the signal transducer and activator of transcription (STAT) pathway. It includes STAT3 and STAT5 [16, 18, 134]. JAK2 phosphorylation enables phosphorylation and dimerization of STAT transcription factors, including STAT1, STAT3, and STAT5a/b. STAT is located in the nuclei and activates regulated genes. EPO-mediated activation of JAK2/STAT5 leads to up-regulation of the anti-apoptotic Bcl-XL and Bcl-2 genes, which then protect pro-erythroblasts from apoptosis [16, 18, 89].

The second cascade consists of the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt) pathways. The PI3K/Akt pathway promotes cell survival and cell growth. Also, PI3K/Akt activates endothelial nitric oxide synthase (eNOS), which promotes the production of nitric oxide (NO), increases blood flow, reduces regional injury, and induces endothelial cell proliferation, migration, and healing [134]. In addition, activation of Akt signaling increases the phosphorylation of STAT5 [157]. It has also been suggested that EPO can stimulate the AMP-activated protein kinase (AMPK) pathway that is located downstream of βCR, and activates eNOS and NO production [134].

The third cascade consists of the mitogen-activated protein kinase (MAPK) pathway. MAPK inhibits GSK3β and reduces inflammation [134].

EPO down-regulates nuclear factorκB (NFκB), and as a result decreases inflammation and edema [134]. NFκB activates the expression of genes which are associated with cell survival, production of inflammatory cytokines, and anti-apoptotic gene pathways [157]. During inflammation, NFκB induces the production of cytokines, like TNF-α, IL-1, IL-6, IL-8, and adhesion molecules that are necessary for leukocyte recruitment to the wound site. It also regulates cell proliferation and apoptosis [61].

All of these signaling pathways are, on the one hand, known for promoting red blood cell proliferation, and, on the other hand, known for vasodilation, insulin-sensitization, and for having anti-thrombotic, anti-inflammatory, and anti-apoptotic actions [89].

2.1.2.4 *EPO in wound healing*

Time is a key factor in wound healing. For optimal wound healing, it is critical that the inflammatory phase is shifted to the proliferative phase as soon as possible. A long inflammatory phase can lead to a delay in wound healing, wound distress, an increased risk of infection, and hypertrophic scars [71]. The wound repair process requires careful management during both inflammatory and proliferation phases. At the beginning of an injury, EPO may not be synthesized. On the one hand, pro-inflamatory cytokines induce the expression and translocation of EPOR and βCR on cell surfaces; on the other hand, some pro-inflammatory cytokines, like TNF-α, may inhibit EPO production. High concentrations of pro-inflammatory cytokines and low concentrations of EPO at the site of injuries prevent binding between EPO and TPR. Studies also show that a low concentration of pro-inflammatory cytokines can induce EPO production and increase EPO-TPR binding; EPO-TPR activates different pathways, for example those which suppress inflammation, promote cell survival, and help to stop the spreading of injury to surrounding cells and tissues [15].

After an injury, the immune system creates vascular thrombosis and edema, which increase the blood flow towards the injury site. Although the immune system destroys pathogens and cleans the wound bed of damaged cells, it also increases hypoxia. The immune response can even cause apoptosis, necrosis, and damage to distant cells and tissues. It has been suggested that EPO helps to regulate and maintain immune homoeostasis. In wounds, macrophages play an important role in releasing pro-inflamatory cytokines and cleaning the wound site. A study has shown that binding of EPO to TRPs on macrophages can block NFκB p65 activation, which then down-regulates the expression of TNF-α, IL-6, and inducible nitric oxide synthase (iNOS) [97]. Moreover, in the innate immune system, EPO and its derivatives tend to shift macrophages from the M1 phenotype to the M2 phenotype, facilitate macrophage phagocytosis, inhibit the maturation of dendritic cells, and down-regulate the inflammatory reactions of mast cells. In the adaptive immune system, EPO directly suppresses lymphocytes and influences the balance of T-helper cell subsets [108]. Also, EPO regulates different adhesion molecules that have anti-macrophage accumulation effects [147]. EPO regulates monocyte chemoattractant protein-1, which suppresses recruitment of monocytes and macrophage transformation [146].

Many studies demonstrate the effect of EPO on wound healing acceleration. EPO stimulates angiogenesis, ECM production, and col-

lagen production; it also regulates inflammatory mediators, has anti-apoptotic effects, and promotes cell survival, which accelerates the wound healing process [12, 54, 57, 66]. EPO promotes phosphorylation of Smad2 and Smad3 in fibroblasts, activates the Smad-dependent TGF-β signaling pathway, induces mRNA expression of TGF-β1 and α-SMA, increases myofibroblast differentiation, and accelerates skin wound closure [126]. EPO also down-regulates Smad-2 expression, which regulates keratinocyte migration and epithelialization [132].

Bader et al. [6] have demonstrated that topical application of EPO accelerates and improves healing of acute and chronic wounds in human skin [6]. Hamed et al. [52, 54] have shown that topical administration of EPO promotes cutaneous healing and decreases the level of inflammatory cytokines in cutaneous wounds of diabetic rats and hairless mice. Their results have shown that topical EPO treatment increases collagen synthesis and deposition, enhances epithelialization, improves angiogenesis and VEGF secretion, inhibits apoptosis, upregulates eNOS, and promotes wound repair and wound closure rate in diabetic wounds [52, 54].

It is said that the effect of EPO on various outcomes in skin is dose-related. For example, Sorg et al. [132] have shown that in cutaneous wound healing in a hairless mouse model, a single high dose of EPO can improve cell migration, wound healing, epithelialization, and microvascular network formation. In addition, it decreases wound cellularity and the time period of the inflammatory phase [132]. However, repeated application of high doses of EPO can impair the repair process and epithelialization, increase wound cellularity, and delay vessel maturation. In addition, it can reduce the migration activity of fibroblasts and keratinocytes [132]. In their study, however, both repetitive-low-dose (RLD) and single high-dose EPO treatment groups showed improvement in wound healing. The RLD group showed increases only in fibroblast migration and did not show differences in epithelialization compared to the control group. This suggests that the effect of EPO on cutaneous wound healing is dose-related [132].

Vascular damage in wound areas can cause abnormal oxygen delivery to tissues, tissue hypoxia, necrosis, and even tissue dysfunction that may endanger organs. One important study has investigated the proangiogenic role of EPO in fibrin Z-chambers that were transplanted subcutaneously in a rat model [57]. Administration of a single dose of recombinant EPO locally into the fibrin matrix significantly increased the expression of the EPOR protein on macrophages, improved granulation tissue formation, and enhanced angiogenesis in granulation tissue [57].

Also, EPO administration up-regulates iNOS expression and fibrin-induced wound healing; these effects of EPO are also reported to be dose dependent [57]. Rezaeian et al. [117] have investigated the effect of EPO on a perfused flap tissue model in mice. In this study, mice

were treated with 3 doses of EPO at 500 IU/kg before, before/after, and after surgery. EPO pre-treatment improved the survival of critically perfused tissue by nitric oxide-mediated arteriolar dilation, protection of capillary perfusion, and VEGF-initiated new blood vessel formation [117]. Rezaeian et al. [118] also show that EPO significantly reduces apoptotic cell death and flap tissue necrosis [118].

Contaldo et al. [23] have designed an experiment to show the effects of EPO on severe ischemic damage in musculocutaneous tissue in a skin flap model in hamsters. Five hours after induction of ischemia, EPO significantly decreased apoptotic cell counts. Tissue pre-treated with single dose EPO showed increased vasodilation, as well as arteriolar and venular blood flow increases, which jointly promote tissue perfusion and oxygenation *in vivo*. There was significant up-regulation of eNOS after EPO pre-treatment. It is suggested that the anti-adhesive, anti-leukocyte transmigration, and anti-inflammatory properties of EPO depend on NO production. NO prevents neutrophile aggregation and adhesion to post-capillary venules. In addition, NO decreases the activation of endothelial cells, inhibits platelet aggregation, and is involved in the relaxation of vascular smooth muscle cells [23].

A study on wound healing in a genetically diabetic mouse model has illustrated that VEGF mRNA and protein expression was significantly increased in the group treated with rHuEPO. Administration of rHuEPO enhanced wound healing and the angiogenic response. It has also been suggested that EPO may stimulate endothelial cell mitosis, granulation tissue formation, and the activation of angiogenic factors [34].

In a study on pigs with type 1 diabetes, topical application of EPO in full thickness burns led to an acceleration of dermal wound healing, through an AQP3-dependent mechanism, which is expressed in the skin [53]. During cutaneous wound healing, topical EPO treatment raises AQP-3 expression, which then promotes ECM production (e.g., production of collagen and hyaluronic acid), enhances angiogenesis and blood flow, stimulates cell migration and proliferation, and increases re-epithelialization by keratinocytes. As a result, wound closure and healing accelerate. Microvascular density and the level of eNOS expression were both significantly increased in the regenerated skin of animals in EPO/Fibronectin treatment groups [53]. However, there is an interaction between the effects of EPO and AQP3 in cutaneous wound regeneration. Blocking AQP3 may minimize the effect of EPO and impair wound healing [53].

2.1.3 *Skin grafts, EPO, and clinical outcomes*

The use of skin grafts is a gold standard treatment for large open wounds (e.g., full thickness burns covering more than 1% of the body's surface) [82]. It is a standardized surgical procedure that is common

in clinical practice. Skin grafts consist of epidermis and a part of the dermis [14]. In this procedure, skin from a healthy part of the body, called the donor site, is removed and transplanted to an injured area of the body (e.g., a burn wound) [45]. After skin grafting, the donor site needs 2 to 3 weeks time to regenerate new skin [14].

However, skin graft treatments are often characterized by some level of scaring at both donor and recipient sites. Scars can cause both psychological and physiological complications, and even lead to post-traumatic anxiety disorders [83] that can change people's lives. As such, researchers are interested in development of treatments that can improve clinical outcomes. One approach under investigation involves administration of EPO to skin graft treatments during surgeries on patients affected by burn injuries, as EPO is hypothesized to enhance the rate and the quality of healing. Here, the biology of burn injuries and skin grafts is discussed, and a focus is placed how they may be impacted by EPO.

Deep second-degree and third-degree burn wounds are characterized by serious burn damage that affects various skin layers, including the epidermis and the dermis. Second degree burn wounds (SDW) specifically feature damage to the epidermis and the stratum reticulare in the dermis, and lead to redness, pain, blisters, and inflammation [83]. After an SDW, the body's regenerative process starts immediately; however, if the SDW is deep, this regenerative process can create areas of excessive and abnormal scars (e.g., hypertrophic scaring).

Third degree burn wounds (TDW), or full-thickness burns, are a kind of skin damage that involves deeper layers of the skin [83]. In TDWs, the wound healing process does not happen spontaneously. Due to severe damage in deeper layers of the skin, patients generally require special treatments in order to recover [45, 82, 83].

Because deep second and third degree wounds are severe (especially when large or affecting sensitive regions), they can require treatment with skin grafts. Transplantation of autologous split-skin grafts within a short period of time after injury can protect tissue from infection, hypertrophic granulation, and later scar tissue formation [45, 82]. These transplants can even protect the body from major risks (e.g., sepsis and death) [91]. Transplanted skin helps the injured area to be metabolically active [14]. Although the harvested skin does not have functional blood vessels, the skin can survive up to four days and exchange materials with the injured area. Injured vessels in the wound area, however, can start to grow, and may even create a vascular network in the transplanted skin [14].

In some cases, when a large area of skin is thermally injured, repetitive skin grafts from the same donor may be necessary. This circumstance is challenging because healthy and available donor skin is needed in order to make repetitive grafts. In addition, after a sample is taken from a donor site, the donor site needs time to regenerate

completely. Therefore, potential treatments that speed the healing process of the donor sites may have important clinical ramifications. EPO has been shown in many research projects to improve vascularization, accelerate wound healing, and regulate inflammation [e.g., 1].

For example, a study has shown that a continuous application of recombinant human erythropoietin (rHuEPO), from 3 hours to 14 days after deep burn injury, improves angiogenesis and wound healing in a mouse model [1]. These outcomes were driven by increases in the expression of CD31 endothelial markers, inducible nitric oxide synthase (iNOS), and the content of nitric oxide (NO) products in the wound area, which augment the content of vascular endothelial growth factors [1]. Similarly, the application of EPO hydrogel together with split skin graft transplants has been shown to complete re-epithelialization, accelerate wound healing, and improve the maturation of scar tissue in 85% of patients after 8 days [123].

In a double-blinded, multi-center clinical trial [47, 90], EPO (150 IU/kg of body weight, per application) was given to thermally injured patients, who were in need of split skin grafting. Application were delivered subcutaneously every other day for at least 3 weeks. A control group was treated with matching placebos. The results of this study showed that EPO had some pro-regenerative effects in patients with burn injuries. The groups treated with EPO generally showed accelerated re-epithelialization and a faster healing process [47, 90].

Here, this dissertation will contribute to the literature on EPO and wound healing by studying the effects of systemic EPO application on the healing process at the donor sites of skin grafts, using new histological and immunohistochemical analyses applied to skin samples collected by the published "EPO in Burns" project [see 47, for details on this study]. Specifically, the potential acceleration of skin regeneration will be investigated. This dissertation will also investigate if the systemic application of EPO can lead to faster stem cell activation, faster re-epithelialization, and better vascularization in split skin donor areas, as measured by cell marker activation. In the following section, details about skin-derived stem cells and stem cell markers will be discussed. Additionally, it will be reviewed how other cell markers can be used to measure re-epithelialization and vascularization.

2.1.4 *Skin-derived stem cells*

Stem cells are recognized by their ability to self-renew, proliferate, and differentiate into different cell types. In the skin, they can be found in epidermic, dermic, and hypodermic tissues [24]. Dermal stem cells divide and rejuvenate the skin. These cells can also regenerate skin tissue through differentiation into different cell types, such as epidermal keratinocytes, dermal fibroblasts, endothelial cells, and cells found on blood vessels [24]. Treatments which up-regulate stem

cell activation may thus have clinical importance. Because of this fact, stem cells with self-renewal characteristics and multi-directional differentiation potential are a target of many research projects and clinical trials, especially in the field of wound healing. Here, the biology of dermal stem cells is described and then some of the clinical literature linking stem cells and wound healing is reviewed.

2.1.4.1 *The biology of dermal stem cells*

Dermal stem cells include: interfollicular epidermal stem cells, hair follicle stem cells, melanocytic stem cells, sebaceous stem cells, pericytes surrounding blood vessels, and mesenchymal stem cells [24].

Interfollicular epidermal stem cells are located in the basal layer of the epidermis, hair follicle stem cells reside in the bulge region and control the hair follicle cycle and hair growth, and melanocytic stem cells are located in the basal epidermis, hair follicles, and may also appear in the dermis [24]. They differentiate into melanocytes, which are responsible for skin and hair pigmantation [24]. Sebaceous stem cells are located in sebaceous glands and differentiate into sebocytes, which produce sebum [24]. Mesenchymal stem cells (MSCs) are located in the dermis [128], and can also be found in various other organs and tissues—such as bone marrow (which is the main source of MSCs), adipose tissue, the umbilical cord, dental pulp, placenta, and endometrium [86, 128]. These cells are described as multipotential fibroblast-like cells [56], with spindle-like morphology, and are plastic adherent [76]. MSCs potentially can differentiate into osteoblasts, adipocytes, chondroblasts, neural cells, smooth muscle cells, Schwann-like cells, and hepatocyte-like cells *in vitro* [62, 76, 138]. Researchers have even shown that dermal MSCs can differentiate into smooth muscle cells with expression of smooth muscle actin (SMA) [138].

In order to identify dermal MSCs, researchers have developed immunohistochemical staining approaches to detect various cell surface markers. Researchers have found that specific markers are expressed on dermal MSCs [these include the CD73, CD90, CD105, CD271, and SSEA-4 markers; 141], while others are not [these include the CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules; 56, 62, 76, 86].

2.1.4.2 *Dermal stem cells and wound healing*

MSCs improve wound healing through paracrine signaling and differentiation into different cell types [128, 164]. Through paracrine signaling, MSCs stimulate neovascularization, re-epithelialization, and recruitment of autologous stem cells (e.g., bone-marrow mesenchymal stem cells) during the process of wound healing [7, 154, 164]. Also, paracrine signaling from bone marrow MSCs can regulate dermal fibroblast proliferation, gene expression, and acceleration of fibroblast

migration. In addition, it can recruit fibroblast to the site of wounds [128]. MSCs can also decrease inflammation and regulate the immune response [62, 76].

Both MSCs and fibroblasts in skin have immunosuppressive effects, which activate cell-cycle arrest, have anti-apoptotic effects, regulate monocyte differentiation into dendritic cells, and promote T-cell survival [56, 76]. The immunosuppressive effects of MSCs and fibroblasts are increased in the presence of pro-inflammatory cytokines, such as IFN-γ, IL-6, and TNF-α [62, 76].

Co-culturing of MSCs and dermal fibroblasts has been demonstrated to increase cell proliferation, accelerate cell migration, up-regulate integrin-α-7 expression, down-regulate the expression of ICAM1, VCAM1 and MMP11 in fibroblasts, and accelerate the wound repair process [128]. Studies have shown that EPO stimulates proliferation of MSCs [7, 13]. Also, EPO can improve recruitment of autologous MSCs to the site of skin injuries [13, 38, 153]. For example, it has been shown that the administration of rHuEPO in deep second-degree scald injury sites in mice and rats increases the expression of CD31, CD90, CD71, and nestin, which promotes hair follicle generation, vascularization, and skin regeneration [38].

2.1.5 *Cell markers*

In this study, the expression of different cell markers was used to evaluate dermal stem cell proliferation, vascularization, re-epithelialization, and wound closure.

