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Local delivery of allogeneic bone marrow and adipose tissue-derived mesenchymal stromal cells for cutaneous wound healing in a porcine model

Summer E. Hanson^{#1,4,5}, Kyle R. Kleinbeck^{#2}, David Cantu², Jaeyhup Kim³, Michael L. Bentz^{1,4}, Lee D. Faucher⁴, W. John Kao^{2,4,5}, and Peiman Hematti^{3,6,*}

¹Division of Plastic and Reconstructive Surgery, Department of Surgery, UW-Madison, School of Medicine and Public Health

²Division of Pharmaceutical Sciences, UW-Madison

³Department of Medicine, UW-Madison, School of Medicine and Public Health

⁴Department of Surgery, UW-Madison, School of Medicine and Public Health

⁵Department of Biomedical Engineering, UW-Madison, College of Engineering

⁶University of Wisconsin Carbone Cancer Center, UW-Madison, School of Medicine and Public Health, Madison, WI 53705, USA

These authors contributed equally to this work.

Abstract

Wound healing remains a major challenge in modern medicine. Bone marrow- (BM) and adipose tissue- (AT) derived mesenchymal stromal/stem cells (MSCs) are of great interest for tissue reconstruction due to their unique immunological properties and regenerative potential. The purpose of this study was to characterize BM and AT-MSCs and evaluate their effect when administered in a porcine wound model. MSCs were derived from male Göttingen Minipigs and characterized according to established criteria. Allogeneic BM- or AT-MSCs were administered intradermally (1×10^6 cells) into partial-thickness wounds created on female animals, and covered with Vaseline® gauze or fibrin in a randomized pattern. Animals were euthanized at 7, 10, 14 and 21 days. Tissues were analyzed visually for healing and by microscopic examination for epidermal development and remodelling. Polymerase chain reaction (PCR) was used to detect the presence of male DNA in the specimens. All wounds were healed by 14 days. MSC-injected wounds were associated with improved appearance and faster re-epithelialization compared to saline controls. Evaluation of rete ridge depth and architecture showed that MSC treatment promoted a faster rate of epidermal maturation. Male DNA was detected in all samples at days 7 and 10, suggesting the

*Correspondence to: University of Wisconsin Carbone Cancer Center, UW-Madison, School of Medicine and Public Health, WIMR 4033, 1111 Highland Ave, Madison, WI 53705, USA. Ph: (608) 265-0106, Fax: (608) 262-1982 pxh@medicine.wisc.edu.

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presence of MSCs. We showed the safety, feasibility and potential efficacy of local injection of allogeneic BM- and AT-MSCs for treatment of wounds in a preclinical model. Our data in this large animal model support the potential use of BM- and AT-MSC for treatment of cutaneous wounds through modulation of healing and epithelialization.

Keywords

mesenchymal stromal/stem cells; tissue regeneration; cutaneous wounds; cell-based therapy

1. Introduction

Cutaneous wounds remain a major challenge in modern medicine, affecting not only physical and psychosocial health of affected patients, but also productivity and healthcare expenditures. Normal wound healing is a complex process based on interactions between many cells, including epithelial cells, endothelial cells, inflammatory cells and fibroblasts as well as resident stromal cells either present in the tissue microenvironment or recruited from circulation. The natural progression in this multistep process includes the transition from hemostasis through inflammation to organized tissue regeneration (Martin, 1997; Stappenbeck and Miyoshi, 2009). As a result, there is a variety of stages during the healing process (inflammatory and reparative) during which one can intervene to affect clinical outcomes.

