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Limiting angiogenesis to modulate scar formation

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ABSTRACT

Angiogenesis, the process of new blood vessel formation from existing blood vessels, is a key aspect of virtually every repair process. During wound healing an extensive, but immature and leaky vascular plexus forms which is subsequently reduced by regression of non-functional vessels. More recent studies indicate that uncontrolled vessel growth or impaired vessel regression as a consequence of an excessive inflammatory response can impair wound healing, resulting in scarring and dysfunction. However, in order to elucidate targetable factors to promote functional tissue regeneration we need to understand the molecular and cellular underpinnings of *physiological* angiogenesis, ranging from induction to resolution of blood vessels. Especially for avascular tissues (e.g. cornea, tendon, ligament, cartilage, etc.), limiting rather than boosting vessel growth during wound repair potentially is beneficial to restore full tissue function and may result in favourable long-term healing outcomes.

Contents

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1. Introduction

Wound repair occurs in every tissue following an injury. The repair process represents one of the most complex biological events, coordinating multiple biological pathways to restore tissue integrity and function. Generally, a scar composed of a disorganised extracellular matrix (ECM) is formed and the original functionality of the tissue is rarely fully restored, potentially resulting in disability [1]. During the process of wound repair, the formation of new blood vessels is imperative for efficient tissue restoration. The presence of a complex vascular bed is vital to the survival of virtually every multi-cellular organism by providing adequate supply with oxygen, nutrients, cells and ensuring the disposal of metabolic waste products. During embryonic development, blood vessels arise *de novo* through differentiation of mesodermal progenitor cells, called angioblasts, which represent the vascular progenitors of endothelial cells. The early vasculature forms by the segregation, migration, and assembly of these angioblasts, a process termed vasculogenesis [2]. Initially, a primitive vascular plexus develops inside the embryo and its surrounding membranes. Angiogenesis, which includes the formation of new blood vessels via sprouting from pre-existing vessels and expansion from pre-existing tubules is responsible for the remodelling and expansion of this network into a complex system that includes arteries, veins, capillaries, and, to a later stage, lymphatics. Finally, through progressive pruning and remodelling a mature circulatory system is established [3]. Ultimately, a fully perfused vascular network is formed which meets the metabolic demands of the tissue. Angiogenesis not only occurs during development but also physiologically in adults during skeletal muscle remodelling [4], during wound healing and tissue regeneration [1]. Overall, angiogenesis and vasculogenesis are not exclusive processes as they constitute complementary mechanisms for postnatal neovascularisation [5,6]. Neovascularisation is critical for successful wound healing and next to vascular endothelial growth factor (VEGF), which is one of the most potent proangiogenic factors, nitric oxide (NO) plays a key role. VEGF increases NO production by increasing the expression of endothelial nitric oxide synthase (eNOS) and further affects endothelial cell migration by decreasing adhesion and organisation. In turn, NO increases VEGF expression in stimulated keratinocytes, resulting in a rapid accumulation of VEGF and NO [7–9]. Dermal endothelial cells respond to VEGF by proliferating and forming capillary tubes and as the capillaries form, endothelial cells expressing endothelial NOS (eNOS) generate even more NO that protects the tissue from hypoxia and ischemia by inducing vasodilation [10].

However, abnormal levels of proangiogenic stimuli potentially also exacerbate scar formation, resulting in dysfunction and pain. Erroneous wound healing not only can manifest itself as delayed healing but also as excessive healing, which is mainly characterised by the deposition of large amounts of ECM and by altered vascularisation and cell proliferation. Generally, the early vasculature formed under situations of high proangiogenic pressure is immature, highly permeable and not fully functional [11,12]. Therefore, during healthy wound healing the initial burst in angiogenesis needs to be tightly regulated as uncontrolled vessel growth can also drive the development of diseases such as arthritis, macular degeneration, psoriasis, and cancer [13]. Further, high vessel ingrowth by high levels of VEGF has been shown to promote scar formation. Interestingly, recent studies suggest that experimental reduction of wound angiogenesis can be beneficial and improve long-term healing outcomes (see Table 1) [14]. Therefore, by controlling the density of blood vessels by therapeutics that partially block this capillary growth might result in a reduced but fully functional vasculature. Moreover, a reduction would potentially both reduce oedema and lessen the need for vascular regression.

The aim of this review article is to give a general overview of the mechanisms driving scar-free and scar-forming healing of wounds in fetal and adult mammalian tissues respectively. Further, we exemplify the potential of limiting scarring by modulating neovessel formation by delivery of antiangiogenic factors to various tissues, including skin, cornea, and musculoskeletal tissues.

2. Adult wound healing

2.1. The phases of wound-healing

Wound healing is a complex and dynamic process which is characterised by four distinct, but overlapping phases: (1) haemostasis; (2) inflammation; (3) proliferation; (4) and remodelling [15,16]. After injury the normal healing response is initiated [17]. Haemostasis results in the formation of a fibrin clot providing a provisional matrix at the site of endothelial injury during the repair process [18,19]. This clot consists of aggregated platelets embedded in a mesh of cross-linked fibrin fibres derived by thrombin cleavage of fibrinogen, together with smaller amounts of plasma fibronectin, thrombospondin and collagen. As activated platelets degranulate, cytokines and growth factors are released. The clot and surrounding wound tissue release pro-inflammatory cytokines and growth factors such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), and epidermal growth factor (EGF). By this early release of growth factors, circulating inflammatory cells, such as neutrophils, macrophages and lymphocytes, are attracted and recruited to the injury site by chemotaxis [18,20,21]. The recruited cells then release another wave of pro-inflammatory cytokines (e.g. tumour necrosis factor [TNF]-α and interleukins) and growth factors (FGF and VEGF) [22,23]. Neutrophils invade the wound site within minutes after injury; next to the initial clearing of invading bacteria and microbes, neutrophils secrete pro-inflammatory cytokines, most likely delivering the earliest signals to activate local fibroblasts and keratinocytes [24]. After several days, neutrophils are phagocytosed by tissue macrophages. Once these are activated, they release a mix of growth factors and cytokines, amplifying the signals released earlier by degranulating platelets and neutrophils. As macrophages clear apoptotic cells, they undergo a phenotypic transition to a reparative state that stimulates keratinocytes, fibroblasts and angiogenesis to promote tissue regeneration, thus driving the transition to the proliferative phase of healing which begins within days after injury [25,26].

The proliferative phase is characterised by capillary growth, collagen formation and the formation of granulation tissue. T-lymphocytes migrate into wounds following the inflammatory cells and macrophages and peak during the late-proliferative/early-remodelling phase. Cellular proliferation and abundant collagen synthesis by fibroblasts support epithelial cell proliferation and migration thereof into the provisional matrix (re-epithelialisation) and subsequent formation of a preliminary tissue [15,21,27].

Table 1
Strategies to modulate angiogenic response in tissue repair.

Author	Tissue	Strategy	Species	Outcome
Savani, Cao et al. [291]	s.c. injection	Active blocking antibodies against RHAMM & CD44	Mouse	Blocked bFGF-induced neovascularisation
Wilgus, Ferreira et al. [134]	Skin	Administration of exogenous VEGF to fetal wounds & neutralisation of VEGF in adult wounds	Mouse	Conversion of scarless to scar-forming phenotype in fetal wounds; reduced vascularisation & scar formation in adult wounds
Galiano, Tepper et al. [120]	Skin	$VEGF_{165}$ in diabetic full-thickness skin wounds of genetically diabetic animals	Mouse	Accelerated wound repair with increased epithelialisation, increased matrix deposition & enhanced cellular proliferation. Upregulation of PDGF-B & FGF-2 in granulation tissue
Romano Di Peppe, Mangoni et al. [122]	Skin	$VEGF_{165}$ gene transfer on excisional wounds of streptozotocin-induced diabetes	Mouse	Accelerated wound closure by promoted angiogenesis
Rossiter, Barresi et al. [124]	Skin	Generation of keratin 5-specicifc VEGF-A deficient mice	Mouse	Well-established skin capillary system of mutant mice; delayed wound healing in adult animals. Keratinocyte-derived VEGF-A might not be essential for skin angiogenesis during embryonic development.
Mori, Kondo et al. [125]	Skin	Generation of β-1,4-galactosyltransferase-I (β4GalT-I) deficient mice	Mouse	Delayed wound healing with reduced re-epithelialisation, collagen synthesis & angiogenesis and reduced VEGF-expression levels.
Canesso, Vieira et al. [292]	Skin	Influence of commensal microbiota on excisional skin wound repair in germ-free (GF) Swiss mice	Mouse	Accelerated wound closure in the absence of commensal microbiota; accelerated wound epithelialisation, decrease in neutrophil accumulation, increase in mast cell & macrophage infiltration in GF mice. Elevated levels of IL-10, VEGF & increased angiogenesis. Reduced scarring & reduced levels of TGF-b1 in GF mice.
Park, Chung et al. [293]	Skin	Effects of low-level light therapy (LLLT) on transplanted human adipose-derived mesenchymal stem cells (hASCs)	Mouse	Enhanced survival of hASCs & stimulated secretion of growth factors
Wang, Han et al. [294]	Skin	Effects of ointment containing HA-fragments in diabetes	Rat	Increased proliferation, migration & tube formation of endothelial cells under high glucose conditions; promoted wound healing by increased angiogenesis
Deodato, Arsic et al. [119]	Skin	Delivery of AAV-vector expressing $VEGF_{165}$ to full thickness excisional skin wounds	Rat	Induction of new vessel formation, reduction of healing time, accelerated remodelling of epidermis & dermis
Liu, Tong et al. [121]	Skin	Liposomal-mediated gene transfer of $VEGF_{165}$ in ischemic skin flaps	Rat	Increased skin flap survival & neovessel formation
Giunta, Holzbach et al. [118]	Skin	Pre-operative $AdVEGF_{165}$ gene transfer in an over-dimensioned ischemic random-pattern-flap model	Rat	Increased skin flap survival & perfusion and decreased necrosis
Takeda, Katagata et al. [123]	Skin	Selective AngII type-1 receptor (AT1) blocker	Rat	Suppressed keratinocyte re-epithelialisation & angiogenesis
Xie, Paras et al. [146]	Skin	Electrospun nanofibrous meshes loaded with VEGF and PDGF-BB as a dual growth factor-releasing nanoparticle-in-nanofiber system for wound healing	Rat	Accelerated wound healing by promoted angiogenesis, increased re-epithelialisation and controlled granulation tissue formation; quicker collagen deposition & earlier remodelling.
Lai, Kuan et al. [147]	Skin	Electrospun collagen (Col) and hyaluronic acid (HA) inter-stacking nanofibrous membranes with programmable release of multiple angiogenic growth factors (VEGF, PDGF, bFGF and EGF) in diabetic skin wounds	Rat	Accelerated wound closure rate, elevated collagen deposition, and increased amounts of mature vessels
He, Zhao et al. [295]	Skin	Cu-doped borate glass microfibers for skin defects regeneration	Rat	Increased angiogenesis, improved collagen deposition, maturity and orientation
Lipp, Bucher et al. [176]	Cornea	Targeting $VEGF_{165}$ in a suture-induced corneal neovascularisation model via topically applied pegaptanib	Mouse	Inhibition of haem- but not lymphangiogenesis
Lee, Leem et al. [296]	Cornea	Subconjunctival injections of bevacizumab after alkali burn injury	Mouse	Bevacizumab significantly decreased neovascularisation and improved corneal transparency
Rush, Bingaman et al. [297]	Cornea	Topical RTKi treatment on EGF-mediated corneal epithelial wound healing	Mouse	Co-administration of RTKi with the synthetic analogue of vitamin K3 (menadione) could minimize blocking of EGFR activity while maintaining the wanted antiangiogenic effects
Abdallah, Louie et al. [298]	Cornea	Topic application of the angiotensin analogue NorLeu3A	Rabbit	Accelerated full-thickness corneal wound healing in a concentration-dependent manner
Kim, Ha et al. [299]	Cornea	Topical application of bevacizumab eyedrops	Human	Reduced corneal neovascularisation (NV) within the first month, increased risk of adverse effects by second month
Dastjerdi, Al-Arfaj et al. [180]	Cornea	Topical application of bevacizumab eyedrops	Human	Short-term topical bevacizumab therapy reduces the severity of corneal NV without local or systemic adverse effects
Kasetsuwan, Reinprayoon et al. [300]	Cornea	Topical bevacizumab used as an adjunctive therapy after excision of primary pterygia	Human	Lower trend for recurrence in the topical bevacizumab group
Ferrari, Dastjerdi et al. [301]	Cornea	Topical application of ranibizumab	Human	Significantly decreased neoangiogenesis and vessel calibre, but not invasion area
Thomopoulos, Harwood et al. [242]	Tendon	Effects of VEGF on canine flexor tendon fibroblasts *in vitro*	*In vitro*	VEGF had no effect on cell proliferation or collagen synthesis, however on endothelial cells
Dallaudière, Lempicki et al. [252]	Tendon	intra-tendinous injection of anti-VEGF mAb bevacizumab® in Achilles and patellar tendinosis	Mouse	Improved and accelerated tendon healing, less collagen fibres disorganisation and decreased neovessel formation

