Published in final edited form as:
Exp Cell Res. 2010 August 15; 316(14): 2213–2219. doi:10.1016/j.yexcr.2010.05.009.

Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair

Anne M. Hocking and **Nicole S. Gibran**
University of Washington, Campus Box 359796, 300 9th Avenue, Seattle, WA 98104, U.S.A.

Abstract

Cutaneous wounds persist as a health care crisis in spite of increased understanding of the cellular and molecular responses to injury. Contributing significantly to this crisis is the lack of reliable therapies for treatment of wounds that are slow to heal including chronic wounds and deep dermal wounds that develop hypertrophic scars. This article will review the growing evidence demonstrating the promise of multipotent mesenchymal stem/stromal (MSCs) for the treatment of impaired wound healing. MSCs are often referred to as mesenchymal stem cells despite concerns that these cells are not truly stem cells given the lack of evidence demonstrating self-renewal *in vivo*. Regardless, abundant evidence demonstrates the therapeutic potential of MSCs for repair and regeneration of damaged tissue due to injury or disease. To date, MSC treatment of acute and chronic wounds results in accelerated wound closure with increased epithelialization, granulation tissue formation and angiogenesis. Although there is evidence for MSC differentiation in the wound, most of the therapeutic effects are likely due to MSCs releasing soluble factors that regulate local cellular responses to cutaneous injury. Important challenges need to be overcome before MSCs can be used effectively to treat wounds that are slow to heal.

Keywords

Wound repair; Mesenchymal stem cells; Multipotent mesenchymal stromal cells; Paracrine signaling; Cell differentiation; Regenerative medicine; Inflammation; Angiogenesis; Epithelialization; Fibroproliferation

Introduction

Cutaneous wounds persist as a health care crisis in spite of increased understanding of the cellular and molecular responses to injury. Contributing significantly to this crisis is the lack of reliable therapies for treatment of wounds that are slow to heal, including chronic wounds due to diabetes mellitus and deep dermal wounds that form hypertrophic scars. Conventional treatments of chronic wounds include topical antibiotics, debridement with or without grafting and compression bandages; advanced therapies include application of bioengineered skin substitutes and growth factors. Despite considerable progress, resistance of chronic

Corresponding Author: Anne M. Hocking, Ph.D., University of Washington, Campus Box 359796, 300 9th Avenue, Seattle, WA 98104, Telephone: 1 206 897 5922, Fax: 1 206 897 5343, ahocking@u.washington.edu.
Nicole S. Gibran nicoleg@u.washington.edu
Anne M. Hocking ahocking@u.washington.edu

wounds to these therapies is not uncommon. In the event of deep dermal injury, there is no effective therapy for the prevention of hypertrophic scar formation. The use of pressure garments, silicone sheeting and steroid injections has only limited success in the reduction of hypertrophic scarring. It is clear therefore that there is an urgent need for novel therapies for both chronic wounds and deep dermal wounds that form hypertrophic scars. This article will review the growing evidence demonstrating the promise of mesenchymal stem cells for the treatment of impaired wound healing.

Definition of mesenchymal stem (or stromal) cells

Mesenchymal stem cells are generally defined as plastic-adherent cells with a fibroblast-like morphology and the capacity for multipotent differentiation *in vitro* [1-2]. These adult stem cells also exhibit immunomodulatory properties and lack significant immunogenicity, both important characteristics for cell-based therapies [3]. Mesenchymal stem cells were first isolated from bone marrow [1-2] but have now been found in many other organs and tissues [4]. Factors contributing to the challenge of precisely defining mesenchymal stem cells include inconsistencies with both nomenclature and the criteria used to identify a mesenchymal stem cell. For example, these cells are commonly referred to as either mesenchymal stem cells, multipotent mesenchymal stromal cells or stromal progenitor cells. This confusing nomenclature exists in large part due to concerns in the scientific community that these cells are not truly stem cells given the lack of evidence demonstrating self-renewal *in vivo* [5-7]. In an attempt to clarify the nomenclature, the International Society for Cellular Therapy (ISCT) in 2006 suggested “that the fibroblast-like plastic-adherent cells, regardless of the tissue from which they are isolated be termed multipotent mesenchymal stromal cells, while the term mesenchymal stem cell is only used for cells that meet specified stem cell criteria. The widely recognized acronym, MSC, may be used for both cell populations as is the current practice…” [5]. Despite this position statement, the term mesenchymal stem cell predominates in the scientific literature. For simplicity, this review will use the acronym MSC as defined by the ISCT and focus primarily on bone marrow-derived MSCs.

