



REVIEW ARTICLE

Stem cell therapy for chronic skin wounds in the era of personalized medicine: From bench to bedside

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KEYWORDS

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Abstract With the significant financial burden of chronic cutaneous wounds on the health-care system, not to the personal burden mentioned on those individuals afflicted, it has become increasingly essential to improve our clinical treatments. This requires the translation of the most recent benchtop approaches to clinical wound repair as our current treatment modalities have proven insufficient. The most promising potential treatment options rely on stem cell-based therapies. Stem cell proliferation and signaling play crucial roles in every phase of the wound healing process and chronic wounds are often associated with impaired stem cell function. Clinical approaches involving stem cells could thus be utilized in some cases to improve a body's inhibited healing capacity. We aim to present the laboratory research behind the mechanisms and effects of this technology as well as current clinical trials which showcase their therapeutic potential. Given the current problems and complications presented by chronic wounds, we hope to show that developing the clinical applications of stem cell therapies is the rational next step in improving wound care.

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Introduction

The skin is the largest organ in the body and the body's first line of immune and physical defense. Types of skin wounds include abrasions, lacerations, punctures, surgical wounds, ulcers, and burn and burn-like wounds, each accompanied by their own set of complications. Cutaneous wounds can be broadly classed in two categories; acute, which heal uneventfully, and chronic, which do not heal for prolonged periods of time. Although not officially defined, this time period without healing is usually between 4 weeks¹ to 3 months.² The frequency of chronic wounds continues to rise with the increasing frequency of diabetes, vascular disease, and obesity, all known to be significant risk factors for chronic wound development.³ At present, chronic wounds cost the U.S. healthcare system about \$25 billion dollars annually.⁴ Given the variety of wounds and their prevalence, it is imperative that functional improvements be made in compensating for inhibited aspects of the healing process.

Cutaneous wound complications include clinical morbidity and mortality, economic burden, and emotional damage. Here, we seek to elucidate current methods for treatment and explore the promising options on the horizon presented by cell-based therapies.

Normal anatomic and histologic features of the skin

Skin is the largest organ, accounting for about 15% of the human body by mass. It serves as a protective barrier against biological and chemical agents, as well as moderating temperature and retaining fluids.⁵ To fulfill these functions, skin is composed of numerous cell types organized into three layers: epidermis, dermis, and hypodermis.

The epidermis is primarily composed of keratinocytes,⁶ Keratinocytes are epithelial cells of ectodermal origin which produce keratin, the major extracellular protein of the epidermis.⁶ The epidermis may itself be subdivided into

four layers according to keratinocyte morphology. The cornified or horny layer (stratum corneum) is the most superficial layer, and is made up of dead anucleated horny cells surrounded by dense keratin extracellular matrix.⁷ This layer decreases water permeability and acts as a mechanical barrier. Deep to the stratum corneum is the granular layer (stratum granulosum), which consists of flattened cells containing abundant intracellular proteins, that renew the contents of the stratum corneum.⁸ Beneath the stratum granulosum, the spinous layer (stratum spinosum) also produces some keratin, but its primary role is strong mechanical adhesion through desmosomes, increasing the structural integrity of skin.⁶ The deepest layer of the epidermis is the basal layer (stratum basalis), consisting of a single layer of columnar or cuboidal keratinocytes sitting atop the basement membrane.⁹ The cells of the stratum basalis are actively dividing and provide replacement cells for all the more superficial layers of the epidermis.⁹ Consistent epithelial thickness is maintained through apoptosis and mitotic control, regulated via numerous cell signaling molecules, and dysregulated in a number of pathologic conditions.⁶ Sweat glands and hair follicles in the epidermis serve thermoregulatory and signaling purposes.

Between the epidermis and the dermis is the basement membrane, a layer of basal cells interconnected by hemidesmosomes and type IV collagen. This structure maintains adherence of the dermis and epidermis in the face of mechanical shear and tensile stress and also allows exchange of cells and fluids between the layers.^{10,11} It also functions as a master regulator for the epidermis, providing developmental signals and establishing cell polarity and directionality for keratinocyte migration.¹⁰

The dermis is the thick layer beneath the basement membrane, consisting of fibroblasts, smooth muscle, nerves, blood vessels, and mast cells. The dermis provides most of the key physical properties of skin, including tensile strength, elasticity, and pliability.⁶ It also provides immune and nutritional support through circulation and somatic sensation via dense innervation. Many of the physical

properties of the dermis are provided by type I collagen, the dominant extracellular protein in this layer, synthesized by fibroblasts.¹² Following synthesis, collagen is crosslinked with elastin fibrils. This crosslinked structure gives this layer its unique strength and flexibility.

Most of the cells in the dermis are mesenchymal in origin, with the exception of nerves and nerve plexuses which are derived from neural crest.¹³ Though the dermis is organized in a less obvious manner than the epidermis, there are consistent depth dependent changes in cell structure and composition.⁶ The dermis contains mitotically capable cells, such as the stem cells present in hair follicles, which renew cells lost to senescence or injury.¹⁴

The hypodermis is a layer of subcutaneous tissue deep to the dermis. This fat provides thermal insulation, buoyancy, and acts as a store of energy.⁵ It also has endocrine functions, producing leptin and converting androstenedione to estrone.¹⁵ The skin has numerous important protective and regulatory functions, and cutaneous wounds can be associated with significant morbidity and mortality. In health, skin is a highly labile tissue, able to undergo regeneration and repair (Fig. 1).

Physiologic cutaneous wound healing

Wound healing is a delicate and complicated process, involving an intricate interplay of cell migration and

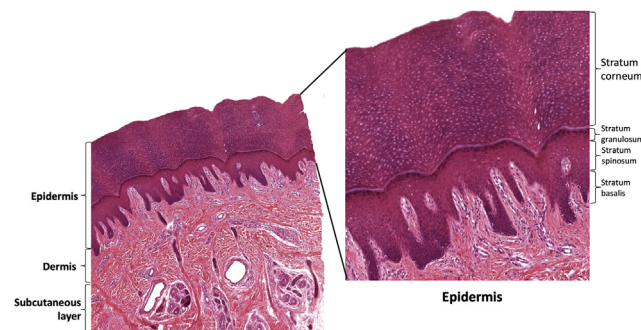


Figure 1 Layers of the skin. Skin consists of numerous cell types organized into 3 primary layers: the epidermis, dermis, and the hypodermis. The epidermis can further be substratified into stratum corneum, granulosum, spinosum, and basalis. The cornified or horny layer (stratum corneum) is the most superficial layer, consisting of dead cells and keratin matrix. Deep to the stratum corneum is the granular layer (stratum granulosum), made up of cells in the process of anucleation and keratin production. Beneath the stratum granulosum lies the spinous layer (stratum spinosum) which anchors the upper and lower layers of the epidermis. The deepest layer of the epidermis is the basal layer (stratum basalis), consisting of a single layer of rapidly dividing columnar or cuboidal keratinocytes sitting atop the basement membrane. Between the epidermis and the dermis is the basement membrane (unlabeled) which maintains adherence between the dermis and epidermis. The dermis is the thick layer beneath the basement membrane. The dermis contains fibroblasts, smooth muscle, nerves, and blood vessels, and provides most of the key physical properties of skin. The hypodermis is the subcutaneous fat layer lying below the dermis.

proliferation, all orchestrated by numerous cytokines. Successful cutaneous wound healing restores the barrier function and tensile integrity of skin. Under normal, healthy circumstances, wound healing proceeds through an inflammatory stage, a proliferative phase, and a remodeling stage.¹⁶ Though somewhat chronological, the different phases overlap, and each phase has important implications for the others. Small disturbances can have major consequences.

