



## Mesenchymal stem cells: potential for therapy and treatment of chronic non-healing skin wounds

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**ABSTRACT.** Wound healing is a complex physiological process including overlapping phases (hemostatic/inflammatory, proliferating and remodeling phases). Every alteration in this mechanism might lead to pathological conditions of different medical relevance. Treatments for chronic non-healing wounds are expensive because reiterative treatments are needed. Regenerative medicine and in particular mesenchymal stem cells approach is emerging as new potential clinical application in wound healing.

In the past decades, advance in the understanding of molecular mechanisms underlying wound healing process has led to extensive topical administration of growth factors as part of wound care. Currently, no definitive treatment is available and the research on optimal wound care depends upon the efficacy and cost-benefit of emerging therapies.

Here we provide an overview on the novel approaches through stem cell therapy to improve cutaneous wound healing, with a focus on diabetic wounds and Systemic Sclerosis-associated ulcers, which are particularly challenging. Current and future treatment approaches are discussed with an emphasis on recent advances.

**KEYWORDS.** adipose stem cells, autoimmune diseases, diabetes, mesenchymal stem cells, systemic sclerosis, ulcer, wound

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**ABBREVIATIONS.** ADSCs, Adipose derived stem cells; APCs, antigen presenting cells; BM-MSCs, Bone Marrow Mesenchymal Stem Cells; EGF, Epidermal Growth Factor; E-MSCs, Endometrium Mesenchymal Stem Cells; FDA, Food and Drug Administration; FGF-2, Fibroblast Growth Factor 2; GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor; GMP, Good Manufacturing Practices; IL, Interleukin; INF- $\gamma$ , Interferon Gamma; MSCs, Mesenchymal stem cells; NK, Natural Killer; PDGF, Platelet derived growth factor; PGE2, Prostaglandin E2; SDF-1, Stromal Cell-Derived Factor 1; SSc, Systemic Sclerosis; SVF, stromal vascular fraction; TGF- $\beta$ , Transforming Growth Factor Beta; TNF- $\alpha$ , Tumor Necrosis Factor Alpha; UCB-MSCs, Umbilical Cord Blood Mesenchymal Stem Cells; VEGF, Vascular Endothelial Growth Factor; vWF, Von Willebrand factor

## INTRODUCTION

A wound is defined as a disruption of the normal anatomic structure and functional integrity of the skin.<sup>1</sup> Chronic or non-healing wounds are wounds that do not progress through the normal wound healing process, resulting in an open laceration of varying degrees of severity.<sup>2</sup> These conditions may be associated to a number of different pathological conditions such as diabetes,<sup>3</sup> venous stasis,<sup>4</sup> autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease,<sup>5</sup> and and Systemic Sclerosis (SSc),<sup>6</sup> for which no definitive therapies are currently available. It has been suggested that all the aforementioned pathological conditions generally lead to a hyper-inflammatory environment that contributes to the impairment of the physiological healing processes.

It has been estimated that wound lesions affect more than 5.7 million people in the US, and that the relative annual health care costs account approximately for 20 billion US dollars.<sup>7</sup> Among the 150 millions of patients affected with diabetes worldwide, 15% suffer from foot ulcerations which often evolve into non-healing chronic wounds.<sup>8</sup> Furthermore, annually, 2.5 million pressure ulcers need treatment in the US,<sup>9</sup> 600,000 patients suffer from venous ulcers<sup>10</sup> and 1.1 million burn injuries require medical attention.<sup>11</sup>

Wound healing is characterized by a multi-step interactive process that leads to the restoration of a functional dermis/epidermis layer and revascularization of the skin.<sup>12</sup> The initial phase of healing is characterized by an

inflammatory reaction aimed at controlling bleeding at the wound site through a complex interaction of activated cells with coagulation proteins and complement mediators. The latter help the recruitment and the infiltration of neutrophils and mast cells that clear the wound from dead cells, debris, foreign particles and bacteria. This process leads to the formation of granulation tissue that favors the transition from inflammation to repair.<sup>12</sup>

Patients with autoimmune diseases, such as diabetes or SSc, have experienced vascular damage, that leads to severe complications such as ulcers often associated with severe pain strongly impairing daily life activities.<sup>13,14</sup> In these patients peripheral ulcerations are sustained by chronic local inflammation, that prevents healing processes.<sup>14,15</sup>

Conventional therapies for non-healing wounds have been primarily targeted on the determinants of such chronic inflammation by enhancing tissue repair through the use of specific growth factors (GFs). Among these, becaplermin, a recombinant human platelet-derived growth factor (PDGF), has been approved for the treatment of diabetic wounds in association with good local care. Limitations to the use of this GFs are: malignancies, infections at the site of the ulcer and the contraindication in the case of ulcers with a diameter > than 5 cm<sup>2</sup> or ulcers that require prolonged treatment ulcers to treat.<sup>16</sup> Furthermore, the clinical outcome of these approaches has been discouraging as the efficacy of these drugs is jeopardized by specific neutralization and/or biological degradation. Finally, a recent Cochrane Database Systematic Review found

that many randomized clinical trials were conducted with a high risk of bias, and that further trials are needed before drawing a firm conclusion that GFs are able to increase the likelihood of a complete wound healing in diabetic foot ulcers.<sup>17</sup>

Cell-based therapies are being gradually introduced into routine medical care to manage skin wounds because they can repair/replace damaged tissue with a normal one due to their natural ability to produce cytokines and molecules necessary for wound healing. Mesenchymal stem cell therapy, with particular focus on adipose derived stem cells (ADSCs), is a novel approach for the treatment of chronic non-healing wounds through: (1) structural repair via cellular differentiation; (2) immune-modulation; (3) secretion of growth factors that drive neovascularization and re-epithelialization; and (4) mobilization of resident stem cells.<sup>11</sup>

### **MESENCHYMAL STEM CELLS DESCRIPTION**

Mesenchymal stem cells (MSCs) are multipotent and self-renewable progenitor cells, identified for the first time in the bone marrow in the '50s as fibroblast precursors. After their discovery, MSCs have been isolated in several tissues, including adipose tissue, bone marrow, umbilical cord blood, peripheral blood, endometrium, dental pulp, dermis, amniotic fluid,<sup>12-15,18,19</sup> as well as in tumors.<sup>20</sup>