Differences in the criteria used to identify MSCs also make it difficult to compare published data. To date, there is no specific cell surface marker unique to MSCs and considerable variability in the cell surface marker expression profile has been observed between described MSC populations. The cell surface marker expression profile for MSCs appears to be influenced at least in rodents by strain and the method used for isolation and expansion [8]. The proteomic profile of human MSCs also demonstrates dependence on the specific culture media used for expansion [9]. Again the International Society for Cellular Therapy has tried to resolve these challenges by proposing three minimal criteria for defining human MSCs: 1) plastic adherence; 2) greater than 95% of the population positive for CD105, CD73 and CD90 expression and greater than 98% of the population negative for CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR surface molecules, and 3) ability to differentiate into osteoblasts, adipocytes and chondroblasts under standard *in vitro* differentiating conditions [10]. In addition, the European consortium Genostem has recently completed a systematic analysis of human bone marrow-derived MSC properties reporting that MSCs are capable of self-renewal, which indicates that these cells are indeed bona fide stem cells [11]. A precise definition of the MSC identity therefore requires development of standardized protocols for isolation and expansion in conjunction with *in vivo* assays to demonstrate ‘stemness’. Elucidation of MSC identity will also be facilitated by the ongoing development of advanced methods for tracking MSCs i*n vivo* [12-13].

The therapeutic benefits of MSCs for treating tissue injury: mechanisms of action

Regardless of the caveats for defining MSCs, abundant evidence demonstrates the therapeutic potential of bone marrow-derived multipotent mesenchymal stromal cells for

repair and regeneration of damaged tissue due to injury or disease. Indeed, MSCs ameliorate tissue damage in almost all of the major organs of the body including heart, brain, lung, liver, kidney, eye and skin [14-20]. These data demonstrating efficacy and broad applicability have motivated the rapid development of MSC-based therapies as indicated by the ninety clinical trials currently listed in the US National Institutes of Health registry of clinical trials (http://clinicaltrials.gov).

Differentiation and paracrine signaling have both been implicated as mechanisms by which MSCs improve tissue repair. MSC differentiation contributes by regenerating damaged tissue, whereas MSC paracrine signaling regulates the local cellular responses to injury. Current data suggest that the contribution of MSC differentiation is limited due to poor engraftment and survival of MSCs at the site of injury. Given these limitations, it has been proposed that MSC paracrine signaling is the primary mechanism accounting for the beneficial effects of MSCs on responses to injury such as inflammation, angiogenesis, and fibroproliferation [14-21]. This hypothesis is further supported by the observation that MSC-conditioned medium also enhances tissue repair [21-22].

Much of the current research now focuses on defining the MSC secretome and identifying the target cells at the site of injury that are responsive to MSC paracrine signaling. Another important priority is to elucidate how the individual target cell types respond to MSC signaling. Proteomic analyses of MSC-conditioned medium indicate MSCs secrete many known mediators of tissue repair including growth factors, cytokines and chemokines [21-22]. *In vitro* and *in vivo* studies indicate that many cell types are responsive to MSC paracrine signaling. In addition, MSC signaling regulates a number of different cellular responses including cell survival, proliferation, migration and gene expression. Interestingly the effects of MSC paracrine signaling appear to be tissue specific. Whereas, MSC treatment of myocardial infarction results in increased angiogenesis [14,21], neovascular growth is inhibited when MSCs are therapeutically administered after corneal injury [19]. These data underscore the importance of crosstalk between cells resident in the injured tissue and the ectopically applied MSCs. Presumably such cell-cell interactions regulate spatiotemporally the composition of the MSC secretome during the repair process. Defining the MSC secretome in the context of the injured tissue microenvironment is therefore essential for optimizing MSC-based therapies.

Bone marrow-derived MSCs improve cutaneous wound repair

Cutaneous wound repair is a complex process requiring coordination of a cascade of cellular responses to injury including inflammation, epithelialization, fibroproliferation and angiogenesis [23]. In adult mammals, the resulting scar tissue is collagen rich and lacking epidermal appendages. Even under optimal healing conditions, normal wound repair is imperfect failing to regenerate skin structure and function. Impaired wound repair results in either chronic wounds that fail to heal or fibroproliferative wounds that form hypertrophic scars. Ideally, MSC-based therapies targeting cutaneous wound repair need to stimulate cellular responses to injury and promote regeneration rather than scar formation.