Table 1 (*continued*)

Author	Tissue	Strategy	Species	Outcome
Zhang, Liu et al. [244]	Tendon	Effect of VEGF injection on Achilles tendon healing	Rat	Improved tensile strength in the early stage of healing was associated with increased expression of TGF-β
Sahin, Tholema et al. [247]	Tendon	in situ freezing model of patellar tendon	Rat	Correlation of impaired biomechanical properties with neoangiogenesis as well as VEGF and MMP-3 expression
Hou, Mao et al. [243]	Tendon	TGF-β1 and $VEGF_{165}$ gene transfer on Achilles tendon healing via BMSCs	Rabbit	Deteriorated tendon properties via neovessel formation & destruction of the collagen network in the VEGF-only group. TGF-β1 suppressed angiogenic effects of VEGF when co-expressed.
Sunding, Willberg et al. [249]	Tendon	Sclerosing polidocanol® injections and ultrasound-guided arthroscopic shaving for patellar tendinopathy	Human	Improved tendon structure and diminished colour Doppler local blood flow
Willberg, Sunding et al. [250]	Tendon	Sclerosing polidocanol® injections into areas with vessel ingrowth to treat midportion Achilles tendinosis	Human	Reduced pain and diminished colour Doppler local blood flow
Alfredson, Ohberg et al. [251]	Tendon	Sclerosing polidocanol® injections into areas with vessel ingrowth in Achilles tendinosis	Human	Promising short-term results and reduced pain
Sone, Kawakami et al. [267]	Cartilage	Administration of VEGF-neutralising antibodies for collagen-induced arthritis (CIA) - (anti-human $VEGF_{121}$ antibody)	Mouse	Significant delay in development of arthritis
Lu, Kasama et al. [268]	Cartilage	Administration of anti-VEGF antiserum for collagen-induced arthritis	Mouse	Delayed onset of arthritis, reduced severity, and diminished vWF (von Willebrand factor) content of arthritic joints
De Bandt, Mahdi et al. [270]	Cartilage	Selective blockade of VEGF and its receptors in the K/BxN model of Rheumatoid Arthritis (RA)	Mouse	Suppressed arthritis and prevented bone destruction by targeting the VEGF-RI pathway
Yoo, Bae et al. [271]	Cartilage	Injection of arginine-rich anti-VEGF Hexapeptide (dRK6)	Mouse	Suppression of dRK6 ongoing paw inflammation and blocking of VEGF-induced production of proinflammatory cytokines
Miotla, Maciewicz et al. [272]	Cartilage	Treatment with soluble Flt-1 VEGF receptor (sFlt)	Mouse	Reduced disease severity in CIA, reduced clinical score and paw swelling, reduced joint inflammation and bone and cartilage destruction
Afuwape, Feldmann et al. [273]	Cartilage	Adenoviral delivery of soluble VEGF receptor 1 (sFlt-1) in CIA	Mouse	Suppressed disease activity I CIA, reduced synovial neovascularisation
Semerano, Duvallet et al. [274]	Cartilage	Vaccination against VEGF in CIA	Mouse	Ameliorated clinical arthritis scores, reduced synovial inflammation and joint destruction, reduced synovial neovascularisation
Grosios, Wood et al. [275]	Cartilage	Effects of angiogenesis inhibitor PTK787/ZK222584 models of arthritis and inflammation	Mouse	Anti-arthritic effects mediated by antiangiogenic actions
Choi, Kim et al. [276]	Cartilage	Effects of anti-VEGFR-1 antibody in CIA	Mouse	Suppression of arthritis, reduced synovial neovascularisation
Hah, Koh et al. [277]	Cartilage	Targeting vascular endothelial growth factor A and angiopoietins in CIA by Double-antiangiogenic protein DAAP	Mouse	Inhibitory effect on arthritis severity and bone destruction, reduced neovascularisation
Bainbridge, Madden et al. [281]	Cartilage	Inhibition of methionine aminopeptidase-2 (MetAP-2) via PPI-2458, a selective non-reversible inhibitor of MetAP-2 in CIA	Mouse	Reduced clinical signs of arthritis in both acute and chronic CIA models with decreased joint inflammation and destruction
Grossin, Weber et al. [283]	Cartilage	Effects of angiogenesis inhibitor TNP-470 in a transgenic mouse model of RA	Mouse	Suppressed arthritis and reduced bone destruction
Kubo, Cooper et al. [262]	Cartilage	Blocking VEGF with soluble Flt-1 (sFlt-1)	*In vitro* rat	Improved chondrogenic potential of mouse skeletal muscle-derived stem cells (MDSCs) in vitro. VEGF-transduced MDSCs caused arthritic change in knee joints, and sFlt-1 improved MDSC-mediated repair of articular cartilage by preventing vascularisation and bone invasion into repaired articular cartilage in osteochondral defects.
Ashraf, Mapp et al. [280]	Cartilage	Effects of dexamethasone, indomethacin and angiogenesis inhibitor PPI-2458 on meniscal transection model of osteoarthritis (OA)	Rat	Dexamethasone reduced, indomethacin had no significant effect on total joint damage score. PPI-2458 treatment reduced synovial and osteochondral angiogenesis, synovial inflammation, joint damage, and pain behaviour.
Lazarus, Doyle et al. [282]	Cartilage	Inhibition of MetAP-2 via PPI-2458 in RA	Rat	Reduced ankle swelling, protection against PG-PS-induced arthritis with improvement of bone structure.
Wang, Da et al. [269]	Cartilage	Effect of monoclonal antibody for vascular endothelial growth factor (VEGF) Avastin® on type II collagen-induced arthritis	Rat	Decreased severity of arthritis, decreased serum levels of VEGF and tumour necrosis factor alpha (TNFα), and decreased VEGF expression in the tissue
Nagai, Sato et al. [279]	Cartilage	Effects of anti-VEGF antibody bevacizumab in an osteoarthritis (OA) model of anterior cruciate ligament transection	Rabbit	Increased collagen type 2 expression in articular cartilage, decreased cartilage degeneration and synovitis
Nagai, Sato et al. [278]	Cartilage	Intravenous administration of bevacizumab for osteochondral defect treatment in rabbit knee joints	Rabbit	Improved repair of articular cartilage in osteochondral defects
Nissen, Boucher et al. [284]	Cartilage	Intra-articular bevacizumab administration for relapsing diffuse-type giant cell tumour treatment	Human	Excellent clinical response with reduced pain and improved range of motion after 2 months follow-up; no treatment side effects

Following robust proliferation and ECM synthesis (e.g. collagens, glycosaminoglycans, proteoglycans, etc.) wound healing enters the final remodelling phase, a process which can last for years. Collagen synthesis and turnover continues and differentiation of fibroblasts into myofibroblasts allows for further wound contraction [15]. Vascular density of the wound returns to normal levels through physiological vessel regression. One critical feature for optimal wound healing is the remodelling of the ECM by depositing collagen in a well-organised network to

regain the architecture of the normal tissue [15]. However, generally the collagen of the scar tissue will rarely become as organised as the collagen found in healthy, adult uninjured tissue [28,29]. Clinically, the maturation and remodelling phase is the most important aspect in wound healing, as impairment in matrix deposition can significantly compromise wound strength and quality. In contrast, if excessive collagen synthesis occurs a hypertrophic scar or keloid can form [27].

In summary, for efficient wound healing the aforementioned phases must occur in a well-orchestrated sequence. Haemostatic and inflammatory mechanisms must be intact, mesenchymal cells must migrate to and proliferate within the site of injury; controlled vascularisation and epithelialisation must occur; and collagen must be synthesised and aligned properly.

2.2. Impaired wound healing

When wound healing is not controlled appropriately, the repair process may become pathogenic, with substantial deposition of ECM components in which normal tissue is replaced with permanent scar tissue. There are various factors that can delay wound healing, thus causing impaired tissue repair, or ultimately non-healing chronic wounds [16,30]. Single or multiple factors may play a role in one or more individual phases, contributing to the overall healing outcome. Local as well as systemic factors can cause impaired or prolonged wound healing by influencing one or more phases of the repair process. Local factors such as oxygenation or infection can influence wound healing directly while factors like age, gender, sex hormones, stress, ischemia, and drugs affect the healing process systemically [16].

The presence of oxygen is necessary for nearly all processes in normal wound healing with regard to aerobic cell metabolism and energy production. Both the absence and the presence of oxygen have effects on wound healing. Oxygen prevents infections, induces angiogenesis, increases keratinocyte differentiation and migration, re-epithelialisation, enhances fibroblast proliferation and collagen synthesis, ultimately promoting wound contraction [31,32]. In contrast, ischaemia causes tissue hypoxia, a crucial element for a failed healing response [33]. However, hypoxia also promotes wound healing via hypoxia-inducible factor 1α (HIF1α) and re-epithelialisation. Similarly, the process of lysine and proline hydroxylation during collagen synthesis is driven by oxygen [34,35]. Reduced tensile wound strength can be a consequence of impaired collagen thermal stability due to inadequate hydroxylation and overall hypoxia has also been shown to inhibit collagen synthesis [36]. Finally, although the induction of angiogenesis is known to be stimulated by hypoxia, appropriate maturation of new capillary networks requires normal oxygen tension [31].

