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A hypoxia response element in the *Vegfa* promoter is required for basal *Vegfa* expression in skin and for optimal granulation tissue formation during wound healing in mice

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Mémoire présenté à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de maître ès sciences (M. Sc.)
en sciences vétérinaires option biomédecine

Décembre 2016

RÉSUMÉ

L'hypoxie contribue à la guérison cutanée via l'induction de HIF-1 (hypoxia-inducible factor-1). HIF-1 gère l'expression de VEGFA (vascular endothelial growth factor A) en se liant au HRE (hypoxia response element) présent au niveau de son promoteur. Par contre, dans le contexte de la guérison cutanée il est incertain si l'hypoxie et HIF-1 contribuent à l'augmentation de l'expression de VEGFA. Pour vérifier cette hypothèse, la guérison cutanée et l'expression de VEGFA ont été étudiées dans un modèle murine, *Vegfa*$^{\delta/\delta}$, possédant un HRE non-fonctionnel dans le promoteur de *Vegfa*. De manière inattendue, le niveau d'ARNm de *Vegfa* présent dans la peau intacte des souris mutantes était diminué. Par contre, le niveau d'ARNm de *Vegfa* dans le tissu de granulation n'était pas altéré par rapport à celui de souris normales. Similairement, le niveau d'ARNm des gènes ciblés par *Vegfa*, *Pdgfb* et *Sdf-1*, étaient aussi comparativement diminués dans la peau intacte des souris mutantes mais aucune différence significative ne fut observée dans le tissu de granulation de plaies. L'analyse histologique des plaies en guérison chez les souris mutantes a démontré un tissu de granulation altéré en quantité et qualité (densité de capillaires). Par contre, la différence dans l'épithélialisation et le taux de guérison cutanée entre les deux populations de souris était non-significative. Les résultats démontrent qu'HIF-1 n'est pas un facteur majeur dans la régulation de *Vegfa* dans le contexte de la guérison cutanée. Cependant, HIF-1 est nécessaire pour maintenir un niveau d'expression basale (*Vegfa* et ses gènes cibles) adéquate et est aussi nécessaire pour la formation d'un tissu de granulation de qualité optimal suivant une blessure cutanée.

Mots-clés : HIF-1α, VEGFA, HRE, peau, guérison, tissu de granulation

ABSTRACT

Hypoxia in skin wounds is thought to contribute to healing through the induction of hypoxia-inducible factor-1 (HIF-1), a key transcriptional regulator of genes associated with healing. HIF-1 regulates the expression of vascular endothelial growth factor A (VEGFA) by binding a hypoxia response element (HRE) in its promoter. Although VEGFA is a key regulator of angiogenesis during healing, whether hypoxia and HIF-1 serve to induce its expression in this context is unknown. To test this hypothesis, we studied VEGFA expression and wound healing in the *Vegfa*$^{\delta/\delta}$ mouse model, which lacks a functional HRE in the *Vegfa* promoter. Unexpectedly, decreased levels of *Vegfa* mRNA were found in intact skin in *Vegfa*$^{\delta/\delta}$ mice, whereas levels in granulation tissue during wound healing were not altered. Likewise, mRNA levels of VEGFA target genes *Pdgfb* and *Sdf-1* were decreased in skin but unchanged in granulation tissue in the *Vegfa*$^{\delta/\delta}$ mice. Decreased *Vegfa* levels in skin could not be attributed to decreased HIF-1 protein expression in the skin of *Vegfa*$^{\delta/\delta}$ mice, and were therefore the consequence of a loss of HIF-1 responsiveness of the *Vegfa* promoter. Histologic analyses of healing wounds in *Vegfa*$^{\delta/\delta}$ mice revealed significant defects in granulation tissue, both in terms of quantity and capillary density, although epithelialization and healing rates were unaltered. It can be concluded that HIF-1 is not a major regulator of *Vegfa* during wound healing. Rather, HIF-1 serves to maintain basal levels of *Vegfa* and VEGFA target genes in intact skin, required for optimal granulation tissue formation in response to wounding.

Keywords: HIF-1α, VEGFA, HRE, skin, wound healing, granulation tissue

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ABBREVIATIONS

2-Oxoglutarate	2-OG
Activator protein	AP
Adenosine triphosphate	ATP
Angiopoietin	ANGPT
Arrest-defective-1	ARD1
Aryl hydrocarbon nuclear translocator	ARNT
Asparagine 803	N803
Basic fibroblast growth factor	bFGF
Basix-helix-loop-helix-Per-ARNT-Sim	bHLH-PAS
Beta-actin	ACTB
c-Jun NH2-terminal kinase	JNK
C-terminal transactivation domain	C-TAD
Carbon dioxide	CO_2
Cluster of differentiation 31	CD31
Cobalt chloride	$CoCl_2$
Delta-like 4	Dll4
Early inflammatory phase	EIP
Egg-laying nine	EGLN
Epidermal growth factor	EGF
Epidermal growth factor receptor	EGFR
Erythropoietin	EPO
Extracellular matrix	ECM
Extracellular signal-regulated kinase	ERK
Factor inhibiting HIF-1	FIH-1
Fetal liver kinase-1	Flk-1

Fibroblast growth factor	FGF
fms-like tyrosine kinase-1	Flt-1
Glucose transporter-1	GLUT-1
Hematopoietic stem cell	HSC
Hematoxylin-eosin-phloxine-saffron	HEPS
Hepatocyte growth factor	HGF
HIF-prolyl hydroxylase	HPH
Hydrogen peroxide	H_2O_2
Hydroxyl ion	OH^-
Hypoxia-inducible factor	HIF
Hypoxia response element	HRE
Insulin-like growth factor	IGF
Interferon-γ	INF-γ
Interleukin	IL
Internal ribosome entry site	IRES
Jagged-1	Jag1
Keratinocyte growth factor	KGF
Kinase domain region	KDR
Late inflammatory phase	LIP
Lysine 532	K532
Macrophage colony stimulating factor	M-CSF
Matrix metalloproteinase	MMP
Membrane type-1 matrix metalloproteinase	MT1-MMP
Mitogen-activated protein	MAP
N-terminal transactivation domain	N-TAD
Neuropilin	NRP
Nitric oxide	NO

Nitric oxide synthase	NOS
Nuclear factor-1	NF-1
Nuclear factor kappa B	NF-κB
Optimal cutting temperature	OCT
Oxygen-dependent degradation domain	ODDD
Peroxide anion	HO_2^-
Phospholipase C	PLC
Placental growth factor	PlGF
Platelet derived growth factor	PDGF
Platelet factor-IV	PF4
Proline 402	P402
Proline 564	P564
Prolyl hydroxylase domain	PHD
Quantitative polymerase chain reaction	qPCR
Quantitative reverse transcription polymerase chain reaction	RT-qPCR
Reactive oxygen species	ROS
Receptor tyrosine kinase	RTK
Ribosomal protein L19	RPL19
Specificity protein 1	Sp1
Stress-activated protein kinase	SAPK
Stromal cell-derived factor-1	SDF-1
Superoxide anion	O^{2-}
Tissue inhibitors of metalloproteinases	TIMPs
Tissue-type plasminogen activator	tPA
Transforming growth factor	TGF
Tumor necrosis factor	TNF
Urokinase-type plasminogen activator	uPA

Vascular endothelial cadherin	VE-cadherin
Vascular endothelial growth factor	VEGF
Vascular endothelial growth factor receptor	VEGFR
Vesiculo-vacuolar organelles	VVO
von Hippel-Lindau protein	pVHL

APPENDIX

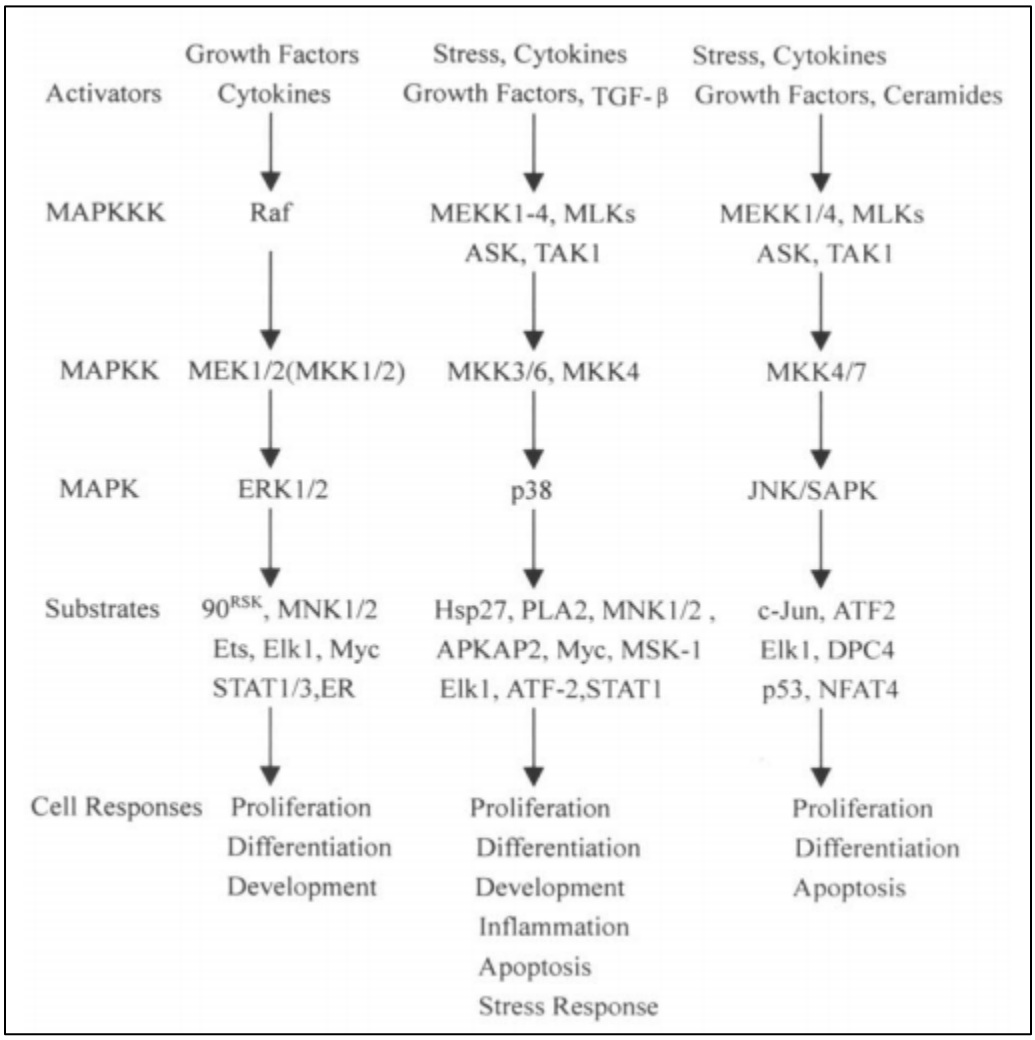

FIGURE S1| MAP kinase cascades in mammalian cells. Overview of the various branches involved in MAP kinase cell signalling. *Image source Zhang and Liu, 2002 figure 1*.

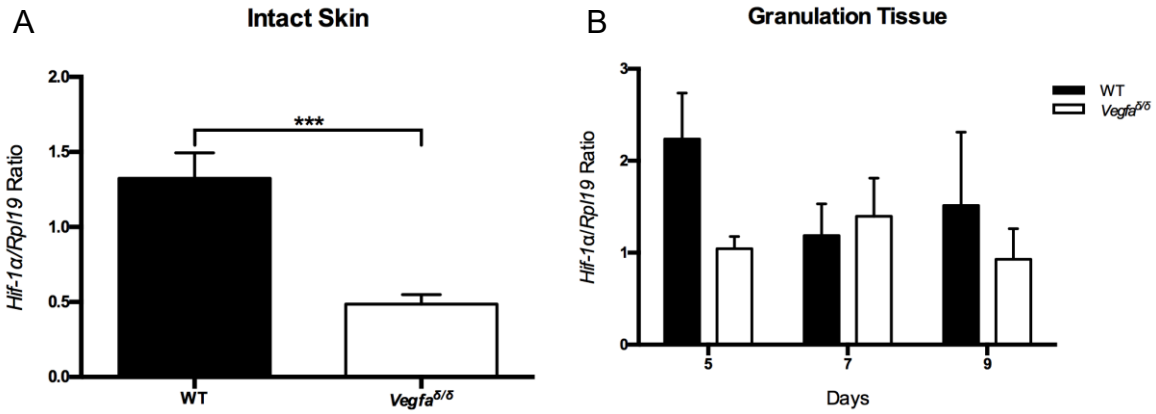

FIGURE S2|*Hif-1α* mRNA levels in intact skin and in granulation tissue in wild type and mutant (*Vegfa*$^{\delta/\delta}$) mice: Quantitative PCR analysis was used to measure mRNA levels of (A) *Hif-1α* in intact skin ***P = 0.0010, WT: 1.3 ± 0.2, *Vegfa*$^{\delta/\delta}$: 0.49 ± 0.06 and (B) *Hif-1α* in granulation tissue. No significant difference was observed when comparing granulation tissue *Hif-1α* mRNA levels between both populations of mice. Messenger RNA levels are relative to that of *Rpl19*. Values represent mean ± SEM (n = 6). A two-tailed unpaired t-test was utilized for the statistical analysis of the intact skin data set. A Sidak-corrected repeated measures 2-way ANOVA was utilized for the statistical analysis of the granulation tissue data set.

CHAPTER 1

INTRODUCTION

Physiology of Wound Healing

The complex series of events involved in the healing of a wound can be divided into 4 phases: hemostasis, inflammation, proliferation and remodelling/scar formation (Figure 1). An alteration of either phase may lead to the development of a chronic, non-healing wound [1-5].

Hemostasis

Tissue injury immediately triggers a transient vasoconstriction (lasting roughly 5-10 minutes) and initiates the activation of the extrinsic coagulation cascade [2, 6-8]. Through

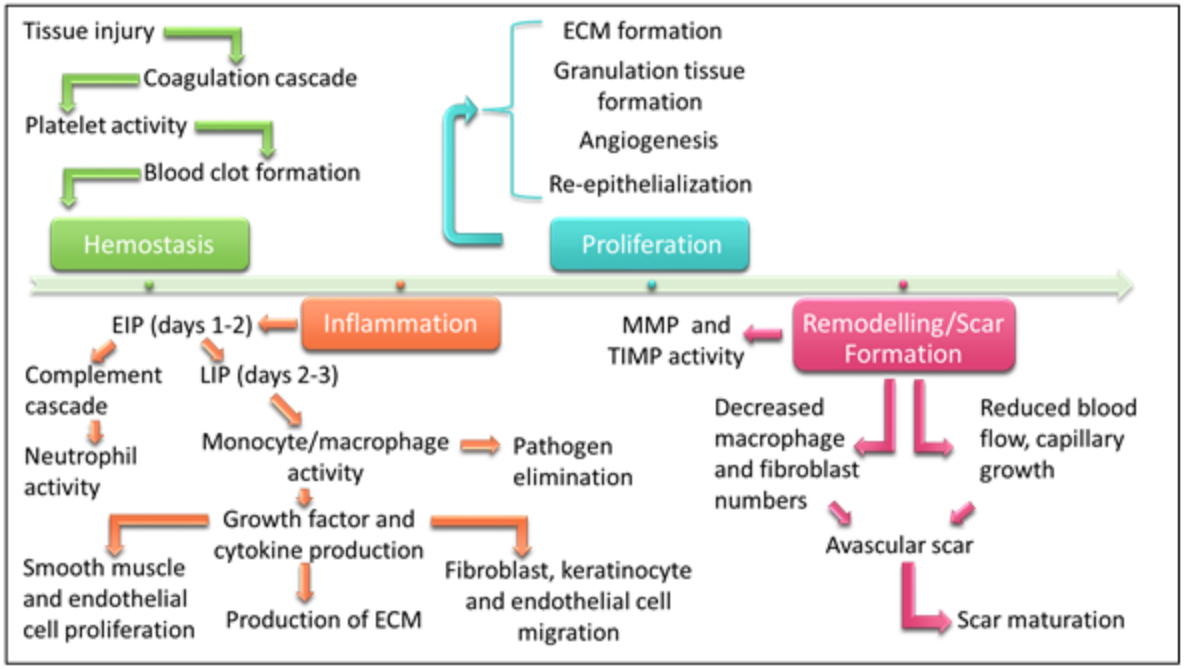

FIGURE 1| Phases of wound healing: Simplified schematic of the phases of wound healing; hemostasis, inflammation, proliferation and remodelling/scar formation. The central arrow denotes the time line along which wound progression occurs. EIP: early inflammatory phase; LIP: late inflammatory phase; ECM: extracellular matrix; MMP: matrix metalloproteinase; TIMP: tissue inhibitors of metalloproteinases

platelet degranulation various growth factors and cell signalling molecules, platelet derived growth factor (PDGF), fibroblast growth factor (FGF), β-thromboglobulin, serotonin, bradykinin, prostaglandins, prostacyclins, thromboxane, histamine, insulin-like growth factor (IGF)-1, epidermal growth factor (EGF), transforming growth factor (TGF)-β and platelet factor-IV (PF4), among others, are released into the wound environment [2, 6, 8]. Approximately 20 minutes post-wounding, through the release of vasoactive substances such as histamine, vasodilation and an increased vascular permeability are favored [8]. The response initiated by these factors favors a healthy inflammatory response by attracting and activating certain cells such as polymorphonuclear leukocytes, macrophages, fibroblasts and endothelial cells, and contributes to hemostasis via the activation of the complement and kinin cascades [2, 6, 8]. Ultimately, platelet aggregation within the fibrin blood clot at the site of injury serves the purpose of diminishing blood loss [2, 6, 7].

