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Therapeutic Strategies for Enhancing Angiogenesis in Wound Healing

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Abstract

The enhancement of wound healing has been a goal of medical practitioners for thousands of years. The development of chronic, non-healing wounds is a persistent medical problem that drives patient morbidity and increases healthcare costs. A key aspect of many non-healing wounds is the reduced presence of vessel growth through the process of angiogenesis. This review surveys the creation of new treatments for healing cutaneous wounds through therapeutic angiogenesis. In particular, we discuss the challenges and advancement that have been made in delivering biologic, pharmaceutical and cell-based therapies as enhancers of wound vascularity and healing.

Graphical Abstract

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Keywords

wound healing; angiogenesis; growth factor therapy; cell-based therapies; stem cell therapy; neovascularization; diabetic ulcers; chronic wounds; non-healing wounds

1. Introduction

In modern times, non-healing, cutaneous wounds have become major medical and social burden in the United States and worldwide. Diabetes is the leading cause of chronic, non-healing ulcers that leave patients at increased risk for limb amputation [1, 2]. It is estimated that chronic wounds cost the U.S. over \$25 billion annually and significantly contribute to the rising cost of healthcare [3–6]. Astonishingly, diabetic ulcers are responsible for 25–50% of the total cost of diabetes treatment [7] and are the most common cause for limb amputations in the United States [8]. Debridement of necrotic tissues, offloading, infection control, surgical revascularization, and limb elevation/compression are often required concurrently and lead to the immense cost and complexity [9, 10]. Additionally, a host of techniques and dressings have been applied with the goal of improving chronic wound healing, including the delivery of growth factors, gene therapy, stem cell therapies and mechanical/pressure-based stimulation [11–15]. Currently, only platelet-derived growth factor-BB (PDGF-BB; Becaplermin/Regranex), is approved in the U.S. for clinical treatment of chronic wounds. However, the mixed efficacy of this treatment has prevented it from achieving widespread clinical adoption [16, 17]. A major aspect of non-healing wounds in diabetes and peripheral vascular disease is a reduced ability to regrow microvasculature through the process of angiogenesis [18]. This disruption of the normal healing process contributes to the dysfunctional healing response and is major therapeutic target for creating new treatments for non-healing and ischemic wounds. In this review, we will examine recent advances in therapies targeting the enhancement of angiogenesis in wound healing.

The healing of wounds is a complex process that greatly depends on the extent and mechanism of injury. Broadly speaking, the wound healing process can be separated into a several stages that include immediate hemostasis, acute inflammation, proliferation and maturation [19]. A key accompanying activity in the proliferation stage is the formation of

new blood vessels, a process known as angiogenesis. In intact tissues, the microvasculature remains in a state of homeostasis where adequate nutrients and oxygen are delivered to the tissues in balance with removal of waste products and carbon dioxide. Upon injury, the microvasculature is disrupted leading to fluid accumulation, inflammation and the development of hypoxia. Hypoxia and inflammatory cytokines activate endothelial cells, leading to recruitment of immune cells. The injured tissue recruits an early influx of neutrophils followed later by macrophages. Macrophages have been recently recognized to polarize into a continuum of phenotypes and the nature of this polarization has a critical effect on wound healing, scar formation and angiogenesis [20]. Classically, the polarization of macrophages ranges in a continuum from a pro-inflammatory M1 phenotype to alternatively activated M2a-d phenotypes that have been associated with anti-inflammatory and wound healing activity [20]. Macrophages are essential to coordinate the angiogenic response in wound healing and produce many factors that regulate new blood vessel growth [21–23]. The M2 phenotype is thought to be more strongly associated with angiogenic activity [24, 25]. The presence of therapeutics and/or biomaterials for delivery of compounds can also induce a response from macrophages. The foreign body response to some biomaterials involves the formation of foreign body giant cells and fibrotic encapsulation of the material [26]. The foreign body reaction to implanted biomaterials can be separated into stages including protein adsorption on blood or biofluid contact, initial cells response/recruitment from cells of the innate immune system including macrophages, production of cytokines and soluble factors that can control inflammation and the recruitment of cells involved in fibrous tissue formation, and finally formation of fibrous capsule around the foreign body [27]. The polarization of macrophages can be altered by interactions with biomaterials and this is a potential strategy for altering the course of wound healing and modulating the result of the foreign body reaction [28, 29].

The injured tissue at this stage undergoes rapid proliferation in a loose provisional extracellular matrix (ECM). In a well healing wound, this granulation tissue is characterized by intense angiogenesis giving it its characteristic pink swollen appearance [30]. Angiogenesis in the provisional matrix is guided by several factors including the development of gradients of hypoxia caused by local microvascular disruption and cytokines produced by macrophages. Among other processes, hypoxia activates the transcription factor hypoxia-inducible factor-1- α (HIF-1 α), which promotes angiogenesis through multiple mechanisms including the regulation of a large number of angiogenesis related genes [31] and the production of angiogenic growth factors including for Ang-2, stromal cell-derived factor-1 (SDF-1) and vascular endothelial growth factor (VEGF-A) [32]. Heparan sulfate proteoglycans also play a key role in regulating the angiogenic activity of many of these growth factors including VEGF-A and fibroblast growth factor-2 (FGF-2) [33, 34].

In dermal wounds with robust healing, the angiogenic activity during the proliferative phase initially creates a disorganized vascular network with vessels of high tortuosity and many dead-end flow pathways, often reaching higher vessel numbers than the skin prior to injury [35]. Following this peak in vessel density, there is increased expression of anti-angiogenic growth factors, such as Sprouty2 and pigment epithelium-derived factor (PEDF), leading to subsequent regression of the vascular network [36, 37]. Maturation of the vascular network to include larger vessels and prevent regression requires the stabilization of the capillaries by

pericytes and vascular smooth muscle cells (vSMCs) [38]. A key growth factor in promoting stabilization of the vascular network is PDGF-BB, which aids in recruitment and differentiation of pericytes [39]. In addition, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), in combination with the receptor Tie2, play a complex role in regulating angiogenesis and maturity of vascular networks [40–42]. Specifically, Tie2 is expressed on pericytes and signaling through Ang-2/Tie2 in pericytes can regulate angiogenesis [43, 44].

Reduced angiogenesis plays a prominent role in the non-healing nature of diabetic foot ulcers and other chronic wounds [45]. Multiple mechanisms are supported for the reduced angiogenesis in these chronic wounds. Diabetic patients with foot ulcers have higher circulatory levels of PEDF in their plasma in comparison to non-diabetic patients and diabetic patients without foot ulcers [46]. In diabetic skin, there is also a marked reduction in syndecan-4 and glypican-1, two key cell surface heparan sulfate proteoglycans that enable effective binding of FGF-2 and other growth factors to their cognate receptors [47, 48]. Similarly, wound exudates isolated from venous leg ulcers have anti-angiogenic properties, even in spite of increased expression of angiogenic factors [49]. These analyses are consistent with a proteomic analysis of venous leg ulcer exudates that found increases in anti-angiogenic proteins in non-healing ulcers [50]. In chronic venous ulcers, there was also increased of proteolysis of VEGF-A [51] as well as increased levels of soluble VEGFR-1, which may serve to neutralize VEGF-A activity [52].

In this review, we summarize the recent progress in the delivery of compounds to wounds with the goal of inducing therapeutic angiogenesis. In particular, we will focus on new systems and approaches for delivering compounds and cells for enhancing angiogenesis in cutaneous wounds. As non-healing wounds are major clinical problem, we will emphasize studies with results relevant to chronic wounds. The review is arranged by the overall therapeutic strategy of using proteins, gene/nucleic acid delivery, small molecules/drug-like compounds or cell therapies (Fig. 1).

2. Protein Therapeutics for Wound Angiogenesis

2.1 Growth Factors

There have been many years of intensive study into the use of growth factors for both inducing angiogenesis and enhancing wound closure in chronic wounds. Endogenous growth factors play a critical role in orchestrating the wound healing process and are essential for effective wound healing [53]. While the delivery of exogenous growth factors has shown benefits to wound closure and angiogenesis in animal studies, almost all growth factor-based agents for wound healing have not shown clear benefits for enhancing wound healing in patients in clinical trials. One hypothesis regarding the failure of these trials is that human patients develop resistance to angiogenic growth factors during the development of vascular disease and the aging process [54]. It is also uncertain whether a single growth factor therapy could be effective for wound healing [55]. The mixed results from clinical trials, combined with the prohibitive cost of the therapies, have slowed the clinical adoption of these treatments, however research is still underway to develop more effective protein therapies for wound healing.

2.1.1 Platelet Derived Growth Factor—Platelet-derived growth factors (PDGFs) are important chemoattractants in the wound healing process. Currently, becaplermin (100 µg/g PDGF-BB) is the only FDA approved growth factor treatments for wound healing [16, 56]. In some clinical trials, becaplermin has been shown expedite wound healing in venous leg ulcers [57, 58]. In a clinical trial on becaplermin for treating diabetic foot ulcers, 48% of patients (29/61) had healed ulcers versus 25% (14/57) in the placebo group with a dosage of 2.2 µg/cm² of ulcer area [59]. Clinical trials in 1998 and 1999 showed statistically significant improvements in this type of chronic wound with becaplermin treatment [60, 61]. In the groups treated once a day with becaplermin, diabetic foot ulcers exhibited decreased wound areas and faster healing rates over the placebo controls. These positive results can be attributed to the ability of PDGF-BB to enhance recruitment of cells to the wound site and the stimulation of an angiogenic response. However, other clinical trials have not found improvements in wound healing with becaplermin treatment [62]. Therapeutic resistance may also play a significant role in preventing the effectiveness of PDGF-BB therapies, thus necessitating higher concentrations and increasing the risk of side effects of the growth factor therapy. In addition, the FDA gave becaplermin a black box warning due to a five-fold increase in death rate among patients that received three tubes of becaplermin and who had pre-existing malignancy [63]. While the risk of getting a new cancer was not increased in patients without cancer, this finding highlights the risk of using pro-growth and angiogenic therapies in patients with an existing cancer. Novel delivery strategies are needed to increase the efficacy of PDGF-BB without increasing the dose and to increase its cost effectiveness [64].

The simultaneous application of multiple growth factors and proteins may provide the extra benefit needed to see therapies applied in a clinical environment. The simultaneous application of PDGF-BB and epidermal growth factor (EGF) has been shown to direct pericyte recruitment to help stabilize blood vessels [65, 66]. Other multiple growth factor studies have shown that the simultaneous application of PDGF-BB and FGF-2 has improved vascular stability in hind limb ischemia models in rats and rabbits [67]. The dual delivery of PDGF-BB and VEGF-A from electrospun nanofiber scaffolds accelerated wound healing in rats and promoted angiogenesis at the wound site [68]. Similar results were achieved in the simultaneous delivery of VEGF-A and PDGF-BB bound by laminin heparin binding domains from fibrin matrices [69]. In another study, researchers created an electrospun nanofiber scaffold of collagen and hyaluronic acid (HA) that released FGF-2, EGF, VEGF-A and PDGF-BB with varying release kinetics. This construct accelerated wound closure and the maturation of blood vessels in the wound beds of rats with streptozotocin-induced diabetes [70]. Finally, combining PDGF-BB with a syndecan-4 proteoliposomes significantly increased its activity in wound closure, angiogenesis and increased the proportion of M2 macrophages in a diabetic mouse model of wound healing [71]. Table 1 summarizes the recent studies using multiple growth factors to enhance angiogenesis in wound healing.

2.1.2 Epidermal Growth Factor—Epidermal growth factor (EGF) is another promising growth factor for wound healing therapies and a known mediator of angiogenesis. Early clinical trials showed reduced healing times and increase re-epithelialization in wounds

treated with recombinant EGF [72, 73], however, concerns over the role of EGF in cancer have reduced enthusiasm for these therapies in the U.S. [74]. Despite this fact, there has been increased interest in EGF treatment to enhance wound healing in chronic wounds in other parts of the world. There are several commercially available forms of recombinant human EGF outside of the U.S. including Easyef (Nepidermin), Regen-D 150, and Heberprot-P. Easyef is available as a spray or ointment and has shown benefits for diabetic foot ulcers in a prospective, open-label trial, crossover study with 89 patients [75]. Regen-D 150 is a gel formulation containing EGF that has shown improvements in wound healing time and reduces the risk of amputation in patients with chronic diabetic foot ulcers [76]. Heberprot-P is a lyophilized formulation of EGF that has been tested in over 100,000 patients in Cuba and has also been shown to increase healing of chronic ulcers and reduce diabetes-related amputations [77]. Finally, clinical trials in Vietnam have supported that recombinant human EGF has positive healing effects on moderate to severe diabetic foot ulcers [78, 79].

Mechanistically, EGF has been shown to enhance angiogenesis through phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) and extracellular signaling-regulated kinase-1/2 (ERK1/2) through VEGF-mediated pathways [80]. EGF can induce production of VEGF-A to enhance tumor angiogenesis in cancer cells [81] and is a powerful chemoattractant for keratinocyte migration to encourage wound re-epithelialization [82] with heparin-bound EGF being the one of the most potent forms [83]. EGF also regulates nuclear factor- κ B (NF- κ B) controlled activation of CCCTC-binding factor (CTCF), a gene regulator that plays a significant role in epithelial cell migration [84]. In mice with a knockout of the NF- κ B p50 subunit, the wound closure percentage was significantly reduced in comparison to wild type (WT) control mice upon EGF treatment [85].

Recently, new delivery methods have been explored to help localize EGF and increase its circulation time [86]. Silk biomaterials have been developed to deliver EGF to promote angiogenesis and wound closure in cutaneous excisional wounds in mice [87, 88]. In a study by Gil et al. three different material structures and two different drug incorporation methods were examined [87]. The silk biomaterial was formulated as a solid film, a porous film, or electrospun nanofibers. Silk biomaterials show wound closure percentages significantly greater than control wounds in a full thickness dermal wound model. The delivery of EGF from silk biomaterials decreased scar tissue formation and increased epithelialization over a Tegaderm control. Another group developed a hyaluronic acid (HA)/collagen sponge that could be used to deliver EGF as a wound dressing [89]. In a wound model in rats, the EGF-containing dressing increased wound closure and angiogenesis in comparison to the non-EGF dressings and control wounds. Finally, to address another major issue in protein therapeutics, dextrin conjugation has been used to reduce the proteolytic degradation of EGF in circulation, thereby increasing its wound healing capacity [90]. By day 11 in a study of full thickness excisional wounds in diabetic mice, neo-dermal tissue generation was greatest in the group that received both 1 μ g/mL and 10 μ g/mL of dextrin-EGF over the control that received 10 μ g/mL of EGF without dextrin conjugation. The dextrin-EGF construct also accelerated wound closure and the formation of granulated tissue at the 10 μ g/mL dose over the control. Further research into novel materials and protective strategies will help develop more effective EGF therapies.