2.1.5.1 *Dermal stem cell markers*

Dermal stem cells are characterized by expression of specific cell surface markers (which will be referred to as "stem cell markers") [139, 159]. Dermal stem cells have been identified in experiments to express: CD10, CD13, CD26, CD44, CD54, CD71, CD73, CD90, CD105, CD106, CD129, CD146, CD271, STRO-1, and SSEA-4 [24, 58]. Other studies have shown that MSCs express different stem cell markers—such as CD73, CD90, CD105, and CD271—but do not express CD14 or CD11b, CD34, CD45, CD79a or CD19, and HLA-DR [62, 76, 86]. Below, the biology of various stem markers relevant to the study of wound healing is reviewed.

CD90:
CD90 or Thy1 (Thymocyte differentiation antigen 1) is a 25–37 KDa glycosylphosphatidylinositol (GPI)-anchored glycoprotein that has been found to be expressed in thymocytes, T cells, hematopoietic stem cells, neurons, cancer stem cells, endothelial cells, and fibroblasts. CD90 is also one of the markers expressed in dermal MSCs [141]. It is suggested that CD90 participates in the self-renewal and differen-

tiation of MSCs [96]. Cells expressing CD90 have been shown to be more susceptible to apoptosis than CD90-negative cells [62]. CD90 is also involved in the production of pro-inflammatory cytokines, such as IL-6 [62].

In studies investigating the use of EPO treatments in rabbit models of full-thickness cartilage defects, EPO-treated groups were shown to have an increase in recruitment of endogenous progenitor/stem cells—such as CD90+ cells—and a decrease in the number of inflammatory cells in synovial fluid, compared with to control group [49]. Studies on the application of rHuEPO in deep second-degree scald injuries in mouse and rat models have shown high expression of MSC markers—such as CD31, CD71, CD90, and nestin—and suggest that EPO can improve skin regeneration [38]. Finally, EPO may stimulate the proliferation of skin MSCs, including CD90+ cells [13]. These results suggest that EPO may improve wound healing through recruitment of CD90+ cells.

CD271:

CD271 is also known as the low affinity nerve growth factor receptor (LNGFR) or the p75 neurotrophin receptor (p75NTR). It is a member of the tumor necrosis factor (TNF) super-family and a key marker used to identify MSCs [2, 86, 141, 159]. CD271 presents in cutaneous nerve fibers, Schwann cells, dermal cells, and keratinocytes [141]. It stimulates neural cell differentiation and survival. In addition, it plays a role in maintaining clonogenicity and function of MSCs [86]. Dermal CD271+ cells show higher proliferation and trilineage differentiation potential than other stem cells, which express markers such as CD44, CD90, and CD105 [2, 58]. Also, studies have shown higher adipogenesis, osteogenesis, and chondrogenesis potential in CD271+ cells compared to CD73+, CD90+, or SSEA-4+ SSCs cells [2, 141, 159].

CD271+ cells in the dermis have multipotent characteristics and adhere to plastic [24, 68, 161]. Such cells also influence the proliferation and differentiation of epidermal stem cells during the healing of cutaneous burn wounds [141, 159, 161].

Three days after burn injury, skin CD271+ stem cells migrate and proliferate into the site of injury [68]. In the early stage of wound healing, the protein level of CD271+ cells is low; it then increases in the mid to late stages of the healing process. This can suggest that CD271+ cells are involved in the re-epithelialization and remodeling phases of wound healing [161].

Zhang et al. [161] have shown that the expression of CD271+ cells increases in burn wound healing. Injection of CD271-over-expressing epidermal stem cells into the site of burn wounds in a mouse model led to an increase in the rate of wound healing [161]. CD271+ dermal cells produce various cytokines and growth factors, as well as neurotrophins that play important roles in wound healing. The expression

rate of TGFβ1, TGFβ2, VEGFα, FGF2, EGF, and PDGFB in CD271+ dermal stem cells has been shown to be elevated relative to the expression rate in CD271- dermal stem cells [68]. CD271 affects wound healing through the differentiation and paracrine action of MSCs [68, 159]. Studies have shown that the number of dermal CD271+ cells are significantly decreased in patients with chronic skin ulcers, compared with healthy patients in the control group [67, 68]. Decreased expression of dermal CD271+ cells can even cause delayed wound healing [68].

CD105:
Endoglin, or CD105, is a cell surface marker and type 1 membrane glycoprotein. It is a part of the transforming growth factor-β receptor (TGF-βR) complex [111, 141]. CD105 is expressed on human endothelial cells [111] and on dermal MSCs [141]. CD105 positive MSCs show increases in stemness level [20] and also have a pivotal role in angiogenesis [141]. Studies have shown that EPO may stimulate the proliferation of skin fibroblosts and MSCs, including CD105+ cells [13]. Some cytokines, like EPO, may even help maintain the hematopoietic potential of CD34+/CD105+ cells [111].

2.1.5.2 *Angiogenesis markers*

Angiogenesis, or the growth of new blood vessels, is a crucial part of a successful skin repair. Other parts of the body communicate with the wound area through blood vessel networks. Blood vessels provide the tissue at the site of wound injury with essential nutrients—e.g., small molecules, like growth factors and cytokines—and permit the disposal of waste materials, thus supporting the regenerative process.

Many cytokines and growth factors, such as EPO, are involved in the process of vascularization and skin regeneration. Here, two cell markers related to angiogenesis and overviewed, and then the role that EPO has been shown to play in angiogenesis is discussed.

CD31:
Cluster of diffrentiation 31 (CD31), also known as the platelet endothelial cell adhesion molecule (PECAM1), is a 130 kDa protein, which is expressed on different cells, such as platelets, neutrophils, monocytes, B-cells, granulocytes, T-cells, NK cells, hematopoietic stem cells, and endothelial cells. In histological analyses, the CD31 marker can be used to identify the existence of endothelial cells in tissue. Detection of CD31 can indicate the presence of angigenesis and integrin activation [10, 81].

During the process of wound healing, new blood vessels are formed in the center of granulation tissue, in order to exchange nutrients and other materials with the regenerating tissue [34]. EPO acts as a mediator of angiogenesis in granulation tissue. Both EPOR and

TPR are expressed on endothelial cells and endothelial progenitor cells (EPCs). As such, EPO can stimulate endothelial cell proliferation, migration, and angiogenesis. [10].

Studies have shown that EPO can increase the expression of some stem cell markers, like CD31. In general, groups treated with EPO have shown an increase of neovasscularization in the wound area compared to control groups [38]. Because CD31 is a cell surface marker associated with vascularization, it can be used to measure the effectiveness of various treatments in increasing the number of endothelial and blood vessels. Later in this dissertation, CD31 expression will be used for this purpose.

VON WILLEBRAND FACTOR (VWF):
Von Willebrand factor (VWF) is a hemostasis glycoprotein that is synthesized by endothelial cells, platelets, and megakaryocytes in the plasma, and the subendothelial matrix [65]. It is located together with vascular endothelial growth factor A (VEGF-A) in endothelial cells [65]. Shortly after a wound injury occurs, activated endothelial cells release VWF at the wound site. VWF acts as a chaperon to coagulation factor VIII (FVIII), and mediates platelet adhesion to the subendothelium during the haemostasis phase [65].

The binding of VWF to platelets produces several pro- and anti-inflammatory mediators [74]. For example, the heparin-binding domain (HBD) of VWF binds to several growth factors, such as VEGF-A, platelet-derived growth factor-BB (PDGF-BB), and fibroblast growth factor-2 (FGF-2) in human plasma. HBD also excites different ECM proteins [65, 101, 109]. Through binding to growth factors, VWF enhances angiogenesis, smooth muscle cell proliferation, and tissue regeneration, thereby accelerating tissue repair and maintaining normal haemostasis [101, 109].

In addition, VWF regulates the inflammation response [60, 74]. For example, Denis et al. [27] have shown that, in a mouse model, VWF-deficient mice experience a decrease in leukocyte recruitment (predominantly in neutrophils) at the begging of inflammatory phase. Moreover, O'Donnell et al., [101] argue that VWF promotes wound healing, and that a lack of VWF can decrease growth factors at the site of injury, and thus delay wound healing [101].

Ishihara et al. [65] have illustrated that, in a mouse model of wound healing, VWF deficiency causes prolonged wound healing, decreases angiogenesis, and decreases the amount of expressed angiogenic growth factors.

In this dissertation, VWF will be used as a proxy measure for neo-angiogenesis during the wound healing process.

2.1.6 *Re-epithelialization and wound closure*

Re-epithelialization is a process in which damaged epithelium is regenerate. During the proliferative phase of wound healing, epidermal cells, like keratinocytes, and dermal stem cells migrate towards the wound area, differentiate, close the wound, and make a new epithelial layer [135]. Successful and complete wound healing depends on re-epithelialization processes. Re-epithelialization is necessary protect the body from the external environment.

Keratinocytes are the most common cells in epidermis and have an essential role in wound closure. Keratinocytes proliferate in the basal layer. These cells are identified in their own proliferative stage by cell markers, such as CK5 and CK14. Differentiated keratinocytes in the spinous, granular, and cornified layers express cell markers, like CK10 and CK1 [107]. As keratinocytes move from the basal layer towards the stratum corneum, they begin expressing different cell markers [107].

In vitro studies have shown that repetitive low-dose EPO treatment, and single high-dose EPO treatment, can increase keratinocyte migration; application of single high-dose EPO has shown significantly faster wound epithelialization [132].

It is suggested that cytokeratin (CK) can be a specific marker of the epithelial cells [163]. So, in this dissertation, the effect of EPO on re-epithelialization at the donor sites of skin grafts will be measured using CK10 and CK14 expression levels. These markers are especially suited to measure epidermal development.

CK14:
Cytokeratin 14 (CK14) or keratin 14 is a member of the type-1 keratin family and is a type of intermediate filament (IF) protein. It forms the structure of the cytoskeleton of epithelial cells, and has a critical role in maintaining the cell nuclei in epidermal cells. Proteins of the keratin family stabilize the skin structure, so that it can tolerate external pressure and mechanical stress, and absorb water [104]. CK14 is an epidermal differentiation marker [73]. Specifically, CK14 is recognized as a keratinocyte proliferation marker in the basal layer of the epidermis, and its expression is down-regulated during cell differentiation [73, 107, 122]. Its expression can be used to detect neo-epidermal structures and epithelialization [73, 107, 122].

CK10:
Cytokeratin 10 (CK10) or keratin 10 is a protein belonging to the type-I cytokeratin family, which is a member of a superfamily of intermediate filament (IF) proteins. CK10 creates intermediate filament structures, that together with actin microfilaments and microtubules, builds the structure of the cytoskeleton of epithelial cells. It can be used as an early marker of keratinocyte differentiation, and helps to

visualize the process of epidermal cell differentiation [33, 73, 106, 107]. Specifically, CK10 has been use to show neo-epidermis formation and epithelialization [122].

α-SMA:
Alpha smooth muscle actin (α-SMA) expresses as a fully differentiated myofibroblast marker [25, 39]. During the tissue repair process, myofibroblasts increase the level of α-SMA expression. TGF-4β also induces the production of α-SMA from fibroblasts, while TNF-α decreases basal α-SMA expression [39]. As such, expression levels of α-SMA can be used to indicate wound contractile features and maturation of the granulation tissue [25, 39].

Studies have shown that EPO increases the expression of α-SMA, increases myofibroblast differentiation, and accelerates skin wound closure [82, 126].

2.1.7 *Research questions to be addressed*

The objectives of this study are to identify if there is a cytoprotective or regenerative effect of systemically administered EPO. Specifically, this dissertation will investigate if healing outcomes are improved at the donor sites of skin grafts in patients who have suffered serious burn injuries when EPO is administered. If so, the aim is to better understand the cellular effects of EPO in such circumstances. Specifically, this study will evaluate:

1. If EPO activates dermal stem cell markers (e.g., CD90, CD105, and CD271) at split skin graft donor sites?
2. If EPO promotes vascularization and up-regulates the expression of CD31 and VWF at split skin graft donor sites? And,
3. If the expression of biomarkers linked to re-epithelialization and granulation tissue formation is greater in the treatment group than in the control group?

2.2 STUDY 1: MATERIALS AND METHODS

In the first study, tissue samples from patients with burn injuries were obtained from: "A multi-centre study on regenerative effects of low-dose erythropoetin in burn and scald injuries (with acronym of EPO in burns)" [45–48]. In this study, patients were randomly allocated to study medication (EPO) or a matched placebo. Tissue samples were processed and stained in order to assay the expression of several key cell markers. The stained tissue was then photographed, and next-generation image processing software was used to quantify expression

levels. Each phase of the study design and research process is detailed below, where all methodological details are described.

2.2.1 *Study design*

The data used in this study were collected as part of a large, prospective, randomized, double-blind, multi-center study, funded by the German Federal Ministry of Education and Research, and fully approved by the designated ethics committee (EudraCT Number: 2006-002886-38, Protocol Number: 0506, ISRCT Number: http://controlled-trials.com/ISRCTN95777824/ISRCTN95777824) [45–48].

Adult patients with deep second-degree (i.e., type 2b) or third-degree burn injuries, who were candidates for skin grafting, were invited to participate in the "EPO in burns" study. Patients were divided randomly into two groups. Patients in the EPO group were treated with EPO as described below, and patients in the other group were administered a matched placebo.

The EPO-treated group received 150 IU of EPO per kg of body weight subcutaneously at the abdomen, starting at day 2 of the study and continuing every other day for 21 days. The EPO treatment was prepared by dissolving multi-dosage vials (NeoRecormon 50,000 IU EPO; Roche Diagnostics GmbH, Germany) into a placebo solution. The placebo group received only the placebo solution without EPO [see Table 2 of: 47, for exact composition of the placebo solution]. Tissue samples from the skin graft donor site (SGDS) were collected at days 2, 10, 12, and 16 [46–48].

2.2.2 *Sample collection*

Split skin grafts from the lateral thigh were harvested at dimensions of 8cm x 8cm, and a thickness of 0.3mm, using a dermatome. Then, samples were obtained using punch biopsies (5 mm) and prepared for histological and immunohistological analyses. Immediately after sampling, skin pieces were placed in liquid nitrogen and then shipped to BBZ (Biotechnologisch-Biomedizinisches Zentrum: Universität Leipzig). At BBZ, the samples were kept in liquid nitrogen until further analysis.

2.2.3 *Embedding tissue in cryomolds (Cryofixation)*

Tissue-Tek cryomolds were prepared and marked with the orientation of the tissue. The cryomolds were filled with chilled Tissue-Tek O.C.T. solution (Leica, Germany) and the frozen tissue was immediately placed in the cryomold and covered with Tissue-Tek O.C.T. solution. The cryomold was then placed into a liquid nitrogen bath until the

Tissue-Tek solution turned white in color and completely froze. The embedded tissue was then stored in liquid nitrogen.

2.2.4 *Sectioning skin tissue with a microtome*

In order to section the skin tissue, a microtome was used. In order to ensure good performance, the microtome was cooled to -20°C. Two drops of Tissue-Tek O.C.T. solution were added to the cold specimen stage and frozen tissue samples were then inserted. The metal holder was then adjusted so that the microtome could accurately section each sample. The sectioning blade was adjusted and the tissue was cut to 10μm thickness. The tissue was then placed on Visium Spatial Slides and used for the next step.

2.2.5 *Haematoxylin-Eosin (HE) staining*

Cryosections were obtained from the skin samples using standard methods, and then fixed in acetone (NeoLab, Germany) for 10 minutes. They were air dried, placed on slides, and a border around each sample was drawn with a Dako pen (Sigma, USA). Next, the slides were put in a ready-made haematoxylin solution (Hämalaun nach Mayer; Carl Roth, Germany) for 10 minutes. After staining, the slides were rinsed with tap water several times, and then kept in tap water for 15 minutes. The slides were washed with deionized water and air-dried. Eosin solution (Carl Roth, Germany) was added to the samples for 1 minute, and the samples were washed with deionized water. In the next step, the samples were put into different concentrations of alcohol (70%, 80%, and 96%, respectively) for 10–20 seconds each, and then 100% alcohol (Roth, Germany) for 3 minutes. The slides were air-dried, covered with a mounting medium, and closed with slide covers. The samples were then checked under a microscope. After staining, the cell plasma appears pink, and the cell nuclei blue or purple. In total, data come from 11 patients (4 EPO, 7 Placebo).

2.2.6 *Immunohistochemistry (IHC)*

Cryosections were prepared as before. The samples were then blocked by incubating in goat serum (in a 1:10 dilution with PBS) for about 20 minutes. Then, the sections were incubated with primary antibodies (see Table 2.1) for one hour at room temperature (25°C). Afterwards, the sections were washed 2 times with PBS for 5 minutes. Next, the slides were placed in a humidified chamber, and were incubated with a secondary antibody, peroxidase-conjugated goat-anti-mouse IgG (Jackson ImmunoResearch, UK; diluted 1:100 in PBS), for 45 minutes. The samples were then rinsed with PBS two times for 5 minutes, and 3-amino-9-ethyl-carbazol substrate (AEC; Sigma, USA) was added to the

Table 2.1: Data associated with immunohistochemical analyses in study 1. For each patient, data from day 2 come from healthy skin samples (positive control), and data from days 10, 12, and 16 come from SGDS samples. IgG1 (BD Biosciences, USA) was used as an isotype control for each sample.

Number of the patients	**Antibody**	**Company**	**Country**
7 (3 EPO, 4 Placebo)	α-SMA	Sigma	USA
12 (5 EPO, 7 Placebo)	CD31	Biozol	Germany
12 (5 EPO, 7 Placebo)	CD90	BD Biosciences	USA
9 (5 EPO, 4 Placebo)	CD105	Abcam	UK
10 (3 EPO, 7 Placebo)	CD271	Miltenyi Biotec	Germany
9 (5 EPO, 4 Placebo)	CK10	Progen	Germany
8 (5 EPO, 3 Placebo)	CK14	Progen	Germany
12 (5 EPO, 7 Placebo)	VWF	Dakocytomation	Denmark

specimens; they were then incubated in a humidified chamber at room temperature for 10 minutes. Next, the sections were washed two more times with PBS. Then, double-distilled water (ddH2O) was added to the slides to reveal the color of the antibody staining. Once the desired color intensity was reached, cell nuclei were counter-stained with Mayer's Haematoxylin (Lillie's Modification; Dako Cyomation, Germany) for one or two minutes. The slides were rinsed with tap water two final times for 15 minutes. The slides were then dehydrated and mounted with glass coverslips. As a result of this process, antibodies were stained dark red, the cell plasma pink, and the cell nuclei blue.