One hallmark of impaired wound healing is a disproportionate fibrotic response resulting in excessive scarring due to uncontrolled production, deposition and contraction of extracellular matrix, which can ultimately lead to organ dysfunction and death [37–39]. Typically, fibrosis is a result of chronic inflammation and various cytokines (e.g. interleukin 13 (IL13), interleukin 21 (IL21), transforming growth factor β-1 (TGF-β1)), chemokines (e.g. monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1-β (MIP-1β), angiogenic factors (e.g. VEGF), growth factors (e.g. PDGF), peroxisome proliferator-activated receptors (PPARs), secreted aspartic proteases (SAPs), caspases and components of the renin-angiotensin-aldosterone system (ANGII) have been identified as important fibrotic regulators and are being investigated as potential therapeutic targets [40–43]. However, currently, there is no effective therapy available for fibrotic diseases such as diabetic nephropathy, liver cirrhosis, fibrosarcoma and vascular fibrosis.

By inducing fibroblasts to synthesise and contract the ECM, TGF-β is a central mediator of the fibrotic response [44]. For example, the pro-fibrotic matricellular protein connective tissue growth factor (CTGF) is induced in endothelial cells by TGF-β. Further, TGF-β induces an ED-A domain containing cellular fibronectin, an alternative-splicing variant of fibronectin expressed during wound healing and fibrotic responses, which triggers the enhancement of alpha-smooth muscle actin (α-SMA) and type I collagen expression [45]. α-SMA is generally restricted to cells of the smooth-muscle lineages, but also fibroblasts have been shown to transiently express α-SMA, which are then referred to as myofibroblasts. Similarly, pericytes expressing contractile smooth muscle actin are ascribed an active role in vascular development and angiogenesis [46]. TGF-β1 is commonly reported to differentiate vascular progenitor cells into pericytes and vascular smooth muscle cells (vSMCs), which are both crucial for successful blood vessel regeneration [47]. Interestingly, type I collagen itself has been reported to have a strong angiogenic activity. It constitutes an optimal substrate for angiogenesis and may thus be considered as the main intermediate matrix constituent driving angiogenesis in mammalian tissues [48–50].

When injected subcutaneously in mice, TGF-β drives the excessive synthesis of extracellular matrix proteins. However, results remain controversial as TGF-β has also been shown to suppress collagen synthesis [51] and while TGF-β seems to promote fibrosis *in vivo* in mice, TGF-β1-deficient mice display an impaired late-stage wound healing phenotype. In addition, treatment of fetal wounds with TGF-β promotes wound closure but also scarring. Finally, treatment of incisional wounds in rats with anti-TGF-β antibodies results in suppressed ECM synthesis [52–56].

3. Scarless fetal wound healing

Research on scarless fetal wound healing has predominantly been performed using skin injury models. Fetal skin wounds heal in a regenerative manner in so far as they restore normal skin architecture including the epithelium and accessory elements such as hairs and glands [57]. This regenerative capability has been demonstrated in all mammalian species studied to date, including humans, rats, sheep, opossums, and monkeys [58–60]. By comparing adult to fetal scarring, it has been shown that in rodents between fetal stage 16 and fetal stage 19 a transition occurs from scarless healing to scar formation following injury [59,61–63]. In humans, the window in which the ability to fully regenerate shifts to a solely reparative wound closure is considered to take place at the end of the second trimester of pregnancy [64]. Not only gestational age, but also the size of the wound affects the repair outcome, with larger wounds requiring repair at an earlier gestational age for scarless healing to take place [65–67].

Several studies have demonstrated that fetal wound healing differs from adult wounds in inflammatory responses, extracellular matrix components, growth factor expression, and overall gene expression profiles (see also Fig. 1) [68–70]. There seems to be consensus that an attenuated immune response is beneficial for scarless healing. Not only is the infiltration of neutrophils greatly diminished but also the number of tissue macrophages is decreased. The paucity of mast cells present is less mature, and they fail to degranulate in response to injury when compared with scar-forming wounds during late gestation [71]. Interestingly, injection of mast cell lysates into E15 wounds in mice disrupted scarless healing, whereas wounds produced at E18, which normally heal with a scar, healed with significantly smaller scars in mast cell deficient $Kit^{W/W\text{-}v}$ mice compared to $Kit^{+/+}$ littermates [71]. Further, quantification and comparison of the immune cells and their chemokines between unwounded human fetal (18–22 weeks) and adult skin (16–56 years) revealed a significant reduction of CD68 positive macrophages and tryptase-positive mast cells in the fetal skin [72]. Moreover, fetal skin contained significantly lower levels of lymphocyte chemoattractants such as the C—C motif chemokine ligands CCL17, CCL21, and CCL27. Since fetal lymph nodes at this gestational age contain sufficient levels of CD45.1-cells comparable to adult lymph nodes and since there is no difference in the vascular network between fetal and adult skin as evidenced by CD31 staining, the

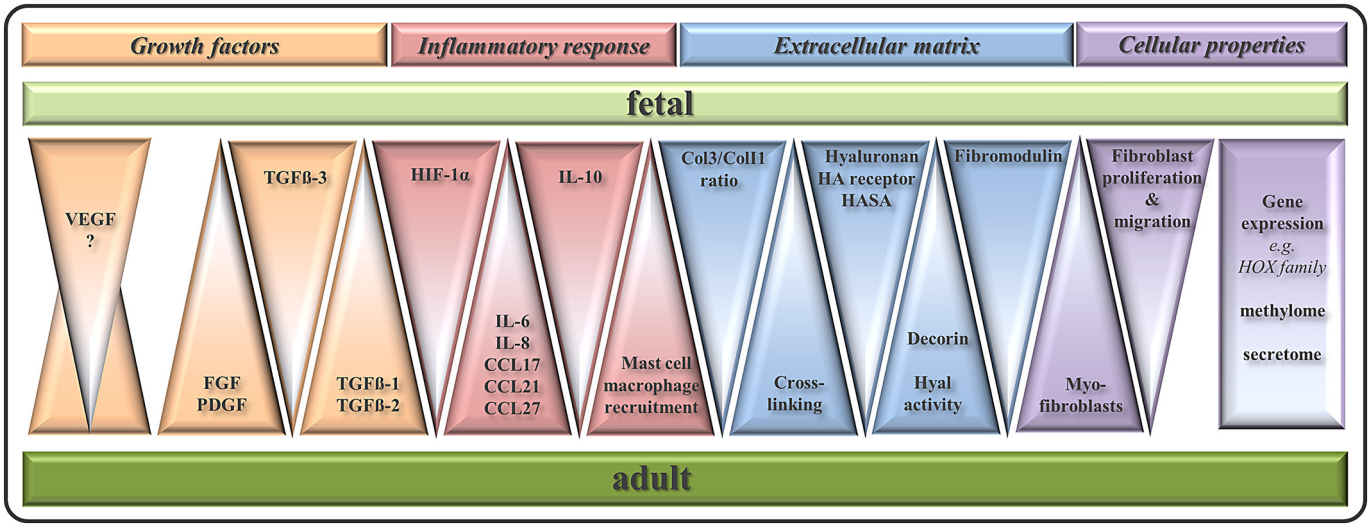

Fig. 1. Molecular and cellular differences between fetal and adult wound healing. Scheme illustrating factors and cellular properties differentially regulated during the transition from scar-free fetal to scar-forming ("adult–like") wound healing. While most factors related to proliferation, inflammation or matrix composition either show a marked increase or decrease (symbolised by triangles) a shift in the overall gene expression/gene methylation and secretome occurs during the transition period involving a large set of genes and proteins. Their impact on scarless healing remains to be fully understood.

attenuated inflammatory response seems to be a result of decreased cell recruitment [72]. In a similar manner Cowin et al. observed that fewer polymorphonuclear leucocytes, macrophages and lymphocytes migrate into fetal skin wounds, although these cells are capable of responding to inflammatory signals in the same manner as in adult wounds [73]. The reduced cellular recruitment and inflammation seen in fetal wound healing may in part be attributed to the observed diminished IL-8 production and IL-8 response to PDGF by human fetal fibroblasts demonstrated *in vitro* and *in vivo* in SCID mice [74]. Strategies to target IL-8 production might therefore present a possible treatment option to modulate the immune response, thus improving wound healing.

Additionally, fetal wounds have been shown to be deficient in IL-6, another pro-inflammatory cytokine involved in monocyte activation and infiltration [57]. In contrast, the anti-inflammatory cytokine IL-10, a major regulator in suppressing the inflammatory response is significantly upregulated during scarless healing [75]. Along these lines, IL-10 overexpression in an adult model of scar formation decreases inflammatory mediators and promotes regenerative healing, whereas wounds in IL-10 knock-out skin grafts showed significant inflammation and scarring [76–78].

Next to differences in immune-regulatory profiles, growth factor expression differs markedly between fetal and adult skin wound healing. In a rat skin wound model, scarless healing correlated with a marked downregulation of FGF isoforms 7 and 10, whereas FGF receptor 2 expression decreased in both scarless (gestational day 16.5) and scar-forming (19.5) wounds [79]. The association of scarless wound healing with downregulation of FGF-7, FGF-10 and IL-8 expression offers therapeutic potential to reduce scarring by altering the wound cytokine and growth factor profile to a profile found during fetal wound healing. A similar beneficial effect on scar reduction as observed by application of neutralising antibodies to both TGF-ß1 and TGF-ß2 to adult excised skin wounds might be achieved by manipulating the activity of FGF-7, FGF-10 and IL-8 in the wound environment [80].

From the 3 known transforming growth factor β isoforms, TGF-ß3 is most abundantly expressed in the embryo, gradually declining during the further course of development with a concomitant increase in the expression of TGF-ß1 and 2 [67,81]. In this context it is interesting to note that TGF-β3 can interact with hypoxia inducible factor 1α (*HIF1A*), a transcription factor known to show a stronger expression in fetal wounds, thereby delaying the immunological response [82]. Evidently, experimental reduction of TGF-β1 levels in adult rat skin wounds using a neutralising antibody markedly improves scarring [80] and the addition of human recombinant TGF-β3 has been demonstrated to reduce scarring in animal models and man [83–86]. However, further investigations have been terminated as phase III clinical trials did not demonstrate an efficacious reduction in skin scarring after application of human recombinant TGF-β3 (Juvista®, Clinical trial NCT00742443).

The capacity for scarless repair has been demonstrated to be intrinsic to fetal tissue and perhaps more specifically to the unique aspects of fetal ECM and the fibroblasts that produce it. Remarkably, transplantation of adult sheep skin into a fetal lamb and subsequent incisional wounding of both the skin graft and the adjacent fetal skin resulted in scar formation of the grafted, but scarless healing of the fetal skin [87]. Similarly, transplantation of fetal tendon into an adult environment responded to injury in a manner intrinsic to fetal tissue [88]. These findings suggest that cell intrinsic properties seem to overrule the fetal environment as the impetus behind the scarless healing phenotype [87]. These cell intrinsic properties not only comprise a unique gene expression profile, but also exhibit a distinct proliferative and migratory behaviour. Fetal dermal fibroblasts displayed a higher proliferation potential compared to adult fibroblasts and fetal anterior cruciate ligament (ACL) fibroblasts migrated twice as fast as adult ACL fibroblasts and produced four times the amount of collagen I compared with adult ACL fibroblasts after 7 days in culture [89,90]. *In vivo*, fetal fibroblasts introduced into an Achilles tendon defect in mice showed a significantly higher expression level of tendon-related extracellular matrix including collagen III and biglycan, the fetal cell sheets sutured to the tendon defect leading to significantly improved mechanical properties compared with grafted adult fibroblasts [89]. Hence, especially the composition of the ECM seems to play a crucial role in wound regeneration. Several major ECM components including collagen and hyaluronan have been indicated to contribute to the remarkable scarless healing ability of fetal skin [91,92]. Compared to adult wounds, fetal lamb skin wounds not only showed increased collagen synthesis with a larger proportion of non-crosslinked forms of collagen, but also a faster deposition of collagen [93]. Conversely, decorin, a proteoglycan known to regulate collagen fibrillogenesis, showed reduced expression in rat fetal scarless skin wound healing [94].