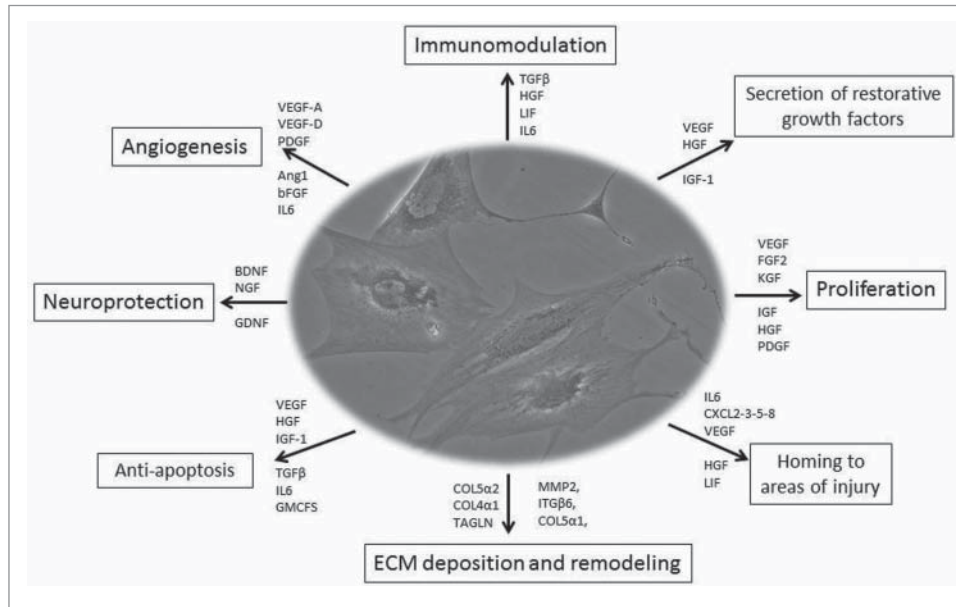
Regardless of their origin, MSCs exhibit a wide differentiating potential, since they are able to give rise to specialized cells of mesodermal origin (i.e., osteocytes, adipocytes, chondrocytes, myoblasts, and tenocytes), and to differentiate into cells of ectodermal origin.<sup>19</sup> Even though MSCs are usually defined by their ability to differentiate into tissues *in vitro*, their trophic, paracrine and immunomodulatory functions are those that may have the greatest therapeutic impact *in vivo*.<sup>22</sup> A large body of medical literature indicates that MSCs are able to repair damaged tissues, because they can migrate toward injured sites in response to inflammation, differentiate into cells and influence the microenvironment

through the release of molecules involved in reparative processes and tissue regeneration such as cytokines (i.e., PGE2, GM-CSF, IL-1, RA, IL-7, IL-8, IL-10, and IL-11), chemokines (as SDF-1) and growth factors<sup>22-25</sup> (**Fig. 1**). In addition, MSCs participate to tissue rescue through pro-angiogenic, anti-fibrotic, and anti-apoptotic pathways<sup>26-28</sup> and a strict correlation between MSCs and blood vessel density in stromal vascularized tissues exists.<sup>29</sup> Stem cells live and reproduce themselves in the morpho-functional unit called niche, in which a huge network of messages (i.e., the "secretome"), is fashioned through the embedded cells.<sup>30</sup> MSCs play a pivotal role in all the phases of the healing process that starts at the wound margin where epidermal cells proliferate and new blood capillaries grow to form granulation tissue. Furthermore, MSCs stimulate endothelial cell recruitment through the secretion of pro-angiogenic factors such as vascular endothelial growth factor (VEGF), modulate tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) production and reduce Natural Killer (NK) cell function in the inflammatory phase, lowering interferon- $\gamma$  (IFN- $\gamma$ ) activity in the process. In the last phase of wound healing, MSCs modulate scar formation through PGE2 secretion, IL-10 up-regulation, IL-6 and IL-8 down-regulation and reduction of collagen production.<sup>31-33</sup> Finally, MSCs have immunomodulatory properties through the production of anti-inflammatory cytokines and the inhibition of CD4+ and CD8+ T cells, B-cells, and NK cells proliferation.<sup>34</sup> On the basis of safety and efficacy in preclinical and clinical preliminary reports,<sup>21</sup> MSC therapy represents a method to treat conditions that currently result in generally poor outcomes or invasive surgery. Indeed, MSC require further investigations to determine *in vivo* distribution of cells and their therapeutic mechanisms, to optimize its use in personalized regenerative medicine.

### **TYPES OF MESENCHYMAL STEM CELL**

In 2006, the Mesenchymal and Tissue Stem Cell Committee of the International Society for

FIGURE 1. Mesenchymal stem cell therapy: role and function Depending on the microenvironment, MSCs are able to secrete several factors which may exert different functions via the release of different types of molecules involved in angiogenesis, immunomodulation, homing, ECM deposition and remodelling, proliferation, anti-apoptosis, and neuroprotection.<sup>26-28</sup>



Cellular Therapy (ISCT) defined the minimal criteria to define the phenotype of MSCs: i) ability to adhere in culture conditions, ii) surface expression of CD105, CD73 and CD90, but not of CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR, and iii) differentiation ability toward osteocytes, chondrocytes and adipocytes.<sup>35</sup> Although ISCT criteria require CD34 negativity, recent reports demonstrate that MSCs originated from adipose tissue express CD34 as a progenitor marker that distinguishes a distinct subset of cells with pronounced differentiation capacity.<sup>36</sup> MSCs can be derived from several tissues, but the best source to develop MSC-based regenerative therapies has not been identified yet.

### **Bone marrow mesenchymal stem cells (BM-MSCs)**

Bone marrow is constituted by a heterogeneous cell population of stromal cells forming the niche responsible for the maintenance of haematopoietic stem cells. *In vitro* culture of

BM-MSCs shows that this population is composed of a mix of tri-, bi-, and mono-potent cells. This heterogeneity could determine the BM-MSCs growth, senescence and differentiation potentials. Recent reports on direct injection of BM-MSCs into injured tissues demonstrated improved repair through mechanisms of differentiation and/or release of paracrine factors.<sup>37-38</sup>

Although bone marrow represents the main source of MSCs, this has some limitations. Indeed, the aspiration of BM-MSCs is an invasive procedure, the amount of cells is modest and their differentiation potential decreases with age.<sup>39,40</sup>

### **Umbilical cord blood mesenchymal stem cells (UCB-MSCs)**

An alternative and attractive source of MSCs is represented by umbilical cord blood that is easier to be collected than bone marrow<sup>41</sup> and shows interesting immunoregulatory properties.<sup>42</sup> Several reports show the therapeutic

potential of UCB-MSCs in humans. There is evidence that UCB-MSCs can improve wound healing and UCB-MSCs CD34+ cells were employed to treat skin wounds refractory to conventional treatment including surgery.<sup>43</sup> Moreover, several clinical trials are ongoing to evaluate the application of these cells in the treatment of burns (clinicaltrials.gov NCT01443689), and chronic diabetic wounds (clinicaltrials.gov NCT01413035).