Cutaneous wounds treated with bone marrow-derived MSCs exhibit enhanced wound repair (Table 1). Administration of MSCs to either acute or diabetic wounds in rodents accelerates wound closure [24-29]. Decreased wound size was also observed when autologous MSCs were applied to human chronic wounds in a pilot study of six subjects [30]. Subsequent focused analyses of wound histology have indicated that MSCs accelerate epithelialization, increase granulation tissue formation and increase angiogenesis [24-29]. In addition, MSC-conditioned medium acts *in vivo* as a chemoattractant recruiting macrophages and endothelial cells to the wound [22]. To our knowledge, only two studies have observed a direct effect of MSC treatment on cutaneous scar formation, although neither study used an

animal model of hypertrophic scarring [24,31]. Both studies showed that MSC treatment increased wound tensile strength with one also reporting a reduction of scarring as measured by histomorphological evaluation [31]. Collectively these data demonstrate that MSC treatment impacts all phases of wound repair including inflammation, epithelialization, granulation tissue formation and tissue remodeling.

Contribution of MSC differentiation to wound repair

In order to follow the fate of exogenously applied MSCs during wound repair, sex-mismatched MSCs expressing green fluorescent protein (GFP) have been used [26,28]; GFP positive MSCs from male mice were administered to wounds on GFP negative female mice [26]. An important observation in these studies is that the percentage of MSC engraftment in the wound is low and decreases with time [26]. Nonetheless, there have been a number of reports suggesting that MSCs differentiate into epidermal keratinocytes, endothelial cells and pericytes *in vivo* [26,28]. There is also evidence that MSCs differentiate into sebocytes in the sebaceous glands in skin adjacent to the wound [29]. Almost all of these studies have relied on the co-localization of GFP with specific cell phenotype markers and report that this is due to MSC differentiation rather than MSC fusion to local wound resident cells as determined by X and Y chromosome fluorescence *in situ* hybridization of MSC-derived cells [26,28-29]. Other investigators, however, have reported that there is no evidence that MSC differentiate into phenotypes typical of resident cutaneous cells in the wound [25]. Such conflicting data may be due to heterogeneity in MSC populations given differences in MSC isolation and expansion in culture. The method of MSC delivery to the wound, which impacts engraftment efficiency, may also be a contributing factor.

MSC paracrine signaling enhances wound repair

Growing evidence indicates that MSC paracrine signaling is the predominant mechanism responsible for enhanced wound repair. MSC-conditioned medium has a similar effect as MSCs on wound repair with accelerated epithelialization [22,32-33]. Importantly, this result is not specific to conditioned medium collected from bone marrow-derived MSCs [22] but has also been observed using conditioned medium from MSCs isolated from adipose tissue [32-33]. In order to elucidate the effect of MSC paracrine signaling on individual cell phenotypes present in the cutaneous wound investigators have resorted to *in vitro* studies using MSC-conditioned medium or paracrine co-culture systems. Confirming the *in vivo* data, MSC-conditioned medium acts as a chemoattractant for macrophages and endothelial cells [22]. Furthermore, MSC-conditioned medium is also a chemoattractant *in vitro* for both epidermal keratinocytes and dermal fibroblasts, implicating MSC paracrine signaling in the recruitment of these specific cell types to the wound [22,33]. In addition to chemotaxis, MSC paracrine signaling may also regulate cell migration in response to injury given recent data showing that dermal fibroblasts complete scratch wound closure faster in the presence of either MSCs or MSC-conditioned medium [34]. MSCs also secrete mitogens that stimulate proliferation of keratinocytes, dermal fibroblasts and endothelial cells *in vitro* [25,32-34]. Further investigation has shown that dermal fibroblasts secrete increased amounts of collagen type I [32] and alter gene expression [34] in response to either MSCs in co-culture or MSC-conditioned medium. Overall, these *in vitro* data suggest that MSCs therapeutically applied to the wound release soluble factors that stimulate proliferation and migration of the predominant cell types in the wound. Consequently MSC paracrine signaling has potential beneficial effects on angiogenesis, epithelialization, and fibroproliferation during wound repair.