Immunohistochemistry validation requires positive and negative controls for antigens and isotype controls for reagents [88]. In experiments, a negative control is used, in order to detect both suspected and unsuspected sources of bias that may lead to spurious causal inferences [80]. Negative controls allow researchers to show that the interaction of the epitope of the target molecule and the paratope of the antibody/affinity reagent creates the reaction visualized [59, 88]. A positive control is a sample that expresses the protein of interest [88]. Immunoglobulin G-1 (IgG1) is the most abundant antibody isotype found in human serum. IgG-1 is used as an isotype control to measure the non-specific binding of antibodies in immunohistochemical analyses [160]. Accordingly, positive, negative, and isotype controls were used in this study, and all results passed quality checks. Table 2.1 provides details on the sample size of patients, and the antibodies used in the IHC analyses.

2.2.7 *Sample photography*

Representative images were taken of each sample at each time point using an Olympus light microscope (Olympus, Germany).

2.2.8 *Image Analysis*

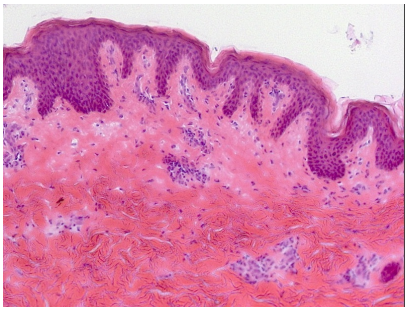
The Orbit image analysis software version 3.64 [133] was used to quantify the relative area of stain types.

To conduct this quantification, each image was opened individually in the software. After that, cell-type classes were set up by manually selecting example regions of each cell type. These target areas were selected using a polygon pen. After selecting example areas for the background, the stained cells, and the non-stained cells, the Orbit model was trained, and then the whole image was classified using the "classify" function. This function lets the Orbit software extrapolate example cell-type classes to the rest of the image and automatically calculates the area of each cell type. For our purposes, three cell-type classes were set up; they include the background (which will be displayed in a purple color), the stained cells (which will be displayed in a blue color), and the non-stained cells (which will be displayed in a green color). After each model fit, the image was visually checked to ensure that the background was accurately distinguished by the purple color, and that the stained cells and the non-stained cells were accurately classified blue and green in color, respectively. Finally, to ensure standardized interpretation of expression between images, a "region of interest" (ROI) was defined by using a pen tool to highlight a constant-width band of tissue, which included the boundary as one side. After the ROI was defined for each image, the relative density of cell types in the ROI was calculated. Figure 2.1 illustrates each of these steps. These data were then saved in an Excel table and were statistically analysed using the R software package [115].

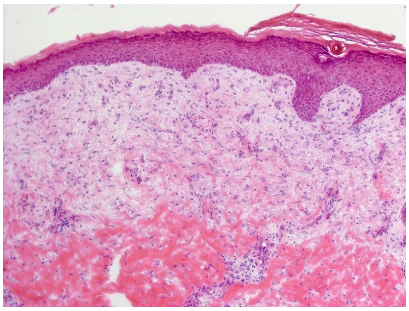
2.3 STUDY 1: RESULTS

2.3.1 *Does EPO activate dermal stem cell markers (e.g., CD90, CD105, and CD271) at the site of skin grafts?*

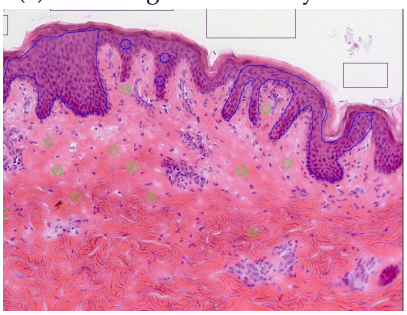
To test if systemic application of EPO improves activation of dermal stem cell markers (e.g., CD90, CD105, and CD271) at split skin graft donor sites, the results of immunohistochemical (IHC) staining are presented in Figure 2.2, 2.3, and 2.4, respectively. IHC staining measures dermal stem cell marker expression, and so the relative levels of stained areas on histological slides can be used to measure the rate and activation of stem cells in the wound healing process.



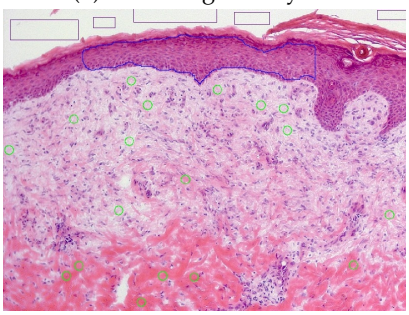
(a) Raw image from healthy tissue.



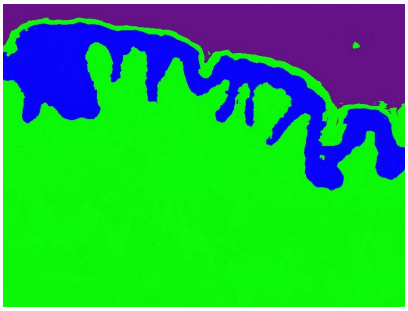
(b) Raw image at day 16.



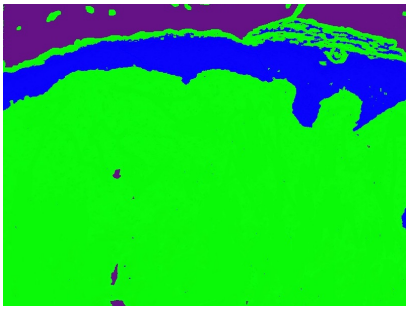
(c) Raw image from healthy tissue with training sets.



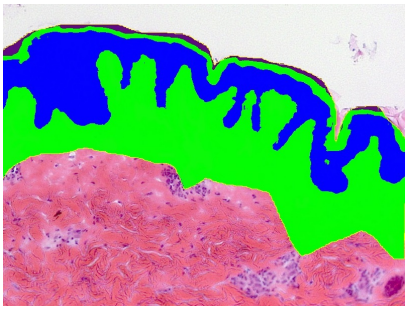
(d) Raw image at day 16 with training sets.



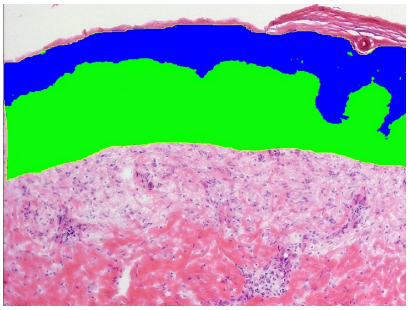
(e) Classified image from healthy tissue.



(f) Classified image at day 16.



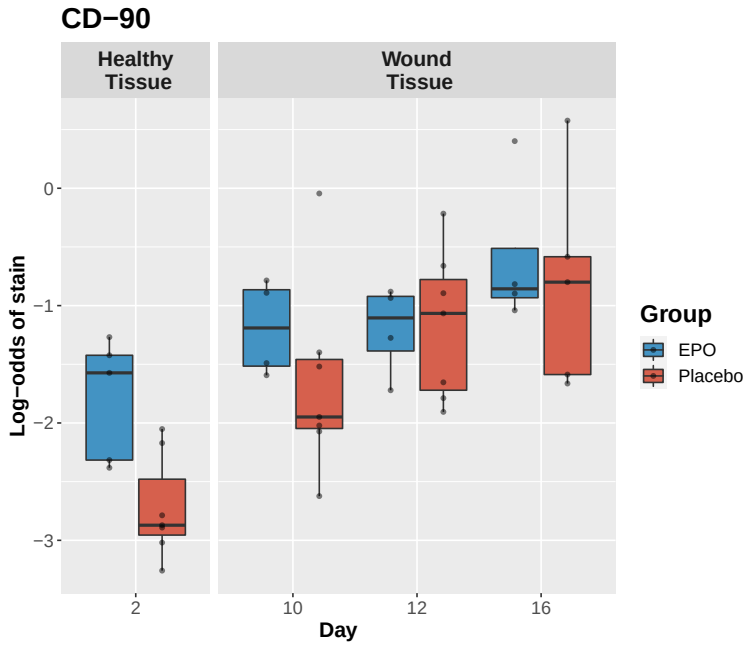
(g) Classified image from healthy tissue with ROI.



(h) Classified image at day 16 with ROI.

Figure 2.1: The steps involved in image analysis with Orbit software.

Figure 2.2: Results of staining for CD90 markers. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CD90 staining a function of treatment group.

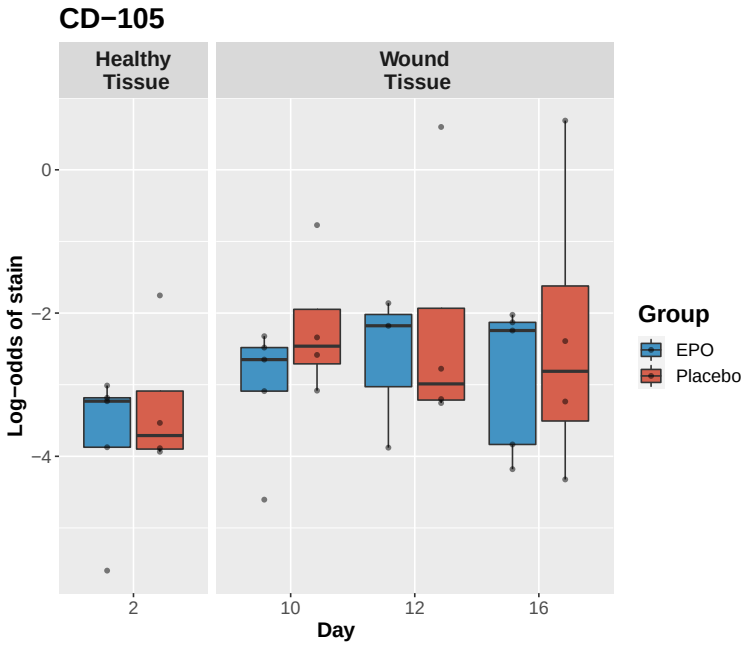


To test if CD90 marker expression is affected by EPO, Figure 2.2 plots the log-odds of CD90 marker expression in tissue samples, as a function of day and treatment group. As wound healing progresses from day 10 to day 16, we find that larger areas become stained in both treatment and placebo groups. However, the data are quite variable, and we find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of CD90 stain is $\beta = 0.26$ ($p = 0.33$). This indicates that EPO has little to no effect on the rate of activation of CD90+ cells during wound healing in these study patients.

To test if CD105 marker expression is affected by EPO, Figure 2.3 plots the log-odds of CD105 marker expression in tissue samples, as a function of day and treatment group. As before, we find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of CD105 stain is $\beta = -0.65$ ($p = 0.21$). This indicates that EPO has little to no effect on the rate of activation of CD105+ cells during wound healing in these study patients.

Finally, to test if CD271 marker expression is affected by EPO, Figure 2.4 plots the log-odds of CD271 marker expression in tissue samples, as a function of day and treatment group. At day 16, the placebo group appears to have slightly higher CD271 expression, but expression levels are highly variable across patients. There is thus no clear effect of EPO on outcomes. Averaging across days, the effect of

Figure 2.3: Results of staining for CD105 markers. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CD105 staining as a function of treatment group.



EPO treatment on expression of CD271 stain is $\beta = -0.14$ ($p = 0.72$). This indicates that EPO has little to no effect on the rate of activation of CD271+ cells during wound healing in these study patients.

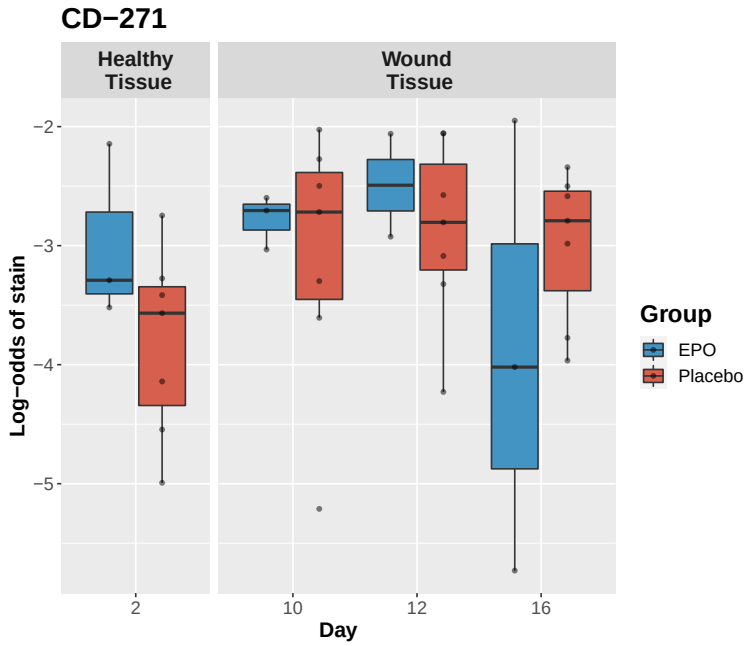
2.3.2 *Does EPO promote vascularization, and up-regulate the expression of CD31 and VWF at the site of skin grafts?*

In order to evaluate vacularization, we used CD31 and VWF expression as endothelial cell markers.

To test if CD31 marker expression is affected by EPO, Figure 2.5 plots the log-odds of CD31 marker expression in tissue samples, as a function of day and treatment group. We find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of CD31 stain is $\beta = 0.001$ ($p = 0.9$). This indicates that EPO has little to no effect on the rate of CD31 expression during wound healing in these study patients.

To test if VWF marker expression is affected by EPO, Figure 2.6 plots the log-odds of VWF marker expression in tissue samples, as a function of day and treatment group. There is higher expression of VWF markers at later days in wound tissue relative to healthy tissue at day 2 in both treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of VWF stain is $\beta = -0.51$

Figure 2.4: CD271 marker expression. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CD271 staining as a function of treatment group.



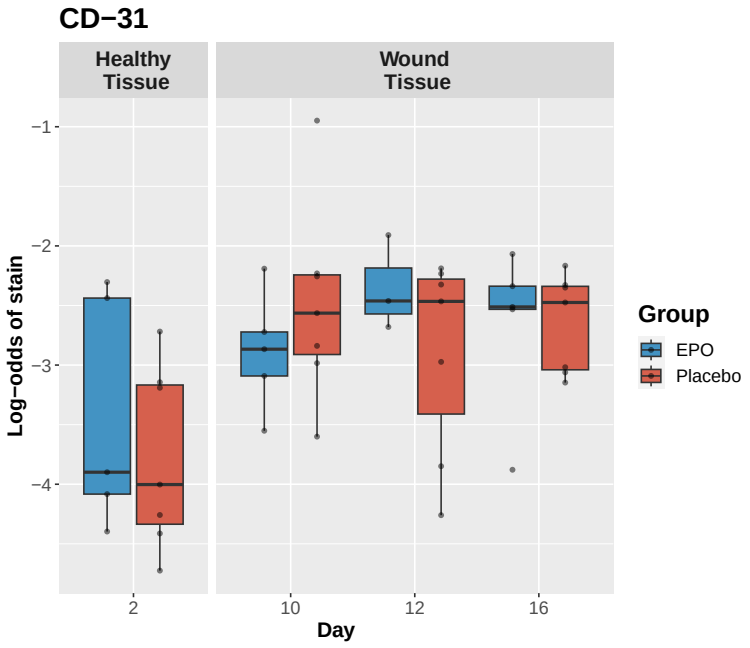
($p = 0.02$). This indicates that EPO has a significant negative effect on the intensity of VWF expression during wound healing in these study patients.

2.3.3 *Does EPO increase the rate of re-epithelialization?*

To test if application of EPO to the site of skin grafts improves overall wound healing, the results of histological staining using hematoxylin and eosin (H&E) are presented in Figure 2.7. H&E staining measures re-epithelialization, and so the relative levels of stained areas on histological slides can be used to measure the rate and completeness of wound healing. As wound healing progresses from day 10 to day 16, we find that larger areas become stained in both treatment and placebo groups. However, we find no detectable differences in H&E staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of H&E stain is $\beta = -0.2$ ($p = 0.95$). This indicates that EPO has little to no effect on the rate or completeness of wound healing in these study patients.

CK10 and CK14 markers can also be used to evaluate the progress of re-epithelialization. CK14 expression represents keratinocyte proliferation in the basal layer, while CK10 expression represents keratinocyte differentiation in the upper-basal layers [73, 107, 122].

Figure 2.5: Results of staining for CD31 markers. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CD31 staining as a function of treatment group.

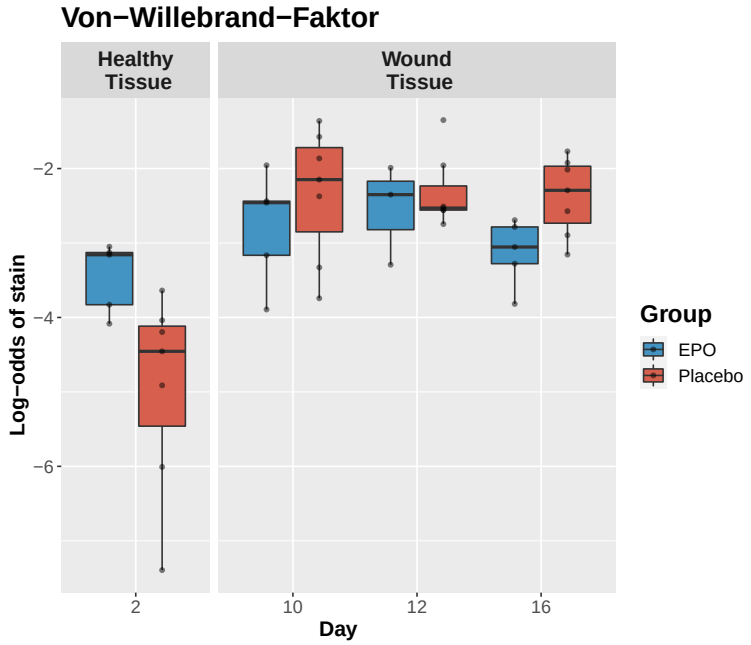


To test if CK10 marker expression is affected by EPO, Figure 2.8 plots the log-odds of CK10 marker expression in tissue samples, as a function of day and treatment group. In both treatment and placebo groups there is slightly higher expression of CK10 markers at later days, but treatment with EPO does not appear to lead to higher CK10 expression. We find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of CK10 stain is $\beta = -0.09$ ($p = 0.9$). This indicates that EPO has little to no effect on the expression of CK10 markers during wound healing in these study patients.