### ***Endometrium mesenchymal stem cells (E-MSCs)***

Also human endometrium represents a promising alternative source of MSCs that can be retrieved after hysterectomy or diagnostic curettage and from menstrual blood.<sup>44</sup>

Meng and co-workers demonstrated that endometrium-derived MSCs (E-MSCs) can be rapidly expanded *in vitro* and differentiated into several functional cells including cardiomyocytes, respiratory epithelium, neuronal cells, endothelial cells, pancreatic cells, myocytes, hepatocytes, adipose cells and osteocytes.<sup>15</sup> Murphy and colleagues demonstrated that E-MSCs show interesting regenerative capacities, especially at ischemic sites, where they are able to induce angiogenesis.<sup>45</sup> Recently, autologous tissue engineered scaffolds using artificial meshes and E-MSCs were prepared for regenerative therapy.<sup>46</sup> They were demonstrated to be suitable for fascial repair.<sup>47</sup> E-MSCs enhance neovascularization, reduce chronic inflammation, support tissue integration – likely because of their capability to modulate tissue response toward foreign materials – and promote distensibility of the artificial mesh.<sup>48,49</sup> Overall, these features make E-MSCs very suitable for wound repair.

### ***Induced pluripotent stem (iPS) cells***

Among the main sources of MSCs that might be used in the repair and regeneration of injured skin, induced Pluripotent Stem (iPS) cells have been used to study disease mechanisms, to test

drugs and to develop personalized cell therapies. iPS cells are a type of pluripotent stem cell artificially derived from a non-pluripotent cell, typically an adult somatic cell, by inducing expression of a defined set of transcription factors<sup>49</sup> or recombinant proteins channeled into the cells via poly-arginine anchors.<sup>50</sup> iPS cells were first produced in 2006 from mouse cells<sup>51</sup> and in 2007 from human cells.<sup>52</sup> MSCs derived from iPS cells (iPS-MSCs) offer the advantages of both MSCs and IPS cells: abundance, passaged >40 times in culture, sustain the self-renewal capacity, and they are also no longer tumorigenic.<sup>53</sup> In wounds, iPS-MSCs have been demonstrated to participate in tissue repair after autologous transplantation without immunological rejection.<sup>53</sup> The transplanted cells in hindlimb muscles and peripheral nerves of mouse model for diabetic polyneuropathy (DPN), ameliorated nerve conduction velocities, plantar skin blood flow, increased the number-to-muscle fiber ratios, suggesting that iPS-MSC transplantation might have therapeutic effects on DPN through secreting angiogenic/neurotrophic factors and differentiation to Schwann cell-like cells.<sup>54</sup> Moreover, recent studies have further support the use of iPS in skin regeneration. The authors successfully used human keratinocyte-derived iPSCs to reconstitute skin *in vitro* for the treatment of recessive dystrophic epidermolysis bullosa.<sup>55</sup> Indeed, active studies in both animal models and future clinical trials need be conducted to develop effective dosing, timing and delivery routes.

### ***Adipose derived mesenchymal stem cells (ADSCs)***

Basic research and preclinical studies of regenerative medicine have been mainly focused on adipose-derived mesenchymal stem cells (ADSCs). In the last years safety and efficacy of implanted ADSCs in different animal models have been investigated and promising preclinical studies with good perspectives for translational approaches are underway.

The adipose tissue is a highly specialized and complex connective tissue with important

functions such as: i) protection or cushion from mechanical injury, ii) insulation against cold, iii) structural and metabolic support as an energy reservoir through fat accumulation, iv) dynamic participation in endocrine physiology. The adipose tissue is considered an important source of restorative growth factors,<sup>56,57</sup> it has multi-lineage differentiation capacity,<sup>58</sup> it can induce immunosuppression of activated immune cells,<sup>59</sup> it is able to homing to areas of injury,<sup>60</sup> and it has *in vivo* differentiation capacity to recreate a physiological condition when transplanted into a pathological microenvironment.<sup>61</sup>

Unlike BM-MSCs, ADSCs can be obtained in large quantities at low risk. They are more abundant on a per gram basis (50,000 vs. 100–1,000) and more easily accessible than BM-MSCs.<sup>62</sup> However, studies have demonstrated that not all fat depots are equal in terms of quality of ADSCs, whereby the percentages of stem cells range from 1 to 10%, most likely depending on the donor and tissue harvesting site. ADSCs harvested from the superficial abdominal depot above Scarpa's layer have been shown to be more resistant to apoptosis than other subcutaneous depots including the arm, hip, and thigh regions.<sup>63</sup> In addition, younger patients appear to have increased induction of their ADSCs than older patients.<sup>64</sup> In humans, subcutaneous adipose tissue can be obtained by liposuction aspirate (preferable option) or during reconstructive surgery. At variance with the latter during which adipose tissue is obtained in solid pieces, the isolation of adipose tissue after liposuction is rather simple, as the procedure yields already finely minced homogeneous tissue fragments on which the enzymatic digestion is more efficient. In 2001 Zuk et al. developed an ADSCs isolation protocol that has become the most widely used up to now.<sup>58</sup> More recently, Bianchi and colleagues<sup>65</sup> described an innovative system, named Lipogems, providing a non-expanded, ready-to-use fat product. This system used mild mechanical forces in a completely closed system, avoiding enzymes, additives, and other manipulations. This innovative enzyme-free technology has been developed to process variable amounts of lipoaspirates, resulting in a non-expanded

adipose tissue product that contains human ADSCs.<sup>65,66</sup>

The functional cells of adipose tissue are adipocytes, which respond to insulin, secrete adipokines such as leptin and adiponectin, and store triglycerides in large, lipid-filled vacuoles.

