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Cutaneous wound healing

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Regenerative Medicine

Potential roles of suppressor of cytokine signaling in wound healing

Wound healing is a dynamic process comprising three overlapping, highly orchestrated stages known as inflammation, proliferation and re-epithelialization, and tissue remodeling. This complex process is regulated by numerous cytokines, with dysregulation of cytokine-induced signaling leading to impaired wound healing. Suppressor of cytokine signaling (SOCS) proteins are a family of eight intracellular proteins which may hold the potential to maintain homeostasis during wound healing through their negative feedback inhibition of cytokine signaling. To date, the roles of SOCS proteins in inflammation, autoimmunity and cancer have been comprehensively illustrated; however, only a limited number of studies focused on their role in wound healing. This review demonstrates the possible links between SOCS proteins and wound healing, and also highlights the potential importance of this family in a variety of other aspects of regenerative medicine.

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Wound healing: current challenges

The process of wound healing is highly complex and requires substantial interaction and coordination between different cell types to succeed in an orderly and timely manner. Issues arising in the coordination or regulation of this process can have severe consequences, in some cases impairing the capacity to complete the process, resulting in wound chronicity.

Chronic, nonhealing wounds are a significant economic burden on public healthcare. According to an investigation into the total (direct and indirect) costs of 22 leading skin disease categories, the expenditure on skin ulcers and wounds in the USA alone was approximately US$12 billion in 2004. Furthermore, regarding direct costs only, skin ulcers and wounds were identified as being the most costly of the 22 skin diseases analyzed, accounting for US$9.7 billion of the US$29.1 billion total annual spend [1]. Nonhealing wounds in the lower extremities represent major clinical and surgical challenges globally due to the significant cost on healthcare resources and medical professionals. Venous ulcers with high recurrence rates are considered to be the most common type of leg ulcer, making up an estimated 70–90% of total cases. In diabetic patients, it has been suggested that nonhealing foot ulcers are the major cause of morbidity, immobility and lower extremity amputation [2]. Indeed, the economic costs associated with diabetic foot care are so considerable that they compare to those of breast and colorectal cancers [3]. On a patient level, diabetic foot ulcers are responsible for the highest proportion of nontraumatic amputations in the USA [3] and, compared with other complications associated with diabetes, amputation has been shown to have the greatest negative effect on a person's quality of life [4,5]. More than 50% of diabetic foot ulcer patients also suffer from peripheral

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arterial disease, which may further affect the ability of the ulcer to heal. However, peripheral arterial disease is salvageable by revascularization, with limb salvage rates increasing to 85% and more than 60% of ulcers healing within 1 year [6].

The wound healing process

The most basic function of skin is to provide a protective barrier against the environment. Wound healing is a dynamic and interactive process which is immediately activated by damage to the skin upon injury. The primary aims of wound treatment are to reduce the healing time as much as possible and to generate aesthetic scar, or even scar free, tissue without compromising skin function.

Initial response after wounding

Before healing can commence, hemostasis must be achieved at the wound to minimize blood loss. Following injury, platelets are recruited to the wound site and embed in a mesh of crosslinked fibrin fibers to form a fibrin clot. The fibrin clot, which acts as a scaffold for infiltrating cells, establishes hemostasis and also provides a provisional extracellular matrix (ECM) to aid the migration of the variety of cells required for the healing process [7]. Following hemostasis, numerous mediators and chemotactic factors, such as PDGF, EGF and TGF-β, are secreted by the platelets and by damaged cells at the wound site in order to recruit inflammatory cells for further debridement [8]. Neutrophils, the first inflammatory cells attracted by these chemotactic signals, infiltrate the wound site for degranulation of the platelet plug and to remove any bacteria present by means of enzyme release and phagocytosis [9], before subsequently undergoing apoptosis within 48 h of complete wound decontamination [10]. Regulation of this stage is crucial as neutrophils also possess the ability to kill healthy cells, which may explain the persistent tissue-destroying nature of chronic wounds [11]. Monocytes and macrophages are then recruited to the wound site by a variety of chemotactic cytokines produced and secreted by activated endothelial cells [12]. After infiltrating the wound site, monocytes differentiate into activated macrophages to provide further cleansing via the phagocytosis of apoptotic neutrophils, microorganisms and fragments of ECM. The activated macrophages also release growth factors which contribute to granulation tissue formation [7].

Generation of hypoxia & pH gradients

Upon blood vessel damage and the disruption of vascular perfusion, large numbers of platelets from the damaged vessels aggregate into the wound site, suddenly increasing the local oxygen consumption and consequently forming a temporary hypoxic microenvironment. The acute hypoxic state initiates wound healing by increasing reactive oxygen species (ROS) activity, in addition to inducing the secretion of a number of cytokines and growth factors, such as VEGF, TGF-β and TNF, which are beneficial for the later stages of wound healing [13]. In normal wound healing, ROS is strictly controlled by the increased expression of ROS-detoxifying enzymes; chronic wounds partially lose this regulatory mechanism thus leading to the excessive production of ROS [14]. Furthermore, the imbalance of ROS mediators may prolong the inflammatory phase [15,16], and the sustained inflammation could lead to impaired chronic wound. *In vitro* and *in vivo* studies have suggested that peroxiredoxin-6, an important mediator of ROS activity, may potentially protect resident cells in chronic wounds [17,18]. KGF, a member of the FGF family derived from fibroblasts during the early stages of wound healing, is also known to increase peroxiredoxin-6 expression by targeting *Nrf2* gene. Therefore, KGF is considered to be directly related to ROS regulation in keratinocytes [19–21].

Being a prominent member of ROS, hydrogen peroxide (H_2O_2) was found to be of importance during the inflammatory phase of wound healing following a breakthrough discovery by Niethammer *et al.* Using a zebrafish model, they found that a tissue-scale gradient of H_2O_2 created by dual oxidase (Duox) activity in epithelial cells contributed to the recruitment of leukocytes to the wound [22]. This observation led to focused attention on the mechanisms by which H_2O_2 gradients mediate leukocyte recruitment in a complex wound healing environment, although a biological rationale for this remains elusive at present. Nevertheless, H_2O_2 has been proposed to be a pivotal molecule during the whole process of wound healing [23]. In addition, oxygenation is required for cell proliferation, bacterial defense and collagen synthesis during wound healing [13]; therefore, an adequate oxygen supply to the wound site in order to meet the increased energy demands is of great importance. A methodology that would enable us to measure the amount of O_2 present after wounding would be beneficial in the elucidation of the role of oxygenation in the different phases of the wound healing process. A breakthrough technique using 2D luminescence lifetime imaging has been reported which allows the visualization and measurement of O_2 pressure (pO_2) *in vivo* and thus may directly facilitate investigations into the spatial distribution of pO_2 [24]. Furthermore, lower pO_2 was detected in chronic wounds compared with that of previously measured in normal wounds, indicating the critical role of oxygenation in physiological heal-

ing process [25]. Alongside the detection of pO_2, the pH values in the wound site of chronic wound samples were measured by Schreml and colleagues. Interestingly, a pH gradients phenomenon was observed and it was further verified that this could be attributed to the increased expression of Na^+/H^+-exhcanger-1, a ubiquitously expressed proton transporter, from the wound center to wound periphery. This indicates that Na^+/H^+-exhcanger-1-induced pH gradients potentially impede wound healing in chronic wounds [25].

Angiogenesis

Angiogenesis (the formation of new blood vessels) occurs as a result of endothelial cell proliferation and migration in response to a variety of chemotactic and angiogenic signals originating from serum and from the ECM microenvironment [26]. Microvascular endothelial cells are considered to be the principal parenchymal cells involved in wound angiogenesis due to their ability to respond to injury through the degradation of basement membrane, proliferation, migration into the stroma, and the formation of the new blood vessels [27,28]. Angiogenesis leads to the development of new capillary networks and helps restore vascular perfusion, subsequently facilitating the delivery of the nutrients and oxygen required to sustain cell metabolism. Thus, the restoration of the vascular perfusion by angiogenesis could potentially relieve the hypoxic microenvironment of a lesion and so any defect that has the potential to interfere with angiogenesis may lead to delayed or impaired wound healing. Furthermore, insufficient vascularization, which has been shown to be related to reduced re-epithelialization and deficient granulation formation, could potentially result in the development of chronic, nonhealing wounds [29]. Therefore, ensuring the normal function of angiogenesis is essential for the wound healing process and, as such, we may be able to improve wound healing by regulating the behavior of endothelial cells and the process of angiogenesis [8].

Stages of wound healing

The wound healing process consists of three highly orchestrated overlapping stages known as inflammation, proliferation and re-epithelialization, and tissue remodeling [14].

The inflammation stage is considered to be the driving force in chronic, nonhealing wounds and, as such, proinflammatory regulators may possess diagnostic and/or prognostic value, in addition to potential as future therapeutic targets [14]. Inflammation starts immediately after injury occurs and generally lasts between 4 and 6 days in the physiological wound healing process [13]. During this time, components of the coagulation cascade prevent the loss of blood and fluids, while inflammatory pathways remove dead cell debris and any bacteria present.

Proliferation and re-epithelialization are of great importance in the wound healing process. Keratinocytes are key to the re-epithelialization phase of the injury response and are involved in the secretion of cytokines for the recruitment of other cells, producing growth factors to improve collagen formation and angiogenesis, and undergoing migration and proliferation to fully enable epithelialization [30]. Keratinocytes have been shown to dramatically change their morphology at the beginning of migration following injury, becoming flat and elongated while 'crawling' along the wound bed [31]. The loss of hemidesmosomes and desmosomes is also observed in migrating keratinocytes [32]. Keratinocytes can exist in two states which are dependent on the status of the epidermis: in healthy epidermis keratinocytes are highly differentiated, but following injury the activated keratinocyte phenotype becomes more prevalent [33–37].

The proliferation and re-epithelialization stage starts, several hours after injury, at the interface between granulation tissue and the fibrin clot with the migration and proliferation of keratinocytes from the cut edges of the wound and from amputated stumps of damaged appendages, such as hairs or sweat glands, to form a barrier against pathogens [11]. Keratinocytes at the cut edges of the wound alter their expression of integrin adhesion molecules in order to reduce adhesion, both to each other and to the basal lamina, and enable movement to the denuted area. At the same time, the following rows of keratinocytes undergo proliferation to ensure sufficient resources for the formation of the epithelial barrier [11,38]. When the intact basement membrane is damaged by wounding, provisional ECM (which is rich of fibronectin, fibrin and vitronectin) acts as a scaffold and facilitates keratinocytes migration [32].

Meanwhile, dermal fibroblasts in the neighborhood of the wound are attracted and activated by chemoattractants, causing them to differentiate into myofibroblasts following the expression α-smooth muscle actin. Myofibroblasts were suggested to be responsible for wound contraction [39] and were valued as a marker of fibrosis and scarring in many pathological tissues [40]. Myofibroblasts are believed to contribute to the contraction and maturation of granulation tissue [41,42], and also to produce and deposit the ECM components that subsequently replace the provisional matrix [8]. Eventually, when the epithelial structure has been reconstituted in the normal healing process, the myofibroblasts undergo apoptosis in order to dramatically reduce their number [43]. In addition, large-scale

angiogenesis, leading to the formation of capillary blood vessels in the deeper connective tissues of the wound site, supplies nutrients and oxygen to meet the metabolic demands of cells involved in tissue repair at this stage.

The final stage of wound healing, tissue remodeling, begins 2–3 weeks after injury and lasts for at least a year. In this stage, granulation tissue gradually converts into mature scar tissue by collagen catabolism [42]. The majority of cell types involved in the former stages of wound healing, such as endothelial cells, macrophages and myofibroblasts, either undergo apoptosis or leave the wound site, and only a few cells remain with collagen and ECM proteins. After 6 months, the repaired tissue is strengthened by remodeling of the major type III collagen to the predominant type I collagen; a process mediated by metalloproteinases secreted by fibroblasts, macrophages and endothelial cells [9]. However, unlike the uninjured skin, the wound will never reach 100% organized collagen form and will never return to perfect skin [44].

Role of cytokines/growth factors in wound healing

Cytokines are a class of small proteins involved in both paracrine and autocrine cell signaling. Cytokines include, among others, chemokines (which promote chemotaxis), interferons and interleukins (which are vital for the function of a healthy immune system) and members of the TNF family (which can induce apoptosis). The cytokines which are produced and released following an immune event can initially dictate whether an immune response is necessary and, if so, whether that response is cytotoxic, humoral, cellular mediated or allergic in nature [45]. Wound healing is tightly regulated by a large number of cytokines and growth factors through various sophisticated signaling pathways. Throughout the wound healing process cytokines and growth factors act as important mediators of differentiation, proliferation, maturation and various other functions of the cells which contribute to wound closure.

A variety of ECM components, cytokines and growth factors are derived from activated keratinocytes during the proliferation and re-epithelialization phase of wound healing, and act as chemoattractants which can then activate fibroblasts, endothelial cells and lymphocytes, as well as neighboring keratinocytes [46]. Some of these cytokines and growth factors, such as IL-1 and TNF-α, regulate activation of keratinocytes, whereas TGF-α also mediates keratinocyte proliferation. Once the wound has healed, dermal–fibroblast-derived TGF-β acts as a regulator to suppress the proliferation of keratinocytes and to induce synthesis of ECM [46]. IFN-γ was found to strongly and specifically induce the expression of keratin-17 [47], a protein expressed in various healthy epithelia that are characterized as contractile tissue. Thus, IFN-γ was suggested to contribute to the contractile nature of keratinocytes in the later stage of wound healing [46]. IL-6 derived from fibroblasts, macrophages, endothelial cells and keratinocytes is another essential cytokine which affects granulation tissue formation, re-epithelialization, angiogenesis, cell infiltration and remodeling. Additionally, IL-6 showed enhanced expression in chronic wounds exhibiting aberrant inflammation, suggesting the importance of the precise control of IL-6 expression patterns in normal wound healing [14]. EGFR is expressed in the basal layer and the first suprabasal layer of adult epidermis [48]. Activation of EGFR through ligand binding could induce keratinocyte proliferation and migration, as well as the degradation of ECM components [49]. However, evidence showed that deficient expression of the downstream signaling protein, STAT-3, induced by EGFR could lead to impaired keratinocyte migration and remodeling [50]. In addition, it was found that the expression of EGFR was reduced in chronic wounds, and that keratinocytes at the nonhealing edge of chronic wounds are incapable of responding to EGF stimulation due to the cytoplasmic localization of EGFR [51], indicating the essential role of EGFR on pathological wound healing. As a result of extensive studies on angiogenesis, many cytokines and growth factors have since been identified as either proangiogenic or antiangiogenic molecules [29]. FGF is a potent mitogen for vascular and capillary endothelial cells [52] and has been shown to stimulate their proliferation, differentiation, migration, invasion and tubule formation ability [53,54]. Another extensively investigated growth factor, TGF-β, was also identified as a critical mediator of angiogenesis due to the fact that it can stimulate endothelial cell differentiation, migration and capillary tubule formation [55,56].

JAK/STAT signaling

The JAK/STAT pathway is an essential cellular mechanism which responds to a wide array of cytokines by transducing their signals to the nucleus and, thus, promoting cell functions such as proliferation, migration, differentiation and apoptosis. Due to the establishment of murine knockout models, the biological significance of JAK/STAT signaling pathway is extensively recognized [57].

To date, four JAK family members (JAK1, JAK2, JAK3 and TYK2) and seven STAT family members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) have been identified in mammals [58].

Each JAK protein consists of four structural domains: an N-terminal FERM (abbreviation of the first four proteins found in this family: four-point-one, Ezrin, Radixin, Moesin) [59], an SH2 domain, a 'pseudo kinase' domain and a C-terminal PTK domain [58]; while STAT family members possess five functional domains: an amino-terminal domain (NH2), a coiled–coiled domain, a DNA-binding domain, an SH2 domain and a carboxy-terminal transcriptional activation domain [60].

Since the JAK/STAT signaling pathway is utilized by numerous cytokines to communicate with the nucleus, any mutation which holds the potential to compromise the regular function of JAK/STAT pathway may affect cytokine-stimulated signaling. Additionally, deregulation of this signaling pathway may cause inflammatory disease, erythrocytosis, gigantism and leukemia [61]. Therefore, mechanisms for the regulation of JAK/STAT signaling to prevent activation beyond the necessary time are of great importance. This is achieved by the use of negative regulators which possess a specific SH2 domain such as SH2-containing phosphatase, protein inhibitors against STATs and suppressor of cytokine signaling (SOCS) [62]. However, compared with SH2-containing phosphatase and protein inhibitors against STATs, which are constitutively expressed [62], SOCS proteins are the only cytokine-inducible inhibitors that may have the potential for use as a biomarker for the dysregulation of cell metabolism.

SOCS

SOCSs are a group of cytokine and growth factor induced proteins containing three common structural and functional domains that negatively regulate cytokine receptor and receptor tyrosine kinase signaling, predominantly via inhibition of the JAK/STAT signaling but also by blocking nuclear translocation and by target protein degradation respectively, in order to maintain the homeostasis of cell function. The group consists of eight family members, cytokine inducible Src-homology 2-containing protein (CIS) and SOCS-1 to SOCS-7, in mammals [63]. Additionally, three family members of SOCS homology were found in both *Drosophila* and *Ciona intestinalis* [64–66].

Discovery of SOCS

SOCS proteins were discovered separately by three independent groups of scientists. In 1997, Starr *et al.* discovered three homologous forms of CIS, namely SOCS-1, SOCS-2 and SOCS-3, and suggested that SOCS-1 plays an important role in the classic negative-feedback loop which regulates cytokine signal transduction [67]. In the same year, Endo *et al.* found that a new JAK-binding protein, which is structurally related to CIS and possesses an SH2 domain, acts as negative regulator in the JAK signaling pathway [68]. Meanwhile, a new protein, known as STAT-induced STAT inhibitor-1, was identified by a third group of scientists, and this protein was also found to be involved in the negative-feedback regulation of cytokine-stimulated JAK/STAT signaling [69]. It was later confirmed that SOCS-1, JAK-binding protein and STAT-induced STAT inhibitor-1 all belong to the SOCS family of proteins [70].