To test if CK14 marker expression is affected by EPO, Figure 2.9 plots the log-odds of CK14 marker expression in tissue samples, as a function of day and treatment group. In both treatment and placebo groups, there is slightly higher expression of CK14 markers at later days, but treatment with EPO does not appear to lead to higher CK14 expression. We find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of CK14 stain is $\beta = -0.723$ ($p = 0.15$). This indicates that EPO has little to no effect on the expression of CK14 during wound healing in these study patients.

α-SMA is a marker of activated fibroblasts or differentiated myofibroblasts and shows the formation of granulation tissue, which accelerates wound closure [112].

Figure 2.6: Results of staining for VWF markers. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is a significant difference in the log-odds of VWF staining as a function of treatment group, with expression being higher in the placebo group.

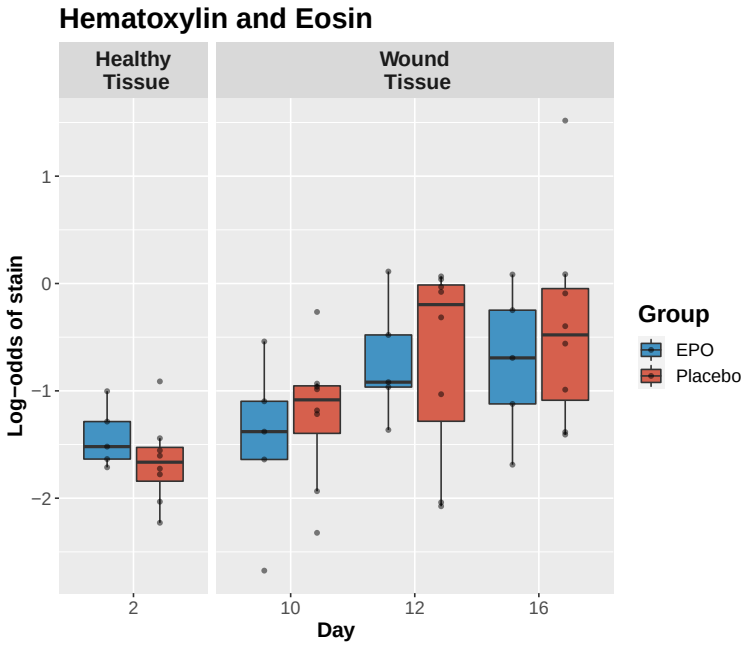


To test if α-SMA marker expression is affected by EPO, Figure 2.10 plots the log-odds of α-SMA marker expression in tissue samples, as a function of day and treatment group. In both treatment and placebo groups, treatment with EPO does not appear to lead to higher α-SMA expression. We find no detectable differences in IHC staining between treatment and placebo groups. Averaging across days, the effect of EPO treatment on expression of α-SMA stain is $\beta = -0.03$ ($p = 0.94$). This indicates that EPO has little to no effect on α-SMA expression during wound healing in these study patients.

2.4 STUDY 1: DISCUSSION

Since 1988, EPO has been approved for medical use in humans [70]. Although EPO was initially used to treat anemia related to chronic kidney disease, nowadays EPO is recognized as a pleiotropic hormone, which has various activities in different cells and tissues [70]. The expression of EPO, and EPO receptors, in hematopoetic and several non-hematopoietic tissues has been the subject of many areas of research. Some studies have shown that EPO can stimulate angiogenesis, mitosis, vascularization, and cell-cycle activation. Also, it is thought to play a role in tissue protection, inhibiting apoptosis and minimizing inflammation in different conditions, such as hypoxia, toxicity, and

Figure 2.7: Results of staining with H&E. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of H&E staining as a function of treatment group.



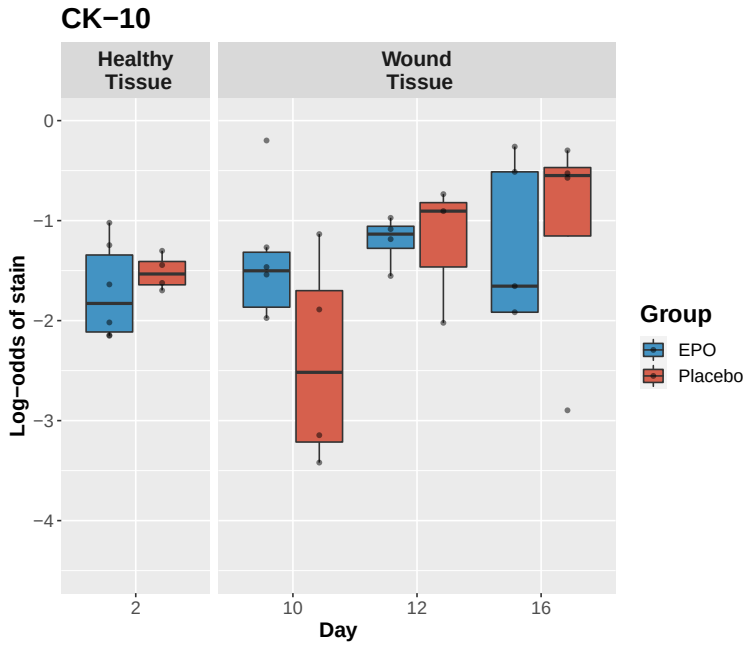
injury [6, 38, 64]. It has even been suggested—in *in vitro* studies and animal models—that EPO might have a potential to restore impaired wound healing by stimulating angiogenesis and cell-cycle in burned tissue [6, 38, 64].

However, data from clinical trials on the effects of EPO on wound healing are comparatively rare [but see 45–48]. This dissertation aims to add to the existing literature by presenting new data testing how experimental treatment with EPO affects wound healing as measured by the expression of various cell markers. In contrast to much past work in *in vitro* and animal models, the current study finds no clear evidence for an effect of EPO on the rate of wound healing as measured by CD31, CD90, CD105, CD271, CK10, CK14, and α-SMA. However, there was some evidence that treatment with EPO led to decreases in expression of VWF, which may have relevance to angiogenesis during wound healing.

2.4.1 *No clear evidence for an effect of EPO on would healing in humans*

As reviewed above, various *in vitro* and animal model studies have suggested that EPO may be involved in wound healing. For example, one study suggested that administration of EPO alone inhibits proliferation of human dermally-derived stem cells (FmSCs) in *in vitro* cultures, but adding IL-6 and EPO together to FmSC cultures

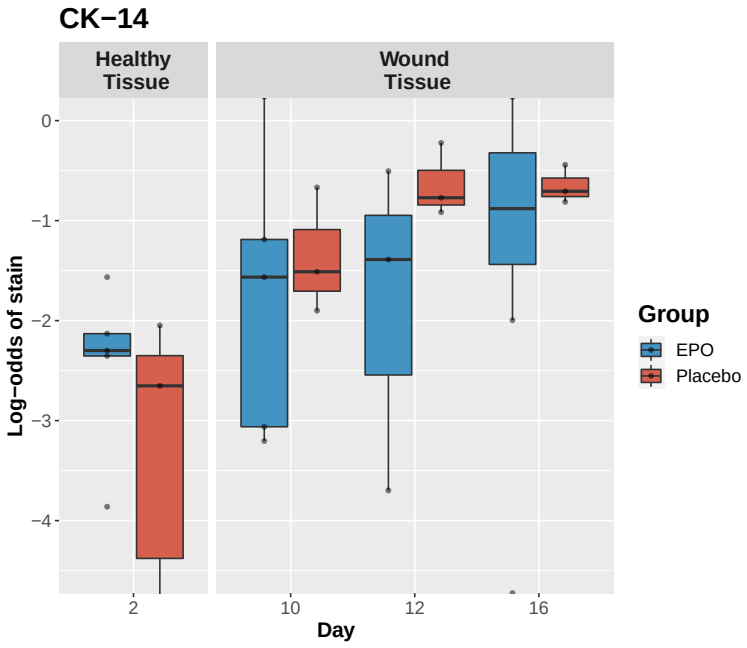
Figure 2.8: Results of staining with CK10. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CK10 staining as a function of treatment group.



in hypoxic conditions leads to increases in stem cell proliferation [6, 45]. In another study, application of rHuEPO in deep second-degree scald injuries in mouse and rat models led to higher expression of MSC markers—such as CD31, CD71, CD90, and nestin [38]. Moreover, a study on the effect of EPO on the proliferation activity of fibroblast and multipotent mesenchymal stromal cells has illustrated that EPO may stimulate the proliferation of skin MSCs, which express CD73, CD90, and CD105 markers [13]. In sum, these findings suggested that EPO may improve skin regeneration through stem cell proliferation [38].

To test if the same results hold in humans, this dissertation explored the effects of experimental treatment with EPO on markers linked to wound healing in a clinical trial setting. The first research question was to investigate if treatment with EPO activates dermal stem cell markers (e.g., CD90, CD105, and CD271). The data, however, show no detectable differences in IHC staining for these markers between treatment and placebo groups. This indicates that EPO has little to no effect on the rate of CD90, CD105, and CD271 expression during wound healing in these study patients. However, in both treatment and placebo groups, there was increased expression of CD90, CD105, and CD271 markers at SGDSs in comparison to healthy skin. This shows that dermal stem cells may proliferate and migrate to wound areas in both treatment and placebo groups. However, there are no

Figure 2.9: Results of staining with CK14. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of CK14 staining as a function of treatment group.

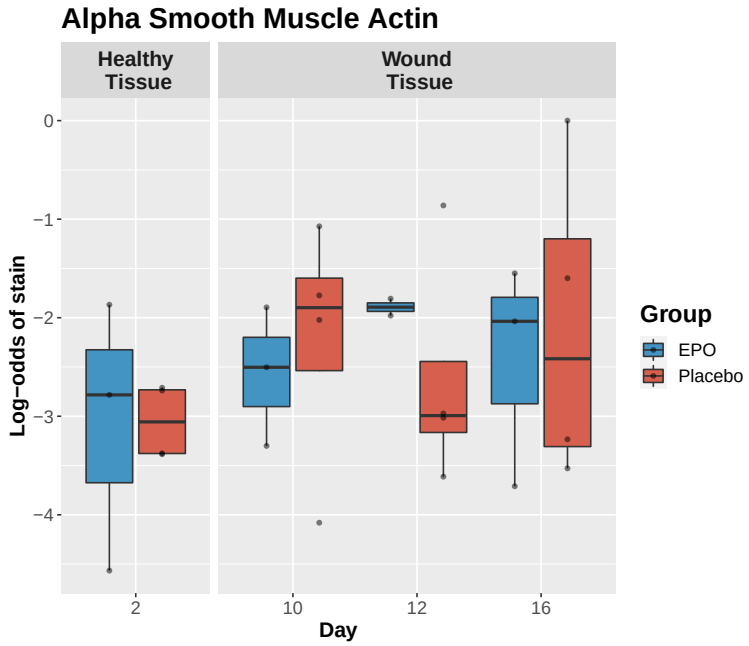


significant differences in marker expression between the placebo and treatment groups.

Another research objective was to investigate if the rate of re-epithelialization in the treatment group was faster than in the placebo group. Wound healing at the donor site of skin grafts normally takes around 2 to 4 weeks to complete, but may take longer in special circumstances. Re-epithelialization typically occurs between the first and fourth week after grafting, but the rates depend on the size of the grafts, the depth of the donor sites, and patient characteristics [3]. The epidermis is the first, and the most critical defense barrier of the body. Therefore, fast and functional re-epithelialization during the wound healing process is essential [122]. A long re-epithelialization period can impair the wound healing process, cause acute or chronic non-healing wounds, and create hypertrophic scars [3, 43].

Keratinocytes, as the most common cell-type in the epidermis, play an important role in re-epithelialization. As keratinocytes move from the basal layer towards the stratum corneum, they begin to express different cell markers [107]. Keratinocytes express CK14 markers during the proliferation phase and express CK10 markers during the differentiation phase. The expression of CK14 and CK10 markers can thus be used to measure the rate of keratinocyte proliferation and differentiation during re-epithelialization [79, 107]. Another marker that is commonly used in research studies to evaluate myofibroblast

Figure 2.10: Results of staining with α-SMA. Data are presented using boxplots. Each color represents a group, and each set of boxes represents a day post treatment. There is no clear difference in the log-odds of α-SMA staining as a function of treatment group.



differentiation, formation of granulation tissue, and wound closure is α-SMA.

In the current study, the effect of EPO on the rate of re-epithelialization at the donor site of skin grafts was investigated using H&E and the three cell-surface markers discussed above. The data show no significant differences in H&E and IHC staining between treatment and placebo groups. This may indicate that injection of EPO has little to no effect on the rate of CK10, CK14, and α-SMA expression during wound healing in these study patients. In both treatment and placebo groups, the epithelial layer was initially formed by day 10 and was complete by day 16.

Much past research has argued that topical and/or systemic application of EPO has anti-inflammatory and anti-apoptotic effects, accelerates epithelialization and vaccularization, and generally improves wound healing [19, 29, 44, 46, 50, 75, 155]. However, the histological and immunohistochemical analyses presented here—which are based on skin samples obtained from a clinical trial—fail to replicate such findings, at least with the specific dosage levels investigated in the "EPO in burns" protocol [46].

Wound healing is a complex process and consists of different stages in which many cells and small molecules are involved. Although, EPO has been suggested to improve wound healing, the biology of wound healing remains complicated. Specifically, our understanding of the biology of EPO and its effects on wound healing is still incomplete.

Future research using different dosages and different analysis methods may help to resolve potentially disparate findings the literature.

2.4.2 *EPO and Von Willebrand factor*

VWF is endothelial cell-specific marker. Studies have shown that VWF promotes angiogenesis, smooth muscle cell proliferation, and tissue regeneration, thereby accelerating tissue repair and maintaining normal haemostasis [101, 109]

A study on cutaneous inflammation in mice has demonstrated that VWF may regulate inflammation [60]. In that study, inflammation in the anti-VWF treated animals was significantly decreased compared to wild-type animals [60]. Also, they observed that high accumulation of VWF in human and murine skin inflammation may indicate that VWF induces skin leukocyte recruitment and vascular permeability [60].

In current study, EPO administration was shown to lead to statistically significant decreases in VWF expression in the treatment group, which suggests lower neovascularization at the site of wounds.

2.4.3 *Limitations of the study*

Pre-clinical experiments using animal and *in vitro* models have suggested that EPO may be effective at improving wound healing outcome. In these studies, EPO is considered to be a tissue protective and anti-inflammatory agent. However, such effects of EPO are not clearly demonstrated in clinical trials.

Some research projects have used animal models in order to study the role of EPO in wound healing in a simple model system. However, the effect of EPO on wound healing may be different between non-human animals and humans, due to differences in biology. Similarly, the results of *in vitro* studies often fail to generalize to *in vivo* contexts [92]. The results of this current study suggest that the effects of *in vivo* treatment with EPO in humans may lead to different outcomes than what has been shown in past work using different research designs.

As many studies have shown, the effect of EPO can depend on dosage and timing regimes [129, 131]. There are many protocols that describe different conditions—i.e., dosage and timing regimens—that are used when testing if EPO affects various conditions or diseases [19, 29, 44, 46, 50, 75, 155]. Low doses, high doses, single doses, or repetitive doses of EPO may have different effects or even side-effects in the body. Also, the dosages needed for EPO to have an effect in non-human animals and humans is normally different. Moreover, the method of administration—i.e., topical or systemic application—may affect the results of EPO treatment in clinical settings [129]. Furthermore, the kind of injury may impact the scope for effects of EPO. For

example, the cutaneous wound healing process at the donor site of skin grafts can depend on various conditions, such as the size of the graft, the depth of the donor site, and patient-level characteristics [3]. The inferences that can be drawn from the current study are therefore limited, as only a single dosage and timing regime was tested, using a single standardized graft profile.

In some studies, EPO appears to have not helped patients, or to have even worsened their situations [92]. Although EPO is commonly regarded as a cytoprotective hormone with many biological effects, administration of EPO and its derivatives may have some disadvantages. For example, administration of EPO may increase the risk of cancer, vascular thrombosis, cerebral ischemia or stroke, congestive heart failure, and heart attacks [92]. Similarly, EPO has been used in cancer patients to treat anemia. However, in such contexts, EPO may have the side effect of inhibiting cancer cell apoptosis through activation of AKT pathways [92]. Moreover, EPO may also increase tumor growth and vacularization of tumors [92]. Some research studies have suggested that systemic application of EPO can increase the risk of thrombosis and cancer development; these risk factors can be dangerous for patients and even life threatening [51, 137]. As such, for EPO to be used as a treatment, there should be strong evidence that the benefits of such treatment outweigh the risks. The current study suggests that systemically administered EPO does not have such strong effects.