Although adipocytes constitute almost 90% of adipose tissue volume,<sup>67</sup> adipose tissue yields a heterogeneous population of many other cell types including ADSCs, preadipocytes, endothelial cells, pericytes, haematopoietic -lineage cells, and fibroblasts. Approximately  $0.5 \times 10^4$ – $2 \times 10^5$  ADSCs can be isolated per gram of adipose tissue.<sup>63</sup>

Most sources indicate that in the SVF, ADSC frequency is of 5.1–20%. Endothelial cells (mature and progenitors) are identified through the expression of CD146+/CD31+/CD144+/VEGFR2+ and could represent from 7% up to ~30% of SVF.<sup>68</sup> Depending on processing, fibroblasts could represent up to 50% of SVF.<sup>69</sup> CD34+ cells are present at large number and could compose up to 63% of SVF. It has also been described that the SVF is composed of nearly 11% CD14+ cells, ~2% CD31+ cells, ~7% CD34+, ~9% CD45+ cells, ~29% CD90+, and ~47% 146+ cells.<sup>70</sup> Other studies indicated that SVF of human adipose tissue contained: endothelial progenitors, pericytes, CD146+/CD34+ transitional cells, and supra-adventitial adipose stromal cells<sup>71</sup> (**Fig. 2**).

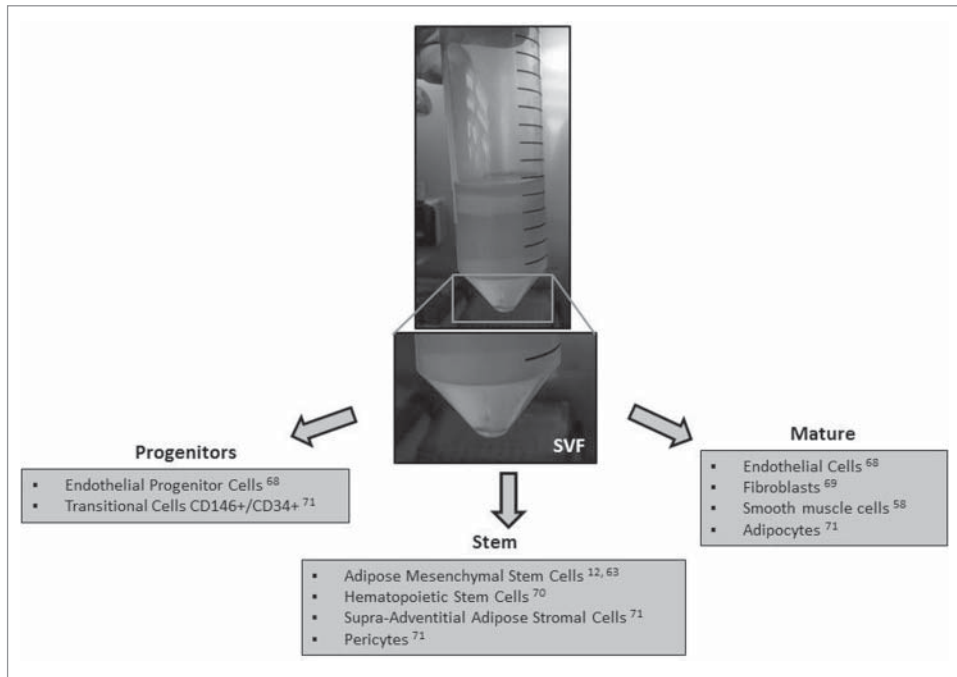
In summary, the stromal vascular fraction (SVF) of adipose tissue is composed of many mature, progenitor and stem cell types. Therefore, depending on adipose tissue processing method, the composition of SVF and relative values of each cell population can differ significantly.

In the following sections we will examine the recent results by ADSC therapy in chronic wound-pathologies as diabetes, and autoimmune diseases, with particular attention for systemic sclerosis (SSc).

## WOUND HEALING

Wound healing is a complex and dynamic process of replacing devitalized and missing

FIGURE 2. SVF features SVF is composed of many mature, progenitor and stem cell types.



cellular structures and tissue layers. The human adult wound healing process can be divided into 3 distinct programmed phases: i) hemostasis/inflammation, ii) proliferation, and iii) remodelling. These phases and their biophysiological functions must occur in the proper sequence, at a specific time, and continue for a specific duration at an optimal intensity by the actions of the main actors represented by all skin cells, keratinocytes, fibroblasts, endothelial cells, nerve cells, immune cells as well as blood cells (i.e., white blood cells, red blood cells, platelets).

1. hemostasis/Inflammation begins at the time of injury and lasts for 24 to 48 hours. This phase begins with hemostasis and leads to inflammation. Platelets form the initial thrombus release growth factors that induce the chemotaxis and proliferation of neutrophils and macrophages, which then become the prominent cell of this phase and release various growth factors and cytokines that change the moderately acellular wound into a highly cellular environment.

2. Fibroblasts proliferate to become the dominant cell of the proliferative phase. They produce collagen, which provides structure to the wound and replaces the fibronectin–fibrin matrix with angiogenesis of new capillaries and with the epithelialization support of Keratinocytes.

3. In the remodelling phase collagen synthesis and degradation reach equilibrium. Fibroblasts organize and cross-link the collagen, wound strength gradually increases, wound contraction occurs, and fibroblast density decrease.

Impaired wound healing occurs for defects in the normal tissue response to injury due to local or systemic factors and to poor treatment of the wound, resulting in chronic skin lesions, or ulcers.

Chronic or non-healing wounds are wounds that do not follow the normal wound healing process, resulting in an open laceration of varying degrees of severity.<sup>72</sup> This disorder may be associated to a number of different pathological conditions such as diabetes,<sup>73</sup> venous stasis,<sup>74</sup> and chronic autoimmune



diseases, such as systemic lupus erythematosus, rheumatoid arthritis, Crohn's disease,<sup>75</sup> and SSc,<sup>76</sup> for which effective therapies are not currently available.