Hyaluronic acid (HA), a glycosaminoglycan found in high concentrations whenever rapid cell movements and proliferation occur, is highly

abundant in the fetal ECM [95,96]. In comparison to adult fibroblasts, fetal fibroblasts exhibit a two- to four-fold increased density of the cell surface HA receptor [97]. In comparison to adult wounds prolonged presence of HA in the matrix of fetal wounds appears to create a 'permissive' wound environment that promotes fetal fibroblast movement and proliferation and inhibits cell differentiation [96]. Enzymatic degradation of hyaluronic acid in fetal rabbit wounds resulted in an adult-like healing response with marked increases in fibroplasia, collagen deposition, and neovascularisation [98,99]. The authors of the study attributed this effect to the generation of biologically active hyaluronic acid degradation products (HADPs), known to be angiogenic and tested their hypothesis by treating fetal wounds with HADPs and different control solutions. Implants from wounds treated with a homogenate showed a dramatic infiltration of fibroblasts and capillaries within a dense collagenous matrix, resulting in a significantly greater neovascularisation when compared to implants treated with control solutions [98].

Expression of fibromodulin (FM), another integral ECM protein, seems to be inversely correlated with scar formation during both fetal skin development and adult wound repair [100]. There is evidence that a significant decrease in expression of fibromodulin, a small leucine-rich proteoglycan involved in collagen assembly, is associated with the transition from scarless fetal-type to adult-type repair with a scar [100]. Whereas fibromodulin expression significantly increased 36 h after injury in rodent E16, it did not in E19 wounds. Thus, abundant fibromodulin levels are associated with scarless fetal repair, whereas "adult repair" is relatively deficient in fibromodulin [100]. Since relative fibromodulin abundance in fetal wounds may affect collagen fibrillogenesis and architecture, a lack of fibromodulin may also account for the compromised regulation of ECM assembly in adult wounds. Fibromodulin loss- and gain-of-function wound models revealed that its loss resulted in fetal rodent skin wounds to heal with a scar. Moreover, administration of fibromodulin protein prevented scar formation in E18 rats, indicating that the addition of fibromodulin alone was sufficient to regenerate scarless, fetal-type wound healing in late-gestation animals. Similarly, gene delivery of fibromodulin was found to promote rat Achilles tendon repair *in vivo* and *in vitro* [101]. However, besides fibrillogenesis, fibromodulin promotes *in vitro* and *in vivo* angiogenesis. FM-deficient mice exhibit significantly reduced blood vessel regeneration in granulation tissues during wound healing [102,103]. Given the proangiogenic effect fibromodulin exerts, these findings indirectly point towards a beneficial effect of angiogenesis during the scarless wound healing process.

Generally, the role of angiogenesis in scarless healing is still controversially discussed. Using a mouse skin injury model, Wilgus et al. found that scarless fetal wounds had lower levels of VEGF and were less vascular than fibrotic fetal or adult wounds [104]. Further, the addition of exogenous VEGF converted the scarless phenotype to a scar-forming one, whereas antibody-mediated neutralisation of VEGF led to a reduction of vascularity and scar formation in adult wounds. In contrast to this finding, Colwell et al. reported an increased expression of VEGF during scarless repair in fetal E16 rat skin when compared to E18 animals [105]. Recently, an unbiased and quantitative proteomic study demonstrated an increased angiogenic response induced by the secretome of human fetal skin fibroblasts. The authors compared multipotent fetal dermal cells (MFDC) with adult dermal cells (ADC) and identified ECM remodelling factors and neoangiogenesis modulators more prevalently to be released by MFDCs [106]. Further, they show that the MDFC secretome stabilises and more efficiently induces the formation of capillary-like networks by endothelial cells *in vitro* [106]. Finally, since angiogenesis is also initiated by macrophages producing proangiogenic factors (see further above), reduced macrophage recruitment to the fetal wound site likely also correlates with reduced angiogenesis.

In summary, the shift in ECM composition during the transition from a fetal scarless to an adult scarring wound phenotype and the corresponding pro- or antiangiogenic effects illustrate vividly the intricate balance of the factors involved in the wound healing process (see Fig. 1). Conflicting data on the expression level of different ECM components and their receptors and ambiguity about the degree of vascularisation during scarless wound healing reflect the complexity of the system.

4. Cutaneous wound healing

4.1. Cutaneous scarring

The skin provides the protective physical barrier of the body and has therefore developed intrinsic mechanisms to protect the organism from a wide range of external insults to enable rapid restoration of tissue integrity and organ-specific function [107–109]. As already discussed, the highly dynamic process of wound healing involves a complex sequence of cellular and biochemical events including the induction of acute inflammation, the rapid proliferation of reparative cells and the formation of a permanent scar composed of fibro-proliferative tissue rich in immature collagen and newly formed blood vessels [110]. Cutaneous scarring is defined as a macroscopic disturbance of the normal structure and function of the skin architecture. Prevention or reduction of scarring after surgery still remains a major goal of plastic surgery and aside from the psychological and social detriments associated with prominent scars, scar tissue can cause restricted joint mobility, impaired growth and loss of organ function. Hope for future therapies lies in the molecular basis of wound healing, for example in blocking "pro-scarring" molecules such as TGF-β1 and -β2, and promoting TGF-β3 and mannose-6-phosphate aiming at creating an environment closer to fetal healing mechanisms [111–113].

Angiogenesis is a key event for cutaneous wound repair to occur. It is stimulated early during the healing response by macrophages through the release of FGF-2, angiopoietin, VEGF-A, and TGF-β [114]. In addition, various matrix metalloproteases (MMPs) play a critical role during angiogenesis, facilitating remodelling of the ECM and allowing sprouting [114–117]. In addition to growth factors and cytokines produced during the early inflammatory phase of wound healing, changes in the tissue environment, such as increased lactate, decreased pH and low oxygen tension also stimulate and regulate angiogenesis. In models of impaired wound healing or severe injury, stimulation of angiogenesis is generally reported to positively affect healing rates [118–122], whereas reduced or inhibited angiogenesis is known to impair these outcomes [123–126]. Nevertheless, several more recent studies show no or only little effects of epidermal healing or wound closure rates by modulation of angiogenesis and studies have even reported improved healing by reducing angiogenesis [104,127,128]. Overall, the importance of neoangiogenesis in the repair process is well documented, its role in promoting scar formation remains less well understood.

4.2. Targeting wound healing and scar formation via VEGF

A plethora of studies demonstrate a pivotal role of VEGF-A during cutaneous wound repair and a detailed description of the studies goes beyond the scope of this review article. For example, Galiano et al. [120] examined the effect of recombinant human $VEGF_{165}$ protein on full-thickness skin wounds in genetically diabetic mice (*db*/*db*). They demonstrated accelerated repair in VEGF-treated wounds with increased epithelialisation, increased matrix deposition and enhanced cellular proliferation after 12 days and an upregulation of PDGF-B and FGF-2 in the granulation tissue. They conclude that topical VEGF is able to improve wound healing by locally up-regulating growth factors important for tissue repair and by systemically mobilising bone marrow-derived cells, including a population that contributes to blood vessel formation. In another study, VEGF-A was specifically inactivated in keratin 5-expressing tissues in mice [124]. Interestingly, the skin capillary system was well established, demonstrating that keratinocyte-derived VEGF-A is not essential for angiogenesis in the skin during

embryonic development. However, healing of full-thickness wounds in adult animals was considerably delayed compared with controls. The authors concluded that keratinocyte-derived VEGF-A plays an important, albeit nonessential role during epidermal wound healing and seems crucial for the development of epithelial skin tumours. Deodato et al. investigated the efficacy of gene therapy of wound healing with an adeno-associated virus (AAV) vector expressing $VEGF_{165}$ [107]. Delivery of $VEGF_{165}$ to full thickness excisional skin wounds in rats resulted in a remarkable induction of new vessel formation, with consequent reduction of the healing time and accelerated remodelling of epidermis and dermis.

However, VEGF may not simply function as a mediator of wound-mediated neoangiogenesis, but instead most likely plays a more diverse role in the wound repair process, potentially influencing scar tissue formation. In the early proliferative phase of wound healing angiogenesis is clearly favoured whereas anti-angiogenic stimuli become more prominent in the subsequent remodelling phase. Blood vessel growth and subsequent regression occurs through competing signals derived from the highly dynamic wound microenvironment acting upon endothelial cells (ECs) in a spatio-temporally controlled manner [129]. The production of pro-angiogenic factors decreases as the oxygen tension within the healing tissue normalises. ECs that have been continually stimulated by pro-angiogenic factors begin to produce and activate inhibitors of angiogenic signalling pathways in a negative feedback mechanism, triggering a so-called "anti-angiogenic switch". As a consequence, pro-apoptotic signalling pathways are activated which in turn lead to systematic EC death and vessel regression [130].

Overall, VEGF/VEGFR-2 signalling, non-canonical WNT signalling, and blood flow-induced signalling are involved in the control of vessel regression. Canonical WNT signalling stabilises the vascular network and promotes EC proliferation and DLL4/NOTCH signalling supports vessel regression by promoting vessel constriction and flow stasis. Whereas ANG1 supports EC survival, ANG2 destabilises the vascular network, driving it into regression in the absence of survival factor activity (e.g., VEGF) [131]. These finely coordinated processes result in an optimally distributed vessel network that ensures an adequate supply of the healing tissue. However, to date very little is known about the endogenous anti-angiogenic mechanisms and stimuli that are responsible for preventing excessive neovascularisation [132]. When tissue has reached homeostatic levels, basal levels of VEGF support the survival of the vascular network in an autocrine fashion by maintaining critical anti-apoptotic AKT signalling in ECs. However, the quiescent vasculature is protected from VEGF overstimulation via modification of signalling events by VE-Cadherin, including its deactivation of overexpressed VEGFR-2 [133]. Importantly, VEGF levels correlate with the amount of scar tissue produced in mouse models of fetal and adult wound healing [129]. For example, systemic treatment with neutralising VEGF antibodies led to a reduction in scar size and normalisation of the collagen fibril structure in adult incisional wounds [134]. Further, abnormal scars, such as hypertrophic scars [135,136] and keloids [137,138], have been shown to express excess levels of VEGF. It is therefore possible that excessive VEGF promotes scar tissue formation by multiple mechanisms such as altering ECM homeostasis towards a state of impaired degradation and excessive accumulation [138]. Also, inhibition of angiogenesis has been reported to be effective in treatment of malignancy of keloids [137]. Along these lines, one remarkable common feature of oral and fetal wounds, both known to heal in a scarless fashion, is a significantly reduced angiogenic response [104,139]. Further, scarless fetal wounds express lower levels of VEGF and by adding exogenous VEGF a scar-free phenotype can be converted to a scar-forming phenotype.