Hemostasis is the first step in wound healing.¹⁷ Platelets exposed to extracellular matrix proteins are activated, causing conformational change and aggregation, as well as release of pro-thrombotic and pro-inflammatory mediators.^{17,18} Formation of a strong and stable cross-linked insoluble fibrin-platelet clot is the end result of secondary hemostasis. Fibrin also has a pro-inflammatory role mediated through activation of monocytes and neutrophils,¹⁹ while platelets are involved in cellular signaling through release of platelet derived growth factor (PDGF) and tumor necrosis factor- α (TNF- α).¹⁸ The insoluble clot is a key element for later stages of repair, acting as a scaffold for the migration of fibroblasts, leukocytes, keratinocytes, and endothelial cells.²⁰

Chemotactic factors produced during hemostasis attract neutrophils and monocytes to the wound. The cellular response is initially dominated by neutrophils, which are later replaced by macrophages. Macrophages play an instrumental role in the inflammatory response: they phagocytize pathogenic organisms and cellular debris, produce regulatory cytokines, influence angiogenesis, and recruit fibroblasts with chemotactic chemicals.²¹ Macrophages produce vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), fibroblast growth factor (FGF), and tumor growth factors α and β .²² The inflammatory phase usually resolves in 2 weeks. A persistent inflammatory response is considered pathologic and is called chronic inflammation. Chronic inflammation is not always characterized by the cardinal signs of inflammation such as redness, pain, or swelling.²¹

The proliferative phase follows the inflammatory phase, though there is significant overlap between the beginning of proliferation and the end of inflammation. Angiogenesis and fibroplasia are the principle components of this phase. The cytokines released during the inflammatory phase recruit vascular endothelial cells, fibroblasts, and keratinocytes.²³ 3–5 days following the initial injury, fibroblasts begin to secrete type III collagen, proteoglycans, and elastin, which forms the granulation tissue.²⁴ Angiogenesis occurs in concert with granulation tissue formation. Vascular endothelial cells, attracted by VEGF, PDGF, and TGF- β , create new capillaries and carry circulating cells and nutrients to the wound.²²

Tissue remodeling follows closure of the wound and can take a year or longer.²⁵ Macrophages, fibroblasts, and endothelial cells are eliminated from the wound via apoptosis, leaving a mostly acellular collagenous matrix, consisting primarily of type III collagen, which is replaced with type I collagen over the next 6–12 months. Fully healed tissue provides a barrier with 60–80% of the tensile strength of the original epithelium (Fig. 2).²⁶

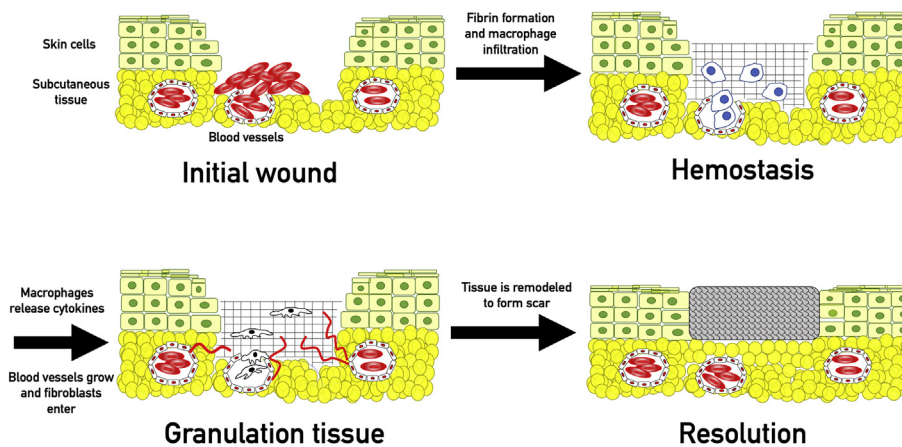


Figure 2 Physiologic wound healing. Following the initial wound, platelets and coagulation factors enter the wound bed from blood vessels and produce a fibrin clot through primary and secondary hemostasis. The fibrin clot is infiltrated by macrophages which function to phagocytose cellular debris and pathogens as well as releasing cytokines. These cytokines (including VEGF, FGF, and PDGF) summon fibroblasts and stimulate angiogenesis. Fibroblasts produce type III collagen for tissue repair. Angiogenesis allows sufficient transport of nutrients to the healing wound bed. Eventually the type III collagen is remodeled into type I collagen, which restores 60–80% of the original strength.

Chronic wounds

Chronic wounds form when the healing process stalls. Mechanisms underlying chronic wound formation vary but can include metabolic diseases (diabetes), failures of blood supply (peripheral vascular disease, radiation injury), medications, or altered immune function. Non-healing wounds are a major issue in healthcare today, an issue which will likely be exacerbated with an aging population. Though prevalence in the general population is about 3:1000, this worsens to about 1:50 for patients older than 75.²⁷ An estimated 500,000 U.S. citizens are afflicted with chronic ulcers.²⁸ Chronic wounds cost the U.S. healthcare system about \$25 billion dollars annually,⁴ about 5.5% of NHS spending.²⁹ On an individual level, chronic wounds are painful, isolating, and emotionally distressing.³⁰ Chronic wounds often lead to further health complications with chronic ulcers preceding 85% of amputations and providing a direct cause for 70%.³¹ Current treatments may also not be effective long term: though treatment for ulcers improves mobility and energy in the short term, one study showed that positive effects were not maintained over a 48 week period.³²

Wound healing is a highly metabolic activity and requires abundant nutrients and oxygen.³³ This necessitates good circulation and explains why chronic wounds are often associated with conditions which decrease blood flow. Non-healing wounds may have less than a quarter of the oxygen tension seen in healing wounds.³⁴ Hypoxemia impairs tissue repair by decreasing metabolic activity of repair cells. It also increases risk of infection by impairing oxygen-dependent killing of microbes.⁴ Ischemia caused by decreased blood flow is often enough to prevent wound healing even in the absence of comorbid risk factors.