The causative mechanisms behind aberrant wound healing are not well understood in either acute or chronic wounds. Acute wounds caused by surgery, trauma or burns can result in hypertrophic scarring and keloid formation due to excessive fibroproliferation in the healing tissue (Ogawa, 2010). Chronic wounds are often related to underlying comorbid conditions and are characterized by inflammatory cytokines, reactive oxygen intermediates and repetitive local injury (Menke *et al.*, 2007; Vande Berg and Robson, 2003). Traditional therapies such as dressings, pressure offloading, local and systemic antibiotics, hyperbaric oxygen and topical growth factors have been only moderately effective in preventing and treating wounds (Mustoe *et al.*, 2006); this is largely due to the loss of normal resident or native cells in the wound bed combined with ongoing inflammation in the regenerating tissue. Cell therapy, which involves the transplantation of autologous or allogeneic cells in order to re-establish the normal architecture of the tissue could provide a novel therapeutic approach (Panuncialman and Falanga, 2007; Herdrich *et al.*, 2008; Hanson *et al.*, 2010).

Mesenchymal stromal/stem cells (MSCs) are multipotent adult progenitor cells of great interest in regenerative medicine due to their very favorable immunological properties and unique regenerative potential (Hanson *et al.*, 2010; Hanson *et al.*, 2010). MSCs, originally isolated from the bone marrow (BM), are defined by expression of a certain set of cell surface markers and lack of expression of hematopoietic markers (Dominici *et al.*, 2006). The other defining characteristic of MSCs is their potential to differentiate into bone, fat and cartilage *in vitro* when grown in appropriate culture conditions. Studies have shown that cells with similar immunophenotypic and functional characteristics can be isolated from most adult tissues including adipose tissue (AT) (Zuk *et al.*, 2001; Beahm *et al.*, 2003).

MSCs isolated from different tissue sources express similar cell surface markers and can differentiate into multiple lineages (da Silva Meirelles *et al.*, 2006). Nevertheless, there is evidence to suggest that these cells are not exactly similar and can be phenotypically and functionally different based on tissue source (Noel *et al.*, 2008). An important property of MSCs in general is their capability to modulate the activation and proliferation of immune cells (Kim and Hematti, 2009; Le Blanc and Ringden, 2007; Le Blanc *et al.*, 2007; Nauta and Fibbe, 2007; Caplan, 2007; Uccelli *et al.*, 2007), which plays an important role in wound healing and scar formation. Such properties have also been the basis for using allogeneic MSCs without consideration of their HLA types for systemic treatment in regenerative medicine, which could be advantageous in the broad application of this approach (Battiwalla and Hematti, 2009).

While there is increasing interest in using MSCs for treatment in a variety of clinical applications including treatment of cutaneous wounds, there are few well-characterized preclinical wound models. Among the common laboratory animal models, the pig has an integumentary architecture similar to humans except for its lack of sweat glands (Sullivan *et al.*, 2001). Porcine skin is most comparable to humans among animal models currently used to study wound healing in terms of dermal thickness, papillary structure, collagen composition, hair follicle density and distribution of dermal blood vessels. The purpose of the current study was to characterize culture-expanded MSCs from BM and AT and evaluate their effect when administered through local delivery in a porcine wound healing model in combination with commercially available dressings.

2. Methods

2.1. Mesenchymal stromal/stem cell isolation and culture

Göttingen Minipigs were used based on animal protocols approved by the University of Wisconsin-Madison Research Animal Resource Center. MSCs were derived from BM and AT of donor males and expanded in culture prior to *in vitro* and *in vivo* studies (Hanson *et al.*, 2010). Briefly, BM-MSCs were isolated from BM aspirate harvested under sterile conditions from the iliac crests of a male donor and washed with phosphate buffered saline (PBS) by centrifugation. Mononuclear cells were isolated by Ficoll-Hypaque 1.073 (GE Healthcare Bio-sciences, Piscataway, NJ, USA) and Leucosep® tube (Greiner Bio-one, Monroe, NC, USA) according to manufacturer protocols. Red blood cells (RBC) were lysed with a 3-min incubation in RBC lysis buffer and mononuclear cells were suspended in alpha minimum essential media (α -MEM) supplemented with 10% fetal bovine serum (Hyclone-FBS, Logan, UT, USA), 1x non-essential amino acids (NEAA; Sigma Inc., St. Louis, MO, USA), 4 mM L-glutamine (Sigma Inc.), and 100U/mL penicillin, 0.01 mg/mL streptomycin sulfate (Sigma Inc.).