Inflammation

The inflammatory phase quickly follows hemostasis and is characterized by the 5 cardinal signs of inflammation; redness, warmth, swelling, pain and loss of function [7]. The inflammatory phase can be further divided into an early inflammatory phase (EIP) (days 1-2) and a late inflammatory phase (LIP) (days 2-3) [2].

Early Inflammatory Phase

The classical and alternative pathways of the complement cascade are activated upon initiation of the EIP. The activation of these pathways ultimately results in the attraction of neutrophils to the wound site via chemoattractants such as TGF-β and/or complement fragments C3a, C5a [2, 9]. Therefore, the first inflammatory cells to reach the wound are neutrophils, which do so within 24-36h after wounding [2, 9]. Neutrophils then begin to eliminate pathogens and foreign matter either by phagocytosis, enzymatic destruction (via degranulation products such as collagenases and elastases) or through the production of free

radicals [2, 6, 8, 9]. Neutrophil activity usually ceases roughly 72 hours post-wounding, once the affected region has been cleared of bacteria and debris [2, 10].

Late Inflammatory Phase

The cell type that predominates this portion of the inflammatory phase and that is essential for normal wound progression is the monocyte/macrophage [2, 8]. Similarly to neutrophils, blood monocytes are attracted to the wound by several chemoattractants (e.g. PDGF, TGF-β) but make a tardier appearance, arriving at the wound site 48-72 hours after injury [2, 9]. Once they have arrived at the wound site, blood monocytes become tissue macrophages. The primary role of macrophages is that of repair. Although they possess phagocytic and enzyme degrading capabilities to control and neutralize potential pathogens, they are the main producers of various growth factors (PDGF, TGF-β, and FGF) that will promote the proliferation of smooth muscle cells, the proliferation of endothelial cells, the initial migration of fibroblasts, keratinocytes and endothelial cells within the wound, as well as the production of the extracellular matrix (ECM) [2, 6-9].

Proliferation

Approximately 3 days after wounding and lasting until 2-4 weeks post-wounding, the proliferative phase follows the inflammatory phase [2, 7, 9]. Fibroblast migration, deposition of ECM and the formation of granulation tissue are all associated with the proliferative phase [2, 7, 9, 11]. Similarly to neutrophils and monocytes, fibroblasts are attracted to the wound by various factors [e.g. PDGF, TGF-β and basic fibroblast growth factor (bFGF)] [2, 7]. The proliferation of fibroblasts results in the production of components (fibronectin, hyaluronan, collagen and proteoglycans) essential for the formation of the new ECM [2].

The newly produced ECM allows for the regulation of differentiation, growth, expansion, and migration of cells [2, 3]. The ECM is able to modify cytokine activity, which is necessary for the regulation of its own components [12]. Overall, the ECM behaves as a

cellular scaffold, permitting the regeneration of wounded skin [3]. The main components of the ECM are collagen, adhesive glycoproteins (fibronectin, laminin, thrombospondin and integrins), and proteoglycans (glycosaminoglycans such as dermatan sulphate, heparan sulphate and hyaluronic acid) [2].

Re-epithelialization is another essential process stimulated during the proliferative phase [9, 11]. Two molecules are primarily responsible for the restoration of an epidermal lining: EGF, whose production is attributed to keratinocytes and platelets, and TGF-α, which is produced mainly by activated macrophages [9].

The proliferative phase also encompasses the production of another key component in the healing of the wound: granulation tissue. Granulation tissue is mainly composed of a network of capillaries and collagen and it serves as a transient dermal substitute until the dermis can be fully repaired [6]. The quality of the granulation tissue within a wound may serve as an indication of the wound health [2]. By 3-5 days after wounding, a healthy granulation tissue bed should appear hyperaemic, moist, shiny and granular [2]. Conversely, the granulation tissue of unhealthy wounds can be described as friable, soft, and of a beefy-red color [2, 13]. Microscopically, granulation tissue is primarily characterized by macrophages, proliferating fibroblasts and capillaries [2, 14].

As the proliferative phase ends, the ratio of hyaluronic acid to chondroitin sulfate is altered (the amount of chondroitin sulfate increases), consequently reducing the migratory/proliferative capabilities of fibroblasts. This favors fibroblast differentiation, thereby denoting the initiation of the remodelling phase [7].

Angiogenesis

Angiogenesis is an essential component of the wound healing process occurring within the proliferative phase and ensures the development of a network of capillaries [2, 6, 11]. Specific growth factors responsible for the creation of these blood vessels are vascular endothelial growth factor (VEGF), bFGF and TGF-β, which can originate from epidermal cells, fibroblasts, macrophages and endothelial cells [1, 9]. Angiogenesis is a process whose

function is directly proportional to oxygen tension levels and that consists of the formation of new blood vessels within an organism [15-18]. Angiogenesis can occur due to endothelial cell splitting, known as intussusception, or by endothelial cell sprouting [19-21]. Angiogenesis differs from vasculogenesis, the latter being responsible for the formation of the very first vascular networks within the viable embryo where mesodermal cells differentiate into angioblasts, the precursors to endothelial cells [22, 23]. Angiogenesis mediated by VEGF is an essential component of many physiological processes such as embryonic development, the ovarian cycle and bone remodelling and it also contributes to pathological conditions such as wound healing, retinopathy and tumor growth and development [17, 21, 24-28]. Many factors are involved in the process of angiogenesis; these include but are not limited to PDGF, TGF-β, tumor necrosis factor (TNF)-α, TNF-β, bFGF, FGF, TGF-α, hepatocyte growth factor (HGF), interleukin (IL)-8, and angiopoietins, as well as one of the most important factors, VEGF [15, 27, 29].

Cell-Cell Junctional Adhesion

Protein complexes present within the endothelium are responsible for stabilizing the vascular endothelial cell lining and ensuring that a proper endothelial cell-cell adhesion exists. These protein complexes are structurally altered throughout angiogenesis in order for this process to successfully reach completion [22]. The major player regulating endothelial cell-cell adhesion is a member of the cadherin family, vascular endothelial cadherin (VE-cadherin) [19, 22].

Endothelial Cell Sprouting

Before endothelial tip cell sprouting can occur, specific steps are required for vascular endothelial cells to become permissive to phenotypic changes. Matrix metalloproteinases (MMP) such as membrane type-1 matrix metalloproteinase (MT1-MMP) and disintegrin as well as VEGF-activated endothelial cell protease secretions are required for the

remodelling/degradation of the sub-endothelial basement membrane surrounding blood vessels [19, 22]. Endothelial cell motility also must be enhanced through the modulation of VE-cadherin. It has been postulated that exposure to VEGF promotes VE-cadherin endocytosis, thereby leading to an increase in endothelial cell motility. Finally, endothelial cell phenotypic specialization, tip cell or stalk cell, is required for angiogenesis to proceed (Figure 2) [22]. This phenotypic specialization is regulated via the Notch pathway [20, 22]. In the absence of VEGF, a balance exists regarding Notch cell signalling between adjacent endothelial cells. A factor named Delta-like 4 (Dll4) is produced by adjacent endothelial cells and activates the neighbouring cell's Notch signalling pathway. In the presence of a VEGF gradient, VEGF will stimulate one of the neighbouring endothelial cells to a greater extent resulting in a higher degree of Dll4 production and therefore will induce a more robust Notch pathway activation. Increased Notch signalling results in decreased expression of vascular

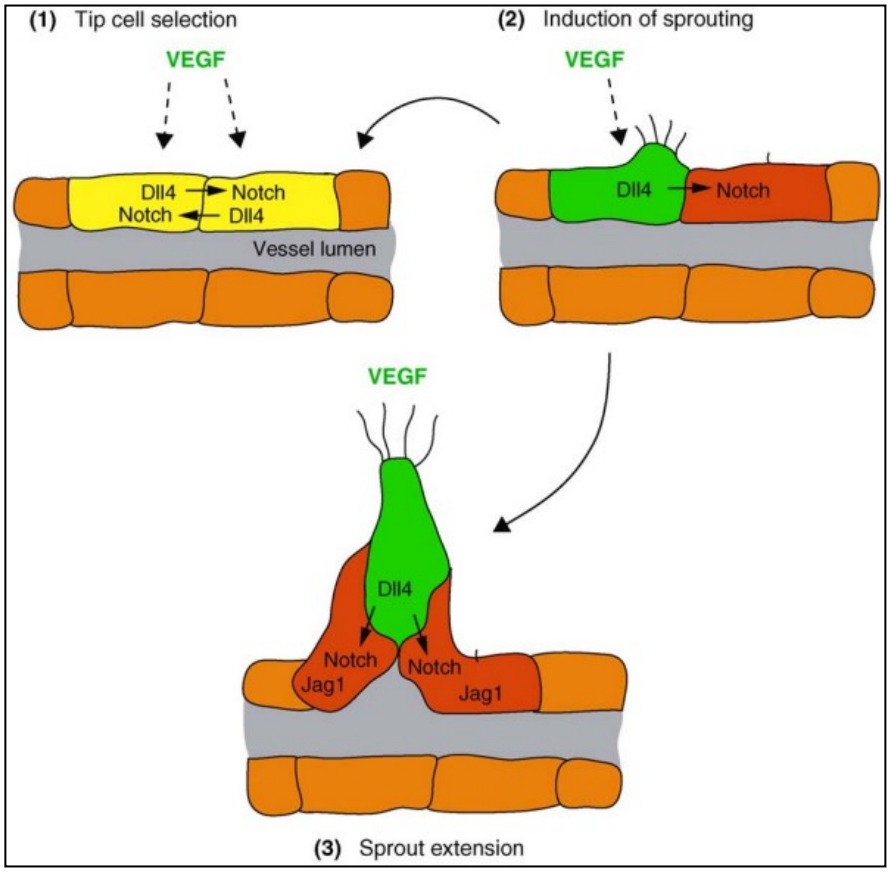

FIGURE 2| Endothelial cell sprouting: Figure demonstrating the influence of VEGF in the specialization of either a tip or stalk endothelial cell phenotype. This decision is mediated through the regulation of Notch signalling between neighbouring endothelial cells. *Image source Eilken and Adams, 2010, figure 2*

endothelial factor receptor (VEGFR)-2 and VEGFR3, increased expression of VEGFR1 and will result in the undertaking of a stalk cell phenotype, whereas the adjacent Dll4-expressing cell will undertake a tip cell phenotype [20, 22]. Stalk cells also express a factor named Jagged-1 (Jag1), which inhibits the characteristics of a motile, sprouting tip cell phenotype [22].

Tip Cell Guidance

Once endothelial cells have undertaken a tip cell phenotype, the sprouting cells' motile filopodia respond to environmental cues which guide the path of new vessel growth [22]. An essential component required for vessel support during its growth is the sheathing of blood vessels by ECM components, primarily mature collagen [18]. The presence of an ECM lacking mature collagen results in newly branched blood vessels that are delicate and poorly assembled [18]. Tip cell guidance is another process in which the importance of heparin-binding domains present in select VEGF isoforms can be observed. VEGF binds to heparin sulfate proteoglycans present in the ECM and through this binding establishes a gradient that aids in attracting tip cell direction [22].

Tip Cell Anastomosis and Tubulogenesis

VE-cadherin is also present on the filopodial tips of sprouting endothelial cells. In this scenario, this cell-cell junctional adhesion complex may aid in the anastomosis of two sprouting endothelial cells. The interaction of both VE-cadherin complexes on their respective tip cells may initiate junction assembly, which will reduce tip cell motility. A phenotypic cell conversion occurs, tip cell to stalk cell, as the process of tubulogenesis commences. The tip cells are introduced into the endothelial cell lining where they become stalk cells and the lumen of the future complete blood vessel is formed [22]. Finally a new round of cell sprouting occurs as other endothelial cells are exposed to and modulated by pro-angiogenic factors [22].

VEGF

Among the growth factors required for the development of a blood vessel network, VEGF was the first to be identified, due to its ability to increase vascular permeability [15, 25, 30]. VEGF is essential for the viability of the developing embryo as well as being an important factor for the proper maintenance and health of the adult vasculature [31, 32]. Embryonic mice genetically engineered to lack one functional *Vegf* allele, $Vegf^{+/-}$, die early in embryonic life, at E9.5-E10.5, mainly due to severe vascular abnormalities that are predominantly cardiovascular related [19, 21, 23, 25, 29, 31, 33-35]. On the other hand, overexpression of *Vegf* (as little as twice the normal amount) leads to death of the organism [25, 31]. Many cell types such as macrophages, glial cells, keratinocytes, endothelial cells, dermal fibroblasts and tumor cells, are capable of secreting VEGF through various pathways [26, 30] (Table I lists factors capable of influencing *Vegf* gene expression). However, keratinocytes remain the major source of VEGF production within cutaneous wounds [30].

VEGF Family

The VEGF family is composed of several members [VEGFA, VEGFB, VEGFC, VEGFD, VEGFE, placental growth factor (PlGF) and VEGFFs (snake venom-derived VEGF related proteins)], but the regulation of endothelial cells seems to be predominantly controlled via VEGFA [19, 21, 31, 35-37]. The action and function of VEGFC and VEGFD predominantly lies in the formation of new lymphatic vessels, (lymphangiogenesis), however, they also behave as influential lymphatic endothelial cell regulatory proteins [23].

TABLE I| Factors capable of influencing *Vegf* gene expression: Table depicting various factors affecting *Vegf* gene expression, whether they are transcription factors, agents influencing transcription factors, or genes. *Image source Jośko and Mazurek, 2004, table 1*

I	Transcription factors	HIF-1α/Arnt, HIF-2α/HLF/HRF, Ap-1, Ap-2, HAF, Sp-1, Sp-3, NFχB, STAT proteins, Smad, p300/CBP, estrogens, progestins
II	Agents influencing transcription factors	IL-1β, Il-6, PDGF, TGF-α/β, TNF-α, EGF, heavy metals (Cd, Co, Ni, Pb), cAMP, Ca^{2+}, PGE_2, PGI, NO, insulin IGF_1, IGF_2, HGF, retinoic acid, Ox-LDLs, angiotensin II, trombine, LIF, steroid hormones, endothelin-1, UV-Ai UV-B radiation, dexamethasone, kardiothropin, organomercurial compound (mersalyl)
III	Genes	ERBB 2, von Hippel-Lindau, oncoprotein E1A, GPCR receptor, Cbfa1/Runx2, VHL, V-SRC, bcl-2

VEGFA

The gene for VEGFA consists of 8 exons and 7 introns which, when subjected to RNA splicing, gives rise to various isoforms [15, 38] (Figure 3). The currently known VEGFA isoforms include $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{148}$, $VEGF_{145}$, $VEGF_{121}$, $VEGF_{189}b$, and $VEGF_{165}b$, where $VEGF_{165}$ was found to be the predominantly active isoform in terms of VEGF activity [15, 31, 36]. Consequently, in the following text describing functional activities related to this growth factor, the use of the term VEGFA will refer to the most biologically active isoform, $VEGF_{165}$. The majority of VEGFA isoforms can exist in either a soluble or ECM bound form, where each form plays a role in dictating the final functional and structural outcome of the vasculature. Soluble VEGFA forms promote vascular hyperplasia, decreased vascular density and poor vascular branching, whereas ECM bound forms favor highly branched vessels and a vasculature characterized by thin walls [31].