As granulation tissue is converted into mature scar tissue a regression of excessive blood vessels occurs over time. This trimming of the vascular network is called "vessel pruning" which is triggered by the shut-off of pro-angiogenic signals (e.g., VEGF) as well as the active engagement of anti-angiogenic signals (e.g., ANG2) [131]. Eventually the number of vessels normalises and returns to a level close to what is observed in uninjured skin [140]. Although not much is known about how vessel regression is regulated in the skin, an association between increased angiogenesis and scarring is notable. As the matrix remodels from a provisional to a more mature composition, the biomechanical properties of the matrix favour the anti-angiogenic phenotype. It is therefore tempting to speculate that the ECM rearrangements during these events can promote scar formation and therefore targeting these events could be exploited to develop novel treatment modalities.

4.3. Drug delivery to skin

The effectiveness of topic application of biotherapeutics, such as growth factors or antibodies, is often limited due to restricted absorption, low stability, and effective elimination prior to penetrating the wound area. Consequently, a large array of biocompatible biomaterials has been developed to allow delivery in a spatio-temporal manner and to improve the bioactivity of the released factors. These include polymeric micro- and nanospheres, lipid nanoparticles, nanofibrous structures, hydrogels and scaffolds [141,142]. Micro and nanospheres are promising systems for the controlled release of peptides and proteins and resemble colloidal systems prepared using natural or synthetic materials, such as poly lactic-*co*-glycolic acid (PLGA), alginate, gelatine, chitosan, as well as other polymer combinations [143]. Further, ECM-inspired matrices have been developed to deliver drugs to skin (and other) wounds. As they can resemble the structure of the native human extracellular matrix on the meso- and microlevel, electrospun fibres have gained increasing interest as therapeutic devices for skin wounds. Such multifunctional wound dressing systems are supposed to provide physical and mechanical protection, prevent invasion of bacteria, allow wound exudate absorption, gas and fluid exchange, maintenance of flexibility and easy removal without adhesion and good biocompatibility [144]. Different materials as well as fabrication techniques are currently applied, including approaches for incorporation of drugs into or drug bonding onto the fibre surface allowing appropriate therapeutic dosing. Generally, they can be loaded with different classes of drugs such as antiseptics, small molecules (e.g. antibiotics, anti-oxidants, anti-inflammatory agents, signalling molecules), macromolecules (e.g. lysozyme, growth factors, plasmids), or cells [145]. For example, Xie et al. [146] developed a dual growth factor-releasing nanoparticle-in-nanofiber system for wound healing. Therefore, VEGF was loaded within nanofibers and platelet-derived growth factor-BB (PDGF-BB)-encapsulated poly(lactic-*co*-glycolic acid) nanoparticles were embedded inside nanofibers, resulting in different release kinetics of the growth factors. In a similar fashion, another group aimed at delivering epidermal growth factor (EGF) and basic FGF for the promotion of early stage epithelialisation and angiogenesis, followed by the release of PDGF and VEGF to assist blood vessel maturation [147].

Taken together, sophisticated drug delivery systems hold promise not only to deliver proangiogenic cues but also to modulate and/or limit neoangiogenesis formation and potentially promote scar-free healing.

5. Corneal scar formation

5.1. The cornea – an angiogenic and immune privileged tissue

The cornea accounts for about 70% of the eye's refractive power and its transparency together with the highly defined curvature allow light to be focused and transmitted to the retina. Therefore, tissue integrity is of particular importance for clear vision and injuries caused by physical impact, chemical insult, or by severe infections can lead to permanent corneal damage, accompanied by opacification and loss of visual acuity [107]. The cornea has five layers (see Fig. 2): the outer epithelium which protects the cornea from the outer world, the Bowman's (basement) membrane, the stroma, Descemet's (basement) membrane, and

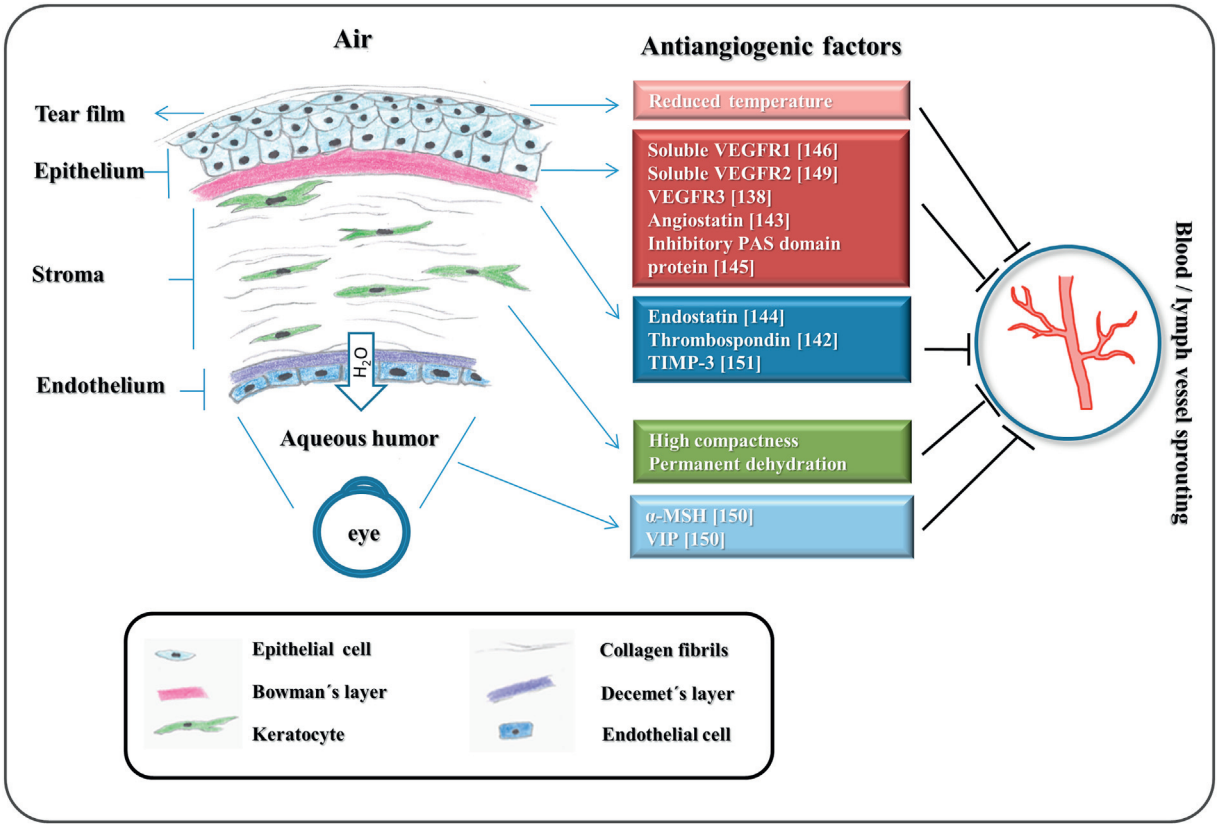

Fig. 2. Corneal anatomy and associated antiangiogenic factors. The cornea comprises 5 layers, the outermost is the epithelium covered with a tear film. The production of soluble VEGFR subtypes within the epithelium leads to sequestration of VEGF and leads, in together with Angiostatin and Inhibitory PAS domain protein to blocking agiogenesis. The Bowman's layer is a basement membrane produced by the epithelium, and exerts antiangiogenic activity by harbouring Endostatin and Thrombospondin-1. The underlying stroma is chacterized by tightly packed collagen fibrils produced by keratocytes. It is constantly dehydrated by the corneal endothelium, actively pumping water to aqueous humor. The auqueous humor also exerts antiangiogenic activity by containing alpha mealnocyte stimulating hormone (α-MSH) and vasoactive intestinal peptide (VIP).

the inner surface endothelium which is in close contact to the aqueous humor, a clear liquid filling the anterior chamber of the eye [148]. The cornea is avascular by nature and does not respond with hem- or lymphangiogenesis to minor inflammatory or other proangiogenic stimuli. This state is referred to as “angiogenic privilege” [149]. Moreover, the cornea is considered to be an immune privileged site, allowing corneal transplantation without graft rejection, making it one of the most transplanted organs with nearly 50.000 transplantations in 2015 in the USA and over 12 million people on waiting lists worldwide [150,151]. The cornea is not only an immune-privileged *site* but also an immune-privileged *tissue*, which resists destruction by the immune system as shown by relatively long survival when transplanted into non-immune-privileged sites [152]. When grafted into a heterotopic site, the alloimmunogenicity of the normal cornea resides within its epithelial and stromal layers, whereas immune privilege was shown to arise from the endothelium. In analogy, the cornea is also an angiogenic privileged *tissue* as such, as it remains avascular when heterotopically transplanted to vascularised sites [152].

A broad spectrum of mechanisms is already known to contribute to these inter-dependent privileges (see Fig. 2): Several redundant, antiangiogenic factors have been localised within the cornea, especially at the inner and outer basement membranes and endothelial/epithelial cells. These include thrombospondin-1 [153], pigment epithelium-derived factor, antiangiogenic extracellular matrix breakdown products such as angiostatin [154] and endostatin [155] as well as receptor antagonists (e.g. interleukin-1 receptor antagonist). Makino et al. have shown that Inhibitory PAS domain protein is expressed in the corneal epithelium, which acts as an inhibitor of hypoxia inducible transcription factors. Thereby, this factor also negatively regulates VEGF-signalling, contributing to the angiogenic privilege [156]. In addition, the aqueous humor and the epithelium seem to contribute to the angiogenic privilege of the cornea by sequestering growth factors. In this regard, it was demonstrated that the corneal epithelium expresses soluble forms of the three major vascular endothelial growth factor (VEGF) receptors (sVEGFR-1, sVEGFR-2, sVEGFR-3), which are assumed to act as decoy receptors to trap VEGF-A, VEGF-C and VEGF-D, thereby contributing to maintaining corneal avascularity [149,156–160]. α-Melanocyte stimulating hormone (α-MSH) and vasoactive intestinal peptide (VIP) were also shown to be present in the aqueous humor and to exert antiangiogenic activity [161,162].

The immune privilege is also a consequence of the absence of blood- and lymphatic vessels in the healthy cornea. Whereas the lack of blood vessels prevents the entry of immune effector cells (e.g., CD4+ alloreactive T lymphocytes, memory T lymphocytes), the lack of corneal lymph vessels blocks the exit of antigenic material or antigen-presenting cells (APCs). However, although the absence of lymphatic-drainage pathways in the eye is indeed important for shielding ocular antigens from the immune system, it does not solely account for immunological ignorance [163]. For example, corneal cells lack MHC class II antigens and the expression of MHC class I antigens is reduced, especially in corneal endothelial cells. This is thought to be due to persistent silencing of the gene encoding the class II transactivator protein [163]. As for the angiogenic privilege, the aqueous humor also plays a major role in suppressing pro-inflammatory processes. High levels of TGF-ß2 lead to reduced macrophage, T-cell and NK-cell activation and confers tolerance to antigen presenting cells [164]. Further, vasoactive intestinal peptide (VIP) inhibits T-cell activation and differentiation [165,166] and α-melanocyte stimulating hormone (α-MSH) in aqueous humor

prevents responding T-cells from secreting pro-inflammatory cytokines (such as IFN-γ) and drives conversion of IFN-γ producing T-cells into regulatory T-cells [167].