Structure & structural-related function

There are three major domains which contribute to the function of SOCS proteins: a conserved central SH2 domain containing an extended SH2 sequence, an N-terminal domain of variable length and divergent sequence, and a carboxy-terminal 40-amino acid module called the SOCS box (Figure 1) [71,72]. Although the function of the N-terminal domain in SOCSs has not been fully elucidated, it is suggested that its helical structure contributes to the stability of the SOCS molecule complex [73]. The SH2 domain of SOCS proteins binds to phosphotyrosine residues on receptor complexes and JAK proteins to regulate the activated JAK/STAT signaling pathway and maintain the balance of cell metabolism. The SOCS box domain is a highly conserved motif comprising two functional subdomains: a BC box recruiting elongins B and C, and a Cul box which regulates Cullin-5 binding to RING-box-2 [74,75]. SOCS box attaches to elongin-B/C followed by binding to Cullin-5, a scaffold protein of an E3 ubiquitin ligase, thereby acquiring the ability to terminate cytokine signaling by binding to target molecules and facilitating proteasomal degradation [74]. It was found that SOCS proteins are relatively short-lived compared with the signaling components they affect, such as the JAK and STAT proteins, and such phenomenon was thought to be attributable to proteasomal degradation through association with their SOCS box [76].

SOCS proteins can be divided into two major subfamilies according to the depth and scope of investigation and their structural similarity. The first subfamily, containing CIS, SOCS-1, SOCS-2 and SOCS-3, has been extensively investigated, whereas study on the other subfamily comprising SOCS-4 to SOCS-7 is still limited. Within each of the two subfamilies, SOCS proteins can be further paired based on similarities in structure and function. The CIS/SOCS-1 to SOCS-3 subfamily are the most investigated and it is found that CIS and SOCS-2 possess similar structures, whereas SOCS-1 and SOCS-3 have similar functions due to their homologous structure. Although inves-

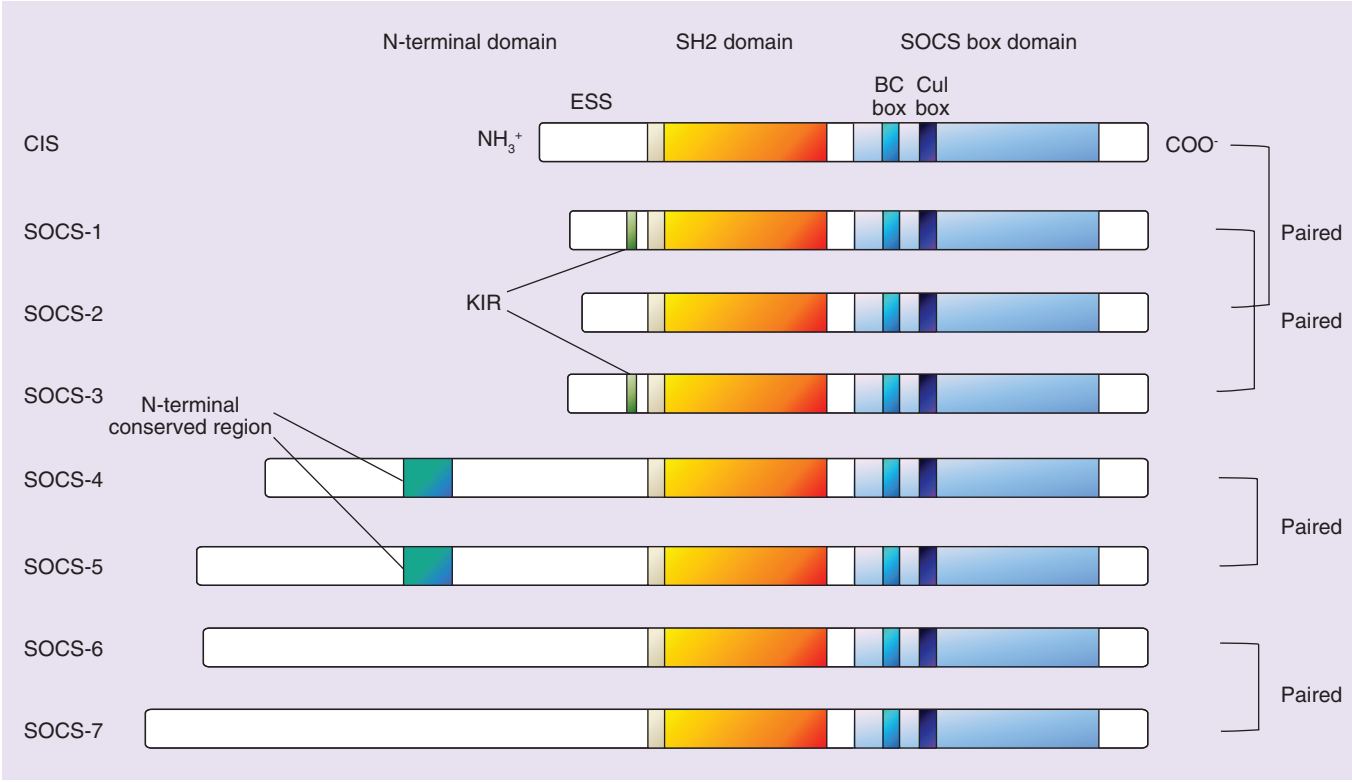

Figure 1. Structure of suppressor of cytokine signaling family members. All SOCS proteins consist of three structural and functional domains which are: a N-terminal domain with variable length of amino acids sequences, a central SH2 domain with ESS and a SOCS box domain containing a BC box and a Cul box at the C-terminus. Each two SOCS family members could be paired due to their structure and function similarity. SOCS-1 and SOCS-3 both possess a unique KIR which inhibit JAK protein activity. SOCS-4 and SOCS-5 both contain a highly conserved region within their N-terminal domain termed as N-terminal conserved region. SOCS-6 and SOCS-7 share more than 50% amino acid identity in SH2 domain and SOCS box domain.
Data taken from [77–79].
ESS: Extended SH2 sequence; KIR: Kinase inhibitory region; SOCS: Suppressor of cytokine signaling.

tigations on the function of the SOCS-4 to SOCS-7 subfamily are limited and require further elucidation, SOCS-4 and SOCS-5 may be paired since they possess the most similarity with regards to structure; with both proteins containing a highly conserved region within their N-terminal domain, termed the N-terminal conserved region. Similarly, SOCS-6 and SOCS-7, the most homologous among SOCS family members, sharing more than 50% amino acid identity in SH2 and SOCS box domains [80], were proposed to be a pair and they are both involved in protein nuclear translocation [81,82]. SOCS family members can also be classified according to their target proteins. CIS/SOCS-1–3 regulates cytokine receptor signaling through the JAK/STAT pathway, whereas SOCS-4 to SOCS-7 are associated with regulation of growth factor receptor signaling [77]. The eight structurally related SOCS family members have been recognized as being able to attenuate cytokine and growth factor signaling by inhibition of JAK tyrosine kinase activity, by blocking signal transduction through competition for the receptor's phosphotyrosine residue, and via the degradation of crucial molecules, such as JAK and the receptor complex, through proteasomal pathway [77]. To date, the structures of the ternary complex of three SOCS family members (SOCS-2, SOCS-3, SOCS-4–ElonginC–ElonginB) have been established for investigating SOCS function [83–87].

Potential roles of SOCS proteins in wound healing

SOCS & epithelial cells

Some studies have indicated that SOCS proteins may regulate the behavior of epithelial cells, one of the key components in the wound healing process. STAT3 is thought to regulate apoptosis in epithelial cells and thus control the remodeling of mammary epithelium [88]. Mice with deletion of SOCS-3 from mammary stem cells or early progenitor cells exhibited aberrant STAT3 activation, suggesting a negative regulatory role for SOCS-3 on STAT3 in the homeostasis of mammary epithelium [89]. Another *in vivo* study showed that SOCS-1 is a negative regulator of epithelial cell proliferation at the stage of mammary devel-

opment [90]. Similarly, SOCS-2-deficient mice restored lactation failure caused by the loss of a single prolactin receptor allele in a STAT5-dependent manner, indicating a negative regulatory role for SOCS-2 toward mammary epithelium development through STAT5 activation [91]. It was suggested that keratinocyte proliferation and migration are strongly disturbed by the presence of SOCS-3 which subsequently contributes to impaired wound healing [92], whereas exacerbated inflammation which characterizes chronic wounds is shown to be related to overexpression of SOCS-3 [93].

SOCS proteins & key molecules in wound healing

To date, studies on the deeper roles of SOCS proteins in wound healing are limited and not well elucidated. However, there are many cytokines and growth factors found to be mediated by SOCS proteins which play an important role in the wound healing process (Table 1). Further investigation of the regulatory roles of SOCS on such cytokines/growth factors and their receptors may provide novel routes for therapeutic intervention.

SOCS & cytokines

An early study discovered that CIS, SOCS-1 and SOCS-3 could inhibit IL-2 signaling by association with the β-chain of the IL-2 receptor and by suppressing the IL-2-dependent activation of STAT-5. In addition, SOCS-1 and SOCS-3 could also associate with JAK-1 and inhibit JAK-1 activation to compromise IL-2-induced signaling. Such findings indicate the important regulatory role of CIS and SOCS-3 on IL-2 signaling [98–100]. Contrary to this, it was found that SOCS-2 increased IL-2-dependent STAT phosphorylation via association with and proteasomal degradation of SOCS-3 [101]. With respect to the wound healing area, IL-2 was found to be able to enhance fibroblast infiltration and metabolism *in vitro*, and was suggested to be beneficial for an immunocompromised wound [94]. Therefore, CIS, SOCS-1, SOCS-3 and SOCS-2 may act synergistically as potential regulators of fibroblast behavior during normal wound healing and may help modulate the inflammatory phase of the wound healing process in immunodeficient patients.

SOCS-1 was found to suppress the signaling of IL-4, an anti-inflammatory cytokine, through inhibition of JAK-1 and STAT-6 [104]. The N-terminus of SOCS-5 is involved in recruiting the IL-4 receptor complex, and it has been suggested that SOCS-5 specifically interacts with the IL-4 receptor α-chain and may functionally affect IL-4-induced STAT activation regardless of receptor tyrosine phosphorylation status [105,112]. During the wound healing process, IL-4, screted by T lymphocytes, basophils and mast cells, is able to induce fibroblast proliferation, collagen production and arginase activity, another critical component that promotes wound healing [94]. Taken together, SOCS-1 and SOCS-5 may affect the re-epithelialization and tissue remodeling phases of wound healing through modulation of IL-4 signaling.

A study has shown that SOCS-3 inhibits the activation of STAT-3 by IL-6, suggesting an inhibitory role for SOCS-3 in IL-6-induced signaling [120]. The establishment of a T-cell and natural killer T-cell SOCS-3 conditional knockout mouse model demonstrated that SOCS-3 regulates the activity of IL-6 via both homodimeric and heterodimeric gp130 receptors [121]. In addition to acting as a negative feedback inhibitor, SOCS-3 was also found to be tyrosine-phosphorylated following stimulation by a number of cytokines and growth factors [117]. Enhanced expression of IL-6, a proinflammatory cytokine, was observed during the inflammatory phase of wound healing. IL-6 is considered to be an essential initiator of the wound healing process due to its mitogenic and proliferative effect on keratinocytes and the chemoattractive function of neutrophils [122]. Thus, SOCS-3 may potentially hold a regulatory role in the inflammatory phase of wound healing.

A previous study showed that SOCS-1 inhibits TNF-α-induced cell apoptosis via regulation of p38 MAPK, indicating an important regulatory role for SOCS-1 in TNF-α signaling [110]. TNF-α was found to be upregulated in chronic wounds and was suggested to result in prolonged inflammation via degradation of the ECM, growth factors and cell receptors by inducing production of matrix metalloproteinases (MMPs) [122]. Thus, SOCS-1 may have the potential to maintain the homeostasis of the inflammation phase of chronic wounds through regulation of TNF-α. In addition, evidence showed that SOCS-3 inhibits IL-1β-induced signaling [95] and that IL-1β exhibited similar expression patterns and synergistic roles to TNF-α in chronic wounds. Taken together, SOCS-3 may play an important role in regulating proinflammatory cytokines in chronic wounds. A further study suggested that SOCS-2 is an important anti-inflammatory regulator and is required for immune responses in diverse pathologies [123]. Another study using a SOCS-7-deficient mouse model demonstrated a negative regulatory role for SOCS-7 in the production of proinflammatory cytokines, such as IL-6 and TNF-α, by mast cells [124]. Therefore, SOCS-2 and SOCS-7 may play opposed regulatory roles in the inflammatory phase of wound healing.

SOCS-1-deficient mice develop complex fatal neonatal defects such as fatty degeneration, necrosis of the liver, infiltration of major organs by inflammatory cells and considerable lymphocyte deficiency, thus indicat-

Table 1. Suppressor of cytokine signaling and essential cytokines/growth factors and receptors involved in wound healing.

Cytokine/growth factor/receptor	Target components in wound healing	Functions in wound healing	SOCS induced by cytokine/ growth factor	SOCS that negatively/ positively regulates downstream signaling	Downstream pathway/ molecule by which cytokine/receptor are negatively/positively regulated by SOCS	Ref.
IL-1β	Endothelial cells,macrophages,leukocytes, keratinocytes, fibroblasts	Inflammation, angiogenesis, re-epithelialization, tissue remodeling, induces keratinocyte, neutrophil and fibroblast chemotaxis, induce neutrophil activation	SOCS-2 SOCS-3	SOCS-3/negative	–	[14,94–97]
IL-2	Fibroblast	Increase fibroblast infiltration and enhance fibroblast metabolism	CIS SOCS-1 SOCS-2 SOCS-3	CIS/negative SOCS-1/negative SOCS-2/positive SOCS-3/negative	IL-2R via STAT-5 Through association with SOCS-3 and degradation Association with JAK-1 and IL-2R/binding to calcineurin	[94,98–103]
IL-4	Macrophages, fibroblasts	Enhance collagen synthesis, induces fibroblast proliferation	SOCS-1 SOCS-2	SOCS-1/negative	Inhibition of activated JAK-1 and STAT-6	[14,69,94,103–105]
IL-4R			SOCS-5	SOCS-5/negative	Inhibition of STAT-6	
IL-6	Endothelial cells,macrophages, keratinocytes, leukocytes, fibroblasts	Inflammation, angiogenesis, re-epithelialization, collagen deposition, tissue remodeling, induce fibroblast proliferation	CIS SOCS-1 SOCS-3 SOCS-5	SOCS-3/negative SOCS-5/negative	–	[14,67,69,94,106–107]
IL-10	Macrophages	Inhibits macrophage activation and infiltration, inhibits TNF-α, IL-1 and IL-6 expression	CIS SOCS-3	–	–	[94,108]

ECM: Extracellular matrix; SOCS: Suppressor of cytokine signaling.

Table 1. Suppressor of cytokine signaling and essential cytokines/growth factors and receptors involved in wound healing (cont.).

Cytokine/growth factor/receptor	Target components in wound healing	Functions in wound healing	SOCS induced by cytokine/ growth factor	SOCS that negatively/ positively regulates downstream signaling	Downstream pathway/ molecule by which cytokine/receptor are negatively/positively regulated by SOCS	Ref.
IFN-γ	Macrophages, keratinocytes	Induces collagenase activity, preventing collagen synthesis and crosslinking	CIS SOCS-1 SOCS-2 SOCS-3	SOCS-1/negative SOCS-3/negative	–	[67,94,109]
TNF-α	–	Regulates collagen synthesis and degradation, increases vascular permeability and homeostasis, provides metabolic substrates	CIS SOCS-1 SOCS-3	SOCS-1/negative	–	[67,94,110–111]
EGF	Keratinocytes, fibroblast	Re-epithelialization, increases fibroblast collagenase secretion, inhibits fetal wound contraction	CIS SOCS-2 SOCS-3 SOCS-4 SOCS-5	SOCS-2/negative SOCS-4/negative	Association with activated EGFR Competing docking site with STAT-3	[14,94,112–116]
EGFR	–	–	SOCS-4 SOCS-5 SOCS-7	SOCS-4/negative SOCS-5/negative SOCS-7/negative	Association with activated EGFR and degradation Association with activated EGFR and degradation Association and degradation	
PDGF	Leukocytes,macrophages, fibroblasts	Inflammation, re-epithelialization, collagen deposition, tissue remodeling, recruits fibroblasts and macrophages, induces collagen synthesis	SOCS-3	–	–	[14,94,117]

ECM: Extracellular matrix; SOCS: Suppressor of cytokine signaling.

Table 1. Suppressor of cytokine signaling and essential cytokines/growth factors and receptors involved in wound healing (cont.).

Cytokine/growth factor/receptor	Target components in wound healing	Functions in wound healing	SOCS induced by cytokine/growth factor	SOCS that negatively/positively regulates downstream signaling	Downstream pathway/molecule by which cytokine/receptor are negatively/positively regulated by SOCS	Ref.
HGF	Endothelial cells, keratinocytes	Suppression of inflammation, granulation tissue formation,angiogenesis, re-epithelialization	SOCS-1 SOCS-3	SOCS-1/negative SOCS-3/negative	Inhibition of STAT-3 activation	[14,118]
TGF-β	Fibroblasts, keratinocytes, macrophages, leukocytes, endothelial cells, ECM	Inflammation, angiogenesis, granulation tissue formation, collagen synthesis, tissue remodeling, leukocyte chemotactic function	SOCS-3	SOCS-3/negative	–	[14,94,119]

ECM: Extracellular matrix; SOCS: Suppressor of cytokine signaling.

ing a crucial role for SOCS-1 in postnatal growth and survival [125–127]. Such devastating defects were attributed to hyper-responsiveness to endogenous IFN-γ and can be prevented following the administration of anti-IFN-γ antibodies or in the presence of an IFN-γ gene-deficient environment. Thus, SOCS-1 appears to be a critical regulator of IFN-γ action [128]. Further investigations *in vivo* demonstrated that the SOCS-box domain was partially responsible for this increased responsiveness to IFN-γ, which can eventually lead to inflammatory disease [129]. A study of SOCS-1 in human keratinocytes suggests that SOCS-1 exerts its inhibitory function against the proinflammatory effects of IFN-γ by not only inhibiting STAT-1 but also via maintenance of ERK-1/2-dependent anti-inflammatory pathways [130]. Inhibition of IFN-γ activity by SOCS-1 was also found to be crucial for the differentiation of Th17 T-helper cells [131]. Since IFN-γ was found to contribute to the enhancement of tissue remodeling and the reduction of re-epithelialization and wound contraction, regulation of IFN-γ is considered crucial to wound healing [94]. Therefore, further investigation of the regulatory role of SOCS-1 on IFN-γ during the re-epithelialization and tissue remodeling stages of wound healing may identify new potential therapeutic targets. Additionally, SOCS-3 was also found to be a negative regulator of IFN-γ-induced signaling though suppression of activated STAT-1, although its inhibitory activity is weaker than that of SOCS-1 [109]; thus indicating another SOCS protein that potentially modulates the later stages of the wound healing process.