2.1.3 Fibroblast Growth Factors—Fibroblast growth factors (FGFs) are a family of growth factors that show great promise for applications in dermal wound healing as they affect a large number of physical processes [91]. There are several different FGF family members expressed during the healing process including FGF-1, FGF-2, FGF-7, FGF-10 and FGF-22 [92, 93]. In general, FGF ligands bind to their receptors in the presence of heparin or heparan sulfate and these signals can promote angiogenesis and proliferation/migration of endothelial cells [94]. The most studied member of the FGF family used in wound healing applications is FGF-2 [95], partly due to its strong association with wound angiogenesis. The other FGFs are more often associated with the enhancement of re-epithelization [93, 96, 97], the most notable being FGF-7. FGF-7 has been shown to be integral in the migration of keratinocytes to the wound area [98] and a lack of FGF-7 delays cutaneous wound healing in mice [99]. Further, the dual delivery of FGF-7 and FGF-2 has been shown to enhance skin wound healing, showing more developed vascular networks than the scaffold control[100].

Multiple studies have reported the benefits of delivery of FGF-2 for wound healing. In freeze injured skin grafts, the delivery of FGF-2 stimulated angiogenesis and accelerated wound healing [101]. More recent studies have corroborated this evidence in a wide range of wound models from skin flaps to incisional hernias [102, 103]. In one study, FGF-2 released from heparin-fibrin composite matrices increased fibroblast proliferation and collagen density, decreased the necrotic area in the skin flap and the vascular density in comparison to control wounds [102]. Research on incisional hernias has shown that tissues treated with FGF-2 after the incision had increased breaking strength and lower hernia development over untreated incisions [103]. In clinical studies, FGF-2 increased wound healing in patients with burns and chronic ulcers [104, 105]. A second clinical study also demonstrated increased wound closure in patients with burns and enhanced strength in wounds treated with FGF-2 [106].

Novel FGF-2 delivery methods have been explored to enhance its wound healing activity. Delivery of FGF-2 from gelatin sheets crosslinked with glutaraldehyde accelerated re-epithelialization, formation of granulation tissue and angiogenesis in comparison to control wounds in a mouse model [107]. For injectable applications, chitosan-heparin hydrogels have been developed to deliver FGF-2 via injection to subcutaneous wound sites [108]. When the gel was injected into the ischemic hind limb of a diabetic mice, there was increased blood vessel formation after four days. Furthermore, there was a significant increase in blood flow and oxygen saturation in the calf and ankle until at least day 28. Diabetic patients have a reduction in cell surface proteoglycans that are essential for robust FGF-2 signaling, including syndecan-4 and glypican-1 [48, 54]. Several studies support that co-delivering syndecan-4 or glypican-1 in a proteoliposome with FGF-2 can markedly enhance its activity for angiogenesis and wound healing in diabetic animal models [47, 48, 54, 109, 110].

The delivery of growth factors and specifically FGF-2 from nanoparticles and nanocrystals has also been investigated [111–113]. Mesoporous silica nanoparticles (MSNs) are a means to create controlled drug release profiles by fine tuning the surface area to volume ratio, pore sizes, and surface properties. Zhang et al. delivered FGF-2 from MSNs with a formulation

that allowed release over 20 days [111]. In addition, Li et al. combined tunability of gelatin microspheres and the structural stability of a collagen/cellulose nanocrystal scaffold shown by Li et al. provided a suitable environment for enhancing angiogenic growth [112]. Microspheres, impregnated with FGF-2, were loaded into a scaffold composed of collagen and cellulose nanocrystals (CNCs), which increased the mechanical stability of the collagen network. In a subcutaneous implantation model, the FGF-2 releasing gelatin microspheres significantly increased the number of new blood vessels compared to FGF-2 from the scaffold alone or control group.

2.1.4 Vascular Endothelial Growth Factor—The vascular endothelial growth factor (VEGF) family is comprised of five separate growth factors, VEGF-A, -B, -C, -D and placenta growth factor (PlGF) [114]. These growth factors are primarily responsible for regulating angiogenesis and lymphangiogenesis in both embryonic and adult development [114]. The VEGF family also plays a significant role in the permeability of blood vessels, vasodilation, and have been shown to stabilize new blood vessel growth during wound healing. However, the application of VEGF therapies in clinical settings has been limited, due in part to the vasopermeability induced by the treatment [115]. Several clinical trials have been performed using recombinant VEGF-A to enhance angiogenesis in critical limb or myocardial ischemia [116–119], but the clinical benefits of VEGF therapy were minor and further studies were not pursued.

During the course of wound healing, the levels of VEGF-A and FGF-2 in the serum have a negative correlation with one another, first high levels of FGF-2 involved in the initial recruitment of endothelial cells followed by high levels of VEGF-A used to stabilize the new vessel growth and provide a prolonged angiogenic signaling period [120]. Losi et al. showed that concurrent delivery of FGF-2 and VEGF-A increased the number of endothelial cells around the immediate vicinity of their scaffold in a hind limb ischemia model, as well as increased perfusion of blood in the hind limbs of rats after ischemic injury [121]. Further, the delivery of granulocyte-macrophage colony stimulating factor (GM-CSF) to diabetic patients with leg ulcers increased the level of VEGF-A produced by macrophages and monocytes [95, 122]. Application of VEGF-A to wounds in db/db mice increased wound closure and angiogenesis [123]. In wounds treated with VEGF-A there was a greater number of endothelial progenitor cells that lead to new blood vessel formation, as well as increased amounts of pro-angiogenic soluble factors.

To further enhance the effect of VEGF-A at the wound site, many therapies also involve the delivery of the growth factor from biomaterials. During degradation, poly(lactic-co-glycolic acid) (PLGA) scaffolds hydrolyze into lactic and glycolic acids [124]. This lactate helps promote angiogenesis and helps recruit endothelial progenitor cells at wound sites and, when combined with VEGF-A, promotes healing [125]. VEGF-A released from PLGA scaffolds lead to significantly greater wound closure in full thickness skin wounds in diabetic mice (>80% of the initial wound area) 10 days post injury compared to controls that received only VEGF-A or the scaffold alone. The PLGA scaffolds promoted angiogenesis at the wound site and the tissue showed greater collagen deposition, dermal remodeling, granulation, and blood capillary infiltration at a greater rate than the control treatments. VEGF-A encapsulated PLGA microspheres and nanoparticles released from chitosan/hyaluronic acid

hydrogels and fibrin scaffolds respectively have also been used to promote angiogenesis in non-healing infected wounds and in diabetic mice [126, 127]. The release of VEGF-A from calcium alginate microspheres has been shown to increase capillary network formation and neovascularization as well in subcutaneous implantation models in rats [128, 129]. When histological analysis was performed, all sites displayed a low level of fibrotic tissue and the microspheres loaded with VEGF-A showed considerable capillary growth and newly formed blood vessels.

Placenta growth factor (PIGF) has also been shown to enhance angiogenesis and can have angiogenic synergy with VEGF-A [130–132]. Mice overexpressing PIGF exhibit increased vascularization in both fetal and adult life [133]. These mice display hypervascularized skin and enlarged blood vessels. Gene transfer of PIGF increases wound angiogenesis in health and diabetic mice [131]. PIGF also promotes chemotaxis of mesenchymal stem cells (MSCs), which may express angiogenic factors or induce increased angiogenesis/angiogenic factor secretion in other cell types. When MSCs were cultured with fibroblast-like synoviocytes, the synoviocytes were found to secrete higher levels of PIGF and induce greater angiogenesis compared to fibroblast-like synoviocyte injection controls [134]. The ECM binding domains from PIGF have also been used to engineer variants of other growth factors (VEGF-A, PDGF-BB and BMP-2) with super-affinity to the ECM, leading to increased repair in chronic wounds in rodents, over their wild-type counterparts [135]. The overall concept is that the localization of the growth factor may be better controlled with super-affinity to the ECM, reducing the side effects of angiogenic therapy. The super-affinity VEGF-A variant promoted angiogenesis with reduced induction of permeability of the vasculature for equivalent wild type VEGF-A treatment [135].

2.2 Non-Growth Factor Protein Delivery

Many other proteins have displayed therapeutic effects on wound angiogenesis in preclinical wound healing models. Insulin is a key example of a protein hormone shown to be a regulator of angiogenesis and wound healing [136, 137]. The delivery of insulin from PLGA microspheres encapsulated in an alginate sponge dressing enhanced healing and reduced scarring from burn injuries in rats by stimulating angiogenesis [137]. In addition, injection of insulin into the skin of mice induced angiogenesis suggesting it is a suitable candidate for ischemic wounds [138]. Furthermore, in a double-blind placebo-controlled clinical trial, the topical delivery of insulin to diabetic skin ulcers resulted in improved wound healing [139]. This result presents insulin as a low-cost alternative to growth factor therapies.

The hormone erythropoietin (EPO) is another non-growth factor protein that has been shown to increase angiogenesis [140–143]. In second-degree burn injury model where a substantial portion of the original vasculature is damaged or lost completely, mice and rats treated with topical application or infusion of EPO displayed rates of angiogenesis that were significantly increased over the control that did not receive EPO [140]. Further, wound closure rates in the rats treated with EPO were accelerated over the control, with the wounds on average 98.8% closed by day 7. In addition, EPO has been used to treat ischemic injuries, where significant revascularization is required to restore adequate blood flow [143, 144], and it is suggested that EPO works synergistically with VEGF to promote angiogenesis at the wound site [145].

On the other hand, EPO has been implicated in tumor angiogenesis in hepatic tumors and Lewis lung carcinoma tumors [146, 147], so similar precautions should be taken as in many growth factor therapies.

Stromal-cell derived factor-1 (SDF-1) is another protein that has been shown to regulate angiogenesis by recruiting endothelial progenitor cells (EPC) to the wound site [148, 149] and promoting sustained cell proliferation [150]. SDF-1 is also a strong chemotactic agent for monocytes, such as macrophages [151], has been shown to promote the local recruitment of monocytes with anti-inflammatory activity enhances the expansion of local vasculature [152]. SDF-1 has also been shown to promote bone mesenchymal stem cells (bMSC) and EPC chemotaxis to wound sites [153, 154]. The migration of bMSCs and EPCs promotes vascularization and in turn helps accelerate the wound healing process. SDF-1 has also been shown to promote angiogenesis in a diabetic mouse model, with a three-fold increase in blood vessel formation over a control group that did not receive SDF-1 [149]. The delivery of SDF-1 significantly accelerated wound closure as well as vascularization in full thickness skin wounds. Finally, in a novel approach, Vågesjö et al. transformed lactobacilli with a plasmid encoding SDF-1 [155]. These bacteria were then applied topically to wound sites in mouse models of hyperglycemia and peripheral ischemia, leading to accelerated wound closure and efficacy of bioavailable SDF-1.

Other non-growth factor-based protein therapeutics have also been explored for enhancing angiogenesis during wound healing. Spidroin, the protein found in spider's silk, has been explored for its advanced scaffolding capacity [156]. Recombinant spidroin (1F9) was used as a cell scaffold to promote the proliferation of cells while undergoing bioresorption. 1F9 could potentially be used in a wide variety of applications. When cultured with 3T3 fibroblasts, the 1F9 scaffold provided a proliferative environment for the cells and upon introduction to a skin wound model in mice, the 1F9 scaffold helped to promote wound healing and angiogenesis. Moreover, 1F9 treated wounds experienced marked increases in wound closure over the controls. Thymosin Beta 4 (T β 4) is another protein has been shown to increase wound healing and angiogenesis in diabetic rats [157] and improve tissue repair. T β 4 accelerated wound closure, with significantly greater wound closing rates over control groups. Further, T β 4 was shown to promote angiogenesis at the wound site as seen by marked increased in endothelial cells and greater neovascularization. Relative capillary density was also increased in rats that were treated with T β 4.

The development of therapeutic resistance is one hypothesis for the reason for the failure of clinical trials for growth factor therapies [48, 110, 158]. In diabetic patient skin, there is a marked reduction in syndecan-4 and glypican-1 [47, 48] proteins that are key in stabilizing the interactions of growth factors with their receptors [109]. Syndecan-4 has been shown to modulate the migration of fibroblasts and keratinocytes in dermal wounds [159]. Delivery of syndecan-4 in a liposomal formulation led to enhanced revascularization in a hind limb ischemia model in mice, diabetic ob/ob mice, and rats [54, 109, 110]. This treatment also enhanced wound closure and angiogenesis in response to treatment with FGF-2 or PDGF-BB (Fig. 2A-E) [47, 71]. Formulating recombinant syndecan-4 in a proteoliposome enhanced the activation of FGF-2 mediated signaling, increased nuclear trafficking of FGF-2 and causes increased recycling of syndecan-4/FGF-2 during uptake [47, 110]. Glypican-1

proteoliposomes also enhanced revascularization in a hind limb ischemia model in WT and ob/ob mice and increased FGF-2 activity on endothelial cells [48]. Monteforte et al. isolated exosomes by immunoselection from glioblastoma cells (one of the most angiogenic tumors), applied them in a mouse hind limb ischemia model and found marked improvement in the recovery of perfusion and formation of blood vessels [160]. Other examples of growth factor co-receptors include the neuropilins (NRP1, NRP2), which help modulate the angiogenic potential of VEGF-A [161–163]. Thus, novel effective protein therapies may involve the delivery of co-receptors to enhance growth factor signaling.

2.3 Peptides

The delivery of short, anti-microbial peptides (AMPs) for therapeutic angiogenesis and wound healing is a promising development in the field [164, 165]. These peptides can be cost effective [166], and may offer an efficacious alternative to traditional growth factor therapies. Cathelicidins are a notable class of natural AMPs, LL-37 being the only human cathelicidin [167–169]. Cherreddy et al. showed improved wound closure in a full thickness excisional wound model in mice with up to a 7.5 mg/wound dose of LL-37 delivered from a tunable, nanoparticle carrier. The wounds that were treated with LL-37 nanoparticles experienced an average wound closure percentage of 90% by day 10, which was significantly higher than the negative control groups, who received either only LL-37 or PLGA scaffold with nanoparticles. Further, LL-37 delivery upregulated IL-6 and VEGF-A production and showed higher re-epithelialization than the control tissues. Other groups have also seen the upregulation of VEGF-A by LL-37 delivery [170]. Concerns remain over LL-37 treatment, as elevated levels of the peptide in circulation can result in the hemolytic activity [171, 172], and, in specific cases, the delivery of LL-37 may promote the growth of other pathogens [173] or inhibit the innate immune response to bacteria [174]. However, engineered hybrid versions of the LL-37 peptide have been shown to mitigate this danger [175]. AP-57 is another natural AMP that is commonly found in the mucosa of the stomach and colon [176]. A dose of 40 µg per treatment of AP-57 from nanoparticles delivered in an amphiphilic polylactic acid (PLA)-Pluronic F127-PLA block copolymer hydrogel was sufficient to accelerate wound closure and promote angiogenesis [177]. The delivery of AP-57 to the wound site increased the number of endothelial cells in the area and increased the vascular density significantly after 14 days *in vivo*.

