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Acellular and cellular approaches to improve diabetic wound healing

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ABSTRACT

Chronic diabetic wounds represent a huge socioeconomic burden for both affected individuals and the entire healthcare system. Although the number of available treatment options as well as our understanding of wound healing mechanisms associated with diabetes has vastly improved over the past decades, there still remains a great need for additional therapeutic options. Tissue engineering and regenerative medicine approaches provide great advantages over conventional treatment options, which are mainly aimed at wound closure rather than addressing the underlying pathophysiology of diabetic wounds. Recent advances in biomaterials and stem cell research presented in this review provide novel ways to tackle different molecular and cellular culprits responsible for chronic and nonhealing wounds by delivering therapeutic agents in direct or indirect ways. Careful integration of different approaches presented in the current article could lead to the development of new therapeutic platforms that can address multiple pathophysiologic abnormalities and facilitate wound healing in patients with diabetes.

Contents

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1. Introduction

1.1. The clinical challenge of diabetic wounds and foot ulcers

Diabetes affects more than 422 million people worldwide, including 29.1 million or 9.3% of the US population [1, 2]. A major problem in diabetic individuals is poor or delayed healing of wounds, and consequently chronic non-healing wounds are one of the major complications associated with diabetes. They are estimated to occur in 15% of diabetic patients and responsible for more than 27% of the annual $176 billion diabetic health care cost in the United State [1, 3]. A particularly important and challenging category of diabetic wounds are diabetic foot ulcers (DFUs) [4]. Annual incidence rates of DFUs are estimated to be 6.3% in diabetic individuals globally [5]. In the U.S., DFUs cause 20% of all diabetic hospital admissions and account for more than 60% of non-traumatic lower limb amputations each year, with only a 40% 5-year survival rate after amputation [1, 6]. With a recent projection [7] that up to 1 in 3 US adults could have diabetes by 2050, the impact and costs associated with chronic diabetic wounds are expected to rise very sharply, motivating the pursuit of novel therapeutics.

1.2. Wound healing in healthy and diabetic individuals

In healthy individuals, a normal wound healing process occurs that consists of three overlapping, yet distinct phases of inflammation, proliferation, and remodeling, with different cell types, growth factors, and cytokines playing important roles at each of the phases to orchestrate smooth progression of the healing cascade. During the inflammatory phase, extravasation of blood components causes the formation of a fibrin clot, which acts as a temporary provisional scaffold for infiltrating cells. Neutrophils are the first cells to arrive at the site of injury to clean and destroy foreign materials, damaged cells, and bacteria. Monocytes are then attracted to the wound site in response to chemoattractants, such as TGF-β and MCP-1, and differentiate into macrophages that initiate the proliferative phase of wound healing, which is characterized by migration and proliferation of different cell types, including endothelial cells, fibroblasts, and keratinocytes that are responsible for angiogenesis, granulation tissue formation, extracellular matrix deposition, wound contraction, and re-epithelization [8, 9]. During the remodeling phase of wound healing, the number of neovessels decline and most of the cells involved in the proliferative phase either leave from the wound or undergo apoptosis, leaving a mature, mostly avascular environment with few cells [10]. In addition, type III collagen that was expressed earlier in the granulation tissue is gradually replaced by stronger type I collagen, which increases the tensile strength of the scar tissue to up to 80% of uninjured skin [11]. For interested readers, further details describing cellular and molecular mechanisms during wound healing have been summarized elsewhere [8, 12–14].

In diabetic wounds, healing is stalled in the inflammatory phase that is characterized by elevated levels of proinflammatory cytokines, proteases, and reactive oxygen species (ROS), as well as cellular dysfunctions [15, 16]. Due to impaired immune responses, such as defective phagocytic and chemotactic activities of granulocytes [17], diabetic wounds are more prone to infection, which results in excessive recruitment of inflammatory cells that produce various ROS and damage structural elements of the extracellular matrix (ECM) [18]. Coupled with elevated levels of proinflammatory cytokines, ROS induces expression of serine proteases and matrix metalloproteinases (MMPs), leading to degradation of the ECM and growth factors, as well as the proteinase inhibitors, which further impairs the wound from mounting an adequate inflammatory response. In addition, ischemia secondary to vascular defects creates chronic wound hypoxia due to lack of proper perfusion, resulting in the release of more ROS [19] that, in turn, increases the proteins' susceptibility to proteolytic degradation [20]. These added proteolytic insults induce severe ECM degradation, which prevents normal matrix-cell interactions necessary for wound repair. As a result, the wound enters into a vicious cycle of defined by its prolonged inflammatory phase and characterized by a dysregulated molecular and cellular wound microenvironment that is not conducive to normal healing responses (Fig. 1). Therefore, correcting wound microenvironment dysfunction by sequestering wound proteases and/or modifying the ECM to more proteolytically-stable and cell-friendly conditions for migration and proliferation could be a viable treatment strategy to tilt the wounds from chronic inflammation towards the normal healing cascade.

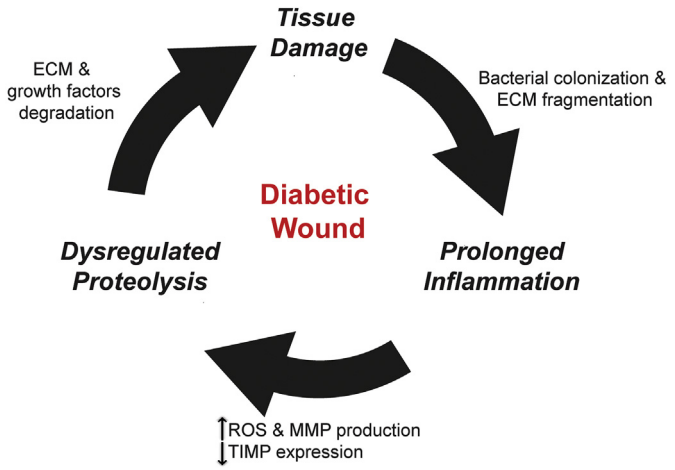

Fig. 1. Vicious cycle of chronic diabetic wound. Once the skin barrier is breached, chronic wounds, including diabetic ulcers, are more prone to infection due to impaired immune responses. Bacterial endotoxins and ECM fragments stimulate excessive recruitment of inflammatory cells, which produce various ROS. The presence of excessive ROS induce expression of serine proteases and MMPs while decreasing the TIMPs in the tissue, leading to dysregulated proteolysis and destruction of the ECM and growth factors that result in further damage to the wound tissues.

In addition to the unbalanced, inadequate local wound microenvironment, dermal cells from chronic diabetic wounds show a decreased response to environmental stimuli, partly due to a lower density of growth factor receptors [18]. Hyperglycemia-induced molecular changes, including formation and accumulation of advanced glycation end products (AGEs), adversely and irreversibly modify both intracellular and extracellular environments [21]. These changes are known to impair both endothelial cell [22] and fibroblast [23] functions, which results in decreased neovascularization and reduced granulation tissue formation and ECM deposition. In addition, AGEs could elicit production of ROS by binding to its receptor (RAGE) expressed in various skin cells including keratinocytes, fibroblasts, dendrocytes, and to a lesser extent in endothelial cells and mononuclear cells [24], and induce the activation of NF-κB [21], which results in pathological gene expression [25] and further impedes the normal activity of these cells during wound healing. Hyperglycemia-induced epigenetic changes in the cells are another source of delay in wound healing responses in diabetic patients. It has been shown that macrophages [26], vascular smooth muscle cells [27], and endothelial cells [28, 29] isolated from diabetic rodents or cultured in high glucose conditions exhibit a persistent proinflammatory phenotype via histone modifications. Therefore, cell-based therapies aimed to correct hyperglycemia-induced changes in cells could present a novel treatment option for diabetic wounds.

1.3. Current treatment approaches for diabetic wounds

Current treatment options for diabetic wounds include pressure offloading, sharp tissue debridement, infection management using antibiotics, and revascularization surgery to restore blood flow [2]. These

treatment strategies are aimed at wound closure rather than addressing the underlying pathophysiology of the wounds, which results in variability of healing effects, extended healing time, and recurrence of wound, leading to treatment failure and amputation [2, 30–34]. Recent breakthroughs in both the scientific understanding of the wound healing process and biotechnology have enabled the introduction of new biological products [30, 31] that have gained FDA approval. Regranex® is sodium carboxymethyl cellulose gel containing becaplermin, a recombinant human platelet-derived growth factor (rh-PDGF-BB) which has shown its efficacy in several studies [35–39]. Cell-based bioengineered skin constructs such as Apligraf® and Dermagraft® have also been shown to significantly reduce healing time for chronic wounds [40, 41]. However, the FDA added a black box warning to the label of Regranex, due to the possible risk of cancer associated with the use of three or more becaplermin gel tubes [42], and the cell-based bioengineered skin constructs have much shorter half-life with stricter storage conditions and higher cost to payer without conclusive superiority in shortening the healing time when compared with the acellular skin construct counterparts [43]. Therefore, there is clearly a great need for improvements in treatment strategies for diabetic wounds. Due to multifactorial origins of wound etiology, there is no single universal mechanism that could explain the persistence of wounds in diabetic patients, and therefore a combination of different therapeutic approaches aimed for correcting multiple deficits simultaneously would help lead to a successful outcome for diabetic wound healing. In the following sections, various reparative and regenerative efforts focused on accelerating diabetic wound healing will be discussed. As a first part of this review, therapeutic approaches targeted on modifying the local wound microenvironment (ECM) using tissue engineering scaffolds in the presence or absence of drugs will be presented. Then, approaches focused on correcting cellular-deficiency using cell-based therapies will be discussed.

2. Drug delivery for diabetic wound healing

Wound bed preparation is a widely accepted concept to remove underlying molecular and cellular barriers to facilitate healing [44]. Thorough wound bed preparation can sometimes be sufficient to induce normal healing responses in complicated wounds. More often than not, however, additional therapies are necessary to restore normal healing responses in chronic diabetic wounds. Systemic drug delivery, via either an oral or intravenous approach, has been a typical route of administration. However, for the treatment of chronic diabetic wounds, systemic administration may not sufficiently deliver drugs to the target tissue due to inadequate perfusion from a deficiency in wound angiogenesis. Moreover, systemic drug exposure could increase the possibility of serious side effects and toxicity in non-target tissues. In order to overcome these shortcomings and risks associated with systemic delivery, topical application of drug on the wound site would be preferable. However, direct topical application of growth factors, cells, or RNA interference (RNAi) delivery is challenged by their biological degradation and poor cell survival in the harsh wound microenvironment. In addition, topical application of bioactive agents such as small molecule drugs and antimicrobials in the form of solutions, creams, or ointments may not be suitable since they could absorb fluid from wound exudates, lose their rheological characteristics, and get washed away [45]. Difficulty maintaining sustained levels of drugs in the targeted area, coupled with diabetes-induced deficiencies in the expression of growth factor receptors in the cells [46], limits the efficacy of delivery of growth factors or small molecules that target specific receptor-mediated signaling pathways. For example, becaplermin gel (Regranex® by Smith & Nephew), the only FDA-approved growth factor for diabetic ulcers [42], has successfully shown its efficacy, however, it was shown that surgical wound debridement was an important factor affecting the efficacy of becaplermin while such a relationship was absent in the placebo group [34]. This suggests that the removal of tissues containing cells that are not responding to the growth factors, possibly due to diabetes-induced deficiency in the expression of growth factor receptors, is an important part of becaplermin treatment, which further highlights the complexity of targeting specific receptor-mediated signaling pathways for the treatment of wounds in diabetic patients.

To combat this persistent challenge, localized delivery of different therapeutics has been introduced over the last two decades by utilizing various biomaterials to prevent the loss of amount and bioactivity of delivered therapeutic compounds while providing a local wound microenvironment that are more conducive for healing. As such, our review in the following section is aimed to provide recent efforts in the development of delivery vehicles in the form of scaffold, rather than focusing on individual therapeutics compounds.