SOCS & growth factors/growth factor receptors

A study showed that the regulatory role of SOCS-3 on TGF-β1 induced SMAD-3-dependent signaling [119]. TGF-β1 exerts its function through the production of cytokines and inflammatory mediators, recruitment of inflammatory cells and macrophages for the purpose of tissue debridement, regulation of fibroblast function, induction of angiogenesis and modulation of the synthesis of proteases and ECM [132]. Its potent ability to stimulate collagen production means TGF-β1 plays an important role in scar formation and in the development of hypertrophic and keloid scars [122]. Other studies have shown that TGF-β1 is able to promote the adhesion and migration of keratinocytes [133] and to regulate keratinocyte proliferation [122]. Although the downstream mechanisms of how TGF-β1 exerts its function on wound healing remains unclear, there might be linkage between SOCS-3 and wound healing through the regulation on TGF-β1 signaling. It was discovered that SOCS-1 and SOCS-3 could inhibit HGF-induced STAT-3 activation [118], indicating their regulatory roles in HGF-dependent signaling. HGF,

which could be induced by TNF-α, IL-1 and IL-6, was considered to promote granulation tissue formation and angiogenesis in wound healing. HGF exerts its function by binding to its receptor tyrosine kinase, c-Met. Chronic wounds were suggested to be attributable to HGF/c-Met pathway dysregulation, and topical application of HGF was recommended as a potential treatment for chronic wounds [14]. Therefore, the precise control of HGF signaling by the implementation of SOCS-1/SOCS-3 may hold potential as another therapeutic option for chronic wounds.

SOCS-2 was found to be able to associate with EGFR at the Tyr^{845} Src-binding site and to decrease STAT-5b phosphorylation stimulated by EGF, indicating its negative regulatory role on EGF-induced signaling [113]. SOCS-4, the expression of which can be induced by stimulation with EGF, is also able to significantly reduce both EGFR expression and EGFR-mediated signaling [114]. SOCS-4 decreases EGFR-dependent STAT-3 activation by promoting degradation of EGFR and by competing with STAT-3 for the phosphorylated Y1092 residue of EGFR [86]. The expression of SOCS-5 was induced in HeLa cells at both transcript and protein level following stimulation with EGF, and the expression of SOCS-5 was found to correlate with the reduction of EGFR levels via proteasomal degradation. It has been further identified that SH2 and SOCS box domains are essential for SOCS-5-dependent inhibition of EGF signaling via interaction with EGFR and for EGFR degradation [112,114]. SOCS-7, also known as NAP4, contains a putative nuclear localization signal and a motif specific to nuclear proteins which could be induced by many cytokines and has previously been shown to bind to activated EGFR via its SH2 domain [115]. It has been established that EGF and EGFR both play crucial roles in the re-epithelialization phase of normal wound healing. Upon injury, EGF derived from platelets, macrophages and fibroblasts is upregulated and is able to accelerate re-epithelialization via the promotion of keratinocyte migration. Once bound to its ligand, EGFR is activated and facilitates re-epithelialization by inducing the proliferation and migration of keratinocytes. However, it was discovered that EGFR was located in the cytoplasm of epidermal cells of nonhealing edges of chronic wounds, instead of at the cell membrane [122]. Since SOCS proteins have the ability to interact with EGFR, to cause internalization of the receptor, and to promote the degradation of EGFR via their SOCS box domain, investigation of SOCS proteins and EGFR in wound healing in *in vitro* and *in vivo* models may hold the potential for the discovery of new therapeutic targets in chronic wound treatment.

SOCS-2 & SOCS-6: negative regulators of SOCS protein activity

Studies have shown that SOCS-2 mediates growth hormone sensitivity by blocking the inhibitory effect of endogenous SOCS-1 [134,135]. Furthermore, Tannahill and colleagues found that SOCS-2 is able to induce cytokine response by reducing the expression of SOCS-3 through the formation of an E3 ubiquitin ligase complex and subsequent proteasomal degradation [101]. Similar evidence demonstrated that SOCS-2 associates with the SOCS box of CIS as ubiquitin ligase [136]. According to mammalian protein–protein interaction trap analysis, SOCS-2 is able to interact with all members of the SOCS family and can target them for proteasomal degradation, further suggesting the dual role of SOCS-2 in inhibiting and facilitating cytokine-induced signaling [137]. SOCS-6 is also defined as a negative regulator of cytokine signaling. It has been suggested that SOCS-6 facilitates proteasomal degradation of the target proteins to which the SH2 domain of SOCS-6 bind [80]. Similar to SOCS-2, SOCS-6 also has the ability to interact with other SOCS family members in a SOCS box dependent manner and acts as a negative regulator of SOCS protein activity [137]. Based on the above mentioned possible linkage between SOCS proteins and critical wound healing molecules, SOCS-2 and SOCS-6 may have dual effect on modulating different stages in the wound healing process either via their original negative regulatory role on cytokine and growth factor signaling or through the unique function of association with their own family members for further proteasome-dependent degradation.

SOCS related downstream signaling pathways

A study demonstrated that SOCS-3 phosphorylation was required for sustained activation of ERK which contributes to cell survival and proliferation functions via the RAS pathway [117]. Similar evidence from a study on SOCS-1 showed that, in addition to negatively regulating IFN-γ-induced JAK/STAT signaling, SOCS-1 also sustained ERK-activated anti-inflammatory pathways in human keratinocytes [130]. Upon insulin treatment, instead of direct inhibition of insulin receptor autophosphorylation SOCS-1 and SOCS-6 were more likely to inhibit insulin-dependent activation of ERK-1/2 [138]. During the wound healing process, ERK has been considered as an important regulator of wound contraction [139] and an *in vivo* study in mice showed that the inhibition of ERK activation led to delayed wound healing [140]. Taken together, SOCS proteins may also mediate wound healing by regulating the activation of ERK.

Conclusion & future perspective

The role of SOCS proteins in inflammation, autoimmunity and in cancer have been comprehensively described in the literature [77,141–142]. However, studies on SOCS proteins and wound healing are limited and so the regulatory role of SOCS proteins in the wound healing process remains unclear. Therefore, SOCS-regulated cytokines and growth factors and their respective receptors, as well as their driven signaling pathways which are important in modulating the behavior of key cell types involved in wound healing, should be considered for more in-depth investi-

Executive summary

Wound healing: current challenges

- Nonhealing wounds represent major clinical and surgical challenges globally due to the significant cost on healthcare resources and medical professionals.

The wound healing process

- Wound healing is a dynamic and interactive process which consists of three overlapping orchestrated stages termed as inflammation, proliferation and re-epithelialization and tissue remodeling.
- Initial response after wounding:
  - Initial response is activated to achieve hemostasis and minimize blood loss.
- Generation of hypoxia and pH gradients:
  - Hypoxia and pH gradients are generated in the early stage of wound healing.
- Angiogenesis:
  - Angiogenesis is an important component in wound healing in order to provide required oxygen and nutrient. Such process is regulated by a variety of cytokines and growth factors.
- Stages of wound healing:
  - Three highly orchestrated overlapping stages in wound healing process are known as inflammation, proliferation and re-epithelialization, and tissue remodeling.

Role of cytokines/growth factors in wound healing

- Many cell types, cytokines and growth factors are involved in such process and their precise regulation are required for normal wound healing.

JAK/STAT signaling

- The JAK/STAT signaling pathway is extensively utilized by a wide array of cytokines and growth factors to transduce signals and to mediate cell functions as the result of biological response. Such signaling pathway is used by wound healing process.

Suppressor of cytokine signaling

- Discovery of suppressor of cytokine signaling (SOCS):
  - SOCS proteins are a family of intracellular proteins containing eight members which primarily exert their function as negative regulators of cytokine-induced JAK-STAT signaling.
- Structure and structural-related function:
  - SOCS proteins contain three functional domains which contribute to different mechanisms of their regulatory role.

Potential roles of suppressor of cytokine signaling proteins in wound healing

- SOCS and epithelial cells:
  - Studies showed that SOCS have direct regulatory roles in epithelial cells.
- SOCS proteins and key molecules in wound healing:
  - SOCS and cytokines:
    - Many evidence suggested that SOCS regulate cytokines which play critical roles in wound healing.
  - SOCS and growth factors/growth factor receptors:
    - Studies showed that SOCS modulate growth factors and their receptors which are involved in wound healing.
  - SOCS-2 and SOCS-6: negative regulators of SOCS protein activity:
    - SOCS-2 and SOCS-6: the ability to regulate SOCS, indicating that they may have dual effect on mediating wound healing process.
  - SOCS-related downstream signaling pathways:
    - SOCS protein was found to interact with key molecules in downstream signaling pathways of wound healing.

Conclusion & future perspective

- In spite of the limited studies on SOCS in wound healing, in-depth investigation on the SOCS-regulated cytokines and growth factors that are important in wound healing may help to understand the potential roles of SOCS on such complicated dynamic process.

gation so as to facilitate a greater understanding of this highly complex, yet fundamentally important, process.

The SOCS family of proteins holds great therapeutic potential given their regulatory roles in numerous key signaling pathways. While the importance of this family has been discussed in relation to wound healing here, the ability of this family to modulate a wide variety of growth factors and cytokine signaling pathways could potentially expand the significance of this family to other areas of regenerative medicine, such as tissue repair. Intense study, focusing specific SOCS relative strategies is needed to fully explore the significance of this important family of proteins.

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Review

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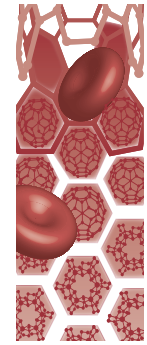

Nanomaterials for wound healing: scope and advancement

Innovative methods for treating impaired and hard-to-heal wounds are needed. Novel strategies are needed for faster healing by reducing infection, moisturizing the wound, stimulating the healing mechanisms, speeding up the wound closure and reducing scar formation. In the past few years, nanotechnology has been constantly revolutionizing the treatment and management of wound care, by offering novel solutions which include but are not limited to: state-of-the-art materials, so called 'smart' biomaterials and theranostic nanoparticles. Nanotechnology-based therapy has recently announced itself as a possible next-generation therapy that is able to advance wound healing to cure chronic wounds. In this communication, the recent progress in advanced therapy for cutaneous wound healing during last 5 years using a nanotechnology-based approach is summarized.

Keywords: nanomaterials (polymer, carbon-based, lipid-contained, ceramic, metallic, metal oxides) • scaffolds with embedded nanomaterials • wound-healing therapy

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Wound healing is a process of repairing damaged tissue, and restoring its integrity. This process involves a series of biochemical and physiological events which distinguishes four distinct, sometimes overlapping phases. The phases are driven by a list of bioactive molecules and mediators (specific for every phase) [1,2]: hemostasis, inflammation, proliferation (proliferation, granulation and contraction) and remodeling. Hemostasis includes vasoconstriction (to limit bleeding, activation of coagulation), and complement cascades (platelet activation, adhesion and aggregation, and clot formation). Inflammation is a defense mechanism in which the wound is cleaned and rebuilding begins through vasodilation and macrophage activation. Slowing in this stage makes inflammation persistent and results in impaired wound healing. Epithelialization, angiogenesis, granulation tissue formation and provisional matrix deposition are the principal steps in the proliferative phase of wound healing. Neovascularization of the wound is a crucial part of the normal healing process so new blood vessels can supply injured tissues with oxygen, nutrients and essential growth factors. Angiogenesis and vasculogenesis are processes of new blood vessel formation either from pre-existing vessesls or by *de novo* generation of endothelial cells. The main feature of remodeling is the deposition of extracellular matrix (ECM) in an organized and well-mannered network, myofibrolasts formation and then contraction of wound [3]. Any alteration and complications during the wound-healing process may lead to a chronic ulcer that fails to heal. In recent years, approximately 84% (number one cause) of diabetic patients were hospitalized due to lower extremity foot ulcers [4]. Many factors can impair wound healing. First, lifestyle (alcoholism and smoking, among others) and age of the subjects have a huge influence on the rate of wound closing (10–14 days). Furthermore, health conditions of patient, such as high cholesterol level, diabetes, peripheral arterial disease, Ehlers–Danlos syndrome, Cutis Laxa, hypothyroidism, homocystinuria and advanced stage of the diseases are

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other group of factors that can delay wound healing [5]. Recent advancement in medicine, pharmaceutical sciences, bioengineering chemistry, nanotechnology sciences has brought several new strategies that minimize any complications associated with wound healing and improve the wound management.

Nowadays, a wide choice of therapy is provided by conventional and modern approaches to wound treatments. Conventional therapy implies debridement and dressing change. Various types of dressing are used for wound healing. The types of dressings can be categorized in three types: traditional dressings, biomaterial-based dressings and artificial dressings [6]. A gauze and gauze/cotton composites with a nonadhesive or an adhesive inner surface, can be considered an example of **t**raditional dressing. Biomaterial-based dressing can mainly be classified as allografts, tissue derivatives and xenografts. Fresh or freeze-dried skin fragments, like scalp tissues or amniotic membrane taken from the patient's relatives or cadavers, represents the most common allograft dressing. These simple examples of tissue derivates and xenografts are dressings derived from different forms of collagen and from pig skin, respectively. Artificial dressings can be in the form of film, membrane, foam, gel, composite and spray. Many are from natural sources but are used in the preparation of artificial dressing (collagen, cellulose, alginate substitutes, fibrin, chitosan, hyaluronic acid, carboxymethylcellulose, collagen, gelatin, polyurethanes and new-age biopolymers) [6–10]. Some biocompatible and biodegradable synthetic polymers (polyglycolic acid, polylactic acid, polyacrylic acid, polycaprolactone, polyvinylpyrrolidone, polyvinyl alcohol, polyethylene glycol) with excellent mechanical properties are also reported to enhance the wound closure, by enhancing re-epithelialization, inducing cell proliferation, migration and differentiation using *in vitro* and *in vivo* wound-healing model [8].

There are commercially created skin substitutes reported for the treatment of skin wounds and injuries [10]. Some of the commercially available wound-healing dressings have been mentioned in a review written by Boateng *et al.* [11]. Wound dressings with capabilities to manage bacterial infection with systemic antibiotic loads are commercially available [8,12]. Advanced therapy includes: tissue-engineered wound beds (decellularized tissue scaffolds, hydrogels, bilayered skin substitutes and fibroblast-seeded or fibroblasts/keratinocyte-seeded scaffold) [10,13,14], proteases, drugs, stem cells (endothelial progenitor and mesenchymal stem cells) [12,14–17], genes [15,18,19] and growth factors therapy (EGF, KGF, TGF1α/β transforming growth factors [TGFs], VEGF, PDGF) [10,13,18] or combination of stem cells/growth factor or gene therapy [15,16]. Other type of advanced wound care strategies are [1,13]: hyperbaric oxygen therapy, vacuum-compression therapy, negative pressure wound therapy, ultrasound noncontact wound-healing device (MIST), electrostimulation, electromagnetic therapy, hydrotherapy, lasers and light-emitting diodes [1,20].

Wound-healing therapy based on utilization of nanomaterials (NMs) has demonstrated new promises and benefits in this field. A variety of nanotechnology platforms have been developed such as fullerenes, nanotubes, quantum dots, nanopores, dendrimers, liposomes, metallic, ceramic and magnetic, nanoemulsions and polymer nanoparticles [21]. Nanomedicine has extensively proven to have excellent potential by utilizing nanoscale objects for drug and gene delivery, biosensors, advanced imaging, tissue regeneration, diagnostics, cancer and other disease treatments [2,22–23]. Unique properties of NMs and their biomimetic advantages and engineering applications for bone, cartilage, vascular, neural and bladder tissue are overviewed in this literature [2,24–26].

NM-based wound-healing therapy

Currently, it is possible to distinguish two types of NMs for wound-healing therapy. NMs which are able to heal due to the features of nano-scaled material or NMs as a cargo for delivering therapeutic agents [2]. Figure 1 represents an analysis of different NM-based treatments in literature for cutaneous wound healing during the last 5 years. In this review, nanotechnology-based products used in wound healing were divided into five groups according to NM composition: polymer, carbon-based, lipids-contained, ceramic, metal and metal oxide nanoparticles and different types of scaffolds with embedded NMs. All types of NMs used for wound care are shown in Figure 2. This figure also outlines the beneficial effects of different NMs on the phase/s of wound healing. Table 1 summarizes the different NMs which have been investigated for management of wound care. It is interesting to mention that there are differently established *in vitro* and *in vivo* would healing models which have been practiced in various studies. These model systems are vital when comparing the healing efficacy of the NM as the size and number of wounds in an animal can influence the outcome. Figure 3 presents whole spectra of *in vitro* and *in vivo* wound model systems exposed to NM treatments, incision types and methods of NM administration.

The effects of different NMs and potency to wound healing are dissimilar, and largely depend on NM physicochemical properties. Some key properties of NMs which can influence the outcome for wound healing are: biomaterial property, size, colloidal sta-

bility, surface functionalization and surface charge. Ability to biodegrade and biocompatibility of NMs has additional advantages over particles which cannot be digested and deposited in body. Other than the physiochemical property, presence of a payload (active ingredient) with NMs having wound-healing activity is definitely beneficial.

Commonly, size of NM defines a mechanism of internalization of NM in the cell. Small size (3–15 nm) provides a passive diffusion pathway for NMs uptake by cells. Transferring cargo directly to the cytoplasm can guarantee safe delivery of its payload to cell. Stability of NMs in the vehicle (pure water, PBs or others) plays an important role for administration of the NMs (injection, IV and topical, among others). Although, NMs with size range 100–200 nm have been used more often than NMs with other dimensions for wound care applications (Table 1). The stability of NMs in the extracellular fluid will prevent aggregation that may help avoid NM cytotoxicity. Impurities (solvent used during the synthesis, stabilizer and/or impurities in the precursor) in NMs also can affect the cell–NMs interaction and affect the outcome. Next, the amount and type of functional group/s on the NM surface can provide bonuses for loading active ingredients and also contribute/s to the surface charge of the material. Last, morphology and shape of the NMs are known to impact the cell nanoparticles interaction and internalization as well. Other than the key features discussed above, crystallinity and shape of the NMs can also play a crucial role in acceleration of wound closure. NM with optimized parameters in a combination with medications or other payload can bring desirable outcome for treatment of complicated wounds (hard-to-heal and ischemic wounds).

The cellular and molecular mechanisms of wound healing are well described in the literature [63,64]. However, to the best of our knowledge only one manuscript identified the mechanism of NMs action or pathway studied the application of NMs in the area of wound healing [65]. Several signaling pathways that can be related to wound-healing process are discussed in [27–30,66,67]: JNK, EMR, WnT, PI3k/AkT/mTOR, TGF-β. The short list of the most common protein/gene regulation below can be suggested for an evaluation during wound healing to outline the mechanism of action: reactive oxygen species (ROS), growth factors (FGF, EGF, SDF-1α, KGF, VEGF, PDGF), NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells), TGF-β/α, TNF, IL (-1, -6, -8 and -10), PGE_2, enzymes (matrix metalloproteinases, myeloperoxidase (MPO), glutathione peroxidase (GPO), protein molecules related to attachment or remodeling of the matrix (angiopoietin-1, L-selectin and intercellular adhesion molecule-1, tissue inhibitors of metalloproteinase, angiogenin, collagen type I and III, fibrinogen) [3].