LL-37 derivatives have also shown promise in enhancing angiogenesis in wounds [178]. LLKKK18 is an 18-mer peptide derivative of LL-37 that displays the most powerful lipopolysaccharide neutralizing activity of all the peptide derivatives of LL-37 [179]. For burn wound treatments, the delivery of LLKKK18 in Carbopol hydrogels reduced the wound size faster than the control, especially in the first 6 days. The level of re-epithelialization was increased in the groups that were treated with LLKKK18. Further, LLKKK18 helps to regulate the levels of reactive oxygen species (ROS), which are present in high quantities in the initial stages of healing following burn injury. Finally, burns treated with LLKKK18 displayed a three-fold increase in the number of new blood vessels at the wound site as determined by α -SMA staining.

Vasoactive intestinal peptide (VIP) is a 28-amino acid long peptide that has angiogenic and wound healing properties [180, 181]. In one study, VIP was loaded into PCL nanospun fibers and delivered to skin wounds on the backs of mice. The wounds that were treated with VIP experienced higher closure percentages than the controls, achieving 96% wound closure after 7 days. As with the delivery of the AMPs mentioned previously, the wounds experienced significant increases in cell proliferation and angiogenesis. The treatment used in this study increased numbers of endothelial cells, levels of VEGF-A and proliferating keratinocytes. Other natural AMPs from non-human sources have been shown to increase angiogenesis and wound healing. The topical delivery of 200 µg/mL of CW49, a peptide derived from frog's skin, has shown to increase angiogenesis *in vitro* and *in vivo*, through HUVEC tube formation assays and a diabetic mouse model respectively [182]. Similarly, tylotoin, a peptide present in salamander skin, showed comparable wound healing capability with EGF in a full thickness dermal wound mouse model [183]. The topical application of 20 µL of 20 µg/mL tylotoin to the wound site twice a day resulted in almost complete closure of the wounds by day 10, similar to a treatment of 20 µL of 100 µg/mL EGF. Combined with tylotoin's induction of endothelial tube formation *in vitro*, suggests that it may play an angiogenic role in wound healing.

Artificial AMPs engineered in lab have also shown promise for wound healing applications. SR-0379 is a key example of the new wave of artificial AMPs [184]. In full thickness skin ulcers in rats, treatment with 200 µg/mL of SR-0379 significantly reduced the wound area over a 60 µg/mL dose of FGF2 after just two days and decreased the wound healing time overall. These results are similar to another artificial AMP, WRL3 [185]. In a MRSA infected burn wound model in mice, the delivery of 4 µg/mL of WRL3, increased angiogenesis, as shown by an increased number of blood vessels upon histological analysis, at the burn site as well as helped mitigate the infection.

2.4 Blood Derived Factors

Blood derived factors present a promising new area of research in the field. Platelets contain growth factors and cytokines that can enhance normal wound healing process and the delivery of a high number of these factors to a wound site could provide a relatively straightforward way to enhance wound healing and angiogenesis. Platelet-rich plasma (PRP) is derived from gradient centrifugation of a patient's whole blood [186], and some components of PRP include PDGF-BB, TGF-β1, FGF-2, VEGF-A, EGF, and platelet factor-4 to name a few [187]. The potential strengths of PRP therapy include the fact that autologous PRP is easily obtainable and that it may mimic the healing process with a combination of growth factors, fibrin and other components, as to a single growth factor therapy. Some studies have supported that PRP can improve neovascularization and wound repair [188], however, the clinical evidence for using PRP in chronic wounds comes from trials with relatively small patient populations, making the current support for use in humans inconclusive [189].

In animal studies, PRP therapy can induce neovascularization and has also been shown to promote graft survival in chondrocutaneous composite grafts in rabbit ears [190]. Treatment with 1 mL of PRP significantly increased the number of endothelial cells and the number of

microvessels per field of view upon histological analysis. The expression of VEGF-A also increased significantly in the PRP group in comparison the control group that did not receive PRP injections. Qui et al. also showed that the controlled release of PRP from a hydrogel composed of poly(d,l-lactide)-poly(ethylene glycol)-poly(d,l-lactide) (PDLLA-PEG-PDLLA: PLEL) triblock copolymer increased cellular migration and tube formation significantly over the control [191]. Further, the controlled release of 0.8 mL of PRP significantly increased the wound closure in rabbits over PRP alone and the control. Additionally, PRP delivered from a PLEL scaffold significantly increased the number of blood vessels in comparison to PRP without the scaffold or the control groups. Therapy with PRP may also promote angiogenesis by modulating the tissue to secrete inflammatory proteins into the surrounding space [192]. In tendon cells from both healthy and tendons exposed to IL-1 β (tendinopathic conditions), the addition of PRP to the culture reduced the secretion of IL-6, IL-8 and monocyte chemoattractant protein 1 (MCP-1). In addition, treatment with PRP increased the production of VEGF-A and hepatocyte growth factor in normal and tendinopathic cells.

Portions of PRP called platelet lysates have been shown to promote angiogenesis and help direct cell migration during the wound healing process [193]. A major function of PRP therapy is derived from its effect on mesenchymal stem cell (MSC) homing and migration. Platelet lysates have been shown to promote significantly faster adhesion and migration of MSCs in culture over the controls. When exposed to platelet lysate, MSCs increase their secretion of VEGF-A and PlGF, both important chemotactic growth factors for endothelial cells. While platelet lysate also promotes endothelial migration, the addition of the secreted proteins from the MSCs in the local environment may even further accelerate the endothelial migration. Finally, platelet lysates have been shown to increase the levels of neovascularization, as shown by the increased expression of PECAM-1 in platelet lysate coated scaffolds over the uncoated scaffolds [193].

Hemoglobin is another protein that researchers have used to improve ischemic wound healing [194, 195]. Hemoglobin carries oxygen throughout the body's circulatory system and oxygen plays a significant role in wound healing, by facilitating aerobic respiration, as well as evolving into ROS. In a rabbit ear ischemic model, the delivery of IKOR 2048 (a chemically modified bovine hemoglobin) was shown to enhance wound repair and increase cellular survival. The local expression of Ki-67 (a marker of cellular proliferation), PECAM-1, VEGF and collagen were also increased upon administration of IKOR 2048 when compared to the saline control. The delivery of 400 mg/kg of IKOR 2048 significantly increased the oxygen tension in the tissue over untreated ischemic tissue, however, the treated tissue could not return it to normal levels. Liposome encapsulated hemoglobin (LEH) delivery to gastrointestinal wounds in rats yielded an increase in burst pressure two days after delivery, over the control groups, demonstrating increased healing.

Blood derived factors present a promising area of research for scientists and clinicians. While longer-term studies are needed to assess the safety of these treatments, the autologous nature of the treatments, the wide range of therapies available, and the auspicious pre-clinical and clinical results indicate PRP treatments could provide a better alternative to

single growth factor therapies alone. Table 2 summarizes the studies using proteins/peptides other than growth factors for enhancing angiogenesis in wound healing.

3. Gene and Nucleic Acid-Based Therapies for Wound Angiogenesis

3.1 MicroRNAs in Wound Angiogenesis

Gene regulation plays a critical role in angiogenesis during wound healing. MicroRNAs (miRNAs) are roughly 21–25 nucleotide long non-coding RNAs that play a powerful role in controlling gene expression by binding to target mRNA and either suppressing mRNA synthesis or causing mRNA degradation [196–198]. Following inflammation, gene expression is regulated to increase angiogenesis and remodeling of the wounded tissue [199]. MiRNAs have been shown to regulate many aspects of wound healing including cell proliferation and migration, collagen biosynthesis and network maturation, inhibition of neovascularization, and improved blood vessel growth [200–206].

3.1.1 MicroRNAs that Target Angiogenesis in Wound Healing—With the power to increase or suppress gene expression, miRNAs have become a target of interest in the field of wound healing. A handful of miRNAs have been shown to specifically regulate angiogenesis within wound healing, summarized in Table 3. These miRNAs are involved in increasing vessel formation and maturity, modulating migration, reducing scar formation, and regulating expression of pro-angiogenic proteins VEGF, GATA2, Ang-2 and PDGF-B, as well as the anti-angiogenic protein TSP-1 [200, 203, 205, 207–209]. Overall, it is becoming increasingly clear that miRNAs play a powerful role in regulating angiogenesis in the context of wound healing.

High expression of various miRNAs has been correlated with decreased angiogenesis. By targeting these inhibitors of angiogenesis, several groups have been able increase angiogenesis in wounds. One method of inhibiting miRNAs is using anti-miRNA or “antagomirs”. Antagomirs are inhibitors of miRNAs that function by binding to specific miRNAs to prevent them from binding to their mRNA targets [210, 211]. Patients with chronic wounds have been found to have a more than two-fold greater expression of miR-92a compared to patients with healing skin wounds [212]. Likewise, overexpression of miR-92a has been shown to result in decreased angiogenesis, however anti-miR-92a antagomir treatment (8 kg/mg injection) enhanced blood vessel growth in an *in vivo* hind limb ischemia model in mice by over 50% [213]. Inhibition of miR-92a also improved wound healing and angiogenesis in a full thickness excisional wound model, specifically through decreasing activity of integrin subunit $\alpha 5$ (Itga5) and sirtuin-1 (Sirt1) [212, 213]. Some antagomir treatments have been modified to be light inducible. The addition of photolabile protecting groups prevents binding with target miRNA until activated by a light source, this light-inducible group has been labeled a “caged” antagomir [212]. This light inducible delivery method enables spatial control of the delivery of the gene therapy [212]. Treatment of diabetic (db/db) mice with an intradermal injection of 1 μ g of light-inducible antimiR-92a in 50 μ L of PBS resulted in double the population of platelet endothelial cell adhesion molecule (PECAM-1) positive cells in the granulation tissue compared to the anti-miR-Control at 11 days post injury (Fig. 3 A). Wound area closure for caged antimiR-92a

with light was on par with non-caged antimiR-92a. Meanwhile, caged antimiR-92a was not effective at improving wound healing, with closure rates similar to the cage antimiR-control (Fig. 3B-G). Identifying miRNAs that regulate wound healing enables a clear path forward with potential antagomir treatment, as seen with miR92-a. In diabetic mouse wounds, levels of miR-26a have been found to be elevated compared to wild type (3.5-fold increase) [214]. To target miR-26a, Locked Nucleic Acid (LNA) anti-miR-26a inhibitors were used. Locked Nucleic Acid (LNA) is a high-affinity RNA analog, which “locks” to the target RNA. Intra dermal injection of 0.63 mg/kg of LNA-anti-miR-26a inhibitors induced angiogenesis, accelerated wound closure by 53% and resulted in an approximately 50% increase in endothelial cells (PECAM-1⁺ cells) in full thickness excisional wounds.

It has been reported that temporary downregulation of miR-200b during wound healing enables angiogenesis [208, 215]. MicroR-200b is thought to inhibit epithelial to mesenchymal transition and target angiogenic cytokines and transcription factors. Treatment of full thickness excisional wounds in mice with lentiviral particles expressing miR-200b (at a titer of 2×10^7 cfu/mL, injected intradermally 1 mm from the wound) induced slower wound healing and reduced blood flow, compared to the control lentivirus treatment. Additionally, reduced numbers of PECAM-1⁺ cells were found in the wound in miR-200b treatment groups compared to the control [208]. Treatment with anti-miR-200b resulted in significantly increased microvessel density (measured via PECAM1+ immunostaining) compared to treatment with the vehicle control in a diabetic (db/db) mouse full thickness excisional wound model [216].

MiRNA profiling of wound healing in C57BL mice identified 54 miRNAs that were increased or decreased more than two-fold compared to miRNA expression levels in normal skin [206]. Wound tissue samples from the wound bed, margin, and normal skin were collected and total RNA was isolated for miRNA microarray analysis. MiR-31, miR-21, miR-203 were upregulated by more than two-fold and miR-429 was down regulated by more than two-fold, confirmed by qPCR. MiR-31 has been identified as a regulator of HIF-1 α , miR-203 targets p63 which is a regulates stem cell maintenance. Meanwhile, miR-203 and miR-429 regulate genes associated with tumors. Treatment with 16 μ g of miR-21 antagomirs dissolved in 100 μ L of PBS injected locally in the surrounding dermis reduced wound closure and collagen deposition. In addition, epithelial cell migration into the wound was reduced with miR-21 targeted antagomirs treatment [206]. In both excisional and burn wound models, expression of miR-29b was decreased by over 50% compared to intact skin at day 7 post wounding [202]. However, treatment with oligonucleotide inhibitors of miR-29b increased HSP47 and COL1A1 gene expression in fibroblasts *in vitro* by more than two-fold. Furthermore, miR-29b lentivirus treatment to increase expression of miR-29b (2×10^7 TU/wound) applied either topically in a diluted Matrigel or injected intradermally in an excisional wound model resulted a decrease in HSP47 and COL1A1 expression by over 50% compared to the control virus treatment. Treatment with miR-29b also reduced scar tissue formation and increased breaking strength of newly formed tissue [202].

MicroRNA profiling of patients with and without diabetes revealed significantly lower levels of circulating miR-191 and miR-200b in patients with type 2 diabetes. Interestingly, diabetic patients with chronic wounds and peripheral arterial disease (PAD) had increased levels

miR-191 and miR-200b, like that of healthy patients [205]. Slightly higher concentrations of miR-191 were found at the wound site compared to plasma levels in diabetic patients with PAD and chronic wounds. To understand the effects of miR-191, tubule formation assays were performed. Tubule formation assays are an *in vitro* assay to assess angiogenic potential, where more tubule formation is indicative of greater angiogenic potential. Treatment with miR-191 reduced *in vitro* tubule formation by over 50% meanwhile, treatment with anti-miR-191 increased tubule formation compared to the scramble control.

Another study found significantly reduced levels of miR-27b in mononuclear blood cells in patients with type 2 diabetes compared to healthy patients [200]. To confirm the role of miR-27b in angiogenesis during wound healing, bone marrow-derived angiogenic cells (endothelial progenitor cells selected via LDL uptake and lectin binding via *Ulex europeus* agglutinin-FITC binding assay) were co-delivered with miR-27 mimics (0.25 nmol in a 1% F-127 pluronic gel applied locally to the surface of the wound). The miR-27 mimic delivery improved wound perfusion, as measured by laser Doppler, in a full thickness excisional wound model in db/db mice. Perfusion was improved by approximately 60% compared to scramble control treatment, to levels similar to that of heterozygous non-diabetic littermate control mice after 8–10 days [200].

Many new miRNA therapies are emerging with the potential to improve angiogenesis in wounds to enhance healing. These therapies are promising, in large part due to the targetable nature of miRNAs using antagomir treatments. However, effective delivery and stability in the wound environment remain challenges for using miRNA-based therapeutics in this area.