Hamed et al. [51] have suggested that topical application of EPO has less risk (e.g., for thrombosis and cancer development) compared to systemic application of EPO. Also, it is easier to apply creams containing EPO directly onto the wound area than it is to administer EPO systemically. By applying EPO topically, hemoglobin levels in the body may be left unaffected, as the plasma concentration of EPO should only increase around the healing area [51]. Gunter et al. [44] have suggested that topical treatment of human diabetic foot ulcers with rHuEPO hydro-gel can accelerate the process of wound healing, while minimizing side-effects [44]. They show that application of rHuEPO topically does not change haematocrit and erythrocyte levels [44]. A possible benefit of local EPO administration is that higher local concentrations of EPO can be achieved. Because EPO has a low binding affinity towards TPR, and such binding is thought to underlie the effects of EPO, a high local concentration of EPO is needed in order to stimulate tissue protective effects [129]. The current study only investigated the effects of systemic treatment with EPO on histological and immunohistochemical outcomes. It remains possible that local treatment with EPO at skin graft donor sites may have more appreciable effects than were found here.

2.4.4 *Conclusions*

It has been suggested that EPO is one of the key pro-regenerative agents in skin regeneration [45]. To test this, a clinical trial was conducted to assess how wound healing is affected by systemic treatment with EPO. IHC analyses failed to uncover any strong effects that differentiate treatment and control groups, with the exception of VWF, which showed signs of decreased expression in the EPO treatment group. These results thus differ from those of Günter et al. [44], who used topical application of EPO. As such, future work is needed to determine if topical application of EPO may better improve wound healing at the donor site of skin grafts. Such studies may also wish to evaluate if the effect of EPO on wound healing is dose dependent.

3

STUDY 2: EPO AND GENE EXPRESSION

3.1 STUDY 2: LITERATURE REVIEW

The aim of the first study was to identify if systemic application of EPO was associated with faster wound healing. However, the research design does not allow us to investigate the mechanisms through which EPO might operate. As such, in order to investigate the mechanisms that might underlie the role of EPO in wound healing, *in vitro* cell cultures of foreskin-derived mesenchymal stem cells (FMSCs) were isolated from human skin samples and cultured under a variety of conditions. Specifically, cells were cultured under control conditions, as well as under conditions where EPO was administered alone, or co-administered with pro-inflammatory cytokines, like IL-6 and TNF-α. The gene expression profiles of FMSCs with and without EPO co-administration was measured and compared. In the following sections, the background literature on EPO and gene expression is reviewed, and the novel experimental results of this study are presented.

3.1.1 *Motivation*

The effect of EPO on tissue regeneration and wound healing has been widely studied. For example, immunohistochemical studies have revealed that rHuEPO gel-treated groups show higher expression of skin regeneration markers than control groups [6, 38]. For many years, scientists have sought to better understand the molecular mechanisms by which EPO may affect tissue repair. Many studies have shown that EPO can accelerate wound healing through anti-inflammatory, immunomodulatory, angiogenic, and anti-apoptotic effects. However, less is known about the molecular mechanisms underlying such effects.

In each of the above-mentioned processes, EPO may affect different pathways by changing gene expression. For example, administration of EPO accelerates cutaneous wound healing in mice, and enhances epithelialization by increasing the expression of TGF-β1 and α-SMA, and elevating the phosphorylation of Smad-2 and Smad-3 [126]. Application of EPO and MSCs in burn wounds in a rat model has led to increases in vimentin, VEGF, and PDGF expression [63]. Moreover, the level of IL-10, which is an anti-inflammatory cytokine, was enhanced. Also, biochemical analysis has shown that IL-1 and COX2 expression were down-regulated when EPO was administered [63].

EPO may also have anti-inflammatory effects and inhibit leukocyte recruitment by preventing the production of pro-inflammatory cy-

tokines, such as TNF-α and IL-6 [51]. TNF-α and IL-6 are involved in different stages of cutaneous wound healing. TNF-α is secreted immediately after injury, and increases the production of pro-inflammatory cytokines, enhances the recruitment of leukocytes, and elevates the inflammatory response. TNF-α has an antagonistic effect on EPO. Similarly, IL-6 inhibits the action of EPO in wound healing [47]. However, studies have shown that a significant increase in fibroblast growth is associated with EPO–cytokine co-administration, even though the stimulation of FMSCs with EPO alone is associated with inhibitory effects on stem cell growth *in vitro* [6]. Similarly, the administration of IL-1β, IL-6, and/or TNF-α with EPO helped to encourage cell proliferation [6, 38].

To better understand the molecular mechanisms underlying the effects of EPO on wound healing, this dissertation will investigate the effects of recombinant human erythropoietin (rHuEPO) on gene expression when administered to FMSCs *in vitro*, both alone and in the presence of pro-inflammatory cytokines. Below several key scientific questions linking EPO administration and gene expression are presented.

3.1.2 *Research questions*

1. Does co-administration of EPO with pro-inflammatory cytokines (e.g., IL-6 and TNF-α) in FMSC cultures produce a larger change in the expression levels of genes linked to wound healing than administration of EPO alone (or control)?

2. Does co-administration of EPO with pro-inflammatory cytokines in FMSC cultures produce a larger effect on the expression levels of genes linked to skin fibrosis than administration of EPO alone (or control)?

3. Does co-administration of EPO with pro-inflammatory cytokines in FMSC cultures produce a larger effect on the expression levels of genes linked to apoptosis than administration of EPO alone (or control)?

3.1.3 *Biology of EPO and other cytokines*

The effects of pro-inflammatory cytokines, like IL-6 and TNFα, have been investigated in various studies of wound healing, skin fibrosis, fibroblast migration, apoptosis, and inflammation. In this section, the biology of IL-6 and TNFα is reviewed. Special attention is then paid to past research that has investigated potential linkages between these cytokines and EPO.

3.1.3.1 *Interlukin-6 (IL-6)*

IL-6 is a pro-inflammatory, pro-fibrotic, anti-fibrotic, and anti-pathogenic cytokine. Depending on context, it may have either pro- or anti-inflammatory properties [71, 100, 149]. IL-6 is produced by multiple cell types, including monocytes, machrophages, Langerhans cells, dermal fibroblasts, endothelial cells, epidermal keratinocytes, mesangial cells, dendritic cells, T-cells, and B-cells [35, 143]. IL-6 has many roles in the body, and is important in acute inflammation, immunoregulation, hematopoeises, and activation and regulation of immune responses [136, 143]. IL-6 stimulates cells, such as macrophages, keratinocytes, endothelial cells, and stromal cells, to release other pro-inflammatory cytokines [71]. Also, IL-6 has been shown to promote chemotaxis of leukocytes (i.e., neutrophils, monocytes, and lymphocytes) to wound sites [71].

3.1.3.2 *IL-6 receptor*

The IL-6 receptor (IL-6Rα) has a membrane form (MIL-6Rα) and a soluble form (SIL-6Rα) [142]. SIL-6Rα is expressed on the surface of infiltrated neutrophils in damaged areas. Binding of IL-6 to SIL-6Rα promotes the endothelial expression of IL-6, induces monocyte chemoattractant protein 1 (MCP1) from endothelial cells, and leads to the infiltration of monocytes. It also shifts the M1 macrophage phenotype to the M2 phenotype, initiates JAK/STAT and MAPK signalling pathways, and stimulates fibroblast migration to wound sites, resulting in a transition from the inflammatory phase of wound healing to the proliferative phase [71].

3.1.3.3 *IL-6 in wound healing*

Injured tissue releases damage-associated molecular patterns (DAMPs)—molecules, which include IL-33, IL-1α, ATP, and DNA. Many DAMPs bind to formyl peptide receptor-1 and Toll-like receptors (TLR) on macrophages and neutrophils at the site of wounds. Binding of ligands to these receptors activates NFκB and MAPK pathways, and stimulates pro-inflammatory cytokine production [71, 157]. As a result, monocytes and macrophages secrete IL-1 and TNF-α at the wound site and promote inflammation. TNF-α then triggers the production of IL-6 from immune cells [71, 157].

Early in the wound healing process, IL-6 increases the adhesion of neutrophils to dermal fibroblasts [35]. IL-6 activates the pro-inflammatory functions of Th-17, $\gamma\delta$ T-cells, and M1 macrophages. At the same time, it activates anti-inflammatory effects of Th-2 and leads Th-2 to differentiate and secrete IL-4 and IL-13 cytokines [71]. IL-4 and IL-13 then shift macrophage phenotypes from M1 to M2. M2 macrophages produce TGF-β, VEGF, and IL-10 to regulate inflammation and improve the reparative process [71, 94].

In the wound healing process, IL-6 is important in shortening the inflammatory phase and triggering the proliferative phase. A long inflammatory phase can cause a longer healing process, increase the risk of infection, produce acute inflammation, and may create hypertrophic scars [71]. In the proliferative phase, IL-6 induces dermal fibroblasts to produce keratinocyte growth factor (KGF), which activates keratinocytes and attracts them to the wound site [35, 71]. Keratinocytes also secrete oncostatin M (a member of the IL-6 cytokine family). This product activates profibrotic STAT3 signaling pathways in dermal fibroblasts [71]. Also, keratinocytes up-regulate TGF-β in fibroblasts. TGF-β increases collagen secretion at the wound site and, together with IL-6, influences the structure of wound closure material, such as the collagen and fibronectin matrix from fibroblasts [71]. IL-6 also induces VEGF secretion, which leads to vascularization. It also improves the interaction of fibroblasts and keratinocytes in the reparative stage [71].

A study on wound healing in an IL-6-deficient transgenic mouse knock out model (IL-6 KO) has shown that the healing process is delayed in IL-6 KO mice compared with the control group. Also, local administration of recombinant IL-6 (rmIL-6) in IL-6 KO mice can reverse delayed wound repair, improve normal re-epithelialization, and produce well-formed granulation tissue, similar in structure to that of a control group of mice without IL-6 knock out [35]. Even in human studies, decreased IL-6 expression from dermal fibroblasts may postpone cutaneous wound healing in elderly people or diabetic patients [35].

On the other hand, over-expression of IL-6 has been shown to lead to skin pathologies and chronic inflammatory diseases, such as rheumatoid arthritis, psoriasis, scleroderma, and systemic lupus erythematosus [35, 100].

3.1.3.4 *IL-6 and EPO*

There have been few studies that explore interactions between IL-6 and EPO during wound healing. However, one study has illustrated that IL-6 and TNF-α may inhibit the production of EPO [140]. In contrast, another study has shown that IL-6 may stimulate EPO production in the liver [98]. Another study has shown that administration of EPO with IL-6 stimulates human dermally-derived stem cell growth *in vitro*; specially, a combination of EPO and IL-6 was shown to up-regulate CD90-cell proliferation [6]. More research is thus needed to better understand such interactions.

3.1.3.5 *TNF-α*

Tumour Necrosis Factor alpha (TNF-α) is a pleiotropic pro-inflammatory cytokine and a member of the TNF super-family. It plays an impor-

tant role in homeostasis and inflammatory disease pathogenesis [72]. TNF-α can have both pro-inflammatory and anti-inflammatory effects. It initiates and expands inflammation through the triggering of the production of IL-6, IL-8, adhesive molecules, chemokines, metalloproteinases, and even additional TNF-α. Also, TNF-α increases the defence reactions of neutrophils, monocytes, macrophages, and lymphocytes [157]. By increasing levels of IL-8, TNF-α decreases keratinocyte proliferation and impairs the reorganization of collagen fibres by fibroblasts [55]. TNF-α also regulates the immune system by producing anti-inflammatory mediators, such as IL-10, corticosteroids, and T-regulatory (Treg) cells [157]. Moreover, TNF-α can suppress the production of EPO in the kidneys [103].

3.1.3.6 *TNF-α receptors*

TNF-α has two different receptors, which activate different signalling pathways in cells. These receptors include TNF-receptor type 1 (p55 or TNFR1) and TNF receptor 2 (P75 or TNFR2) [157]. TNF-α binds to both TNFR1 and TNFR2, however, these receptors have different actions and can activate different signalling pathways. For example, TNFR1 has both a neurodegeneration signalling effect and a neuroprotection effect *in vivo*, while TNFR2 shows only a neuroprotective effect [69].

The binding of TNF-α to TNFR1, which is expressed on almost all cells, activates NFκB and initiates pro-inflammatory pathways [40, 95, 157]. TNFR1 contains death domain motifs, while TNFR2 does not [69]. The binding of TNF-α to TNFR1 elicits several signal transduction pathways, which activate inflammation and proliferation through activation of signal complex I. Then, signal complex I activates NFκB, extracellular signal-regulated kinases, the stress-activated MAP kinases p38, and c-Jun N-terminal kinase (JNK) [157].

In addition, the binding of TNF-α to TNFR1 induces IL-6 and IL-10, which are both signalled through the JAK-STAT pathway. The action of IL-6 depends on the environment, as it may show both pro-inflammatory and anti-inflammatory reactions, depending on context [40, 95], as discussed above.

Similarly, TNF-α binding to TNFR2 may either up-regulate or down-regulate Treg cell activity. In addition, TNF-α binding to TNFR2 activates cell migration, proliferation, neural survival, tissue regeneration, immune system modulation, and inflammation suppression [157].

TNF-α/TNFR2 maintains cell survival and enhances cell proliferation through the activation of different pathways, such as the NFκB pathways, MAPK pathways, PI3K/Akt pathways, and STAT5 phosphorylation. In addition, it increases Treg cell sensitivity and stability [157]. NFκB pathways transcribe genes involved in cell survival and cell proliferation. Activation of PI3K/Akt pathways maintains cell survival and increases cell proliferation, cell adhesion, and migration [157].

3.1.3.7 *TNF-α in wound healing*

After TLRs are activated, NFκB initiates the production of pro-inflammatory cytokines, such as TNF-α and IL-6 from immune cells. This response, in turn, activates phagocytosis mechanisms in macrophages and neutrophils, which clean the wound area of dead cells, tissue debris, and pathogens [40, 95].

TNF-α plays an important role in each wound healing phase. It is secreted to the wound site in the early stage of wound healing by macrophages (as the main source) and neutrophils [103]. A high level of secretion of TNF-α into a wound site can increase inflammation, prolong the inflammation phase, and intensify acute inflammation [121].

TNF-α, however, also induces the production of IL-10 from monocytes. IL-10 has anti-inflammatory effects and prevents excessive inflammation by inhibition of both NFκB activation and IL-6 production. In addition, it initiates cell proliferation and tissue remodelling [40, 95].

TNF-α also triggers nitric oxide (NO) production. It has been argued that NO has tissue protective effects and plays an important role during inflammation. NO increases blood pressure homeostasis, increases blood flow, and accelerates wound healing [103]. Blocking TNF-α can block NO synthesis [103].

Finally, TNF-α has an important role in wound closure, and can have regulatory effects on fibroblast activation during the inflammatory phase. TNF-α induces the production of proteoglycan and fibronectin from the fibroblasts that form the extracellular matrix structure in the proliferation phase of wound healing [121]. During this phase, fibroblasts also proliferate and secrete substantial amounts of ECM, TGF-β, and collagen into the wound site [121]. The increased amount of ECM at the damaged site promotes wound closure and creates scars, and sometimes even hypertrophic scars.

3.1.3.8 *TNF-α and EPO*

Yang et al. [157] have argued that an ideal way of controlling inflammation is to use TNFR1 antagonists or sTNF inhibitors, which block the binding of TNFR1 and sTNF, without disrupting the TNFR2 signalling pathway. In addition, Günter et al. [45] have argued that metalloproteinases and TNF-α should be antagonized due to their inflammatory and anti-proliferative effects. EPO, as a proliferative factor, can antagonize the effect of TNF-α [45]. EPO has been shown to decrease inflammation and pro-inflammatory cytokine production; EPO and transforming growth factor β-3 (TGF-β-3) may increase proliferation, formation of granulation tissue, and neodermis structure [45].

A study using a rat brain microvascular endothelial cell model has shown that EPO can increase cell survival; however, TNF-α promotes

apoptosis [148]. Another study using a rat model has suggested that EPO prevents the accumulation of macrophages, thereby decreasing TNF-α levels and preventing tissue damage from the apoptotic effects of TNF-α. The same study argues that EPO shifts TNF-α receptors from the apoptotic receptor TNFR1 to the proliferative receptor TNFR2 by downregulating TNF-α receptors and upregulating TNFR2 transcriptions [103].

3.1.4 *EPO in gene expression*

In many research studies, EPO has been shown to have effects on certain gene expression and signalling pathways. For example, local administration of EPOa/MSCs in burn wounds in rat models has been shown to significantly up-regulate expression of IL-10, VEGF, PDGF, and vimentin. Also, IL-1 and COX2 are significantly down-regulated [63]. Another study has shown that application of EPO up-regulates the expression of TGF-β1 and α-SMA (α-smooth muscle actin). Also, EPO increases the phosphorylation of Smad-2 and Smad-3 in damaged skin compared with non-damaged skin. EPO may activate Smad-dependent TGF-β signalling pathways when TGF-β is blocked by anti-TGF-β antibodies [126]. EPO-treated groups have shown increased iNOS expression in granulation tissue during wound healing. Expression of iNOS increases wound strength, epithelialization, collagen synthesis, and wound closure [57].

The cytoprotective effects of EPO depend on the modulation of different signal transduction pathways, such as apoptosis pathways, the JAK pathway, the STAT pathway, the PI3K pathway, the AKT pathway, the WNT pathway, and pathways involving forkhead transcription factors, NFκB, and GSK3β [57, 92]. When EPO binds to EPOR, the receptor undergoes a conformational change in which two intracellular monomer domains are pulled together, enabling cross phosphorylation via the binding of Jak2 kinase. Phosphorylation of Jak2 kinase initiates signal transduction cascades in colony forming unit erythroid (CFUe) that phosphorylate tyrosine residues on the cytoplasmic region of EPOR [16, 18]. Jak2 is activated in the cytoplasmic domain of EPOR, and induces different signalling pathways via several adaptor proteins, such as phosphoinositide 3-kinase (PI3K), signal transducer and activator of transcription 5 (STAT5), p42/44 mitogen-activated protein kinase (MAPK), and nuclear factor κ B (NFκB) [89].