In the US alone, an estimated 24.7 million people have been diagnosed with type II diabetes mellitus (T2DM), about 7.6% of the total population.³⁵ Diabetes impacts every step of the wound healing process. Peripheral

neuropathy and vascular disease increase risk of skin breakdown. Ulcers in diabetic patients show abnormal cytokine signaling, resulting in decreased immune cell presence.³⁶ Decreased immune cells increase prevalence of secondary infections, a serious complication of diabetic ulcers.³⁷ Cytokine signaling creates complex feedback loops. Diminished macrophage presence and impaired macrophage function in diabetic wounds is both a cause and result of altered signaling.³⁸

Diabetic macrophages release significantly less VEGF than macrophages from healthy individuals.³⁹ Decreased VEGF expression may be a major factor contributing to defective diabetic healing, supported by the fact that administration of topical VEGF has been shown to improve healing time in diabetic, but not control, subjects.⁴⁰ Insulin receptors (InsR), downregulated in T2DM, may also play a role in wound healing. InsR expression levels were inversely correlated to protein expression levels in diabetic wounds and upregulation of InsR receptors improved healing time.⁴¹ Adipose tissue derived stem cells (ASCs) play an important role in cutaneous wound healing, and diabetic patients have functionally defective ASCs resulting in inadequate growth factor release and decreased presence of ASCs in wound sites.⁴² Diabetic patients also show an inadequate cellular response to hypoxia. In normal wound healing, hypoxia summons endothelial progenitor cells (EPCs) which participate in neovascularization to increase blood flow and oxygen delivery. EPCs harvested from diabetic patients showed decreased hypoxia-induced adhesion, migration, and proliferation, resulting in impaired capillary formation and persistent ischemia.⁴³ Finally, diabetic keratinocytes function abnormally, leading to decreased re-epithelization and persistence of wounds.⁴⁴

Tissue repair is a carefully coordinated process, organized through a communication system of growth factors, cytokines, and chemokines. It is therefore unsurprising that disruptions in the cytokine profile may be a primary issue in chronic wounds. The role of VEGF in wound healing has

already been mentioned. In fact, decreased VEGF has been noted in many chronic wound beds.⁴⁵

Wound healing requires a balance of inflammation and repair, and increased release of pro-inflammatory cytokines may be as damaging as decreased production of growth factors. Inflammation is a key step in the healing process but is usually self-limited. The micro-environment within nonhealing wounds (regardless of underlying pathology) is one of uncontrolled inflammation. Nonhealing wounds show significantly increased levels of the pro-inflammatory cytokines IL-1, IL-6, and TNF- α .⁴⁶ Fibroblasts seeded with pro-inflammatory wound fluid demonstrated impaired proliferative response compared to those seeded with fluid from healing wounds.^{46,47} Increased inflammation upregulates elastase in the wound, resulting in the degradation of exogenously added therapeutic growth factors TGF- β and PDGF.⁴⁸ Decreasing the concentration of the pro-inflammatory cytokines in a wound has been shown to enhance speed of healing.⁴⁹

Cellular response to cytokines may also impact wound healing. Senescent cells are often unable to respond adequately to growth signals.⁵⁰ Increasing senescent cells in wound beds decreased rate of healing.⁵¹ All these findings suggest that altered cell signaling, predominantly increased pro-inflammatory cytokines and decreased anti-inflammatory and angiogenic cytokines, plays a key role in chronic wound formation. The intricacy of the healing process complicates attempts to pinpoint a specific clinical target, and the great range of possible underlying pathologies makes it difficult to cover the entire spectrum with current medical techniques (Fig. 3).

Standard therapy for chronic wounds

Standard wound care attempts to prevent complications arising from epithelial breakdown (such as infection or fluid loss) as well as to create an environment that is conducive

to healing. Wound care and dressings are a mainstay of treatment. Debridement (removal of necrotic or otherwise nonviable tissue) is important for exposure of healthy tissue and reduction in the inflammatory effects of necrotic tissue.⁴ Dressings are used to prevent infection and promote healing processes. Different dressings, such as moist occlusive dressings, dry gauze dressings, semi-permeable films, and hydrogels, have various beneficial effects including improved re-epithelialization, increased moisture, thermal insulation, or fluid absorption.⁵² Offloading of pressure and management of secondary infections are also important aspects of treatment.⁵³

Skin substitutes are biologic substances that act as synthetic skin. They have been widely and effectively used in surgical defects and large surface area burns. High cost has prevented their adoption for chronic wound care, though an economic analysis showed long term cost savings when used to treat recalcitrant chronic wounds.⁵⁴

Vacuum assisted closure, or sub-atmospheric pressure dressing, has been shown to increase blood flow and decrease swelling by siphoning excess fluid from the wound bed.⁵⁵ It also facilitates removal of bacteria from wounds which decreases rates of infection.⁵⁶ Treatment with hyperbaric oxygen has been proposed to enhance angiogenesis and immune function, but is associated with serious adverse oxygen toxicity.⁵⁷ Furthermore, studies have shown only an incremental improvement in diabetic ulcer healing over a 1 year period with hyperbaric oxygen therapy.⁵⁸

As previously discussed, altered cytokine profile is a critical issue in many chronic wounds. Efforts have been made to normalize the micro-environment of the wound bed via application of exogenous growth factors. Platelet derived growth factor (PDGF) attracts and activates fibroblasts, endothelial cells, and epithelial cells.⁵⁹ Application of high dose ($1.0 \mu\text{G}/\text{cm}^2$ ulcer area) topical PDGF improved healing of pressure ulcers in diabetic patients, though lower doses showed no effect.⁶⁰ A large randomized control study supported this finding, as 50% of ulcers treated with