3.2 Delivery Methods

3.2.1 Free Viral Vector Delivery—Adeno-associated viruses (AAV) are non-enveloped viruses containing linear single stranded DNA. These viruses are characterized by low immunogenicity and the need for a helper virus to replicate [217]. AAVs are an attractive method for gene therapy particularly due to their safety, as they show little evidence of being pathogenic [217, 218]. Recombinant adeno-associated viruses (rAAV) encoding human vascular endothelial growth factor-165 (VEGF165) was delivered in a db/db diabetic mouse wound model in which two full thickness longitudinal wounds were made on the dorsum of the mice. Recombinant AAV-VEGF165 treatment, delivered by intradermal injection of $\sim 10^{11}$ particles, resulted in a significant increase in angiogenesis after 7 and 14 days compared to the control delivery of rAAV-LacZ. Recombinant AAV-VEGF165 increased angiogenesis, epidermal regeneration, and granulation tissue thickness at the wound site by over two-fold at both 7 and 14-day time points [219].

The angiopoietins are another family of regulators of angiogenesis and vasculogenesis. Specifically, Ang-1 important for vessel stabilization, maturation, and remodeling [220]. Delivery of rAAV encoding Ang-1 more than doubled granulation tissue thickness after both 7 and 14 days in a diabetic mouse incisional wound model, and nearly tripled capillaries present in the wound samples in rAAV-Ang-1 treated mice compared to the delivery of rAAV-LacZ after 14 days [209]. In these studies, it was demonstrated that VEGFR-2 was more than doubled as a result of rAAV-Ang-1 treatment compared to rAAV-LacZ treatment, but VEGF mRNA expression did not differ between treatment groups [209]. Overall, it

appears that AAV delivery of VEGF and angiopoietin are both effective treatments for improving angiogenesis and wound healing in diabetic mouse models of wound healing.

3.2.2 Scaffolds and Hydrogels Employed in Gene Delivery—A broad range of scaffold and hydrogel delivery systems have been employed for delivering gene therapies targeting angiogenesis in wound healing, each delivery system with unique benefits for gene-based therapies. The delivery systems include simple fibrin scaffolds, gel-in-gel systems that enable sequential release, electrospun gels, multilayer gels, and hydrogels functionalized with peptides to encourage nuclear translocation.

Nitric oxide (NO) is a regulator of endothelial cell growth, vasomotor tone and angiogenesis [221]. Previously, it has been demonstrated that there is a decrease in the production of nitric oxide (NO) in wounds of patients with diabetes [222]. Endothelial nitric oxide synthase (eNOS) can be delivered to increase NO production and enhance wound healing. Delivery of adenoviral eNOS (3×10^7 pfu) in a fibrin scaffold (60 mg/mL) led to a sustained production of eNOS for two weeks compared to delivery of the free virus in a diabetic rabbit ear excisional wound model [223, 224]. Additionally, the fibrin scaffold yielded a reduction in the volume of inflammatory cells by approximately half from the 7-day time point to the 14-day time point [223].

Rab18, a gene involved in trafficking and regulating secretion, had a 40-fold decrease in expression in wounded keratinocytes under high glucose conditions [225]. A hyperglycemic rabbit model was used to test the co-delivery of Rab18 and eNOS plasmid DNA. Fibrin microspheres were encapsulated in a fibrin gel (60 mg/mL) to deliver the plasmids (10 μ g of Rab18 plasmid DNA). This delivery system built on the use of a fibrin scaffold to enable release of eNOS plasmids from the fibrin gel followed by a secondary wave of release of Rab18 plasmids from the fibrin microspheres within the gel. The combination of Rab18-eNOS DNA delivery increased wound closure to approximately 90% after 14 days whereas eNOS delivery alone had only reached approximately 70% wound closure [225]. This model is useful in delivering multiple treatments in sequence to maximize therapeutic effects.

Elastin-like-polypeptide (ELP) systems have also been used for the sequential delivery of genes such as eNOS and IL-10 [226]. IL-10 is an anti-inflammatory cytokine that is present in ischemic tissue [227]. Like the fibrin microspheres in a fibrin gel, this system was designed to deliver IL-10 plasmids quickly followed by delivery of eNOS from ELP microspheres within the delivery platform. Co-delivery of IL-10 (10 μ g) and eNOS (20 μ g) and delivery of eNOS alone (20 μ g) encoding plasmids resulted in increased blood vessel density after two weeks in a subcutaneous implant model compared to IL-10 plasmids. In a hind limb ischemia model in mice, perfusion almost doubled in eNOS and IL-10/eNOS treatment groups in comparison to saline controls, ELP scaffold alone, or IL-10 treatment [226].

Electrospun poly-L-lactide (PLA) and polycaprolactone (PCL) have been used to create scaffolds for gene delivery of keratinocyte growth factor (KGF) [228]. It was found that PLA/PCL composite scaffolds promoted the highest levels of cell adhesion to the scaffold, indicating that the scaffold could support cell growth to enable angiogenesis and wound healing [228]. In a full thickness excisional mouse wound model, treatment with KGF

plasmids (approximately 3.4 µg DNA/mg scaffold) in a PLA/PCL scaffold placed on the wound resulted in a two-fold increase in re-epithelization of excisional wounds significantly at day 5 after wounding compared to untreated wounds. Greater proliferation, and improved epidermal thickness were seen after 7 days compared to scaffolds with control plasmids [228].

Bilayer dermal equivalents have also been used for gene delivery in full thickness wounds. Dermal equivalents are useful in providing a template for the dermis to use as it heals. Bilayer dermal equivalents have been used to deliver VEGF-165 to full thickness burns in a porcine wound model [229]. The porous collagen-chitosan/silicone bilayer membrane dermal equivalents (BDE) loaded with plasmid VEGF-165 (50 µg per mg scaffold) demonstrated sustained release for up to 4 weeks *in vitro*. *In vivo* results showed significantly more vessel formation in the VEGF-BDE compared to blank BDEs or plasmid DNA alone, with the greatest improvements at 21 days being an 80% increase in vessel density compared to the control [230]. As well as significantly more mature vessels at 7, 14, and 21 days post wounding. This demonstrates the benefits of combinatorial therapies providing both a tissue scaffold and gene delivery in the wound.

Modification of the HIF1- α gene by truncating the oxygen dependent domain from the gene (HIF1- α - ODD) has provided a novel gene to deliver for increasing angiogenesis [231, 232]. Under normoxic condition, oxygen binds to HIF1- α and eventually results in degradation of the HIF1- α protein. Removal of the ODD from HIF1- α prevents oxygen binding and subsequent degradation, thereby stabilizing the protein [233]. Once stabilized, HIF1- α can induce expression of VEGF longer, with the goal of increasing angiogenesis. Delivery of HIF1- α DNA with a deleted oxygen-sensitive degradation domain (HIF1- α - ODD) improved vessel maturity as measured by the number of vascular structures displaying PECAM-1 and smooth muscle actin (SMA) [232]. The HIF1- α - ODD treatment resulted in the greatest number of vessels displaying both PECAM-1 and β -SMA compared to VEGF-A protein treatment or the fibrin matrix alone in an excisional wound mouse model at the 7-day time point. In another study, the HIF1- α - ODD gene was delivered with poly (L-lysine)-graft-poly (ethylene glycol) (PLL-g-PEG) functionalized with one of two peptides, either a TG-peptide designed to sustain release of the DNA or a poly-arginine peptide designed to aid in nuclear translocation [231]. The poly-arginine modified PLL-g-PEG delivery of HIF1- α - ODD resulted in the greatest increase in VEGF-A, PECAM-1, and smooth muscle alpha-2 actin (ACTA2) in wounds of normal mice and diabetic rats seven days after wounding, compared to all other treatment groups.

3.2.3 Electroporation and Sonoporation Enhanced Delivery—Electroporation uses electrical pulses to induce temporary pores in the cell membrane, allowing the passage of DNA into the cell. Sonoporation uses ultrasound to create cavitation bubbles, which in turn create pores in the cell membrane [234]. The major benefit of electroporation and sonoporation is that they do not require virus particles to deliver DNA to cells [235, 236]. Use of electroporation during delivery of plasmid TGF- β 1 significantly increased wound closure three and five days post wounding in a full thickness excisional wound model in diabetic (db/db) mice [237]. Approximately five-fold more endothelial cells were observed

in the granulation tissue at the center of the wound bed compared to TGF- β 1 plasmid treatment without electroporation, indicative of angiogenesis.

Minicircle DNA is a type of plasmid DNA in which the bacterial backbone has been removed to reduce the potential for immunotoxic responses [238, 239]. Minicircle-VEGF165 DNA (20 μ g) delivery via sonoporation improved wound healing compared to the null plasmid control diabetic mouse treatment group in a full thickness excisional wound model [240]. Wound closure was significantly improved at day six after wounding, almost to the same level as non-diabetic mice controls. Blood perfusion and endothelial cell staining indicated that VEGF165 gene delivery via sonoporation increased capillary density and blood perfusion in the wound site compared to null plasmid control treated diabetic mice. Similar studies came to the same conclusion, ultrasound delivery of plasmid VEGF165 gene in a diabetic mouse excisional wound model yielded strong increases in blood perfusion in the wound compared to EGF gene delivery and control diabetic mice (untreated) [241]. These studies demonstrate the potential for electroporation and sonoporation to boost gene delivery once targetable genes have been identified for angiogenesis in wound healing.

4. Drug and Drug-Like Compounds for Enhancing Wound Angiogenesis

4.1 Strategies using delivery of small molecules to promote angiogenesis and rescue impaired wound healing in animal models

A dynamic angiogenic response is critical for wound healing. Numerous investigators have focused on the development of innovative approaches for therapeutic angiogenesis to aid in healing of chronic wounds [242–245]. Recent advances in our understanding of chronic wound biology have led to the development of small-molecular therapies that promote angiogenic activity and can modulate the repair process with exciting potential for clinical application. Previously, small molecules have been used to remedy diabetic impaired wound healing in animal models [246–249]. Nitric oxide (NO) has been identified as one molecule that plays a vital role in wound healing. NO levels increase significantly following skin injury and then gradually decrease as wound healing progresses [250, 251]. Han et al. synthesized nitric oxide releasing nanoparticles (NO-np) by encapsulating in a hydrogel-glass composite a mixture of tetramethyl orthosilicate, polyethylene glycol, chitosan, glucose and sodium nitrite in sodium phosphate buffer (0.5 mol/L) [250]. The nitrite within the matrix reduced to NO to allow for stable release of NO from nanoparticles. Han et al. found that topical application of NO-releasing nanoparticles increased angiogenesis in mice excisional skin wound model utilizing implanted silicone ring. Generation of dense vascularization of wounded area was observed, while non-treated wounds experienced minimal new blood vessel formation by measuring expression of CD34. NO-releasing nanoparticles also increased levels of TGF- β in wounds know to play an important role in promoting angiogenesis during wound repair. In addition to angiogenesis, NO also mediates inflammatory response in wound healing regulating recruitment and infiltration of inflammatory cells like inflammatory macrophages [252–254]. Macrophages play a significant role in the initiation and progression of wound healing, but an uncontrolled inflammatory response in the long-term can be counterproductive for tissue repair. Further

studies need to be performed to evaluate long-term dosage response of NO-releasing nanoparticles to determine long-term therapeutic efficacy.

4.2 Adenosine Triphosphate

Most of the energy-requiring processes, including mitotic and biosynthetic activities of cells during wound healing, either directly or indirectly occur via hydrolysis of adenosine triphosphate (ATP). ATP is used throughout the wound healing process during various metabolic processes involving protein and lipid biosynthesis, growth factor production, signal transduction and mitosis. There are changes in enzymatic and metabolic activity throughout the wound healing process. Reduced blood flow and decreased oxygen supply at wound site often results in ischemic and potentially hypoxic conditions which can disrupt the balance of energy production and utilization in cells. This imbalance can result in the depletion of high-energy phosphate ATP that in turn can decrease the cellular energy supply, potentially hindering the healing process [255]. Although the delivery of ATP is a potential strategy to enhance wound healing. Extracellular ATP released during cell damage and apoptosis has been shown to activate damage-associated molecular patterns (DAMP) signaling eliciting an inflammatory immune response. [256] Therefore it is important to utilize a vehicle to deliver soluble ATP to mediate an undesired immune response.

Encapsulation of ATP in small unilamellar lipid vesicles (ATP-vesicles) accelerated the healing of full-thickness skin wound in nondiabetic and diabetic rabbit models [257, 258]. Early *in vivo* studies evaluating the treatment of full-thickness skin wounds with highly fusogenic lipid vesicles containing magnesium–ATP showed that intracellular delivery of ATP significantly enhanced wound healing [257]. Wang et al. created wounds on the ventral side of each ear using a stainless-steel punch. Ischemic ears were generated via artery ligation at the base of ear. Immunostaining for PECAM-1 in the non-ischemic ear wound after six days showed that wounds treated with ATP-vesicles contained significantly higher number of small capillaries compared to saline-treated group 14.3 ± 2.7 capillaries/field versus 5.5 ± 1.2 capillaries/field observed respectively. The delivery of ATP-vesicles (10 mM) to ischemic ear wounds in rabbits initiated early angiogenesis in the wound bed to promote faster wound closure and generation of better developed granular tissue and re-epithelialization compared to nontreated rabbits [258]. In addition to the presence of capillary loops in the deeper layers of the wound bed in the ATP-vesicles–treated group histological analysis confirmed an increased presence of neutrophils, macrophages and lymphocytes in the ATP-vesicle treated group in comparison to saline-treated group six days after injury.

A follow up study by Wang J. et al. performed in diabetic rabbits observed similar results to the study published a year earlier [258]. Full thickness and ischemic ear wounds were created in alloxan-induced diabetic mice. There was a significant difference in the wound closure times between ATP-vesicle treated groups in both ischemic and non-ischemic ears. Like their first paper, treatment with ATP-vesicles increased closure rate of wound significantly exhibiting wound closure as early as day 9 for ATP-vesicle treated mice compared to day 12 for mice treated with saline only. When ischemic wounds of diabetic mice were treated with ATP-vesicles, the average closure time was 15.3 days in comparison

to 19.3 days for control wounds [259]. Histological analysis after two days showed early angiogenic activity the presence of a few small capillaries and numerous inflammatory cells including neutrophils, lymphocytes, and macrophages were present in granulation tissue of ATP-vesicle treated wounds. Unfortunately, Wang et al. did not measure the formation of mature vascular networks. After 17 days, saline-treated wounds showed incomplete re-epithelialization and less granulation tissue formation, while the ATP-vesicles treated group showed full granular tissue coverage and complete re-epithelialization of the wound indicating ATP-vesicles have the potential to initiate formation of a new vasculature network in injured tissue to restore blood circulation in wound area critical to facilitate ECM production and epithelialization, but this was not confirmed. [259]. These findings indicate that the intracellular delivery of ATP enhanced wound healing in normal and diabetic wounds and offers a potential option for treating chronic non-healing wounds. These studies evaluated the angiogenic potential of ATP-vesicles several days post injury. Future studies that investigate the therapeutic potential of ATP-vesicles in wound healing should further evaluate the long-term effect of ATP-vesicle treatment on angiogenesis in wound healing, and how presence or lack of a stable vasculature alters progress of wound healing. Although the mechanisms by which intracellular ATP delivery enhances wound healing are not fully understood, it is apparent that the availability of ATP is an important aspect of the healing process.