All of these signalling pathways, on the one hand, promote red blood cell proliferation, and, on the other hand, are known for vasodilation, insulin-sensitization, and for having antithrombotic, anti-inflammatory, and anti-apoptotic actions [89]. Jak2 phosphorylation enables phosphorylation and dimerization of STAT transcription factors, including STAT1, STAT3, and STAT5a/b [89]. STAT is located in nuclei and is activated through the JAK/STAT pathway. It reg-

ulates the expression of many genes, mostly those related to cell survival, cell proliferation, cell differentiation, immune response, and hematopoiesis [116]. EPO-mediated activation of JAK2/STAT5 leads to up-regulation of the anti-apoptotic Bcl-XL and Bcl-2 genes, which then protect pro-erythroblasts from apoptosis [89]. It has been suggested that EPO increases the expression of anti-apoptotic genes (e.g., Bcl-xl and Mcl) and decreases the expression of pro-apoptotic genes (e.g., Bax, Bid, and Bim) [93]. The AKT pathway also inhibits transcription of apoptotic genes by controlling FOXO3a, and therefore increases cell survival [92].

The aim of the current study is to first investigate how EPO impacts gene expression and affects various biological pathways in foreskin-derived stem cells (FMSCs), and then study how such effects may be impacted by co-administration of cytokines like IL-6 and TNF-α.

3.1.5 *Human foreskin-derived mesenchymal stem cells (FMSCs)*

Foreskin tissue is excised during routine circumcisions, and was once considered to be nothing more than a biological waste material [99, 102]; however, foreskin tissue is now recognized to be a readily available source of multipotent mesenchymal cells [99]. Foreskin-derived stem cells have self-renewal properties and mesenchymal multilineage differentiation characteristics [9, 130].

Somuncu et al. [130] have shown that human foreskin stem cells are positive for CD14, CD29, CD34, CD44, CD45, CD73, and CD90 markers, and negative for the CD31 marker. Also, human foreskin stem cells differentiate into adipocytes, chondrocytes, osteocytes, and epithelial and neural cells [130]. Another study has shown that human dermal foreskin also expresses CD73, CD90, CD105, CD271, and SSEA-4 markers [141], which are identified as MSCs markers.

Cell cultures, especially skin-cell cultures, are used as a biological model in scientific research. FMSCs have been widely used in *in vitro* studies as feeder cell lines for human embryonic stem cells. Likewise, they can be use to study human disease mechanisms and wound healing processes, and be used in tissue engineering projects, regenerative medicine, and drug testing [127, 130].

In this dissertation, FMSCs were isolated from human foreskin tissue and stimulated with different cytokines (e.g., EPO, TNF-α, and IL-6) in *in vitro* cultures in order to test the research objectives mentioned above.

3.2 STUDY 2: MATERIALS AND METHODS

In the second study, we will explore how gene expression is affected by EPO and trauma cytokines. To do this, FMSCs were obtained from foreskin tissue samples, and then cultured in one control and six differ-

ent treatment conditions. RNA was then isolated, and gene expression was analysed using an Illumina BeadChip microarray. Finally, standard bioinformatic pipelines were used to analyse the data, identify gene pathways, and measure the effects of each treatment condition on gene expression. Below, methodological details are provided.

3.2.1 *Obtainment of tissue samples*

Foreskin tissue samples were collected from the plastic surgery waste material of three patients—with written consent—from a private clinic (Pediatric and Ambulatory Surgery, Elster Passage, Zschochersche Straße 48, 04229 Leipzig). Skin tissue was cleaned and disinfected with Betaisodona solution (Mundipharma, Germany) and ethanol solution. Immediately after sampling, the skin pieces were placed in sterile phosphate buffered saline (PBS; Gibco, Germany) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Germany) and stored at 4°C until use.

3.2.2 *Isolation of FMSCs*

The skin samples were cut coarsely into rough pieces. They were completely immersed in Betaisodona solution for 20 minutes and then rinsed with PBS until they were free of brown staining. After disinfection, the subcutaneous fatty tissue was removed with scalpel and forceps. The final samples were cut into pieces of about 5 x 5 mm, and were incubated in Dispase II (Sigma-Aldrich, Germany) for 3h at 37°C or overnight at 4°C. Dispase is a protease, which helps to separate intact epidermis from dermis effectively. The epidermal layer was removed and the dermal layer was then digested with 0.1% collagenase A (Roche, Germany) for 10h at 37°C with gentle agitation. Collagenase helps cell dissociation. The digested tissues were passed through 70μm mesh filters in order to separate cells, and were then centrifuged at 600g for 5 min at room temperature.

Next, the supernatant was discarded, and the pellet was resuspended in cell culture medium, which consisted of high glucose DMEM supplemented with 10% fetal calf serum (FCS; Gibco, Germany), and 1% penicillin/streptomycin (Biochrom, Germany). Then, cells were counted with trypan blue dye (Invitrogen, USA) and a Neubauer hemocytometer under a microscope. Afterwards, the cells were seeded into Petri dishes (TPP, Germany) which contained the culture medium. The cells were then maintained in an incubator, which was supplied with a humidified atmosphere of 5% CO_2 and 20% O_2, balanced with N_2 in a tri-gas incubator (Thermo Fisher Scientific, Germany) at 37°C.

3.2.3 *Subcultivation*

After approximately 7–14 days, the confluent FMSCs grew in Petri dishes. The resulting cells were washed twice with PBS and kept in trypsin (trypsin 0.05% with EDTA 0.01%; Biochrom, Germany) for 3–5 minutes. When the cells became detached from the surface, the cell suspension was transferred to a sterile centrifuge falcon tube (TPP, Germany) and 5 ml of fresh cell culture medium were added to the cells. The cell suspensions were centrifuged for 5 min at 1800 rpm. Afterwards, the supernatant was carefully discarded. The cell pellet was resuspended in cell culture medium. Next, the cells were counted with a Neubauer haemocytometer in the presence of trypan blue. Finally, the cells were cultured in T-75 cell culture flasks (TPP, Germany) and were incubated at 37°C, 20% O_2, and 5% CO_2 in an incubator.

3.2.4 *Cell proliferation*

After passaging the cells 5 times, the cells were cultured in a T-75 flask at a density of 75.000 cells/cm^2. In passage 6, approximately 225.000 cells were added to the T-75 flask and incubated with prepared medium and cytokines (see Table 3.1, for details on treatment compositions). The medium was changed on days 3 and 5. On day 7, the viable cells were detached by trypsin, and were counted using a hemocytometer chamber and trypan blue dye. Then, they were used for RNA extraction.

Table 3.1: The treatments administered to each group. The medium consists of 89% DMEM, 10% FCS, and 1%Pen-Strep. EPO (Human recombinant EPO; Sigma, USA) was prepared with concentrations of 20ng/ml, TNF-α (ReliaTech, Germany) and IL-6 (ReliaTech, Germany) were prepared with concentrations of 10ng/ml.

Materials	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6	Group 7
EPO				7,2μl	7,2μl		7,2μl
TNF-α		3,6μl	3,6μl	3,6μl			3,6μl
IL-6			3,6μl	3,6μl		3,6μl	
Medium	72ml	36ml	36ml	36ml	36ml	36ml	36ml

The formula for Group 1 is a control, and will produce enough solution for 6 replications. The formulas for Groups 2–7 are treatments, and will produce enough solution for 3 replications each.

3.2.4.1 *RNA Isolation*

To isolate RNA, we used an RNeasy mini kit (Qiagen, Germany). First, the samples were stabilized using an RNA stabilization reagent.

Then, the samples were disrupted and lysed using RLT buffer. In the next step, the samples were homogenized to shear genomic DNA and reduce the viscosity of the lysate. Following this, ethanol was added to adjust binding. The samples were added to the RNeasy spin column, so that the RNA could be absorbed by the membrane. Any contaminants were removed with simple wash spins (buffers: RW1 and RPE). The samples were eluted with ready-to-use RNA in water. The amount of isolated RNA was quantified using a Nanodrop ND1000 spectrophotometer (Thermo Scientific, USA).

3.2.4.2 *Illumina BeadChip Microarray*

Before microarray analysis, RNA integrity and concentration were examined on an Agilent Fragment Analyzer (Agilent Technologies, USA) using the RNA kit (Agilent Technologies, USA) according to the manufacturer's instructions. Illumina BeadChip analysis was conducted at the microarray core facility of the Faculty of Medicine, University of Leipzig (Germany). In total, 250ng of RNA per sample was precipitated with GlycoBlue (Invitrogen, USA) as a carrier, and dissolved at a concentration of 100–150 ng/μl prior to probe synthesis, using the TargetAmp Nano Labelling kit for the Illumina BeadChip (Epicentre Biotechnologies, USA). Then, 750ng of cRNA were hybridized to Human HT-12 v4 expression BeadChips (Illumina, USA) and were scanned on the Illumina HiScan instrument according to the manufacturers specifications. Raw data on 47.323 probes were extracted, and quantiles were normalized using Illumina GenomeStudio. Expression values were background subtracted.

3.2.5 *Statistical analyses*

Each cell culture and microarray experiment was replicated three times, and the analysis incorporates this fact. Statistical evaluations and data visualisations were completed using the R software package [115].

In the first step of the analysis, heatmaps were constructed to visualize patterns of gene expression—i.e., up-regulation and down-regulation—as a function of individual ID and treatment condition. This analysis allows us to check for consistency in expression responses between samples from the same individuals, and allows us to visually see how expression is affected by treatment condition at a broad level. See Figure 3.1 for an example of how to interpret these heatmaps.

In the next step of the analysis, the up-regulation and down-regulation of genes as a function of treatment condition is evaluated using the `eBayes` function from the `limma` R package [120], which provides support for linear models for microarray data. After each analysis of gene expression was completed, log-fold-change values (which are

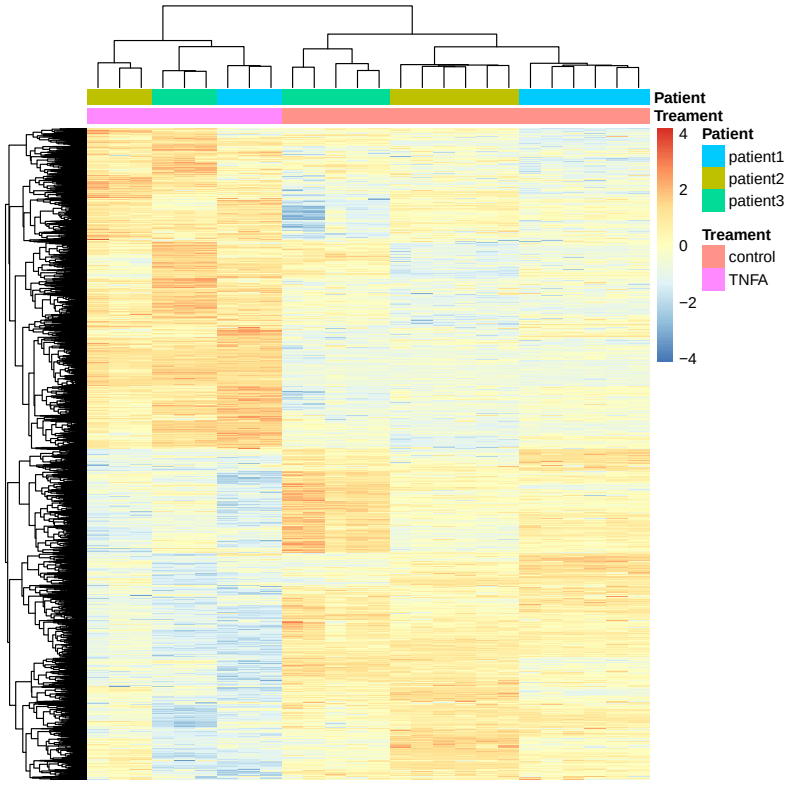


Figure 3.1: Heatmap of gene expression (TNF-α versus control) as a function of individual ID and treatment condition. A clustering algorithm is used to sort the matrix. Note that the top row of colored labels represents unique individuals, the second row of colored labels represents treatment conditions, and the color of each cell in the matrix represents gene expression levels. This analysis reveals that multiple samples taken from the same individual show highly consistent expression patterns. For example, note how within individuals the same genes are consistently expressed or not expressed. Next, note that, across individuals, treatment with TNF-α has consistent effects on the expression of genes.

indicative of differential expression) and q values (which are indicative of statistical significance) were calculated, and then visualized using standard volcano plots. Here, q values less than 0.05 were regarded as significant. See Figure 3.2 for an example of how to interpret a volcano plot.

Gene set enrichment analysis and gene ontology (GO) analysis were conducted using the R statistical environment version 4.0.2 [115], and the `gage` 2.38.8 package [85]. KEGG pathways and GO categories that present q-value lower than 0.05 were considered significant. Pathway figures and GO graphs were created using the `pathview` package version 1.28.1 [84] and `ggplot2` version 3.3.2 [150]. Only the top five GO categories for up- and down-regulated genes were plotted [78]. See Figure 3.3 for an example of how to interpret gene ontology analysis.

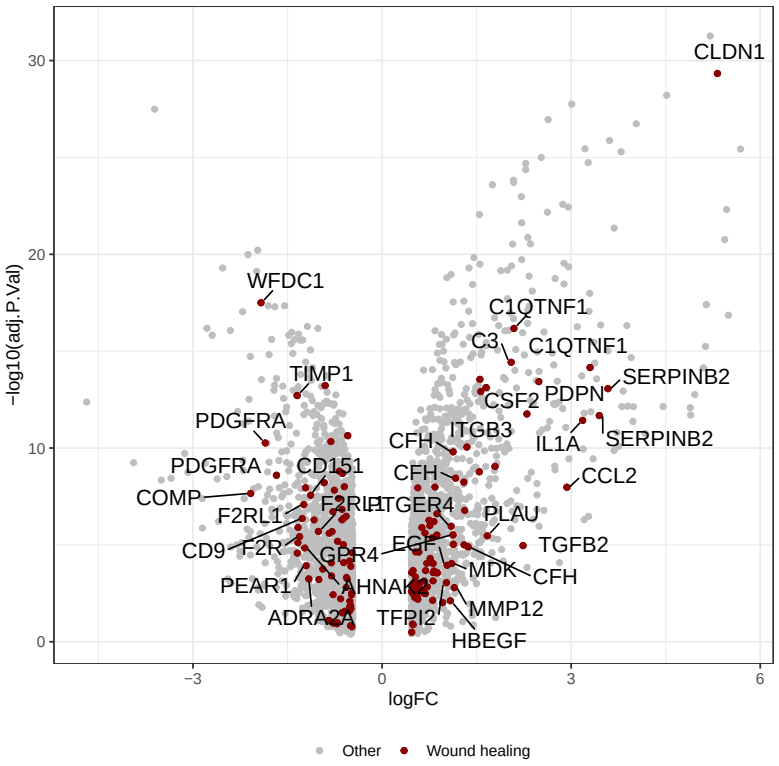


Figure 3.2: Volcano plot of gene up- and down-regulation as a function of treatment with TNF-α relative to control. Each point represents a gene. The value of the point along the x-axis represents the log-fold-change in expression as a function of treatment. The value of the point along the y-axis represents the statistical significance of the expression difference. Genes that appear higher up on the y-axis, and to the far left or right on the x-axis, appear to be reliably affected by the treatment. It is also possible to highlight the subset of genes with specific gene ontology characteristics. For example, the genes plotted here in red are linked to wound healing.

3.3 STUDY 2: RESULTS

Earlier in the chapter, the principal research questions of this study were presented. Specifically, we asked if co-administration of EPO with inflammatory cytokines (e.g., IL-6 and TNF-α) in FMSC cultures produces a larger change in the expression levels of genes linked to wound healing, skin fibrosis, and apoptosis than administration of EPO alone (or control).