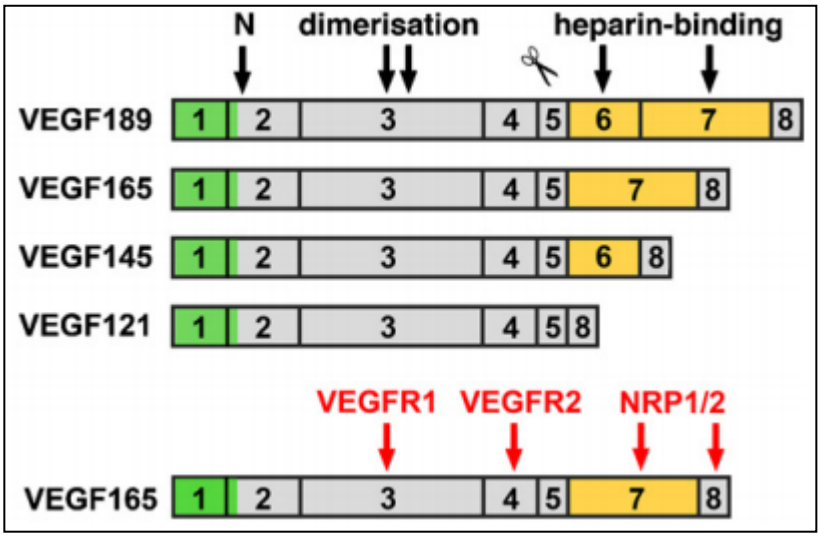

FIGURE 3| VEGFA isoform structures: Major VEGFA isoforms found within humans (mice isoforms possess one less amino acid). Exon 1 encodes for the signal sequence [15]. Exon 3 confers the ability to dimerize as well as interact with VEGFR1 [15, 38]. VEGFR2-binding is enabled by the presence of exon 4 within the fully formed protein structure [15, 38]. Exon 5 ensures that VEGFA isoforms are vulnerable to plasmin cleavage [15, 38]. Exon 6 and 7 are heparin-binding domains. The presence/absence of these two domains explains why some VEGFA isoforms, such as $VEGF_{189}$, are mainly sequestered in the ECM via binding to various heparan-containing ECM components, as opposed to $VEGF_{121}$ which is a fully diffusible VEGFA isoform [38]. Furthermore, a portion of exon 7 and all of exon 8 are responsible for the ability to bind to coreceptors NRP1/NRP2. This interaction is required for enhanced VEGFR2 cell signalling via the binding of VEGFA to its receptor [38]. *Image source Raimondi and Ruhrberg, 2013, figure 1.*

$VEGF_{165}$

$VEGF_{165}$ possesses intermediary, exon inclusions allows this isoform to be bound to the ECM as well as being secreted, and therefore optimal structural characteristics compared to other VEGFA isoforms, thereby making this isoform the predominant mediator in carrying out the various functions of VEGFA [27]. To further enforce that $VEGF_{165}$ is the predominant functionally active VEGFA isoform, mice solely expressing $VEGF_{164}$ (mouse VEGFA isoforms possess one less amino acid) do not possess any morphological vascular differences when compared to wild type mice [21, 27, 31]. $VEGF_{165}$ possesses a heparin-binding domain and therefore is mitogenically active. A portion of $VEGF_{165}$ is secreted, however a large portion of $VEGF_{165}$ remains bound to the cell surface and ECM [27, 39]. Similarly to $VEGF_{189}$, $VEGF_{165}$ bound to the ECM can be released as a secreted form via the action of plasmin [27].

VEGF Receptors

There exists 4 different VEGF receptors; VEGFR1, VEGFR2, VEGFR3 and Neuropilin-1/Neuropilin-2 (NRP1/NRP2) [27]. VEGFR3 is not a suitable receptor for VEGFA, but rather behaves as a receptor for VEGFC and VEGFD and therefore is the only receptor that will not be outlined [21, 27, 33].

VEGFR1

VEGFR1, also known as fms-like tyrosine kinase-1 (Flt-1), is a receptor tyrosine kinase (RTK) [27, 33, 40]. Ligands capable of interacting with this receptor include VEGFA, PlGF and VEGFB [27]. *Flt-1* expression is influenced by oxygen tension levels because it is a hypoxia-inducible gene [33, 34, 41, 42]. Alternative splicing of *Flt-1* can result in the expression of a soluble form of the receptor that acts as a negative regulator of VEGFA activity through its ability to bind and sequester the ligand VEGFA [19-22, 27, 31]. Therefore VEGFR1 has been deemed a “decoy receptor” [19, 27, 31, 43]. Although this receptor primarily functions as a

"decoy receptor" it still plays an important role in monocyte chemotaxis, inflammation, increasing vascular permeability, cell migration, hematopoietic stem cell (HSC) repopulation and the induction of matrix metalloproteinase-9 (MMP-9), urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) [21, 23, 25, 27, 31, 33, 37]. Plasminogen activators (uPA and tPA) are responsible for the transformation of plasminogen to plasmin, a predominant fibrinolytic agent, necessary for fibrin dissolution and consequently permitting wound re-epithelialization [44]. MMP-9 on the other hand results in the excision of collagenous anchors tethering keratinocytes to the basal lamina [44].

VEGFR2

VEGFR2, also known as kinase domain region (KDR) or fetal liver kinase-1 (Flk-1), is structurally identical to VEGFR1, however the affinity of VEGFR2 for its ligand is 10 times lower than is that of VEGFR1 [21, 27]. Known ligands for this receptor include VEGFA, VEGFC and VEGFD. VEGR2 signalling is the main route through which VEGFA can carry out its principal functions [22, 25, 27, 31, 36, 37, 43]. *Flk-1*$^{-/-}$ embryonic mice suffer the same fate as *Flt-1*$^{-/-}$ mutants, which is embryonic death at E8.5-9.5 however vascularization does not occur in the former [21, 25-27, 33, 34]. Oxygen tension levels alter the expression of *Flk-1*, which is down-regulated under hypoxic conditions [23]. However, receptor phosphorylation is increased under low oxygen tension levels [31]. Upon ligand binding, six tyrosine residues, 951, 996, 1054, 1059, 1175 (of particular importance), and 1214, located within VEGFR2, are phosphorylated thereby permitting the subsequent activation of various signalling pathways, such as extracellular-regulated kinase (ERK) 1/2 [19, 31]. Activation of this receptor has been associated with many functions such as endothelial cell migration, maturation, proliferation, survival, differentiation and vasculogenesis [19, 33].

NRP

NRP1 is a non-tyrosine kinase receptor that influences neuronal activity and whose normal ligand belongs to the collapsin-semaphorin family [21, 27, 31, 35, 36]. NRP1 influences the effects of angiogenesis, explaining the specific interactions between NRP1 and select VEGF family members (VEGFA, VEGFB, VEGFE and PLGF), whereas NRP2 regulates lymphangiogenesis [21, 22, 36, 37]. Neuropilin receptors can act as powerful VEGFA signalling enhancers when co-expressed on cells with VEGFR2. All VEGFA isoforms are capable of interacting with NRP1 via the inclusion of exon 8 in their protein structure [21, 31]. Exon 7, a heparin-binding domain within the VEGFA protein structure, along with the heparin-binding domain found within the NRPs, leads to the formation of a surface whereby VEGFA/NRP can interact with VEGFRs via the mediation of heparan [21]. This interaction is capable of promoting an altered presentation of the VEGFA ligand to VEGFR2 in a manner that enhances the signal pathway induced by receptor stimulation. Like *Flt-1* and *Flk-1*, *Nrp-1*$^{-/-}$ embryonic mice are not viable and are characterized by vascular abnormalities, demonstrating the necessity of the enhanced VEGFA signal transduction induced by this coreceptor [27, 33, 36].

VEGFA Function

VEGFA possesses two main functions. The first is to increase the nutrient supply to tissues and the second is playing a role in waste management by increasing the removal rate of waste products [15, 45]. VEGFA accomplishes these fundamental functions via three processes: vasodilation, angiogenesis and increased vascular permeability [15, 21, 45].

Vasodilation directly supports both of VEGFA's main functions. VEGFA is a potent vasodilator capable of dilating blood vessels very rapidly and to a large degree [15, 27]. The increase in blood vessel diameter will result in increased blood flow, thereby allowing more oxygen and glucose to reach the target tissue as well as increasing the removal rate of waste products such as carbon dioxide (CO_2), H^+ and lactate [15]. The mechanism underlying the VEGFA-induced vasodilation is based upon the action of nitric oxide (NO) [15, 27, 33]. VEGFA

binds to its receptor, VEGFR2, which will then activate the phospholipase C (PLC) pathway. The end result of the activation of the PLC pathway is an increase in intracellular Ca^{2+} that interacts with calmodulin to stimulate nitric oxide synthase (NOS) and therefore NO production. NO is then capable of freely diffusing through the endothelial cells and acting on the smooth muscle cells surrounding the blood vessels and relaxing them, leading to vasodilation [15]. NO is also part of a positive feedback loop because NO is capable of stimulating VEGFA expression [33].

VEGFA is one of the most potent angiogenic factors since it is capable of driving all the important processes required for angiogenesis, including endothelial cell proliferation and migration, basement membrane degradation, tube and lumen formation, increased vascular permeability, and new vessel formation [15, 22, 26, 46-48]. This growth factor is able to stimulate the growth of new vessels, whether they are arteries, veins or lymphatics [27]. In the context of wound healing, angiogenesis allows essential nutrients and oxygen to be delivered to mitotically active cells, thereby increasing healing rates [30]. VEGFA is also capable of upregulating other factors, such as PDGFB and stromal cell-derived factor-1 (SDF-1), which play an important role in angiogenesis [49-51]

VEGFA is capable of increasing the hydraulic conductivity of fluid as well as increasing the diffusive permeability of certain macromolecules by altering the number or length of pores present in the vasculature [15, 27]. The increase in vascular permeability allows for the extravasation of certain proteins, fibrinogen and plasminogen, within the area surrounding the wound. The presence and deposition of these factors within the ECM directly aids subsequent tissue healing by creating a suitable ECM substrate for tissue growth as well as by creating an environment that is pro-angiogenic [15]. The mechanism of VEGFA-induced increased vascular permeability is similar to that of vasodilation. VEGFA will bind to its receptor, VEGFR2, thereby leading to the activation of PLC and, ultimately, to an increase in intracellular Ca^{2+}. However, the increase in Ca^{2+} does not stimulate downstream factors but, rather, it directly affects endothelial cells [15, 27]. Endothelial cell exposure to increased Ca^{2+} levels will result in contraction of the cells, clustering of vesicles inside the cells, and the

formation of vesiculo-vacuolar organelles (VVO) leading to an increased permeability to fluid and macromolecules [15, 25].

VEGFA Expression

Oxygen tension levels play a very important role in the ability of the HIF-1 complex to mediate VEGFA gene expression [27]. However, hypoxia-regulated VEGFA expression is not the only pathway capable of stimulating this gene, which is expressed within nearly all tissues in the adult [25, 52] (Table I). Hypoglycemia has been found to stimulate VEGFA expression [33, 53, 54]. Furthermore, acidosis is also documented to lead to increased VEGFA levels [55]. Similarly to HIF-1α, many growth factors are able to stimulate VEGFA gene expression; these include EGF, TGF-α, TGF-β, TNF-α, keratinocyte growth factor (KGF), IGF-1, FGF, bFGF and PDGF [18, 25, 27, 33, 34, 56, 57]. Cytokines, IL-1α, IL-6 and IL-8, also play a role in the up-regulation of this gene [25, 27, 33, 56]. Chemical induction of VEGFA expression is not the only manner in which VEGFA expression is stimulated. Physical forces such as a simple stretch can increase VEGFA gene expression [25].

Reactive oxygen species (ROS) are also mediators able to increase VEGFA expression [15, 57, 58]; fibroblasts, endothelial cells and macrophages are all stimulated to express VEGFA due to ROS exposure [58]. Furthermore, hydrogen peroxide (H_2O_2) is capable of directly stimulating the VEGFA promoter resulting in increased expression and secretion levels of this growth factor by keratinocytes and macrophages [18, 59]. This HIF-1 independent H_20_2-induced VEGFA expression is mediated via the interaction of Sp1 with the VEGFA promoter [57, 59].

Located within the *Vegfa* promoter are multiple binding sites for different transcription factors such as HIF-1, activator protein (AP)-1, AP-2, Sp1, nuclear factor (NF)-1, nuclear factor kappa B (NF-Kb) and Sp1-related factors, signifying a control of VEGFA expression other than hypoxia via the activation of different signalling cascades [16, 34, 46, 60]. The MAP kinase signalling pathway documented to induce *Vegfa* expression is that of ERK1/ERK2 pathway [34, 61]. The ERK1/ERK2 pathway has been shown to increase *Vegfa*

gene expression via the binding of transcription factors AP-2 and Sp1 to their appropriate recognition DNA sequences within the *Vegfa* promoter [29, 56]. Moreover, the active combinatory effect of Sp1 alongside AP-2 has been documented to increase *Vegfa* expression [46, 52]. Macrophage colony stimulating factor (M-CSF) secreted by various cell types including endothelial cells, fibroblasts, tumor cells, and monocytes, has been found to upregulate *Vegfa* expression via the activation of the ERK pathways [46, 62].

Remodelling and Scar Formation

The process of remodelling has already begun as the first signs of granulation tissue formation appear within the wound bed. Therefore the fourth and final wound healing phase begins around 1 week post-wounding and can last ≥1-2 years [2, 9]. The ECM is continuously being remodelled, due to constant remodelling of collagen. This dynamic state is present until an equilibrium is reached at roughly 21 days post-wounding [2, 7]. MMPs are responsible for proteolysis and for controlling cell migration through the ECM [2, 63]. Initially MMPs are beneficial to the wound environment as they promote angiogenesis, cleanse the wound via debridement, and aid in epithelialization [5]. MMPs vary considerably as several different types exist, each with their own target. Interstitial collagenases alter collagen I, II, and III, gelatinases degrade amorphous collagen and fibronectin and stromelysins alter components of the ECM such as laminin [2]. Uncontrolled MMP activity impairs wound healing since MMPs degrade collagen, essential for proper healing [2, 63]. Therefore MMPs are usually found in the form of zymogens requiring an activating catalyst (proteases such as plasmin) found only in regions of injured tissue [2].

As the wound progresses in its remodelling, certain changes can be observed. There is an increased activity of MMP inhibitors [tissue inhibitors of metalloproteinases (TIMPs)] and reduced MMP activity, mainly due to the activity of TGF-β [2, 9]. A reduction in the number of macrophages and fibroblasts can be observed as can a reduction in cellular metabolic activity [2, 9]. Blood perfusion to the wound site is reduced as well [2, 9]. Therefore, the granulation tissue is slowly replaced by avascular scar tissue and, as the scar matures,

collagen fiber diameter increases and type III collagen is replaced by type I until a ratio (collagen I : collagen III) of 4 : 1 is achieved [2, 6, 7, 9]. In addition to the superior organizational state of the collagen fibers, cross-linking of these fibers also occurs, thereby increasing the tensile strength of the scar tissue as the wound heals [6, 9]. The wound tensile strength within the first week of healing is minimal. In humans, within 4-6 weeks post-wounding the wound tensile strength increases to 30-50% of the strength of intact skin and 60% wound tensile strength is achieved by 6 months [4]. The maximum tensile strength reached in a healed wound approximately 1 year after wounding is 80% of the strength of healthy intact skin [2, 4, 7].

Oxygen and Wound Healing

Oxygen and Cell Function

Energy Production

Essential intracellular processes such as biosynthesis, movement, transport and proliferation depend on an energy source in the form of adenosine triphosphate (ATP) [18, 59, 64]. ATP is synthesized via oxidative phosphorylation, requiring oxygen for its completion, in amounts that are necessary to satisfy each cell's purpose [18, 41, 58, 59].

Defence against Pathogens and ROS Production

Respiratory burst refers to the production of ROS such as peroxide anion ($H0_2^-$), hydroxyl ion (OH^-) and superoxide anion (O^{2-}), which are produced by various leukocytes and serve to destroy invading pathogen and control against infection [18, 41, 58, 59]. This process is highly dependent on the presence of oxygen in the environment [41, 57-59]. The active enzyme responsible for this process, NADPH-linked oxygenase, requires a high concentration of oxygen in order to be fully active since the production rate of the toxic radicals is directly

proportional to the degree of enzyme activity (50% activity at 45-80 mmHg and maximal activity may require oxygen levels >300 mmHg) [18, 41, 59].

PDGF, EGF, TNF-α, and IL-1β are capable of increasing the synthesis of ROS through fibroblast and leukocyte stimulation via Rac1 [57, 59]. ROS production alters the behaviour and function of cells/processes via its influence on increasing cytokine release, stimulating angiogenesis and increasing cell motility [18, 41, 58].