Adipose tissue-derived MSCs were isolated from subcutaneous tissue excised from the flank of the same donor males. The excised fat was rinsed with PBS then minced and digested with collagenase/PBS (Sigma Inc.) at 37 °C for 45 min. The sample was neutralized with an equal volume of α -MEM and centrifuged at 5000x g for 5 min. Red blood cells were lysed by 3-min incubation in RBC lysis buffer and mononuclear cells were suspended in supplemented α -MEM as above and plated on tissue culture flasks. After adherence of

stromal cells to plastic plates over two days, the culture media was changed to remove non-adherent cells. Cells were cultured in a 37 °C incubator with 5% CO₂ in a 95% humidified atmosphere. Media was changed every three days and cells were expanded until passages 4-6, at which time they were used for flow cytometry, differentiation experiments and *in vivo* administration.

2.2. Fluorescent activated cell sorting (FACS) analysis

For fluorescent activated cell sorting (FACS) analysis, single cell suspensions were stained and analyzed using FACScalibur™ flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) within 24 h of staining. We used FlowJo software (Tree Star, Ashland, OR, USA) to analyze acquired data. Antibodies used included: anti-CD14 allophycocyanin (APC), anti-CD29 phycoerythrin (PE), anti-CD44 PE and anti-CD90 PerCP-Cy5.5 (all from eBioscience, San Diego, CA, USA). For surface staining, Fc receptors were blocked with Fc Receptor Blocking Agent (Miltenyi Biotech, Auburn, CA, USA) for 15 min at 4 °C. Surface antibodies were added and incubated for 30 min at 4 °C in the dark, and then cells were washed with wash buffer (2% FBS in PBS) and fixed with 1% paraformaldehyde (PFA) in PBS until ready for analysis. Control staining with directly-labeled isotype-matched monoclonal antibodies was performed in all FACS experiments.

2.3. Differentiation of cells toward osteogenic and adipogenic lineages

Cell differentiation into osteogenic and adipogenic lineages and subsequent detection was performed using established methodologies (Hanson *et al.*, 2010). Briefly, BM- and AT-derived MSCs were treated with either osteogenic medium or adipogenic medium (Miltenyi Biotech) for 14 days, with media changes every 3-4 days. Cultures were assayed for mineral content by Alizarin Red S staining (Acros Organics, Newark, NJ, USA) and for lipid accumulation by Oil Red O staining (Sigma Inc.). Controls included cells treated with standard culture media for 14 days.

2.4. Wound model and surgical procedure

Both male and female Göttingen pigs were used (average wt 30-35 kg) in our experiments. A male pig was used for allogeneic MSC derivation and eight female pigs were used for the surgical procedure, treatment and tissue harvest. General anesthesia was induced with tiletamine/zolazepam (Telazol™, 4.4 mg/kg intramuscular) and xylazine (2.2 mg/kg intramuscular), then maintained with isoflurane while continuously monitoring pulse oxygenation, respiration and heart rate. Under general anesthesia, pig backs were shaved, prepped with povidone iodine, and the subcutaneous tissues infiltrated with 50 mL of 0.25% bupivacaine w/ 1:100 000 epinephrine solution for analgesia, hemostasis and volume resuscitation. Wounds were produced with an electric dermatome set (Zimmer, Inc., Warsaw, IN, USA) at 0.030" depth (Kleinbeck *et al.*, 2009). We used partial thickness wounds, which can result in hyperpigmented or hypertrophic scars, thus allowing us to observe the impact of MSCs and dressing on healing outcomes. All scars were expected to heal secondarily without significant contracture since the deeper dermal architecture remained intact. Epinephrine solution 1:100 000 on non-adherent gauze was applied briefly

to the wound bed for hemostasis. The autograft skin was used as a grid with 2 cm × 2 cm squares created for wound definition.