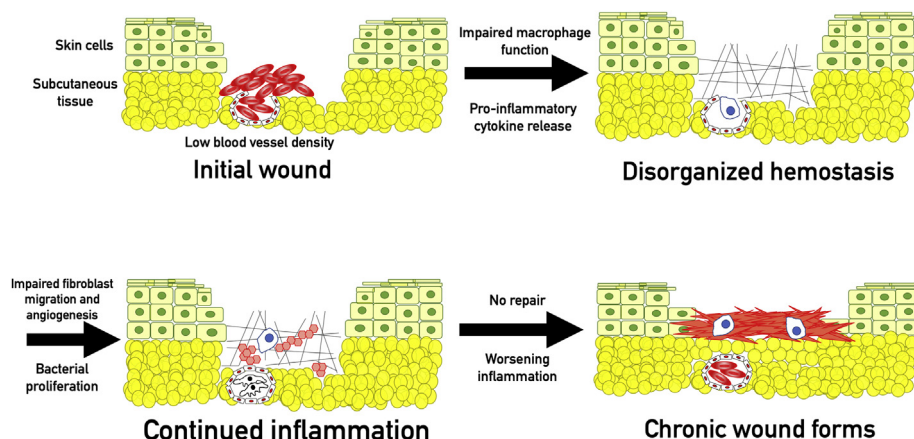


Figure 3 Chronic wounds. Impaired macrophage function impairs phagocytosis, resulting in continued pathogenic colonization of the wound. In chronic wounds, macrophages also malfunction in their signaling role, resulting in increased pro-inflammatory cytokine production. Impaired communication and persistent infection lead to a prolonged inflammatory state, which inhibits fibroblast migration and angiogenesis. Eventually, persistent infection, hypoxemia, and insufficient tissue repair results in a chronic inflammatory state. Chronic inflammation creates a self-reinforcing positive feedback loop that leads to the formation of a chronic wound.

high dose PDGF healed over a 20 week period compared to 36% in the control group.⁶¹ Fibroblast growth factor (FGF) enhances angiogenesis and promotes migration and activation of fibroblasts.⁵⁹ FGF has been shown to markedly accelerate wound healing in diabetic mice,⁶² though clinical trials showed no benefit to topical FGF over placebo.^{63,64} Vascular endothelial growth factor (VEGF) increases vascular permeability to allow migration of endothelial cells and lymphocytes as well as stimulating angiogenesis.⁶⁵ Mice treated with an anti-VEGF antibody almost completely lacked granulation tissue formation and had severely decreased vessel density.⁶⁶ Conversely, wounds on diabetic mice treated with high dose topical VEGF showed increased granulation tissue formation and increased vessel density. Topical VEGF also corrected other cytokine levels, normalizing FGF and PDGF levels to that of non-diabetic mice.⁴⁰ VEGF and FGF may also have synergistic effects. Co-stimulation with VEGF and FGF significantly increased angiogenesis *in vivo* when compared to either growth factor alone.⁶⁷ However, the wound models used in laboratory trials may not replicate clinical conditions. In general, laboratory wounds are new, small, and sterile, differing significantly from the environment in most clinical cases.

Though topical growth factors have shown promise for the treatment of chronic wounds, the difficulty of achieving correct dosages may limit clinical efficacy. Furthermore, multiple growth factors may be required to achieve optimal results, further increasing the complexity of treatment. Topically applied growth factors may also not penetrate to the wound base in deeper wounds.⁶⁸ Growth factors often need to be changed daily, which interrupts standard compression treatment.⁶⁸ A further complication is that the environment in chronic wounds is actively hostile to exogenous growth factors. Increased neutrophil activity in nonhealing wounds boosts elastase activity, which has been shown to degrade exogenously added TGF- β and PDGF- β .⁴⁸ The difficulties associated with growth factor application have made clinical translation problematic, despite promising lab findings. Though current therapies are efficacious in preventing secondary complications of chronic wounds, treatments are often unable to correct micro-imbalances and achieve true resolution.

Endogenous stem cells in cutaneous wound healing

Adult stem cells play a crucial role in all stages of cutaneous wound healing. The inflammatory stage is characterized by migration of neutrophils and macrophages. While some of these leukocytes are pulled directly from circulating blood, studies have shown that bone marrow-derived stem cells (BMSCs) also play an important role,⁶⁹ homing to injured tissues before proliferating and differentiating into required lineages.⁷⁰ Mast cells, important directors of the inflammatory phase, have also been shown to arise from precursor stem cells present in the skin.⁷¹

The role of stem cells in the proliferative phase is more obvious. Division and differentiation of tissue-specific adipocyte stem cells (AdSCs) regenerate damaged or lost tissue.⁷² Interfollicular and hair follicle bulge epithelial

stem cells proliferate and differentiate into cell lineages of keratinocytes for re-epithelialization.⁷³ BMSCs may also contribute to fibroblast populations in wounds: up to 20% of fibroblasts may be of migratory BMSC lineage.⁷⁴

Revascularization can occur via angiogenesis, the proliferation of endothelial cells in pre-existing blood vessels, or through vasculogenesis, which is the *de novo* creation of blood vessels by differentiation of endothelial progenitor cells (EPCs). Interestingly, angiogenesis is major mechanism of revascularization. Only 4% of vascular cells in wound sites arise from EPCs.⁷⁵ Endothelial progenitor cells are still critical to wound healing but exert their effects primarily through secretion of growth factors, rather than proliferation.⁷⁶ Hematopoietic stem cells (HSCs), derived from bone marrow, also play a role in production of new endothelial cells.⁷⁷

Many pathologies related to chronic wounds impact stem cell functioning. EPCs from diabetic patients displayed impaired migration to wound sites and adhesion to TNF-activated endothelial cells.⁷⁸ Diabetic EPCs also demonstrated decreased response to hypoxia, resulting in decreased vascularity in wound sites. Hypoxia usually induces vessel growth in control animals, but actually decreased vessel growth in diabetic animals.⁴³ This feedback loop reinforces and chronically prolongs the hypoxic state, which alters the functions of many cells required for tissue repair. Fibroblasts in diabetic patients are also less responsive to growth factors, showing decreased proliferation when stimulated with PDGF, IGF, or EGF.⁷⁹ Age is another risk factor for non-healing wounds. An epigenetic study in mice revealed upregulation of pro-inflammatory genes in the HSCs of older subjects.⁸⁰

Every phase in wound healing is mediated by stem cell proliferation and signaling. Impaired stem cell functioning therefore leads to chronic wounds. As stem cells directly interact with the wound environment in a complex and multifactorial manner, clinical approaches which utilize them could theoretically be very beneficial. Cell based treatments are a clear and rational next step in chronic wound care.

Growth factors in stem cell-based skin repair

Stem cells both produce and are regulated by growth factors. Growth factors have variable roles, but are also highly specific, with some growth factors only acting on a single receptor of a single cell type.⁸¹ Activated growth factors can have a number of different effects, including direct protein activation, upregulation of genes regulating protein production, or migration and chemotaxis.⁸² Growth factor signaling is necessary for stem cell response in wound healing.