In this section, these research questions will be addressed. As a first step, gene expression is visualized directly using heatmap plots. These plots help to ensure that the microarray analyses were conducted correctly, and that expression levels of each gene were consistent across samples from the same individuals. Next, volcano plots are used to visualize the extent and statistical significance of up-regulation and

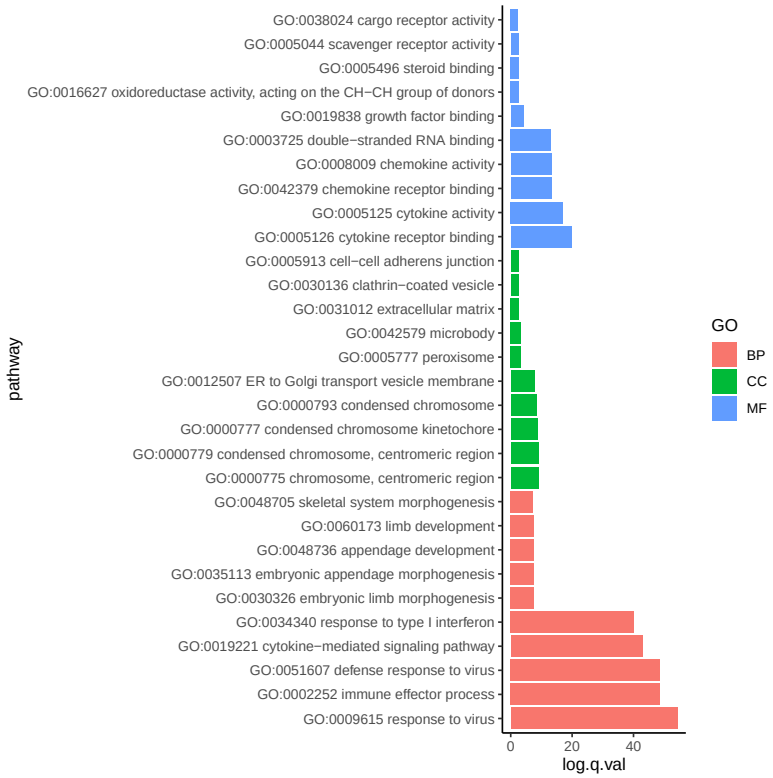
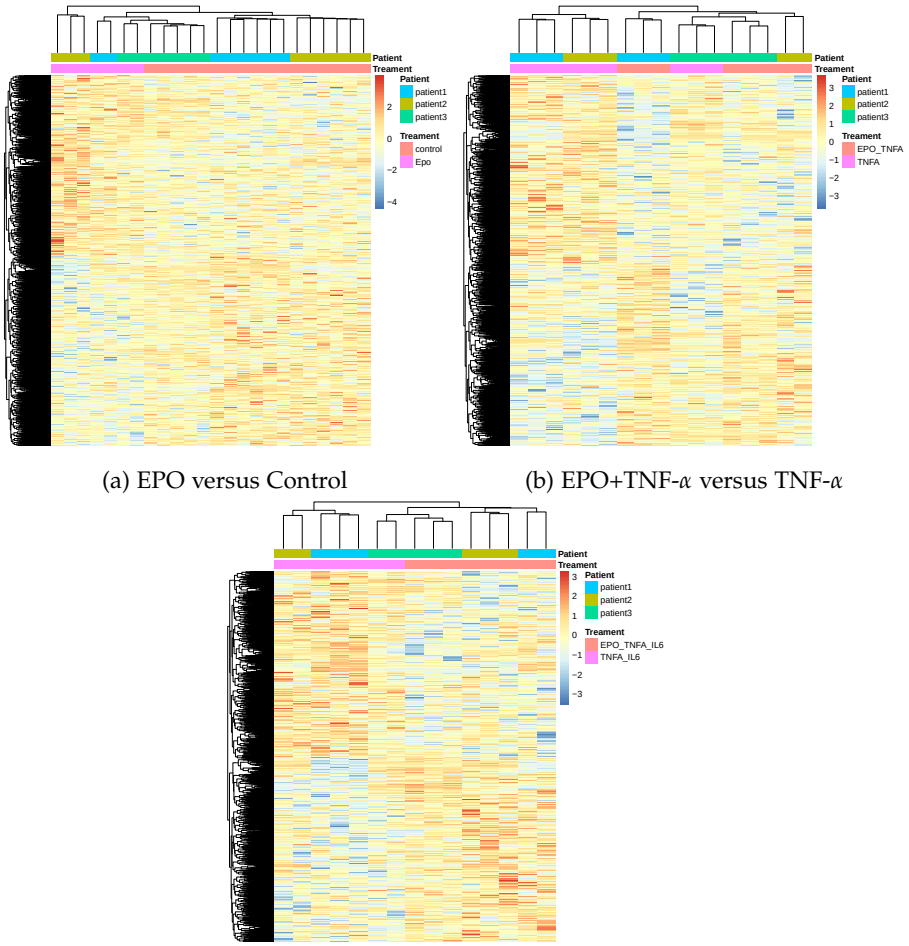


Figure 3.3: Bar plot representing the results of a functional enrichment analysis (TNF-α versus control). The pathways listed here are the ten most reliably up-regulated in each of the three gene ontology super categories (i.e., biological process, BP; cellular component, CC; and molecular function, MF).

down-regulation of the set of genes included in the microarray. These plots can also be used to visualize expression changes in specific genes of interest (e.g., genes involved in wound healing). Finally, because gene expression levels are linked in complex biological pathways, a functional enrichment analysis is conducted to investigate if specific pathways are significantly up- or down-regulated by treatment with EPO and/or other cytokines.

3.3.1 *The effect of cytokines on general gene expression*

Here, heat maps are used to visualize raw gene expression data. These plots directly represent the individual- and gene-specific expression levels in a matrix where expression is represented with color. Further, clustering methods are used to organized genes that have similar expressions patterns into nearby rows in the matrix. The color of the boxes represents the intensity of gene expression. Here, red represents genes with high expression levels, yellow represents genes with moderate expression levels, and blue represents genes with low expression levels.



(a) EPO versus Control

(b) EPO+TNF-α versus TNF-α

(c) EPO+TNF-α+IL-6 versus TNF-α+IL-6

Figure 3.4: Heatmaps of gene expression as a function of individual ID and treatment condition in the final study. A clustering algorithm is used to sort each matrix. Note that the top row of colored labels represents unique individuals, the second row of colored labels represents treatment conditions, and the color of each cell in the matrix represents gene expression levels. These analyses reveal that multiple samples taken from the same individuals show highly consistent expression patterns. For example, note how within individuals the same genes are consistently expressed or not expressed. Next, note that, across individuals, treatment with EPO plus cytokines has consistently different effects on the expression of genes than cytokines alone.

Figure 3.4 plots the results of the microarray analysis. It is clear that multiple samples from the same individuals cluster together and that treatment groups cluster together, indicating that the analysis functioned correctly. Across individuals, treatment with EPO plus cytokines has consistently different effects on the expression of genes than cytokines alone. In the next section, the specific differences will be analyzed quantitatively.

3.3.2 *The effect of cyctokines on expression of wound-healing related genes*

Gene profiles for 387 genes related to wound healing were downloaded from the Gene Ontology Browser (dataset GO:0042060) [41]. The expression changes in these genes were compared with the results of other genes in the microarray analysis. It is specifically tested if any wound healing genes are significantly up-regulated or down-regulated, using linear models for microarray data [120]. Gene expression differences between treatment conditions were visualized using volcano plots, which represent expression differences as a log-fold-change and statistical significance as a negative-log-adjusted-p-value (i.e., a q-value).

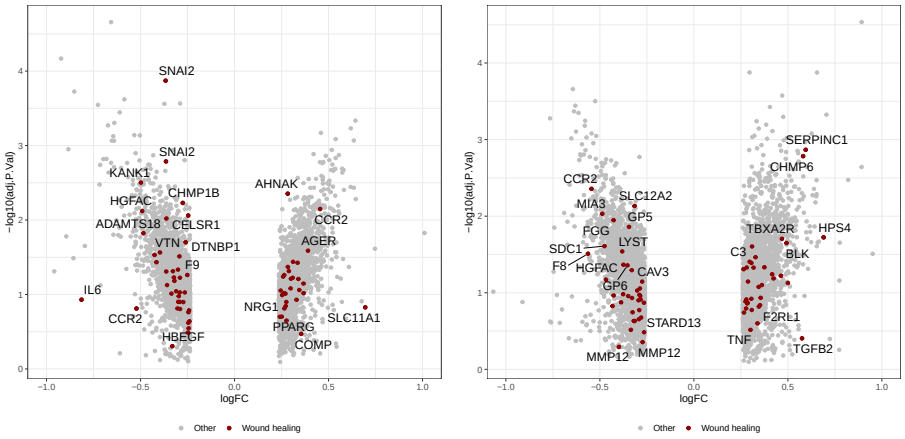
Figure 3.5 plots the results of changes in gene expression as a function of treatment with EPO. There are three comparisons: 1) EPO versus control, 2) EPO+TNF-α versus EPO, and 3) EPO+TNF-α+IL-6 versus TNF-α+IL-6. The first case gives the effect of EPO on gene expression in isolation, the second case gives the effect of EPO on gene expression in the presence of TNF-α, and the third case gives the effect of EPO on gene expression in the presence of both IL-6 and TNF-α. The most up-regulated genes are located on the right, the most down-regulated genes are located on the left, and the genes with the most statistically significant differences in expression are located at the top of the plot. Labels are given for the most differentially expressed genes.

In the base condition, EPO appears to have effects on several wound healing genes. For example, AHNAK, CCR2, AGER were up-regulated. SNAI2 was highly significantly down-regulated. KANK1, CHMP1B, HGFAC, and ADAMTS18 were down-regulated.

In the second condition, where EPO was administered with TNF-α, EPO also appears to have effects on wound healing genes, but the specific wound healing genes that are up- or down-regulated changes, indicating that TNF-α modifies the effect of EPO. For example, SERPINC1 and CHMP6 were highly significantly up-regulated. Also, TBXA2R, HPS4, and BLK were up-regulated. CCR2, SLC12A2, MIA3, GP5, and FGG were down-regulated.

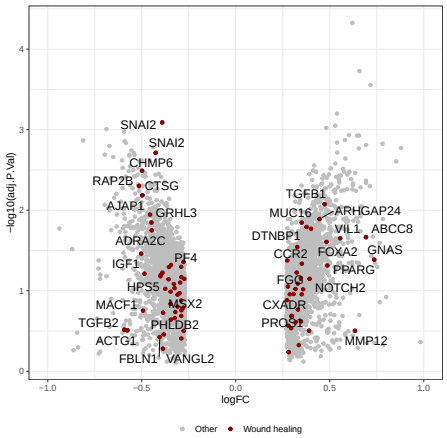
In the third condition, where EPO was administered with TNF-α and IL-6, EPO continues to have effects on wound healing genes, but the specific wound healing genes that are up- or down-regulated again changes, indicating that IL-6 further modifies the effect of EPO. For example, TGFB1, MUC16, ARHGAP24, and VIL1 were up-regulated. SNAI2, CHMP6, RAP2B, and CTSG were down-regulated.

It is interesting to note that log-fold change (both positive and negative) appears greater on average in the case where EPO was co-administered with both IL-6 and TNF-α. See Figure 3.5(c). This indicates that the effect of EPO on overall gene expression may be greater in the presence of key cytokines.



(a) EPO versus Control

(b) EPO+TNF-α versus TNF-α



(c) EPO+TNF-α+IL-6 versus TNF-α+IL-6

Figure 3.5: Volcano plots of gene up- and down-regulation as a function of treatment with EPO in three different contexts. Each point represents a gene. The value of the point along the x-axis represents log-fold-change in expression as a function of treatment. The value of the point along the y-axis represents the statistical significance of the expression difference. Genes that appear higher up on the y-axis, and to the far left or right on the x-axis, appear to be reliably affected by the treatment. It is also possible to highlight the subset of genes with specific gene ontology characteristics. For example, the genes plotted here in red are linked to wound healing. Wound healing genes with log-fold expression changes greater than 0.25 are labeled.

3.3.3 *The effect of cyctokines on expression of skin fibrosis related genes*

The study was continued by repeating the analysis with a focus on genes linked to skin fibrosis. Gene profiles for 125 genes related to skin fibrosis were downloaded [from: 42]. The expression changes in these genes were compared with the results of other genes in the microarray analysis. It is specifically tested if any skin fibrosis genes are significantly up-regulated or down-regulated, using linear models

for microarray data [120]. The results of these analyses are presented in Figure 3.6.

In the base condition, EPO appears to have effects on several skin fibrosis genes. For example, CCR2, CX3CR1, HPS4, and PDZK1 were up-regulated. DGKA, NR4A1, ALOX15, and CCR2 were down-regulated.

In the second condition, where EPO was administered with TNF-α, EPO also appears to have effects on skin fibrosis genes, but the specific skin fibrosis genes that are up- or down-regulated changes, indicating that TNF-α modifies the effect of EPO. For example, EN1 was highly significantly up-regulated. Also, DGKA, TNFSF13, SERPINF2, and HPS4 were up-regulated. CCR2 was highly significantly down-regulated. SDC1, LSP1, PDGFRA, and SHH were down-regulated.

In the third condition, where EPO was administered with TNF-α and IL-6, EPO continues to have effects on skin fibrosis genes, but the specific skin fibrosis genes that are up- or down-regulated again changes, indicating that IL-6 further modifies the effect of EPO. For example, TGFB1 was highly significantly up-regulated. VEGFB, CCR2, and GNAS were up-regulated. CNR1, AGT, ALOX15, and PDZK1 were down-regulated.

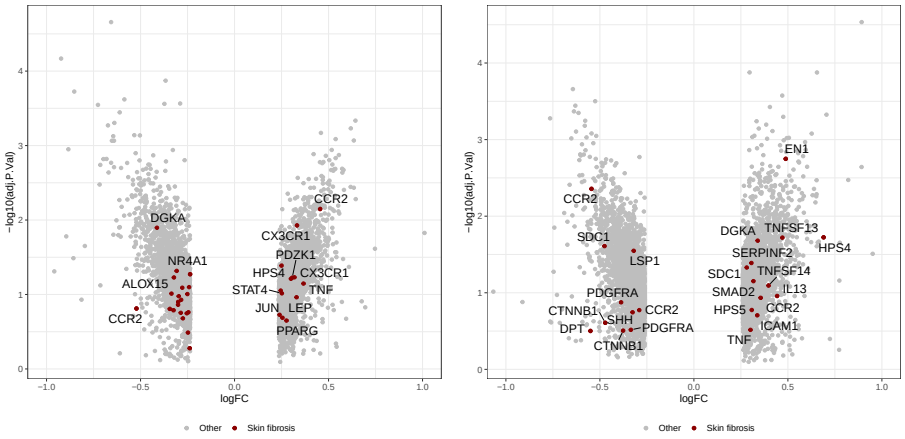
3.3.4 *The effect of cyctokines on expression of apoptotic process related genes*

The study was continued by repeating the analysis with a focus on genes linked to apoptotic processes. Gene profiles for 1,978 genes related to apoptotic processes were downloaded from the Gene Ontology Browser (dataset GO:0006915) [41]. The expression changes in these genes were compared with the results of other genes in the microarray analysis. It is specifically tested if any genes involved in apoptotic processes are significantly up-regulated or down-regulated, using linear models for microarray data [120]. The results of these analyses are presented in Figure 3.7.

In the base condition, EPO appears to have effects on several apoptotic genes. For example, XKR5, PIK3CG, TRPC5, MAPK7, and ZMYND11 were up-regulated. SNAI1, SNAI2, IL6ST, ADNP, and LTK were down-regulated.

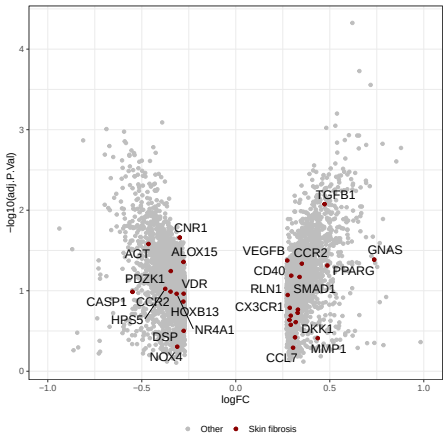
In the second condition, where EPO was administered with TNF-α, EPO also appears to have effects on genes involved in apoptotic processes, but the specific genes that are up- or down-regulated changes, indicating that TNF-α modifies the effect of EPO. For example, DHODH, EN1, RELT, and NQO1 were up-regulated. IL6ST, ABL2, TNFRSF8, FGG, and MADD were down-regulated.

In the third condition, where EPO was administered with TNF-α and IL-6, EPO continues to have effects on genes involved in apoptotic processes, but the specific genes that are up- or down-regulated again changes, indicating that IL-6 further modifies the effect of EPO. For example, MAPK8IP3, DFFB, FMR1, ATG3, and EPHA7 were



(a) EPO versus Control

(b) EPO+TNF-α versus TNF-α



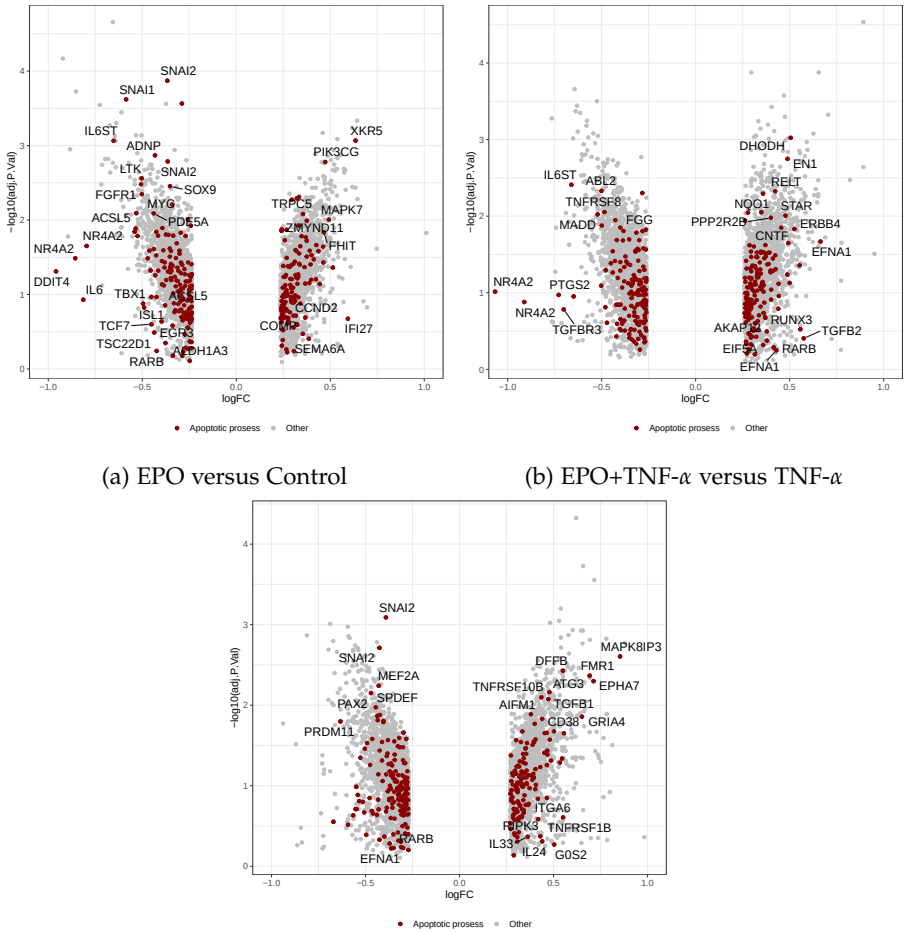
(c) EPO+TNF-α+IL-6 versus TNF-α+IL-6

Figure 3.6: Volcano plots of gene up- and down-regulation as a function of treatment with EPO in three different contexts. Each point represents a gene. The value of the point along the x-axis represents log-fold-change in expression as a function of treatment. The value of the point along the y-axis represents the statistical significance of the expression difference. Genes that appear higher up on the y-axis, and to the far left or right on the x-axis, appear to be reliably affected by the treatment. It is also possible to highlight the subset of genes with specific gene ontology characteristics. For example, the genes plotted here in red are linked to skin fibrosis. Skin fibrosis genes with log-fold expression changes greater than 0.25 are labeled.

up-regulated. SNAI2 was again highly significantly down-regulated. MEF2A, SPDEF, PAX2, and PRDM11 were down-regulated.