Each wound was intradermally injected with 1×10^6 MSCs/1 mL PBS derived from BM or AT. The concentration of cells and volume administered has been shown to be widely variable in the literature, with the number of cells in different studies ranging from 1×10^5 to 2×10^6 cells per wound (Falanga *et al.*, 2007; Badiavas and Falanga, 2003; Yoshikawa *et al.*, 2008; Blanton *et al.*, 2009). In the current study, we administered 1×10^6 male cells into the four quadrants of the partial thickness wound by local injection to position the cells directly in the deep dermal layer without distorting the wounded tissue. A 1-mL saline injection was used as control. Dressing treatments including bismuth impregnated Vaseline gauze (Xeroform™, Coviden, Mansfield, MA, USA), fibrin sealant (Tisseel™, Baxter, Deerfield, IL, USA) with non-adherent gauze (Telfa™, Coviden) and autograft skin was then immediately applied. These treatments were placed randomly in different experiments and separated by the epidermal autograft grid to prevent overlap of dressings (Figure 1). Figure 1 shows the wound grids and an example of dressing placement. Xeroform and fibrin/Telfa are readily-available FDA approved wound treatments and therefore were selected for this study. The polymer dressings shown in the end wound positions of Figure 1B were still under preparation and were not analyzed in this study. Treatments were overlaid with gauze, self-adherent wrap (Coban™, 3 M, St. Paul, MN, USA) and tape. Animals were treated with intramuscular buprenorphine twice daily until dressing removal. Pigs were housed independently with no movement or diet restrictions. Outer dressings were removed on postoperative day 7. No additional primary or secondary dressings were applied thereafter. Animals were euthanized with Buethanasia-D 0.2 mL/kg IV for tissue collection at post-operative days 7, 10, 14 and 21. Two female pigs were used during each period. Tissues were harvested immediately following euthanasia and either cryopreserved in PCT solution and liquid nitrogen or fixed in 10% buffered formalin for at least 48 h.

2.5. Gross wound examination

At euthanasia, gross analysis was performed and photographs taken of each wound to assess for epithelialization, autograft take, wound contraction, granulation, infection and scarring. We used the widely accepted clinical method of wound assessment, the Vancouver Scar Scale (VSS), which takes into account four physical characteristics: height, pliability, vascularity and pigmentation (Sullivan *et al.*, 1990; Vercelli *et al.*, 2009). Scar assessment was performed in a blinded fashion.

2.6. Histological evaluation and measurement of epidermal maturity

Tissue specimens were obtained from the various treatment sites at different time point and from unwounded tissue. Tissues were paraffin-embedded, sectioned and stained with hematoxylin and eosin. The slides were observed in a blinded fashion under a standard light microscope. For every slide, qualitative observations were made on epidermal growth, epidermal maturity, granulation tissue thickness and extracellular matrix (ECM) maturity, and dermal inflammation. Epidermal maturity was determined by epidermal confluence, rete ridge thickness determined by maximum and minimum epidermal pegs, and remodeling region/granulation tissue ECM maturity was determined by prominence and orientation of

collagen bundles. For each tissue section, five individual regions were observed with even spacing over the width of the section. Viewing regions were evenly spaced across the length of the biopsy, and aligned and oriented in the same manner for every section examined to ensure consistency of analysis. Maximum and minimum epidermal thicknesses were measured for each viewing region using the National Institutes of Health ImageJ processing and analysis software (Kleinbeck *et al.*, 2009; Faucher *et al.*, 2010; Kleinbeck *et al.*, 2010). The mean of all ten viewing regions (five from each pig) was reported as a representative value of each *n*.