Oxygen Levels in Healing Wounds

Within the first few days after wounding, oxygen levels are drastically reduced giving rise to a hypoxic environment [59]. A significant decrease in oxygen levels is present approximately 48 hours following injury and maximal hypoxia (≤ 10 mmHg) is attained close to 4 days post-wounding [65]. Oxygen tension levels vary within the wound itself with levels as low as 10 mmHg recorded at the center of the wound and levels of 60 mmHg at the wound periphery [18, 41]. Hypoxia is primarily the consequence of the traumatic destruction of the vasculature as well as the augmentation of oxygen consumption rates due to increased cellular density and metabolic activity [18, 41, 53, 58, 59, 66]. Acute hypoxia favors wound healing because the low oxygen tension environment promotes fibroblast proliferation, keratinocyte motility, procollagen synthesis, angiogenesis, ROS production and an increase in the expression of various growth factors such as TGF-β1, PDGF and VEGFA [41, 58, 59, 64, 67, 68]. Furthermore, low oxygen tension levels have been documented as the predominant inducer of VEGFA expression [23]. However, hypoxic conditions that persist (chronic hypoxia) negatively impact wound healing since minimum oxygen tension levels of 30-40 mmHg are required for efficient wound healing mechanisms [41, 58, 66]. Therefore angiogenesis, re-epithelialization, fibroblast collagen deposition and the ability of leukocytes to control the presence of pathogens, are all impaired under hypoxic conditions [18, 41, 58, 66]. Moreover, fibroblast proliferation rates decrease, procollagen synthesis decreases, and TGF-β1 expression is downregulated [41, 58, 59]. Hence, the continued presence of hypoxia has been linked to delayed wound healing [68]. In a normally healing wound, oxygen tension levels

return to normal and stabilize over time as a result of angiogenesis due to the influence of VEGFA [41, 59]. Hypoxic conditions have been shown to favor the stability of *Vegfa* mRNA due to the prolongation of the gene transcript's half-life, normally approximately 30 minutes, which is extended by 3-8-fold [32, 53, 69, 70]. Similarly to HIF-1α, VEGFA possesses an internal ribosome entry site (IRES) that enables this growth factor to be adequately expressed at the protein level in spite of hypoxic/stressful conditions [26, 33, 42, 53, 60]. Therefore under low oxygen tension levels, VEGFA levels ultimately increase due to increased mRNA stability, increased transcription rates, and increased translation rates [16, 32, 33, 42, 56].

Hypoxia-Inducible Factor-1

Structure

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor that induces the expression of a plethora of genes important for cell survival, erythropoiesis, metabolic regulation, cell proliferation and vascular biology [41, 59, 71, 72] (Table II). HIF-1 was first discovered in the nuclear extracts of liver cells, Hep3B cells, exposed to hypoxic conditions. It was discovered that the HIF-1 complex interacted with a specific DNA sequence, 5'-TA**CGTG**CT-3', located within the enhancer region of the erythropoietin (EPO) gene [73]. Furthermore, the consensus sequence, 5'-R**CGTG**-3', was not only found to be present at the level of the EPO enhancer region but was also located within the DNA of various genes such as VEGFA, angiopoietin (ANGPT)-1, ANGPT2, PDGFB, glucose transporter-1 (GLUT-1) and SDF-1 [18, 71, 74, 75]. The protein subunits (α and β) constituting the HIF-1 complex are members of the basic-helix-loop-helix-Per-ARNT-Sim (bHLH-PAS) protein family that utilize the bHLH and PAS motifs to dimerize and consequently become functionally active [17, 18, 72, 73, 76]. The bHLH is not only necessary for proper heterodimerization between HIF-1 subunits but also possesses a "basic" region responsible for HRE binding, subsequently resulting in the promotion of target gene expression [71, 72, 76, 77]. HIF-α subunits contain three possible

TABLE II| Target genes of the HIF-1 complex: Genes are categorized according to their cell function contribution upon activation. The gene of particular importance for all the necessary steps of angiogenesis is VEGFA. *Modified image source Ke and Costa, 2006, table 2.*

HIF-1 target genes	
Function	Gene (abbreviation)
Erythropoiesis/ iron metabolism	Erythropoietin (EPO)
	Transferrin (Tf)
	Transferrin receptor (Tfr)
	Ceruloplasmin
Angiogenesis	Vascular endothelial growth factor (VEGF)
	Endocrine-gland-derived VEGF (EG-VEGF)
	Leptin (LEP)
	Transforming growth factor-β3 (TGF-β3)
Vascular tone	Nitric oxide synthase (NOS2)
	Heme oxygenase 1
	Endothelin 1 (ET1)
	Adrenomedulin (ADM)
	α_{1B}-Adrenergic receptor
Matrix metabolism	Matrix metalloproteinases (MMPs)
	Plasminogen activator receptors and inhibitors (PAIs)
	Collagen prolyl hydroxylase
Glucose metabolism	Adenylate kinase-3
	Aldolase-A,C (ALDA,C)
	Carbonic anhydrase-9
	Enolase-1 (ENO1)
	Glucose transporter-1,3 (GLU1,3)
	Glyceraldehyde phosphate dehydrogenase (GAPDH)
	Hexokinase 1,2 (HK1,2)
	Lactate dehydrogenase-A (LDHA)
	Pyruvate kinase M (PKM)
	Phosphofructokinase L (PFKL)
	Phosphoglycerate kinase 1 (PGK1)
	6-phosphofructo-2-kinase/gructose-2,6-bisphosphate-3 (PFKFB3)
Cell proliferation/survival	Insulin-like growth factor-2 (IGF2)
	Transforming growth factor-α (TGF- α)
	Adrenomedullin (ADM)
Apoptosis	Bcl-2/adenovirus EIB 19kD-interacting protein 3 (BNip3)
	Nip3-like protein X (NIX)

isoforms, whereas only one possible configuration for the HIF-β subunit exists [72]. Furthermore, knockdown experiments have demonstrated that the HIF-1α isoform is of predominant importance for target gene expression when exposed to hypoxic conditions [77]. Figure 4 depicts the essential domains required for proper protein function as well as the residues that are post-translationally modified in order to modulate the activity of this transcription factor.

HIF-1α

The importance of HIF-1α is unequivocal since embryonic lethality arising at E10.5 accompanied by the development of a faulty vasculature characterizes *Hif-1α*$^{-/-}$ mice [17, 71, 77, 78]. HIF-1α possesses two transactivation domains located at each protein extremity,

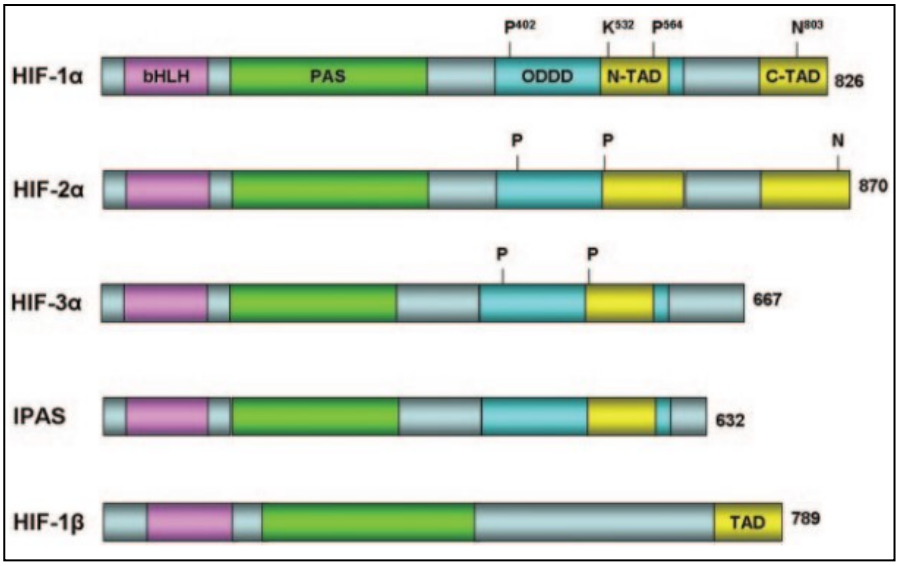

FIGURE 4| HIF-α isoforms and protein domains: Graphical representation of the protein structures of the various components of the HIF-α subunits (HIF-1α/HIF-2α/HIF-3α) and HIF-1β subunit. The important functional domains are color coded between different subunits and the HIF-1α residues of importance are depicted in the image. These important proline residues that affect HIF-1α stability are conserved within the other HIF-α subunits as depicted in the image. The structure of the alternatively spliced HIF-3α subunit, IPAS, is also shown. *Image source Ke and Costa, 2006, figure 1.*

named N-terminal transactivation domain (N-TAD) and C-terminal transactivation domain (C-TAD) [72, 77]. The transactivation domains permit the recruitment of essential transcription enhancing co-activators such as p300/CBP and Ref-1 [72, 77]. Oxygen levels do not seem to alter the transcription rate of this gene [72, 77]. HIF-1α expression levels are influenced by the activity of various transcription factors such as AP-1, NF-1, NF-κB and constitutive expression is predominantly maintained via Sp1 [77]. HIF-1α is ubiquitously expressed in all murine tissues and its expression is upregulated in the presence of various growth factors such as IGF-1, IGF-2, EGF and bFGF [71, 72, 77, 79]. Furthermore, due to the presence of an IRES located within the 5' untranslated region of *Hif-1α*, translation rates are not affected by variable oxygen tension levels, as compared to a generalized reduction in protein synthesis under hypoxic conditions [18, 32, 53, 72, 77]. Moreover, oxygen tension levels alter the stability of HIF-1α/HIF-2α/HIF-3α, via their oxygen-dependent degradation domain (ODDD) [18, 72, 77]. As a result, under normoxic conditions at the cellular level, minimal HIF-1α protein levels are observed [34].

HIF-1β

HIF-1β, also known as the aryl hydrocarbon nuclear translocator (ARNT), is constitutively expressed and is therefore present at stable levels under hypoxic/normoxic conditions [18, 72, 76, 79]. HIF-1β also possesses a transactivation domain however this domain has not been shown to have a significant effect on the positive modulation of HIF-1 gene target induction [77]. Similarly to null *Hif-1α* mutants, embryonic *Hif-1β*$^{-/-}$ mice are non-viable and develop an abnormal vasculature [71].

HIF-1 Complex Stability

The stability of HIF-1α is strongly influenced by ambient oxygen tension levels. Under normoxic conditions enzymes capable of hydroxylating specific HIF-1α amino acid residues results in the ability of a polyubiquitinylating complex, VCB-Cul2-E3 ligase complex, to bind and mark the protein for proteasomal degradation [72]. However, once oxygen is removed from the environment (hypoxia), HIF-1α is no longer marked for proteasomal degradation due to the absence of hydroxylated HIF-1α amino acid residues [72]. This therefore allows HIF-1α to migrate to the nucleus where dimerization with HIF-1β occurs and target gene expression is promoted via the recruitment of co-activators [72]. A detailed explanation of all enzymes and factors influencing HIF-1α stability is found in the figure captions of figures 5 and 6.

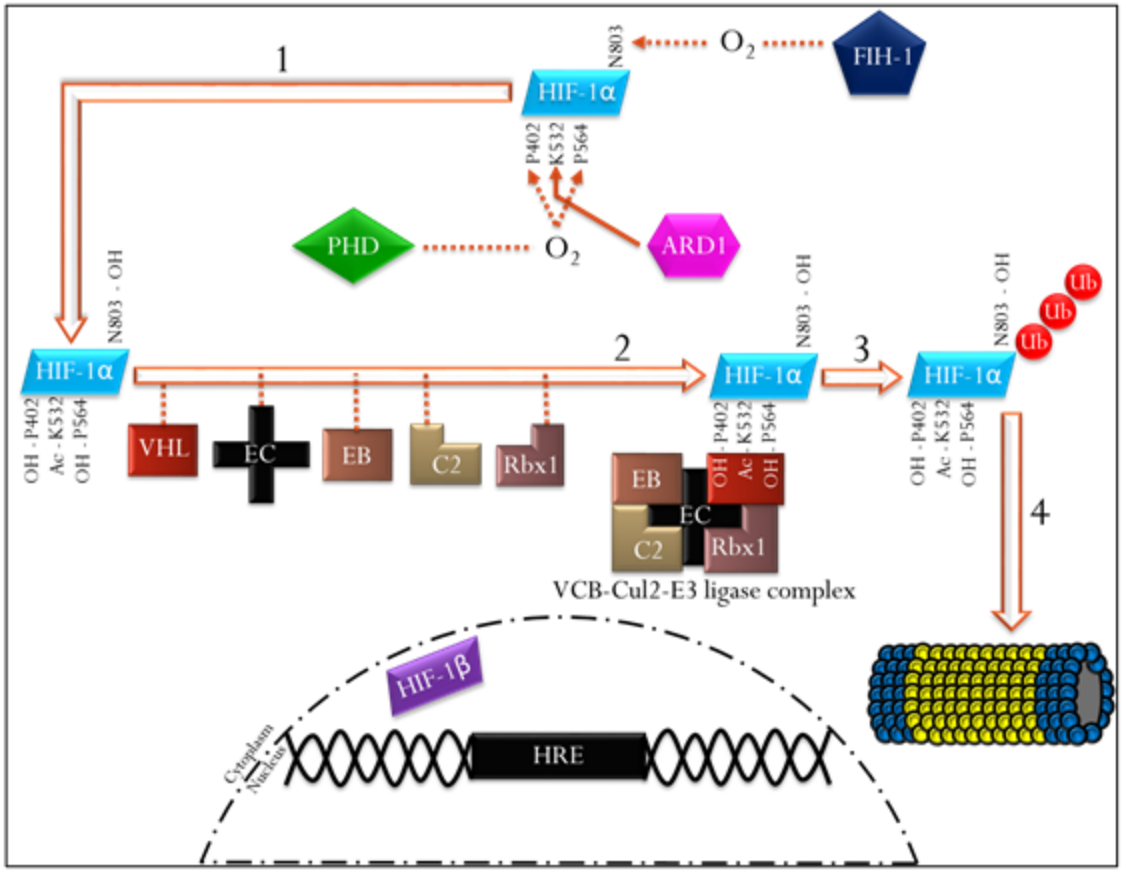

FIGURE 5| HIF-1α stability under normoxic conditions: In the presence of adequate oxygen tension levels (normoxia ≈21% O2), oxygen can be utilized by various enzymes (PHD and FIH-1) to hydroxylate HIF-1α residues, including P402, P564 (via PHD) and N803 (via FIH-1) [72, 80]. Hydroxylation of the proline residues alongside acetylation of HIF-1α residue K532 via ARD1 favors pVHL binding to HIF-1α [72, 76]. The VCB-Cul-E3 ligase complex, once fully formed, polyubiquitinylates the hydroxyproline residues and hydroxylated lysine residues found in HIF-1α and HIF-1α is consequently degraded by the 26s proteasome [72]. This effective degradation process ensures that the half-life of HIF-1α is very short and, as a result, this protein is found within the cell for <5 minutes [17, 77, 80]. Therefore HIF-1α will not be capable of translocating to the nucleus and dimerizing with HIF-1β in order to form the fully functional HIF-1 complex [18, 72, 80]. Furthermore, hydroxylation of residue N803 in HIF-1α via FIH-1 ensures that target gene expression does not ensue by eliminating p300/CBP coactivator recruitment that is necessary for transcription activation [72, 80].

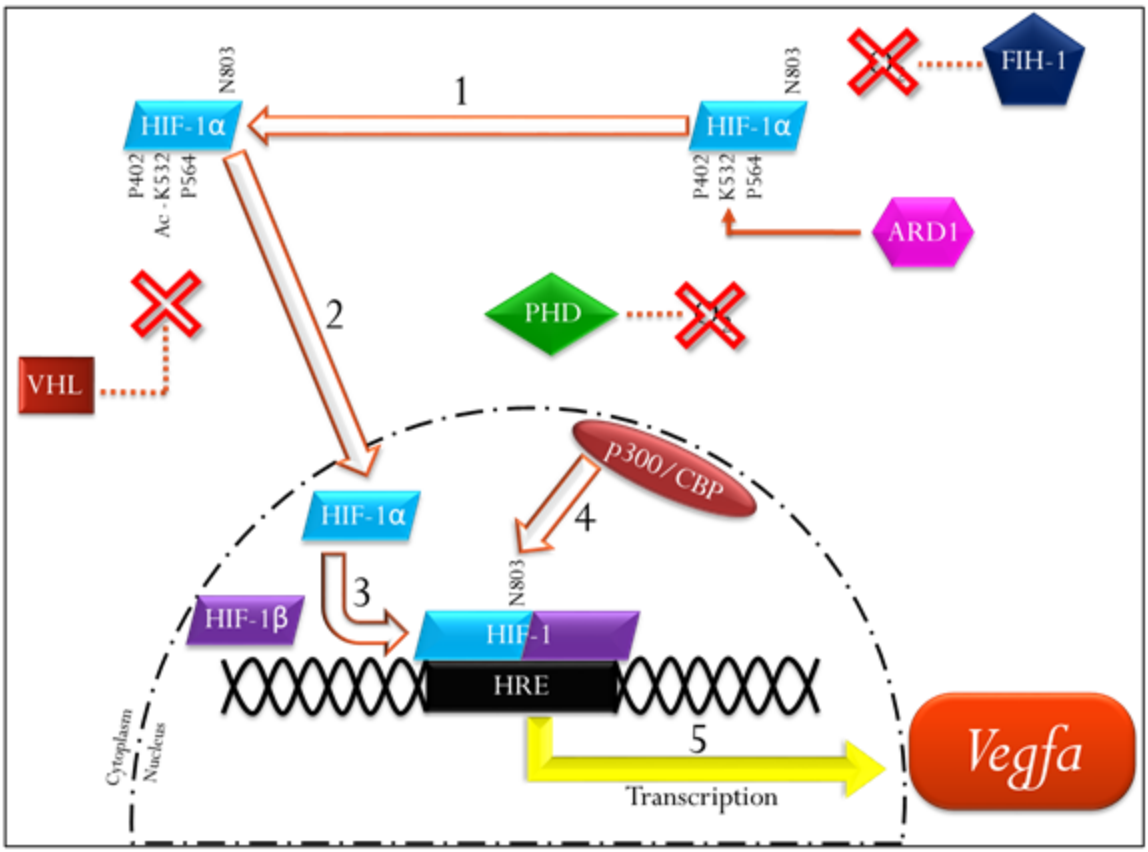

FIGURE 6 | HIF-1α stability under hypoxic conditions: In the presence of an environment nearly devoid of oxygen (hypoxia), enzymes regulating HIF-1α stability and activity, PHD and FIH-1, no longer possess the substrate oxygen. Therefore, the proline and lysine residues found in HIF-1α are not post-translationally modified, thereby eliminating any incentive for pVHL to bind and polyubiquitinylate HIF-1α. HIF-1α is no longer marked for proteasomal degradation and is free to translocate to the nucleus and dimerize with HIF-1β to form the functional HIF-1 complex [41, 72, 80]. The degree of hypoxia will determine the level of HIF-1 activity, where the complex will be half-maximally active at concentrations of 10-14 mmHg of oxygen and fully active at levels <3 mmHg [65]. HIF-1 is then capable of recognizing the HRE of its various target genes [41, 72] and binding via the basic regions found within each HIF-1 complex subunit [72]. Through the recruitment of essential coactivators such as CBP/p300, transcription of the target effector genes (list in Table II) can be fully stimulated resulting in gene expression of factors such as VEGFA [72].