3. Acellular approaches

3.1. Tissue-engineered scaffolds: vehicles for overcoming obstacles

Wound healing involves dynamic interactions between cells, the ECM, and growth factors. The ECM is a non-cellular component in all tissues and organs. It is a major constituent of the skin layer and is comprised of structural proteins, such as collagens, fibronectin, laminin, proteoglycans, glycosaminoglycans, and other non-structural matricellular proteins. In addition to providing a structural support for cells, the ECM acts as a reservoir of growth factors that are rapidly mobilized to stimulate cell proliferation and migration upon injury [47]. In diabetic wounds, proteolytic dysregulation leads to degradation of ECM components, receptors, and growth factors that are essential for healing. There have been numerous efforts to develop tissue-engineered scaffolds to mimic structural and/or functional aspects of the native ECM. Once placed in the wound, the tissue-engineered scaffolds should be able to withstand the harsh proteolytic wound microenvironment and act as a temporary provisional matrix that allows infiltration of cells so that normal cellular activities can take place to promote matrix deposition, angiogenesis, and ultimately wound healing. In addition, the scaffold matrices can be used as drug delivery vehicles by loading them with therapeutic molecules or cells. In this section, acellular tissue engineering scaffolds that are sourced from different materials will be discussed.

3.2. Naturally-derived scaffolds

Natural scaffolds sourced from tissue ECM or ECM components have been widely used to promote wound healing due to their high degree of biocompatibility and mimicry of the physiological milieu. Scaffolds formed from isolated ECM components, such as collagen and hyaluronic acid (HA), are among the most widely used natural polymers for the development of acellular matrices for wound healing [48]. However, when used in isolation, they fail to recapitulate the complex nature and structure of the native ECM. Collagen-based scaffolds show poor stability due to their susceptibility to proteolytic degradation in the diabetic wound microenvironment. Additionally, collagen contraction is a major concern after implantation, and may result in ineffective wound covering and closure [49]. HA is rapidly degraded by hyaluronidases, whose activity is known to be elevated in the serum of diabetic patients [50], and may be further fragmented by free radicals in the wound tissue [51]. Therefore, decellularization of allogeneic and xenogeneic tissues to obtain their natural ECM to serve as a scaffold has gained popularity as such scaffold contains proteins and other polysaccharides to deliver

cues for cellular migration and survival. Some of the notable advantages of using decellularized ECM-derived scaffolds to treat chronic wounds are their ability to bind to growth factors, promote angiogenesis, confer an antibacterial effect to the wound by natural degradation, and reduce collagenase and gelatinase activities [52–55].

3.2.1. ECM-derived scaffolds

Each tissue has an ECM with a unique composition and structure, conferring a tissue-specific microenvironment that differs in mechanical properties and biochemical composition in each organ. Therefore, the most ideal matrix is the one that most closely resembles the structure and function of the native ECM it is replacing. For this reason, acellular dermal matrices derived from donated human skin tissue have gained popularity for the treatment of diabetic wounds. There have been several clinical studies demonstrating a significantly accelerated healing with allograft acellular dermal scaffolds over conventional standards of care, such as sharp debridement or moist wound therapy [56, 57]. In addition, these allograft scaffolds are classified as banked human tissue for transplantation and therefore the FDA approval is not required, resulting in fewer restrictions on their clinical application. However, because of the possible risk of disease transmission and the relatively higher cost to produce them due to the limited availability of human cadaveric skin, the possibility of using xenogeneic acellular dermal matrices sourced from porcine and bovine tissue have been actively pursued. Some of the most widely characterized decellularized xenogeneic ECM scaffold products that are used for the treatment of diabetic ulcers are derived from small intestinal submucosa (SIS, most notably OASIS® by Smith & Nephew) [58–60] and urinary bladder matrix (UBM, Matristem® by ACell) [61–63], both of which are of porcine origin. The SIS scaffold is comprised mainly of type I collagen and retains glycosaminoglycans including hyaluronic acid [64] and various growth factors [65–69] even after sterilization, and can be configured into sheets, powders, and multilaminate forms. When compared with clinical outcomes from standard wound care, patients with diabetic foot ulcers whose wounds were managed with SIS consistently showed improved healing outcomes in terms of reduction in wound size, as well as faster and enhanced wound closure [58], an increase in the number of ulcer-free months, and decreases in the probability of developing complicated ulcers and amputation [59].

UBM is another well-characterized ECM-derived acellular matrix. In addition to retention of multiple growth factors that play key roles in tissue regeneration and healing, the unique advantage of the UBM scaffold is that it retains an intact basement membrane component, which anchors epithelial tissues to the ECM in the target organs and helps promote angiogenesis and wound healing [70, 71]. UBM has been shown to enhance healing of recalcitrant wounds. Kimmel et al., reported that three diabetic foot ulcer (DFU) patients each presented with a necrotic wound, a non-healing surgical wound, or an infected wound probed to bone were all successfully healed with application of different forms of UBM [72]. The efficacy of UBM was further confirmed in a more recent prospective longitudinal study [62], which demonstrated that chronic DFUs treated with UBM in conjunction with standard local wound care healed significantly faster with decreased rate of recurrence as compared with standard local wound care alone.

Decellularized ECM derived from fetal bovine dermis has recently shown successful treatment outcomes of non-healing wounds originated from various etiologies including diabetes [73–75]. The unique advantage of this matrix is the high level (~30%) of type III collagen content in the tissue [76]. Type III collagen is the first type of collagen produced during embryonic development [77] and during the early phase of wound healing. In humans, the fetal dermis is comprised of about 20% of type III collagen while adult dermis contains about 10% [77], and it was shown that lack of type III collagen is associated with less regenerative wound healing capacity with more scar tissue formation [78], suggesting the important role of type III collagen during wound healing. Indeed, a recent study comparing the healing capacity of fetal bovine dermis with that of bilayered living skins substitute [79] on 40 diabetic foot ulcer and 28 venous stasis ulcer patients showed that the fetal bovine dermis healed both types of ulcer faster than the living skin substitute, highlighting the significance of type III collagen during recalcitrant wound healing.

The clinical efficacy of xenogeneic acellular matrix products, such as SIS and UBM, have largely been positive, however, the ECM derived from these organs have been shown to contain trace amounts of host DNA and more importantly, α-Gal epitopes which are the major barrier for xenotransplantation from non-human mammals to humans [80, 81]. Although the amount of remnant DNA and α-Gal epitopes in the porcine ECMs is thought to be too little to cause any adverse reaction [80], immune response and xenogeneic disease transmission cannot be completely ruled out. Recently, acellular ECM derived from porcine adipose tissue using different decellularization methods have been reported with more effective elimination of xenogeneic epitopes [82].

In addition to the porcine adipose tissue, human adipose tissue has gained attention as a potential source of scaffold for application in soft tissue augmentation [83, 84]. Although the adipose-derived ECMs have not been extensively investigated for the treatment of wound healing, the rich content of native ECM components including collagen, and the relative ease with which large quantities may be safely obtained [85], adipose-derived ECMs are particularly appealing for their applications in diabetic wound healing.

Most of the decellularized ECMs described above are known to sequestrate cytokines that play essential roles during the wound healing cascade, and structural proteins that confer antimicrobial and antiproteolytic activities by incorporation into the wound tissue and/or by natural degradation [52–55]. Therefore, these acellular matrices are essentially drug delivery systems with built-in bioactive agents that promote wound healing. In order to harness the full potential of these matrices, incorporation of additional biochemical agents or cells that are removed during decellularization process may enhance the wound healing potential and could be interesting avenues for future drug or cell delivery research. For instance, ECMs are known to play a significant role in the regulation of growth factor signaling, and the delivery systems that correctly recapitulate the ECM's growth factor regulatory functions showed successful healing outcomes in diabetic animal models [55]. Thus, utilizing the ECM-derived scaffolds to harness their natural growth factor binding capacity could prove to be a useful strategy for cytokine-based therapy. Furthermore, the ECM-derived matrices could potentially be used as a vehicle for cell delivery to the wound site. Hoganson et al. recently showed in their *in vitro* study that decellularized porcine peritoneum-derived ECM microparticles seeded with primary dermal human fibroblasts could be utilized as paracrine and cellular delivery therapies when delivered in a collagen gel [86].

Acellular ECM-derived matrices possess unique advantages to be harnessed as a delivery vehicle for growth factors or cells to the wound tissue. Further development and clinical trials will be required to prove their usefulness as primary therapeutics for diabetic wound healing.

3.2.2. Non-ECM-derived scaffolds

Non-ECM-derived natural scaffolds that have been investigated for the treatment of diabetic wounds include non-mammalian polysaccharides, such as chitosan and alginate, and self-assembling peptide nanofibers.

Chitosan has been widely used for wound dressing due to its ability to facilitate hemostasis [87, 88] and granulation tissue formation [89].

The presence of primary amine groups in the chitosan confer an overall cationic charge, which destabilizes and permeabilizes microbial membranes [90]. Therefore, chitosan possesses high potential to be utilized as a safe alternative to antibiotics for prevention and treatment of infection in diabetic ulcers. It has been shown to exhibit effective antimicrobial activity against *Styphylococcus Aureus* and *Pseudomonas Aeruginosa*, the two most common and virulent strains of bacteria that are found in diabetic foot infections [91, 92], when used alone or in combination as an antibiotic delivery vehicle [93, 94]. Lysozymes in cytoplasmic granules of neutrophils and macrophages hydrolyze chitosan to release N-acetyl-D-glucosamine, which is known to possess antibacterial properties [95], increase the functions of inflammatory cells and macrophages, and stimulates fibroblast proliferation and collagen deposition [96, 97]. In addition, the molecular structure of chitosan is similar to the glycosaminoglycans in the basal membrane and ECM, and this allows interactions between chitosan and extracellular adhesive molecules such as laminin, fibronectin, and collagen [98], making it an ideal scaffolding material for the treatment of diabetic ulcers. Due to its ability to bind to certain organic compounds, high susceptibility to enzymatic hydrolysis, ease of chemical modification, and intrinsic antimicrobial activity, chitosan is a promising material for use as a drug delivery vehicle in diabetic wound healing [99]. By modifying its physical forms into powder, microparticles, nanoparticles, or nanofibers, chitosan could be used to encapsulate or immobilize bioactive substances or enzymes and open up a new avenue for drug delivery in diabetic wound healing [100–102]. For instance, antioxidant enzymes or known antioxidant phytochemicals could be delivered in diabetic wounds using a chitosan-based delivery system to simultaneously attenuate oxidative stress and infection. Nguyen et al. demonstrated that a curcumin-loaded chitosan/gelatin composite increased antibacterial activity against *Pseudomonas Aeruginosa in vitro* and led to greater wound closure in a rabbit excisional wound model [97]. Thus, by blending with other synthetic or natural polymers, chitosan-based composites could prove to be a viable option to create inherently antimicrobial scaffolds where wound infection is a major concern, such as diabetic wounds or burn patients.