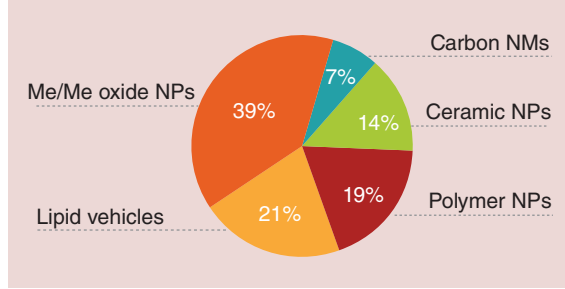

Figure 1. Number of publications regarding wound-healing therapy using different nanomaterials. This figure reveals that highest number of publications explored with metal/metal oxide NMs and the least number of publications tested carbon NMs for their efficacy in wound care. Lipid vehicles and polymer NPs have also been studied for different applications in wound repair.
NM: Nanomaterial; NP: Nanoparticle.

Polymer NM therapy

Nanoparticles used for wound-healing applications are often based on polymer materials that have been used as dressings [8,10] or have successfully proven themselves for drug delivery, bioimaging and biosensing assays applications [31]. For example, poly (lactide-co-glycolide) (PLGA), polycaprolactone (PCL) and PEG are three synthetic polymers that have been used to engineer biomaterials for wound care applications. PLGA-curcumin nanoparticles showed twofold increase in wound-healing capability compared with that of PLGA or curcumin. Curcumin has shown its abilities for anti-inflammatory, antioxidant and anti-infective properties [68,69]. PLGA nanoparticles loaded with curcumin quenched ROS, inhibited MPO, down-regulated the expression of anti-oxidative enzymes like glutathione peroxidase and NF-κβ that minimized the inflammatory responses, expedited re-epithelialization and improved granulation tissue formation [70]. It is important to note that the illustration provided in this study [70] explains the phases of wound healing regulated by the NMs. A different study [71] used PLGA nanoparticles to deliver recombinant human EGF(rhEGF) in order to enhance full-thickness diabetic wound closure. Nanoparticles showed a sustained release of rhEGF for 24 h. Sustained release of rhEGF over time improved total healing effect via mouse fibroblast proliferation in *in vivo* model. A similar study with PCL nanoparticles loaded with enoxaparin capped with chitosan were investigated for wound-healing application where particles were applied topically using the gel ENOXA [32]. Such treatment displayed improved wound healing in diabetic animals due to drug stability, and good skin penetration without any toxic

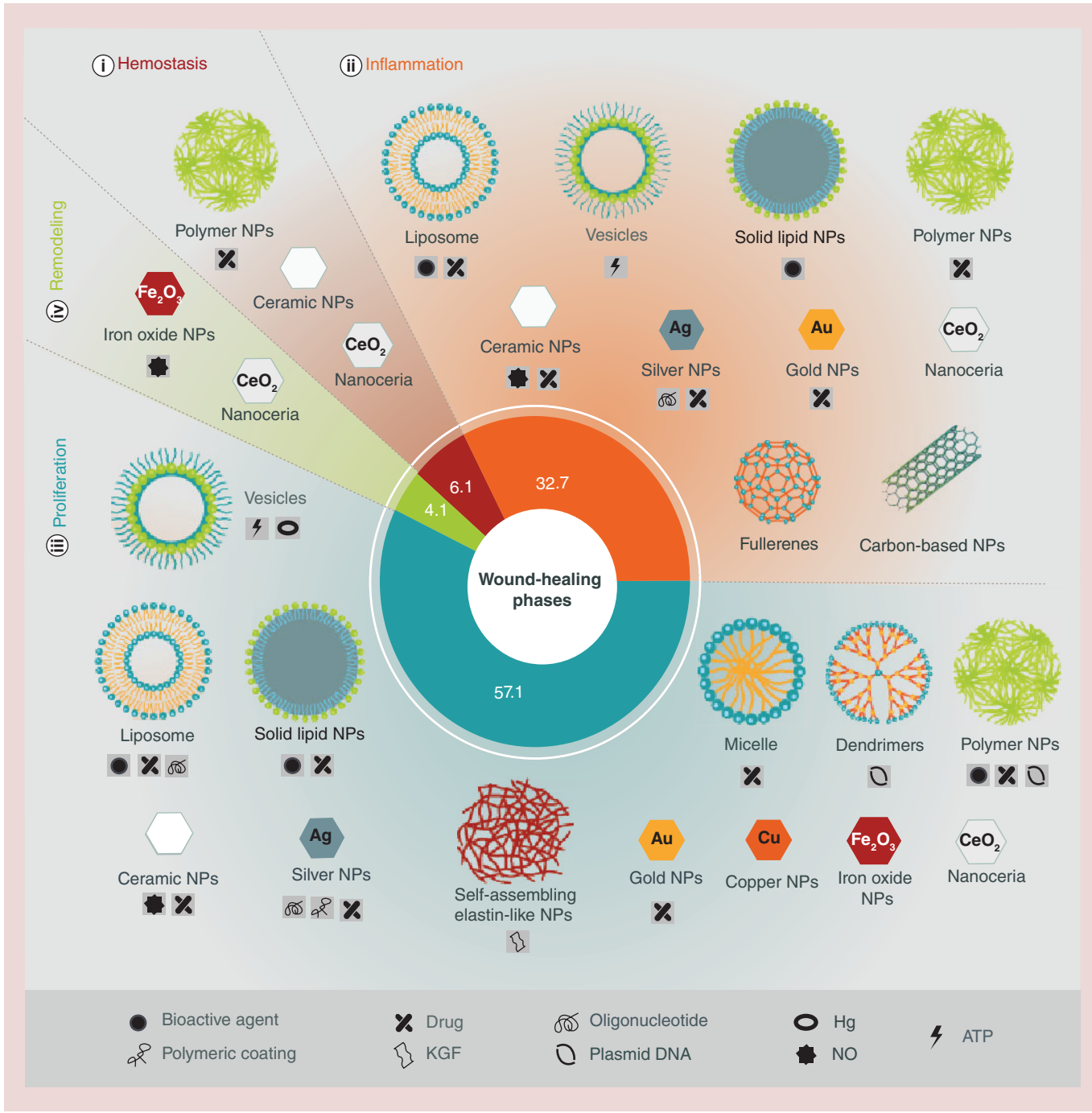

Figure 2. Various nanomaterials showed beneficial effect in the different phases of wound healing. This figure divides nanomaterials into four groups based on which phase of wound healing they effected/corrected. Most of the nanomaterials have shown an enhanced wound healing by accelerating the proliferation (phase III) then inflammation (phase II) whereas very few nanomaterials are known for help in hemostasis and remodeling. Interestingly, cerium oxide NPs have displayed the beneficial effects in all four phases of wound healing.
NP: Nanoparticle.

effect. The study of hypericin (Hy)-loaded PCL-PEG nanoparticles on infected *in vivo* cutaneous wounds showed minimum expression of TNF. Modulations of signal molecules production brought better epithelialization and collagen deposition [33].

Other types of polymers, biodegradable poly(b-amino esters) (PBAEs) and copolymers of maleic acid were also widely investigated for gene delivery and drug release systems for wound-healing applications [72,73]. Intradermal administration of the PBAEs

nanoparticles in a murine wound model was tested in a recent study [74]. Sonic hedgehog gene (SHH) loaded into the nanoparticles showed facilitated angiogenesis and tissue regeneration by activating angiogenic signaling pathways. Expressions of angiogenic factors (two isoforms of VEGF and some chemokines such as SDF-1α were especially high in skin tissue samples at early time points of wound healing. The amine end-modified PBAEs did not show cytotoxic effects *in vitro* and was an effective vehicle to deliver SHH *in vivo* [74]. PBAEs NMs, pH sensitive system, correct pH at wound site that enhance antibacterial defense.

Chitosan which is mentioned above is another naturally occurring polysaccharide that has been extensively researched for biomedical applications due to its biocompatibility, biodegradability, mucoadhesivity and anti-infection activity [34]. Chitosan nanoparticles were shown to have significant bactericidal effects on many different types of bacteria and no cytotoxic effect on mouse fibroblast cells [35]. In this study, chitosan particles were reported to modulate the inflammatory response in human gingival fibroblasts. IL-1β-stimulated prostaglandine E2 (PGE_2) production was inhibited by chitosan through the JNK (c-Jun N-terminal kinases) pathway [65].

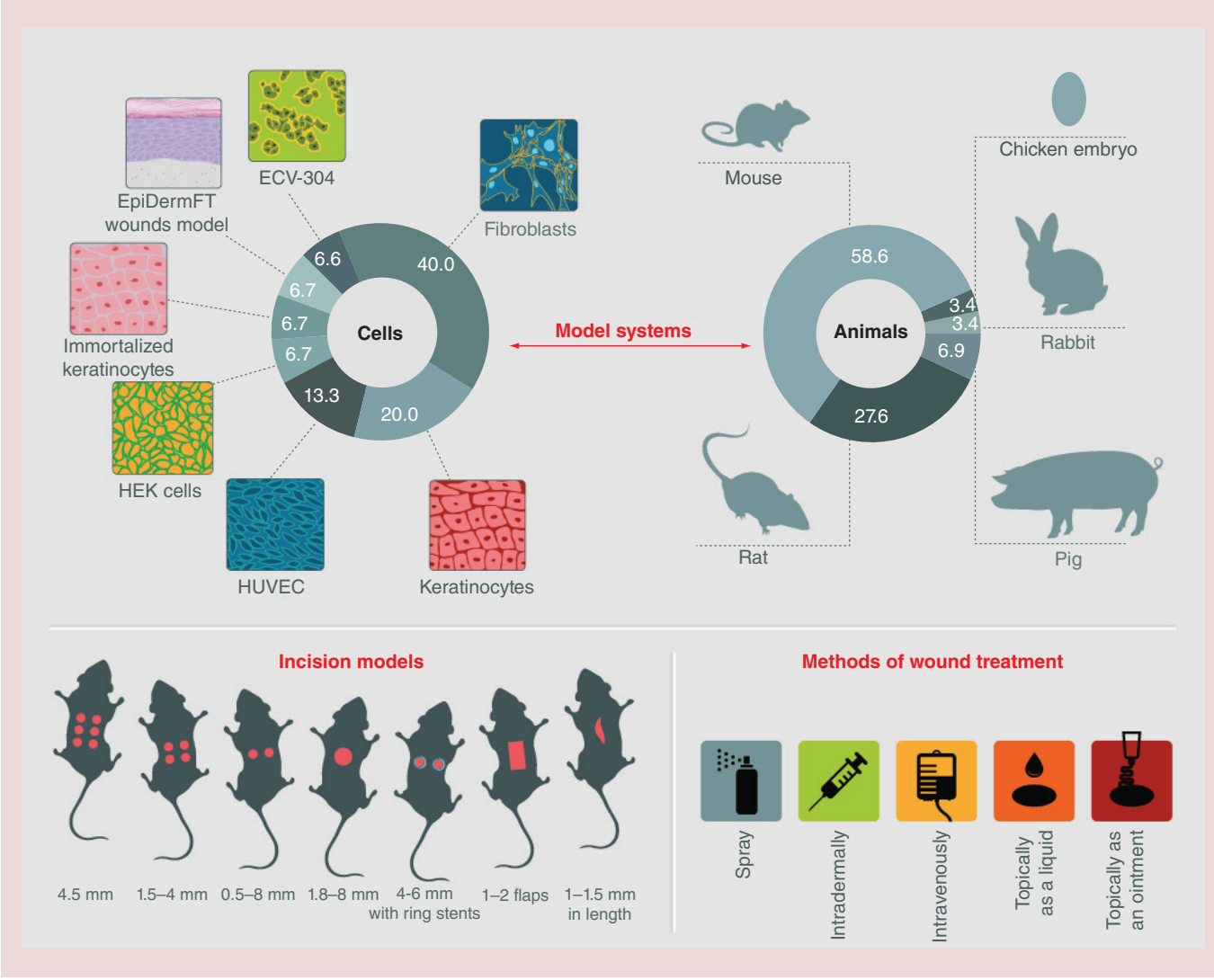

Figure 3. Wound model systems, incision models and methods of wound treatment. Model systems used for *in vitro* and *in vivo* studies to explore the effects of nanomaterials on the wound-healing process are infographically presented. EpiDermFT (*in vitro* model) is the epidermal full-thickness model, containing epidermis and dermis and represents close equivalent of skin but this model is expensive and time consuming to prepare. Therefore, fibroblasts and keratinocytes isolated from mice or humans are very popular as *in vitro* models. Mouse and rat models prevail among other animal models as mice and rats are readily available, easy to handle and are cost effective. In this figure, we have also listed different incision models and administration methods used in literature for wound-healing applications.

Table 1. Different nanomaterial samples studied for wound-healing application.

NM composition	NM characteristics (size, PdI, Z pot, EE)	Treatment parameters	Model systems	Benefits	Ref.
Curcumin-PLGA	177 nm, 0.105, -23 mV, 89%	33 μg/μl, intradermally	6–7-week-old RjHan:NMRI female mice	Anti-inflammatory activity, improved re-epithelialization and granulation tissue formation	[27]
rhEGF-PLGA	194 nm, 0.18, 86%	1 μg rhEGF, spray	8-week-old male Sprague–Dawley rats	Stimulated fibroblasts proliferation, better tissue repair at intermediate and advanced stages	[28]
Enoxaparin-PCL-chitosan	496 nm, 20 mV, 98%	2 mg/g gel ENOXA, topically	6–8-week-old male Wistar rats	Improved wound healing via correction of hemostasis and inflammation	[29]
Hy-PCL-PEG	50 nm, -9 mV, 75%	50 μl of 0.124 μM (HY) topically	Female Wistar rats	Downregulated TNF expression, upregulated VEGF expression, better epithelialization, keratinization and development of collagen fibers	[30]
SHH-PBAEs	238 nm, 21.3 mV	15.5 μg/μl, intradermally	6-week-old female athymic mice	Promoted angiogenesis and tissue regeneration	[31]
KGF–ELP fusion protein	500 nm, 0.04	7.5 μg/μl fibrin gel contained 0.036 nM NPs	Genetically diabetic male B6.BKS(D) -*Leprdb*/J mice	Induced granulation, enhanced re-epithelialization, prevented scar formation	[32]
PAM-RG4/minicircle VEGF165 DNA complexes	115 nm, 23 mV	20 μg subcutaneously at bilateral sites	8-week-old male C57BL/6J mice	Diabetic wounds closure at rate similar to normal ones by rapid collagen deposition, well-organized dermal pattern and higher extent of mature blood vessel formation	[33]
C_{60} modified with hexa-dicarboxyl, tris-dicarboxyl and gamma (γ)-cyclodextrin	20 nm–1 um for CD-, hexa-C_{60} and 100–160 nm for tris-C_{60}	25–100 μg/ml in medium for 4–24 h	HEK cells	Altered cytokines (IL-1, -6, -8, TNF-α) response and their release kinetics	[34]
MWCNs, GO, RGO	50–60 nm COOH-, 60–90 nm NH2-MWNT, -24 mV, +27 mV; 172 nm, 0.179, -56.5 mV	24–48 h, 5–100 μg/ml	NIH-3T3 and primary human dermal fibroblasts	Dose-dependent viability and wound closure	[35,36]
Clarithromycin-Ch-fatty acids micelles	250–300 nm, <0.5, 39–44%	0.05–0.5 mg/ml	Normal human dermal fibroblasts	Induced cell proliferation	[37]
Gll-Phl-Chol-α-gal liposomes	Submicromic size, 100 mg/ml	10–100 mg NPs on the pad of a dressing	3-month-old knockout pigs for the 1,3GT gene	Wound closure acceleration via rapid recruitment of macrophages, effective cytokine production; extensive angiogenesis, advanced collagen deposition and granulation	[38,39]

db/db: Diabetes mice; ECM: Extracellular matrix; EE: Encapsulation efficiency; EGCG: Epigallocatechin gallate; GO: Graphene oxide; HY: Hypericin; ICR: Institute for Cancer Research ; MWCN: Multiwalled carbon nanotube; N/A: Not available; NM: Nanomaterial; NMRI: Naval Medical Research Institute; NO: Nitric oxide; PdI: Polydispersity index; Phl: Phospholipids; RGO: Reduced graphene oxide; ROS: Reactive oxygen species; TMSO: Tetramethylorthosilicate; TNF: Tumor necrosis factor; Z pot: Zeta potential; model systems include cellular *in vitro* systems used for cytotoxicity tests.

Table 1. Different nanomaterial samples studied for wound-healing application (cont.).