It has been suggested that all these diseases generally contribute to the generation of a hyper-inflammatory environment that further impairs the physiological healing processes. Chronic ulcers affect over 6 million people in the United States, with an incidence that is expected to grow mirroring the increasing mean age of general population and the number of subjects affected by diabetes. Chronic ulcers strongly affect the quality of life and productivity of the patients, representing a financial burden to the health care system. The average cost of treatment per patient is about \$ 1,000 and in 46% of patients the healing process lasts 26 weeks, in 15% of subjects may last up to 2 y<sup>77</sup>

Cell-based therapies are slowly gaining ground in routine medical care and, especially, in wound management of skin. They offer promise for the repair and/or replacement of damaged tissue and the restoration of lost functionality because they possess many of the criteria necessary for wound healing.<sup>78</sup>

### **ADSCS APPLICATION FOR WOUND HEALING THERAPY**

The ability of ADSCs to play a role in wound healing seems strictly related to their anti-inflammatory properties, which also aids the tolerance of transplanted cells even in the case of allogenic ADSC both in acute and in chronic conditions, as they exhibit pleiotropic immune regulatory activities (e.g., inhibit the function of different immune cell subpopulations of the innate and adaptive immunity). These properties are mediated by the release of soluble paracrine factors and by direct cell-to-cell interactions with professional antigen presenting cells (APCs) such as dendritic cells,<sup>79</sup> T cells,<sup>80,81</sup> B cells<sup>82</sup> and macrophages.<sup>83</sup> ADSCs are able to block APCs maturation in a contact-dependent manner, to induce the expression of anti-inflammatory cytokines such as IL-10 and to enhance TGF- $\beta$  activity.<sup>57</sup>

The application of ADSCs in wound repair and tissue regeneration has been demonstrated in a number of experimental models both *in vitro* and *in vivo*. ADSCs in cutaneous wounds significantly accelerated the re-epithelization by promoting human dermal fibroblast proliferation through direct cell-cell contact or via paracrine secretion of a variety of growth factors. In a full thickness excisional injury model in rats, ADSCs were shown to enhance neovascularogenesis and to accelerate wound closure via secretion of VEGF-A, hepatic growth factor, and FGF-2,<sup>84</sup> and thus promoting subsequent angiogenesis and proliferation of keratinocytes or dermal fibroblast.<sup>57</sup> This study also validated the differentiation potential of ADSCs into endothelial and epithelial cell types, supporting the applicability of ADSCs to tissues regeneration.

### **FOCUS ON ADSCS APPLICATION FOR WOUND HEALING IN DIABETES**

Impaired wound healing is a major clinical problem in diabetic patients leading to limb amputation in several cases.<sup>85</sup> Cell-based therapies are promising in this field and ADSCs are good candidates.<sup>84</sup> Recent preclinical studies, including animal models of diabetes, showed the beneficial effect of ADSC administration in promoting wound healing.<sup>86</sup>

Kuo and colleagues investigated the effect of ADSCs transplantation into streptozotocin-induced diabetes rodent model wounds. Results revealed that the complete wound healing time was significantly decreased in the ADSC-treated group compared to controls. Moreover, histological examination revealed that the ADSC-treated group showed a significant reduction in the pro-inflammatory reaction, with significantly increased levels of EGF, VEGF, rPH, and Ki67 expression with increased angiogenesis via vWF and VEGF expression. The authors hypothesized that ADSC treatment significantly stimulates neo-angiogenesis and increases tissue regeneration through paracrine and autocrine mechanisms.<sup>87</sup>

Kim et al demonstrated that the administration of ADSCs enhanced wound healing in a mouse model, and that ADSCs promote human dermal fibroblast proliferation, not only by cell-to-cell direct contact, which was confirmed by co-culture experiment, but also by paracrine activation through secretory factors, resolved by transwell co-culture and culturing with conditioned medium of ADSCs.<sup>88</sup> Therefore, it was postulated that ADSCs did not directly influence wound healing as previously thought, but worked indirectly via local mediators.

Indeed, the release of growth factors at the wound site favors the activation of resident cell function, the recruitment of cells, and the differentiation into resident cell types. In fact, the ability of topically applied ADSCs to differentiate into endothelial and epithelial cells as well as their capacity to release large amounts of proangiogenic growth factors have been described.<sup>89</sup>

Evidence of an effective treatment with ADSCs in diabetic ulcers derives from several clinical trials.<sup>90</sup> Nevertheless, there are some limitations to the use of autologous ADSCs, due to an altered phenotype of MSCs in diabetic patients: when characterized phenotypically and functionally, diabetic MSCs were less potent than normal ones, with a decreased expression of VEGF-A and chemokine receptor CXCR4 in fibroblast positive ADSCs. High expression of fibroblast markers associated with reduced expression of VEGF-A may affect the effectiveness of autologous cell therapies in diabetic patients. Therefore, in diabetic patients an allogenic donor could be the optimal source.<sup>91</sup>

### ***FOCUS ON ADSCS APPLICATION FOR WOUND HEALING IN SYSTEMIC SCLEROSIS***

Scleroderma, or systemic sclerosis (SSc), is a chronic multisystem autoimmune disease characterized by vasculopathy, diffuse fibrosis of skin and various organs and immune abnormalities. Patient suffering for SSc have often

hand disability for the presence of digital ulcers that severely interfere with daily life.<sup>8</sup>

Although the pathophysiology of SSc is undoubtedly complex and remains incompletely understood, progresses have been made in elucidating at least some of the multiple mechanisms which are likely to contribute to the vascular and fibrotic alterations.<sup>92</sup> Most research on the changes in vascular and fibrotic features in SSc has focused on the MSCs with conflicting results.<sup>93</sup> BM-MSCs from patients with SSc are similar to those from healthy donors in terms of their phenotype and capacity to differentiate into adipogenic and osteogenic lineages,<sup>94</sup> showed an upregulation of pericyte-specific markers and a decreased proliferation capacity.<sup>95</sup> It has also been shown that SSc MSCs constitutively over-express and release pro-angiogenic mediators compared with healthy control MSCs.<sup>96</sup>

In 2013 an open-label and single arm study was performed in 12 SSc patients in order to evaluate the number of adverse events related to SVF injection.<sup>97</sup> This procedure improved manifestations of peripheral vasculitis as Raynaud's phenomenon, ameliorate digital ulcers and consequently had an impact on hand pain. These results suggest that SVF may improve vasomotor tone and microvascular perfusion. This hypothesis is further substantiated by the significant reduction of avascular areas and dystrophic capillaries evaluated using nailfold capillaroscopy.