4.3 Statins

Statins are 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors primarily used to treat hypercholesterolemia and atherosclerosis. Over the past decade, researchers have shown that, in addition to their lipid-lowering effect, statins possess pro-angiogenic properties that can potentially protect against ischemic injury and stimulate angiogenesis [260]. Although still under investigation, early studies suggest that statins angiogenic activity is mediated via the Akt/PI3K pathway to regulate proliferation of endothelial cells and capillary morphogenesis, and to modulate secretion of critical factors such as VEGF-A in multiple cell types [261]. In an early study evaluating the therapeutic potential of simvastatin to enhance wound healing, Bitto et al. used simvastatin to treat full-thickness wounds in female *db/db* mice. Intraperitoneal injection of diabetic mice with simvastatin (5 mg/ kg) increased VEGF-A protein and gene expression in diabetic mice with excisional wounds, following 3 and 6 days of treatment [262]. Improved wound healing and increased angiogenesis were observed in simvastatin treated diabetic wounds by day 12. Immunostaining used to detect PECAM-1 in injured tissue sections revealed an increase in PECAM-1 positive staining and nearly double the microvasculature density in simvastatin-treated group compared to the untreated control group [262]. Additionally, daily treatment with simvastatin also improved the tensile strength of damaged tissue and enhanced production of nitric oxide in wound, nitric oxide as previously discussed is a molecule shown to play a significant role in wound healing process [250, 251].

A similar study conducted a few years later by Asai et al. observed that the wounds of *db/db* mice treated topically with simvastatin (50 µg/wound) exhibited more than 90% re-epithelialization of injured tissue and accelerated wound closure with significantly smaller wound areas observed by day 7 compared to control. Immunostaining for PECAM-1 showed

a marked increase in neovascularization in treated wounds on day 14 (Fig. 4A, B). In addition to enhanced wound vascularity, immunostaining for lymphatic endothelial cell specific marker lymphatic endothelial hyaluronan (HA) receptor-1 (LYVE-1) displayed the presence of new lymphatic vessel at the margins of the wound (Fig. 4C, D) [246]. Presence of new lymphatic vessels suggests recovery of the lymphangiogenic function, which is important for maintaining tissue homeostasis and macrophage infiltration during an immune response [263]. Treatment with simvastatin resulted in an increased population of macrophages M2 (alternatively activate) macrophages expressing IL-13 with a significantly smaller population of TNF- α expressing M1 macrophages indicating that simvastatin treatment did not elicit an undesired inflammatory response that could potentially hinder wound healing. Simvastatin treated group also exhibited increased protein expression of VEGF-C in simvastatin treated wounds. Gene expression for PDGF-B, eNOS and FGF-2 were also significantly upregulated by simvastatin treatment [246].

More recently, improved drug delivery systems have been utilized to enhance the angiogenic and wound healing potential of statins for treating chronic wounds. Lyophilized wafers composed of sodium carboxymethyl cellulose and methyl cellulose have been developed as potential wound dressings for administering simvastatin and other small molecules to chronic wound sites [263]. Moisture retentive dressings are desired for treating chronic wounds to absorb wound exudate and manage the moisture balance [264]. Lyophilized wafers are a viable candidate for wound dressings that require a moist environment such as mucosal wounds and venous ulcers [265]. When applied to wound sites, they absorb wound exudate and form a gel to maintain a moist environment [263, 265]. Hydrogels have also been developed that possess desired mechanical properties that maintain optimum moisture in the wound area and aid in wound healing process. An ideal wound dressing would exhibit slower drug release from the medicated dressing to help prolong the action and release of the active drug at wound site. A recent study by Yasasvini et al. evaluated the *in vivo* wound healing efficacy of polyvinyl alcohol (PVA) hydrogels loaded with simvastatin encapsulated chitosan microparticles. Simvastatin loaded chitosan microparticles were generated via modified ionic gelation method and loaded hydrogels were prepared by chemical cross linking method.[266] Cutaneous wounds were created in rats followed by treatment with varying concentration of simvastatin-chitosan microparticle loaded PVA hydrogels (2.5, 5 and 10 mg). Treatment with low dose simvastatin hydrogels (2.5 mg/patch) every 7 days for 21 days displayed increased rate of wound closure compared to control non-treated wounds and medium and high dose treated wounds (5 mg and 10 mg respectively) [266]. In rat wounds treated with low dose hydrogels, histological analysis revealed granulation tissue with complete epithelialization 21 days after wounding. Recent studies have shown that utilizing drug delivery systems to administer statins have increased drug efficacy as a therapeutic in wound healing. Unfortunately, there is still very little research that explores how these systems enhance statins angiogenic potential *in vivo*. Previous studies exploring the angiogenic potential of free statins have shown that topical administration of statins promotes vascular formation in wound injury. The field would further benefit from exploring how target drug delivery systems enhance this effect. Additionally, although novel drug delivery systems offer a strategy for sustainable controlled drug release to facilitate a prolonged therapeutic effect of drugs and aid in long-term wound recovery, further

characterization of the *in vitro* and *in vivo* toxicity and efficacy of simvastatin-loaded delivery systems is needed.

4.4 Deferoxamine

In chronic wounds the healing process is often impaired and many growth factors like VEGF, which are essential to angiogenesis and the wound repair process are downregulated [267, 268]. As part of the wound repair mechanism, the wound microenvironment can become hypoxic due to injury of the microvasculature. This hypoxia can induce cytokine production and cell recruitment to accelerate wound closure [247, 269]. Hypoxia-inducible factor-1 (HIF-1) is involved in regulating the response of cells to altered oxygen levels and plays a key role in controlling hypoxia-mediated events in wound healing [247, 269]. During wound repair, the active subunit HIF-1 α regulates various processes including cell metabolism, proliferation, survival, and angiogenesis targeting important pro-angiogenic genes such as VEGF-A and stromal cell-derived factor 1- α (SDF-1 α) [247]. SDF- β also known as C-X-C motif chemokine 12 (CXCL12), has been shown to be beneficial in promoting wound healing in *in vivo* models.[155] The chemokine CXCL12 binds receptors on surface of immune cells to regulate inflammatory response during healing process.

Lactobacillus reuteri bacteria (*L. reuteri*) transformed with a plasmid encoding for the chemokine CXCL12 to produce prolonged overexpression of CXCL12 by sustaining a pH regulated hypoxic wound microenvironment were shown to be beneficial to wound healing when topically administered to full-thickness wounds in mice with peripheral ischemia following hind-limb ischemia procedure and hyperglycemia.[155] Improvements in wound healing and blood perfusion were observed in both hyperglycemic and ischemic mice. Therefore, downstream effectors of HIF-1 particularly CXCL12 or SDF-1 β are promising targets for wound healing therapeutics.

Deferoxamine (DFO) is an iron chelator, which has been used to mimic oxygen deprivation in cells and induce the accumulation of HIF-1 α . Hou et al. explored the effect of DFO treatment on neovascularization in diabetic wounds, and the expression of HIF-1 α and downstream genes. Full-thickness excision cutaneous wounds were generated on dorsal skin of rats with streptozotocin-induced diabetes and treated with the vehicle control group (PBS), DFO (10 mg/mL) or VEGF-A (0.5 nM) [247]. The DFO-treated group exhibited accelerated wound closure at 7, 10 and 14 days compared to the vehicle and VEGF-A treated groups [247]. Histological analysis showed that DFO treatment stimulated formation of microvessels around the newly formed granulation tissue seven days after wounding. Vessel density was assessed via vWF immunostaining at 7 and 14 days. The number of vessels per field was approximately four times greater in DFO treated wounds compared to the vehicle only group, and slightly higher than the VEGF-A treated groups with 20 and 15 vessels/field respectively. Western blot analysis of tissue lysates showed that HIF-1 α was associated with DFO treatment enhancing neovascularization. Protein expression of HIF- α was significantly elevated in DFO treated wounds and VEGF protein expression was also found to be higher in the DFO-treated group compared to vehicle control group. Protein expression levels of SDF-1 α were elevated at day 7 compared to both VEGF-A and vehicle treated groups. These results suggest the potential of DFO to enhance neovascularization in wound healing through increasing HIF-1 α activity. A later study by Ram et al. found that

treating wounds of diabetic induced rats with DFO ointment (0.1%) also enhanced wound healing and angiogenesis. Wounds treated with DFO experienced greater wound closure from day 7 to 19 compared to control group. Real time PCR and western blot analysis showed that gene and protein expression of HIF-1 α , VEGF-A, SDF-1 α , TGF- β 1 and IL-10 were significantly upregulated in wounds treated with DFO from days 3 to 14 [270]. In contrast, the relative mRNA expressions of pro-inflammatory cytokines TNF- α , MMP-9 and IL-1 β were significantly downregulated at days 7 and 14 post-wounding. Additionally, DFO treatment was observed to decrease protein levels of tumor necrosis factor alpha (TNF- α) post-wounding from day 3 onward and increased protein levels of IL-10 compared to controls at day 3 and onward. Indicating that treatment with DFO does not induce an early inflammatory response *in vivo*. DFO also induced angiogenesis in newly formed granulation tissue [270]. Although density of microvessels was not quantified, an overall larger number of small blood vessels were observed in regenerated tissue of DFO-treated group throughout the 19-day study compared to control group.

Researchers have tried to enhance the therapeutic potential of DFO using transdermal delivery systems that can obtain deeper penetration into the wound bed. Duscher et al. developed a transdermal drug delivery system that used reverse micelle encapsulation of DFO by nonionic surfactants (polysorbate 80 and sorbitan monolaurete) within a polyvinylpyrrolidone (PVP) biodegradable matrix and disperse in ethyl cellulose for slow release [271]. Pressure ulcers were induced in db/db mice by placing two ceramic magnets for three separate ischemia/reperfusion cycles. Each cycle consisted of placement of magnets for 3 or 6 hours followed by release or reperfusion for 3 or 6 hours. Treatment of pressure ulcers with transdermal DFO (1%) improved healing and stimulated complete wound closure by day 27 compared to day 39 for control group. Histological analysis showed that localized treatment with DFO transdermal delivery improved the dermal thickness of healed diabetic ulcer and promoted neovascularization within the wound bed (Fig. 5A-D). Immunostaining for PECAM-1 showed that treatment with transdermal DFO had more than twice the number of PECAM-1 positive capillaries. Sustained local delivery of DFO treatment was also found to increase early VEGF protein expression in diabetic ulcers at 24 and 48 hours. Pretreatment for 48 hours with transdermal delivered DFO prior to ulcer induction visibly reduced tissue necrosis and prevented ulcer formation later. Dysregulation of the HIF-1 α /VEGF signaling can be caused by excessive ROS production a central problem in diabetic wound healing Levels of ROS were negatively affected by pretreating with DFO. Dihydroethidium (DHE) is a superoxide indicator used to measure *in situ* generation of ROS. DHE immunofluorescent staining showed that ROS levels decreased dramatically in DFO pretreated db/db mice. Thus, DFO is a promising therapy for rescuing impaired wound healing in diabetic patients and novel delivery systems offer a potential strategy for improving treatment efficacy.

4.5 Natural Compounds

In addition to tissue healing and regeneration, another goal of wound therapy is to promote adequate healing while minimizing scar complications such as imbalanced collagen synthesis [272]. Astragaloside IV (AS-IV) one of the main active ingredients in Astragali Radix derived from the root of *Astragalus membranaceus* has long been exploited in

Traditional Chinese Medicine for its healing and anti-scarring effects [272, 273]. Chen et al. observed that AS-IV (0.5%) locally delivered at the wound site of full-thickness skin excision wounds in rats stimulated quicker recovery of wounds with minimal scarring at day 30 compared to blank control wounds. A lower ratio of type I/III was observed in the AS-IV group compared to that of the blank control group demonstrating treatment was able to regulate collagen synthesis and reduce scar formation during the wound remodeling process (Fig. 6C). Consistent with the reduction in scarring, AS-IV treatment stimulated angiogenesis at wound site. A few blood vessels could be observed in healed tissue of AS-IV treated group 30 days after wounding (Fig. 6A-B) [272].

To reduce the dosing frequency and enhance therapeutic efficacy, pharmaceutical carriers for the topical application of AS-IV have been investigated. One study examined the use of solid lipid nanoparticle-enriched hydrogel (SLN-gel) prepared by a solvent evaporation method to encapsulate AS-IV (0.5% AS-IV/gel) and locally delivered it to full-thickness skin excision wounds in rats [273]. They found that treating wounds with solid lipid nanoparticle-enriched hydrogel loaded with AS-IV (AS-based SLN-gel) dramatically enhanced wound healing capabilities compared to a blank SLN-gel, and free AS-IV solution treated groups at days 4 and 12 after wounding. AS-IV solution and AS-based SLN-gel both stimulated angiogenesis at the wound site. Histological analysis of the wound beds showed a higher density of newly formed blood vessels in both AS-IV solution and AS-based SLN-gel, while no blood vessels were observed in control and only a few microvessels in the SLN-gel carrier only treated group [273].

In addition, AS-IV has been used to functionalize wound dressings to promote healing and prevent scar formation in burn wounds. Shan et al. developed silk fibroin/gelatin electrospun nanofibrous dressings loaded with AS-IV (0.5 mg). A homogeneous solution of a suitable blending ratio of SF/gelatin and AS-IV was flowed through a needle at a fixed rate of 0.01 mL/min. under a high applied voltage, 15kV, and electrospun to produce nanofibrous dressing. Treatment with AS-IV loaded dressing significantly accelerated wound healing compared to blank control (normal saline) groups at 7 and 15 days post-wounding in a partial-thickness burn wound model in rats [274]. Minimal scarring was observed in repaired tissue of both AS-IV loaded nanofibrous dressing and AS-IV solution treated wounds. In addition, immunostaining for PECAM-1 showed increased blood vessels in both treatment groups. Newly formed vessels were observed in the newly formed granulation tissue 31 days post-wounding in both AS-IV treatment groups. Treatment with AS-IV enhanced the number and dimensions of blood vessels compared to no vasculature formed in blank control and very few micro vessels in blank nanofibrous dressing [274]. AS-IV is a promising candidate for treating impaired wound healing. Previous studies have shown its ability to promote wound re-epithelization, angiogenesis and regulate extracellular matrix remodeling in chronic and burn wounds. Although a poorly water-soluble drug, it has been successfully incorporated into novel biocompatible carriers that allow for sustained drug release to induce increased tissue regeneration and inhibit scar formation during wound healing.