NM composition	NM characteristics (size, PdI, Z pot, EE)	Treatment parameters	Model systems	Benefits	Ref.
Lipoid–PEG/Ocdp–curcumin and quercetin liposomes	112–220 nm, -9–13 mV, 0.23–0.37, 56–71%	20 μl for 3–6 h, topically daily for 3 days	New born pig skin using Franz diffusion cells; 5–6-week-old female Hsd:ICR(CD-1) mice	Inhibited detrimental activity of ROS; MPO accumulation and leukocyte infiltration; reduced edema formation; increased fibroblasts proliferation and production of collagen and elastin	[40]
Phospholidip-PEG-Hb vesicles	250 nm, 35 g Hb/dl	10 g/dl in 0.9% NaCl	DDY mice	Improved healing via enhanced oxygenation, angiogenesis and vasculogenesis	[41]
ATP-phospholipid-DOTAP, trehalose vesicles	120–160 nm	N/A	8–10-week-old New Zealand white rabbits	Rapid wound healing by correction of immune response, speeding up granulation and re-epithelialization	[42]
Liposomes-encapsulated Hb	250 nm	2 ml (20% in saline)/kg by tale's intravenous infusion at 2, 4 and 5 days	8-week-old Balb/c mice	Collagen synthesis	[43]
Solid lipids-morphine-poloxamer 188 NPs	185–200 nm, 0.25, 100 ± 20%	50 μl of 125–250 μg/ml	EpiDermFT wounds models-human full-thickness skin equivalents	Promoted re-epithelialization	[44]
rhEGF-Poloxamer-Tween-80-Precirol ATO 5-Mygliol (SLN-rhEGF and NLC-rhEGF)	332–357nm, ≈0.32, -33–-35mV, 74–96%	10–20 μg in 20 μl of vehicle (0.5% w/v carboxymethylcellulose in 0.9% w/v saline) administered topically twice a week	Balb/C 3T3 A31 fibroblasts, human foreskin fibroblasts, human immortalized keratinocytes (HaCaT); 8-week-old male db/db and genetically diabetic db/db mice (BKS.Cg-$m^{+/+}$Leprdb/J)	Speeded up wound healing via restoration of inflammation and re-epithelialization	[45]
Chondroitin sulfate-silver and acharan sulfate-silver	5.8–6.2 nm, -16.9 mV	100 mg ointment daily	6-week-old male ICR mice	Accelerated the deposition of granulation tissue and collagen	[46]
Silver and oligonucleotide ([5'-HS-(CH2)6-TAATGCTGAAGG-3])-silver	50 nm	N/A	Adult male Balb/cmice	Improved the congestion, inflammatory cell infiltration, fibroblast proliferation and new collagen synthesis	[47]

db/db: Diabetes mice; ECM: Extracellular matrix; EE: Encapsulation efficiency; EGCG: Epigallocatechin gallate; GO: Graphene oxide; HY: Hypericin; ICR: Institute for Cancer Research ; MWCN: Multiwalled carbon nanotube; N/A: Not available; NM: Nanomaterial; NMRI: Naval Medical Research Institute; NO: Nitric oxide; PdI: Polydispersity index; Phl: Phospholipids; RGO: Reduced graphene oxide; ROS: Reactive oxygen species; TMSO: Tetramethylorthosilicate; TNF: Tumor necrosis factor; Z pot: Zeta potential; model systems include cellular *in vitro* systems used for cytotoxicity tests.

Table 1. Different nanomaterial samples studied for wound-healing application (cont.).

NM composition	NM characteristics (size, PdI, Z pot, EE)	Treatment parameters	Model systems	Benefits	Ref.
Bryonia laciniosa leaf extract-silver	15 nm, -32.3 mV	0.09 mg NPs in gellun gum gel daily for 14 days	Male Wistar rats, TE 353.Sk	Immunomodulation via cytokines (IL-6, -10) level lowering; scar reduction by keratinocytes migration, cell proliferation, ECM production, reduced inflammation	[48]
BSA-Thrombin-γ-Fe_2O_3	20 nm	70 mg/ml in mixture containing fibrinogen and a $CaCl_2$ solution	Male Wistar rats	Improvement of skin tensile strength adhesion, reducing stitch-induced scarring	[49]
'Metabolized' Fe_2O_3	20–40 nm	4 μl of 42.2 g/l in distilled water (pH 7–7.5)	Wistar rats	Accelerated wound closure with esthetic scar formation	[50]
Copper	33.8–119nm	0.2 g copper-methylcellulose-based ointment	Female mice of the SHK line	Regenerating activity	[51]
EGCG-alpha lipoic acid-gold	3–5 nm	0.07 mg NPs/g of ointment	Hs68 fibroblasts, 8-week-old male BALB/c mice	Regulated the angiogenic effects, modulated inflammation	[52]
EGCG-gold	N/A	Gas injection	Wild-type and streptozotocin-induced diabetic mice	Increased VEGF and collagen I and III protein expression	[53]
Chloroauric acid-gold	25–50 nm, -13.2 mV	5–20 μg/ml	HUVECs and ECV-304, chick embryo model	Proangiogenic activity: promoted cell migration, new blood vessel formation	[54]
Cerium oxide	160 nm	1–2% daily	8-week-old female Sprague–Dawley rats	Increased wound tensile strength amount of collagen and hydroxyproline production	[55]
	3–5 nm	0.5–10 μM, daily topically	Human keratinocyte cells, murine dermal fibroblasts, 3–4-month-old male C57BL/6 mice	Prevented infection, clearing debris, increased density of blood vessels, enhance the proliferation and migration of keratinocytes and fibroblasts	[56,57]
	3–5 nm, -14.1–17.8	0.1–1 μM	HUVECs	Modulated intracellular oxygen level, activated HIF-1a	[58]
$CaCl_2$-collagen-beta-glycerol-phosphate	50–200 nm	100 μl with electrical conductivity of 13 mS/cm, topically and intravenous injection	8-week-old female Balb/c mice	Decreased wound size via contracture by calcium release calcium in pH-dependent manner	[59]

db/db: Diabetes mice; ECM: Extracellular matrix; EE: Encapsulation efficiency; EGCG: Epigallocatechin gallate; GO: Graphene oxide; HY: Hypericin; ICR: Institute for Cancer Research ; MWCN: Multiwalled carbon nanotube; N/A: Not available; NM: Nanomaterial; NMRI: Naval Medical Research Institute; NO: Nitric oxide; PdI: Polydispersity index; Phl: Phospholipids; RGO: Reduced graphene oxide; ROS: Reactive oxygen species; TMSO: Tetramethylorthosilicate; TNF: Tumor necrosis factor; Z pot: Zeta potential; model systems include cellular *in vitro* systems used for cytotoxicity tests.

Table 1. Different nanomaterial samples studied for wound-healing application (cont.).

NM composition	NM characteristics (size, PdI, Z pot, EE)	Treatment parameters	Model systems	Benefits	Ref.
NO-TMSO-PEG-chitosan	N/A	5 mg, topically	Human dermal fibroblasts, 6–8-week-old female Balb/C and NOD.SCID/NCr mice	Stimulated migration and proliferation of fibroblasts, and collagen type III expression, increase vascularization, lessened inflammation, upregulated expression of genes associated with extracellular matrix formation and VEGF	[60,61]
Curcumin-TMSO-PEG-chitosan	222 nm, 81.5%	7.5 mg/ml in coconut oil, 50 μl topically daily for 7 days	Keratinocytes PAM212, zebrafish embryos, 6–8-week-old Balb/C mice	Antimicrobial effect; accelerated wound closure via well-formed granulation tissue, enhanced collagen deposition, new vessel formation and re-epithelialization	[62]
SiO_2	15–80 nm, 0.15	2–15 μl, 30–52% in distilled water (pH 8.5–9) by a brush or a micropipette on one or two edges of the wound	Wistar rat model	Bleeding control; tissue repair; esthetic healing	[50]
SiO_2, Na_2O, CaO and P_2O_5	30–60 nm, 1 μm	Ointment (18 wt% of bioactive glass powder + vaseline), topically	Specific pathogen-free and chemical-induced diabetic male Sprague–Dawley rats	Promoted the proliferation of fibroblasts and growth of granulation tissues, stimulated production of two growth factors, VEGF and FGF2	[62]

db/db: Diabetes mice; ECM: Extracellular matrix; EE: Encapsulation efficiency; EGCG: Epigallocatechin gallate; GO: Graphene oxide; HY: Hypericin; ICR: Institute for Cancer Research ; MWCN: Multiwalled carbon nanotube; N/A: Not available; NM: Nanomaterial; NMRI: Naval Medical Research Institute; NO: Nitric oxide; PdI: Polydispersity index; PhI: Phospholipids; RGO: Reduced graphene oxide; ROS: Reactive oxygen species; TMSO: Tetramethylorthosilicate; TNF: Tumor necrosis factor; Z pot: Zeta potential; model systems include cellular *in vitro* systems used for cytotoxicity tests.

The N-acetyl glucosamine from chitosan is a component of dermal tissue as well as elastin. Elastin is a protein that plays a role in structural, mechanical and cell signaling [36]. A recent study of synthesized chimeric nanoparticles formed via spontaneous self-assembling of elastin-like peptides (ELP) [37] has revealed its perspective for wound healing. ELP particles, KGF and ELP particles with KGF loaded (KGF–ELP) in fibrin gel and fibrin gel itself were tested and compared both *in vitro* and *in vivo* models. ELPs itself increased almost fivefold in fibroblast proliferation [37]. One-time administration of ELP-KGF particles improved re-epithelialization and granulation within wound sites of diabetic mice in comparison to the controls.

Dendrimers, highly branched polymers in particular polyamidoamine (PAMAM) have been successfully used for cellular delivery of plasmid DNA, forming a stable complex by limiting its degradation. For example, arginine-grafted cationic dendrimer was tested for wound healing in diabetic and normal mice as a vehicle to deliver minicircle plasmid DNA encoding VEGF. This polycomplex (PAM-RG4) provided rapid proliferating basal cells and collagen deposition. Furthermore, less immature blood vessel formation resulted in diabetic wound recovery comparable to wound healing for normal mice [38]. Dendrimers have structure with internal cavities and surface channels which are favorable for accommodation of small molecules. This unique feature of dendrimers to carry high amount of drugs can be used for treatment of hard-to-heal wounds required medicine administration. In addition to the delivery of plasmid, research was also conducted toward development of oligonucleotide delivery nanovehicles for wound-healing application. Sirnaomics, Inc. has been working on developing the effective formulation contained siRNA, targeting for TGFβ1 and Cox-2 (cyclooxygenase-2), encapsulated into HK polymer nanoparticles. The nanoparticles in methylcellulose formulations demonstrated pain reduction with antifungi activity, wound-healing acceleration and minimization of scar formation [33]. Other, noncoding RNAs known to regulate inflammation (146a), proliferation of cells (21, 15b, 222/221) and remodeling (29a) phases can be tested for their efficacy to heal wound faster [39–41,43].

Carbon-based NM therapy

Carbon NMs, including fullerenes, carbon nanohorns and carbon nanotubes and graphene have gained interest in nanomedicine due to their versatile uses in advanced imaging, tissue regeneration and drug or gene delivery [42,44] although their biocompatibility remained controversial [42]. Fullerenes and carbon nanotubes displayed positive results in wound healing via correcting inflammatory and proliferative phases. Since fullerenes are superpowerful antioxidants, they are capable of scavenging and detoxifying ROS and reactive nitrogen species [44]. Fullerenes can be functionalized to avoid aggregation, and alter their solubility and toxicity using hexa-dicarboxyl, tris-dicarboxyl and gamma (γ)-cyclodextrin (CD) [45]. Modified fullerenes were explored to study immune cell responses in keratinocytes. Pro-inflammatory cytokines (IL-1, -6 and -8) expression and release kinetics were analyzed. Cytokines TNF-β, GROβ (the IL-8-related chemotactic cytokine), and RANTES (CCL5/regulated on activation, normal T-cell expressed and secreted) level after exposure to different fullerene derivates which regulate keratinocyte proliferation, differentiation, migration, apoptosis and angiogenesis in cutaneous wound-healing models were also evaluated. The release of the cytokines was influenced by functionalization of the fullerene's surface [45]. For example, tris-C60 significantly reduced inflammatory cytokine secretions in a dose/time-dependent manner, whereas CD-C60 led to an increase in IL-6 and IL-8 secretions. The nature of fullerenes altered cell cycle as well as the proliferation capacity of cells [45]. The behavior aspects of other carbon materials such as graphene (GO, RGO) and carbon nanotubes were tested on murine fibroblasts [75,76]. The cells were exposed to a layer made of multiwalled carbon nanotubes (MWCNs), functionalized MWCNs, graphene oxide and reduced graphene oxide for 24–48 h. This was done to study cell viability, morphology, adhesion, spreading and proliferation patterns. No cytotoxic effects or difference in the cell's proliferation and adhesion were observed for various substrates. However, cells were spread broader on the substrates contained MWCNs. The substrates with a high water contact angle demonstrated less focal adhesions per cell, whereas gene transfection was better on the relatively less rough substrates [76].

Lipids contained NM (micelles, vesicles and liposomes) therapy

Chitosan fatty acids micelles were suggested as prospective carriers to deliver hydrophobic drugs in the curing of complicated wounds [77]. Clarithromycin-loaded (clarithromycin-Ch-fatty acids) micelles were fabricated via self-assembling of chitosan and mixture of oleic and linoleic acids. The micelles were characterized for their size and polydispersity, cytotoxicity on normal human dermal fibroblasts. Moreover, their mucoadhesive properties, and drug loading capacity were also investigated. In fact, the micelles displayed good biocompatibility, ability to induce cell proliferation, and about 20-times higher clarithromycin loading capacity compared with saturated solution in water [77].

A very interesting approach of accelerating wound closure was invented using liposomes [78,79]. Submicroscopic liposomes (Gll-Phl-Chol-α-gal) were prepared from mixtures of lipids, extracted from rabbit red blood cell membranes and loaded with α-gal epitopes. Carbohydrate antigen, α-gal epitope (Galα1–3Galβ1–4GlcNAc-R), binds to the anti-Gal antibody within a wound and activates the cascade of the compliments, which cleave chemotactic factors, C5a and C3a. Local increasing concentrations of chemotactic factors in a wound site induced rapid recruitment of macrophages followed by their migration into the wound site [78,79]. Next, α-Gal nanoparticles were bound to the receptors on the membranes of infiltrated macrophages and activated the secretion of prohealing cytokines that accelerated healing of the wound. Wounds in pigs that were treated topically with nanoparticles by thin fluidic film showed accelerated rate of wound closure by more than 25% [78].

Liposomes (lipoid-PEG/Ocdp-curcumin and quercetin liposomes) are a mixture of soybean phospholipids, triglycerides, fatty acids and contained PEG and/or octyl-decyl polyglucoside and loaded with curcumin and quercetin were studied to treat *in vitro* and *in vivo* models of full-thickness skin defects [46]. The used phytodrugs possessing antioxidant and anti-inflammatory properties were able to prevent skin ulceration and enhance early regeneration of wounds. Several bionanovesicular formulations facilitate the drugs delivery and allowed effective modulation in inflammation by neutralizing detrimental activity of ROS, preventing MPO accumulation and leukocyte infiltration. In another study, hemoglobin-loaded phospholipid bilayer vesicles coated with polyethylene glycol (HbVs) enhanced the oxygenation of ischemic cutaneous wounds [47,48]. At the first postsurgery day, oxygen saturation reached 15% higher level in ischemic wounds after treating wound with HbVs. Tissue survival was improved by 24% and wound-healing rate was accelerated twofold on the 6th postoperation day. The immunohistochemical assessments revealed higher capillary density and higher endothelial nitric oxide synthase 3 (eNOS) expression in the wounds exposed to HbVs. Another study compared hemoglobin (Hb) encapsulated nanocarrier with red blood cells for cutaneous wounds healing [47]. The Hb-liposomes prevented ischemic wound formation by suppressing inflammation and accelerating granulation. However, there were no supporting data for new blood vessel regeneration with treatment of liposomes in this study [48]. Skin defects were significantly (30%) reduced under the Hb-liposomes infusion via rapid fibroblast proliferation and collagen deposition [47].

In another interesting approach, adenosine triphosphate (ATP)-vesicles were synthesized and tested for wound-healing application where vesicles were applied using nonionic cream. ATP-vesicles were constructed from phospholipids, DOTAP (liposomal transfection reagent) and trehalose. They were mixed with a nonionic vanishing cream (Dermovan) for application. As expected, ATP-vesicle was shown to provide an energy source for cell survival, enhanced granulation and re-epithelialization in rabbit's diabetic wounds, for both nonischemic and ischemic models [80]. These vesicles were able to correct immune cells response which is delayed in diabetic wounds by stimulating the infiltration of neutrophils, lymphocytes and macrophages at early stage of postwounding. The significant acceleration of wound-healing rate was observed in comparison to the controls (saline solution, empty vesicles, Mg-ATP, cream) for nonischemic (16.5%) and ischemic wounds (20.7%). These results indicate that transfection agent can be also used for efficient delivery of other molecules having wound-healing capabilities such as drugs, oligonucleotides and microRNA, among others.

As mentioned earlier, lipid-based nanoparticles were able to promote re-epithelialization as well as solid lipid NPs (SLNPs). For example, SLNPs with encapsulated morphine, were reported to control pain and accelerated keratinocyte migration, proliferation and differentiation [49]. Two other formulations, SLNPs and nanostructured lipid carries (NLCs) were loaded with rhEGF which demonstrated effectiveness in enhanced wound closure at topical administration to diabetic mice. RhEGFs released from the nanoparticles during 72 h of treatment were bioactive and significantly induced cell proliferation *in vitro* (two types of fibroblasts and keratinocytes) compared with control nanoparticles. Similar cellular uptake and subcellular distribution were detected for both formulations. A reduction in polymorphonuclear leukocytes, domination of damaged tissues regeneration and formation of new connective tissue and blood vessels were exhibited by the nanoformulations. Wounds treated with rhEGF (free or encapsulated) revealed approximately 13% faster wound healing than the control groups (empty nanoparticles or vehicle) with new epithelium covering more than 50% of the wound [50]. The approach of delivering growth factor locally to the wound site using lipid particles works very well as it was discussed above in terms of polymer nanoparticles.

Metallic & metal oxide nanoparticle therapy

Different types of metal or metal oxide nanopartilcles were analyzed for their wound-healing application, silver nanoparticles is one of them. Silver nanoparticles (AgNPs) as well as silver have proven to exhibit antimicrobial activity against a broad range of microbes including fungi, different types of bacteria, yeast and even

viruses [51], reducing or preventing wound infection. The antibacterial activity of AgNPs showed correlations with physical properties such as shape and size of nanoparticles [81]. For example, trihedral-shaped particles showed higher antimicrobial activity than spherical and rod-shaped particles [81]. Silver has anti-inflammatory properties [82] and minimize ROS production depending on size and concentration of nanoparticles [83]. AgNPs were also reported to improve tensile properties of repaired skin by influencing collagen alignment [52]. Silver nanoparticles modified with chondroitin sulfate and acharan sulfate were recently demonstrated to stimulate wound recovery, and accelerate collagen deposition and new tissue formation in the wound area [54]. A recent study compared bare silver nanoparticles and nanoparticles coated with oligonucleotide ([5′-HS-(CH2)6-TAATGCTGAAGG-3]) (20–200 nm) in a full-thickness skin wounds model of 4.5-mm diameter [53]. The idea of nanoparticles surface modification was to improve the biocompatibility of nanoparticles by delaying the release of silver ions and minimizing their toxic effect on tissues. Surface functionalized nanoparticles regulated inflammatory response and enhanced the proliferative phase of wound healing via rapid fibroblasts and collagen deposition [53]. Green synthesis of AgNPs using Bryonia laciniosa leaf extract improved the nanoparticles cytocompatibility and efficacy toward wound healing in comparison with marketed cream with silver sulfadiazine [84]. Results showed an acceleration of rapid wound epithelialization and wound closure. Interestingly, scarless healing was reported by modulating pro-inflammatory cytokines (IL-6 and IL-10).