Optimizing MSC engraftment in cutaneous wounds

Optimizing MSC engraftment in the cutaneous wound is critical for the development of effective MSC-based therapies. Studies in models of brain and heart injury indicate that

engraftment efficiency is directly influenced by the MSC delivery protocol [12]. MSC engraftment was shown to be dependent on timing of delivery, number of cells delivered and site of delivery. Early delivery of MSCs after injury resulted in a higher rate of engraftment, as did increased number of MSCs delivered [12]. Unfortunately the effect of MSC delivery protocol on engraftment in the cutaneous wound has not been well documented. Heterogeneity between MSC populations, differences in wound models and varied MSC delivery protocols in published studies make it difficult to determine the impact of timing of delivery, number of cells delivered and site of delivery on MSC engraftment to wounds. To date, all published reports have delivered MSCs immediately after cutaneous injury [25-29] or within 24 hours of wounding [24]. The only exception has been a study that demonstrated that applying MSCs to human chronic wounds of more than one year duration reduced wound size [30]. This is also the only published report that has correlated the number of cells applied and reduction in chronic wound size [28]. Reported methods for MSC delivery to wounds include injection and topical delivery using a range of vehicles such as PBS [25], matrigel [26], fibrin polymer [30] and human amniotic membrane grafts [35] (Table 1). Either intravenous injection for systemic delivery or intradermal injection combined with topical administration for localized delivery were effective methods for administering MSCs to wounds, albeit with low rates of engraftment [24,26,28]. At the time of injury, MSC administration via intradermal injection at the wound edge in combination with topical application of MSCs embedded in matrigel resulted in approximately 30% engraftment in day 7 murine wounds [26,36]. Engraftment decreased over time with less than 10% in day 14 wounds and less than 5% in day 28 wounds. MSC engraftment to wounds was significantly higher when MSCs were isolated from “superhealer” MRL/MpJ mice, which are known for their regenerative capacity in response to injury [27]. This high engraftment efficiency correlated with enhanced responses to injury such as increased granulation tissue formation and angiogenesis in the PVA sponge model of cutaneous wound repair. Further investigation implicated sFRP2, a known inhibitor of Wnt signaling, as a mediator of MSC proliferation and engraftment.

Donor immunogenicity and the local wound environment are two other factors potentially contributing to MSC engraftment. Whereas many studies suggesting MSCs are immunosuppressive, others report that allogeneic MSCs elicit a host immune response that results in reduced engraftment [37]. Two studies report that in rodent wounds there were no significant differences in percent engraftment between allogeneic and syngeneic MSCs [25,36]. How the local wound environment influences MSC engraftment is also under investigation. Since chronic wounds may be subject to metabolic perturbations such as ischemia or hyperglycemia, one must consider the effects of these environmental factors on MSC responses. A recent study analyzing the effect of ischemic conditions on MSC survival *in vitro* [38] suggests that hypoxia has no effect but glucose deprivation results in a significant reduction in MSC viability. Interestingly, hypoxia only has an effect if the exposure was prolonged and in combination with nutrient deprivation, when cultured under these conditions almost all of the MSCs died [39]. Paradoxically, in animal models of cardiac injury, preconditioning MSCs to hypoxia *in vitro* results in higher rates of engraftment *in vivo* [12]. In addition, a recent study suggests that conditioned media from adipose-derived MSCs cultured under hypoxic conditions is more effective at accelerating wound closure compared to conditioned medium harvested from MSCs cultured in normal physiological levels of oxygen [33]. Subsequent experiments implicated the growth factors, VEGF and bFGF as potential mediators of this increased efficacy of hypoxic MSC-conditioned medium [33]. It is clear however that much work remains to be done to determine the impact of the local wound environment on MSC engraftment. To date, it is unknown how components of the chronic wound environment such as bacterial biofilms, chronic inflammation and oxidative stress affect MSC engraftment.

MSC-based therapies targeting repair AND regeneration in the wound

MSC-based therapies for cutaneous wounds may be administered using two different approaches. The first strategy involves application of MSCs directly on to the wound surface while the second relies on administration of MSC-conditioned medium to the wound. Application of cells has the potential advantage of promoting regeneration via MSC differentiation in addition to repair via MSC paracrine signaling. In contrast, although the use of MSC conditioned medium presumably targets repair only, although this strategy does circumvent challenges with MSC engraftment to the wound.