Alginate is a polysaccharide isolated from the cell walls of various species of brown algae. It was shown that diabetic wounds in rats treated with calcium alginate showed milder inflammation, faster epithelialization, increased fibroblast density, and increased collagen I/III ratio in the wound [103]. Due to a wide array of complications associated with the disease, exudates from chronic diabetic foot ulcers show increased expression of inflammatory mediators and MMPs [104], causing wound bed and ECM damage as well as maceration of periwound skin that are implicated in enlargement and perpetuation of the wounds. Therefore, exudate management in diabetic wounds is very important to reduce the healing time and diminish the chances of infection in the wound. Because alginate dressings can absorb 15 to 20 times their weight in wound fluid, they are an ideal scaffold to treat chronic diabetic wounds, which are at a risk for increased wound exudates. When an alginate dressing is in contact with exudates from the wound, an ion exchange occurs between the divalent cations in the alginate dressing and the sodium ions in the exudates or blood [105], resulting in the formation of a gel that helps maintain a moist wound microenvironment [106]. In addition to its application as a wound dressing, alginate has shown promising potential as a delivery system for cell [107, 108] and small molecule and macromolecular drugs [109] for wound healing. Recently, Tellechea et al. [110] showed that injectable alginate gels encapsulated with neuropeptide substance P (SP), outgrowth endothelial cells (OEC), or a combination of both all significantly accelerated wound closure in a streptozotocin (STZ)-induced diabetic mouse model when compared with alginate alone, with a combination of OEC and SP being the most effective, suggesting alginate as a safe and novel delivery system for diabetic wound therapeutics. In the presence of divalent cations such as Ca^{2+}, Sr^{2+}, Ba^{2+} or Zn^{2+}, alginate solution forms a non-antigenic, biocompatible hydrogel that is suitable for tissue engineering and drug delivery applications [111, 112]. Because alginate gelation requires the presence of divalent cations as a crosslinker, encapsulation of proteins or growth factors can take place under very mild conditions without the usage of high temperature or harsh chemical crosslinking agents [113], which enables incorporation of proteins, DNA, and cells into alginate matrices with a full retention of their biological activity [114]. In addition, by chelating the divalent cations using EDTA or sodium, the alginate hydrogel can easily be converted back to a solution [113] to release the encapsulated cargo. Therefore, alginate has been used to encapsulate numerous proteins and growth factors [114], but of particular importance, due to their immediate applicability for the treatment of diabetic wounds, bFGF and VEGF were shown to enhance local angiogenesis [115–117]. In fact, alginate has emerged as one of the more popular materials for VEGF delivery. An *in vitro* study [113] reported that a high-yield (>97%) encapsulation of VEGF was achieved using a Ca^{2+} crosslinked alginate matrix with a sustained release of encapsulated VEGF at a constant rate for 14 days, highlighting the potential of alginate as a sustained drug delivery vehicle for diabetic wound healing. However, the inability to tune the release rate has been a major limitation of using alginate as a delivery vehicle. A more recent study showed that by mixing populations of alginate particles formed by crosslinking with Zn^{2+} and Ca^{2+} at various ratios, the release rate of the encapsulated VEGF can be tuned [118].

Self-assembling peptide scaffolds made from natural amino acids have unique properties which make them a promising class of biomaterial for the treatment of diabetic wounds [119–122]. Self-complimentary oligopeptides are comprised of alternating hydrophobic and hydrophilic amino acids, and spontaneously self-assemble into 3D hydrogel scaffolds woven from nanofibers once exposed to physiological media or salt solutions [123, 124], and hydrogel scaffolds presenting different properties can be designed by varying the length, pattern, and sequence of amino acids [125]. Since the formation of the 3D hydrogel is mediated through noncovalent interactions including hydrogen bonds, ionic bonds, Van der Waals', and hydrophobic interactions, harmful chemical crosslinkers are not required to initiate the gel formation, and the degradation products are amino acids that can be readily metabolized by the cells [126] to synthesize proteins, such as collagen, enzymes, and cytokines in the healing wound. Moreover, the sol-gel transition takes places at physiological conditions with a high water content, which allows the diffusion of a wide range of molecules, making the peptide hydrogel very attractive for the encapsulation of cells, drugs, or proteins for drug delivery [127–129]. Indeed, the self-assembling peptide nanofiber based hydrogels have been used as 3D scaffolds for diabetic wound healing [119–122] and as protein delivery vehicles for myocardial infarction [130–132]. Narmoneva and colleagues showed that wounds treated with RAD16-II self-assembling peptide nanofibers (AcN-RARADADARARADADA-CNH^2) significantly accelerated wound closure, neovascularization, and overall wound healing with enhanced repair tissue strength in a diabetic mouse model [120]. Furthermore, a significant increase in VEGF expression and the number of perfused neovessels was observed in the wound treated with RAD16-II nanofibers [119], indicating its strong pro-angiogenic capacity. It is particularly interesting that the nanofiber-induced endothelial cell activation and the wound angiogenesis were mediated by integrins through low-affinity binding to the RAD motif, which has close homology to RGD binding domain [119]. The sequence of the self-assembling peptide nanofibers can be easily altered or functionalized through peptide bonds to confer specific biological activities [133, 134]. It has recently been shown that proliferation and migration of keratinocytes and fibroblasts can be modulated by functionalizing RAD16-I peptide nanofibers (AcN-RADARADARADARADA-CNH^2) with a fibronectin motif (RGDS) or collagen type I motif (FPGERGVEGPGP) [133]. In a more recent study published by Kim et al. [122], the authors demonstrated that by conjugating the sequence of substance P (RPKPQQFFGLM) to the RAD16-II nanofibers, wound closure, collagen

deposition, and angiogenesis were all significantly improved in a diabetic rat model. In that study, substance P was delivered to the wound either as a simple mixture with nanofibers or was conjugated with nanofibers, and although both groups were more effective in recruiting endogenous mesenchymal stem cells than the non-treated group, the authors claimed that the conjugated group was more powerful in terms of sustained delivery of substance P, whereas the non-conjugated mixture provided a burst release at an earlier time point [122].

Wound healing is a dynamic process that requires contributions from different growth factors at different time points. Thus, incorporation of multiple growth factors and/or bioactive molecules could prove to be a viable strategy to augment diabetic wound healing. For instance, wound infection is one of the major contributors of chronicity in diabetic wounds and peptide nanofibers comprised of or modified with cationic amino acids, such as arginine or lysine, are known to have strong antimicrobial effects [135–139] against major bacterial strains found in diabetic wounds including *Staphylococcus Aureus, Pseudomonas Aeruginosa, Escherichia coli,* and *Methicillin-Resistance Staphylococcus Aureus (MRSA)*. Therefore, by utilizing multi-functionalized self-assembling peptide nanofibers that contain antimicrobial cationic residues, while encapsulating bioactive molecules, such as drugs and proteins, could alter the diabetic wound microenvironment to overcome the stalled inflammatory stage and drive towards healing.

3.3. Synthetic scaffolds

Cells respond to a variety of different stimuli including mechanical strength, porosity, and topography of the local microenvironment, and one of the advantages of using synthetic materials as wound healing scaffolds is the tunability of the material's physical and chemical properties to modulate these parameters by adjusting polymer compositions, variation, and arrangement of constituent monomers. Moreover, synthetic materials are capable of delivering bioactive agents, such as drugs or growth factors, in a controlled manner by tailoring the degradation profile [140]. Synthetic polymeric biomaterials are often cheaper and easier to produce with a more uniform quality, offer better functionality and a longer shelf-life as compared with biologically derived scaffolds. However, most of these synthetic polymers are not bioactive and therefore, these polymers are often used in combination with other natural or synthetic polymers to improve functionality [141] and different fabrication techniques are employed to render them biologically functional to enhance cell adhesion, proliferation, and degradability for tissue scaffold and drug delivery purposes. In particular, electrospinning has become one of the more popular techniques to fabricate polymeric nanofiber scaffolds since their high surface area-to-volume ratio offers more surface area that promotes cell adhesion/proliferation, prevents fluid accumulation, and facilitates gas exchange [142].

In this section, recent efforts in the development of synthetic polymeric scaffolds for drug delivery in wound healing applications will be discussed. Although there are a wide variety of synthetic polymers to choose from, polyesters and polyurethane are among the most commonly used biocompatible synthetic polymers for the creation of 3D tissue scaffolds and drug delivery applications [140, 143, 144]. As such, only these two classes of polymers will be discussed here. Readers who are interested in other types of synthetic polymers should refer to [145].

3.3.1. Traditional synthetic scaffolds

Among the synthetic polymers, polyesters including polylactic acid (PLA), poly (lactic-co-glycolic acid) (PLGA), and poly (ε-caprolactone) (PCL) are the most widely used to create 3D scaffolds, due to their good biodegradability [141, 146, 147]. They are FDA approved for medical applications due to their high biocompatibility; their degradation products are nontoxic and can be easily metabolized by the Krebs cycle [148, 149], making them ideal materials for drug delivery vehicles.

Platelet-rich plasma (PRP) has gained considerable attention in clinics to promote healing in orthopedics applications due to the release of multiple growth factors and cytokines stored in the alpha granules of platelets [150] and have a significant potential to be used for the treatment of diabetic wounds. However, the short half-life and rapid deactivation of the growth factors, coupled with harsh proteolytic diabetic wound microenvironment call for a better delivery system to overcome these obstacles. He and colleagues [151], recently showed that PLA hydrogel (PLA-PEG-PLA) loaded with PRP showed a significant increase in the number of newly formed and mature blood vessels, as well as faster wound closure, better reepithelization and collagen formation compared with PLA hydrogel-alone and PRP-alone control groups in the full-thickness excisional wound model in rats.

Electrospinning of PLA has also been utilized to entrap different therapeutic molecules, including anti-inflammatory, antioxidant, and antimicrobial agents [152–157], or non-viral DNA plasmid encoding keratinocyte growth factor [158] to promote wound healing. Electrospun PLA nanofiber mashes closely mimic the native topography of the wound bed, which prevents loss of moisture and proteins necessary for proper healing, and facilitates the removal of wound exudates [142, 147]. In addition, hydrophobic drugs that are only soluble in organic solvents can be mixed in the solution of PLA before electrospinning for homogeneous distribution in the nanofibers [147]. Curcumin, an active ingredient of turmeric, is a well-known antioxidative, anti-inflammatory, and anti-infective agent, and has been shown to improve healing in diabetic wounds [159]. However, due to its hydrophobicity with limited stability and photosensitivity, curcumin has limited therapeutic application [160]. Several groups have used different polyesters to overcome these obstacles and locally deliver curcumin to promote wound healing [153, 161, 162]. Park and colleagues showed that PLA nanofiber mats loaded with curcumin significantly increased closure rate of full-thickness excisional wounds in mice as compared with gauze covered or curcumin-free PLA nanofiber mat controls [153]. Interestingly, even in the absence of curcumin, the PLA nanofiber mats significantly promoted wound closure when compared with gauze cover control, suggesting that PLA nanofiber mats alone act as wound healing agents [153], possibly due to the nature of electrospun nanofiberous morphology, which is known to have high surface area to volume ratio, promoting absorption of wound exudates and exchange of gases and nutrients to promote cell proliferation [163, 164].

Chereddy et al. showed that curcumin encapsulated by PLGA nanoparticles (NP) promoted healing in a silicone-splint, full-thickness mouse wound model [162]. Interestingly, the study showed that, albeit less profoundly than the curcumin encapsulated PLGA NP group, the curcumin-free PLGA NP group showed significantly increased wound closure and collagen deposition in the wound than the no treatment group at day 10 post-wounding, suggesting that the PLGA NP itself acts as a wound healing agent [162]. Indeed, lactates released from the PLGA are known to activate several molecular pathways to promote endothelial cell migration and tube formation *in vitro* [165–167], as well as promote recruitment of circulating vascular progenitor cells and vascular morphogenesis *in vivo* [168, 169]. Porporato et al. reported that, unlike PLA, PLGA when implanted subcutaneously, exhibited sustained release of lactate, resulting in accelerated angiogenesis and wound healing [170], indicating that PLGA itself could be utilized as a source of sustained release of lactate for the treatment of diabetic wounds. Because of this, any future studies using PLGA as a delivery vehicle for any therapeutic agents should always consider including a separate drug-free PLGA control group to avoid the misinterpretation of drug efficacy.

PCL is another polyester that has shown great potential in biomedical applications, including drug delivery in diabetic wound healing.