Other than silver, iron oxide was also explored for wound-healing applications [55]. Thrombin-conjugated γ-Fe_2O_3 nanoparticles accelerated the healing of incisional wounds significantly via relative improvement of skin tensile strength and reducing stitch-induced scarring [56]. 'Metabolizable' iron oxide nanoparticles purchased from Alfa Aeser and stabilized by citric acid, peptized with NH_4OH were also tested as a new adhesive/suturing material for dorsal skin wound repair for the organs which provided fast hemostasis without bleeding syndrome and inflammation [57]. Recently, copper nanoparticles have been shown to induce pro-inflammatory mediators that increase microvessel formation [58]. In this study, copper nanoparticles with methylcellulose-based ointment were applied to a 60 mm^2 full-thickness wound of mice skin [85]. The authors have also investigated the effect of nanoparticle size and crystalline copper content on wound healing. The fastest primary adhesion was found for nanoparticles with the biggest sizes (100–119 nm), whereas the highest regenerating activity (51.7%) was detected for nanoparticles (119 nm) with the lowest crystalline copper content (0.5%). Two other samples containing 96 and 94% crystalline copper also accelerated wound healing by 44.8 and 37.9%, respectively.

Application of gold nanoparticles (AuNPs) in the field of diagnostic and imaging tool, antitumor agent, drug delivery system, photothermal and photodynamic therapies was explored [59,86]. It is interesting to mention that gold nanoparticles, in combination with other drugs or simply alone have also been tested for wound-healing capabilities. The combination of AuNP and antioxidants epigallocatechin gallate (EGCG) and alpha lipoic acid were able to modulate two phases of wound healing: inflammatory and angiogenesis. These occur at the molecular level in wounds of diabetic mice. The proangiogenic properties *in vitro* and *in vivo* wound models were revealed for biosynthesized gold nanoparticles (b-Au-HP) [87]. Antioxidant activity of AuNPs occurs by controlling ROS generations (hydrogen peroxide and superoxide anion) [60]. Exposure of endothelial cells (HUVECs, ECV-304) to b-Au-HP resulted in induced cell proliferation with increased mitosis in a time-dependent manner. Moreover, treatment of the chick embryo model with AuNPs promoted new blood vessel formation in a dose-dependent fashion and time-dependent migration of HUVECs cells into the wound area. Another group [61] used gas-injection by the GNT GoldMed™ Liquid Drug Delivery System to treat diabetic wounds with the mixture of EGCG and AuNPs. Such formulation increased VEGF and collagen I and III protein expression in the wound area while speeding the rate of murine wound healing.

Metals from lanthanide group and its oxide or hydroxide revealed their regenerative potential. OmegaGenesis attained improved angiogenesis using europium hydroxide nanorods [62] to aid in the healing of a variety of wound types. The liquid suspension of nanorods was formulated using a cream or gel to enhance healing. The nanorods suspension can be utilized in combination with pain relief and anti-infective to enhance the wound-healing process. Another rare earth oxide nanoparticle, cerium oxide nanoparticle (160 nm) was reported to improve wound healing activity in *in vivo* model [88] by inducing an amount of hydroxylproline content and collagen production, which increased wound tensile strength and reduced wound closure time. Other groups [89,90] investigated nanoceria's (cerium oxide nanoparticles) effect on wounds using 10 μM nanoceria suspension with particles of size <20 nm. Nanoceria showed an increase in speed of cutaneous wound healing in mice models. The migration of major skin forming cells was enhanced in a concentration-dependent manner [89–91]. A rapid infiltration of inflammatory cells into the wound area and significantly greater density of blood vessels were

reported for nanoceria-treated mice, as compared with the control [90]. Moreover, it has been shown that nanoceria are able to induce proangiogenesis by stabilizing HIF-1a expression and altering gene regulation via modulation of intracellular oxygen level [91].

As mentioned, reports on metal/metal oxide nanoparticles have revealed potential in the field of wound care, including reduction/prevention of infection, induced migration of cells needed for angiogenesis or faster healing and increase in the strength of the healed skin. However, the use of metal/metal oxide nanoparticles in medicine brings concerns about the safety aspect of the metal/metal oxide nanoparticles to environment and living systems which are not clear. A recent publication warns about topical treatment of burn and wounds, and reported that toxicity has correlation with size of the AgNPs [92]. Viability of normal human dermal fibroblasts had strong correlation with size of the nanoparticles. Small AgNPs (4.7 nm) were much more cytotoxic than large NMs (42 nm). Moreover, small nanoparticles induced oxidative stress and depleted glutathione level [92]. Toxicity of different nanoparticles including titanium dioxide, zinc oxide, magnesium oxide, silver and gold was tested on mice skin cells in comparison with triglyceride-coated nanoparticles [86,93]. Coating increased the nanoparticle's biocompatibility, cell metabolic activity, ATP level and decreased ROS generation. Interestingly, it is reported that metal nanoparticles were more toxic than metal oxide nanoparticles [86]. In addition to cytotoxicity, biodistribution and rate of NMs clearance should also be evaluated.

Ceramic NM therapy

There are several examples of the use of ceramic material contained silica, and its derivates, calcium salts and hydroxyapatite in the form of nanoparticles for wound healing. Collagen-coated calcium-based nanoparticles that were synthesized using $CaCl_2$ with beta-glycerol-phosphate had the potential to modulate calcium homeostasis and the pH of milieu which accelerates skin wound healing [94]. It is proven from literature, that even a small change in pH may promote or inhibit bacteria growth, and enzymes prevalence, and alter the oxygen supply [95]. Nevertheless, the authors concluded that decreasing the open wound size occurred via contracture [94].

Nitric oxide (NO)-releasing nanoparticles that were synthesized using a mixture of tetramethyl orthosilicate (TMOS), polyethylene glycol, chitosan, glucose and sodium nitrite. These nanoformulations promoted angiogenesis and enhanced vascularization. NO stimulated migration and proliferation of fibroblasts, and collagen type III expression in the affected area that increased the rate and degree of granulation [96]. In another study, these NMs showed enhanced vascularization, well-organized tissue deposition, reduced inflammation and improved wound closure [97]. Similarly, TMSO-based nanosuspension with curcumin encapsulation, demonstrated antimicrobial effects toward gram-negative pathogens and an accelerated wound-healing process [98]. There were four appropriate controls used in this study: curcumin, bare nanoparticles, vehicle and silver sulfadiazine. There were delays in wound healing observed for each control. Significant impacts of curcumin-TMSO NMs on wound closures were observed via well-organized granulation, enhanced collagen deposition and maturity and increased neovascularization within the wound site. It has been reported that NMs accelerated wound closure by about 34% in comparison to the control [98].

Silica nanoparticles (SiO_2 NPs) have been suggested as innovative suturing and adhesive alternative to Dermabond (2-octyl cyano-acrylate) and Ethicon 4/0 [57]. SiO_2 NPs synthesized by the Stober method and purchased from Aldrich (silica Ludox TM-50), were tested for nanobridging of full-thickness dorsal skin and hepatic injury in the Wistar rat model [57]. These NPs in distilled water or as a powder were deposited into the bleeding area and two edges of the wound were manually kept together for a while. Hemostasis occurred in 1 min and thin granulation tissue was observed on day 3 postsurgery of the skin. In the case of hepactectomy, the PVA membrane with SiO_2 powder was placed on the bleeding section of the hepatic lobe. Nanobridging on the rat's beating heart was also performed using silica Ludox TM-50 which was brushed on the heart surface as an adhesive for 3D-porous polysaccharide biodegradable hydrogel. These studies have shown that silica nanoparticles as nanobridging material have a lot of potential for liver, spleen, kidney, heart and lung surgeries. This study did not test the toxicity of the silica NM. Toxicological data of the silica material would complete the study.

Bioactive glass is another material which has been commercialized for their dental or bone regenerative applications. It was reported that healing of the cutaneous wounds in normal and diabetes-impaired rats can be accelerated by bioactive glasses. For instance, bioactive glass samples [99] were able to promote the proliferation of fibroblasts and growth of granulation tissue, and stimulated the production of growth factors such as VEGF and FGF2. Rate of wound healing depended on the composition of bioactive glass. Commonly, samples were based on SiO_2, Na_2O, CaO and P_2O_5 with irregular bulks of 60 nm spheres, and relatively dispersible nanoparticles in surface (about 30 nm). Every sample was mixed with melted Vaselin to obtain an ointment and can be applied topically [99].

Scaffolds with NM-embedded therapy

In a series of papers about the development and applications of functionalized biomaterials as bedding, scaffolds, gel and dressings with embedded NMs of various kinds are listed. This direction represents a combination of conventional (scaffold, dressing) and advanced therapy (NMs). Several interesting and recent examples are discussed in this section and schematically presented in Figure 4.

A recent study investigated some of these simple and combined approaches [100]. The pad-dry-cure technique, cotton fabrics with loaded suspension of powdered silver nanoparticles (12–22 nm, 0.163, –28 mV) showed great potential for healing similarly to the controlled cream (Dermazin) [100]. In an alternative study, a flexible polyethylene cloth with distributed nanocrystalline silver particles was tested in both *in vitro* and *in vivo* wound models [101]. No signs of toxicity and cells deaths were observed in the healed skin samples of patient's biopsies, although the authors noticed temporary reduction of mitochondrial functionality. Inclusion of AgNPs into the dressing led to the fast regeneration of cutaneous layer *in vivo*. AgNPs (10–30 nm) deposited onto bacterial cellulose nanofiber arranged in a form of network has displayed 99% reduction of *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth via slow Ag^+ release from the composite. It also allowed attachment and growth of epidermal cells within the wound site [102]. In another study, authors tested a new cationic biopolymer guar gum alkylamine with AgNPs [103]. The enhanced wound closure was promoted via hydration of wound surface and induction of the proliferative phase of wound healing [103]. A similar combined strategy was approved for preclinical study of fibrous mats/scaffolds as a wound dressing [104]. The mat produced via electrospinning using PVA and chitosan oligosaccharides with loaded AgNPs (15–22 nm) displayed no toxicity and antibacterial activity toward *E. coli* and *S. aureus* within the wound site [104]. Covalently cross-linked alginate fibrous hydrogel promoted the regeneration process by promotion of fibroblast migration to the wound area and reduction of the inflammatory phase. This material improved the quality as well as rate of the healing process [105].

The wound healing potential of chitosan-copper and zinc nanoparticle's (dispersion of 30–40 and 30–70 nm) composites was evaluated [106] in adult rats in another study. Antibacterial and anti-inflammatory effects were observed in the treated group. Another investigation [107] using similar nanocomposites based on chitosan and copper nanoparticles (50 nm) reported increases in VEGF, TGF-β1 and IL-10 expression. In conclusion, facilitation of all components of the proliferative phase of the wound healing process was revealed [107]. Another study investigated chitosan nano-dressing composed pectin and TiO_2 nanoparticles (20–40 nm) [34] which provided excellent antimicrobial properties with good biocompatibility and a control level of moisture. These composite materials were suggested for wound-healing applications based on their demonstrated healing efficiency *in vivo*.

Surface-modified magnetite (Fe_3O_4@C_{16}) nanoparticles with vegetal origin, eugenol and limonene, were used to prepare wound dressings with both microbicidal and antiadherence properties, which are significant for wound regeneration [108]. Similar to this study, the incorporation of gentamicin-loaded ZnO nanoparticles (polyhedral-shaped particles, 15 nm) in chitosan three-component gel, was offered for wound dressing [109]. Incorporation of nanopowder with antibiotics into a chitosan gel matrix provided a slow release rate of the drug. Synergic effects of antibacterial activity and combinations of beneficial features for wound-healing application enhanced the growth inhibition for *S. aureus* and for *P. aeruginosa*, as compared with the gentamicin control. The composite may also serve as a water source to restore a humid environment within the wound interface while providing a cooling sensation and soothing effect [109].

As we can see, plenty of researchers were interested in the development of dressings and scaffolds with embedded metallic nanoparticles based on silver, copper, zinc and magnetite, whereas only a few publications have discussed scaffolds embedded with polymer NMs in wound-healing application. Biomimetic skin substitutes comprised poly(L-lactic acid) scaffold with immobilized type I collagen. Scaffolds were embedded with PCL nanoparticles loaded with indomethacin or polyester urethane nanoparticles (218 and 196 nm, respectively) and had a positive effect on cellular growth as well as tissue regeneration which potentially enhanced healing response [110].

A promising therapeutic platform capable of sustaining release and intracellular delivery of siRNA has been presented in a recent study [111] which used a biodegradable and porous polyurethane scaffold (composed of a polyol component that is 60% PCL, 30% poly(glycolide), 10% poly(D,L-lactide) and a hardening component-lysine triisocyanate). This formulation and delivery of siRNA were able to knock down the overexpressed pro-inflammatory genes in chronic nonhealing skin wounds.

Chitosan-poly(ethylene oxide) nanofibrous electrospun scaffold with embedded PLGA nanoparticles has been evaluated *in vivo* for wound healing [112]. PDGF and VEGF encapsulated PLGA nanoparticles were also tested for wound-healing applications [112].

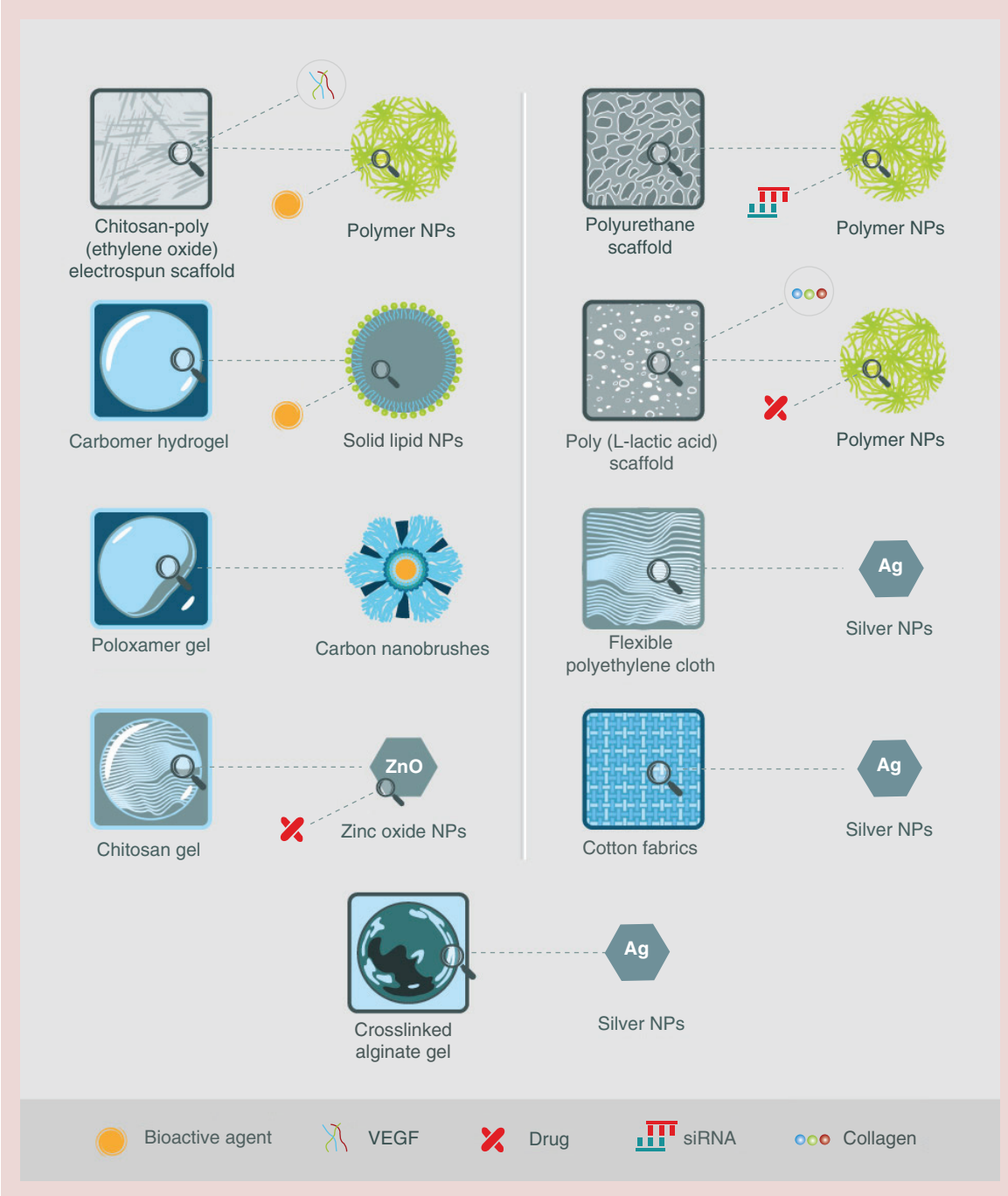

Figure 4. Examples of scaffolds with embedded nanomaterials for wound-healing treatment. Wound healing scaffold represents gel-type, polymeric, fibrous or spongiform 3D matrix with loaded substance exhibiting wound-healing property and with embedded nanomaterial.
NP: Nanoparticle.

Few studies also explored carbon nanocomposite and lipid nanoparticles embedded scaffolds for wound-healing applications. Carbon nanobrushes embedded in a biocompatible poloxamer gel was able to enhance growth capabilities, stimulate wound closure and repair injured tissue [113]. Astragaloside IV, possesses anti-inflammatory activity and was able to accelerate wound healing, and reduce scars. Astragaloside IV-enriched solid lipid nanoparticles incorporated in carbomer hydrogel was examined for their wound-healing abilities using *in vitro* (immortalized human fibroblast and keratinocyte cell lines) and *in vivo* (rat full skin excision) models. Such dressing improved the migration and proliferation of keratinocytes, strengthened

wound healing and inhibited scar formation *in vivo* by speeding up wound closure [114].

Conclusion

Reviewed herein are potential applications of NM-based approaches in the area of cutaneous wound care for the last 5 years. This overview reveals the benefits of using NMs in wound healing. It is important to note that any outcome of NM therapeutics depends on the NM formulation, doses and methods of application. Beneficial effects of different NMs/NM embedded scaffolds for wound-healing applications have been reported; however, the molecular mechanisms/signaling pathway of NM action on wounds were not clearly understood. Better understanding of the signaling pathway will elucidate the actions of NMs on the wounds, and will help to establish nanotechnology-based wound-healing therapy. Understanding the cellular response and analysis of cell signaling pathways involved in wound-healing with varying physicochemical features of NMs may open up new routes for novel nanotherapeutics. As NMs are highly active compared with its bulk, toxicity of NMs must be taken into consideration in every case before its usage in wound care products.