2.7. DNA extraction of male MSCs injected into female pigs

Genomic DNA was extracted from the harvested tissue using a spin column protocol according to manufacturer guidelines (Qiagen, Inc, Valencia, CA, USA). Briefly, each 50 μm tissue section of harvested pig (approximately 25 mg) was exposed to proteinase K solution (20 μL proteinase K solution/180 μL ATL buffer solution) for 7 h and heated in a water bath at 56 °C with periodic vortexing. After lysis of the harvested tissue, 200 μL of buffer AL and 200 μL ethanol (100%) were added. The mixture was then added to the DNeasy Mini Spin column (QIAGEN Inc. Valencia, CA, USA) and centrifuged for 1 min at 6000x *g*. The flow-through was discarded and 500 μL of AW1 buffer added and centrifuged at the same times and speed mentioned previously. Next, 500 μL of AW2 buffer was added and centrifuged for 3 min at 20 000x *g*. The flow through was similarly discarded and 200 μL of buffer AE added for the final DNA elution. The mixture was allowed to incubate for 1 min at room temperature and then centrifuged for 1 min at 6000x *g*. The eluted DNA solution was stored at -20 °C until polymerase chain reaction (PCR) was performed.

2.8. Polymerase chain reaction

To determine whether MSCs remained in the local tissue wound bed following injection, a primer was selected for a chromosome Y (CY) probe specific to *sus scrofa* DNA (swine) based on previous work in fluorescence *in situ* hybridization (FISH) (Rubes *et al.*, 1999). A forward (5'-AATCCACCATAACCTCATGGACC-3') and reverse (5'-TTCTCCTGTATCCTCCTGC-3') primer for the male-specific sequence (X12696) was designed to amplify a 377-base pair (bp) CY-specific fragment. The 377-bp CY-specific fragment was used to amplify and demonstrate the presence of Y-chromosome DNA in female pigs.

2.9. Gel electrophoresis

A 2% gel was prepared using 2.5 g Agarose and 0.5x TBE buffer (125 mL) and ethidium bromide (7.5 mL). An E-Gel® Low Range Quantitative DNA Ladder (Invitrogen) was used for band comparison (175 ng/10 μL) as flanking DNA ladders (20 μL) for each gel run (as indicated below). E-Gel sample loading buffer (4 μL) was added for tracking during gel electrophoresis and 20 μL was added to each well with an empty well spaced in between each run. Gel electrophoresis was performed at 20 V for 10 min and then 100 V for 1 h. The gels were immediately imaged using a ChemiDoc™ XRS transilluminator system (BioRad Laboratories, Inc., Hercules, CA, USA) at maximal contrast and medium brightness.

2.10. Statistical analyses

Two pigs were used for each period. For gross analysis and scar assessment, data from both pigs at each time point, injection type and dressing were analyzed as a pooled mean $\pm$ standard error. For histological analysis, data from all five viewing regions and both pigs at each time point, injection type and dressing were analyzed as a pooled mean $\pm$ standard error. Data were compared by repeated measures using ANOVA to examine the impact of cell type (or saline), dressing type and time. A p -value of < 0.05 was considered significant. All analyses were performed using SAS statistical software version 9.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Göttingen Minipig mesenchymal stromal cell characterization

Mesenchymal stromal/stem cells were harvested from bone marrow and adipose tissue of a healthy male donor and expanded to passages 4-6 (Figure 2A, left column). Cells were positive for available MSC markers such as CD29, CD44 and CD90 and negative for hematopoietic markers such as CD14 (Figure 2B). MSCs subjected to adipogenic and osteogenic culture conditions were shown to differentiate into adipocytes and osteocytes, respectively, after 14 days in culture. Oil Red O staining showed lipid vacuoles stained red (Figure 2A, middle column) while Alizarin Red S staining showed deposits of calcium crystals stained orange to brown (Figure 2A, right column), confirming their identity as MSCs according to accepted criteria (Dominici *et al.*, 2006).