3.3.5 *Functional enrichment analysis*

Because of the large variety of gene-specific effects, it can be hard to interpret how treatment with EPO affects specific biological processes.



(a) EPO versus Control

(b) EPO+TNF-α versus TNF-α

(c) EPO+TNF-α+IL-6 versus TNF-α+IL-6

Figure 3.7: Volcano plots of gene up- and down-regulation as a function of treatment with EPO in three different contexts. Each point represents a gene. The value of the point along the x-axis represents log-fold-change in expression as a function of treatment. The value of the point along the y-axis represents the statistical significance of the expression difference. Genes that appear higher up on the y-axis, and to the far left or right on the x-axis, appear to be reliably affected by the treatment. It is also possible to highlight the subset of genes with specific gene ontology characteristics. For example, the genes plotted here in red are linked to apoptotic processes. Apoptotic process genes with log-fold expression changes greater than 0.5 are labeled.

As such, functional enrichment analysis is used to study how specific biological pathways as a whole are affected by treatment with EPO. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analyses were performed to test for differentially expressed gene pathways. Q-values of less than 0.05 were considered significant.

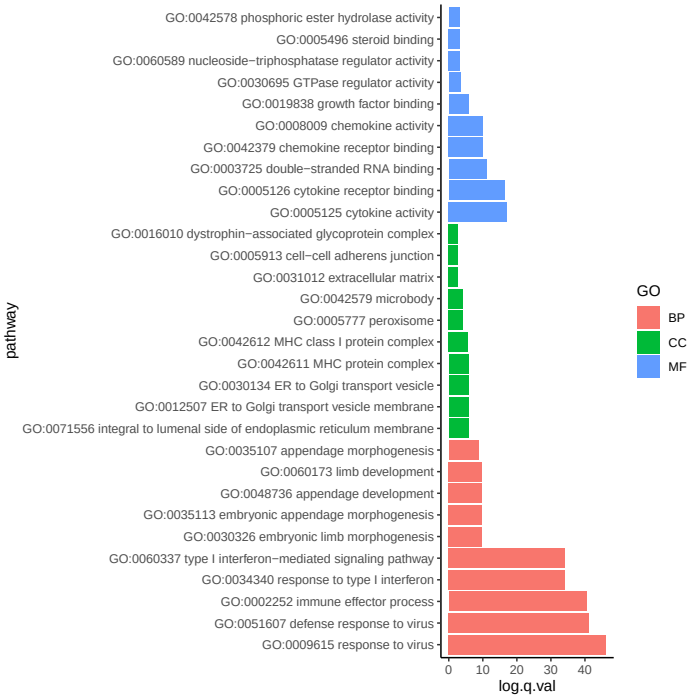
No significant effects were observed in the EPO versus control contrast, the EPO+TNF-α versus TNF-α contrast, and the EPO+TNF-α+IL-

6 versus TNF-α+IL-6 contrast. However, significant up- and down-regulation was observed in the EPO+TNF-α+IL-6 versus control contrast and the EPO+TNF-α versus control contrast (Figure 3.8). Bar plots present the distributions of the most enriched GO terms, the functions of the genes involved and the relationships between them, and the number of genes involved. The plot includes pathways involved in biological processes (BP), cellular component (CC), and molecular function (MF) categories.

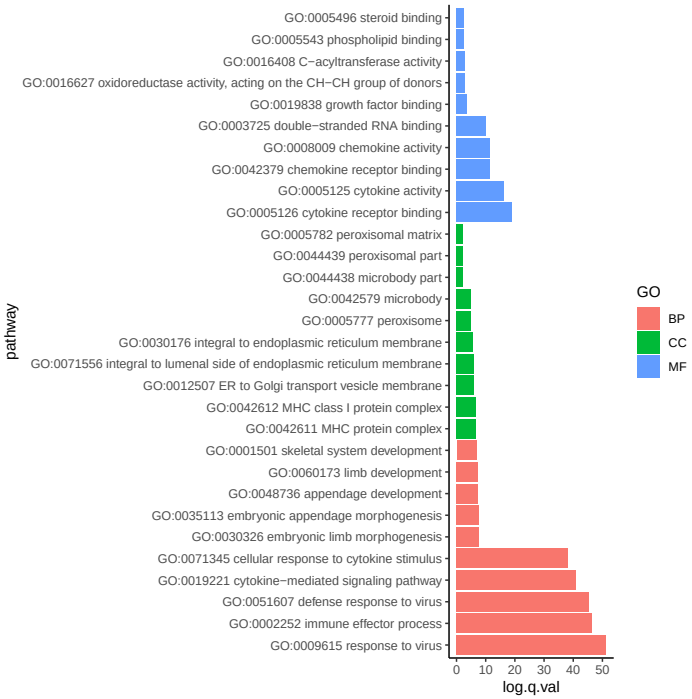
The most significantly enriched KEGG pathways are shown in Tables 3.2 and 3.3.

Table 3.2: The most significantly up-regulated KEGG pathways.

	EPO vs control	EPO+TNF-α vs TNF-α	EPO+TNF-α+IL6 vs TNF-α+IL-6	EPO+TNF-α vs control	EPO+TNF-α+IL6 vs control
hsa00532 Glycosaminoglycan biosynthesis					✓
hsa03008 Ribosome biogenesis in eukaryotes				✓	✓
hsa03030 DNA replication					✓
hsa03050 Proteasome				✓	✓
hsa03440 Homologous recombination					✓
hsa04060 Cytokine-cytokine receptor interaction				✓	✓
hsa04061 Viral protein interaction with cytokine				✓	✓
hsa04062 Chemokine signaling pathway				✓	✓
hsa04064 NF-kappa B signaling pathway				✓	✓
hsa04110 Cell cycle				✓	✓
hsa04115 p53 signaling pathway					✓
hsa04141 Protein processing in endoplasmic reticulum				✓	✓
hsa04145 Phagosome					✓
hsa04210 Apoptosis				✓	✓
hsa04215 Apoptosis - multiple species				✓	✓
hsa04217 Necroptosis				✓	✓
hsa04218 Cellular senescence				✓	✓
hsa04380 Osteoclast differentiation				✓	✓
hsa04514 Cell adhesion molecules				✓	✓
hsa04612 Antigen processing and presentation				✓	✓
hsa04620 Toll-like receptor signaling pathway				✓	✓
hsa04621 NOD-like receptor signaling pathway				✓	✓
hsa04622 RIG-I-like receptor signaling pathway				✓	✓
hsa04623 Cytosolic DNA-sensing pathway				✓	✓
hsa04625 C-type lectin receptor signaling pathway				✓	✓
hsa04640 Hematopoietic cell lineage				✓	✓
hsa04650 Natural killer cell mediated cytotoxicity				✓	✓
hsa04657 IL-17 signaling pathway				✓	✓
hsa04658 Th1 and Th2 cell differentiation				✓	✓
hsa04659 Th17 cell differentiation				✓	✓
hsa04660 T cell receptor signaling pathway				✓	✓
hsa04662 B cell receptor signaling pathway				✓	✓
hsa04664 Fc epsilon RI signaling pathway				✓	✓
hsa04668 TNF signaling pathway				✓	✓
hsa04670 Leukocyte transendothelial migration				✓	✓
hsa04926 Relaxin signaling pathway					✓
hsa04978 Mineral absorption					✓
no effects significant	✓	✓	✓		



(a) EPO+TNF-α versus control



(b) EPO+TNF-α+IL-6 versus Control

Figure 3.8: Bar plots of the most significantly enriched pathways in 3 different categories: biological processes (BP), cellular component (CC), and molecular function (MF).

Table 3.3: The most significantly down-regulated KEGG pathways.

	EPO vs control	EPO+TNF-α vs TNF-α	EPO+TNF-α+IL6 vs TNF-α+IL-6	EPO+TNF-α vs control	EPO+TNF-α+IL6 vs control
hsa00030 Pentose phosphate pathway				✓	✓
hsa00071 Fatty acid degradation					✓
hsa00190 Oxidative phosphorylation				✓	
hsa00280 Valine, leucine and isoleucine degradation				✓	
hsa00562 Inositol phosphate metabolism				✓	✓
hsa00640 Propanoate metabolism				✓	✓
hsa00830 Retinol metabolism				✓	✓
hsa00980 Metabolism of xenobiotics by cytochrome P450				✓	✓
hsa00982 Drug metabolism - cytochrome P450				✓	✓
hsa00983 Drug metabolism - other enzymes				✓	
hsa01200 Carbon metabolism				✓	✓
hsa04137 Mitophagy - animal				✓	✓
hsa04140 Autophagy - animal				✓	
hsa04146 Peroxisome				✓	✓
hsa04150 mTOR signaling pathway					✓
hsa04310 Wnt signaling pathway				✓	✓
hsa04350 TGF-beta signaling pathway				✓	✓
hsa04360 Axon guidance				✓	✓
hsa04371 Apelin signaling pathway					✓
hsa04520 Adherens junction				✓	✓
hsa04550 Signaling pathways regulating pluripotency				✓	✓
hsa04713 Circadian entrainment				✓	✓
hsa04714 Thermogenesis				✓	
hsa04727 GABAergic synapse					✓
hsa04810 Regulation of actin cytoskeleton				✓	✓
hsa04910 Insulin signaling pathway				✓	✓
hsa04919 Thyroid hormone signaling pathway				✓	✓
hsa04921 Oxytocin signaling pathway				✓	
hsa04923 Regulation of lipolysis in adipocytes				✓	
no effects significant	✓	✓	✓		

3.4 STUDY 2: DISCUSSION

In past research, Bader et.al, [4, 6] have shown that EPO administered alone strongly inhibits human dermally-derived stem cell proliferation, but little is known about the mechanisms that drive such effects. There is some evidence that adding cytokines (e.g., IL-6 and TNF-α) when EPO is administered may change the effect of EPO. For example, co-administration of EPO with TNF-α and IL-6 led to increases in stem cell proliferation [4, 6, 38]. But again, however, the mechanisms underlying such effects are yet to be well understood.

The current study was designed to uncover if and how EPO affects the expression of various genes, and how such effects may be modified by the availability of cytokines. Specifically, it was tested if administration of EPO alone, and co-administration of EPO with TNF-α and IL-6, can change the expression of genes and pathways specifically linked to wound healing, skin fibrosis, and apoptosis. The comparison between EPO and control conditions shows that EPO indeed appears to have effects on several genes involved in wound healing, skin fibrosis, and apoptotic processes. Also, administration

of EPO with TNF-α led to different level of expression in the same set of genes, indicating that TNF-α modifies the effect of EPO. We see similar results for co-administration of IL-6, which further modifies the effect of EPO. It appears that the effect of EPO on overall gene expression may be greater in the presence of key cytokines.

On a gene-by-gene basis, there is some evidence in the current study that adding cytokines changes the effect of EPO. For example, there were some differences in which genes were up-regulated versus down-regulated, but effect sizes were small, and only a few genes were shown to have significant differences in expression as a function of treatment with EPO. Many of the genes whose expression was affected by EPO in this study are involved in inflammatory processes. For example, CCR2 recruits monocytes and macrophages to wound areas, and is involved in inflammatory responses during wound healing. CCR2 deficiency has been shown to impair monocyte recruitment to the site of tissue injuries, decrease inflammatory gene expression, and impair wound healing [151]. AGER or AGE can inhibit neutrophil migration, and prolong wound healing by inducing production of large amounts of inflammatory cytokines, such as TNF-α; it may also increase oxidative stress, which impairs re-epithelialization and the wound healing process [113].

In wider-scale GO and KEGG pathway analyses, no strong effects were found linking EPO to pathway changes, whether or not cytokines were co-administered. There were no differences in the EPO versus control contrast, the EPO+TNF-α versus TNF-α contrast, or the EPO+TNF-α+IL-6 versus TNF-α+IL-6 contrast. This suggests that the gene expression changes induced by EPO are not well described by the pathways included in GO or KEGG analyses. Nevertheless, EPO might still have important effects on the biology of wound healing, skin fibrosis, and apoptotic possesses, as gene expression changes need not be part of GO or KEGG pathways to be clinically relevant.

It is notable, however, that effects of TNF-α and IL-6 versus control were present. For example, many GO and KEGG pathways showed changes in both EPO+TNF-α versus control and EPO+TNF-α+IL-6 versus control conditions. This suggest that the study design was successful, in that the highly active cytokines, TNF-α and IL-6, significantly changed the activity of various pathways. *However, co-administration of EPO did not lead to any changes above-and-beyond what could be explained by cytokine treatment alone.*

The study presented here thus calls into question the idea that EPO has significant impacts on gene expression in FMSCs. However, there are some limitations to this study. Future research would benefit from use of a larger number of samples from a larger number of diverse research participants. Different dosages of EPO, and different time periods of exposure, may give the researcher different information about the effects of EPO on gene expression. Finally, we saw that EPO

has some effects on gene expression, however, such effects do not appear to aggregate to the larger pathway scale. Nevertheless, EPO may have important biological effects on wound healing and related processes that are not captured by the GO and KEGG analyses.

Although many studies have shown various effect of EPO, there is still much to learn about the molecular and cellular effects of EPO *in vivo* and *in vitro*. Co-administration of EPO with different cytokines (rather than just TNF-α and IL-6) may provide new insights. More work is still needed.

3.4.1 *Conclusion*

This study is the first to examine the role of EPO administration, and co-administration of EPO with TNF-α and IL-6 cytokines, on genome-wide gene expression levels in FMSCs. Treatment with EPO alone, and co-administration with TNF-α and IL-6, leads to changes in gene expression, but no statistically significant effects in GO or KEGG signaling pathways were observed. However, when treatment groups were compared to the control condition, there were some changes in expression pathways. Taken together, these data suggest that EPO does not seem to have strong effects on gene expression, while TNF-α and IL-6 do. Future work is needed to determine if application of EPO with other cytokines, or at other dosage levels, leads to larger-scale changes in GO or KEGG signaling pathways.

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Meimanat Fathi

Curriculum Vitæ

Expertise

Biotechnology and stem cell biology, erythropoietin and wound healing, cell culture, 3D retinal organoid culture, cell isolation from tissue, DNA/RNA extraction, PCR/Realtime PCR, ELISA assays, Brdu assays, western blot assays, immunohistochemistry, histological image analysis, animal experimentation, good manufacturing practice (GMP), R software.

Education

Jan.2017–Present **PhD student, Biotechnology**, *Faculty of Natural Sciences*, Leibniz Universität Hannover (LUH), and Department of Cell Techniques and Applied Stem Cell Biology, University of Leipzig. Germany

Dissertation, *The effects of EPO on cutaneous wound healing, dermal stem cell marker activation, and gene expression patterns*

Sep.2011–Dec.2013 **Master of Science, Genetics**, *Azad University. Iran*

Thesis, *The effect of naloxone-alum adjuvant on IL-17 cytokine in an HIV-1 multi-epitopic model*

Sep.2007–Aug.2011 **Bachelor of Science, Genetics**, *Azad University. Iran*

Professional Experience

Research and Employment

Aug.2022-Present **Wissenschaftliche Mitarbeiterin**, *Paul Flechsig Institute of Brain Research*, University of Leipzig, Germany

Aug.2021-Aug.2022 **Researcher**, *Department of Cell Techniques and Applied Stem Cell Biology*, University of Leipzig, Germany

Nov.2020-Aug.2021 **Wissenschaftliche hilfskraft**, *Paul Flechsig Institute of Brain Research*, University of Leipzig, Germany

Oct.2016-Dec.2019 **Wissenschaftliche Mitarbeiterin**, *Bionethos Innovation GmbH. Klinga, Germany*

May.2013-Feb.2015 **Researcher**, *Pasture Institute. Tehran, Iran*

Jan.2010-Apr.2013 **Researcher**, *Medical Sciences Research Center, Tehran Medical Branch of Azad University. Tehran, Iran*

March.2009-Aug.2011 **Researcher**, *Research Center of Cardiac Surgery and Transplantation, Shariati Hospital. Tehran, Iran. Cardiac, Vascular and Stroke Research Institute, McMaster University. Canada*

Publications

2021 **Fathi M.**, Ross CT., Hosseinzadeh Z. Functional 3-dimensional retinal organoids: Technological progress and existing challenges. *Frontiers in Neuroscience*. 15, 668857. 1–9. DOI:10.3389/fnins.2021.668857

2021 **Fathi M.**, Nezamzadeh R., Abdollahpour-Alitappeh M., Yazdi MH., Khoramabadi N, Mahdavi M. Formulation of a recombinant HIV-1 polytope candidate vaccine with naloxone/alum mixture: Induction of multi-cytokine responses with a higher regulatory mechanism. *Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS)*. 129, 8. 480–488. DOI:10.1111/apm.13122

2014 **Fathi M.**, Nezamzadeh, R., Mahdavi, M. Studying the effects of naloxone-alum adjuvant mixture on cytokines in model of multi-epitope vaccine in HIV-1. 4, 2. 7–14. *Journal of Chemical Health Risks*. DOI:10.22034

Computer skills

Microsoft office, LaTex, EndNote, Citavi, R statistical software, Axiovision and Orbit histological analysis software, Online marketing, Professional photography

Languages

English *C2*

German *B2*

Farsi *Native speaker*

LIST OF PUBLICATIONS

Fathi M., Ross CT., Hosseinzadeh Z. 2021. Functional 3-dimensional retinal organoids: Technological progress and existing challenges. *Frontiers in Neuroscience*. 15, 668857. 1–9. DOI:10.3389/fnins.2021.668857

Fathi M., Nezamzadeh R., Abdollahpour-Alitappeh M., Yazdi MH., Khoramabadi N, Mahdavi M. 2021. Formulation of a recombinant HIV-1 polytope candidate vaccine with naloxone/alum mixture: Induction of multi-cytokine responses with a higher regulatory mechanism. *Acta Pathologica, Microbiologica et Immunologica Scandinavica (APMIS)*. 129, 8. 480–488. DOI:10.1111/apm.13122

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