Future perspective

Conventional therapy in wound-healing care is dressing based. Traditional dressings are intended to provide wound cover, bleeding arrest, fluid adsorption, moistening or/and drying, infection protection and dead tissue removal. NMs, due to their unique properties open a new array of wound-healing products. NMs can modify each phase of wound healing as they possess antibacterial and anti-inflammatory activities, proangiogenic and proliferative properties. NMs are able to correct expression level of some important proteins and signal molecules to enhance wound healing. Thus, NMs or the combination of materials at both micro and nanoscales, may become beneficial enough to overcome most of the challenges that exist in wound care management. Distinguishing the functions of conventional materials and novel NMs in wound-healing therapy may lead to successes in managing complicated wounds, such as chronic and ischemic ulcers by a combination of nanomicro hybrid materials. Development of novel biocompatible and biodegradable NMs, which are able to correct all phases of wound healing, can be a future goal for researchers working in this area.

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Executive summary

Advantages over conventional materials

- Nanomaterials (NMs) therapy seemed to be more beneficial than conventional therapy in wound care. NMs embedded scaffolds are the future of wound care products.
- NMs are able to modify one or more wound-healing phases.

Toxicity & mechanism of action

- Cyto- and gene-toxicity of NMs must be taken into careful consideration before applying any NM for therapy.
- Signaling pathways of wound healing should be investigated in details and be distinguished for each wound-healing phase in the presence or absence of NMs.

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Short Communication

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Regenerative Medicine

Micronized cellular adipose matrix as a therapeutic injectable for diabetic ulcer

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Background: Despite the clinical potential of adipose-derived stem/stromal cells (ASCs), there are some clinical difficulties due to the regulation of cell therapies. **Materials & methods:** Micronized cellular adipose matrix (MCAM) injectable was prepared through selective extraction of connective tissue fractions in fat tissue only through mechanical minimal manipulation procedures. **Results:** It retained some capillaries and ASCs, but most adipocytes were removed. The presence of viable ASCs, vascular endothelial cells was confirmed and ASCs of MCAM kept intact mesenchymal differentiation capacity. In diabetic mice, skin wounds treated with MCAM showed significantly accelerated healing compared with phosphate-buffered saline-treated ones. **Conclusion:** The proven potential of MCAM to accelerate healing in ischemic diabetic ulcers may offer a simple, safe and minimally invasive means for tissue repair and revitalization.

Keywords: adipose stem cells • diabetic ulcer • extracellular matrix • flow cytometry • ischemia • lipoaspirates • minimal manipulation • tissue revitalization • vascular endothelial cells • wound healing

Adipose tissue is structurally complex, harboring a variety of cells within its lobulated fibrous septal network. Through enzymatic digestion, this network can be disintegrated and its heterogeneous complement of indigenous cells, or so called stromal vascular fraction (SVF), may be isolated. Because adipose-derived stem/stromal cells (ASCs) are important SVF residents with mesenchymal multipotency [1,2], SVF has been strategically engaged as a supplement to enhance fat engraftment [1,3–4]. However, if dissociated SVF cells are injected separately, rather than properly integrated into grafted fat, unexpected migratory and/or phenotypic outcomes may result, creating adverse complications (i.e., ectopic fibrosis and lymphadenopathy) [5]. Currently, a number of vehicles for delivery of cells are available, such as injectable biomaterial scaffolds, 3D spheroidal cell cultures and engineered cell sheets [6–8]. These sophisticated constructs help prevent untoward cell migration, thus avoiding lost or undesired contributions by cellular and extracellular matrix (ECM) componentry.

Decellularized ECM of various tissues or organs may serve as bioactive scaffolding, thereby facilitating tissue remodeling and repair [9–11]. Although adipocytes account for >90% of fatty tissue by volume, the native ECM of fat provides a niche for other cellular subsets (e.g., ASCs, vascular endothelial cells and pericytes), enabling biologic functions that are shared in part with acellular dermal matrix.

As niche components, stem cells generally lie in wait for changes in microenvironment. Stem cells isolated from the tissue, however, are already activated in an unphysiological microenvironment, and thus extra care needs to be taken in controlling the fate and behaviors of those cells in clinical utilization. Since 2005, policies of the US FDA aimed at preventing potential contamination and genetic alteration of

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stem cells [12] and enzymatic isolation and cultural expansion of ASCs were not considered as 'minimal manipulation', although isolated or cultured ASCs are already extensively used in a number of clinical trials.

For this investigation, a bioactive injectable comprised of functional ECM and resident cells (including ASCs) was formulated through minimal manipulation of adipose tissue. Given the combination of micronized connective tissue, viable ASCs and vascular endothelial cells generated, we have applied the term micronized cellular adipose matrix (MCAM). In this study, injectable MCAM was tested for its therapeutic value for wound repair of diabetic skin ulcer. Wound healing impairment in diabetic patients is a significant clinical issue affecting millions of patients worldwide. The major underlying pathology is noted to be chronic inflammation and ischemia based on peripheral vascular dysfunction, where tissue-resident stem cells are considered to be depleted. Although numerous products for wound dressing with bioactive ECM or growth factors are available, the clinical effects on diabetic ulcer are very limited. Thus, we sought to characterize and evaluate MCAM, which contains human adipose derived stem cells and vascular endothelial cells in their original niche of ECM, as a potential therapeutic tool for stem cell-depleted pathological conditions.

Materials & methods

Mouse tissue preparation

C57BL/6JJcL mouse inguinal fat pads were harvested, washed and weighed. One gram of fat pads was cut into tiny pieces with surgical scissors (continuous fine mincing for 5 min). After transfer to a tube containing 2.5 ml cooled phosphate-buffered saline (PBS), the morcellated tissue was thoroughly shaken several times and then centrifuged (800 × *g*, 5 min). MCAM was extracted as tissue sediment, and floating fatty tissue was also sampled.

Human tissue preparation

Lipoaspirate was obtained from a healthy 23-year-old female donor (BMI = 24) submitting to abdominal liposuction under general anesthesia. The study protocol was approved by our Institutional Review Board. Once soft tissues were infiltrated with a solution of saline plus epinephrine (1:1,000,000), subcutaneous fat was suctioned (-500 to -700 mmHg) using a conventional liposuction machine equipped with a 2.5-mm (inner diameter) cannula. MCAM was extracted from the lipoaspirate with the above described micronization and centrifugation, and floating fat was also sampled.

Whole-mount staining

Whole-mount staining was performed with Wheat germ agglutinin (WGA) Alexa Fluor 488 (Life Technologies, CA, USA), lectin PNA Alexa Fluor 594 (Life Technologies) and Hoechst 33342 solution (Dojindo, Kumamoto, Japan) as instructed by manufacturers. Images were then acquired via confocal microscope (Leica DMIRE2; Leica Microsystems, Wetzlar, Germany).

Flow cytometry

Mouse MCAM and roughly excised inguinal fat were separately prepared. Each sample was digested in 0.1% collagenase (Wako, Osaka, Japan) Hank's balanced salt solution (HBSS) by incubation in a shaking water bath (37°C, 30 min). To remove the collagenase, SVF cells were washed with PBS for three-times. After filtration, SVF cells were washed and stained with the following antibodies and corresponding isotype controls: Anti-CD45-Viogreen (Miltenyi Biotec, Bergisch Gladbach, Germany), Anti-CD34-Biotin (eBioscience, Inc., CA, USA), Rat IgG2a Kappa Control Biotin (BD Biosciences, CA, USA), Streptavidin-APC (BD Biosciences), Anti-CD31-PE (BD Biosciences), Rat IgG2a Kappa Control PE (BD Biosciences). Samples and controls were then analyzed by flow cytometry (MACSQuant Analyzer 10; Miltenyi Biotec). Gating for each signal was set to eliminate 99.9% of the cells in corresponding isotype control. CD45 gating was applied first, and CD45-negative portion was further analyzed for CD34 and CD31 signals.

Cultured ASCs of human origin

Human ASCs in floating fat and in MCAM were cultured separately. Floating fat was digested with collagenase (as above) [12], and isolated SVF cells were seeded in Dulbecco's Modified Eagle Media (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 IU penicillin and 100 mg/ml streptomycin. Explant culture was performed for human MCAM to generate MCAM-derived ASCs.

Multilineage differentiation assay

Assays of adipogenic, osteogenic and chondrogenic lineages were conducted as follows: adipogenic differentiation: ASCs were incubated for 21 days in DMEM containing 10% FBS, 0.5 mM isobutyl-methyl-xanthine, 1 M dexamethasone, 10 µM insulin and 200 µM indomethacin; osteogenic differentiation: ASCs were incubated for 21 days in DMEM containing 10% FBS supplemented with 0.1 mM dexamethasone, 50 mM ascorbate-2-phosphate and 10 mM glycerophosphate (Nacalai Tesque Inc, Kyoto, Japan); and chondrogenic differentiation: a micromass culture system was

utilized, as previously reported [13], incubating ASCs in a 15 ml tube for 21 days in DMEM containing 1% FBS supplemented with 6.25 mg/ml insulin, 10 ng/ml TGFβ-1 and 50 nM of ascorbate-2-phosphate. Regular growth medium was used to plate controls. The three lineages were analyzed qualitatively via Nile red (adipogenic), von Kossa (osteogenic) and Alcian blue (chondrogenic) differential staining and gauged quantitatively by AdipoRed assay (Lonza, Basel, Switzerland), Calcium-E test (Wako Pure Chemical Industries Ltd, Osaka, Japan) and micromass diameter.

Healing of diabetic ulcers in mice models

Care of B6-db/db mice (BKS.Cg/Lepr^{db}/m/JCL, 8-week old male) was conducted in accordance with institutional guidelines, using a protocol approved by the Animal Experimental Committee of University of Tokyo. Under general anesthesia (isoflurane inhalation), the B6-db/db mice were depilated and two full-thickness cutaneous wounds (6 mm each) were created on both sides dorsally, using skin punch devices. A donut-shaped silicone splint was then placed to prevent wound contraction and secured by interrupted 6–0 nylon sutures. MCAM prepared from one inguinal fat pad of a wild-type B6 mouse was injected by 29-G needle into four differing points of subcutis at wound peripheries (n = 4). Injection of PBS served as control. Treated wounds and splints were covered by transparent sterile dressings. Wounds were photographed on days 0, 2, 4, 7, 9, 11 and 14, determining their areas by Photoshop CS5 (Adobe Systems, CA, USA).

Statistical analysis

Results were expressed as mean ± standard error of the mean. To compare capacity for multilineage differentiation, Student's *t*-test was applied; and paired *t*-test was invoked for comparing wound sizes. p-values < 0.05 were considered statistically significant.

Results

Microstructure of micronized cellular adipose matrix

After mouse, adipose tissue was morcellated (100–400 μm maximum dimension), suspended in PBS and centrifuged, tissues were separated into yellowish floating adipose tissue (floating fat) and whitish bottom sedimentation (MCAM) (Figure 1A, left). Scanning electron microscopy (SEM) confirmed a scarcity of adipocytes in MCAM, whereas adipocytes were abundant in floating fat (Figure 1A, right). The lobular structure of floating fat was maintained, with MCAM consisting primarily of connective tissue and collagen bundles.

Whole-mount imaging highlighted functional features of MCAM (Figure 1B). As noted by scanning electron microscopy, MCAM was lacking in mature adipocytes (i.e., the large, round WGA$^+$ (wheat germ agglutinin) green cells seen in floating fat). However, Hoechst$^+$ nucleated cells persisted in MCAM at rather high density. In addition, MCAM retained branches and segments of vessels and identifiable capillaries.

Cellular content of SVF isolated from mouse MCAM

Fluorescence-activated cell sorting analyses were performed to delineate cellular composition, once SVFs were individually isolated from floating fat and MCAM through collagenase digestion (Figure 2). SVFs of fat and MCAM were characterized through a combination of surface markers and classified into four subpopulations; hematopoietic cells (mainly white blood cells; CD45$^+$), vascular endothelial cells (CD45$^-$/CD31$^+$/CD34$^+$), ASCs (CD45$^-$/CD31$^-$/CD34$^+$), and other cells (CD45$^-$/CD31$^-$/CD34$^-$). The SVF of floating fat consisted largely of CD45$^-$ nonhematopoietic cells (~75%), with ASCs accounting for half of the nonhematopoietic fraction. The SVF of MCAM contained all four subpopulations (including ASCs) albeit in differing ratios. ASCs accounted for 13% of MCAM-SVF cells, with hematopoietic cells, vascular endothelial cells and other cells constituting 57.9, 0.7 and 28.3%, respectively. The original protocol was designed for optimal adipose digestion, which may explain the less efficient digestion of MCAM connective tissue and its different composition of cells (ASCs and vascular endothelial cells).

Multilineage differentiation capacity of ASCs isolated from human MCAM

MCAM and floating fat were also extracted from the human lipoaspirate. Cultured ASCs derived from human floating fat and MCAM were compared in terms of capacity for differentiating into three mesenchymal lineages: adipogenic, osteogenic and chondrogenic. After 3 weeks of induction, ASCs of both floating fat and MCAM displayed similar degrees of multilineage differentiation. No morphologic differences in Nile red, von Kossa or Alcian blue staining were detected (Figure 3A), nor did quantification of lipid content, calcium deposition and micromass diameter (reflecting adipogenesis, osteogenesis and chondrogenesis, respectively) differ significantly (Figure 3B). Thus, it was indicated that MCAM contained ASCs with similar differentiating capability to those obtained from regular adipose tissue.

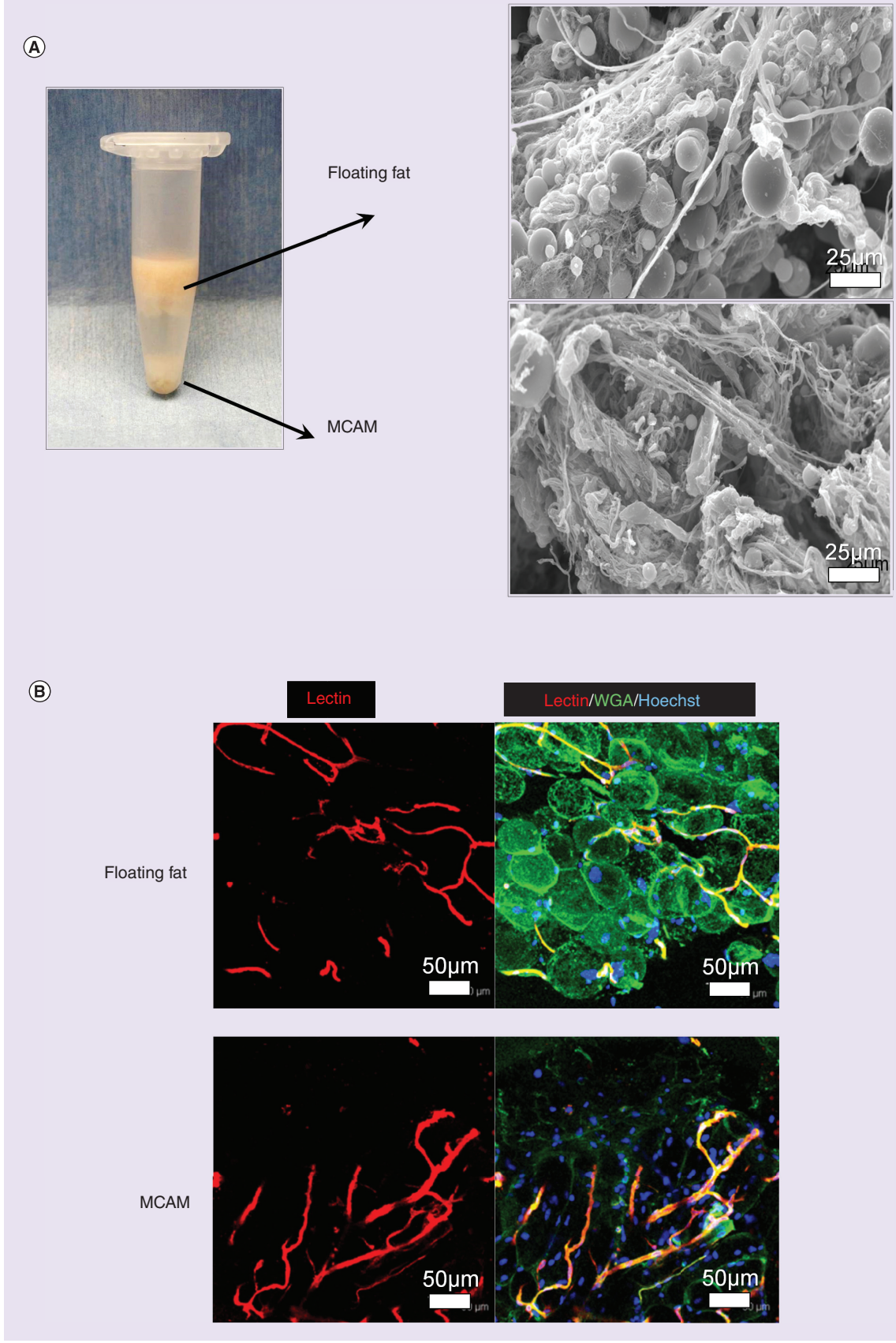
A
Floating fat
MCAM
25μm
25μm
B
Lectin
Lectin/WGA/Hoechst
Floating fat
MCAM
50μm
50μm
50μm
50μm

Figure 1. Microstructure of floating fat and micronized cellular adipose matrix (see facing page). **(A)** Once minced inguinal fat pad of mouse was centrifuged, micronized cellular adipose matrix (MCAM; whitish sediment) separated from floating fat. Scanning electron microscopy revealed highly fibrous nature of MCAM (vs floating fat), with few mature adipocytes. Bars = 25 μm. **(B)** Whole-mount staining of lectin (vascular endothelial cells: red), wheat germ agglutinin (cell membranes: green) and Hoechst (nuclei: blue); mature adipocytes scarce in MCAM, but microvascular structures in connective tissue remained intact. Bars = 50 μm.
MCAM: Micronized cellular adipose matrix; WGA: Wheat germ agglutinin.
For color images please see online at www.futuremedicine.com/doi/full/10.2217/RME.15.48

Therapeutic effects of mouse MCAM for diabetic ulcers

Subcutis of full-thickness dorsal skin ulcers inflicted in diabetic mice was injected at wound peripheries with MCAM prepared from wild-type mice, using pBS injections as control. Healing of diabetic ulcers was significantly more rapid with MCAM (vs pBS) injection, with 64% smaller wound size on day 4 ($p = 0.0082$) and 65% smaller size on day 7 ($p = 0.0043$; Figure 4). At the close of week 2, closure was essentially completed in MCAM-treated ulcers, whereas wound beds of pBS-treated ulcers remained hyperemic.