A number of different growth factors are known to have important roles in cutaneous wound healing. Hypoxic wound environments induce expression of HIF-1 α , which upregulates FGF, and HIF-2 α , which upregulates VEGF expression. VEGF plays numerous roles in revascularization. VEGF concentration directs and guides differentiating angioblasts to cause budding of vessels.⁸³ FGF increase recruitment and replication of mesenchymal stem cells to support the structure of new capillaries.⁸⁴ Growth factors

control keratinocyte migration and de-differentiation in ECM remodeling in epithelial wounds, allowing them to multiply and fill in the wounded area.⁸⁵ Lack of growth factors in chronic wounds has been associated with decreased stem cell function of many lineages. One strategy to mimic or restore growth factor signaling in the wound to promote improved stem cell function is to enable the slow release of the growth factor or an inducer molecule from a hydrogel or dressing. A citrate-based thermoresponsive hydrogel was shown to protect the bioactivity of stromal cell-derived factor 1 alpha entrapped within the hydrogel and allow its slow release for at least 21 days with significant improvement in wound closure rate in diabetic mice.⁸⁶ In another study, the slow release of copper ions from the same material demonstrated increased blood vessel formation, likely mediated via VEGF release.⁸⁷

Therefore, although delivery of growth factors can be difficult, due to depth of penetration, concentration, or degradation by the wound environment, carefully designed biomaterials and small growth factor inducing molecules can be used to harness their potential. Viral transduction to temporarily over-express growth factors may also potentially solve many of the aforementioned issues.⁸⁸ Viral transduction may present an efficient strategy to obtain maximum benefit from both stem cell and growth factor therapy.

Growth factors and stem cells have a complimentary relationship. Without adequate production of and response to growth factors, stem cells are not able to perform their tasks in wound healing. It is therefore insufficient to examine only the direct effects of stem cells in cutaneous wound healing; local growth factors must be examined for their role in reinforcing and directing stem cell activity.

Stem cell sources for clinical use

Numerous stem cell lines have been investigated for their clinical potential. Embryonic stem cells (ESCs) were initially investigated for skin healing for their ability to self-renew and divide into keratinocytes.⁸⁹ Though ESCs have the potential to differentiate into many cell types including skin progenitors,⁹⁰ studies have shown that they can generate tumors, and their use remains controversial.⁸⁹

Human induced pluripotent stem cells (iPSCs) are created from differentiated adult somatic cells. Multiple terminally differentiated cells are capable of being reverse-engineered to pluripotency. iPSCs can be derived from adult mouse fibroblasts by treatment with Oct3/4, Sox2, c-Myc, and Klf4. iPSCs also can differentiate into tissues from all 3 germ layers *in vivo*.⁹¹ Keratinocytes have proven even more effective than fibroblasts for production of iPSCs: keratinocytes transduced with OCT4, SOX2, KLF4, and c-MYC produced iPSCs more quickly and efficiently than fibroblasts.⁹²

The ability to induce pluripotency is only half the challenge. To be therapeutically effective, researchers must be able to direct stem cell differentiation into specific cell types and lineages. iPSCs have shown promise here as well. Researchers have induced differentiation of iPSCs into fibroblasts⁹³ and keratinocytes⁹⁴ that are functionally indistinguishable from embryologically derived cells or adult

cells of the same lineage. iPSCs also demonstrate promising immunogenicity. Human iPSCs introduced to foreign lymphocytes elicited IL-10 (anti-inflammatory) production.⁹⁵ In addition to their ease of harvest and favorable immune properties, iPSCs avoid the ethical controversy surrounding ESCs.

Tissues which continue to divide throughout life (labile) must maintain stem cell populations even in adulthood. These adult stem cells can be harvested and demonstrate regenerative potential. One such population is hematopoietic stem cells (HSCs), blood cell progenitors expressing the CD34 marker, usually residing in the bone marrow. Though HSCs mostly produce blood cells, they have also been shown to produce epithelial cells and hepatocytes.⁹⁶ HSCs may also be a major source of fibroblasts during tissue repair.⁹⁷ HSCs express a specialized form of CD44 which binds very strongly to E-selectin, resulting in powerful homing to sites of inflammation.⁹⁸ It may be possible to deliver HSCs to the site of injury simply by injection into the bloodstream, simplifying clinical treatment.

Mesenchymal stem cells (MSCs) are the progenitor cells for connective tissue and can be found in nerves, adipocytes, umbilical cord blood, and bone marrow.⁸⁹ They have a variety of favorable characteristics for use in tissue engineering, including rapid proliferation and wide differentiation capacity.⁹⁹ Similar to iPSCs, MSCs lack immunological reactivity.¹⁰⁰ Injected bone marrow derived MSCs have been shown to improve ulcer healing rate in diabetic patients compared to HSCs.¹⁰¹ MSCs derived from adipose tissue (AdSCs) are of particular clinical interest, as they share many of the properties of bone marrow derived MSCs (BMSCs) but can be obtained less invasively.¹⁰² AdSCs display a similar cytokine profile to BMSCs.⁹⁹ Furthermore, *in vitro* results suggest that AdSCs also play a pro-angiogenic role.⁹⁹ However, mice treated with BMSCs in a toxic shock model showed a survival advantage over those treated with AdSCs, suggesting a comparative advantage for MSCs in resolution of inflammation.¹⁰³

One of the least invasive potential sources of pluripotent stem cells is urine. Zhang et al discovered that select cells isolated from urine (USCs) were capable of forming progenitor-like cells with the ability to differentiate into urothelial, endothelial, smooth muscle and interstitial lineages.¹⁰⁴ Human USCs have been shown to be efficacious for chronic wound repair. hUSCs cultured in bacterial cellulose significantly improved wound healing in rats, with stem cell treated wounds showing increased epithelialization and depth of healing within one week.¹⁰⁵ Additionally, hUSCs significantly improved angiogenesis. All these effects (wound healing, collagen formation, and vessel formation) could be improved even further with application of silicate biomaterial (bioglass).¹⁰⁶ Further innovation in hybrid cell and biomaterial based technologies could ensure adequate wound regeneration even with stem cells harvested in an entirely non-invasive manner, such as these urine derived stem cells. Interestingly, with additional reprogramming, USCs could even be reprogrammed to form human neural progenitor cells which have showed promise for repair of devastating spinal cord injuries.¹⁰⁷ Although studies of USCs have showed extreme promise, there are multiple problems still associated with their use, including varying efficiency in cultures and problems with stem cell isolation,¹⁰⁸

heterogeneity in cell markers potentially implying decreased purity of cell lines,¹⁰⁹ and most importantly, their functional differentiation and long term effectiveness has not yet been carefully studied.¹¹⁰

In the next section, we will argue that the major mechanism by which stem cells improve wound healing is through cytokine signaling. Cytokine signaling takes place via production of extracellular vesicles, called exosomes. Recent studies have investigated removing stem cells from the picture entirely, and replacing them with exosomes, with promising results.^{111–113} A recent study showed that exosomes isolated from USCs effectively improved diabetic wound healing in rats through increased angiogenesis.¹¹⁴ Exosome treatments have the advantage over stem cells of not being living, replicating cells, which makes their application potentially simpler and safer. In addition, using exosomes could allow mass production of treatment as they may not need to be personalized to prevent immune rejection. However, exosomes face additional challenges including more difficult production and harvesting and full understanding of exosome effects has still not been completely elucidated.¹¹⁵

There have been many advances in the field of stem cells in the last 20 years, including harvest from adult tissues, induction of pluripotency, or even completely non-invasive harvesting or urine derived stem cells. Exosomes take this one step further by removing the cells from the equation entirely and treating directly with the secreted structures which impact wound healing. With the increased availability of various cell lines, *in vivo* experiments have begun to demonstrate the mechanisms and benefits of stem cell treatments for chronic wounds (Table 1).