Herbal remedies have been helpful in the treatment of dermatological disorders such as skin lesions, eczema, burns and hypertrophic scars [275–277]. One example is the plant, *Centella*

asiatica that contains various active compounds such as centelloids, asiaticoside, madecassoside, asiatic acid and madecassic acid that have been found to be beneficial in wound healing [275]. Ointments containing varying doses (0.1 to 1%, w/w) of these compounds or a combination have previously been shown to enhance wound repair [275, 278–280]. Asiaticoside derived from *Centella asiatica* possesses diverse pharmacological effects including angiogenic promoting activity and wound healing enhancing capabilities. Kimura et al. observed that topical treatment of burns in mice with asiaticoside at lower doses of 10^{-8} to 10^{-12} (w/w) enhanced burn wound healing. Treating with ointment for 20 days containing various doses of asiaticoside significantly reduced burn wound area on days 6 to 18 compared to vehicle control (petroleum jelly only) with no significant difference observed between three concentrations (10^{-8} , 10^{-10} and 10^{-12} w/w) [278]. Kimura's group also treated the burn surface with polyethylene filter pellets containing three different concentrations of asiaticoside 10 pg, 1 ng, and 100 ng. Asiaticoside (10 pg and 1 ng/filter pellet) decreased polymorphonuclear leukocytes infiltration at wound site at 1 and 3 days, while treatment with all three doses (10 pg, 1 ng, and 100 ng/filter pellet) increased the number of macrophages present in wound at 1, 5 and 7 days post treatment compared to control. Examination of cytokine levels in the exudates from asiaticoside-treated burn wounds showed increased levels of IL-1 β at multiple time points compared to control vehicle treated group [278]. Topical application of asiaticoside also increased level of monocyte chemoattractant protein-1 (MCP-1) in exudates of burn wound-treated mice compared to control group [278]. Histological analysis of regenerating skin of burn wound-treated mice showed increased numbers of proliferating cells, as assessed by Ki-67 staining, and increased in number of VEGF expressing cells in the healing wound at day 9 [278]. Several other studies demonstrated that low doses of asiaticoside stimulate angiogenesis by enhancing keratinocyte production of MCP-1 that in turn increases VEGF-A levels in chronic wounds [278, 279].

The poor solubility of asiaticoside in aqueous medium is a major limiting factor in the drug's therapeutic efficacy [279–281]. To improve the pharmacological effect of free asiaticoside, carriers have been developed to increase wound healing potential. Phaechamud et al. performed the chick chorioallantoic membrane assay (CAM) to evaluate the angiogenic effect of chitosan-aluminum monostearate composite sponge dressings loaded with varying concentrations of asiaticoside. Homogenous mixture of chitosan solution and asiaticoside was fabricated via lyophilization technique, frozen, freeze dried, and treated with dehydrothermal treatment to stabilize structure of lyophilized sponge dressing. Phaechamud et al. showed that asiaticoside exhibits dose-dependent angiogenic activity *in vitro* [279]. The average angiogenesis score increased with increasing concentration of asiaticoside. The average angiogenesis score was calculated as the ratio of total number of embryos treated to evaluable embryos left after a 12-day study. Embryos treated with varying concentration of asiaticoside 40 μ g, 80 μ g, 160 μ g, and 320 μ g generated the highest overall angiogenesis scores 1.25, 1.25, 1.83, and 2.12 respectively. In comparison to VEGF positive control which at the following concentrations 100ng, 200ng, and 300ng scored 0.43, 0.17, and 0.2 respectively.

In another study, full-thickness skin wounds in rats were treated with asiaticoside-loaded porous polyvinyl alcohol (PVA) microspheres [280]. The asiaticoside-microspheres

enhanced wound healing compared to untreated wounds or those treated with free asiaticoside solution. Asiaticoside microspheres enhanced wound healing accelerating closer rates compared to free drug significantly at day 7 and day 14 and minimized scar formation. In the rats treated with asiaticoside microspheres, immunohistochemical staining for PECAM-1 showed a higher density of microvessels in comparison to the other treatment groups. Overall, treatment with asiaticoside reduced scar complications, stimulated re-epithelialization and collagen deposition, and promoted wound angiogenesis to improve wound healing. Although free asiaticoside has been previously shown to possess properties that enhance healing and reduce scar formation, its lack of solubility is a major limitation and new carriers for its delivery would be beneficial for improving its bioavailability.

Materials derived from natural processes have also been shown to have significant pro-angiogenic activity in wound healing. Dextran hydrogel-based dressings were highly proangiogenic in burn injury in mice and pigs [282, 283]. In comparison to control wounds, there was increased neutrophil infiltration in wounds treated with dextran hydrogels [282]. In addition, there was increased infiltration of endothelial cells with treatment with the dextran hydrogel dressings. Bioactive glass microfibers doped with cupric oxide also increased angiogenesis and wound healing [284]. These microfibers degrade to hydroxyapatite under physiological conditions in seven days. In a rat wound model, these fibers increased wound closure and vessel area during healing.

4.6 Hyaluronan Oligosaccharides

Hyaluronic acid (HA), also known as hyaluronan, is a glycosaminoglycan (GAG) that is a long unbranched polymer comprised of repeating D-glucuronic acid and N-acetyl-D-glucosamine disaccharide units, bound through alternating β -1, 4 and β -1, 3 glycosidic bonds. HA is a functional component of connective tissue ECM that plays a critical role in cell signaling, tissue development and maintenance of tissue health and homeostasis [285, 286]. Unlike many other GAGs, HA is not sulfated or covalently attached to core protein as a proteoglycan [287]. Previous research has implicated hyaluronan as a potential therapeutic capable of both stimulating and inhibiting angiogenesis depending on its form and concentration [287, 288]. Physiological responses to hyaluronic acid is highly influenced by HA molecular weight, which is classified into low (< 5 kDa), intermediate (60–800 kDa) and high (> 800 kDa) molecular weight groups [289]. Physiological concentrations of native long-chain, high molecular weight HA have been shown to induce anti-inflammatory and anti-angiogenic activity *in vivo* and *in vitro* [249, 288, 290, 291]. In particular, HA oligomers consisting of 2–10 disaccharides are highly bioactive and capable of stimulating neovascularization in animal wound models [249, 292]. Although the size-dependent effects of HA on biological processes are not completely understood, the size of HA can have a considerable influence on receptor activation and its downstream signaling. In an injured or disease state, aberrant conditions can cause the degradation of high molecular weight by hyaluronidases and ROS into low molecular weight HA which further depolymerized to form oligomeric HA [293, 294]. Byproducts can then bind several cell surface receptors such as CD44, HA-Mediated Motility (RHAMM), hyaluronic acid receptor for endocytosis (HARE), lymphatic vessel endothelial hyaluronic acid receptor 1 (LYVE1), layilin, toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) to influence receptor activation and their

downstream signaling. CD44 is a cell-surface receptor for hyaluronan implicated in the regulation of endothelial cell functions, and tumor angiogenesis [295, 296]. Although the role of CD44 in angiogenesis is still being investigated, CD44 has been shown to possess anti-angiogenic properties impairing tube formation *in vitro* and *in vivo* [296, 297]. CD44 mediates HA-dependent cell signaling involved in the regulation of endothelial cell proliferation, migration, adhesion, and tube formation required for angiogenesis. The interaction between HA and CD44 receptor is, in part, dictated by the size of the HA molecule that can influence CD44 clustering and activation. Oligomeric HA can competitively bind to CD44 receptor in place of anti-angiogenic high molecular weight HA to act as an antagonist to CD44, triggering intracellular signaling to promote endothelial cell proliferation, and migration to stimulate neovascularization *in vitro* [287, 291].

Previous studies have shown that topical delivery of exogenous HA oligomers to injured tissue promotes wound repair *in vivo* [249, 292]. Gao et al. topically treated murine excisional dermal wounds with HA oligomers (50 µg/per wound) encapsulated in a slow-releasing gel of ethylene-vinyl acetate copolymer. Treatment with HA oligomers accelerated wound healing, with the treated wounds exhibiting similar closure rates and recovery as those treated with VEGF-A (10 µg per wound). The HA oligomer treated wounds had increased numbers of blood vessels. Compared to the PBS and HMW-HA treated mice, there was a significantly larger number of blood vessels in the wound bed. The group treated with HA oligomer encapsulated gel by day 6 had approximately 4 times the number of blood vessels per field area of those wounds treated with PBS and HMW-HA. By day 8 post wounding HA oligomer gels contained more PECAM-1 positive blood vessels although this number was less significant than at day 6. Additionally, treatment with HA oligomers induced the formation of new lymphatic endothelium. Immunostaining for LYVE-1, a marker for lymphatic endothelial cells, was upregulated and dispersed throughout vessels in the wound bed of mice treated with HA oligomers, VEGF and HMW-HA [292]. Overall density of lymphatic vessels in HA oligomer-treated mice was significantly greater than PBS-treated mice on day 4 and 8. Although the lymphatic vessel density was less compared to high molecular weight HA and VEGF-A, suggesting that HA oligomers are superior in stimulating angiogenesis over lymphangiogenesis. Administration of HA oligomers was found to affect the expression of numerous factors involved in wound repair including endothelial cell proliferation, cell migration and collagen synthesis. Treatment with HA oligomers upregulated gene expression of endothelial eNOS, an important atheroprotective molecule, and cell adhesion molecules, E-selectin and integrin β3 at early time points compared to control [292]. In addition to inducing angiogenesis during wound repair of dermal injury, oligomeric HA treatment effects the mRNA expression of key endothelial mediators and regulators of collagen deposition.

A recent study also supports the use of HA oligomers for treating diabetic wounds. Ointment prepared by emulsification containing HA oligomers (0.15%), petroleum jelly (2.4 g), stearic acid (2.4 g), glycerol monostearate (1.6 g) and liquid paraffin wax (2.5 g) was topically administered to wounds created in rats with streptozotocin-induced diabetes [249]. Treatment with HA oligomers containing ointment significantly decreased wound size and induced formation of granulation tissue and re-epithelialization of wound surface by day 14 after wounding [249]. In addition to enhancing wound healing, HA oligomer treatment

improved blood flow in diabetic wounds as measured by laser doppler imaging. Histological analysis of wound tissue 14 days post-wounding showed increased numbers of capillaries in the HA oligomer groups in comparison to control wounds [249]. Treatment with HA oligomer containing ointment was found to not affect total Src and ERK1/2 expression in wound tissue, but levels of phospho-Src and phospho-ERK1/2 increased significantly [249]. Additionally, oligomeric HA treatment increased expression of TGF- β 1 protein.

Overall, hyaluronan has proven to be a versatile bioactive molecule playing a significant role in the initiation and progression of biological processes and pathological conditions. Shorter HA fragments like oligomers are crucial mediators of intracellular signaling cascades triggering activation of surface receptors such as CD44 to regulate inflammatory and angiogenic responses [287]. Topical administration of oligomeric HA is a promising therapeutic strategy to promote wound healing and angiogenesis in animal models of chronic and diabetic wounds [249, 292]. Evidence suggests treatment with HA oligomers is beneficial in promoting neovascularization in animal wound models, but additional studies are needed to further profile the adverse effects of prolonged HA oligosaccharide treatment including potential unwanted cell activation and prolonged inflammation. Tables 4, 5 and 6 summarize the results of studies using small molecular therapies to enhance wound healing and angiogenesis.

5. Stem Cell Based Therapies for Wound Angiogenesis

5.1 Challenges and Strategies for Therapeutic Cell Delivery

Stem cell therapies are an emerging and promising approach for treating diseases not well addressed by conventional therapies. Several stem cell types have been explored to facilitate angiogenesis and wound healing. Adult stem cells including adipose-derived stem cells (ASCs), bone marrow-derived mesenchymal stem cells (MSCs) and induced pluripotent stem cells (iPSCs) have been explored for their potential paracrine effects, immune response modulation, and their ability to differentiate into vascular lineages.

The mode of delivery of cells to the wound can have profound effects on the cell state and regenerative activity [298]. Multiple strategies have been tested to overcome the general lack of engraftment and cell viability associated with cellular therapeutics [299]. Many researchers have utilized porous scaffolds constructed out of various polymers and hydrogels. Primarily, these scaffolds function to improve cellular engraftment so that they can elicit a prolonged effect at the site of injury. Ideally, scaffold materials should be non-immunogenic, non-cytotoxic and facilitate a phenotype in the delivered cells that supports their regenerative properties. For wound healing applications, resorbable scaffolds are often used so that endogenous tissues can grow to replace the scaffold area. Furthermore, scaffolds benefit from having a level of porosity that can allow for cellular infiltration of their network and migration toward the site of injury. To promote this outward migration, integrin-binding/cell adhesion sites can be added to the material surface. While each of these characteristics can help with cellular viability and integration into host tissue, some cellular therapies have found success without the use of a scaffold. The following section summarizes and highlights several differing approaches for promoting angiogenesis and wound healing through therapeutic cellular delivery.

5.2 Adipose Derived Stem Cells

Adipose-derived stem cells (ASCs) are a promising therapeutic cell type for wound healing as they are multipotent and can achieve a vascular phenotype, however their success has been hindered due to poor survivability at the wound microenvironment [299]. Numerous studies have been conducted to optimize ASC delivery and achieve a robust therapeutic effect in various wound healing models (Table 7). Zamora et al aimed to expedite wound healing through the use of human ASCs differentiating into a supportive vascular role on PEG-fibrin (FPEG) gels [300]. ASCs were derived from discarded burn skin samples and cultured on a 3D PEG-fibrin gel. After three days in culture, ASCs formed tubule-like structures like those created by endothelial cells, however they lacked PECAM-1 and vWF expression. Gene expression of pericyte markers including for alpha actin-2 (ACTA2), PDGFRB, neural/glial antigen-2 (NG2), angiopoietin-1 (ANGPT1), and angiopoietin-2 (ANGPT2) were detected via RT-PCR. This result was confirmed via immunostaining of NG2 and PDGFR- β , suggesting a supportive vascular phenotype. To test the wound healing potential of ASCs, researchers employed a full-thickness excisional wound model in rats. Although wounds appeared to close at similar rates, histological analysis revealed that vascularization of the wound bed occurred earlier and to a higher degree in the ASC-FPEG treated group. A human specific mitochondrial antigen was used to determine if these differentiated ASCs played a direct role in wound healing and vascularization. Human cells were found on the outer layer of medium sized vessels in the wound bed, ostensibly performing a similar role to that of pericytes or vSMCs. In ASCs grown on FPEG scaffolds *in vitro*, VEGFA gene expression was found to increase over the culture period along with VEGF-A protein expression over the course of 15 days. These results indicate that, under specific conditions, ASCs can differentiate into cells that both encourage neovascularization through VEGF secretion and stabilize vessels through pericyte-like interactions.