HIF-α Residues

HIF-1α stability and activity is influenced by specific post-translational modifications of certain residues encompassed within its ODDD: proline 402 (P402), proline 564 (P564) and lysine 532 (K532) [72, 77]. Modifications of asparagine residue (N803), within its C-TAD, also result in variable HIF-1α activity [72].

Prolyl Hydroxylase Domain

Prolyl Hydroxylase Domain (PHD), also known as HIF-prolyl hydroxylase (HPH) or Egg-laying Nine (EGLN) is a member of the 2-oxoglutarate (2-OG) dependent dioxygenases, enzymes capable of hydroxylating certain residues via molecular oxygen [18, 72]. Three isoforms of this enzyme have been discovered: PHD1/HPH3/EGLN2, PHD2/HPH2/EGLN1 and PHD3/HPH1/EGLN3 where PHD2 is the most potent in terms of its ability to hydroxylate HIF-α residues [72]. The proper function of PHD requires two cofactors, ascorbate and iron (Fe^{2+}). Iron chelators and metal irons such as, Co^{2+}, Ni^{2+} and Mn^{2+} are able to significantly reduce PHD enzyme activity by altering the availability and stability of the ferrous ion [72, 76]. PHD enzymatic function relies on splitting molecular oxygen and consequently hydroxylating HIF-1α-containing P402 and P564 residues, thus promoting the interaction between HIF-1α and the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex [18, 72, 80].

VCB-Cul2 E3 Ligase Complex

PHD-mediated hydroxylation of P402 and P564 found in HIF-1α allows for specific binding of HIF-1α to pVHL. Once pVHL complexes with HIF-1α, other factors necessary for the complete formation of the pVHL ubiquitin E3 ligase complex (VCB-Cul2 E3 ligase complex) can be recruited. Elongin-C, elongin-B, cullin-2 and Rbx1 are recruited to form the VCB-Cul2 E3 ligase complex [72, 76]. HIF-1α is consequently polyubiquitinylated and therefore marked for proteasomal degradation [18, 72, 76, 77, 80]. The phenotype for a null mutation of the

gene encoding for pVHL, a tumor suppressor gene, has been linked with contributing to the progression of certain tumors [33, 71]. An inactive pVHL would increase HIF-1α stability and therefore increase HIF-1 complex formation, thereby leading to the activation of genes favoring tumor progression, such as the angiogenic factor VEGFA [71].

Arrest-Defective-1

Arrest-defective-1 (ARD1) is an acetyltransferase capable of post-translationally modifying K532 found on HIF-1α [72, 76]. Acetylation of this residue favors the interaction of HIF-1α with pVHL [72, 76]. Gene expression and translation of this protein are both downregulated under hypoxic conditions, which may increase HIF-1α stability; however the activity of this enzyme is not influenced by oxygen tension levels [72].

Factor inhibiting HIF-1

Factor inhibiting HIF-1 (FIH-1) is a 2-OG-dependent dioxygenase, like PHD [18, 72]. Asparagine residues found within the C-TAD (N803 in HIF-1α and N851 in HIF-2α) can be hydroxylated via FIH-1, thereby preventing p300/CBP coactivator recruitment necessary for successful gene target expression via the HIF-1 complex [18, 72, 76, 77, 80]. Oxygen tension levels do not affect expression levels of FIH-1 and FIH-1's function does not alter HIF-1α/HIF-2α's stability [72, 76]. However, the formation of a ternary complex between FIH-1, pVHL and HIF-1α leading to the recruitment of a histone deacetylase via pVHL facilitates FIH-1's transcriptional repression of HIF-1 induced gene expression [72].

HIF-1 complex activity

MAP Kinase Pathway

Serine/threonine kinases are proteins that make up the mitogen-activated protein (MAP) kinase signalling cascade family [34, 81]. There exists an abundance of factors capable of triggering the various branches of the MAP kinase signalling cascade such as growth and stress factors, hormones, and ECM components [34]. The p42/p44 (also known as extracellular signal-regulated kinase: ERK2/ERK1) MAP kinase pathway as well as the stress-activated protein kinase (SAPK) MAP kinase pathways, p38 and c-Jun NH2-terminal kinase (JNK), are all components categorized under MAP kinase signalling (Figure S1) [34, 81, 82].

ERK1/ERK2 Pathway of the MAP Kinase Family

Various components are capable of activating the ERK pathway, such as ROS, Ca^{2+}, VEGFA, PDGF and TGF-β [59]. Activation of the ERK pathway leads to the phosphorylation of HIF-1α and increases the transactivational capacity of HIF-1 [46, 59, 77]. The phosphorylation of HIF-1α residues does not seem to affect the stability of this subunit but rather entices HIF-1β to interact with its counterpart, thereby allowing dimerization to further carry out its target gene expression activation [59, 76]. Furthermore, direct phosphorylation of the coactivator p300/CBP via the ERK pathway has been shown to positively influence the transcription-inducing activity of the HIF-1 complex [77].

Wound Healing Models and Measurements

Various animal wound models exist in the literature ranging from incisional, excisional, burn and granulation tissue models [83]. For this study an excisional wound model was selected for the same reasons outlined by Galiano et al [83]. The wounds are easily

harvested and specimens can be analyzed via immunohistochemistry or molecular profiling [83]. Furthermore, an excisional mouse wound model allows for a simpler analysis of wound healing processes such as epithelialization, granulation tissue formation, scar formation, contraction and angiogenesis which was of particular importance for this study [83]. Wound location selection, dorsum of mice, was based upon the area most unlikely to be affected by post-operative auto-inflicted trauma. Auto-inflicted trauma to experimentally created wounds would skew future data analyses regarding wound healing rates as well as gene expression profiles.

A macroscopic as well as a histological approach was utilized in this study in order to quantify and evaluate wound healing in the murine model. Macroscopically, surface area was measured, using Image J software, from digital photographs taken from a standardized distance from the wound, on pre-determined post-operative days, in order to determine the rate of wound healing. With regards to the histological analysis of wound healing, specific parameters (epidermal tongue length, epidermal gap, wound width) were used to evaluate wound healing, reflecting measurements found within the literature [84]. As the wound heals new epithelium originating from the wound margins will proliferate towards the center of the wound until a complete new epithelial cover is formed. The new epidermis originating from the wound margins is denoted as the epidermal tongue and therefore each wound analyzed histologically will have a left and right epidermal tongue. The distance in between both epidermal tongues, still uncovered by new epidermis, is denoted the epidermal gap. Finally, the distance between wound margins (thereby including the left epidermal tongue, epidermal gap and right epidermal tongue) represents the wound width. In this study these histological parameters were used to calculate the percentage of neo-epidermal coverage. This calculation resulted in the addition of the left and right epidermal tongues divided by the wound width. It was decided to present the data in this manner as it depicted the information in a more accurate and logical manner.

RESEARCH HYPOTHESES

It is well documented that a hypoxic environment allows for the formation of the active HIF-1 complex, which stimulates the induction of certain genes necessary for wound healing such as *Vegfa*, a potent angiogenic agent. However, whether HIF-1 acts to induce *Vegfa* expression in the context of cutaneous wound healing has not been established. This study aimed to compare cutaneous wound healing between wild type and mutant mice incapable of inducing *Vegfa* expression via HIF-1, in order to better understand the contribution of HIF-1-induced *Vegfa* expression with regards to the healing process. The following hypotheses were formulated:

1) A hypoxia response element (HRE) at the level of the *Vegfa* promoter is required for normal *Vegfa* expression in cutaneous wounds.

2) HIF-1 induced *Vegfa* expression is required for optimal *Vegfa* downstream target expression within a cutaneous wound healing setting.

3) HIF-1 induced *Vegfa* expression is required for optimal cutaneous wound healing progression, including wound healing rates and granulation tissue formation.

RESEARCH OBJECTIVES

The objectives of this study were to determine the effect of a lack of HIF-1-induced *Vegfa* expression on cutaneous wound healing within $Vegfa^{\delta/\delta}$ mice when compared to their wild type counterparts.

Objective 1: To compare mRNA and protein expression levels of various pro-angiogenic factors (*Vegfa, Hif-1α, Sdf-1, Pdgfb*) between wild type (WT) and mutant ($Vegfa^{\delta/\delta}$) mice throughout the course of cutaneous wound healing

- Real-time qPCR of *Vegfa, Sdf-1, Pdgfb* and *Hif-1α* throughout the course of wound healing
- Western blot of HIF-1α and VEGFA within intact skin

Objective 2: To compare the rate of cutaneous wound healing between WT and mutant mice

- Measure the surface area of healing wounds on days 0, 3, 7, 10 and 14

Objective 3: To examine the differences in wound healing progression between WT and mutant mice, at a histological level

- Measure various histological features (epidermal tongue length, epidermal gap, wound width) to estimate progression of epidermal coverage;
- Measure the surface area of granulation tissue in cross sections of wound samples;
- Calculate the capillary density within the granulation tissue of healing wounds

CHAPTER 2 - Article in preparation for submission to PLoS One

A Hypoxia Response Element in the *Vegfa* Promoter is Required for Basal *Vegfa* Expression in Skin and for Optimal Granulation Tissue Formation During Wound Healing in Mice

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[5] Conceptualization, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing – original draft preparation

Role of HIF-1 in Basal VEGFA Expression in Skin

Keywords: HIF-1α, VEGFA, HRE, skin, wound healing, granulation tissue

ABSTRACT

Hypoxia in skin wounds is thought to contribute to healing through the induction of hypoxia inducible factor-1, a key transcriptional regulator of genes associated with healing. Hypoxia inducible factor-1 regulates the expression of vascular endothelial growth factor A (VEGFA) by binding a hypoxia response element in its promoter. Although VEGFA is a key regulator of angiogenesis during healing, whether hypoxia and hypoxia inducible factor-1 serve to induce its expression in this context is unknown. To test this hypothesis, we studied VEGFA expression and wound healing in mutant mice that lack a functional hypoxia response element in the VEGFA promoter. Decreased levels of VEGFA mRNA were found in intact skin of mutant mice whereas levels in granulation tissue during wound healing were not altered. Likewise, mRNA levels of VEGFA target genes, platelet-derived growth factor B and stromal cell-derived factor-1, were decreased in skin but unchanged in granulation tissue of mutant mice. Decreased VEGFA mRNA levels in skin of mutant mice could not be attributed to decreased hypoxia inducible factor-1 protein expression, and were therefore a consequence of the loss of hypoxia inducible factor-1 responsiveness of the VEGFA promoter. Histologic analyses of healing wounds in mutant mice revealed significant defects in granulation tissue, both in terms of quantity and capillary density, although epithelialization and healing rates were unaltered. We conclude that hypoxia inducible factor-1 is not a major regulator of VEGFA during wound healing; rather, it serves to maintain basal levels of VEGFA and its target genes in intact skin, required for optimal granulation tissue formation in response to wounding.

INTRODUCTION

Hypoxia within skin wounds arises primarily in response to traumatic destruction of the cutaneous vasculature and augmentation of cellular oxygen consumption rates due to temporary increases in cellular density and metabolic activity [1-4]. Acute hypoxia favors wound healing because low oxygen tension levels promote angiogenesis, fibroplasia, epithelialization, and extracellular matrix (ECM) synthesis [1, 2, 4-6]. These effects are regulated, at least in part, by hypoxia-induced increases in the expression of various growth factors including transforming growth factor (TGF)-β1, platelet-derived growth factor (PDGF) and vascular endothelial growth factor (VEGFA) [1, 2, 4, 6].

Hypoxia triggers the accumulation of hypoxia-inducible factor (HIF)-1, the cellular hypoxia sensor, which is the key element in the process of oxygen homeostasis and in the re-establishment of blood vessels in hypoxic areas [1, 4]. HIF-1 is a heterodimeric transcription factor that induces the expression of a plethora of genes that mediate adaptive responses to hypoxia such as angiogenesis and cell proliferation/survival [1, 4, 7]. It exerts its transcriptional regulatory effects by binding a site named the hypoxia-response element (HRE) found within the promoters of various genes including *VEGFA,* angiopoietin (*ANGPT)1, ANGPT2, PDGFB,* glucose transporter (*GLUT)-1* and stromal cell-derived factor (*SDF)-1* [3, 7-9].

Angiogenesis is a physiological process whereby new blood vessels form from pre-existing vessels [10, 11]. In the wound environment, these new vessels are required to deliver essential nutrients and oxygen to mitotically active cells, thereby ensuring that healing progresses normally [12]. VEGFA is a key regulator of angiogenesis in a variety of developmental and physiological processes including wound healing [13, 14]. VEGFA is also capable of upregulating other factors, such as PDGFB and SDF-1, which play an important role in angiogenesis [15-17]. HIF-1 is thought to be a major regulator of *Vegfa* expression in several cell types, and acts via a well-characterized HRE in the *Vegfa* promoter [7, 18]. However, whether HIF-1 acts to induce *Vegfa* expression in the context of cutaneous wound healing has not been established. In the current study, we tested this idea *in vivo* using the

Vegfa$^{\delta/\delta}$ mouse model, which lacks a functional HRE in the *Vegfa* promoter, and therefore cannot respond to hypoxia/HIF-1 with an increase in *Vegfa* transcriptional activity [19]. We predicted that *Vegfa* upregulation in response to wounding would fail to occur in mutant mice, and that wound healing would be compromised due to poor angiogenesis and the presence of granulation tissue of inferior quantity and/or quality. While our experimental goal was achieved, the outcome of our study demonstrated that, contrary to prediction, HIF-1 is required for basal expression of *Vegfa* in intact skin, rather than in granulation tissue during wound healing.

MATERIALS AND METHODS

Mice

Vegfa^{tm2Pec} (hereafter *Vegfa*$^{\delta/\delta}$) mice were as originally described [20]. Wild type and mutant mice used for all experimentation were male and ranged from 6-8 weeks in age. All mice were healthy upon commencement of experimentation. Animals were housed individually under standardized conditions with controlled temperature, humidity, and a 12-hour-day/12-hour-night light cycle. Animals had free access to water and standard mouse chow. This study was conducted at the Faculté de médecine vétérinaire in strict accordance with the guidelines for the care and use of laboratory animals, as sanctioned by the Canadian Council on Animal Care. The protocol was approved by the Comité d'éthique de l'utilisation des animaux de l'Université de Montréal (Permit Number: Rech-1635). All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

Part I: Molecular profile

For the first part of the study, 6 *Vegfa*$^{\delta/\delta}$ and 6 wild type mice were used. On day 0, mice were anesthetized by isoflurane inhalation 20 minutes after subcutaneous administration of Metacam™ (meloxicam, 4mg/kg – Boehringer Ingelheim, St. Joseph, Missouri, USA). The dorsum was clipped and prepared aseptically. Three full-thickness excisional wounds were made to below the level of the *panniculus carnosus* (muscle layer was removed), one on each side of midline and a third one centrally located just caudal to the other two wound sites, using a sterile, disposable, 6 mm-diameter biopsy punch and scissors; the excised tissue (intact skin) was kept as a day 0 sample. Wounds were left uncovered to heal by secondary intention.