***In vivo* experiments and mechanisms of stem cell treatment**

Since stem cells are involved in all stages of wound healing, it is logical that stem cell treatments exert their effects through divergent mechanisms (Fig. 4).

Angiogenesis

Ischemia is often one of the major pathologies underlying impaired wound healing. Recent studies have shown that stem cell treatments can improve blood vessel formation and healing in ischemic settings. Injection of CD34 + EPCs into ischemic wounds on diabetic mice significantly improved vascular density and healing time.¹¹⁶ Another study using topically applied fetal-derived CD133 + cells showed similarly accelerated wound closure and increased capillarization.¹¹⁷ Stem cells also showed the potential to improve underlying ischemia. CD133 + cells injected into mice with ligated femoral arteries enhanced blood flow to the ischemic limbs via increased neovascularization.¹¹⁸ Injected AdSCs in irradiated mice improved skin perfusion and vessel density.¹¹⁹ Interestingly, vessel density in AdSC treated mice nearly doubled as compared to control mice, but only 2.6% of the vessels in the wound site expressed AdSC markers, implying that the major mechanism of increased angiogenesis was not based on proliferation of stem cells. This conclusion is supported by another study which demonstrated that new vascular structures were not derived from transplanted stem cells, though stem cells were seen to migrate to the wound bed and increase rate of healing.⁷⁵

Table 1 Stem cell sources in pre-clinical trials.

Stem cell source	References	Advantages and disadvantages
Embryonic stem cells	90	Advantages: Ability to differentiate into any cell line Disadvantages: Controversial harvesting, potentially tumorigenic
Induced pluripotent fibroblasts	91,93,95	Advantages: No ethical problem with harvesting, immunogenicity. Disadvantages: Induction of pluripotency may take longer and not as efficient.
Induced pluripotent keratinocytes	92,94	Advantages: No ethical problems with harvesting, improved production of iPSCs compared to fibroblasts. Disadvantages: May be difficult to harvest and isolate from samples, slower growth and replication.
Hematopoietic stem cells	96–98	Advantages: CD44 allows homing to sites of inflammation, potentially allowing injection rather than topical application. Disadvantages: Have only shown ability to differentiate into blood cells, hepatocytes, and fibroblasts
Bone marrow mesenchymal stem cells	99–101	Advantages: Rapid proliferation, wide differentiation capacity, anti-inflammatory effects. Disadvantages: More difficult to harvest than AMSCs.
Adipocyte mesenchymal stem cells	99,100,102	Advantages: Ease of harvest, rapid proliferation, wide differentiation capacity, angiogenic properties. Disadvantages: Decreased proliferation and density compared to BMMSCs. May have poorer anti-inflammatory effects.
Urine derived stem cells	104–108	Advantages: Extreme ease of harvest, ability to form urothelial, endothelial, smooth muscle, or even neural lineages. Disadvantages: Varying isolation efficiency, increased heterogeneity of cell lines, lack of data on long term effectiveness of USC grafts

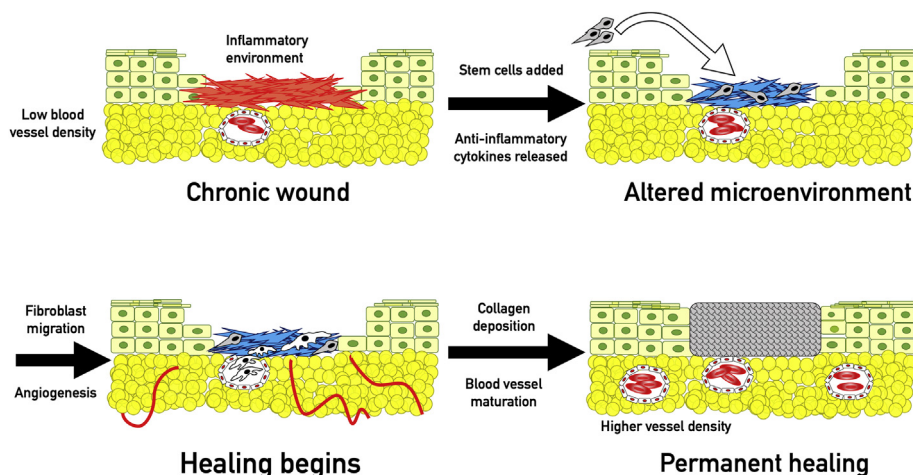


Figure 4 Effects of stem cells. Stem cells improve the conditions in chronic wounds primarily through alteration of the microenvironment. Stem cells have been shown to produce pro-angiogenic and anti-inflammatory cytokines, especially IL-10, TGF- β , and VEGF. They also reduce the concentration of pro-inflammatory cytokines, mainly IL-1 β , TNF- α and IL-6. Resolution of inflammation allows proliferation of fibroblasts and blood vessels, eventually resulting in repair as previously detailed.

Cytokine signaling has been suggested to be the major mechanism of improved angiogenesis in stem cell treatments. One study found that CD133 + stem cells applied to wounds secreted large amounts of VEGF-A and IL-8 and increased rate of healing, while application of anti-VEGF-A or IL-8 antibodies rendered treatment ineffective.¹¹⁷ ASCs also increased capillary density in diabetic mice via augmented expression of HIF-1 α and VEGF-A.¹²⁰ Paracrine signaling from exogenous stem cell treatment improves vascularization and healing even in the anti-angiogenic environment of chronic wounds.