While particular cellular treatments are meant to facilitate cell survival or differentiation so that they might have a direct effect on wound healing, others can have an effect via paracrine signaling. In papers by Garg et al and Kosaraju et al, pullulan-collagen hydrogels were created that increased bone marrow-derived mesenchymal progenitor cell (BM-MPC) recruitment to injury sites [301, 302]. Pullulan is a non-immunogenic, hemocompatible, material comprised of polysaccharides. To test progenitor cell recruitment, Kosaraju et al used a parabiosis model in which the vasculature of a GFP-expressing reporter mouse and a wild-type mouse were connected. This model allows for the transfer of cells between mice to be observed. Two dorsal excisional wounds were created on the backs of the wild-type mice. These wounds were treated with ASC-seeded hydrogels, an injection of ASCs in PBS, or a PBS control injection. Cells from the unwounded, GFP expressing, mice then migrated to the injury sites on the WT mice. BM-MPCs comprised of 23%, 8.4%, and 2.1% of the total cells recruited to the injury site in ASC hydrogel implant, ASC injection, and PBS control treatments respectively. Furthermore, a subpopulation of these BM-MPCs was further identified using single cell gene expression profile analysis. This population included the expression of known provasculogenic and remodeling genes such as angiogenin (ANG), endosialin (CD248), NANOG, NF κ B1, stem cell antigen-1 (SCA1), and extracellular superoxide dismutase (SOD3). This enhanced wound healing gene profile was expressed by

a greater amount of BM-MPCs for the ASC hydrogel condition compared to ASC injection and PBS control.

Conditioned media was collected from ASCs cultured on hydrogels or ASCs cultured using glass or plastic cell culture surfaces[302]. Conditioned media harvested from hydrogel-cultured ASCs significantly increased migration and proliferation in isolated BM-MPCs. Expression of genes relating to wound healing, cellular recruitment, and neo-vascular genes including chemokine ligand 2 (CCL2), matrix metalloproteinase-3 (MMP3), hepatocyte growth factor (HGF), hypoxia inducible factor 1 α (HIF1 α), and endoglin (ENG) was significantly elevated in BM-MPC cultures with hydrogel cultured conditioned media compared to control media. In addition, they observed increased protein levels of HGF and MMP3. Conditioned media from ASCs grown in hydrogels increased the tube formation by BM-MPCs in comparison to control groups. Overall, this study showed that ASCs grown on this pullulan-collagen scaffold enhanced angiogenesis through paracrine mechanisms and increased recruitment of circulating BM-MPCs to the wound area.

5.3 Bone Marrow Derived Mesenchymal Stem Cells

Bone marrow derived mesenchymal stem cells (MSCs) are also promising candidates as a cellular therapy for enhancing wound healing (Table 8). MSCs have been found to modulate inflammation at wound sites as well as influence surrounding cells to encourage regeneration as opposed to fibrosis [303–306]. In one study, researchers used a full-thickness cutaneous wound model in rats to study the wound healing potential of rat MSCs [307]. The cells were treated with dexamethasone and ascorbic acid (vitamin C) and cultured to create a tightly packed, confluent, sheet of cells. After 12 days, the cell-aggregate layer began to detach from the culture surface and was used in place of a scaffold for therapeutic delivery. Evaluation of gene expression showed that pro-wound healing markers collagen 1 (COL1) and transforming growth factor beta (TGF- β) were significantly elevated in cells that grew in the aggregate form, suggesting superior wound healing potential. A full thickness cutaneous wound model was performed in rats to test wound healing. The cell aggregate was placed on the wound bed with three layers of sterilized porcine small intestine submucosa to protect the cells from the wound dressing. Wound size after four weeks was significantly smaller (~50% smaller) for the aggregate group compared to the topically delivered cell suspension and intravenously delivered cells. Furthermore, capillary density was found to be greatest in the aggregate group.

Inflammation was assessed for each group through RT-PCR and immunofluorescence staining. After week two, gene expression showed significantly lower TNF- α and IL-1 β levels compared to controls. Additionally, cell aggregate groups showed lower inflammatory marker levels compared to the other two treatment groups. Gene expression for inducible nitric oxide synthase (iNOS) was found to be higher for the aggregate group compared to both the control and other treatment groups. Taken together, these results indicate a lower level of inflammation and a pro-healing wound environment. Furthermore, the presence of CD45+ lymphocytes was reduced and BM-MSC engraftment was improved at the wound bed after two weeks. This study did not show that MSCs were directly involved in the formation of new vasculature, instead the authors attribute the improved wound healing to

inflammatory regulation. Furthermore, this work represents a departure from a substantial portion of tissue engineering research which utilizes an independent scaffold for cell survival and engraftment by achieving a positive effect with a relatively simple cell aggregate approach. This method avoids issues that are normally considered with synthetic scaffolds such as degradation/absorption, cytotoxicity, and immune response. While this technique proved effective in dermal wound healing in this work, further investigation has opened the door to other wound healing arenas such as cardiac tissue repair, with areas soon to follow. [308, 309]

While MSCs have been shown to act as modulators of immune response, they are still susceptible to the potentially cytotoxic environment of the wound bed. Geesala et al sought to compliment the anti-inflammatory, pro-regenerative, function of MSCs by including a scaffold composed of polyethylene glycol and polyurethane (PEG-PU) [310]. Reactive oxygen species can impair stem cell function and reduce wound healing [311, 312]. This group aimed to modulate ROS levels and increase the efficacy of their cellular treatment through the combined activity of MSCs and a PEG-PU scaffold. The PEG-PU scaffold was synthesized using castor oil, a substance that contains elevated levels of vitamin E. In vehicle control studies, vitamin E has shown to increase the activity of the antioxidant enzyme, Cu/ZnSOD. Preliminary work was performed to create the interpenetrating polymer network, test scaffold biocompatibility, and to ensure cellular migration into and out of the scaffold. The protective role of the scaffold was assessed by varying concentrations of hydrogen peroxide to induce oxidative stress in cells that were cultured either with or without scaffolds. Each of the cell-only groups exhibited a dose dependent drop in proliferation based on an MTT assay. All scaffold groups showed increased proliferation compared to the cell-only groups when they were MSCs treated with 1 μ M and 10 μ M H₂O₂. Hoechst staining showed markedly fewer apoptotic nuclei between the scaffold and non-scaffold groups.

The scaffolds were then tested in a full-thickness excisional wound model in C57BL/6J mice. The study consisted of four treatment groups including an untreated control, scaffold alone, free MSCs, and MSCs embedded in the scaffold. By day 7, the MSC-scaffold group appeared to have the greatest level of wound closure, with approximately five times the number cells at the site of injury than the control. Significant upregulation of pro-inflammatory cytokine gene expression was observed in the MSC-only group compared to both the scaffold-only and MSC-scaffold groups, indicating that the scaffold may reduce inflammation and could protect transplanted cells an immune response. The presence of ROS in the tissue was evaluated using oxidation sensitive dihydroethidium dye staining. These results indicated that the scaffold-only and MSC-scaffold groups had the least ROS activity while the MSC-only group had the greatest. Consistent with these findings, gene expression for the antioxidant enzymes catalase and GPX2, a glutathione peroxidase, was significantly elevated for the MSC-scaffold group compared to free MSCs.

To test successful engraftment at the wound site, MSC-indicative fluorescent markers including CD133-PE and CD90.2-APC were used on tissue samples. The MSC-scaffold group showed the largest amount of fluorescence at days 7 and 10, suggesting better engraftment was achieved with the scaffold. The MSC-scaffold group sections contained a

significantly greater amount of PECAM-1 stained cells compared to the scaffold and MSC-only groups. In addition, there was increased gene expression for angiogenesis related genes including those for VEGFR2, VEGFR3, and Tie-2 from tissues harvested from the wound site. Overall, the study demonstrates a novel and potentially effective strategy for combining cellular therapeutics with hydrogel scaffolds to improve the inhospitable area of the wound site to allow for expedited healing.

5.4 Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSCs) have recently received significant attention as a cell type for improving angiogenesis and wound healing. Kim et al differentiated iPSCs into separate populations of endothelial cells and vSMCs [313]. They compared the angiogenic capability of these iPSC-derived vascular cells to primary somatic cells of the same type. They first performed a co-culture tube formation assay using vascular cells differentiated from iPSCs, cells differentiated from ECs, and HUVECs/hvSMCs at varying ratios. Both differentiated cell groups exhibited cooperation between ECs and vSMCs and greater tubule area compared to the HUVEC/hvSMC group (Fig. 7 A, B). They then injected the cell groups subcutaneously into athymic nu/nu mice suspended in a mixture of Matrigel and collagen I. After two weeks, they found increased perfused vessel area and vascular density in the groups treated with the iPSC-derived cells in comparison to groups treated with the gel alone or somatic endothelial cell/vSMCs in the gel. Through proteomic profiling of the secreted factors they found that iPSC-derived vascular cell produced more pro-angiogenic soluble factors. They also implanted the cells in a dermal wound model in nu/nu mice and found that the iPSC-derived vascular cells were superior in their ability to induce wound healing and angiogenesis in the wound bed. Lee et al found that iPSC-derived lymphatic endothelial cells also enhanced wound healing and vascular growth [314]. Using ear and dorsal wound models in nu/nu mice, they observed incorporation of the cells into the lymphatic vessel network and a rise in lymphovasculogenesis and lymphangiogenesis. In addition, the iPSC-derived lymphatic endothelial cells induced enhance wound healing in comparison to control (PBS) or human primary lymphatic endothelial cell-treated wounds. Zhang et al also tested the ability of exosomes produced by iPSC-derived MSCs to induced enhanced wound healing and angiogenesis [315]. After confirming the activity of the exosomes through proliferation/migration and tube formation assays, exosomes were subcutaneously introduced in a dorsal cutaneous injury model in rats. Wound closure after two weeks was significantly higher for the exosome-treated group compared to media controls and they observed an increase in collagen and elastin deposition. Furthermore, wound epithelialization and vessel density were significantly increased while scar width was significantly decreased. Overall, these works support that iPSC-derived cells have potential for enhancing wound healing and angiogenesis.

5.5 Other Stem Cell Populations

A number of other stem cell types have also been investigated in wound healing and angiogenesis. Placenta-derived mesenchymal stem cells (PMSCs) wound models have been studied in both rats and mice [316, 317]. Kong et al. employed a diabetic, Goto-Kakizaki, rat model with full thickness dorsal skin wounds. Fluorescently labeled PMSCs were injected intradermally at the wound site and monitored until all wounds closed. Compared to non-

treated controls, PMSC-treated wounds demonstrated expedited wound closure as well as increased vascular density as the wound site. Furthermore, PMSCs were confirmed to incorporate into the host vascular network visualized through immunofluorescence histology. Similarly, Abd-Allah et al. employed a skin wound model in wild type mice for 7 and 12 days. Like the previous experiment, PMSCs delivered either intraperitoneally or intraregionally induced more rapid healing when compared to the untreated group. Additionally, RT-PCR detected higher expression of angiogenic and wound healing factors including Integrin $\beta 1$, Integrin $\beta 3$, and a decrease in ICAM expression. RT-PCR was also used to detect the presence of human cells in the form of albumin and GAPDH expression. An ELISA also revealed greater VEGF in the PMSC-treated tissues. Both of these studies lend merit to PMSCs as wound healing agents by not only demonstrating expedited closure but also engraftment of PMSCs into the functional tissues at the wound bed. With the relative ease of harvesting this type of stem cell, PMSCs could seemingly see wider use in similar studies and applications in the future.

Skin progenitor cells have been explored as a therapy for wound healing and angiogenesis [318, 319]. Ke et al. sought to examine the effects of skin-derived precursor cells (SKPs) on diabetic wound healing in T2D mice. They hypothesized that SKPs could aid in the healing process of chronic wound disease states by improving vascularization in the area via differentiation and integration of SKPs. A collagen sponge was used as a scaffold to aid the integration of SKPs into the dorsal, full-thickness, skin wound of diabetic mice. Greater wound closure was observed for the SKP/collagen as well as the collagen only groups compared to the saline control at day 7 but slight difference was observed between the groups at day 14. Furthermore, greater capillary densities were observed in the SKP/collagen group via α SMA and isolectin staining of histology at the wound bed. The authors also observed potential neuronal regeneration alongside vascular formation through the addition of nestin staining. SKPs appear to have a vast potential in the wound healing arena as they can differentiate towards three crucial wound heal cell lineages (vSMCs, ECs, and neurons). Paired with an effective scaffold, difficulties involving engraftment and cell survival can be mitigated and the effect of SKPs can be enhanced as seen in the previously discussed article. Altogether, these cells present another promising option for an easily sourced adult stem cell therapy for wound healing.

6. Conclusion

The development of effective therapeutics for enhancing wound angiogenesis, particularly for non-healing or complex wounds, requires the ability of the compound to work in the harsh environment of a poorly healing wound. As discussed in this review, many compounds have shown great promise in enhancing wound healing and angiogenesis in animal models of wound healing. In the past, many wound healing treatments that have been successful in preclinical models ultimately fail to provide benefits to patients when tested in clinical trials. Fundamentally, the field suffers from the lack of a validated preclinical animal model of chronic wound healing that correlates well with human wound healing in the clinic. While many approaches have been used to reduce wound healing in animal models, there is no consensus as to the model with the best correlation to compromised human wound healing [320]. A change in approach and experimental mindset is needed in how studies are

designed for assessing promising wound therapies. *In vitro* studies should be targeted not solely at assessing activity in healthy cells but cells either from diseased patients or in combination with treatments that inhibit the angiogenic or wound healing response. The baseline assumption should be one of compromised response and therapeutic resistance, rather than healthy healing. New treatments should be designed or evaluated in the context of their ability work in compromised wound environments.

Ultimately, effective wound healing is a temporal process that can potentially be compromised by disease or infection in a number of ways. In diabetic wound healing, studies have identified many compromising factors for wound healing including the loss of heparan sulfate proteoglycans [47, 48, 54], alterations in inflammatory response [321], increased proteolysis[322] and alterations in ROS signaling [323], to name only a few. It is difficult to imagine therapy with any single agent to be able to address more than a few of these factors, pointing to the need for combination therapies that address the issues of a patient's particular wound and diagnostic assays to delineate when a particular compound should be used. In this context, the biology and compromised mechanisms of wound healing must be factored into the design and development of treatments and delivery platforms for addressing chronic wounds.

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Figure 1.
Summary diagram of the cellular and molecular components of wound healing and wound angiogenesis (created using BioRender and Adobe Illustrator).

Figure 2. Wound angiogenesis enhancement by syndecan-4 proteoliposomes. (A) von-Willebrand Factor (vWF) staining in the wound bed. Bar = 250 μ m. (B) Quantification of the small and (C) large vessels in the wound bed. (D) PECAM-1 fluorescence immunostaining for (red), α SMA (green) and nuclei (blue). Bar = 50 μ m. (E) Quantification of PECAM-1⁺/β-SMA⁺ vessels in the wound beds. * $p < 0.05$ versus control group. † $p < 0.05$ versus control and S4PL groups ($p < 0.05$). Used with permission from [71].