5.2. Mechanisms of corneal wound healing and scar formation

Corneal wound healing involves a complex cascade of cellular events and is orchestrated by a complex network of growth factors. Damage to the cornea can result in opacification and impaired vision. However, smaller and superficial wounds often heal with "*restitutio ad integrum*". The exact mechanisms leading to full restoration or scarring of the injured cornea remain poorly understood. Interestingly, the different corneal layers have different capacities and mechanisms to regenerate after injury or to form scars, respectively. The epithelium, the layer most prone to injury, is constantly self-renewing and has the highest regenerative capacity as epithelial cells are replenished every 7–10 days. This self-renewal is based on a population of corneal epithelial stem cells derived from the limbal palisade, the transition zone between cornea and sclera. From there, stem cells migrate towards the central cornea and maturate towards functional corneal epithelial cells [168]. A variety of growth factors, such as EGF, TGF-β, hepatocyte growth factor (HGF) and keratinocyte growth factor (KGF) are involved in epithelial corneal healing [169]. Damage of this limbal stem cell reservoir, e.g. by chemical trauma, leads to overgrowth of the adjacent conjunctiva, indicating that the limbus is not only a stem cell source but also an important barrier maintaining compartmentalisation of the eye [107].

Corneal scaring usually occurs, if next to the epithelium also the underlying stroma is damaged. Particularly any damage to the basement membrane increases the risk of scar formation. Corneal injury extending into the corneal stroma causes fluid influx and fibrin deposition, leading to swelling and subsequent local loss of transparency. During normal corneal stromal repair, fibroblasts and myofibroblasts deposit multiple elements of the ECM including type III collagen, fibronectin, tenascin C and glycosaminoglycans, facilitating the migration of fibroblasts [107]. If these fibroblasts and myofibroblasts remain active, excessive matrix deposition and fibre misorientation lead to opacification. The main challenge in stromal wound healing is therefore to restore the exceptionally regular collagen organisation, which is initially lost at the site of injury.

The endothelium has the lowest mitotic activity and lowest regenerative capacity among all corneal layers. Small injuries of this layer lead to endothelial cell migration towards the site of damage and to cellular enlargement. An alternative response to injury is observed, when the endothelial cells undergo endothelial-to-mesenchymal transition (EnMT), resulting in excessive proliferation, loss of cell-cell contacts, cell polarity and potentially to deposition of ECM, again leading to opacification. This process has been shown to rely on TGF-β, FGF-2, IL-1β and involves NFκB activation [107,170].

Inflammation is a common response to damage of all corneal layers. Contrary to general belief, the healthy cornea indeed harbours CD45+ leukocytes, which are constantly renewed and originate from the bone marrow [171,172]. Moreover, potentially antigen presenting Langerhans cells were described to reside within the cornea [173]. Generally, corneal inflammation is a very complex process involving virtually all cell types present in the tissue and is driven by a large variety of growth factors. Briefly, immune cell recruitment after corneal injury is mediated by pro-inflammatory cytokines released from epithelial cells and corneal keratocytes at the injured site. Being attracted by Il-1, Il-6 and TNFα and several other cytokines, leukocytes enter the stroma from the limbal blood vessels and migrate towards the wound site. After the initial wave of neutrophil invasion, macrophages extravasate from the limbal vessels, infiltrate the stroma from superficial to deeper layers and migrate towards the corneal centre [174]. These macrophages remove debris and apoptotic cells at the wound site. However, they have also been shown to be mediators of angiogenesis after severe and prolonged corneal injury by excessive production of VEGF-A, -C and -D [175].

5.3. Angiogenesis as pharmacological target to prevent corneal scar formation

5.3.1. Targeting VEGF-signalling

Pathologic ingrowth of blood and lymphatic vessels into the cornea not only reduces visual acuity directly but also is the main risk factor for immune-mediated graft rejection after corneal transplantation. VEGF-A plays a central role in the induction of pathological corneal neovessel formation and binds to the VEGF receptors VEGFR-1 and -2, thus mediating not only hem- but also lymphangiogenesis [176]. Therefore, inhibition of VEGF-A signalling is considered to be a promising strategy for prevention of vessel ingrowth into the cornea and antibody-mediated inhibition of this pathway is already in clinical use. The most commonly used inhibiting antibodies targeting VEGF-A signalling are bevacizumab and ranibizumab. Both recognize all VEGF isoforms, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$ [177], whereas the aptamer pegaptanib only binds to $VEGF_{165}$ inhibiting hemangiogenesis in the retina as well as in the cornea [178]. Topical application of bevacizumab eye drops has been shown to effectively reduce corneal neovascularisation after injury or pterygium surgery without adverse systemic effects [179–183]. Similar results were obtained with ranibizumab eye drops [184]. However, most of the published anti-VEGF trials were uncontrolled studies including small patient cohorts, and the observed reduction in corneal neovascularisation appeared to be incomplete and transient [180,184,185].

Pterygium is a degenerative and proliferative fibrovascular disorder of the ocular surface; usually a triangular- or wing-shaped tissue extending from the conjunctiva onto the cornea. Topical application of 0.05% bevacizumab has been shown to reduce the recurrence rate of pterygium 3 months post-treatment by trend in a cohort of 22 patients in a randomized controlled trial [182]. Further, in alkali-burned corneas blocking of VEGF by bevacizumab treatment also inhibited TGF-β expression and improved corneal transparency [186].

5.3.2. Receptor tyrosine kinase inhibitors & targeting the renin-angiotensin system

Most angiogenic factors, including the families of fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), stimulate angiogenesis through activation of their specific tyrosine kinase receptors. The antiangiogenic receptor tyrosine kinase inhibitor (RTKi), 3-[(4-bromo-2,6-difluorophenyl) methoxy]-5-[[[[4-(1-pyrrolidinyl)butyl]amino]carbonyl]amino]-4-isothiazolecarboxamide hydrochloride, was originally designed to inhibit VEGFR-2-mediated angiogenesis in various cancers. In addition to VEGFR-2, RTKi also inhibits other proangiogenic receptor tyrosine kinases, such as FGF receptors 1 to 3 (FGFR1–3), Tie-2, and ephrin receptor B4 (EphB4) [187]. Thus, this molecule may be a valuable tool to prevent unwanted corneal angiogenesis leading to scar formation. However, *in vitro* and *in vivo* studies on human corneal epithelial cells and in mice demonstrated that RTKi alone hampered corneal wound healing due to an off-target effect on EGFR signalling. In order to mitigate the inhibition of EGFR activity, the authors co-administered RTKi with the synthetic analogue of vitamin K3, menadione, thereby maintaining EGFR activity without compromising the desired antiangiogenic effects [188].

Angiotensin (Ang) peptides have been demonstrated to modulate cellular proliferation, angiogenesis, and dermal repair, thus playing a potential role in scar formation [189–191]. Particularly the interaction of angiotensin-(AS 1–7), a cleavage product of angiotensin regulates processes potentially relevant for scar formation. It negatively

modulates leukocyte migration, inflammatory cytokine expression and macrophage function. Moreover, activation of the Mas-receptor by angiotensin-(AS 1–7) exerts an anti-proliferative effect on smooth muscle cells, cardiomyocytes and liver tissue [192–194]. In a study on Dutch pigmented rabbits it was shown that the Renin-Angiotensin system is a potential target to pharmacologically prevent corneal scar formation. Topic application of the angiotensin analogue NorLeu^{3}A accelerates full-thickness corneal wound healing in a concentration-dependent manner. This was associated with rapid resolution of oedema, reduced inflammation, and reduction in wound leakage duration. Further, only for the treated incisions a near-normal ECM architecture without evidence of fibrosis was observed [195]. Most likely, this effect is exerted by activation of the Mas-receptor (MasR), which had previously been shown to be expressed in corneal basal and superobasal epithelial cells [196]. Most importantly, the angiotensin analogue not only promotes re-epithelialisation and inhibits fibrosis but also leads to an avascular healing phenotype. Again, this may be due to the activation of MasR, which had been shown to reduce angiogenesis in prostate tumours by reducing the expression of proangiogenic factors such as VEGF and placental growth factor [197].

5.3.3. Drug delivery to the cornea

Being the outermost ocular layer, topic drug delivery is the most commonly used route to deliver pharmacologic substances to the cornea. Other than in the remainder of the eye, clearance via blood- and lymph vessels is negligible, due to the avascular nature of this tissue. The exposed part of the eye is covered by a thin fluid layer, the precorneal tear film. The thickness of this film varies with the frequency of blinking and ranges from 1 to 100 μm [198]. Due to anatomical constraints, the volume that can be administered to the eye is limited to approximately 30 μL. Therefore, the effective clearance system of the front of the eye represents a major hurdle for providing constant drug concentrations. The tear film was shown to have a turnover rate of 16%/min, leading to massive clearance of any drug delivered topically [199]. The precorneal residence of an ophthalmic solution can be increased by the inclusion of viscosity enhancing polymers. Various polymers have been used to increase solution viscosity, including poly(vinyl alcohol), poly(vinylpyrrolidone) and various cellulose derivatives [200]. Further, the application of drug loaded hydrogels is considered a potential way to circumvent excessive clearance [201]. Finally, recently a study demonstrated that nanoencapsulation of bevacizumab is able to extend the bioactivity of the mAb in a controlled manner, potentially allowing the administration of lower doses and/or less frequent applications [202].

As a variety of antiangiogenic factors is known to be involved in maintaining a healthy, avascular status in the cornea, it is tempting to target these factors by gene therapy. Along these lines, several genes have been targeted, such as VEGF, angiostatin, endostatin, vasohibin, decorin or pigment epithelium–derived factor to modulate the angiogenic response [203]. Various ways of gene delivery have been exploited, including delivery by lentiviral, adenoviral or adenoviral associated vectors (AAV) [204]. Lentiviral vectors have been shown to effectively deliver relevant genes to the cornea (e.g. IL-10) in animal studies [205–207]; however, as they integrate into the host genome, clinical translation of lentiviral vectors is prohibited due to the risk of insertional mutagenesis [208]. Adenovirus based gene delivery to the eye was extensively studied *in vitro* and in animal experiments. After entry to the cytoplasm, the virus undergoes endosomal lysis and releases its genome. As there is no integration into the host genome the expression of the transgene is usually only temporary [203]. Nevertheless, in a mouse model of alkali burn, subconjunctival delivery of vasoinhibin-1 gene transfected adenoviruses lead to reduced neoangigenesis in the cornea, possibly by reducing expression of VEGFR-2 [209]. Finally, AAV represent a modern, safe alternative to other virus subtypes used for gene delivery, with reduced risk of host gene damage, also able to transfect non- proliferating cells. Recently, AAV gene transfer was shown to effectively transduce human corneal fibroblasts [210].