3.2. Macroscopic wound healing assessment

During the 3-week period, all treatment sites were evaluated for gross wound healing (Figure 3). The assessment, modeled after the Vancouver Scar Scale, included visual inspection for vascularity, pigmentation, evidence of infection and rejection, as well as palpation for height, contracture and pliability. There were no outward signs of infection in any of the treatment groups, including no erythema, purulent drainage or exudate. There was no evidence of rejection or abnormal pathology associated with the allogeneic MSC injections, including desquamation, bullous reaction, vascular thrombosis and teratoma (Bradley *et al.*, 2002). Additionally, none of the pigs showed any signs of systemic illnesses such as fever, chills, weight change and diffuse rash. The rate of epithelialization was grossly similar for MSC-treated and saline control wounds dressed with bismuth gauze (Xeroform) and fibrin sealant (Tisseel); all wounds were healed by day 21 and all treatments trended towards improved cosmesis, as indicated by lower scar scores (Figure 4). Wounds covered with Xeroform revealed differences in scar assessment among the BM-MSC, AT-MSC and saline treatments at postoperative day 7; however, there was no statistical significance by day 21 among the treatment groups (BM-MSCs 1 ± 0.5 ; AT-MSCs 0.5 ± 0.5 ; saline control 1 ± 0). Wounds covered with fibrin sealant were nearly identical at postoperative day 7 and displayed significant variability in cosmetic outcome as healing progressed, with both MSC-treated groups showing improved scar scores at day 21 compared to controls (BM-MSCs 1 ± 0 ; AT-MSCs 0.5 ± 0.5 ; saline control 3 ± 0.8 , $p < 0.01$).

3.3. Histological wound healing assessment

Histopathological analysis of the tissue sections from all treatment groups at each time point were examined for a variety of epidermal characteristics associated with wound healing (Figure 5). The unwounded skin shown microscopically in Figure 5 (left column) demonstrates a tightly packed, dark-stained and relatively thin epidermis. Beneath this layer is the dermis, comprised of supportive and connective tissue (largely collagen bundles), as well as hair follicles, sweat glands and other functional structures. This normal architecture was disrupted by with wounding and the goal of the healing process is to re-establish this anatomy. The early phases of wound healing are characterized by proliferation and disorganization as a zone of granulation or remodeling tissue. This was most evident in the Autograft group since the horizontal gray line (x) where the partial thickness graft was replaced on the wounded skin. Similarly, this remodeling zone was observed in the dermal-epidermal junction (arrow) in the other slides. The healing epidermis, more so than the normal epidermis, had an undulating appearance with intermittent, regular protrusions of the epidermis into the underlying dermis known as rete ridges or pegs. An example of a rete ridge is shown by the double arrows in Figure 5. Often, as tissue remodels, the irregular appearance of the epidermis remains more prominent than unwounded skin. At day 7 of our study, all treatments showed varying degrees of epidermal healing and thickness, which persisted over time as remodeling occurred. The day 7 slides in Figure 5 are characterized by a large remodeling zone and highly irregular epidermal projections. As the epidermis thinned and matured, the rete ridges were retained. By day 21, the dermis more closely resembled that of unwounded skin in collagen organization compared to the initial remodeling zone of granulation tissue.

Epidermal thickness is illustrated in Figure 6. It shows the average maximum and minimum values of epidermal thickness over 10 viewing regions for each of the treatment groups; wounds covered with Xeroform are shown in Figure 6A while wounds covered with fibrin are shown in Figure 6B. The qualitative trends observed microscopically are again demonstrated. The erratic epidermal architecture and remodeling zone correlated with a greater epidermal thickness in all treatment groups at day 7. Over time, the epidermis thinned similarly in all wound covered with Xeroform, regardless of injection type (Figure 6A). Alternatively, the wounds covered with fibrin glue showed restoration of rete ridges (a hallmark of healing), with significantly greater epidermal thickness in the MSC treated groups compared to saline control (* Figure 6B). There was no difference between AT- and BM-MSC treated wounds in the fibrin glue/non-adherent gauze dressing group. To summarize, healing occurred in all treatment and control sites; however, variations in dermal and epidermal architecture observed in the MSC-treated groups could have correlated with faster remodeling and epidermal maturation and improved clinical scar characteristics.