Post-operatively, mice were injected subcutaneously with 1 ml of warm, 0.9% sterile saline solution for fluid replacement in accordance with CEAU mouse surgical protocol. All mice were placed on a warming pad until mobile and were then returned to their cage

(cardboard paper bases were used as substrate) where they were further warmed until fully awake. Mice received a subcutaneous dose of Metacam™ (4mg/kg, SID) on postoperative days 1 and 2.

Animals were anesthetized as per wound creation surgery on days 5, 7 and 9; at each of these times, a single wound was randomly selected for harvest. On day 9 the animals were euthanized by carbon dioxide (CO_2) inhalation after wound harvest. Wound sampling consisted of creating a new 8 mm-diameter wound encompassing the initial wound to ensure that the entire region of interest was collected. Tissue samples were embedded in Optimal Cutting Temperature compound (OCT - Tissue-Tek, Torrance, CA, USA), snap-frozen in liquid nitrogen and then stored at -80°C.

Real-time RT-PCR

Wound tissue samples were thawed and the peripheral and deeper wound sections were discarded, keeping only granulation tissue. Conversely, the totality of day 0 samples (i.e., intact skin) was kept. Total RNA was isolated and purified using the RNeasy Fibrous Tissue Mini kit (Qiagen Sciences, Maryland, USA), following the manufacturer's instructions. Synthesis of cDNA was done using a SuperScript VILO cDNA synthesis kit (Thermo Fisher Scientific, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was carried out in a Bio-Rad CFX96 Touch Real Time PCR Detection System, using the SSoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), following the manufacturer's instructions. Custom oligonucleotide primers were designed via Life Technologies, Inc (Table 3) and standard curves were generated by serial dilution of a preparation of total cDNA. Expression levels of genes were calculated relative to the housekeeping gene Ribosomal Protein-L19 (*Rpl19)* [21].

TABLE III| Primers used for real-time qPCR

Gene	Forward 5' – 3'	Reverse 5' – 3'
Vegfa	GGAGACTCTTCGAGGAGCACTT	GGCGATTTAGCAGCAGATATAAGAA
Pdgfb	GAGGGGGATCCCATTCCTGA	GCCCCATCTTCATCTACGGA
Sdf-1	TTCTTCGAGAGCCACATCGC	TCAGCCGTGCAACAATCTGA
Hif-1α	GGAGCCTTAACCTGTCTGCCACT	CGCTTCCTCTGAGCATTCTGCG

Western Blot

Intact skin and granulation tissue samples were weighed then placed in extraction buffer (Tissue Protein Extraction Reagent [T-PER® – Thermo Scientific, Rockford, IL, USA)] supplemented with a protease inhibitor tablet [Complete - Sigma-Aldrich, Mannheim, Germany]) as per manufacturers' instructions, and homogenized using a PowerGen 125 (Fisher Scientific) tissue homogenizer. The solution was then centrifuged at 10,000 xg for 5 minutes at 14ºC. The protein concentration of the supernatants was measured using the Bradford protein assay. Twenty microgram (µg) protein samples were separated on a 12% agarose gel then transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P Transfer Membranes - EMD Millipore Corporation, Billerica, M, USA). Membranes were blocked in 5% milk for 1 hour at room temperature and then incubated overnight in a 5% albumin solution containing the primary antibody at a dilution of 1:500. The primary antibodies used to detect HIF-1α and VEGFA were obtained from Santa Cruz Biotechnology (sc-53546 and sc-507, respectively). The following day, the membranes were incubated for 60 minutes in a 12.5% milk solution containing horseradish peroxidase (HRP)-conjugated secondary antibody, and detection of immunoreactive proteins was performed with a chemiluminescent HRP substrate (Immobilon – Millipore) and the Chemidoc™ MP imaging system (Bio-Rad Laboratories, Inc.). Beta-actin (ACTB) (β-actin - sc-47778; dilution 1:500,000; Santa Cruz Biotechnology) was used as a loading control. Signal intensity measurements were

obtained using Image Lab™ software version 5.2.1 (Bio-Rad Laboratories, Inc. Hercules, CA, USA).

Part II: Wound healing assay

Eight mutant (*Vegfa*$^{\delta/\delta}$) and 8 wild type mice were used for the second part of the study. The same anesthetic, surgical and analgesic protocols applied in part I of experimentation were followed in part II, except two wounds were created on either side of midline using a sterile, disposable 10 mm-diameter biopsy punch and a scalpel blade instead of three. The excised tissue (intact skin) was kept as a day 0 sample. Wounds were photographed (baseline measurement) and were left uncovered to heal by secondary intention.

Mice were immobilized and each wound was photographed using a digital camera alongside a scale bar, on postoperative days 3, 7, 10 and 14. The wound surface area was calculated in pixels, by a blinded observer, using ImageJ software (http://imagej.nih.gov/ij/), and expressed as a percentage of the original wound surface area to determine the rate of healing. On day 7 the animals were anesthetized as per wound creation surgery, and the right-sided wounds were collected. Newly created wounds through harvesting were left to heal by secondary intention. On day 14, the animals were euthanized by CO_2 inhalation then the left-sided wounds were collected. All samples were harvested with a biopsy punch to include the remaining wound and the surrounding skin, placed flat on a piece of filter paper, subcutis side down, then divided in half. One half was fixed in 10% neutral-buffered formalin solution for 24h then stored in 70% alcohol at 4^{o}C until processing in paraffin wax for histology. The second half was embedded in OCT then snap-frozen in liquid nitrogen and stored at -80°C until real-time (RT)-qPCR and Western Blot assays.

Histological analyses

Serial sections taken at the greatest wound width were stained with hematoxylin, eosin, phloxine and saffron (HEPS) stain. The neo-epidermal coverage, based on the percentage of the total wound width covered by new epithelium (sum of the epidermal tongues migrating from the right and left wound margins divided by the wound width), and the remaining wound width (epidermal gap length) were calculated for each section. Measurements were determined relative to a constant reference (thickness of the intact epidermis adjacent to the wound). Each measurement for each criterion was taken twice and averages were used for statistical analyses. Total wound width was measured on days 7 and 14, whereas neo-epidermal coverage and the remaining wound width were measured on day 7.

The area of granulation tissue was quantified in day 7 wound samples using Zen Pro 2012 software and an Axioimager M1 microscope (Zeiss, Toronto, Ontario, Canada). Capillaries within the granulation tissue were counted; only blood vessels running perpendicularly to the wound surface were included in the count. The average number of capillaries was then divided by the granulation tissue area measurement obtained for that section, to obtain the capillary density.

Statistical analyses

Statistical testing was done using Prism v6.0d software (GraphPad software, Inc. La Jolla, CA, USA). The threshold of statistical significance for all analyses was defined as $P \leq 0.05$. The statistical analyses applied to specific data sets are detailed in the corresponding figure legends.

RESULTS

Part I: Molecular profile

Vegfa mRNA levels in wild type and in mutant mice lacking a functional HRE in the *Vegfa* promoter (*Vegfa*$^{\delta/\delta}$) were determined in both intact skin and granulation tissue throughout the course of wound healing. *Vegfa* mRNA levels in intact skin from *Vegfa*$^{\delta/\delta}$ mice were significantly lower compared to those of their wild type counterparts (Figure 7A) however, unexpectedly, no differences in *Vegfa* mRNA levels in granulation tissue were detected between genotypes during wound healing (Figure 7B).

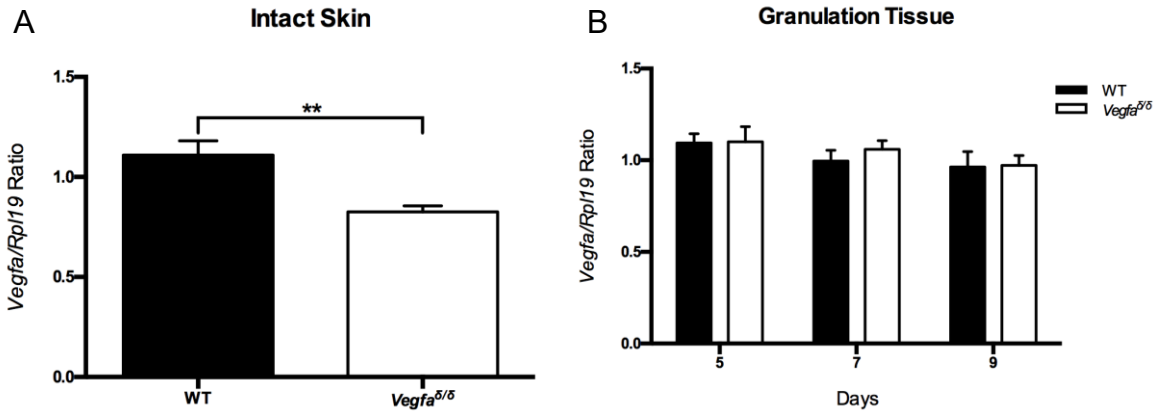

FIGURE 7| *Vegfa* mRNA levels in intact skin and in granulation tissue in wild type and mutant (*Vegfa*$^{\delta/\delta}$) mice: (A) *Vegfa* mRNA levels in intact skin. (B) *Vegfa* mRNA levels in granulation tissue at the indicated days post-wounding. Values represent means ± SEM (n = 6). A two-tailed unpaired t-test was used to analyze data from intact skin. A Sidak-corrected repeated measures 2-way ANOVA was used to analyze data from granulation tissue. **: values are significantly different, P = 0.0044.

Messenger RNA levels of downstream VEGFA angiogenic target genes, *Pdgfb* and *Sdf-1*, were subsequently measured to verify if they reflected levels of *Vegfa*. *Pdgfb* and *Sdf-1* mRNA levels in intact skin mirrored *Vegfa* mRNA levels, being significantly lower in mutant mice than in wild type mice (Figure 8A, 8C). Neither *Pdgfb* nor *Sdf-1* mRNA levels in granulation tissue differed amongst genotypes during the healing process (Figure 8B, 8D).

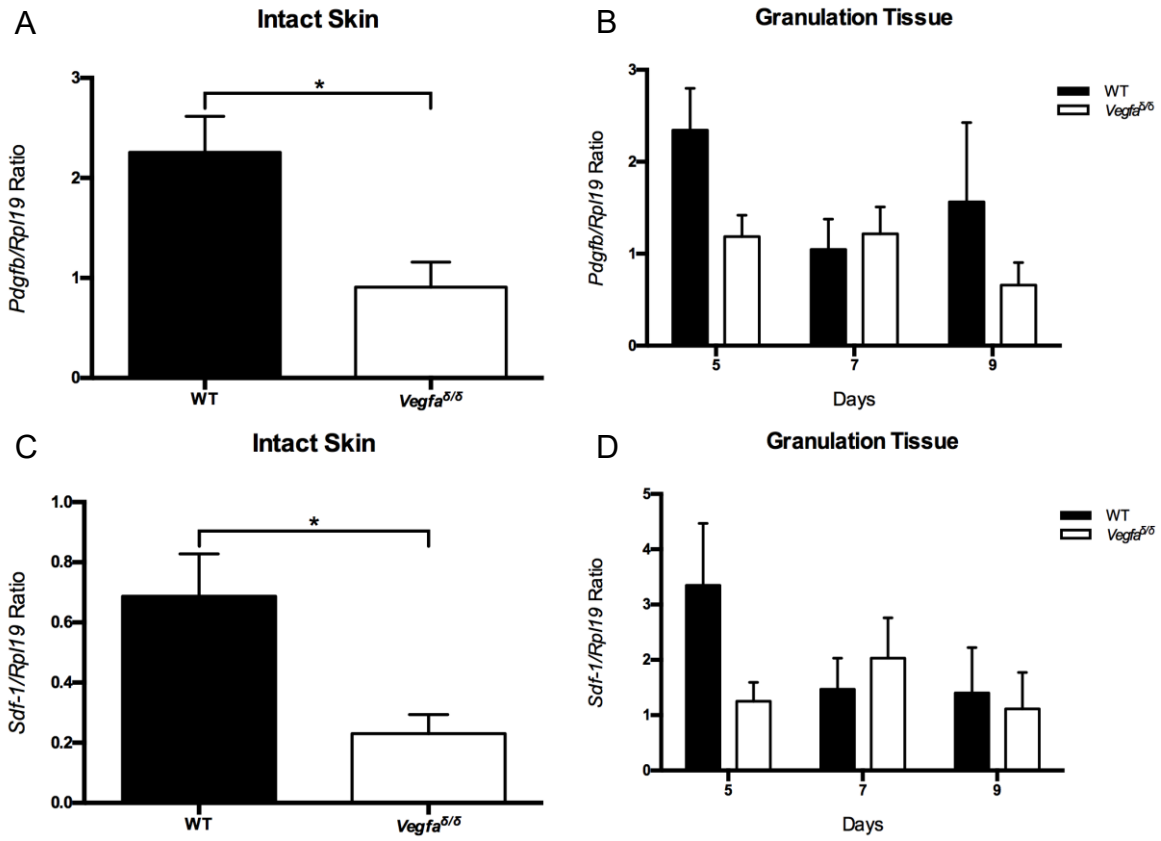

FIGURE 8| *Pdgfb and Sdf-1* mRNA levels in intact skin and in granulation tissue in wild type and *Vegfa*$^{\delta/\delta}$ mice: (A) *Pdgfb* mRNA levels in intact skin, *: values are significantly different, P = 0.0119. (B) *Pdgfb* mRNA levels in granulation tissue. (C) *Sdf-1* mRNA levels in intact skin, *: values are significantly different, P = 0.0145. (D) *Sdf-1* mRNA levels in granulation tissue. Messenger RNA levels are relative to that of *Rpl19*. Values represent means ± SEM (n = 6). A two-tailed unpaired t-test was used to analyze data from intact skin. A Sidak-corrected repeated measures 2-way ANOVA was used to analyze data from granulation tissue.

HIF-1α and VEGFA expression in intact skin were analyzed by immunoblotting. Whereas HIF-1α expression did not differ between genotypes, VEGFA expression in intact skin was significantly lower in mutant mice than in wild type mice (Figure 9C).

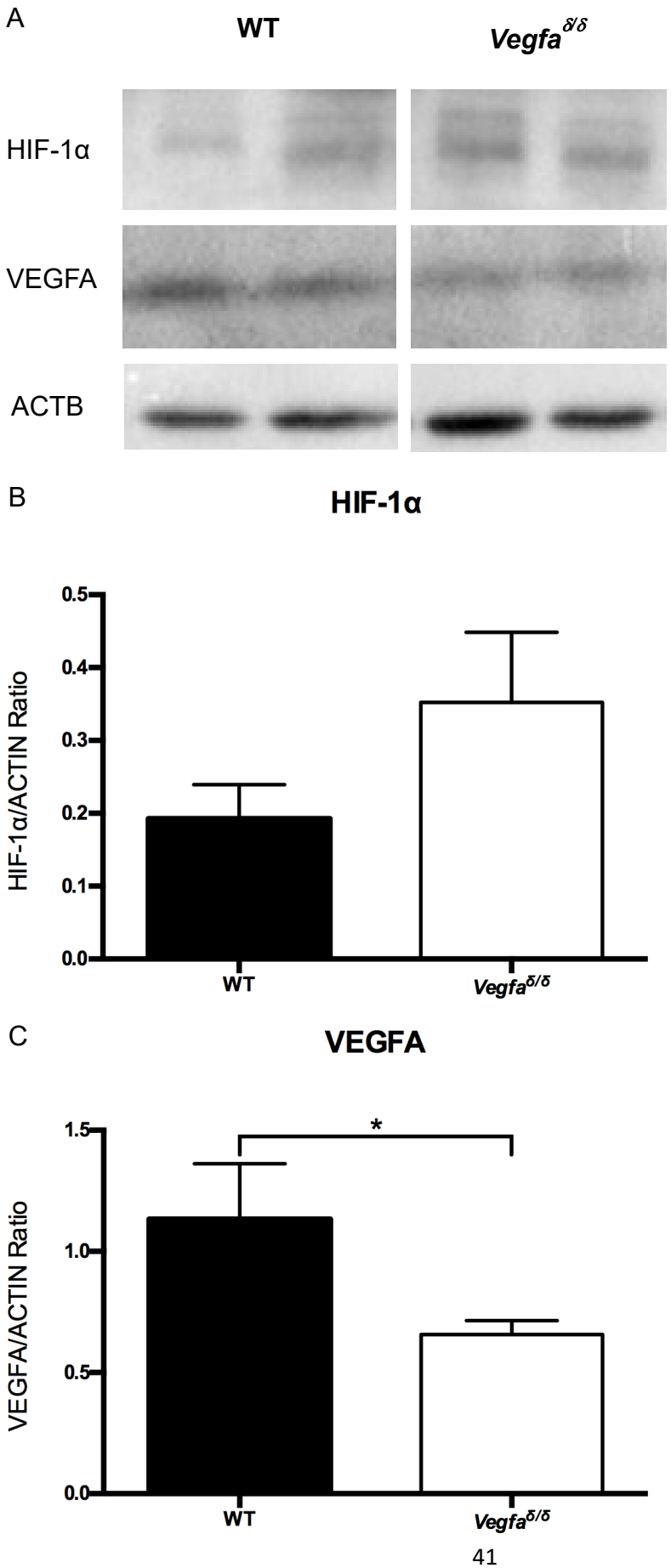

FIGURE 9| HIF-1α and VEGFA expression in intact skin of wild type and *Vegfa*$^{\delta/\delta}$ mice: (A) Representative immunoblot analyses of HIF-1α and VEGFA expression in intact skin (n = 2/genotype). (B) Quantification of HIF-1α immunoblots (n = 6/genotype). Data were analyzed using a two-tailed unpaired t-test. (C) Quantification of VEGFA immunoblots (n = 6/genotype). Data were analyzed using a Mann-Whitney test. *: values are significantly different, P = 0.0152.