Because of its low melting temperature and superior viscoelastic properties, it is one of the easiest synthetic polymers to process into a wide range of shapes and sizes for implants and devices [171]. In addition, PCL possesses very high drug permeability with a relatively slow degradation rate with less acidic byproducts as compared with other polyesters [141], making it an ideal vehicle for drug delivery applications. It has been shown that curcumin-loaded PCL nanofibers significantly increased the rate of wound closure in a diabetic mouse model, and reduced the inflammatory response from mouse peritoneal macrophages [161], suggesting the possibility of its usage as a drug delivery vehicle in the diabetic wound environment. However, PCL is known to be poorly hydrophilic, which reduces cell adhesion, migration, proliferation, and differentiation [172]. Although the aforementioned study [161] showed good viability of fibroblasts cultured on PCL nanofibers after 48 hours, the study was focused mostly on the drug delivery vehicle aspect of PCL. If the synthetic scaffold would serve the dual purpose of a cell-friendly wound healing scaffold as well as drug delivery vehicle, it is important to consider other physical parameters to improve cell-material interactions. For instance, it has been shown that the orientation of PCL nanofiber alignment is very important to influence cell adhesion and migration [173]. Xia and coworkers showed radially aligned scaffolds show faster migration of dural fibroblasts than the scaffolds with randomly aligned fibers, resulting in an increase in the area covered by cells on the *ex vivo* model for the surgical repair of a small dural defect [173].

The local delivery of antibiotics to an infected wound site using PCL has been shown to improve healing in a mouse model. Teoh and colleagues [174] used 3-dimensional a polycaprolactone–tricalcium phosphate (PCL–TCP) mesh to deliver gentamicin sulphate (GS). In this study, the group created a full thickness excisional wound inoculated with 35 ul of *P. aeruginosa* (10^9-10^{10} CFU/ml) on the back of each mouse and treated with either PCL-TCP-GS (treatment) or sterilized gauze as a control. The treatment group showed increases in wound closure, neovascularization, and collagen deposition in the wound at day 7, with the bacteria count in the treatment group below detectable limits as early as 1 day while the control group still showed 10^6 CFU after 14 days post-wounding [174].

Polyurethane (PU) is a widely used synthetic polymer for wound dressing because it is non-toxic, non-adherent, and non-allergenic [175], while possessing a good barrier function, oxygen permeability, and overall wound healing capacities [176]. Therefore, it is not surprising that PU was one of the early synthetic materials tested as an artificial skin substitute for the treatment of full-thickness wounds [177]. PU is comprised of alternative soft and hard segments and the polymer properties, such as physical properties and biodegradability, can be tailored by varying raw materials and synthetic methods [144, 178]. In order to control porosity and degradability to improve the suitability of PU for tissue engineering and drug delivery purposes, different manufacturing techniques and copolymers such as PCL, PGA, PEG, and lactic acids are commonly used as soft segments [179–182]. Different bioactive compounds have been loaded onto PU-based dressings or scaffolds to promote wound healing. The two most widely applied therapeutic compounds are anti-infective agents, such as silver [178, 183], and growth factors [184, 185], both of which have been shown to improve healing in diabetic [183, 185] or infected [178] wound models in rodents.

Recently, Duvall and colleagues [186] have shown that delivery of siRNA targeting prolyl hydroxylase domain protein 2 (PHD2) from a PU-based scaffold (polyester urethane) significantly increased the size and number of vessels in the scaffold. PHD2, when inactivated naturally or by siRNA, is known to activate hypoxia-inducible factors 1 alpha (HIF1α) to mediate the transcription of pro-angiogenic genes, such as VEGF or FGF-2 [187]. The authors claimed that the sustained RNAi-induced modulation of transcription factors, such as HIF1α, controlled a battery of related genes and could produce more robust effects on tissue regeneration as compared with the delivery of a single growth factor, which is the current standard [187]. Unlike most of the local *in vivo* siRNA applications injected in saline with no regard for sustained bioactivity, sustained local delivery from PU scaffolds could enhance the use of siRNA for drug delivery and regenerative applications [188].

3.3.2. Novel synthetic scaffolds

Most of the scaffolds derived from natural components or synthetic polymers that have been discussed here so far rely on either passive provision of a cell-friendly microenvironment to promote cell infiltration and/or direct delivery of therapeutic molecules through careful manipulation of physical parameters to achieve a better release profile of incorporated molecules. Although these traditional approaches have achieved a great deal in improving the healing of recalcitrant wounds, such as diabetic ulcers, there still is room for improvement.

Park and Gerecht developed a synthetic, gelatin-based hydrogel that can cause local *in situ* hypoxia when subcutaneously injected into rodents [189]. By conjugating ferulic acid to a polymer backbone, such as gelatin or dextran [190], a hypoxia-inducible (HI) hydrogel can be formed via oxygen consumption in a laccase-mediated reaction. In this study [189], gelatin was chosen as the polymer backbone for its superior support for vascular morphogenesis. The *in vitro* study showed that the HI hydrogel encapsulating endothelial-colony-forming-cells (ECFC) supports sustained hypoxia up to 50 hours within the matrix, with accompanying significant increases in tube formation and the expression of pro-angiogenic genes, such as HIF1α, MMPs, VEGF, and VEGFR2, in the encapsulated ECFCs as compared with nonhypoxic gels (Fig. 2) [189]. The HI hydrogel without the ECFC encapsulation showed gradual increase in oxygen level after reaching the nadir of hypoxia after 30 minutes, indicating that ECFCs affected O_2 levels within the matrix [189]. In *in vivo* study using both mice and rats, the density and the size of the microvasculature were higher in the hypoxic hydrogel with more perfused vessels that anastomosed with the host vasculature to a greater extent than in the nonhypoxic hydrogels [189], suggesting the possibility of using HI hydrogel as an acellular matrix for the treatment of diabetic wounds where angiogenic capacity is severely impaired.

Customary acellular treatment strategies aim to improve healing of diabetic wounds have been mainly focused on maintaining a moist wound microenvironment through conventional or tissue engineered dressing scaffolds with additional control of infection and correction of cellular deficits by delivering antibiotics, small molecule drugs, and growth factors. However, one important characteristic of diabetic wounds that has been less actively pursued to improve the healing response is oxidative stress. Diabetic tissues contain lower levels of antioxidant compounds and enzymes as compared with non-diabetic counterparts [191, 192], implicating the roles of these molecules in wound healing. Indeed, studies have shown that local delivery of antioxidant compounds, such as glutathione [193], vitamin C [194], and vitamin E [195] improve healing in diabetic wounds. Ameer and colleagues recently synthesized an intrinsically antioxidant, novel biodegradable polyester-based biomaterial by incorporating vitamin C into their previously characterized poly(diol citrate) polymer network [196]. This newly synthesized polymer, poly(1,8-octanediol-co-citrate-co-ascorbate (POCA) showed its antioxidant activity *in vitro* and *in vivo* [196]. In more recent studies, the same group utilized citrate-PEG thermoresponsive antioxidant hydrogel (poly-(polyethyleneglycol citrate-*co*-N-isopropylacrylamide, PPCN) to encapsulate copper ions [197] or SDF-1 [198] to enhance the healing of the wound in a diabetic mouse model. In these studies, the authors claimed that the antioxidative properties of the PPCN synergistically played a role in reducing oxidative stress to promote wound closure and angiogenesis [197, 198], suggesting a potential usefulness of the antioxidant matrix as a novel candidate for the treatment of chronic diabetic wounds and opens up a new avenue of research for the biomaterials and tissue engineering community.

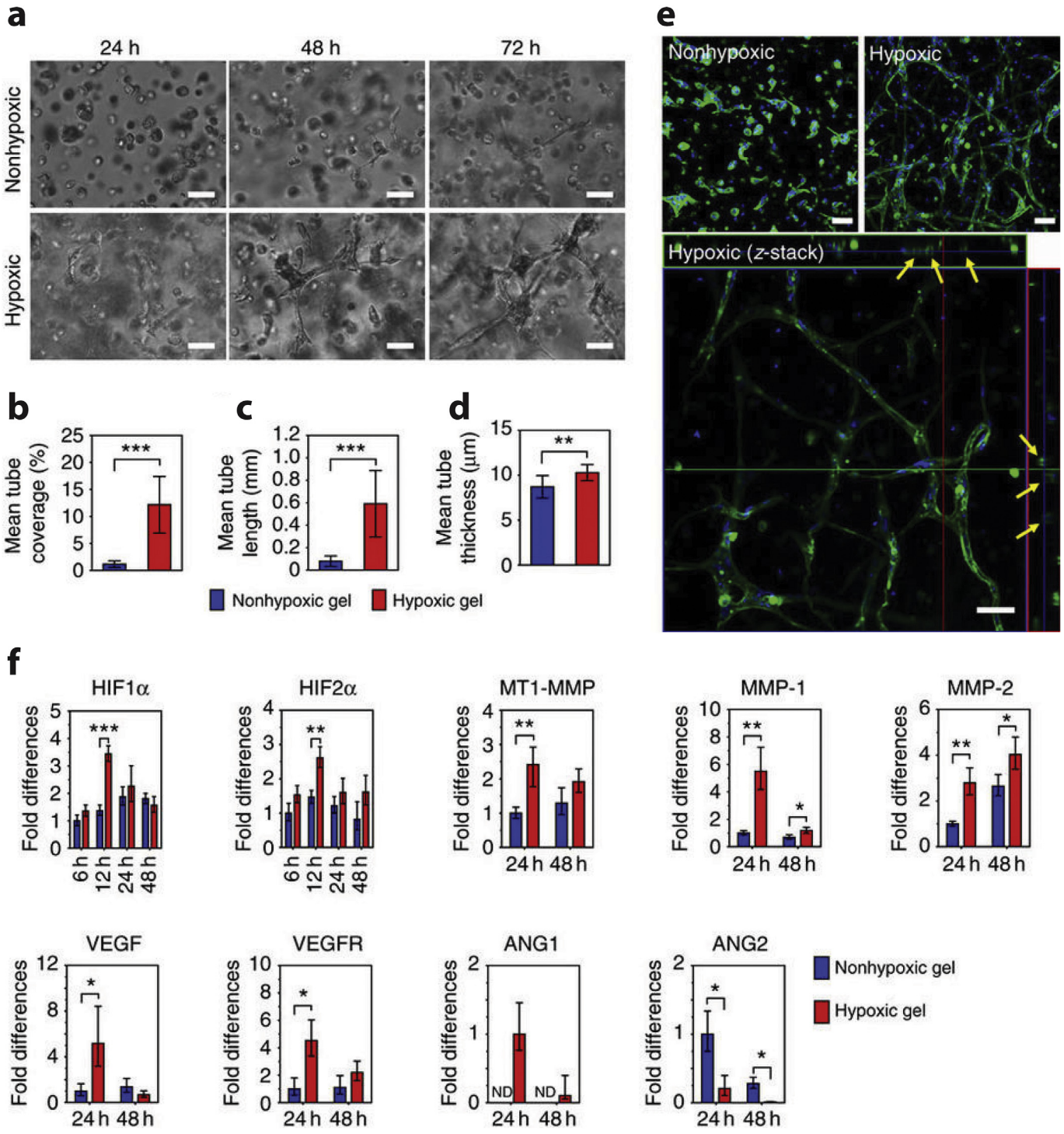

Fig. 2. Hypoxia inducible (HI) hydrogel as a pro-angiogenic microenvironment. Vascular tube morphogenesis by endothelial-colony-forming-cells (ECFC) within the HI hydrogels. (a) Light microscope images of ECFCs encapsulated within HI hydrogels during 3 days of culture. Quantitative analysis of vascular tube formation by ECFCs shows (b) mean tube coverage, (c) tube length, (d) tube thickness, and (f) expression of various genes involved during angiogenesis. (e) Confocal microscopic images of ECFCs encapsulated within nonhypoxic and hypoxic gels; confocal z-stacks and orthogonal sections show lumen formation (arrows) within the vascular network (phalloidin in green; nuclei in blue). Scale bars: 50 um, Error bars: standard deviation, $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Adapted from [178].