Discussion

For this investigation, our injectable MCAM by design was a formulation of bioactive ECM and functional ASCs, prepared by mechanical mincing and elimination of adipocytes from adipose tissue. Sharp-bladed instruments, such as scissors, easily micronized the connective tissue in fat without significantly altering its basic structure, and viability of cells was retained. Approximate specific gravities of human adipocyte and connective tissue obtained from human lipoaspirates were 0.85–0.87 and 1.1–1.2, respectively, underscoring that MCAM regularly sediments upon centrifugation of processed samples.

There is an abundance of evidence affirming the biologic utility of ECM, namely the capacity to regulate proliferation and differentiation of tissue-resident stem cells [14]. Acellular ECM products, whether dermal or fatty by nature, have proven therapeutic in clinical and experimental contexts [9–11,15–16], providing biocompatible substrates or scaffolding and trophic/growth factors needed to accommodate and recruit stem/progenitor cells. In addition to supplying ECM essentials, our MCAM injectable also incorporates an array of viable cells (ASCs, vascular endothelial cells and more) that engage in wound healing.

To date, such scaffolds are designed to mimic native environments, ensuring original cell functions are maintained for optimal cell expansion and tissue regeneration [17–19]. ASCs in MCAM occupy their original ECM niche, ready to assume original roles. As shown by whole-mount staining and flow cytometry, vessels and capillaries of MCAM, as well as ASCs and other stromal cells, retained their natural states and positions. Functional aspects of ASCs in MCAM were also well preserved, as partly indicated by differentiation assays.

ASCs are thought to be potent sources of trophic factors and to have multilineage differentiation capacity. Nonetheless, in transplantation of dissociated (suspended) ASCs, local retention was found to be poor (many disappearing in 1 week), thus nullifying therapeutic intent or resulting in unexpected stem cell behaviors [5,7,20]. As the size of injectable MCAM ranges 100–400 μm, the ECM of MCAM may also provide better mechanical support and anchorage for ASCs to avoid their rapid and seemingly detrimental migration.

Our *in vivo* results suggest that MCAM does impact the healing of diabetic ulcers by accelerating tissue repair. Although similar therapeutic effects have already been reported with use of isolated and cultured ASCs in diabetic or generic refractory ulcers [21–24], substantial procedural manipulations (i.e., enzymatic digestion and isolation or culture of cells) to possibly change biological properties of ASCs are not required for MCAM preparation, eliminating potential regulatory concerns. In skin wound healing models, significant differences are usually observed at a specific and limited time range [25,26], because the skin wound closes eventually in any model (thus no difference at later stages). Our objective was to design a safe and functional injectable, requiring little in the way of preparation. In this study, MCAM obtained from wild-type healthy mice was administered into diabetic mice and it is a limitation of this study that MCAM from diabetic mice was not evaluated. Although ASCs from diabetic patients may not have the same function as those from healthy subjects, ASCs are known to be relatively immunoprivileged and may work as a temporary drug to release trophic factors and help wound healing by allogeneic use.

Fat grafting is claimed to have comparable clinical effects, promoting wound healing of refractory ulcers, such as those seen postirradiation [27–29], and providing a remedy for stem-cell depleted conditions such as chronic ischemia [30,31], systemic sclerosis [32] and scar contracture [33–35]. Improvement of such problematic conditions suggests that the clinical benefits may be partly attributable to functional ASCs. However, if volumetric restoration is not desired, as in transplantations for tissue revitalization/fertilization, the volume occupied by adipocytes within the graft would signifi-

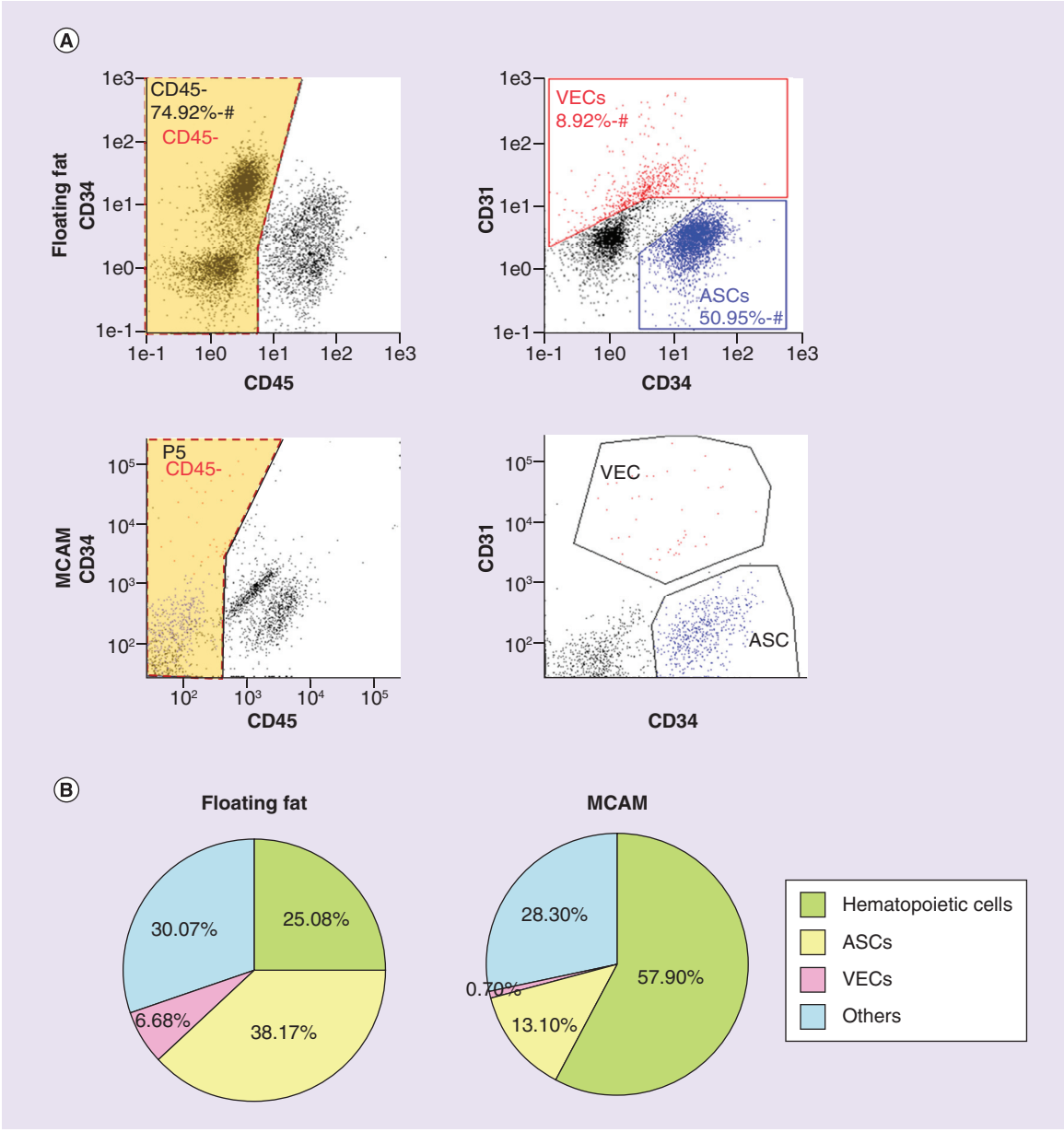

Figure 2. Cellular content of stromal vascular fraction isolated from mouse floating fat and micronized cellular adipose matrix. (A) SVFs from floating fat and that from MCAM were isolated through digestion by collagenase, identifying content as follows: hematopoietic cells (primarily WBCs; $CD45^+$), VECs ($CD45^-/CD31^+/CD34^+$), ASCs ($CD45^-/CD31^-/CD34^+$) and other cells ($CD45^-/CD31^-/CD34^-$). **(B)** ASCs in MCAM account for 13.1% of SVF cells. Hematopoietic cells, VECs and other cells comprise 57.9, 0.7 and 28.3%, respectively. More ASCs (38.2%) and VECs (6.7%) are evident in SVF of floating fat by comparison.
ASC: Adipose-derived stem/stromal cell; MCAM: Micronized cellular adipose matrix; SVF: Stromal vascular fraction; VEC: Vascular endothelial cell.

cantly decrease the density of ASCs and ECM. In these cases, ASCs alone or in conjunction with ECM (as in MCAM) may provide sufficient therapeutic effect, as we have shown.

Conclusion

MCAM is an autologous/allogeneic injectable of bioactive ECM and functional cellular components generated through minimal manipulation of adipose tissue. Smaller-sized and more homogeneous (50–100 μm) particles may be preferable as injectables, but as we already learned, such preparations may jeopardize the viability of cells. Further studies are needed to verify the hypothesized mechanism underlying the efficacy of MCAM and further optimization of preparation methods. Therapeutic effect of MCAM in other stem

cell-depleted states, including irradiation damage and fibrous diseases will also need to be tested. Besides, we still need to elucidate the specific functions of adipocytes in clinical fat grafting and to see what benefits of fat grafting will be lost when we use the product like MCAM, where mature adipocytes are virtually absent. Our efforts here attest to the therapeutic potential of MCAM in ischemic diabetic ulcers, offering a novel mode of tissue repair and revitalization with a minimally invasive approach.

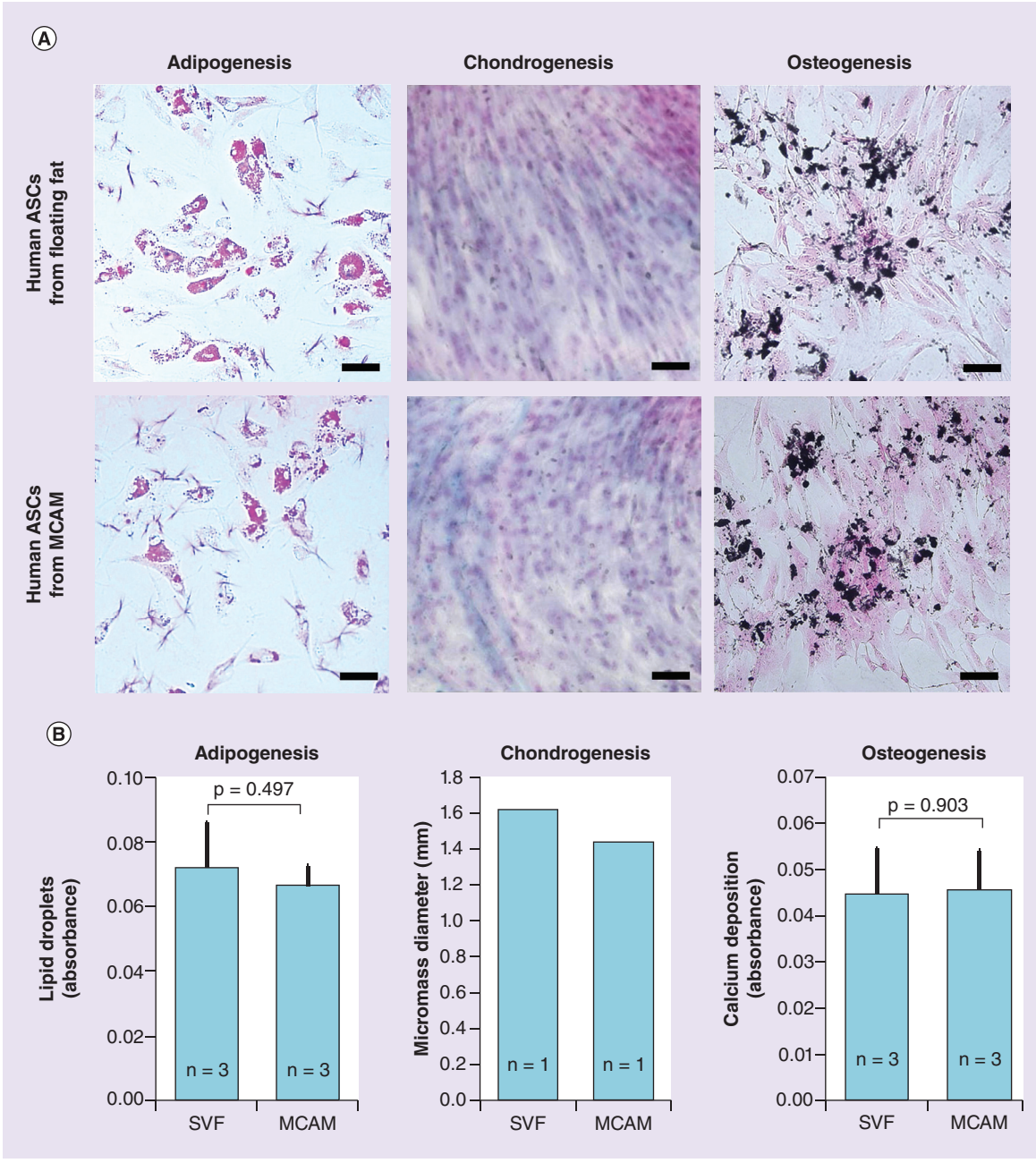

Figure 3. Multilineage differentiation capacity of cultured adipose-derived stem/stromal cells isolated from human floating fat and micronized cellular adipose matrix. (A) Microscopic images of differential induction: cultured human ASCs of floating fat and MCAM yielded similar adipogenic, chondrogenic and osteogenic differentiation; lineage-specific differentiation delineated by Nile red, Alcian blue and von Kossa stains, respectively. Scale bar = 100 μm. **(B)** Quantitative analysis of cellular differentiation: capacity for multilineage differentiation, as indicated by accumulated lipid (adipogenesis), micromass diameter (chondrogenesis) and calcium deposition (osteogenesis), showing no significant differences between fractions. ASC: Adipose-derived stem/stromal cell; MCAM: Micronized cellular adipose matrix; SVF: Stromal vascular fraction.

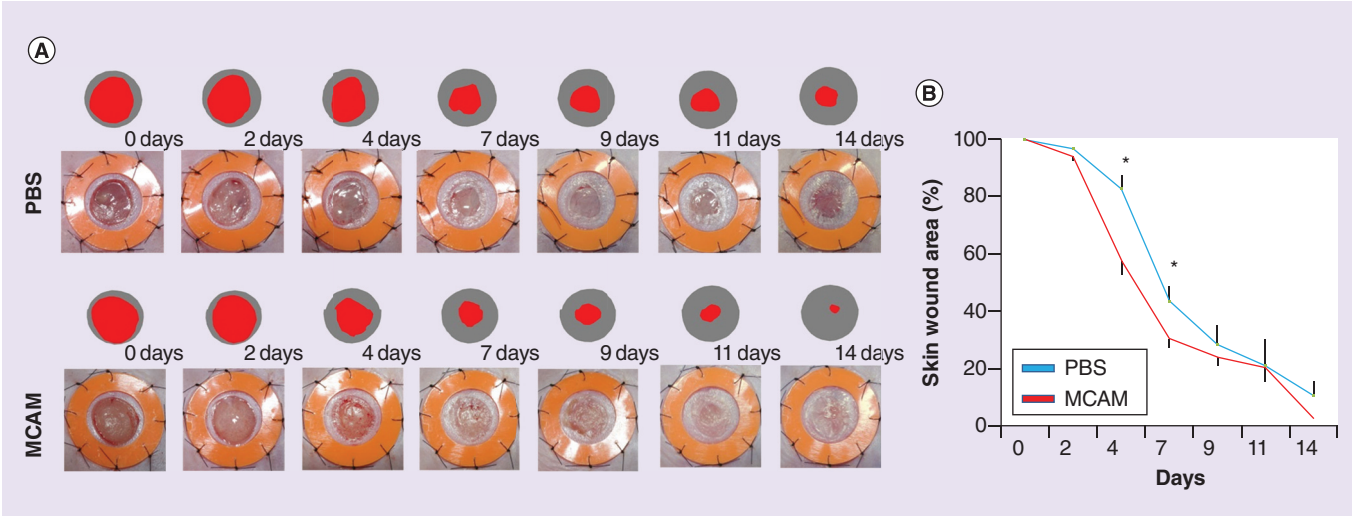

Figure 4. Therapeutic mouse micronized cellular adipose matrix injection of diabetic ulcers. (A) Representative photos of cutaneous wounds (6 mm) created on dorsal areas of diabetic (db/db) mice (n = 4) and treated by local injection of PBS or MCAM on day 0, with 2-week follow-up; ulcerated surface areas determined via software. **(B)** Comparison of wound surface areas (determined digitally): significantly reduced ulcer size in MCAM-injected mice (vs PBS-injected controls), day 4 ($p = 0.0082$) and day 7 ($p = 0.0432$).
MCAM: Micronized cellular adipose matrix; PBS: Phosphate-buffered saline.

Future perspective

Regulatory organizations in many countries regard SVF isolated from adipose tissue as a 'more than minimal manipulated' biological drug, of which uses are strictly regulated due to its associated safety issues. Although therapeutic potential of ASCs in the SVF was extensively studied, cell suspension of ASCs may not be the optimal means in order to maximize its therapeutic effects and avoid unfavorable migration. If injectable tools containing ASCs can be prepared through minimal manipulation and show comparable therapeutic effects, it would be a potential alternative. Also, as ASCs are relatively immunoprivileged, allogeneic ASCs may be clinically used, for example, as a temporarily-working drug to release cytokines. It may be a good news for diseased patients whose ASCs cannot function as those of healthy patients. In this study, we focused on the characterization and therapeutic effects of MCAM as the first step to develop a new cellular/tissue product. MCAM accommodates viable ASCs within their natural niche and may be valuable in

Executive summary

Micronized cellular adipose matrix definition and preparation

- MCAM is the abbreviation for micronized cellular adipose matrix. It is an injectable cellular matrice product extracted from the adipose tissue.
- MCAM is obtained as the pelleted material from centrifugation after finely mincing and fragmentation of fat tissue.

Microstructure of MCAM

- Comparing with the intact fat tissue, MCAM includes few adipocytes but a substantial number of adipose derived stem cells (ASCs) within its abundant connective tissue.
- Microvascular structures in extracellular connective tissue of MCAM remained intact.

Analysis of cellular composition

- MCAM retained ASCs and vascular endothelial cells.
- ASCs within MCAM were viable and maintained similar mesenchymal differentiation capacity compared to those ASCs obtained through the standard collagenase digestion method.

Regenerative potential

- In diabetic mice model, excisional skin wounds treated with MCAM showed accelerated healing compared to phosphate-buffered saline-treated wounds.

Conclusion

- MCAM is an injectable tissue product contains both functional stem cells and extracellular matrix. MCAM is prepared through minimal manipulation and thus can be readily used without regulatory issues. MCAM may offer a safe and effective option in chronic wound repair and tissue revitalization.

treating stem cell-depleted conditions such as chronically inflammated tissues/ulcers and radiated tissue damage. Another future study is needed to explore the effectiveness of MCAM compared with other cellular formulations such as freshly isolated SVF and cultured ASCs.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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