Part II: Wound healing assay

The overall decrease in wound surface area over time did not differ significantly between wild type and mutant mice (Figure 10A). The majority of wounds had fully healed by day 14 of experimentation and therefore day 14 samples were not included in the analysis of this part of the study.

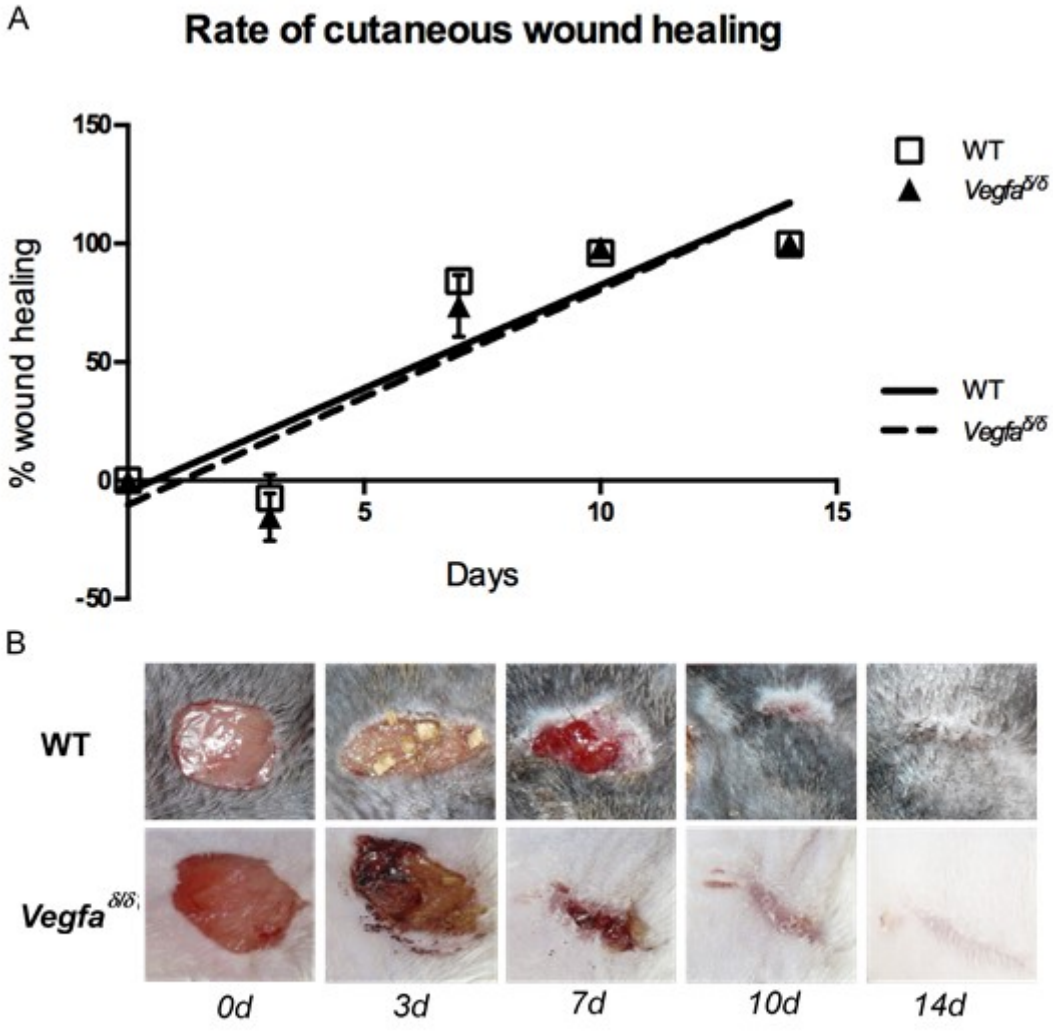

FIGURE 10 | Healing kinetics and macroscopic appearance of wounds in wild type and *Vegfa*$^{\delta/\delta}$ mice: (A) Percent wound healing over time. Values represent means ± SEM (n = 8 for WT; n = 7 for *Vegfa*$^{\delta/\delta}$). Data were analyzed using one-way ANOVA. (B) Representative photographs of the healing wounds at the indicated days post-wounding (0d = immediately post-operative).

Since VEGF is known to contribute to keratinocyte migration [21], histometric analyses were done to examine potential epidermal differences in the wounds of wild type and mutant mice (Figure 11A). Quantification of neo-epidermal tongue lengths and epithelial gaps showed no difference between the wounds of wild type and mutant mice at day 7 (not shown). Consequently, no significant difference in neo-epidermal coverage was found between the two populations of mice (Figure 11B).

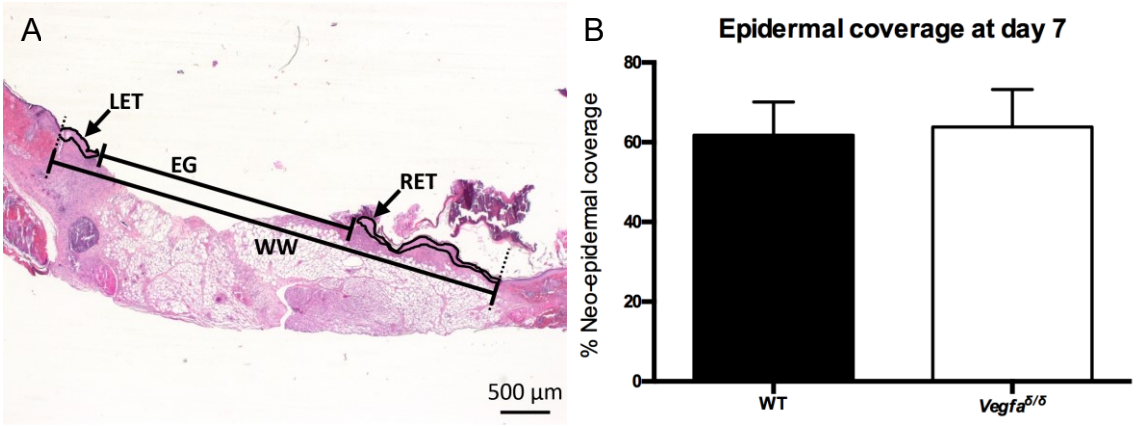

FIGURE 11| Epithelialization of healing wounds in wild type and *Vegfa*$^{\delta/\delta}$ mice: (A) Representative photomicrograph of a 7 day-old wound in a wild type mouse. Measurements of the left (LET) and right epidermal tongues (RET), epidermal gap (EG) and wound width (WW) were used to evaluate the progress of wound healing. (B) Quantitative analysis of neo-epidermal coverage in the healing wounds of WT and *Vegfa*$^{\delta/\delta}$ mice. Data were analyzed using a two-tailed unpaired t-test. Values represent means ± SEM (n = 8 for WT; n = 6 for *Vegfa*$^{\delta/\delta}$).

Because VEGFA is a major stimulator of angiogenesis in response to the hypoxia generated by wounding, the blood vessel-rich granulation tissue present within 7 day-old wounds was examined. Granulation tissue surface area was measured in cross-sections as an approximation of granulation tissue volume (Figure 12A). A significantly smaller surface area of granulation tissue was present in the wounds of mutant mice (Figure 12B). Furthermore, there was a trend towards reduced capillary density within the granulation tissue of 7 day-old wounds in the mutant mice compared to the wild type mice (P = 0.0602) (Figure 12D).

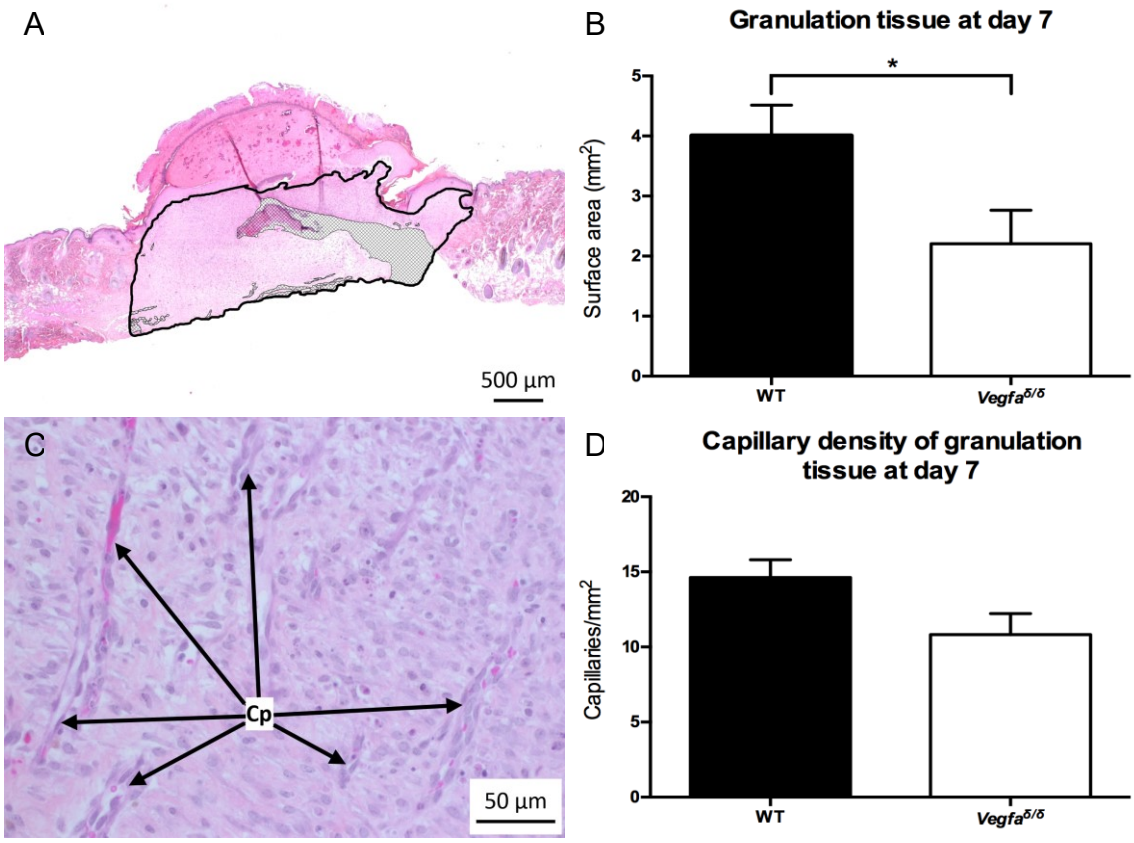

FIGURE 12| Content of granulation tissue and capillaries in wild type and *Vegfa*$^{\delta/\delta}$ mice: (A) Representative photomicrograph of a 7 day-old wound in a wild type mouse with the granulation tissue circumscribed. Adipose tissue or muscle found within the demarcated zone (grey hatched area) was excluded from the surface area measurement. (B) Surface area of the granulation tissue in 7 day-old wounds, *: values are statistically different, P = 0.0354. Values represent means ± SEM (n = 8 for WT; n = 6 for *Vegfa*$^{\delta/\delta}$). A two-tailed unpaired t-test was used to analyze the data. (C) Representative photomicrograph showing examples of new capillaries (Cp) within the granulation tissue of 7 day-old wounds. (D) Capillary density (Cp / mm^2 of granulation tissue). Values represent mean ± SEM (n = 8 for WT; n = 6 for *Vegfa*$^{\delta/\delta}$). A two-tailed unpaired t-test was used to analyze the data.

DISCUSSION

Hypoxia associated with cutaneous wounding triggers the expression of a number of genes required for the healing process, including the key angiogenic factor VEGFA [4, 7]. Although it is established that HIF-1 can regulate *Vegfa* expression in hypoxic cells via a HRE [7], that this regulatory process drives *Vegfa* expression in the context of cutaneous wound healing has long been presumed, but not actually demonstrated. In the present study, we employed the *Vegfa*$^{\delta/\delta}$ model, in which the lack of a functional HRE in the *Vegfa* promoter provided the ideal system to determine the contribution of HIF-1 to *Vegfa* expression in wounded skin.

Contrary to our hypothesis, *Vegfa* expression was not compromised in the granulation tissue in the wounds of *Vegfa*$^{\delta/\delta}$ mice. The simplest explanation for this finding is that HIF-1 is simply not a regulator of *Vegfa* in the context of wound healing. Indeed, many additional factors and signaling processes are known to regulate *Vegfa* expression and may be relevant in granulation tissue. These include epidermal growth factor, platelet-derived growth factor and interleukin-1α, which are released in response to wounding [14, 22]. Physicochemical stimuli such as acidosis, hypoglycemia, reactive oxygen species and mechanical stretching all stimulate *Vegfa* expression [14, 23, 24, 25]. Likewise, the extracellular signal-regulated kinase (ERK)1/ERK2 pathway induces *Vegfa* expression through the binding of transcriptional factor complexes such as adaptor complex 2 and specificity protein 1 to the *Vegfa* promoter [26, 27]. Whether these or other signaling processes drive *Vegfa* expression in granulation tissue will be grounds for further study. Another explanation for unaltered *Vegfa* expression in the granulation tissue of *Vegfa*$^{\delta/\delta}$ mice is that compensatory mechanisms (perhaps including the aforementioned *Vegfa* regulatory processes) may have been induced to augment *Vegfa* expression, thereby masking any deficit caused by deficient HIF-inducibility of the *Vegfa* promoter. Further experiments will be required to test this possibility.

In this study, we readily detected HIF-1α protein in intact skin, confirming previous reports of constitutive expression in several functionally diverse cell types including those

populating skin [28, 29, 30]. The decreased levels of *Vegfa* expression in the skin of *Vegfa*$^{\delta/\delta}$ mice therefore indicate that one role of the constitutively expressed HIF-1α is to ensure proper basal expression of *Vegfa*.

Despite normal expression of *Vegfa* (and *Pdgfb* and *Sdf1*) in their granulation tissue, wounds in *Vegfa*$^{\delta/\delta}$ mice featured a reduced abundance of granulation tissue, and capillary density was decreased within the granulation tissue. Although we cannot provide a definitive explanation for this, deficient *Vegfa* expression in the intact skin of *Vegfa*$^{\delta/\delta}$ mice (i.e., prior to wounding) provides a plausible explanation. Upon wounding, decreased VEGFA levels present locally within the skin may have led to delayed and/or reduced capillary and granulation tissue formation in the early phases of healing, leading to the changes observed 7 days post-wounding in the *Vegfa*$^{\delta/\delta}$ model. This would suggest that a certain level of VEGFA expression in intact skin is required for an optimal response to wounding and a rapid onset of the healing process. Surprisingly, in spite of defects in the granulation tissue, the wounds of *Vegfa*$^{\delta/\delta}$ mice healed at the same rate as the wounds of wild type mice. This might be explained by the highly contractile nature of wound healing in mice [31]. Indeed, several studies of cutaneous wound healing in mice employ stents/splints to prevent wound contraction [32, 33], thereby permitting a better analysis of the epithelialization process. Future studies of the *Vegfa*$^{\delta/\delta}$ mice could employ stenting in order to more conclusively determine if the granulation tissue defects specifically affected epithelialization.

In summary, this study describes a novel role for HIF-1 in the expression of *Vegfa* in skin. Constitutively expressed HIF-1α maintains a basal level of *Vegfa* expression, which is required for optimal granulation tissue formation following wounding.

ACKNOWLEDGEMENTS

The authors thank Dr. Guy Beauchamp for help with statistical analyses, as well as Meggie Girard, Dr. Mayra Tsoi and Dr. Gustavo Zamberlam for help with the mouse colony.

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CHAPTER 3

DISCUSSION AND FUTURE EXPERIMENTS

Since the mice used in this study were capable of producing and activating HIF-1, no differences in HIF-1α upregulation in response to wounding were expected between wild type and mutant mice. However, HIF-1-induced *Vegfa* expression in the mutant mice was impossible due to genetic modifications. Considering that the only genetic difference between the two populations of mice was the ability to express *Vegfa* via a HIF-1 pathway, differences observed throughout the experiment were interpreted as being the result of an absence of this HIF-1 dependent *Vegfa* induction.