In addition to the antioxidant compounds, antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPX), peroxiredoxin (PRDX), and catalase play an important role during cutaneous wound healing [199]. It has been shown that the systemic administration of antioxidant enzyme mimetics, including SOD-mimetic [200] and SOD/catalase-mimetics [201] promotes healing in diabetic and irradiated [201] wounds. However, the antioxidant compounds lack ROS specificity and therefore could be indiscriminately scavenging ROS, including those which are physiologically essential, when delivered to the wound site. In addition, they react with ROS stoichiometrically and are converted to inactive forms after reaction [199]. The antioxidant enzymes, on the other hand, are ROS-specific and not

consumed in their ROS-neutralizing reactions. However, when locally delivered, they are prone to proteolysis in the harsh diabetic wound microenvironment and furthermore, delivering only one kind of antioxidant enzyme could lead to conversion of one ROS to another ROS. For instance, SOD converts superoxide to hydrogen peroxide, which is then normally converted to oxygen and water by glutathione peroxidase (Gpx), peroxiredoxin (Prdx), or catalase, but when only SOD is delivered, there could be too much hydrogen peroxide build up in the wound tissue where it is known to have reduced levels and activities of Gpx and catalase with diabetes [202, 203]. Therefore, a different approach to address oxidative stress is needed.

Recently, attempts have been made to utilize the endogenous antioxidant system to improve diabetic wound healing [204, 205]. Nuclear factor (erythroid-derived 2)-like 2, more commonly known as Nrf2, is a transcription factor that serves as one of the most important cellular pathways in detoxification and cellular protection against oxidative stress [206, 207], and is considered as a master regulator of the antioxidant response [206]. The mode of Nrf2 activation is very amenable to pharmacological modulation (Fig. 3). It has recently been shown that an Nrf2/Keap1 pathway is dysfunctional in chronic hyperglycemic conditions, which could be partially responsible for the delayed healing in diabetic wounds [205], and either pharmacological activation of Nrf2 [205] or reducing the expression of its suppressor Keap1 using topical siRNA [204] has shown to enhance the healing in the wounds of both type 1 [205] and type 2 [204] diabetic mouse models. In addition, it has been shown that Nrf2 activation promotes bacterial resistance in mice [208]. There are numerous natural and synthetic compounds that are known to activate Nrf2 pathways, including curcumin, sulforaphane, synthetic triterpenoids, and fumaric acid esters among others [209], but these compounds themselves are harmful chemicals and their mode of action is via covalent modification (alkylation) of Keap1 by increasing oxidative stress, making systemic administration less favorable due to the off-target effects, such as random alkylation of multiple proteins required for normal homeostasis [209]. Therefore, local delivery of the Nrf2 activator would be more preferable to take advantage of Nrf2 pathways to promote antioxidant status in the diabetic wounds. Curcumin, due to its inherent antioxidant and anti-inflammatory properties has been extensively used to treat diabetic wounds using different synthetic polymeric vehicles, as discussed earlier [149, 161, 162]. However, there are many different Nrf2 activating compounds from natural and synthetic origins, and by carefully selecting biological and/or synthetic polymers, these compounds can be encapsulated or conjugated to a polymer backbone to create intrinsic antioxidant scaffolds for sustained activation of Nrf2 to improve healing in diabetic wounds. For instance, dimethyl fumarate is the first FDA-approved Nrf2 activating compound that is currently being used for the treatment of multiple sclerosis [210] and its active metabolite, monomethyl fumarate, has the potential to be conjugated via its readily available carboxylic acid moiety to a polymeric backbone containing primary amine groups, such as gelatin, to form an intrinsically antioxidant, Nrf2 activating hydrogel scaffold.

Once the skin barrier is breached, wounds in diabetic patients are more prone to fall into a vicious cycle of wound chronicity as shown in Fig. 1. In order to break out of this vicious cycle, multiple treatment modalities addressing different deficiencies simultaneously will increase the chances of healing. For this, it is important to tilt the harsh wound microenvironment to a more pro-healing condition to restore the healing potential of the major players involved in the wound healing process, including the ECM, cells, and growth factors. By blending natural and synthetic polymers introduced in this section, it would be possible to create composite scaffolds possessing multiple properties, i.e. an artificial ECM that is comprised of a proteolytically stable composition

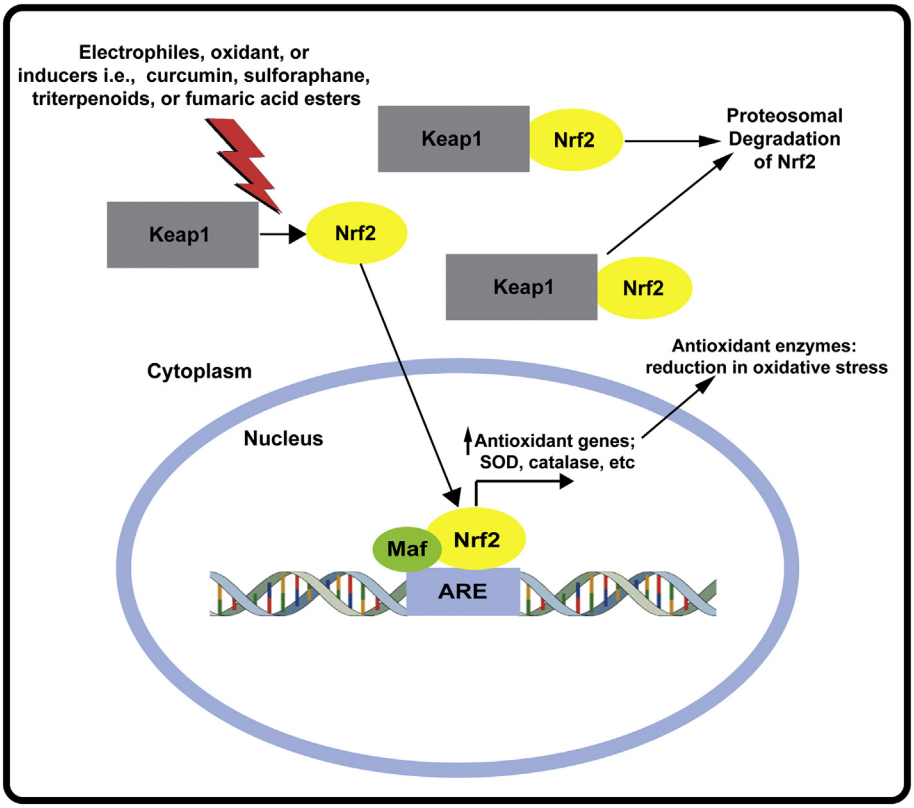

Fig. 3. Schematic showing the activation of Nrf2. Nrf2 normally resides in the cytoplasm, bound with its repressor Keap1, which directs Nrf2 to proteosomal degradation under normal physiological conditions. In the presence of inducers such as oxidative stimuli or other pharmacological modulators, Nrf2 is liberated from Keap1 and translocates to the nucleus, where it activates the transcription of multiple cytoprotective genes, including antioxidant and anti-inflammatory genes.

with antimicrobial and/or antioxidant properties by design. In addition, incorporating cells back to the acellular ECM may further enhance the chances of restoring the healing potential of diabetic wounds.

4. Cells as therapeutics for diabetic wound healing

The complexity of the multi-step process of wound healing and skin regeneration has limited the success of both single drug and gene therapies. Even when these therapies have been designed to specifically home to the wound site, the necessity and precision with which these drugs must recapitulate their physiological spatial and temporal presentation is a barrier, which has not yet been overcome. Further, the traditionally defined trajectory of wound healing is significantly altered or halted in diabetic patients, rendering additional difficulty in designing, delivering, and timing therapies. The resident cells in the diabetic wound are often critically impaired and do not respond well to traditional growth factor or drug therapy. To circumvent these shortcomings, cell therapy has been implemented and has seen success both at the bench and in the clinic. Therapeutically delivered cells may participate directly in a regenerative capacity or, perhaps in a more frequently observed fashion, may provide paracrine or trophic signals which alter the wound molecular microenvironment towards a pro-regenerative state. Cell therapy may contribute to immune modulation, cell differentiation, angiogenesis, ECM production, growth factor production, and wound contraction [211]. Although cell-based approaches to wound healing continue to be studied and translated with enthusiasm, several important considerations must be taken into account moving forward, in particular towards healing of diabetic wounds. Such considerations include cell source, cell healing mechanism, timing and frequency of delivery, and vehicle for delivery and cell retention at the wound.

4.1. Cell dysfunction in diabetic wound healing

An introduction to cell function in the wound healing process has been described in the preceding sections. Here, we will describe how chronic diabetic wounds are derailed from the typical wound healing pathway by cellular dysfunction. It is critical to understand that wound healing involves cells local to the wound, as well as cells homed systemically [212]. These cells may differentiate from local and recruited multipotent or progenitor populations, which, for proper fate decisions, require a unique wound microenvironment that necessitates numerous cues be presented in a specific spatiotemporal manner. The microenvironment in diabetic wounds plays a major role in cellular dysfunction as well as the non-healing nature of chronic wounds. In particular, diabetes causes impaired wound healing by affecting one or more biological mechanisms in the wound healing process. These impairments are most often triggered by hyperglycemia, AGEs, chronic inflammation, micro or macro circulatory dysfunction, hypoxia, neuropathy, or impaired neuropeptide signaling [21, 213]. Research uncovering cellular dysfunction resulting from chronic and diabetic wounds has revealed significant impairments or alterations in nearly all cell types involved in wound healing.

Cellular dysfunction begins with the first cells recruited to the wound. Neutrophils and macrophages have impaired adhesion, chemotaxis, phagocytic properties, and deregulated cytokine production, including tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and vascular endothelial growth factor (VEGF) [31, 212]. These issues manifest with recurrent infection common in patients with chronic wounds, as well as alter the biochemical microenvironment by deregulation of cytokine and growth factor production. Because recruited neutrophils and macrophages fail to fully succeed in their role of clearing local pathogens and promoting local cell proliferation, additional pro-inflammatory cells are recruited. Constant influx of neutrophils and macrophages leads to prolonged inflammation, which significantly contributes to delays in wound healing [212]. Ultimately, IL-1β and TNF-α are increased in diabetic wounds with upregulation resulting from both cellular dysfunction as well as hyperglycemia [194]. Pro-inflammatory cytokines result in increased production of matrix metalloproteinases (MMPs), which play a crucial role in ECM remodeling and cell migration [212]. Indeed, concentrations of MMPs are up to 60 times higher in chronic wounds, thus severely inhibiting critically important cell migration events, ECM regulation, and can lead to breakdown of growth factors [214]. The chronic pro-inflammatory state and constant influx of neutrophils and macrophages causes an imbalance between proteases and their inhibitors, thus preventing optimal regeneration and leading to a bevy of problems including reduced potential for stem cell differentiation and reduced granulation tissue formation, alongside impaired angiogenesis [12, 212].

Cells responsible for granulation tissue formation, ECM deposition, and angiogenesis are also impaired. For example, local fibroblasts are senescent, as evidenced by decreased proliferative response to growth factors such as transforming growth factor β1 (TGF-β1) and platelet-derived growth factor (PDGF), and have impaired migration, VEGF production, and response to hypoxia [215–217]. This effect lies, at least in part, with a decrease in growth factor receptors [18]. Keratinocytes also exhibit a decreased response to growth factors [218]. As such, growth factor therapy may not provide the intended effect on *in situ* cells, as they are functionally impaired. Additionally, critical roles, such as proliferation and migration are deregulated and cell apoptosis is increased [31]. The role of keratinocytes and fibroblasts in angiogenesis is also impaired [212]. To further exaggerate this issue, endothelial progenitor cells (EPCs), which are most often recruited from the bone marrow, show decreased numbers in diabetic and chronic wound patients [219], decreased mobilization to the wound bed through reduction in stromal cell-derived factor 1 (SDF-1) [220], as well as general reduction in response to stimuli, such as growth factors and hypoxia [194], and exhibit impairments in proliferation, adhesion, tube formation, and growth factor secretion [221, 222].

Other progenitor and stem cell populations home to the wound in physiological conditions. However, significant reduction in both stem cell recruitment and activation persist in diabetic patients. A hyperproliferative and non-migratory epidermis results in constant cycling of resident epidermal stem cells, which leads to depletion of the stem cell population and significant impairment in wound healing [223].