Currently the modest differentiation capacity of MSCs *in vivo* limits the potential of therapeutically administered MSCs to promote regeneration rather than scar formation in response to cutaneous injury. Possible strategies to enhance MSC differentiation include genetic manipulation of the MSCs and/or creation of a wound environment that is permissive for differentiation. Recent studies have introduced transcription factors into MSCs in order to direct differentiation toward specific cell fates [40]. To date, there are no published reports targeting specific cutaneous cell phenotypes but such advances would be clearly beneficial for the regeneration of epidermal appendages, innervation and pigmentation after injury.

The third therapeutic option: recruitment of endogenous MSCs to the wound

Another strategy for administering MSC-based therapies is to enhance recruitment of endogenous bone marrow-derived MSCs to the site of injury. It is clear that this approach requires an understanding of endogenous MSC trafficking in response to injury. Chimeric bone marrow transplantation models have demonstrated that bone marrow-derived cells home to uninjured skin and are actively recruited to cutaneous wounds. To date, the majority of these studies have relied on expression of enhanced green fluorescent protein (eGFP) to track bone marrow-derived cell trafficking *in vivo*. eGFP+ bone marrow-derived cells have been observed in both the epidermis and dermis of uninjured skin [41-43]. Bone marrow-derived cells in the epidermis are often associated with hair follicles with a number of studies reporting localization to the hair follicle bulge region [41,44], while eGFP+ cells in the dermis resemble spindle-shaped dermal fibroblasts [42].

In response to cutaneous injury, endogenous bone marrow-derived cells are mobilized and recruited to the wound. During wound repair, the bone marrow contributes inflammatory cells, endothelial progenitor cells and fibrocytes to the wound [20]. Data from chimeric bone marrow transplantation models indicates that epidermal keratinocytes and dermal fibroblasts in the healing wound may also be derived from the bone marrow [41-44]. This contribution to the epidermis and dermis in uninjured skin and wounds was also observed when bone marrow is reconstituted with chimeric MSCs [42,45].

Although the mechanism and the signals responsible for MSC homing to uninjured skin and wounds is not well understood, it is likely involves the complex interplay of adhesion molecules, chemokines, chemokine receptors, extracellular matrix proteases and tissue inhibitors of matrix metalloproteinase [12]. One study has demonstrated that intradermal injection of the chemokine, SLC/CCL21 at the wound edge increases recruitment of intravenously injected MSCs and significantly accelerated wound closure [28]. The neuropeptide, substance P has also been recently implicated in the induction of MSC mobilization into the circulation after injury [46].

Conclusion

Multipotent mesenchymal stromal cells (MSCs) have shown promise as therapeutic agents promoting tissue repair and regeneration in response to injury and disease. Advantages of

MSC-based therapies include relative ease of both isolation and expansion in culture prior to transplantation. Although MSC differentiation has been reported at sites of injury, the low engraftment efficiency suggests that MSC paracrine signaling not differentiation is primarily responsible for the therapeutic effects of MSCs.

There has been much interest in the development of MSC-based therapies for cutaneous wounds that promote both rapid wound closure and healing without scar formation. To date, MSC treatment of acute and chronic cutaneous wounds results in accelerated wound closure with increased epithelialization, granulation tissue formation and angiogenesis. In addition, MSCs have been reported to differentiate into epidermal keratinocytes, endothelial cells and pericytes in the cutaneous wound despite low engraftment efficiency. However, there is currently no evidence that MSCs treatment promotes regenerative “scarless” healing due to MSC differentiation to replace damaged skin.

A number of challenges need to be overcome before MSCs can be used to effectively treat difficult to heal wounds. First, there needs to be a consistent definition of MSC identity with thorough characterization prior to therapeutic administration. In addition, MSC delivery and engraftment needs to be optimized in order to increase both MSC paracrine signaling and differentiation in the wound. Understanding factors that promote MSC differentiation into skin phenotypes in vivo will also be critical for tailoring MSC-based therapies to prevent or reduce scarring. Questions also remain about how exactly MSC paracrine signaling improves wound repair. Identification of MSC-derived signals, target cells and their responses in vivo is needed. Further investigation is also required to determine whether the MSC secretome is regulated spatiotemporally due to crosstalk with resident wound cells during the repair process. The effects of the wound microenvironment such as ischemia, oxidative stress and metabolic perturbations on the MSC phenotype and secretome also remain unknown. Addressing such challenges and questions will determine whether MSC-based therapies can successfully prevent or cure wounds that are currently difficult to treat.