Figure 3.

(A) Expression of miR-92a in healthy and diabetic mice, and in acute and chronic human wounds. (B) Percent wound closure over time in full thickness excisional wound model. (C) Photographs of wound area over time for each treatment group. (D) Distance between epithelial tips as a measure of re-epithelialization. (E) Amount of granulated tissue, measured as an area. (F) Distance between the ends of the panniculus carnosus. (G). Representative images of hematoxylin and eosin staining, indicating granulation tissue (gt),

epithelium (e), and ends of the panniculus carnosus (arrow heads). Used with permission from [328].

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Figure 4. Effects of simvastatin on vascularity and lymphangiogenesis in full-thickness excision skin wound of db/db mice. (A) Neovascularization 14 days post-wounding at the wound margin of simvastatin-treated or vehicle-treated db/db mice. (B) Quantification of percent of vascularity. (C) Lymphangiogenesis 14 days post-wounding at the wound margin of simvastatin-treated or vehicle-treated db/db mice. (D) Quantification of percent of lymphatic vascularity. Green corresponds to LYVE-1-positive, newly formed lymphatic vessels. Blue fluorescence indicates DAPI-labeled nuclei. Scale bar = 100 μm. Used with permission from [246].

Figure 5. Deferoxamine-treated diabetic ulcers exhibit increased neovascularization and improved dermal thickness. (A) Immunohistochemical staining for newly formed capillaries via endothelial cell marker PECAM-1 (red). Scale bar = 10 μ m. (B) Quantification of PECAM-1⁺ pixels per high-power field (HPF). (C) Picrosirius red staining used to assess dermal thickness of completely healed diabetic mice ulcers. Scale bar = 10 μ m. (D) Quantification of picrosirius red-positive pixels per HPF. Used with permission [271].

Figure 6.

Anti-scar effects of astragaloside IV on rat full-thickness skin excision wounds 30 days after wounding. (A) Masson's trichrome staining of granulation tissue to assess collagen synthesis and blood vessels formation. Collagen and blood vessels identified by red and green arrows, respectively. (B) Effect on angiogenesis assessed by presence of blood vessels in granulation tissue indicated by black arrows. (C) Sirius red staining to evaluate collagen composition in healed tissue. Type I collagen indicated by white arrow, and type III collagen indicated by pink arrows. Blank control refers to the no treatment group. Used with permission from [272].

Figure 7. The angiogenic potential of hiPSC-derived and hESC-derived ECs (red) and vSMCs (green) to HUVEC/vSMC controls in a co-culture tube formation assay were compared. Equal numbers of cells across each group were cultured on Matrigel and allowed to form tubules overnight. Multiple EC to vSMC ratios were tested across all groups including 100:0, 60:40 and 40:60. (A) Fluorescent microscopy images of the tube formation assay. (B) Quantification of the tube area for the cells in the tube formation assay. *p < 0.05 versus 100:0 HUVEC group. Scale bar = 100 μ m. Used with permission [313].

Table 1.**Multi-Growth Factor Approaches for Enhancing Wound Healing and Angiogenesis**

Growth Factors	Results	Exp. Model	Ref.
PDGF-BB and VEGF-A	Promotes angiogenesis at the wound site, with significantly increased numbers of capillaries in the wound bed	Rats	[68]
PDGF-BB and FGF-2	Increased the levels of neovascularization significantly	Rats	[67]
PDGF-BB, FGF-2, VEGF-A and EGF	Accelerates wound closure and enhances the maturation of blood vessels in the wound bed in diabetic rats	Diabetic Rats	[70]
PIGF domains and VEGF-A	Chimeric protein promoted angiogenesis with reduced vascular permeability	Rats	[135]
PDGF-BB and IGF-1	Increased numbers of blood vessels at the wound site, Increased VEGF expression	Rats	[324]

Table 2.

Proteins, Peptides, and Blood Derived Factors for Enhancing Wound Healing and Angiogenesis

Treatment	Function	Exp. Model	Ref.
Insulin	Accelerates wound healing, promotes keratinocyte migration, increases angiogenesis	Mice/Rats	[136–138]
Erythropoietin	Delivery increases the rates of neovascularization and increases levels of PECAM-1 ⁺ cells at the wound site	Mice/Rats	[140–143]
hGH	Increases re-epithelialization, increases angiogenesis, and increases infiltration of T-lymphocytes	Diabetic Rats	[325, 326]
Syndecan-4	Enhances the revascularization, wound closure, and angiogenesis in response to treatment with FGF-2 and PDGF-BB	ob/ob Mice/Rats	[159]
Glypican-1	Increases revascularization in hind limb ischemia model and increases FGF-2 activity in endothelial cells	ob/ob Mice	[48]
SDF-1	Promotes recruitment of endothelial progenitor cells and anti-inflammatory monocytes. Helps to accelerate the wound healing process and promotes bMSC migration to the wound site.	Mice	[148, 153]
Spidroin	Increases proliferation of cells at the wound site to promote wound closure and angiogenesis	Mice	[156]
Tβ4	Promotes faster wound closure rates, greater neovascularization at the wound site, and greater capillary density.	Diabetic Rats	[157]
LL-37 (peptide)	Upregulates VEGF-A production, increases wound closure percentage, increases rates of re-epithelialization	Mice	[91–92, 94]
CW49 (peptide)	Increases angiogenesis <i>in vivo</i> , HUVEC tube formation <i>in vitro</i>	Diabetic Mice	[171]
Tylotoxin	Accelerated wound closure (comparable to 100 µg/mL EGF dose), induces tube formation <i>in vitro</i>	Mice	[172]
SR-0379	Decreased wound healing time, accelerated wound closure	Rats	[173]
WRL3	Increased angiogenesis in burn model as shown by increase number of blood vessels per field of view	Mice	[174]
Ap-57 (peptide)	Increase wound closure rates and promotes angiogenesis	Mice	[93, 95]
VIP	Increases wound closure percentages, cell proliferation and angiogenesis at the wound site	Mice	[180, 181]
PRP	Increases the number of PECAM-1 ⁺ endothelial cells and the number of microvessels in the wound site. Increases the wound closure rate and MSC migration to the wound site	Rabbits	[190, 191, 193]
Hemoglobin	Increases cell survival, oxygen tension in the tissue, greater macrophage infiltration and tissue granulation.	Rats	[194, 195]

Table 3.**Gene Therapies for Enhancing Wound Healing and Angiogenesis**

Gene/miRNA microRNA	Function	Exp. Model	Ref.
miR-21	Supports wound contraction and collagen network maturation	Wound Model in Mice	[206]
miR-23a	Reduction of miR expression, increased MET expression and increased angiogenic potential	<i>In vitro</i>	[204]
miR-26a	Inhibiting miR-26a induced angiogenesis, and increased wound closure (by 53%)	Wound Model in db/db mice	[214]
miR-27b	Improves bone marrow derived angiogenic cells by suppressing TSP-1. Overexpression reduces directional migration of mMSCs.	Wound Model in db/db mice/Skin burn model in mice	[200, 201]
miR-29b	Reduces collagen biosynthesis during wound healing via inhibition of HSP47 expression, also suppressing angiogenesis, leading to reduced scar formation	Wound and Burn Model in Mice	[327]
miR-92a	Overexpression blocks angiogenesis and antagomirs enhance blood vessel growth. Inhibition improves wound healing and angiogenesis.	Hind limb Ischemia Mice and Wound Model in db/db Mice	[213, 328]
miR-184	Reduces neovascularization, reduces FOG2, PDGF-B, and PPAP2B	Matrigel plug assay in Mice miR profiling in diabetic	[203]
miR-191	migration and angiogenesis via ZO-1 regulation	Modulates cell patients/ <i>in vitro</i> tubule formation assay	[205]
miR-199a-5p	Downregulation of miR increases Ets-1- MMP1 pathway expression and angiogenesis	Wound model in WT and Ets-1 KO mice	[329]
miR-200b	Inhibition of miR-200b increased GATA2 wound model in and VEGFR2 leading to improved wound angiogenesis and closure	Excisional WT and db/db mice	[208, 215]

Table 4.

Small Molecules for Enhancing Wound Healing and Angiogenesis in mice

Treatment	Results	Mechanism of Action	Exp.Model	Ref.
Simvastatin	↑ VEGF mRNA ↑ Endothelial Cells ↑ NO products ↑ Collagen synthesis	Inhibition of HMG-CoA reductase Pleiotropic Effects	db/db Mice	[262]
Simvastatin	↑ Endothelial Cells ↑ LYVE-1 ↑ Macrophage infiltration ↑ VEGF-C ↑ PDGF-β, eNOS, FGF-2 mRNA	Inhibition of HMG-CoA reductase	db/db Mice	[246]
Deferoxamine	↑ VEGF protein ↑ Endothelial Cells ↓ Skin Necrosis/ Apoptosis ↓ ROS Prevented Ulcer Formation	Iron chelator Increases HIF-1α transactivation Upregulates angiogenic factors	db/db Mice	[271]
Asiaticoside	↑ Macrophage Infiltration ↑ VEGF, MCP-1, and IL-1β ↑ Cell Proliferation	Inhibition of TGF-β receptors Increased NO production Increased TNF-α production	Mice	[278]
HA oligomers	↑ Endothelial Cells ↑ LYVE-1 ↑ Fibroblast proliferation ↑ eNOS/procollagen mRNA ↓ MMP-9/ MMP-13 mRNA	Binds to CD44 receptor promoting pro-mitotic effects on endothelial cells and angiogenesis	Mice	[292]

Table 5.

Small Molecules for Enhancing Wound Healing and Angiogenesis in Rabbits

Exp.Model	Treatment	Results	Mechanism of Action	Ref.
Rabbit	Mg-ATP delivered in unilamellar lipid vesicles	↑ Endothelial Cells ↑ Inflammatory Cells ↑ Re-epithelialization	Regulates production of pro- and anti-angiogenic factors that activate various receptors such as A2B receptors	[258]
Diabetic Rabbit	Mg-ATP delivered in unilamellar lipid vesicles	↑ Granulation Tissue ↑ Re-epithelialization ↑ Inflammatory cells	Regulates production of pro - and anti-angiogenic factors that activate various receptors such as A2B receptors	[259]

Table 6.**Small Molecules for Enhancing Wound Healing and Angiogenesis in Rats**

Exp Model	Treatment	Results	Mechanism of Action	Ref
Rat	Simvastatin loaded chitosan microparticles in PVA hydrogel	↓ Wound size Complete epithelialization (d21)	Inhibition of HMG-CoA reductase Pleiotropic Effects	[266]
Rat	Astragaloside IV Topically applied	↓ Scar Formation ↑ Skin Wound Tensile Strength ↑ Microvessel Density ↓ Ratio of Type I/III collagen	Alters activity of VEGF receptors regulating pathways involving PKB/PI3K	[272]
Rat	Astragaloside IV Solid lipid nanoparticle in hydrogel	↑ Blood Vessels ↑ type I collagen deposition	Alters activity of VEGF receptors regulating pathways involving PKB/PI3K	[273]
Rat	Astragaloside IV in Silk fibroin/gelatin electrospun nanofibrous dressing	↓ Scar Formation ↑ VEGF ↑ numbers of blood vessels Type I collagen deposition	Alters activity of VEGF receptors regulating pathways involving PKB/PI3K	[274]
Rat	Asiaticoside in PVA/ethyl cellulose microspheres	↑ Blood Vessels Minimal Scarring Organized Collagen I Fibers	Inhibition of TGF-β receptors Increases NO and TNF-α	[280]
Diabetic Rat	+/- DFO +/- VEGF Intraperitoneal Injection	↑ Capillary Density ↑ HIF-1α, SDF-1α and VEGF	Iron chelator Increases HIF-1α transactivation Upregulates angiogenic factors	[247]
Diabetic Rat	DFO Ointment	↑ HIF-1α, VEGF, SDF-1α, TGF-β1 and IL-10 ↓ TNF-α, MMP-9, and IL-1β	Iron chelator Increases HIF-1α transactivation Upregulates angiogenic factors	[270]
Diabetic Rat	HA Oligomers in Ointment	↓ Wound Size ↑ Blood Flow ↑ Capillary Density ↑ p-Src, p-ERK and TGF-β1	Binds to CD44 receptor promoting pro-mitotic effects on endothelial cells and angiogenesis	[249]

Table 7.**Adipose-Derived Stem Cell Therapies for Enhancing Wound Angiogenesis**

Cell Type	Scaffold	Treatment	Result	Exp.Model	Ref.
mASCs	Pullulancollagen hydrogel	None	↑Recruitment of BM-MPCs ↑Provasculogenic gene expression	Mice	[302]
hASCs	Pullulancollagen hydrogel	Capillary Seeding	↑Wound healing speed ↑Vascularity	Mice	[301]
hASCs	PEG-fibrin gel	None	↑Remodeling speed ↑Vessel formation	Rats	[300]
pASCs	Integra collagen scaffold	None	↑Vascular density ↑Number of mature vessels	Pigs	[330]
rASCs	Cell sheet	Ascorbic acid	↑Wound closure speed ↑Vessel density	Rats	[331]
hASCs	None	Low-Level Light Therapy	↑VEGF and HIF-1 β secretion ↑Cell survival ↑Perfusion recovery	Mice	[332]
rASCs	ncl-PADM	None	↑Elastic modulus of graft ↑Cellular and vascular infiltration	Rats	[333]

Table 8.**Cell-Based Therapies for Enhancing Wound Angiogenesis**

Cell Type	Scaffold	Treatment	Result	Ref.
rBM-MSCs	Cell Aggregate Matrix	Dexamethasone/ Ascorbic Acid	↑ Wound closure ↑ Capillary density ↓ Inflammation	[307]
mBM-MSCs	PEG-PU	None	↑ Anti-inflammatory cytokines ↑ Wound closure ↓ Oxidative stress ↓ Pro-inflammatory cytokines ↓ ROS at wound site	[310]
rBM-MSCs	Graphene Foam	None	↑ Wound closure ↑ Neo-vascularization ↑ VEGF and FGF-2 mRNA ↓ Fibrotic scar formation	[334]
hiPSCs	HA Hydrogel	VEGF and SB-431542	↑ Cellular integration into vasculature	[335]
hiPSCs	None	VEGF, BMP-4, and FGF-2	↑ Tubule formation ↑ Vascular density ↑ Wound closure	[313]
PMSCs	None	None	↑ Wound closure ↑ Vascular density ↑ Integrin β1, Integrin β3, VEGF ↓ ICAM	
SKPs	Collagen Sponge	None	↑ Capillary regeneration	[336]
Monocytes	iMonogrid Gel	Monocytes treated toward M2 phenotype	↑ Vascular density	[337]