Alternatively, gene silencing strategies are under debate, using antisense oligonucleotides, morpholino oligomers, siRNAs, or shRNA, which might be useful for targeting proangiogenic factors in the cornea [203]. In summary, although a promising approach, controlling corneal neoangiogenesis by gene therapy is still in its infancy and awaits clinical translation.

6. Musculoskeletal tissues

Musculoskeletal diseases are one of the most prevalent health problems worldwide and are often accompanied by serious disability and compromised quality of life for patients. Further, as the increase in longevity raises the average age of the population musculoskeletal conditions are projected to affect approximately one quarter of the population [211,212]. Therefore, there is a growing socio-economic need for effective and reproducible strategies to repair musculoskeletal tissue in general. Compared to tendons, where the healing process often results in the formation of scar with inferior tissue quality (see Fig. 3), bone has a remarkably high regenerative capacity and generally heals in a scar-free manner with the newly formed bone tissue exhibiting all the characteristic of normal, uninjured bone [213,214]. Angiogenesis plays an essential role in bone healing as newly formed blood vessels provide oxygen and nutrient supply to the highly metabolically active callus. They further provide a route for inflammatory and stem cells to enter the site of injury. However, unfavourable local conditions such as soft tissue injury, inadequate blood supply, mechanical instability or extensive bone tissue loss after trauma or tumour resection may result in delayed healing, non-union or persistent bone defects. Numerous small and large animal preclinical studies have demonstrated the positive effects of promoting angiogenesis during bone regeneration which has been reviewed extensively elsewhere [214–216]. Generally, impaired VEGF signalling results in an ischaemic environment at the regeneration site, which consequently can result in non-unions or delayed unions and it was shown that treatment with exogenous VEGF after injury promotes angiogenesis and ultimately tissue regeneration [217]. However, as mentioned further above, strong angiogenic pressure can promote the formation of immature, non-perfused vessels, resulting in impaired wound healing. A recent study has demonstrated that treatment with teriparatide (recombinant parathyroid hormone [rPTH]), next to its anabolic effects on bone tissue, also inhibits arteriogenesis and promotes bone allograft integration by limiting fibrosis [218].

Skeletal muscle repair is also a highly synchronised process with a remarkable ability to regenerate fully vascularised and innervated contractile muscle tissue [219,220]. While there is robust evidence that VEGF is required for the angiogenic response, the mechanisms underlying VEGF secretion from skeletal muscle in disease and after injury has yet to be fully understood [221].

There is general consensus that driving neoangiogenesis generally leads to an improved healing outcome in muscle and bone. However, this is less clear for bradytrophic tissues such as tendon and cartilage. Ultimately, functional tendon and cartilage regeneration encompasses the full restoration of the biological, biochemical and biomechanical properties, which are often impaired by spontaneous healing events (see Fig. 3). Usually, a connective scar tissue forms at the injury site and the replaced tissue does not function adequately. As adult tendon and cartilage both resemble a avascular tissue, inhibition of neoangiogenesis and VEGF signalling might provide a potential target for novel treatment strategies.

6.1. Tendons

Tendons transmit force generated by muscles to the skeleton, are able to withstand tension and store and restore elastic energy [222]. This is possible due to specific biomechanical properties resulting

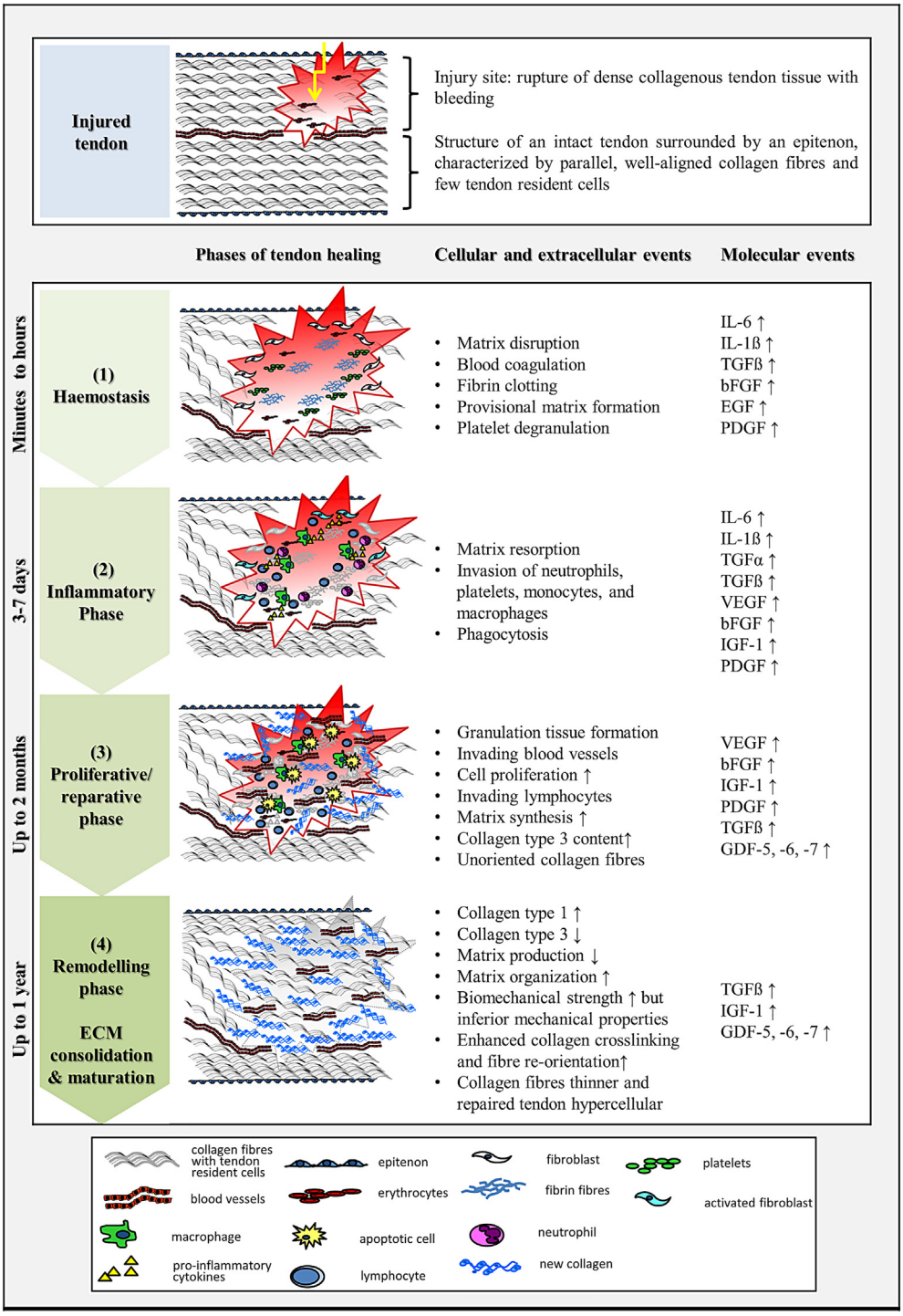

Fig. 3. Phases of tendon healing. A trauma or chronic degenerative process causes the rupture of the dense collagenous tendon tissue and leads to matrix disruption and bleeding. Tendon healing is accomplished by 4 well-orchestrated and overlapping phases. (1) The first step of tendon healing starts with haemostasis, where further matrix disruption takes place, platelets degranulate, blood coagulates and a fibrin clot forms a provisional matrix. Several growth factors and cytokines are secreted. (2) Secondly, inflammatory processes commence, leading to matrix resorption, invasion of inflammatory cells, such as neutrophils and macrophages. Numerous growth factors (e.g. TGFα and ß, VEGF, bFGF) and inflammatory cytokines (e.g. IL-6, IL-1ß) orchestrate these processes. This inflammatory phase lasts up to 1 week, followed by a (3) phase of cell proliferation and reparative extracellular matrix synthesis, dominated by type III collagen expression and generation of isotropic collagen fibres. In this phase blood vessels and lymphocytes invade the injury site, accompanied by a high expression of VEGF and other growth factors. The proliferative phase is generally completed around 2 months after injury. (4) The final phase of tendon healing - the remodelling phase - takes at least up to 1 year and involves extracellular matrix consolidation and maturation. More type I collagen and less type III collagen are synthesised and total matrix production is reduced. Enhanced matrix organisation with increased collagen cross-linking and fibre re-orientation leads to an improvement in biomechanical strength. However, newly formed tendon tissue is characterised by thinner and less oriented collagen fibres, hypercellularity and a generally inferior biomechanical strength compared to a healthy tendon.

from an exquisite, anisotropic and hierarchical organisation of the ECM [223]. Tendons and ligaments represent bradytrophic tissues which are poorly vascularised and innervated and they are characterised by a very poor regenerative capacity [224], which in part is most likely also due to the low number of tissue-resident cells and very limited rates of tissue turnover within the core region of adult tendons [225]. Generally, it is suggested that poor vascularity may prevent adequate tissue repair following trauma, leading to further weakening of the tendon [226,227].

As for other tissues, neoangiogenesis in tendon healing requires the coordinated release of synergistic growth factors. VEGF expression has already been shown to be expressed in injured Achilles tendons and the production of two isoforms of VEGF ($VEGF_{121}$ and $VEGF_{165}$) after injury supports their role in tendon repair [228,229]. In addition, Achilles tendons have been shown to rupture most commonly within a zone of hypo-vascularity. Therefore, general proangiogenic, VEGF-mediated therapies have been proposed to promote tendon healing [230]. However, hypervascularity is a clinical sign of tendon degeneration. Upon injury, the highly organised macromolecular structure is disturbed and a highly vascularized connective scar tissue with inferior biomechanical properties forms. Therefore, in contrast to highly vascularized tissues, such as skin or bone, neovascularisation following tendon injury is not necessarily a sign of functional tissue repair. Whether increased vascularisation drives degenerative processes such as loss of collagen fibre orientation, ectopic formation of bone, fat or cartilage, or if it is a consequence of these pathological changes remains unclear. However, as also discussed for cutaneous and corneal wound repair, it is tempting to speculate that a balanced manipulation of the angiogenic response during tendon healing could support the functional regeneration of tendons and ligaments [224]. Interestingly, several conservative treatment options e.g. eccentric training or cryotherapy decrease pathologically increased capillary tendon flow without deteriorating the local tendon microcirculation in Achilles tendinopathy. A decrease in capillary flow is therefore proposed to have beneficial physiologic effects on tendinopathy [231,232]. Further, tendon-resident cells express proteins with antiangiogenic properties. Oshima et al. have demonstrated that the C-terminal domain of Tenomodulin (TNMD) exhibits both antiangiogenic and anti-tumour activities when expressed in a secreted form, indicating a crucial role in maintaining an antiangiogenic state in tendon tissue [233]. Further, TNMD has recently been demonstrated to limit the formation of a fibrovascular scar during early events in tendon healing [234]. Finally, at the gliding area of adult tendons, the antiangiogenesis factor endostatin is highly expressed [235].