With frequency and prevalence of cellular dysfunction in the diabetic wound, a unimodal drug or growth factor therapy is less likely to succeed than in a healthy patient. Understanding the underlying biological phenomena leading to cell impairment can enable design of rational therapies with a higher likelihood of success. Therapies encapsulating and correcting multiple dysfunctional manifestations are a potentially powerful option. As such, cell therapy with multipotent potential has been studied fervently over the last decade.

4.2. Cell source/cell type

Numerous cell types play critical roles throughout the wound healing process. As such, understanding the role of each cell type is paramount when designing cell-based therapies. Researchers have studied the effects of many cell types ranging in source and potency from autologous dermal fibroblasts to embryonic stem cells. Studies have been conducted in animal models, as well as human clinical trials. While results are promising, regulatory hurdles are difficult to navigate and additional large scale clinical trials are still needed. Few recent studies have focused on mature cell delivery, with the focus mainly on multipotent progenitor and stem cells. Two broad, but crucial, considerations for cell therapy are cell type and cell source. Cell delivery will be discussed in Section 4.3.

4.2.1. Multipotent stem cells

Within the two broad classifications of cell type and cell source, an impressive array of studies has been published. Therapeutic use of

bone marrow derived cells was reported for diabetic wound healing over a decade ago. Bone marrow mononuclear cells (BM-MNCs), separated from bone marrow aspirate, contain multiple cell populations including mesenchymal stem cells (MSCs) and EPCs, as well as growth factors and cytokines. A combination of local and systemic, intra-arterial, injection of BM-MNCs reduced wound size or led to a completely healed wound while increasing vascularization in two separate case studies [224]. Since publication of these studies, the use of bone marrow derived cells has advanced significantly towards utilizing more defined and characterized cell populations to (i) enable more consistent results and (ii) because numerous reports have indicated benefits of using defined, BM-MSC, populations over less defined, BM-MNC, treatments [225–227]. These results have been confirmed in both animal [226, 227] and human studies [225], in which wounds healed faster and exhibited higher degrees of perfusion when BM-MSC treated subjects were compared to BM-MNC treated subjects. When retention times were compared, BM-MSCs could be retained up to 21 days after implantation in an impregnated artificial dermis, while BM-MNCs do not appear past 7 days post-implantation [227]. Based on multiple reports, improved angiogenesis seems to contribute to the enhanced therapeutic effect of BM-MSCs over BM-MNCs. Although similar outcomes have been seen in multiple studies, another study, which compared whole bone marrow, whole bone marrow cultured cells, and BM-MSCs concluded that whole bone marrow had the greatest therapeutic benefit, as determined by increased in vitro tube formation, greatest positive effect on fibroblast migration in a scratch wound assay, and best healing response *in vivo* [228]. The results from these studies culminate in several interesting conclusions and bring up several points of discussion. Perhaps most obviously, cell therapy has potential to aid in wound healing. However, experimental design, including differences in model used, cell harvest technique, cell purification, and culture conditions, are all critical towards making comparisons between studies. One issue in the field of diabetic wound healing is the wide variety of techniques used. It is critical to consider these differences when making comparisons between studies. Lastly, characterization is critical to ensure repeatability and to develop patient-specific therapy. As such, more defined cell populations, such as MSCs rather than whole bone marrow aspirate, have received more attention.

While characterization techniques continue to improve with respect to defining specific MSC populations, this group of multipotent stem cells has traditionally been defined by their ability to differentiate into adipogenic, chondrogenic, and osteogenic lineages [229]. However, other lineages, including endothelial, have been observed and MSC source tissue is important to consider as gene expression is dependent on tissue source [230, 231]. With this in mind, MSCs can be found in bone marrow, adipose tissue, peripheral blood, umbilical cord blood, dental pulp, muscle, skin, placental tissue, and others [232]. In addition to BM-MSCs and BM-MNCs, other MSC and multipotent cell populations from bone marrow and other sources have been applied therapeutically to promote diabetic wound healing.

Peripheral blood derived cells do not require invasive extraction, a common complaint with BM cell therapy, as they can be mobilized and activated by granulocyte colony-stimulation factor (G-CSF) injection. Peripheral blood mononuclear cells (PB-MNCs) have been used effectively primarily in treatment of diabetic limb ischemia rather than diabetic ulcers, but nevertheless have been effective in improving blood perfusion and, importantly, aiding in limb salvage in the clinic [233–235]. While not the primary focus of treatment, PB-MNCs have also been shown to promote healing of diabetic ulcers [235]. When peripheral blood progenitor cells were directly compared to BM-MNCs, similar improvements in regard to limb salvage and degree of ischemia were seen [236].

Another cell classification that can be mobilized by G-CSF administration is hematopoietic stem cells (HSCs) [237]. Although less studied towards wound healing applications, HSCs, which may differentiate into any blood cell type including critical immune cell regulators of wound healing, home to the wound and play a role in controlling proliferation and migration of epidermal and dermal MSCs, and have displayed the ability to improve chronic wound pressure sores in the clinic and promote formation of collateral circulation in diabetic ischemic limbs [237, 238]. Importantly, these cells can be patient derived, thus circumventing potential immunological incompatibility. Other than patient derived HSCs, umbilical cord blood (UCB) can serve as a source of HSCs, as well as MSCs. Obtaining these cells following patient consent is non-invasive and simple, but, for obvious reasons, they cannot be used autologously. However, they are a less mature cell type, and thus have greater differentiation and proliferation potential [230]. Human UCB-MSCs have enhanced wound repair in diabetic mice, potentially as a result of increased TGF-β [239], and have also been shown to accelerate wound healing in SCID mice [240]. In [240], UCB-MSCs differentiated into keratinocytes, as indicated by co-localization of keratin 19 or pan-keratin with BrdU in the wound tissue. Human umbilical cord perivascular cells, which have a higher proliferative rate and frequency than BM-MSCs, increased thickness and organization of the repaired dermis and induced complete re-epithelialization. Of note, wound strength was also increased over control treatments at early time points, indicating cell delivery plays a significant role early in the wound healing process [241].

Potential allogeneic cell sources tested for wound healing applications are not strictly limited to UCB-derived cells. Placenta-derived MSCs (PMSCs) have seen success in accelerating wound healing by enhancing angiogenesis in diabetic rats, with wound closure enhanced and microvessel density increased. PMSCs were shown to localize to the wound tissue and incorporate into the host vasculature as well as contribute in a paracrine fashion. By subjecting PMSCs to wound-mimetic hypoxic conditions, bioactive levels of pro-angiogenic factors including VEGF, hepatocyte growth factor (HGF), basic fibroblast growth factor (bFGF), TGF-β, and insulin-like growth factor 1 (IGF-1) were secreted. PMSCs can also develop an endothelial-like phenotype when exposed to the proper cues *in vitro*, confirming the potential direct contribution to neovascularization observed *in vivo* [242]. Another studied discussed the beneficial effects of PMSCs with respect to their role in immunomodulation [243]. Human PMSCs were injected around diabetic rat wounds and accelerated wound healing through control of pro-inflammatory signaling, by decreasing TNF-α, IL-1, and IL-6, and promoting secretion of anti-inflammatory factors, such as IL-10. Indeed, the beneficial effects seen upon PMSC administration could be partially abrogated by IL-10. In an *in vitro* co-culture study using PMSCs and dermal fibroblasts, PMSCs displayed potent immunomodulation through suppression of pro-inflammatory (lipopolysaccharide) induction of NF-κB [243]. Immunomodulation may provide significant benefit towards allogeneic clinical use of PMSCs, as they may avoid rejection upon injection or implantation. PMSCs are readily available and easy to access, as they are considered medical waste. Their potential benefits certainly warrant further study, although significant screening and pre-clinical and clinical trials will be needed to confirm their safety and therapeutic potential. Additional immunomodulatory effects of multipotent cell populations are discussed later in this section.

While the initial wave of cell-based therapies for treatment of diabetic wound healing focused on bone marrow derived cells, more recent studies have shifted focus to adipose derived stem (or stromal) cells (ASCs). ASCs are more abundant and more accessible than bone marrow derived cells. Compared with BM-MNCs, ASCs have been shown to more effectively heal diabetic foot ulcers in a rat model [244]. In direct comparison with BM-MSCs, ASCs were equally able to promote reperfusion in a murine model of hind limb ischemia, but ASCs were observed as a more potent promoter of neovessel formation. Interestingly, this effect was facilitated by upregulation of MMPs, with other angiogenic factors remaining unchanged in conditioned media from the two cell sources [245]. Other groups have reported ASCs have enhanced granulation

tissue formation, promoted re-epithelialization through secretion of pro-angiogenic factors, such as epidermal growth factor (EGF) and VEGF, and improved proliferation, particularly in fibroblast populations and basal epidermal layers [246, 247]. Supplementary positive therapeutic effects may be due to direct engraftment and incorporation into granulation tissue [246], engraftment into both the epidermis and dermis [248] and accumulation in the subdermal layer followed by migration to subcutaneous layers, with retention up to 8 weeks post-implantation [247]. A recent report from Shi et al [244]. specifically demonstrated localization of transplanted ASCs and found they could fuse with epidermal cells, although this observation was described as 'occasional'. They also show that transplanted cells could differentiate into keratinocytes, by use of co-localization with a fluorescent tag and both K5 and K14 keratinocyte markers. Engraftment as epidermal cells increased within 3 days and continued up to 2 weeks. Further, ASCs were able to functionally incorporate into host vessels and even differentiate into CD31 positive endothelial cells [244]. Another interesting observation posited by several studies describes the immunomodulatory effect of ASCs. ASCs may act in an anti-inflammatory capacity [247–249], as shown by a reduction of CD45 positive cells in the wound bed [247], as well as inhibition and suppressed cytokine secretion by T cells [249]. Immunomodulation and anti-apoptotic effects have even been hypothesized to outweigh specific angiogenesis and fibroblast recruitment in aiding wound regeneration [248]. Such immunomodulatory effects have also been observed in other stem cell populations, including BM-MSCs, which can shift the wound microenvironment from degradation towards tissue synthesis [250], alter the pro-inflammatory state by suppressing T-lymphocyte proliferation [251], inhibit dendritic cell maturation and function [252, 253], and suppress B cell proliferation, chemotaxis, and differentiation through production of soluble factors [254]. These properties highlight the potential for BM-MSCs and ASCs for allogeneic use, as they may be able to avoid immune rejection.

There is motivation for allogeneic cell therapy, as autologous cells derived from diabetic patients have severe impairments (see Section 4.1). In addition to those cells mentioned in Section 4.1, other cell types from diabetic subjects have exhibited dysfunction in wound healing applications. In a study comparing diabetic wound healing potential of ASCs from non-diabetic mice and ASCs from diabetic mice, cells from non-diabetic mice significantly increased the healing rate over those from diabetic mice by improving re-epithelization, promoting keratinocyte proliferation and granulation tissue formation, as well as encouraging dermal regeneration. While cells from non-diabetic mice had a clear benefit, diabetic cells still outperformed control treatments and promoted a similar level of neovascularization as non-diabetic cells [255]. Additional studies have indicated higher levels of apoptosis and senescence as well as limitations in differentiation potential of diabetic ASCs [256], as well as reduced ASC proliferation and migration, decreased secretory capacity, and a diminished effect on resident keratinocyte and fibroblast proliferation and migration [257]. Similar observations have been observed in BM-MSCs, with particular defects in proliferation, paracrine signaling, as shown through decreased secretion of VEGF and IGF-1, anti-apoptotic properties when exposed to hypoxia and serum deprivation conditions to mimic the harsh wound environment, and myogenic differentiation [258]. Other bone marrow-derived populations, such as EPCs, are less angiogenic and more pro-inflammatory, in terms of T-cell activation and IL-12 production, as a result of hyperglycemia [259]. This is an interesting result, as angiogenic potential of diabetic ASCs does not seem to be significantly impaired [255]. While these results have been consistent in the bulk of the literature, one study has shown less functional impairment when BM-MSCs from diabetic patients are subjected to an array of *in vitro* studies and compared to healthy BM-MSCs. Davies et al. showed that indeed gene expression of diabetic and healthy BM-MSCs differed, but the two cell types did not differ phenotypically and functionally with respect to immunomodulation and migration potential. Both cell types have comparable colony forming ability and growth kinetics, suggesting that autologous BM-MSCs can be obtained with high enough cell number to develop effective treatments. *In vitro* scratch assays also showed no difference in rate or extent of healing. While pro-inflammatory markers, in particular IL-6, showed upregulation in gene expression, secretion of pro-inflammatory cytokines was unchanged between cell types and both populations responded similarly to exposure to pro-inflammatory cytokines, with an ability to suppress T-cell activation [260]. These initially confounding results again highlight the need for standardization of cell culturing and characterization techniques, as well as the need for large scale clinical trials, as cells extracted from small patient populations may not always comprise a truly representative cohort.