In 2014, an Italian group performed a study on finger injection of ADSCs in 15 SSc patients, having a long-lasting digital ulcer in only one fingertip, unresponsive to intensive systemic and local treatment. An improvement in healing time, a significant reduction of pain intensity and an increase in the number of capillaries has been showed after a 6-month follow up period.<sup>98</sup>

Other authors reported data on the therapeutic effects of MSC local or regional transfer in patients with SSc suffering from ischemic lesions in their fingers or limbs. Although only few cases were described, it is noticeable that the adopted procedures have constantly induced an improvement on ischemic lesions.<sup>99</sup>

### **FOCUS ON ADSCS APPLICATION FOR WOUND HEALING IN AUTOIMMUNE DISEASES**

ADSCs have been proposed as candidates for the treatment of wound healing in of different immune-mediated diseases,<sup>100,101</sup> however the greater experience is available with non-ADSCs (generally, BM and UCB-MSCs).<sup>102</sup>

Studies on preclinical efficacy of ADSCs in autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis (mainly collagen-induced arthritis), Crohn's disease (experimental colitis), experimental autoimmune hearing loss, experimental autoimmune thyroiditis, experimental autoimmune encephalomyelitis (model of multiple sclerosis), and immune thrombocytopenia have been carried out in different animal models<sup>103–124</sup> (**Table 1**). The current evidences of cellular therapy for autoimmune diseases in humans are mainly based on non-controlled trials. Few clinical studies on the immunomodulatory properties of ADSCs have been performed; they are mainly case reports based on compassionate-use treatments for rheumatoid arthritis, multiple sclerosis, polymyositis, autoimmune inner ear disease, atopic dermatitis, and autoimmune thrombocytopenic purpura. Moreover, the adipose tissue-derived SVF<sup>58</sup> has been successfully administered instead of *ex vivo* culture-expanded ADSCs in rheumatoid arthritis and multiple sclerosis, showing encouraging results.<sup>124</sup> Also in the case of autoimmune diseases other than diabetes, MSCs have an altered phenotype and function. An allogenic rather than autologous MSC-based therapy might be preferable for treatment. Indeed, autologous MSCs are characterized by an early senescence, but they preserve immunomodulatory properties that support their use anyway.<sup>125</sup>

### **TISSUE BIOENGINEERING**

Regarding skin wound healing, tissue engineering is making strides in creating new biomedical skin substitutes.<sup>126</sup> Indeed, the combination of cell therapy with biomaterials is one of the main challenges to treat wound

healing. Numerous studies of biomaterials for wound dressings have been performed for the improvement of the functions that support wound healing. Materials for wound dressings are required to have good biocompatibility, wound-sealing capability, and to maintain a humid environment to inhibit drying of the wound. Additionally, fabrication is desired for sponge, film, and gel forms to adjust to the wound shape or size. Wound dressings are also required to have an absorption capability of wound exudates fluid, which includes important growth factors to stimulate cells of the immune system. As reported by our group, silk fibroin can possess all these properties and combination of human ADSCs with silk fibroin resulted in accelerated wound healing of diabetic ulcers in impaired db/db diabetic mice.<sup>89</sup>

Moreover, topical application of human ADSCs seeded on a silk fibrin-chitosan scaffold was shown to improve wound repair and these cells were shown to differentiate and contribute to fibrovascular, endothelial, and epithelial components of the reconstituted tissue.<sup>127</sup> Very recent evidence coming from animal models suggest that also allogenic ADSC sheets,<sup>128</sup> or ADSC spheroids assembled on polymer membranes could be a future therapeutic approach.<sup>129</sup> The advantage of such bioengineering techniques is to provide ADSCs with a more favorable milieu for cytokine and chemokine production, as demonstrated by animal models.<sup>129</sup> Furthermore, the use of antibacterial materials such as chitosan may reduce the risk of bacterial growth, thus reducing the incidence of side effect.<sup>130</sup>

### **CLINICAL STUDIES WITH ADSCS AS CELL-BASED THERAPY**

Stem cell research is in its early stages of development and the market is therefore still behind. Approximately 4 million people affected by wound healing impairments for diabetes, autoimmune disease or burns in the US would benefit from cell therapy products.

The US Food and Drug Administration (FDA) defines somatic cell therapy as the administration of autologous, allogenic or

Table 1. Studies on preclinical efficacy of MSCs in animal models for autoimmune diseases

Investigators	MSC type	Animal disease model	Major findings after MSC transplantation	Ref.
Choi EW et al, 2012	Human ADSCs	Systemic lupus erythematosus	Significantly higher survival rate with improvement of histologic and serologic abnormalities and immunologic function, and decreased incidence of proteinuria	103
Choi EW et al, 2015	Human ADSCs	Rheumatoid arthritis	Significantly lower anti-mouse collagen II autoantibody levels, cartilage damage, IL-6 and keratinocyte chemoattractant serum levels	104
Park MJ et al, 2014	Human ADSCs	Systemic lupus erythematosus	Significantly reduction of ICOS(+)CD44(+) follicular helper T cells, Th1 cells and Th17 cells, increment of Foxp3-expressing regulatory T cells	105
Gonzalez MA et al, 2009	Human ADSCs	Rheumatoid arthritis	Significantly reduced the incidence and severity of experimental arthritis by down-regulating the 2 deleterious disease components: the Th1-driven autoimmune and inflammatory responses.	106
Zhou B et al, 2011	Human ADSCs	Rheumatoid arthritis	Prevention and treatment of collagen-induced arthritis by significantly reducing the incidence and severity of experimental arthritis, inhibiting the production of various inflammatory mediators, decreasing antigen-specific Th1/Th17 cell expansion, and inducing the production IL-10.	107
Serratrice N et al, 2014	Human micro-fat	Systemic Sclerosis (SSc)	Significantly reversed dermal and epidermal sclerosis with a significant increase of the local vascularization	108
Gonzalez MA et al, 2009	Human ADSCs	Crohn's disease	Significantly amelioration of the clinical and histopathologic severity of colitis, abrogating body weight loss, diarrhea, and inflammation and increasing survival	109
Gonzalez-Rey et al, 2009	Human ADSCs	Inflammatory bowel diseases	Down regulation of the Th1-driven inflammatory responses and a wide panel of inflammatory cytokines and chemokines and up-regulation of IL-10, acting on macrophages. Protection from severe sepsis by reducing the infiltration of inflammatory cells in various target organs and by down regulating inflammatory mediator production.	110
Goncalves Fda, C, et al, 2014	Murine ADSCs	Crohn's disease	Histological evaluation demonstrated decreased colonic inflammation based on reduced crypt loss and reduced infiltration of inflammatory cells with	111

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Table 1. Studies on preclinical efficacy of MSCs in animal models for autoimmune diseases (*Continued*)