Inflammation

Ongoing inflammation is another trait of the non-healing wound. Although controlled inflammation is an important and necessary phase of wound healing, chronic inflammation damages the wound bed and inhibits the proliferative phase and tissue remodeling.¹²¹ Chronic inflammation also increases protease activity, degrading collagen and preventing repair.⁸⁹ Stem cell treatments have been proven to be immunomodulatory and aid in resolving the inflammatory state. In vitro, MSCs secreted the anti-inflammatory cytokine IL-10, which decreased T-cell reactivity to antigen presenting cells.¹²² The role of IL-10 was further supported by a study which showed that addition of anti-IL-10 antibodies inhibited MSC immunosuppression.¹²³ In addition to their own secretions, stem cells also alter the signaling of nearby cells: MSCs cultured with dendritic cells decreased secretion of the pro-inflammatory cytokine TNF- α and increased secretion of IL-10. T-cells cultured with MSCs produced less IFN- γ and IL-4.¹²⁴ Macrophages can be activated to either pro- or anti-inflammatory phenotypes, and MSCs induce differentiation into the M2 (anti-inflammatory) form.¹²⁵ ASCs also promoted M2 differentiation and decreased inflammatory response to lipopolysaccharides.¹²⁶

Injection of CD-34 + umbilical cord blood cells into a wound decreased expression of the pro-inflammatory factors IL-1 β , TNF- α , and IL-6 and increased IL-10 expression and products.⁴⁹ ASCs produce both IL-10 and TGF- β , which decreases inflammation by inducing differentiation of regulatory T-cells.^{127,128} Infusion of ASCs into a graft-versus-host mouse model decreased GVHD, an immunogenic reaction.¹²⁹

Chronic inflammation can also be due to persistent infection. Stem cell treatment may paradoxically improve pathogen clearance even while modulating the inflammatory response. MSC treatment promoted clearance of bacterial products via increased phagocytic ability of host immune cells and improved survival in septic mice.¹³⁰ ASCs have been shown to produce macrophage colony stimulating factor which recruits phagocytic cells.¹³¹

One of the mechanisms by which chronic inflammation prevents wound healing is through overproduction of reactive oxygen species, causing excessive free radical stress. An imbalance of free radical production can overwhelm local stem cells and prevent wound healing.¹³² Exogenously added stem cells can help to return balance to the system and promote healing. A recent study showed that injection of MSCs helped alleviate lung injury in paraquat poisoning in rats.¹³³ Paraquat is an herbicide which causes cell damage through production of reactive oxygen species. Reduction in lung injury was accomplished through previously discussed signaling mechanisms, such as decreased TNF- α , IL-1 β , and IL-6 production.

Stem cell treatment can reduce inflammation through a myriad of different effects. Direct IL-10 secretion modulates the inflammatory response and increases differentiation of anti-inflammatory cells. Alterations of local cell signaling decrease inflammatory cytokine secretion from leukocytes. Though stem cell treatment decreases inflammatory signaling, pathogen clearance is actually enhanced, decreasing mortality in a sepsis model. Reduction of

inflammation modifies the chronic wound environment and promotes healing.

Differentiation and fibrosis

Proliferation of endogenous stem cells is a critical step in cutaneous wound healing. Exogenously added MSCs have shown the ability to differentiate into keratinocytes¹³⁴ and epithelial cells.¹³⁵ However, numerous studies have found that stem cell treatments show low levels of engraftment *in vivo*, often making up less than 1% of differentiated cells.¹³⁶ Yet functional improvements have been observed despite minimal exogenous stem cell differentiation in various modalities.¹³⁷

Though stem cell treatments do not contribute significantly to wound healing via direct differentiation, multiple studies have demonstrated stem cell mediated improvements in function of nearby cells. MSCs co-cultured with dermal fibroblasts increased fibroblast proliferation and migration.¹³⁸ Another study demonstrated increased fibroblast collagen deposition when cultured with CD34 + umbilical cord blood derived cells.⁴⁹ MSC conditioned medium applied to an excisional wound increased keratinocyte migration and proliferation.¹³⁹ Stem cells also improved tissue remodeling: a study found that repeated topical application of MSCs yielded a basket-weave collagen organization very similar to healthy skin.¹⁴⁰ Enhanced fibrosis and tissue reorganization are crucial to preventing recurrence and reversion of chronic wounds.

Decreased scar formation

Though fibrosis and scar formation is considered a normal part of physiologic wound healing, excessive or early fibrosis can actually prevent proper healing. An obvious example would be keloid or hypertrophic scars,¹⁴¹ however, a similar process can happen with large burns, trauma, or significant surgical interventions. Even large chronic wounds can cause problematic scars after healing, which has been shown to increase recurrence.¹⁴² These large scars sometimes require surgical correction, which triggers additional inflammatory reactions and potentially increased scar or chronic wound formation. Stem cells have been proven efficacious as a non-surgical intervention for scar formation by reducing the ratio of type I to type III collagen. Topical application of BMMSCs overexpressing TGF- β significantly reduced scar depth and density in a rabbit model.¹⁴³ Further work in this area may demonstrate that cell-based therapies have a possible role in prevention or treatment of scar formation as well as epithelial breakdown.

Clinical applications

Stem cell treatments have proven efficacious in rodent models. A few representative examples include the following: bone marrow in a collagen matrix improved rate of wound healing in acute wounds on rats,¹⁴⁴ ASCs injected into diabetic rats improved skin graft survival and decreased wound size within 1 week,¹⁴⁵ ASCs seeded on a dermal matrix improved wound healing and blood vessel

density in a full thickness wound model,¹⁴⁶ and a recent study found that subcutaneous injection of ASCs significantly improved wound healing in diabetic rats through decreased pro-inflammatory factors.¹⁴⁷

One of the earliest clinical applications, in 2003, examined topical application of BMMSCs for chronic wounds. They demonstrated complete closure of wounds in all 3 patients, despite wounds previously persisting for more than 1 year with standard therapy.¹⁴⁸ A 2005 case study had similar results: topical treatment with autologous bone marrow cells reduced wound size and improved vascularity in a recalcitrant ulcer in a patient with T2DM.¹⁴⁹ BMMSCs delivered in a novel fibrin spray improved chronic wound healing in patients as well as in diabetic mice.¹⁵⁰

One of the challenges of stem cell therapy is growing sufficient numbers of cells for treatment. A follow up to the fibrin spray study found that nanofiber-expanded bone marrow cells, which increased number of cells, were still effective for treatment of patients with chronic venous ulcers.¹⁵¹ Administration of these cultured cells improved the cytokine profile of the wound bed, increasing pro-angiogenic markers and vascularity. Cultured BMMSCs applied to patients with intractable dermatopathies of various origins resulted in complete healing for 18 of 20 patients.¹⁵² Intramuscular injections of ASCs improved vascularity, pain, and walking distance in 10/15 diabetic patients with non-healing ulcers.¹⁵³

Pressure wounds are often persistent and difficult to treat with current practices, in addition to frequent recurrence. In 3 patients with non-healing sacral pressure ulcers, treatment with CD34 + stem cells decreased wound volume by 60% from baseline in 3 weeks.¹⁵⁴ In another study, topical application of bone marrow derived stem cells fully healed pressure ulcers in 19 of 22 treated patients within 3 weeks, despite prior persistence for more than 4 months. Remarkably, none of the ulcers recurred for at least 1 year following treatment.¹⁵⁵