6.1.1. VEGF-based strategies in tendons

Generally, in the past most strategies to improve tendon repair were aiming at increasing vascularisation. For example, platelet rich plasma (PRP) preparations are frequently being applied to treat tendinopathies. Among many other growth factors, VEGF is abundantly present in PRP, suggesting that enhancement of neovascularisation might be one of the working mechanisms and especially leukocyte-rich PRP has been suggested to be an effective treatment for tendinopathies [236]. However, it remains to be seen if indeed the proangiogenic activities of PRP are genuinely responsible for an improvement and the effectiveness of PRP in general is still under debate [237]. For example, de Vos et al. [238] could not demonstrate that PRP injection results in an improved tendon structure and an increased degree of neovascularisation in Achilles tendinopathy.

Increased expression of growth factors is particularly prominent in the early phases of tendon healing [239], leading to increased cellularity and tissue volume. In one study, the temporal accumulation of VEGF mRNA at the repair site of a canine intra-synovial flexor tendon repair model was quantified [240]. A significant accumulation of VEGF mRNA at the site of injury with peak levels at post-operative days 7 and 10 was found which return to baseline levels by day 14. Interestingly, VEGF mRNA accumulation temporally precedes and is spatially distinct from the vascular ingrowth itself, suggesting that cells within the tendon repair site are also involved in molecular processes modulating angiogenesis during the early phase of tendon healing [241]. Another study demonstrates high levels of the two splice variants $VEGF_{120}$ and $VEGF_{165}$ to be expressed in ruptured Achilles tendons during the healing process, whereas levels in normal Achilles tendons are negligible [228].

Several *in vivo* studies performed in dogs and rabbits aimed at improving tendon regeneration by administration of VEGF. One study evaluated the effects of single growth factors as well as a combination of growth factors on cell proliferation and collagen deposition in canine flexor tendon fibroblasts *in vitro* [242]. However, results were controversial and in another study delivery of a gene encoding $VEGF_{165}$ even had detrimental effects on Achilles tendon healing in rabbits [243]. In contrast, Zhang et al. evaluated the effect of exogenous vascular endothelial growth factor (VEGF) on tendon healing in an Achilles tendon transection model in rats and observed significantly improved tensile strength of repair tissues in the early course of tendon healing (up to 2 weeks post-surgery), which was associated with increased expression of transforming growth factor-β [244]. However, the positive effects subsided within 4 weeks post-surgery.

Recent studies reveal an important role of VEGF-induced angiogenesis in degenerative tendon diseases. However, the exact mode of action how VEGF influences mechanical properties remains poorly understood. It is known that hypoxia, inflammatory cytokines and mechanical loading increase the expression of VEGF in tenocytes [245]. For example, cyclic stretching of rat Achilles tendon cells with a frequency of 1 Hz *in vitro* resulted in an increased expression of $VEGF_{121}$ and $VEGF_{165}$, whereas a low frequency (0.5 Hz) reduced VEGF expression to control levels. In addition, the expression of HIF-1α was increased [246]. Since VEGF has also the potential to stimulate the expression of matrix metalloproteinases (MMPs) and inhibit the expression of tissue inhibitors of matrix metalloproteinases (TIMPs) in various cell types (e.g. endothelial cells, fibroblasts, chondrocytes), it might play a significant role for the pathogenic processes during degenerative tendon diseases. Sahin et al. propose a time-dependent correlation of HIF-1/VEGF-induced, and MMP-3-mediated angiogenesis during tendon healing, resulting in impaired biomechanical properties due to ECM rearrangements [247]. Therefore, a therapeutic modulation of neoangiogenesis by influencing the level of VEGF and consequently of metalloproteinases (e.g. MMP-3) might be a promising target to treat degenerative tendon diseases.

As discussed previously, embryonic and fetal healing is characterised by scar-free healing. In one study partially lacerated fetal lamb flexor tendons were analysed at 7 time points after injury and were compared to equally lacerated adult sheep tendons. In the fetal animals, no subcutaneous scarring was present, the digital sheath and tendon healed two weeks after injury and a smooth, gliding surface was reconstituted. In contrast, in adult sheep tendons a dense subcutaneous scarring was obvious. The digital sheath healed by 4 weeks and the tendon gap by 6 weeks, but a smooth gliding surface was not restored [248]. Additional studies could provide valuable insight into fetal, regenerative pathways which could be targeted to improve tendon healing or even achieve functional regeneration.

The potential to improve tendon healing by modulation of the vasculature is also supported by clinical results demonstrating that a locally administered (in the area with neovascularisation) sclerosing drug has a beneficial effect on chronic mid-portion Achilles tendinosis [245,249–251]. Further, a preclinical study in mice suggested that a single, intra-tendinous injection of the antiangiogenic monoclonal antibody bevacizumab improved and accelerated tendon healing [252]. All treated samples showed better joint mobilisation with thinner tendon diameters, less collagen fibre disorganisation and less neovessel formation compared to control samples.

In summary, in order to develop rational strategies to improve tendon healing via a well-balanced angiogenic response, we need a thorough understanding of the molecular and cellular networks driving tendon neovascularisation and pruning of the vascular plexus during the remodelling phase of tendon healing.

6.2. Cartilage

Similar to tendons and ligaments, cartilage is one of the few hypo- or avascular tissues found in nature. By contrast, adjacent tissues including muscle, bone and synovium are well vascularised. Microcapillaries in these surrounding tissues never invade cartilage under physiological conditions and the antiangiogenic properties of cartilage are conferred

by multiple molecular mechanisms [253]. For example, tenomodulin is localised to the sites of attachment of muscle to skeletal tissues that delimit the extension of the vasculature [233]. Next to Tnmd, chondromodulin-1 (ChM-1/Lect1) is specifically localised in the avascular zone of cartilage during endochondral bone formation. Both, the bioactivity and localisation of ChM-1 and Tnmd, indicate that these molecules may be relevant in the maintenance of an antiangiogenic state [233]. However, ChM-1-null mice demonstrated normal cartilage, with no reported abnormal cartilage vascularisation [254]. Interestingly, Tnmd has also been suggested to play a role in limiting retinal neovascularisation [255]. Next to Tnmd and ChM-1, additional proteins exerting antiangiogenic properties have been detected in cartilage, including endostatin [256,257], the matricellular glycoprotein SPARC [258], and thrombospondins 1 and 2 [259]. However, the exact mechanisms of the antiangiogenic effects of these proteins are complex and partially tissue-specific and their contribution to the avascular nature of cartilage and its maintenance remains largely unclear.

6.2.1. Targeting synovial angiogenesis in osteoarthritis

During osteoarthritis (OA), angiogenesis is increased in the synovium, menisci, and osteophytes. Further, the articular cartilage loses its resistance to vascularisation and vessel ingrowth occurs at the osteochondral junction. As a consequence, ossification in osteophytes and the deep layers of articular cartilage occurs. Increased levels of VEGF have been reported in the articular cartilage, synovium, synovial fluid, subchondral bone, and serum during later stages of osteoarthritis (OA) [260,261] and inhibition of VEGF signalling is being explored as a treatment option to limit progression of OA and also rheumatoid arthritis [262,263]. Importantly, recent *in vivo* studies in mice have examined the role of intra-articular administration of VEGF demonstrating independently that VEGF can elicit characteristic features of OA [264,265]. Angiogenesis might facilitate inflammation, hence contributing to the structural disease progression in OA. Therefore, limiting blood vessel (and nerve) ingrowth has gained significant attention in treating OA [266]. So far, studies have assessed antiangiogenic therapies by specific inhibition of VEGF through anti-VEGF antibodies [267–270], peptides [271], sFlt-1 [272,273], as well as vaccination against VEGF [274] for the treatment of rheumatoid arthritis (RA). However, inhibiting VEGFR-1 signalling appears to be more effective than targeting VEGFR-2 [275,276]. Another study evaluated the anti-arthritic effect of the chimeric decoy receptor double-antiangiogenic protein (DAAP) in collagen-induced arthritis, which can both bind VEGF-A and angiopoietins and block their actions. DAAP had a much greater inhibitory effect than VEGF-Trap or Tie2-Fc on arthritis severity and bone destruction accompanied by significantly diminishing pathologic abnormalities such as angiogenesis and macrophage infiltration [277].

In preclinical studies, intravenous application of the humanized monoclonal anti-VEGF antibody bevacizumab resulted in improved cartilage repair of osteochondral defects in rabbits. The bevacizumab-treated group showed repair sites filled mostly with hyaline cartilage after 3 months compared to fibrocartilage and bone formation in control groups [278]. Further, early low-dose bevacizumab treatment showed reduction of articular cartilage degeneration and osteophyte formation in an OA rabbit model with increased expression of type II collagen, aggrecan, and chondromodulin-1 in chondrocytes, decreased Runx2 expression in the subchondral bone, and suppressed expression of MMP-13 and ADAMTS-5 in the synovium [279]. However, as the mAb was administered during the early phases of OA progression, anti-VEGF therapy potentially is more feasible for patients suffering from post-traumatic OA. Further, given the potential adverse events of systemically administered bevacizumab, local administration with a suitable drug delivery system is potentially more feasible.

sFlt-1, the endogenously produced soluble splice variant of VEGFR-1, binds to and inhibits VEGF. In an osteochondral defect model, Flt-1 improved the chondrogenic potential of mouse skeletal muscle-derived stem cells and prevented neoangiogenesis after transplantation to an osteochondral defect [262]. In addition, blocking angiogenesis by inhibition of methionine aminopeptidase type 2 (MetAP-2) and thrombospondin-1 have been demonstrated to show promising results in suppressing VEGF signalling during OA and RA [280–283].

Although there are currently no ongoing clinical trials, limiting VEGF-signalling or angiogenesis in general is a promising and exciting modality to treat OA or RA and to improve cartilage repair. It's feasibility is partially underscored by a case report, demonstrating beneficial outcomes after the injection of bevacizumab into the knee joint to treat pigmented villonodular synovitis [284]. To date, however, the only antiangiogenic agents to be granted FDA approval are the monoclonal anti-VEGF-A antibodies bevacizumab (Avastin®, Genentech) and ranibizumab (Lucentis®, Genentech) and the VEGF-binding domain-Fc fusion protein Ziv-aflibercept (Zaltrap®, Sanofi-Aventis) [285,286], none of which have been approved for treatment of OA or RA.

7. Conclusion and future perspectives

Wound healing is a multi-facetted, tightly controlled process and includes the formation of a perfused vascular plexus meeting the demands of the affected tissue. Although angiogenesis is a central aspect of the wound healing process and is generally imperative to promote tissue injury repair, if taking place in a uncontrolled fashion it can spur scarring and promote pathological processes such as fibrosis, tumour progression, or various ocular diseases. Although our understanding of the processes controlling the balance between *physiological* and *pathological* angiogenesis has increased, much remains to be learned. We need to further investigate the spatio-temporal events regulating blood vessel formation and regression and the cross-talk between inflammation and angiogenesis in order to promote regenerative healing.

Several antiangiogenic approaches show promise to limit or control an excessive angiogenic response in various tissues (see Table 1). These could potentially be improved by adopting smart drug delivery systems [142,202,287,288] and scaffolds [289,290] to further enhance their effectiveness and to achieve the goal of functional regeneration. Especially for avascular tissues, such as tendon and cartilage, limiting neoangiogenesis in a controlled manner potentially could be the key to effective treatments in the future.

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Declaration of interests

All authors declare no competing interests.

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