Investigators	MSC type	Animal disease model	Major findings after MSC transplantation	Ref.
Zhou Y et al, 2011	Human ADSCs	Autoimmune hearing loss	down-regulation of IL-6 and TNF and by the up-regulation of IL-10 and IL-4, IFN, and apoptotic T cells Significantly improved hearing function and protected hair cells, decreased the proliferation of antigen-specific Th1/Th17 cells and induced the production of IL-10 in splenocytes, and the generation of antigen-specific CD4(+) CD25(+) Foxp3(+) Treg cells with the capacity to suppress autoantigen-specific T-cell responses.	112
Choi EW et al, 2011	Human ADSCs	Autoimmune thyroiditis	Significantly lower absorbance of thyroglobulin autoantibody; significant decrease in the proportion of CD3+ and CD11b; dramatically decreased of lymphocyte infiltration in the thyroid glands, and of the production of proinflammatory cytokines and down-regulation of Th1 cytokines.	113
Choi EW et al, 2014	Human ADSCs	Autoimmune thyroiditis	Significantly reduction of canine thyroglobulin autoantibodies in serum and of the infiltration of T-lymphocytes between the follicles of the thyroid glands. significantly increment of FoxP3 expression in submandibular lymph nodes.	114
Constantin G et al, 2009	Murine ADSCs	Autoimmune encephalomyelitis	Significantly amelioration of the disease course and reduction of both demyelination and axonal loss, and induction of Th2-type cytokine shift in T cells, secretion of bFGF-, BDNF-, PDGF-AB. with increased number of endogenous oligodendrocyte progenitors.	115
Payne NL et al, 2013	Human ADSCs	Autoimmune encephalomyelitis (EAE)	Significantly reduction in peripheral T-cell proliferative responses, reduction in pro-inflammatory cytokine secretion as well as a preferential inhibition of Th17-mediated neuroinflammation. Inhibition of the phenotypic maturation, cytokine production and antigen presenting capacity of bone marrow-derived myeloid dendritic cells,	116
Scruggs BA et al, 2013	Human ADSCs	Autoimmune encephalomyelitis	ADSCs from older donors failed to ameliorate the neurodegeneration associated with EAE, and mice treated with older donor cells had increased CNS	117

Table 1. Studies on preclinical efficacy of MSCs in animal models for autoimmune diseases (Continued)

Investigators	MSC type	Animal disease model	Major findings after MSC transplantation	Ref.
Yousefi, F et al, 2013	Human ADSCs	Autoimmune encephalomyelitis	Intraperitoneal route showed a more pronounced effect in maintaining the splenic CD4+CD25+FOXP3+ T cell population, increased of IL-4 secretion, decreased IFN- $\gamma$ secretion and reduced cell infiltration in brain more effectively as compared to the intravenous. route. Intraperitoneal route showed a more pronounced effect in maintaining the splenic CD4+CD25+FOXP3+ T cell population, increased of IL-4 secretion, decreased IFN- $\gamma$ secretion and reduced cell infiltration in brain more effectively as compared to the intravenous. route.	118
Semon JA et al, 2013	Murine SVF and ADSCs	Autoimmune encephalomyelitis (EAE)	Intraperitoneal administration of ADSCs significantly ameliorated the severity of disease course. SVF effectively inhibited disease severity and was statistically more effective than ADSCs. Both cell therapies also demonstrated a reduction in tissue damage, a decrease in inflammatory infiltrates, and a reduction in sera levels of IFN- $\gamma$ and IL-12.	119
Zhang X et al, 2014	Murine ADSCs	Autoimmune encephalomyelitis (EAE)	Significantly ameliorate the disease course, autoimmune mediated demyelination and cell infiltration through the regulation of the inflammatory responses, however, mice treated with autologous ADSCs showed no therapeutic improvement on the disease progression.	120
Tafreshi AP et al, 2014	Murine ADSCs	Autoimmune encephalomyelitis (EAE)	Glycogen synthase kinase 3 $\beta$ (GSK3 $\beta$ ) was absent in radial astrocytes and significantly lower in neurons of EAE animals. The loss of phosphorylated GSK3 $\beta$ in radial glia and neurons in EAE spinal cords was concurrent with radial glia migration and astrogliosis. This disturbance in the expression of inactive GSK3 $\beta$ was recovered in neurons, but not in the radial glia, after treatment of EAE mice with ADSCs capable of inducing a Th2 shift.	121
Semon JA et al, 2014	Human ADSCs and BM-MSCs	Autoimmune encephalomyelitis (EAE)	Intraperitoneal administration of all cell types significantly ameliorates the severity of disease.	122

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Table 1. Studies on preclinical efficacy of MSCs in animal models for autoimmune diseases (*Continued*)

Investigators	MSC type	Animal disease model	Major findings after MSC transplantation	Ref.
Bassi EJ et al, 2012	Human ADSCs	Autoimmune diabetes	SVF was as effective as the more commonly cultured BMSCs and ADSCs. All cell therapies demonstrated a similar reduction in tissue damage, inflammatory infiltrates, and sera levels of IFN $\gamma$ and IL-12, with the latter significantly lower in SVF-treated than BMSC-treated or ASC-treated mice. Down regulation of the CD4(+) Th1-biased immune response and expansion of regulatory T cells (Treg) in the pancreatic lymph nodes. Within the pancreas, inflammatory cell infiltration and IFN- $\gamma$ levels were reduced, while insulin, pancreatic duodenal homeobox-1, and active TGF- $\beta$ 1 expression were increased.	123
Xiao J et al, 2012	Human ADSCs	Immune thrombocytopenia (ITP)	Significantly increment of platelets level in the ITP mice. In the ADSC group, IFN- $\gamma$ , IL-2, and IL-17 significantly decreased, while IL-4, IL-10, and TGF- $\beta$ 1 increased.	124

xenogenic non-germ cells excluding blood products for transfusion, which have been manipulated, processed, propagated, expanded, selected *ex vivo*, or drug-treated.<sup>131</sup> Cell therapy products are considered as drugs, so they follow the same regulations, adhering to the Current Good Manufacturing Practices (GMP), which establish minimum quality requirements for their manipulation. The key points of the current FDA regulation for cell therapy products include: demonstrations of preclinical safety and efficacy; no risk of transmission of infectious or genetic diseases from donors; no risk of contamination or other adverse effects of cells or sample processing; specific and detailed determination of cell type, purity and potency of the final product; *in vivo* safety and efficacy of the final product.<sup>132</sup> Clinical applications using ADSCs for wound healing, burns, diabetic foot and chronic limb ischemia are underway throughout Asia, Europe and North and South America. Some of these can be found on the clinicaltrials.gov website [clinicaltrials.gov (Accessed on 20 November 2015)], where 10 studies are listed under the search term “adipose stem cell” AND “wound” (as of 20 November 2015). Of these, 7 studies actually are recruiting patients, 1 is completed, 1 is active and 1 is unknown because it has not been updated (clinicaltrials.gov) (**Table 2**).