Acknowledgments

We thank Leigh Martin, Lara Muffley and Andria Smith for excellent technical assistance, Dr. Frank Isik for initiating this project, Dr. Jan Johnson and Jeffrey Bradley for critical comments on the manuscript. Our work on MSCs and wound repair has been supported by grant number R01 GM073624 from the NIH National Institute of General Medicine and by grant number P30 DK017047 from the NIH National Institute of Diabetes and Digestive and Kidney Diseases.

Abbreviations

MSC	multipotent mesenchymal stromal cell and/or mesenchymal stem cell
GFP	green fluorescent protein

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Table 1

Summary of MSC treatment protocols, outcome and potential mechanism of action

Wound model	Delivery method	Results	Mechanism	Ref.
Murine excisional wound treated with bone marrow stromal progenitors (diabetic mouse model)	Topical delivery - single treatment with 7.5×10^5 cells	Enhanced epithelialization, granulation tissue formation and angiogenesis	No evidence of MSC differentiation	[25]
Murine excisional wounds treated with murine bone marrow-derived GFP+ MSCs (diabetic mouse model)	Topical delivery using fibrin spray	Accelerated wound closure	MSC differentiation into blood vessels	[30]
Murine excisional splinted wounds treated with allogeneic or syngeneic bone marrow-derived GFP+ MSCs	Topical delivery in matrigel and four intradermal injections - single treatment with 1×10^6 cells	Accelerated wound closure and increased epithelialization, cellularity and angiogenesis	MSC differentiation into epidermal keratinocytes	[26, 36]
Murine excisional splinted wounds treated with bone marrow-derived MSC-conditioned medium	Topical delivery and subcutaneous injection of MSC-conditioned medium	Accelerated wound closure with increased numbers of macrophages and endothelial progenitors	MSC paracrine signaling	[22]
Murine model of granulation tissue deposition treated with bone marrow-derived GFP+ MSCs	Implantation of MSC loaded PVA sponges - single treatment with 2.5×10^5 cells	Increased deposition of granulation tissue	MSC differentiation into wound vasculature	[27]
Murine excisional wounds treated with bone marrow-derived GFP+ MSCs	Systemic delivery - single treatment with 1×10^6 cells	Accelerated wound closure	MSC differentiation into keratinocytes, endothelial cells and pericytes	[28]
Murine excisional wounds treated with human adipose-derived MSCs	Topical delivery using collagen gel solution - single treatment with 1×10^6 cells	Accelerated wound closure	Not addressed	[32]
Murine excisional wounds treated with human adipose-derived MSC-conditioned medium	Topical delivery using collagen gel solution mixed with MSC-conditioned medium	Accelerated wound closure	MSC paracrine signaling	[33]
Rat incisional wounds treated with syngeneic or allogeneic bone marrow-derived MSCs	Systemic or local injection - single treatment with 5×10^6 cells or 2×10^6 cells once daily for four days	Increased wound bursting strength with increased collagen content	Not addressed	[24]
Rat excisional wounds treated with BrdU labeled bone marrow-derived MSCs	Intravenous injection - single treatment with 1×10^6 cells	MSCs localized to hair follicles, sebaceous glands, blood vessels and dermis	MSC differentiation into keratinocytes	[29]
Rabbit incisional wounds treated with human bone marrow-derived MSCs	Intradermal injection – single treatment with 1.5×10^6 cells	Increased wound tensile strength and reduced scarring	Not addressed	[31]
Rabbit excisional wounds treated with rabbit bone marrow-derived MSCs	Topical delivery – grafted with human amniotic membranes loaded with 2×10^5 cells	Accelerated wound closure	Not addressed	[35]
Human acute and chronic wounds treated with	Topical delivery using fibrin spray	Reduction in chronic wound size in humans	Not addressed	[30]

Wound model	Delivery method	Results	Mechanism	Ref.
autologous bone marrow-derived MSCs				

MSC, multipotent mesenchymal stromal cell and/or mesenchymal stem cell; GFP, green fluorescent protein