Multipotent adult progenitor cells (MAPCs) can be collected from bone marrow and other tissues and have similar characteristics to MSCs, but with more potential for expansion [261]. In addition to relatively similar differentiation potential, immunomodulatory effects, such as suppression of T-cell proliferation, correlate with results observed in MSCs [262]. When compared to MAPC derived vascular progenitors and unselected BM cells, undifferentiated MAPCs restored blood flow and muscle regeneration in mice with limb ischemia. The benefits of this cell therapy were attributed to both direct and trophic effects on blood vessel and muscle regeneration. While some therapeutic benefit was seen with MAPC derived vascular progenitors and unselected BM cells, their impact was transient. Further, BM cells showed a sustained inflammatory response, reminiscent of cells local to chronic wounds [263].

In physiological wound healing, local stem and progenitor cells make significant contributions to skin regeneration. Such cells have been studied in their capacity as therapeutics as well. MAPCs from the skin, termed dermis-derived multipotent cells (DMCs) accelerated healing in a rat wound model through paracrine secretion and cell engraftment [264]. Stem cell populations in the bulge of hair follicles are immune privileged and thus could allow for allogeneic use [265]. Transplanted hair follicle dermal cells have shown potential clinical benefit by their ability to repopulate the mouse haematopoietic system for up to one year [266], thus warranting investigation for wound healing applications. Epidermal stem cells have also been shown to augment diabetic wound healing in a murine model through activation of the Notch signaling pathway [267].

4.2.2. Endothelial progenitor cells

The preceding paragraphs have mentioned the importance of vascular regeneration and its association with accelerated or enhanced wound healing, but were more focused on using multipotent stem cells and not necessarily focused on specifically inducing angiogenesis. However, an estimated 30-40% of diabetic foot ulcers result from ischemia, highlighting the importance of vascular regeneration in wound healing or wound prevention [268]. Fetus derived CD133+ EPCs were shown to enhance wound healing in diabetic mice with ischemic hind limbs and limb ulcers through paracrine activation of angiogenesis, through secretion of VEGF and IL-8, and Wnt signaling, but not a high degree of cell engraftment. Maintenance of 'stemness', as indicated by CD133+ cells, was crucial to therapeutic success as CD133- cells, which represent a more mature endothelial phenotype, were not as effective in wound healing. CD133+ cells were effective in activating Wnt signaling in the host, which can have reciprocal effects on angiogenesis, and their paracrine signaling was effective in blocking Wnt antagonists [269]. Use of an RGD-g-PLLA scaffold to deliver EPCs facilitated wound healing and helped promote engraftment, survival, and retention of delivered cells, up to 4 weeks, *in vivo*. EPCs incorporated into the host vasculature and host cells, including fibroblasts and endothelial cells, were able to grow in the scaffold [270]. A more thorough discussion of the benefits of scaffold-based cell delivery is presented in Section 4.3. A recent publication from Lee at al. [271] describes an interesting finding that Lnk deficient EPCs can promote proliferation, migration, and tube

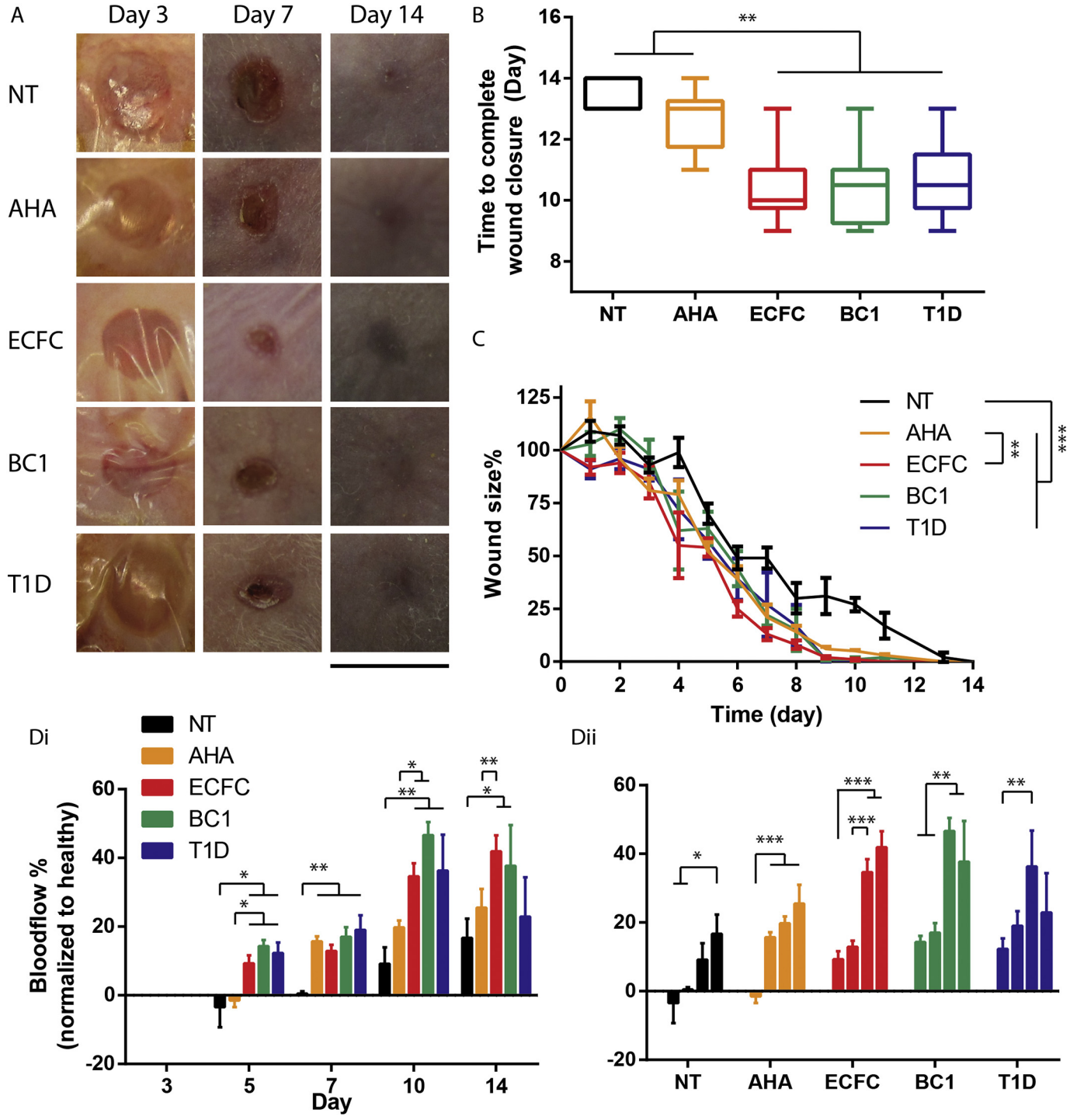

Fig. 4. Engineered vascular networks for diabetic wound healing (A) Closure of wounds treated with acellular (no treatment (NT), hydrogel (AHA)) and cellular (ECFC, BC1, T1D) constructs and wounds receiving no-treatment. Representative planimetry photos of wounds at day 3, 7 and 14 (B) Time to full closure of the wounds. (C) Wound closure progression along the treatment period. (Di) Blood flow profile of different treatment groups at the same time point and (Dii) blood flow profile progression over time within each group. Note that here we consolidated all NT controls together from all other control groups because no significant differences were observed among them. Scale bar is 1 cm. N = 12 for ECFC, EVC-BC1 and EVC-T1D N = 6 for AHA and N = 42 for NT. Two way ANOVA post test for significance *p < 0.05, **p < 0.01, and ***p < 0.001. EVC (early vascular cell); BC1 (control iPSC line); T1D (experimental iPSC line). Reproduced from [260].

formation, as well as inhibit inflammation, as evidenced by decreased cytotoxic T cells, macrophages, and neutrophils, and activate host myofibroblasts, thus effecting numerous cell populations at various stages throughout the healing process. Lnk-negative cells had a more significant therapeutic effect than Lnk-positive cells, highlighting the potential for cell alteration and cell augmentation as methods for enhancing cell-based treatment options [271].

4.2.3. Pluripotent stem cells

Gerecht and collegues have recently published a study of hyaluronic acid-based hydrogel delivered vascular networks from EPCs (specifically endothelial colony forming cells, ECFCs) and induced pluripotent stem cell (iPSC) derived early vascular cells (EVCs)[272]. In this study, EVCs were derived from both healthy

and diabetic donors and were tested for their efficacy towards wound healing in a diabetic (STZ-induced) mouse model. All cell groups showed accelerated wound healing over both acellular hydrogel and control groups with cell groups also facilitating more rapid reperfusion, particularly during the first week of treatment (Fig. 4). Alongside reperfusion, granulation tissue formation and appropriate regression underlie the potential benefits of the cell groups. The ECFC group exhibited the highest retention in the wound area of the cell groups, which may be attributed to their more mature nature over the iPSC-derived groups. Another key finding in this work is that both diabetic and healthy patient-derived EVCs performed similarly in vivo, matching earlier *in vitro* finding that diabetic patient-derived EVCs exhibit similar functionality as healthy EVCs and can respond to hypoxia, which is a critical parameter of the diabetic wound environment [273]. This finding reveals that reprogrammed cells may be less susceptible to diabetic impairment, and thus have potential for autologous use. Epigenetic changes caused by chronic hyperglycemia may be reversed upon reprogramming, with DNA methylation markers exhibiting low levels (<5%) in reprogrammed cells, which may continue to be reduced upon passaging [274, 275].

While studies of pluripotent stem cell populations have not been extensive, this area of research represents an emerging and exciting field with potential for patient specific therapy. One study developed a patient specific iPSC-derived skin equivalent composed of both fibroblasts and keratinocytes [276]. Further, conditioned media from embryonic stem cell (ESC)-derived EPCs aided in re-epithelization and wound tensile strength [277], and application of undifferentiated ESCs in an STZ-induced rat model accelerated diabetic wound healing during the early stages. Undifferentiated ESCs promoted re-epithelialization, thicker granulation tissue, angiogenesis, proliferation of fibroblasts, and growth factor and fibronectin upregulation. Transplanted cells engrafted and survived in the wound area up to day 9. Critically, no tumor formation was associated with undifferentiated ESCs, although no long term study was analyzed [277].

4.2.4. Critical considerations for cell-based therapies

While it is unlikely that undifferentiated ESCs will be used directly in the clinic due to their controversial ethical issues as well as non-autologous nature and potential for tumor formation, their utilization *in vitro* and in pre-clinical models brings about several key talking points surrounding optimization of cell therapies. The first of these points pertains to cell potency. Pluripotent, multipotent, progenitor, and terminally differentiated or mature cells have all been used in applications for healing diabetic wounds. The benefits of multipotent cell therapies are likely due to their heterogeneous cell populations which facilitate cell-cell signaling and a broader range of secretory and trophic factors that have potential to influence multiple stages of wound healing. Identifying specific effects of these cells is more difficult and characterization prior to implantation is often inconsistent.