### LIMITATIONS

For translational medicine, safety remains a major issue. First of all, although stem cells have been largely studied *in vitro* and in animal models, *in vivo* mechanisms are largely obscure and the biological implications in humans still remain to be proven. Another limitation in the use of MSCs is that they are found in a very small amount in the tissue of origin, thus requiring expansion protocols *in vitro*. During the protocol the risk of contamination of the cells is low, but still possible. Furthermore, also the risk of exposure to prions and of immunological should be considered. These risks are due to the fact that supplements often have an animal origin and they could be overcome with the use of synthetic media. Another risk is the

exposure to toxic agents such as endotoxin. Then, unpredictable fluctuations of the *milieu* of MSCs during expansion or after transplantation, could affect their biological functions.<sup>133</sup> Although acute toxicity seems not to be a major concern, based on findings in >2,000 patients so far exposed to MSCs,<sup>134</sup> and fewer to ADSCs,<sup>135</sup> evidence from animal models suggests that the approach might be associated with loss of endogenous tumor surveillance. In fact, descriptions of the possible involvement of ADSCs in tumorigenesis have been reported *in vitro* e *in vivo*,<sup>135-137</sup> and biological mechanisms are poorly understood. ADSCs are appealing thanks to their ability to proliferate and differentiate also secreting cytokines and growth factors and their immunoregulatory function. Overall, these characteristics may expose the patients both to oncogenic/tumor-supporting risk and to ectopic differentiation risk.<sup>138</sup> There is growing evidence that MSC treatment is safe in humans, although cancer recurrence after fat grafts have been reported in patients whose history was notable for sarcoma,<sup>139</sup> and breast cancer.<sup>140</sup> Finally, the possibility that cell expansion could give rise to genomic abnormalities is still debated, although major concerns derive more from the possibility of an accelerated cell senescence, that could expose the patient to more side effects or to less efficacy. Indeed, Importantly, long-term safety data are lacking and implementation of devoted registries is critical, especially regarding tumor surveillance.

Therefore, despite the encouraging data regarding therapeutic applications, the opportunity of stem cell therapy should be carefully evaluated in each patient, balancing the risks and benefits of a relatively novel technique.

### CONCLUSIONS

In recent years, basic and translation research held great hope for this new field with significant progress in the modulation of stem cell commitment *in vitro* and providing protocols for targeted clinical applications. Recent advances in bioengineering and nanotechnology



Table 2. clinical studies from clinicaltrials.gov as of 20 November 2015

Study	Condition	Status	Location	Phase	Identification number
1 Adipose Derived Regenerative Cellular Therapy of Chronic Wounds	Diabetic Foot; Venous Ulcer; Pressure Ulcer	Rank	Tower Outpatient Surgical Center, Los Angeles, California, USA	II	NCT02092870
2 Adipose-Derived Stromal Cells (ASC's) and Pressure Ulcers	Pressure Ulcer	Recruiting	Mayo Clinic in Florida, USA	I	NCT02375802
3 Autologous Adipose-Derived Stromal Cell Delivered Via Intramuscular Injections for the Treatment of Critical Limb Ischemia	Critical Limb Ischemia	Recruiting	Ageless Institute, Miami, Florida, USA	II	NCT02099500
4 Autologous Adipose-derived Stem Cells (ASCs) for the Treatment of Perianal Fistula in Crohn Disease: A Pilot Study	Perianal Fistula; Crohn Disease	Recruiting	Paolo Bertoli, Bergamo, Italy	II	NCT02403232
5 To Evaluate the Safety and Efficacy of IM and IV Administration of Autologous ADMSCs for Treatment of CLI	Critical Limb Ischemia	Recruiting	Kasiak Research Pvt Ltd, Thane, Maharashtra, India	I and II	NCT02145897
6 The Role of Lipoaspirate Injection in the Treatment of Diabetic Lower Extremity Wounds and Venous Stasis Ulcers	Diabetic Wounds Venous Stasis Wounds	Unknown †	Veterans Affairs Medical Center, Washington D.C, District of Columbia, USA	I	NCT00815217
7 Treatment of Hypertensive Leg Ulcer by Adipose Tissue Grafting	Skin Ulcer	Recruiting	University Hospital, Caen, France	I	NCT01932021
8 Feasibility Study of the TGI Adipose-derived Stromal Cell (ASC)-Coated ePTEF Vascular Graft	Lower Limb Ischemia	Active, not recruiting	University of Louisville Hospital, Kentucky, USA	I and II	NCT01305863
9 Safety of ALLO-ASC-DFU in the Patients With Diabetic Foot Ulcers	Diabetic Foot Ulcer	Completed	Asan Medical Center Seoul, Republic of Korea	I	NCT02394886
10 A Study to Evaluate the Safety of ALLO-ASC-DFU in the Subjects With Deep Second-degree Burn Wound	Burn	Recruiting	Hallym university Medical Center, Seoul, Republic of Korea	I	NCT02394873

have allowed researchers to manipulate micro-environments in increasingly precise spatial and temporal scales, recapitulating key homeostatic cues that may drive regeneration. MSCs are able to secrete a large number of trophic factors capable of repairing the recipient tissue through angiogenic, anti-apoptotic and anti-fibrotic mechanisms. In this context, adipose tissue is emerging as a clinically relevant and easy to harvest source of multipotent progenitors to develop regenerative therapies. The application of ADSCs in wound repair and tissue regeneration has been shown in a number of experimental models both *in vitro* and *in vivo*. The positive outcome obtained with this therapeutic approach, although promising, is limited to small cohorts of patients and needs to be confirmed in larger and controlled studies.

Based on the interesting available evidence in the literature, we are confident that MSCs cell therapy will be a promising and important strategy for chronic wound repair in a next future. Understanding the dynamics that regulate MSCs homeostasis, especially their anti-inflammatory effect and immunomodulatory capacity, has led to challenge a number of consolidated beliefs on their therapeutic mechanisms. Since the precise mechanism which allows this effect is not completely understood, more studies, focused on the role of adult stem cells in wound healing, are needed in order to address this question and improve the efficacy of this therapy.

Furthermore the possibility to obtain a final cell product containing viable adipocyte, pre-adipocyte, and stem cells, eliminating problems related to enzymatic digestion and other manipulations, exhibits a great appeal not only for its application in plastic and reconstructive medicine, but also in research, and regenerative medicine.

#### **DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

No potential conflicts of interest were disclosed.

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