Though the number of positive results is encouraging, most of these studies have lacked effective randomization, positive placebos, or, in some cases, any control groups at all. A few larger randomized control trials have showed promise as well. Application of ASCs seeded in fibrin glue to patients with chronic perianal fistulas resulted in healing for 17 out of 24 patients, while only 4 out of 25 patients in the control group (fibrin glue without ASCs) displayed full healing in the same time frame.¹⁵⁶ In a study of 96 diabetic patients with critical limb ischemia (CLI) and non-healing foot ulcers, treatment with BMMSCs (treatment groups were randomly assigned) showed improved brachial pressure index and significantly decreased limb amputations within 3 months of treatment.¹⁵⁷ Another study of CLI in diabetic patients demonstrated 100% healing in patients treated with bone marrow derived MSCs, as well as significantly improved quantitative and qualitative measures including painless walking time, ankle brachial index results, and MRA analysis when compared to controls.¹⁰¹ Injection of BMMSCs into nonhealing foot ulcers of 12 diabetic patients significantly improved wound size, walking distance, and fibroblast proliferation over a 3 month period compared to those injected with saline.¹⁵⁸ A recent study using bone marrow derived mesenchymal stem cells on diabetic foot ulcers showed significantly improved re-

epithelization within a week of a single topical treatment with a much lower cell density by using single pass stem cells.¹⁵⁹ This finding has important ramifications for one of the major problems involved in stem cell clinical treatments: the time required to amplify the initial sample. Because stem cells lose some of their stromal characteristics during the amplification process, cells harvested early during the expansion process are potent enough to improve wound healing even at lower density. Further research into this quality vs. quantity issue will need to be done to elucidate optimal *in vivo* amplification of stem cells for clinical treatments.

A double blinded, randomized clinical trial with 97 participants examining the long term efficacy of bone marrow aspirate for CLI recently closed, though results have not yet been made publicly available.¹⁶⁰ Another interventional trial examining the efficacy of ASCs for treatment of

diabetic ulcers, venous ulcers, and pressure ulcers has concluded, but results have not yet been published.¹⁶¹ A study investigating the effects of allogeneic stem cell treatment on burn wounds and the maximum safe dosage for treatment is still ongoing.¹⁶² Experimental results from case reports and randomized control trials have demonstrated the efficacy of stem cell techniques for chronic wound treatment (Table 2).

Limitations and future directions

The difficulties associated with clinical stem cell treatment are significant. Stem cells are heterogeneous. The technology to standardize or monitor standardization on a large scale is not currently available. Cells from different sources or different hosts can vary in proliferative capacity,

Table 2 Clinical stem cell treatments for chronic wounds.

Year	Reference	Study design (number of participants)	Stem cell source and modality of treatment	Result
2003	109	Case report, proof-of-principle (n = 3)	Bone marrow cells applied topically in isotonic NaCl solution	Complete healing of previously recalcitrant ulcer in 3/3 participants
2005	110	Case report (n = 1)	Bone marrow cells applied topically in isotonic NaCl solution	Improvement in epithelization and vascularization within 1 week
2007	111	Case report, experimental treatment (n = 8)	Cultured MSCs delivered in collagen sponge artificial dermis	Complete healing of previously recalcitrant ulcer in 8/8 participants
2008	113	Case report, experimental treatment (n = 20)	Cultured MSCs delivered in fibrin spray	Improvement of ulcer in 18/20 patients, healing positively correlated with number of cells applied
2009	117	Randomized control trial (n = 35)	Cultured ASCs in fibrin glue	Perianal fistula healed in 17/24 patients in treatment group, 4/25 in control
2009	119	Randomized control trial (n = 24)	Cultured BMMSCs	Significant improvements in ulcer size in experimental group
2010	118	Randomized control trial (n = 96)	Concentrated BMMSCs applied directly to wound bed	Significantly decreased limb amputations among patients in treatment group at 90 days post treatment
2011	116	Case report, experimental treatment (n = 22)	BM-MNCs suspended in saline solution injected into "wound pocket"	19/22 pressure ulcers healed in 21 days, no recurrence in 19 months follow-up
2012	114	Case report, experimental treatment (n = 15)	ASCs injected intramuscularly	Improved vascularity and pain in ischemic ulcers at 6 months follow up
2014	115	Case report, experimental treatment (n = 3)	CD34 + BMCs injected into wound bed	Improvement in wound size in 3/3 patients
2015	121	Phase 2 randomized clinical trial (n = 25)	ASCs applied topically to wound	Results not yet published
2016	120	Phase 2 randomized clinical trial (n = 97)	Bone marrow aspirate injected into ischemic tissue	Results not yet published
2018	157	Case report, proof of principle (n = 3)	Early passage MSCs applied topically	Significantly improved healing within 1 week of application in 3/3 participants
2018	122	Phase 1 safety trial (n = 20)	Allogeneic stem cells topically added to burns	Study still ongoing

differentiation, and even mRNA expression levels.¹⁶³ For minimal immunogenicity, stem cells should be harvested from the patient. However, stem cell function is frequently impaired in diabetic or elderly patients.¹⁶⁴ Though expansion procedures allow production of large quantities of effective stem cells from a small original sample, these procedures are costly and time consuming.

Economic barriers to the adoption of these therapies are also formidable. The classic industry-led drug development model may not be an appropriate paradigm for stem cell-based therapies. Current pharmaceutical manufacturing techniques for small molecules are difficult to adapt to cellular manufacturing.¹⁶⁵ Venture capitalists, viewing the field as too young to have profitable short term returns, have been unwilling to invest heavily in regenerative technology.¹⁶⁶ The costs associated with cell-based therapy are frontloaded, so return on investment requires patience.¹⁶⁷ Finally, intellectual property and patent law surrounding stem cells present new challenges that many companies are unable or unwilling to navigate.¹⁶⁵ Governmental regulations for cell-based therapies are still in flux and are further complicated by the heterogeneity of the subject matter.¹⁶⁸

Nonhealing wounds are a major challenge in healthcare today and will continue to become more problematic with an aging population. Many factors, including impaired macrophage function, fibroblast function, or altered cytokine signaling, lead to chronic wound formation. Current treatments fail to address the magnitude and complexity of the problem.

Stem cell treatments are a novel solution. Stem cells exert their effects primarily through cytokine signaling, creating an anti-inflammatory and pro-angiogenic environment. This environment contributes to healing and improves underlying pathologies, decreasing recurrence. In laboratory and clinical studies, stem cells obtained from various sources improved healing of stubborn wounds. The effects have often been startling: wounds which had previously persisted for more than a year healed within a matter of weeks. Though widespread adoption of these techniques has been complicated by the costs and complications associated with large-scale production of cell products, cell-based therapy for non-healing wounds is a field with great potential. In fact, as the population ages and the costs associated with chronic wound care increase, can we afford not to improve our treatments?

Conflict of interests

The authors declare no conflict of interests.

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