Another important consideration is the specific healing mechanism elicited by cell therapy. Many of the observed benefits of cell therapy are due to paracrine effects, with many studies showing poor and transient engraftment and survival of delivered cells. More detail on improving this cell retention and direct contribution will be discussed in Section 4.3. While many studies have shown therapeutic benefit of application of conditioned media, and thus secreted factors, there have been reports of negative clinical outcomes from these studies [278]. As a workaround, the relatively newly applied concept of therapeutic exosomes has seen recent interest. MSC exosomes have been shown to induce proliferation of both normal and chronic wound fibroblasts and increase tube formation of HUVECs *in vitro*. Exosomes can also induce expression of growth factors in a dose-dependent manner. Critically, when conditioned media in this study was depleted of exosomes, rather than growth factors, beneficial effects were impaired [278]. Use of exosomes as a cell-derived therapy is a burgeoning area of research with many potential benefits.

Considering the many cellular defects from diabetic subjects, cell source is a vital parameter for understanding cell therapy. The use of autologous cells has benefits in minimizing regulatory hurdles and eliminating issues of rejection, but cell dysfunction may reduce clinical impact of these cells, and patient age may significantly affect stem cell populations and require extensive *in vitro* expansion prior to transplantation [279]. Reprogramming, as previously discussed, may reduce epigenetic changes caused by hyperglycemia and minimize cell dysfunction. Other mechanisms for correcting cell dysfunction will be discussed below in Section 4.3 and represent another method for improving cell-based therapies. While immune rejection, screening, and regulatory issues underscore limitations to translation of allogeneic therapies, the immunomodulatory effect of some multipotent stem cell populations has encouraged further study. Even tissue source may alter clinical efficacy. Many early studies focused on bone marrow derived cells, but the trend has shifted towards more easily obtained, cheaper, and more abundant, ASCs [250]. Further difficulty in drawing comparisons across studies comes from alterations in clinical and pre-clinical wound model, cell delivery location, delivery frequency, and delivery vehicle. Increased standardization in all of these areas will benefit translational potential of cell-based therapies.

4.3. Optimizing cell delivery and maximizing therapeutic potential

In order to increase the efficacy of cell therapy, delivery mechanisms must be optimized to ensure cell viability, full potential of paracrine function, and engraftment or differentiation into functional cells of the regenerated skin. Traditional transplantation methods of intravenous (IV), subcutaneous (SC), intramuscular (IM), and topical injection have all seen some pre-clinical and clinical success.

Intravenous cell delivery can promote wound healing through recruitment to the wound site. One study found a direct contribution to regeneration via transdifferentiation as keratinocytes, endothelial cells, and pericytes [280]. Another report identified that both direct engraftment and paracrine effects contribute to accelerated wound healing following IV delivery [244]. Improvements in healing over controls in these experiments are clear, but when compared with other delivery mechanisms, IV delivery typically does not fare as well. Topical or local injection either with or without a hydrogel vehicle consistently showed added therapeutic benefit [239, 264, 281], with fewer studies showing minimal differences between local and systemic delivery [282]. It is important to note that in [282], IV therapy was administered once daily for 4 days, while local injection was only administered once, raising the question of whether or not IV therapy can truly match topical transplantation in therapeutic benefit. Another important consideration in regards to IV administration is the functional impairment in stem cell homing to the chronic wound environment, which has limited study of IV delivery for healing of diabetic wounds [283].

Local application of cells, including topical application to the wound bed [284], IM injection [225, 235, 277], SC injection [285], and intradermal injection [286] have been used extensively. Combinations of delivery mechanisms have also been used including intra-arterial and IM co-administration [224], combined topical and IM delivery [287], and intra-dermal injection concomitant with topical application of cell encapsulated growth factor reduced matrigel [286]. Clinical benefit has been observed with all of the above delivery methods, but direct comparisons are difficult because cell concentration, delivery volume, number of injections, and frequency of injections all vary between studies. What does appear clear is that increasing cell survival or number of effective viable cells has often proven beneficial, whether that be through multiple administrations, increasing cell concentrations, or through use of a delivery vehicle [247, 284–286, 288].

One unique approach to deliver cells and enhance their clinical benefit is through delivery as aggregates rather than single cell suspensions.

Aggregate delivery has been show to increase the rate of wound closure through production of ECM proteins and secreted soluble factors. *In vitro* analysis also revealed upregulation of genes and proteins related to migration, proliferation, angiogenesis, and ECM deposition in aggregate versus monolayer culture [289]. While not directly studied in [289], cell-cell interactions within multicellular aggregates along with secreted factors may aid in survival upon implantation. Cell-cell and cell-ECM interactions are critical in regulating cell function, including survival [290]. Armed with this knowledge, many groups have developed delivery vehicles that promote cell-ECM interactions and may also provide protection from the harsh chronic wound microenvironment.

To maximize the therapeutic effect of cell-based treatments, both paracrine and differentiation or direct contribution to regenerated tissue is required. Towards this end, artificial skin substitutes and cell-laden scaffolds have been utilized. Two cellularized skin substitutes, Apligraf (formerly Graftskin) [41] and Dermagraft [40], are FDA approved for diabetic foot ulcer treatment [213]. These important products began to develop the framework for tissue engineered scaffolds and have illuminated many important design parameters for success in diabetic wound healing, but will not be discussed in detail in this review. The use of allogeneic cells within these products has not prevented FDA approval, but establishing autologous grafts may have added benefit in the clinic. Human skin equivalents with iPSC derived fibroblasts and keratinocytes represent an opportunity for patient specific therapy [276]. Improvements to multicellular skin substitutes will also likely benefit from 3D printing, which can facilitate complex spatial patterning and has the potential to create highly biomimetic engineered therapeutics [291].

Use of acellular scaffolds has been described in detail previously (See Section 3). In regards specifically to stem cell-based therapies, a commercially available collagen sponge with a silicone artificial dermis (Pelnac) was among the first to see the benefit of a delivery vehicle for BM-MSCs when compared with intra-dermal injection in the peri-wound space [292]. Other artificial dermis materials have been used effectively to deliver BM-derived cells [227, 293], ASCs [246], and immortalized human MSCs [294]. ECM proteins and glycosaminoglycans, which play a key role in wound healing and skin regeneration, have also been used to deliver stem and progenitor cells, including fibrin [241, 281, 295, 296], collagen [269, 288, 297], HA [272], or combinations such as fibrin and collagen [298] and collagen/HA [299]. Other naturally derived polymers, including silk [300], and synthetic polymers, such as PLLA [270] have been utilized. These reports motivate further study of cell delivery vehicles and bring about several talking points for these future studies. In particular, therapeutic benefits are often seen at earlier time points, with non-scaffold cell injections ultimately achieving similar cell numbers in the wound area [297]. Achieving long term cell viability is still an important unmet goal that will help ensure the highest degree of wound healing. In [297], another important point is discussed. Differentiation and fate decisions of delivered cells did not differ significantly between administration methods. Scaffolds, which can control or promote fate decisions may be an area of interest in the future. Scaffold properties, such as stiffness [296], also determine success, and should be taken into consideration when designing wound healing cell-delivery materials. Finally, pre-culture time is an important parameter that has not been discussed thoroughly. The advantages or disadvantages of pre-assembled structures, with established cell-cell and cell-ECM interactions, compared with single cell delivery vehicles may be important to consider moving forward.

Cellular dysfunction in diabetes and the chronic wound environment has been made abundantly clear throughout this review. One final method for improving cell therapy is by correcting aberrant cell behavior. An understanding of fundamental pathways and biological dysfunction coupled with advancements in the available tools to control cell behavior have facilitated advancements in this area of research. Simply pretreating MSCs in different oxygen conditions can improve their efficacy, for example by reducing apoptosis with hyperoxic treatment [301] and increasing paracrine secretion with hypoxic preconditioning [302]. Other enhancements of multipotent stem cell populations have been seen by inhibition or overexpression of certain regulators of cell dysfunction. Early growth response factor (EGR-1) plays a role in insulin resistance in type II diabetes. EGR-1 and its target genes are upregulated in diabetic ASCs and have functional impairment in wound healing compared with healthy ASCs. shEGR-1 can restore the wound healing ability of diabetic ASCs [303]. ASCs are also known to have impaired production of SDF-1 in diabetic subjects. To correct this impairment, overexpression of SDF-1 in ASCs was shown to promote wound healing in diabetic mice [304].

EPC dysfunction and impaired recruitment is also common in diabetes. Use of gene therapy to upregulate manganese superoxide dismutase, which plays an important role in resistance to oxidative stress, accelerated wound healing [305] and topical application of sonic hedgehog, which can directly promote EPC proliferation, migration, adhesion, and tube formation, also enhanced wound healing [306]. As discussed previously, reprogramming may reduce epigenetically induced dysfunction from hyperglycemia [274, 275] and modified, Lnk-deficient, EPCs can accelerate wound healing [271].

Correcting fibroblast dysfunction is also important in maximizing skin regeneration. One study in particular showed diabetic fibroblasts were less effective in wound closure and exhibited activation of the delta isoform of protein kinase C (PKCδ), which contributes to disruptions in insulin signaling. Pharmacologic inhibition or siRNA knockdown of PKCδ restored insulin signaling and improved wound healing [307]. Diabetic fibroblasts are also defective in their response to hypoxia. Thangarajah et al., suggest that chronic exposure to high glucose induces a decrease in transactivation of hypoxia-inducible factor 1 alpha (HIF-1α), which is caused by impaired binding to the co-activator p300. Treatment with deferoxamine (DFO) normalized HIF-1α/p300 interactions and thus promoted neovascularization and wound healing in diabetic mice [308, 309].

Potential for continued work in the area of cellular enhancement is high, with new mechanisms underlying function and dysfunction in wound healing being uncovered at a high rate. The continued development and refinement of gene editing technologies, such as CRISPR/Cas9, may also prove beneficial in this area of work.

5. Conclusion

Despite recent advances, treating diabetic wounds to heal in a timely manner still remains a challenge due to their multifactorial etiology. Therefore, integrating multiple therapeutic approaches is necessary to facilitate the healing process. As such, there have been numerous studies testing the feasibility of using different materials for the delivery of various bioactive agents, including growth factors, siRNAs, microRNAs, small molecules, or cells. However, a majority of these studies have mainly focused on *in vitro* studies with very limited *in vivo* validations, which call for further pre-clinical and clinical testing for safer and smoother transition to the bedside. In addition, most of the *in vivo* wound healing studies introduced in this review used rodents, with a particular focus on mouse models. Because rodents are loose skin animal, their mode of wound healing is predominantly by the process of skin contraction whereas humans have tight skin and heal more slowly by re-epithelization and granulation tissue formation [310]. Therefore, this difference should be kept in mind when comparing rodent-derived data with human skin wound healing.

Due to the dynamic nature of the wound healing process, it may be necessary to deliver multiple bioactive agents that possess different properties, or require different release profiles, necessitating the development of new composite scaffolds that can accommodate each bioactive component while maintaining the optimal wound

microenvironment. There are already many different types of scaffold materials, therapeutic agents, and cells to choose from. Thus, it is not that there is a shortage of armamentarium for the wound care practitioners but finding the "right" or "better" combination of delivery vehicles and therapeutic agents through integration of these available resources could be a bigger challenge because it requires a deep understanding of multiple disciplines. As such, investigating the use of a combination of products in different categories, e.g., growth factors and cell-based tissue constructs [312], could provide more useful data for clinicians. Numerous studies using different approaches have been discussed in this review, however, less studied is the direct comparison of efficacy among the currently available products on the market [43, 311]. In addition, when evaluating new experimental approaches, including more commercially available products as control groups rather than comparing the efficacy with just the standard of care, would yield more practical results to get a sense of how close the field is towards solving the diabetic would healing problem.

With the advances in our conceptual understanding of the pathogenesis of diabetic wounds at the cellular and molecular levels, it becomes more evident that a more holistic approach is needed to address multiple culprits (infection, oxidative stress and chronic inflammation, etc.) that are responsible for undermining proper functioning of major players (different cell types, growth factors, cytokines and ECM, etc.) involved during the wound healing process, and the recent breakthroughs in tissue engineering scaffold design and stem cell research hold great promise to help improve quality of life and prevent amputations in diabetic patients.

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