Vegfa is a gene capable of influencing expression levels of various other factors, such as *Pdgfb* and *Sdf-1* [50, 51, 85, 86]. The results shown in figures 7 and 8 suggest that HIF-1 is required for basal *Vegfa* expression in skin, itself influencing the cutaneous expression of other transcriptional factors, such as *Pdgfb* and *Sdf-1*. *Hif-1α* mRNA levels were also measured in intact skin and in granulation tissue over the course of wound healing and displayed a similar expression profile when compared to *Pdgfb* and *Sdf-1* (Figure S2). It has been documented that the expression of various genes, such *Pdgfb* and *IGF-1/2*, are capable of upregulating *Hif-1α* expression levels [79, 87]. Therefore, it is possible that the significant difference in *Hif-1α* mRNA levels observed in figure S2 can be explained by downregulated mRNA levels of *Pdgfb* and/or other *Hif-1α* expression influencing genes in $Vegfa^{\delta/\delta}$ mice that were not included in the scope of this study. The results from the molecular profile assay imply that absence of a HIF-1 induced *Vegfa* response alters *Vegfa* expression within the context of skin biology. Given the importance of the HIF-1/VEGFA axis, it was expected that *Vegfa* upregulation in response to the hypoxia caused by wounding would be blunted in mice lacking the HRE within the *Vegfa* promoter however, this was not observed. This result may be explained by two potential hypotheses. It is possible that a HIF-1 induced *Vegfa* response within the context of cutaneous wound healing is not necessary for the production of *Vegfa* in granulation tissue. Another possibility is that alternate *Vegfa* induction pathways,

activated by wounding, must have compensated for the effect of the mutation in the *Vegfa*$^{\delta/\delta}$ mice.

Indeed, many additional factors and signaling processes are known to regulate *Vegfa* expression and may be relevant in granulation tissue. These include but are not limited to EGF, PDGF and IL-1α, which are released in response to wounding [25, 27]. Physicochemical stimuli such as acidosis, hypoglycemia, ROS and mechanical stretching all stimulate *Vegfa* expression [25, 33, 55, 57]. Likewise, the ERK 1/2 pathway induces *Vegfa* expression through the binding of transcriptional factor complexes such as adaptor complex 2 and specificity protein 1 to the *Vegfa* promoter [34, 61].

Wound healing environments are a complex mix of growth factors, cytokines and cell populations [2]. During acute cutaneous wound healing a transient acidosis is present, due to the accumulation of lactic acid, consumption of cellular oxygen and accumulation of local CO_2 [88]. All 4 phases of the wound healing process (hemostasis, inflammation, proliferation, remodelling/scar formation) are subjected to various influences of mechanical forces [89]. In a cutaneous wound, fibroblasts, myofibroblasts, endothelial cells and epithelial cells are affected by mechanical forces acting upon granulation tissue, whether it be intrinsic or extrinsic in nature [89]. Platelet degranulation within the hemostasis phase immediately results in the release of growth factors and cell signaling molecules (i.e. PDGF, EGF, FGF, TGF-β) [2, 6, 8]. Neutrophils dominate the EIP and produce ROS in order to eliminate pathogens and foreign matter [2, 6, 8, 9]. Macrophages dominate the LIP and through their role of repair release various growth factors (PDGF, TGF-β, FGF) [2, 6, 8, 9]. PDGF, EGF, IL-1β and TNF-α are capable of increasing the synthesis of ROS through fibroblast and leukocyte stimulation via Rac1 [57, 59]. The aforementioned text not only demonstrates how these processes/factors induce *Vegfa* expression, but also how they are present within a cutaneous wound setting. In order to demonstrate the importance of the ERK1/2 pathways within the world of cell signaling, the epidermal growth factor receptor (EGFR) signaling pathway within keratinocytes will be studied. In an unstimulated environment, EGFR ligands (TGF-α, amphiregulin, heparin binding-EGF, epiregulin) are continuously being produced and EGFR activation is stabilized via an autocrine/paracrine feedback loop involving the activation of

ERK1/2 [90]. However, in a stimulated environment, an influx of pro-inflammatory cytokines, TNF-α, interferon-γ (INF-γ) stimulate their respective receptors leading to the production of the EGFR ligands previously mentioned, ultimately leading to the activation of ERK1/2. Furthermore, cell-matrix and cell-cell interactions via fibronectin and e-cadherin leads to stimulation of the EGFR and consequently ERK1/2. Finally, keratinocytes respond to the presence of redox stress through the production of intracellular ROS, leading to the inhibition of protein tyrosine phosphatases thereby maintaining an active EGFR resulting in ERK1/2 activation [90]. It can be seen that various circumstances can easily lead to the activation of ERK1/2 within a single cell type, which may potentially result in the activation of *Vegfa*.

Given all the *Vegfa* signal transduction inducers; whether it be acidosis, mechanical stimulation, or chemical stimulation (cytokines, growth factors, ROS) it is plausible to hypothesize that the stimulation of alternate pathways, other than HIF-1, may have ensured adequate *Vegfa* mutant mouse (*Vegfa*$^{\delta/\delta}$) expression if such expression was sub-optimal. Whether this is achieved through the stimulation of ERK1/2 remains to be examined.

The fact that normal *Vegfa* mRNA levels were attained in the granulation tissue of *Vegfa*$^{\delta/\delta}$ mice, attests to the importance of this growth factor in the wound healing setting. Nevertheless, in spite of the normal levels of *Vegfa* expressed by mutant mice in response to wounding, the temporal profile of other cytokines involved in wound healing may have been influenced by the low baseline *Vegfa* levels in intact skin as well as the time required for mutant mice to upregulate the aforementioned alternate *Vegfa*-inducing pathways. Indeed, peak levels of *Pdgfb, Sdf-1* (Figure 8) and *Hif-1α* (Figure S2) mRNA were delayed in mutant mice relative to wild type mice in response to wounding.

Oxygen tension levels do not seem to alter HIF-1α transcriptional and translational rates rather, they influence the stability of this complex subunit leading to its elimination via proteasomal degradation under normoxic conditions [34, 72, 77]. Consequently, measurement of HIF-1α at the protein level (Figure 9B) provides a more accurate portrait of HIF-1's biological activity when compared to the mRNA level (Figure S2). Although hypoxia should, theoretically, not be present in intact skin, figure 9B shows a certain level of basal HIF-1α protein expression in intact skin, as has been reported elsewhere [91, 92] . HIF-1α

protein levels in intact skin did not differ between wild type and *Vegfa*$^{\delta/\delta}$ mice, which refutes the possibility that the observed differences in *Vegfa*/VEGFA expression may be attributed to altered HIF-1α levels, and further supports the conclusion that HIF-1 activity is required for basal *Vegfa* expression in murine skin. Interestingly, the levels of VEGFA angiotropic protein in the skin of *Vegfa*$^{\delta/\delta}$ mice were significantly lower (Figure 9C), further supporting the conclusion that HIF-1 is necessary for basal murine cutaneous *Vegfa* expression. Lower levels of VEGFA protein may potentially lead to aberrant regulation of vessel function, possibly contributing to the observed phenotype, as previously suggested by Oosthuyse *et al* [93] who attributed the smaller birth size and lower weight gain of *Vegfa*$^{\delta/\delta}$ mice to insufficient vascular growth.

It was originally hypothesized that dampened upregulation of *Vegfa* in response to the hypoxia generated by wounding would compromise healing in the *Vegfa*$^{\delta/\delta}$ mice, partly as a result of delays or decreases in angiogenesis and, consequently, deficient granulation tissue. However, based on the molecular data obtained in the first part of this thesis, it then seemed possible that the compensatory induction pathways ensuring sufficient *Vegfa* mRNA levels throughout repair would, in fact, allow healing to progress normally. It was observed with the wound progression assay that the mutant mice did not suffer delays in healing, however the quantity and the quality of the granulation tissue within 7-day-old wounds were deficient.

Observations suggest that decreased basal levels of *Vegfa* and its downstream targets in the intact skin of *Vegfa*$^{\delta/\delta}$ mice resulted in deficient granulation tissue formation and angiogenesis during wound healing. Normal expression of *Vegfa* in the granulation tissue of *Vegfa*$^{\delta/\delta}$ mice likely mitigated this to some extent, resulting in a relatively mild phenotype with no measurable decrease in the rate of wound healing. In summary, it appears that a certain threshold of basal *Vegfa* is required for the formation of optimal granulation tissue in response to cutaneous wounding.

Surprisingly, in spite of defects in the granulation tissue, the wounds of *Vegfa*$^{\delta/\delta}$ mice healed at the same rate as the wounds of wild type mice. This might be explained by the highly contractile nature of wound healing in mice [83]. Wound contraction consists of the

process in which the wound area is decreased in size through the action of fibroblasts and differentiated fibroblasts, myofibroblasts [94]. The granulation tissue within the wound bed plays an important role in the process of wound contraction through the shortening of newly deposited collagen fibers [95]. Wound contraction is an essential process in the healing of a wound via secondary intention, influenced by the location of the wound, the wound size and the mobility of the skin and deeper tissues [94, 95]. Indeed, many groups use stents to prevent wound contraction in their studies of wound healing in mouse models so as to enable the observation of epithelialization defects [83]. The degree of wound contraction between both populations of mice was not directly measured, therefore it is not known whether or not sub-optimal mutant mouse granulation tissue (quantity and capillary abundance) reduced the efficacy of wound contraction. However, due to a lack of insignificant macroscopic changes regarding murine wound healing, the amount of collagen deposited within the granulation tissue of mutant mice was still sufficient to permit an adequate level of wound contraction.

In conclusion, this study provides new evidence suggesting that hypoxic regulation of *Vegfa* is not required for cutaneous wound healing to proceed in mice. However, HIF-1 induced gene expression is necessary for adequate *Vegfa* mRNA levels in intact skin. Although a relative decrease in *Vegfa* mRNA levels in intact skin did not delay wound healing as observed macroscopically, optimal granulation tissue formation and composition was significantly altered. Given that *Vegfa* mRNA levels in granulation tissue were comparable between the two populations of mice, it seems that both the mRNA levels of *Vegfa* as well as its influence on target genes such as *Pdgfb, Sdf-1* (decreased mRNA levels) in intact skin had a significant impact on the initial formation and development of granulation tissue within a cutaneous wound. Furthermore, this study demonstrates the importance of *Vegfa* within a cutaneous wound setting. A certain level of *Vegfa* gene expression redundancy exists and, therefore, it is possible that sub-optimal *Vegfa* mRNA levels in the intact skin of mutant mice led to rescue by alternate non-hypoxic *Vegfa*-inducing pathways, thereby ensuring a normal rate of cutaneous wound healing.

This study presents certain limitations. As previously stated, a portion of wound healing within mice originates from contraction of the wound site [96]. The wounds created experimentally in this study were not stented and therefore the portion of wound healing arising from contraction was not eliminated. The effect of HIF-1 induced *Vegfa* expression on re-epithelization of murine cutaneous wounds was therefore difficult to evaluate and interpret. A HIF-1 induced *Vegfa* response may be necessary for adequate re-epithelialization, however murine wound contraction may have possibly masked this result leading to insignificant differences in the macroscopic rate of wound healing (Figure 10) and histological neo-epidermal coverage (Figure 11). It would therefore be necessary to repeat the experimental protocols with stented wounds in order to fully evaluate the effect of a lack of a HIF-1 induced *Vegfa* response regarding re-epithelialization of murine cutaneous wounds. Furthermore, the quantity of granulation tissue was calculated by considering the amount of surface area measured using computer software. The positioning of the skin samples directly impact the granulation tissue surface area measurements and therefore poor specimen positioning leading to poor measurement recordings is another potential experimental limitation. It is for this reason that the utmost care was taken to properly position the specimens prior to embedding them in paraffin wax in order to reduce error due to poor orientation. The results obtained demonstrate that significant changes in gene expression were found in early stages of wound healing. The timeline chosen for this study was not a limitation in itself as it permitted obtaining gene profile expressions of select genes over a longer period. It is now known that future experiments should include more time points within the early stages of wound healing.

While anti-CD31 antibody is often used to demonstrate the presence of endothelial cells in tissue sections, new capillaries were easily identified on HEPS-stained sections of wound samples in the current study. Furthermore, anti-CD31 staining of histological slides led to inconsistencies in vessel identification (insufficiently or excessively stained) and therefore was not an appropriate technique for slide analysis. In order to reduce the occurrence of capillary count inconsistencies, counting was performed multiple times (5 times in total) and an average count was taken for statistical analyses. In addition, for all

processes of capillary counting, the same criterion was applied; the blood vessel diameter needed to be of adequate size (diameter corresponding to one erythrocyte) and only blood vessels perpendicular to the wound bed were considered new capillaries. Erroneous capillary slide counts would be possible if these criteria had not been applied correctly or if unstained capillaries falling within these criteria were not included. This would lead to a skewed capillary density when considering the number of capillaries per mm^2 of granulation tissue.

Phenotypic differences between wild type and mutant mice were observed. Homozygous mutant mice obtained from $Vegf^{+/\delta}$ breeding pairs were all growth retarded. The weight of mutant mice at the start of experimentation was approximately 35% inferior to that of their wild type counterparts. The skin of mutant mice seemed somewhat flaccid compared to that of the wild type mice, as observed during surgery. Neurological deficits (twitches, uncontrolled movements) were also observed in some mutant mice. In order to improve the experimental design for future experiments, as previously mentioned, cutaneous stents would be necessary. The use of stents would not only eliminate the contribution of wound contraction to wound healing, but would also aid in controlling the phenotypic skin inconsistencies found between both mice. It was observed that the positioning of the skin post-operatively had affected the manner in which the wound healed. As the skin of mutant mice lacked a certain level of rigidity, wounds tended to deform as the wound healed. Deformed wounds would not only alter the evolution of the healing process (altered epithelization and wound contraction) but may have also influenced the histological analyses of this study (granulation tissue surface area and neo-epidermal coverage). Stenting would therefore aid in holding the periphery of the wound margins in place, allowing for a more accurate interpretation of the rate of wound healing and neo-epidermal coverage. Another modification of the experimental design would be to not include any neurologically affected mice, thereby eliminating any possible wound healing influences attributed to neurological deficits.

This study has exposed interesting new advances in cutaneous wound healing research, in particular that basal *Vegfa* expression in skin seems to depend on HIF-1α expression levels. Furthermore, the formation of granulation tissue of optimal quality also

seems to depend on basal *Vegfa* expression, which in turn is dependent on HIF-1α expression levels. It is also possible that pathways other than HIF-1α that directly/indirectly stimulate *Vegfa* expression may be upregulated during the wound healing process in order to compensate for reduced levels of basal *Vegfa* in mutant mice.

As presented in the introduction, the wound healing process is highly dynamic and complex. Cell populations and cytokine/growth factor production vary with time and minor environmental alterations may modify the manner in which a wound heals [2]. For this reason, I believe the next experiment that should be carried out to further understand the data obtained thus far is a DNA microarray. The data suggests that significant differences in mRNA levels (*Vegfa, Sdf-1, Pdgfb, Hif-1α*) were observed in intact skin between wild type and mutant mice, however no significant difference was observed when comparing granulation tissue mRNA levels between both populations of mice. It therefore may be interesting to perform a DNA microarray in intact skin and in granulation tissue at earlier time points, perhaps day 0, 2 and 4. This would allow for the observation of which factors are upregulated/downregulated, at what time point these changes occur and within which population of cells. This data may shed light on the pathways and genes that are truly important for the expression of *Vegfa* within a healing cutaneous wound. Chemically-induced hypoxia using cobalt chloride ($CoCl_2$), was attempted during this study, however Western blot results showed that the $CoCl_2$ trials were ineffective. Possible reasons for this failure include overly thick tissue explants, insufficient concentrations of $CoCl_2$ medium, or an insufficient culture time. In order to determine which pathways may have potentially upregulated *Vegfa* expression with regards to wound healing, the use of specific signal transduction inhibitors in an *in vitro* model would be necessary. Altering the CoCl2 experimentation protocol with the use a ERK1/2 selective inhibitor, FR180204, may be a viable option in researching how gene expression, in particular *Vegfa*, would be altered in the absence of the downstream effects of ERK1/2 [97]. Therefore, tissue explants from both populations of mice would be exposed to culture rich medium necessary for wound healing. This medium would also contain $CoCl_2$ in order to mimic a hypoxic environment as well as the ERK1/2 inhibitor, FR180204. Tissues can then be cultivated at select times in order to

measure *Vegfa* mRNA level variations. It is to be expected that *Vegfa* mRNA levels within mutant mice would remain at sub-optimal levels, comparable to those observed within intact mutant mouse skin, due to the elimination of ERK1/2 *Vegfa* induction.

On a larger scale, due to an aging global population, elevated treatment costs and a proportion of diabetic and obese individuals that continues to rise, the identification of pathways essential for normal wound healing is of upmost importance [18]. The data presented in this thesis provides new information on the manner in which a cutaneous wound responds to a deficient hypoxia-driven *Vegfa* response.

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