3.4. PCR analysis of male DNA in female tissue

To determine the presence and persistence of MSCs in the porcine wound tissue, we designed a sex mismatch between the donor and recipient animals. Female Gottingen Minipigs were injected with culture-expanded BM- and AT-derived MSCs from a male donor at wounded and unwounded tissues during the initial wounding procedure (day 0). A CY-377 amplicon was generated using standard PCR cycling. A representative gel is shown

in Figure 7. Qualitatively, the CY amplicon was demonstrated in each of the treatment groups and there did not appear to be much difference in the banding patterns of each of the experimental groups and dressings. Interestingly, unwounded tissue harvested immediately following intradermal MSC injection ($t = \text{day } 0$) showed amplification levels comparable to the male tissue sample (positive control, no cell injection). There were no significant differences between BM- and AT-MSCs observed at days 0 or 7. There was a decrease in the amount of DNA signal by day 7 and again by day 10. By day 14, qualitatively, the band pattern associated with CY-377 amplicon was consistent with the non-injected female tissue (negative control, data not shown).

4. Discussion

This study illustrated the feasibility and potential effectiveness of locally administered MSCs from two different sources in a large animal model. Göttingen Minipigs have been used extensively in dermatologic, neurological, cardiovascular and endocrine research in a variety of acute and chronic disease processes (Johansen *et al.*, 2001; Larsen *et al.*, 2005; Dame *et al.*, 2009; Agay *et al.*, 2010). Further, it is a widely accepted model in pharmacology for investigating dermal delivery and absorption of several FDA approved medications due to relative similarity in skin structure compared to humans (Qvist *et al.*, 2000; Dame *et al.*, 2008; Markert *et al.*, 2009). In this pilot study, we characterized BM- and AT-derived MSCs in terms of their characteristics *in vitro* as well as their impact on cutaneous healing *in vivo* following local injection in a Göttingen Minipig model. To the best of our knowledge, this is the first study to characterize and compare simultaneously different tissue-derived MSCs, BM and AT from the same donor for wound healing applications. The primary limitation of this study was the small number of animals for each time point, an inherent problem with large animal models due to cost, thus limiting statistical significance. However, when investigating wound models, porcine skin is far superior to small animal models such as rat and mice. In addition, another drawback could be that this was not a model of chronic, non-healing wound or hypertrophic scar. Nevertheless, there are few large animal models examining cutaneous pathology. Future models should include radiation burns and a diabetic porcine model since both inflammation and injury are potentiated in these wounds. This study was designed to characterize these cells and investigate the effect of MSC-treatment on the normal healing process. As a result, the pre-clinical model developed in the current study could be used to investigate many variables that might be challenging to address in human clinical trials such as the effect of tissue of origin on the potential safety and efficacy of MSCs, defining optimal cell dose, and the effect of different clinically available dressings on outcomes of MSC therapy. Further understanding of how MSCs can affect the underlying pathophysiology of wounds and how they in turn are affected by the wounded and diseased tissues of interest in this large animal model could provide the rationale for future well-designed clinical studies.

While all wounds examined in our study were completely healed by day 21, there was temporal variation observed in both histological remodeling and macroscopic scar assessment among the treatment groups. Wounds covered with Xeroform gauze showed significant variability early on and the rate of healing and maturation of the remodeling area occurred faster in MSC-treated wounds. Conversely, the wounds dressed with fibrin sealant

and non-adherent gauze began with a cosmetically unfavorable wound score at day 7. This could be due to the interactions between the administered fibrin, an extracellular matrix protein, with natural clotting factors and other transudate normally expressed in wounded tissue. This interaction translated macroscopically to a hyperpigmented, less pliable wound observed at day 7, but perhaps facilitated epithelialization by serving as a scaffold for new tissue ingrowth. This could have been responsible for the significantly better scar scores by day 21 in the cell injected groups, supporting the use of ECM-based dressings complimentary to cell therapies. Notably, there was no difference between AT- and BM-derived MSC-treated groups with respect to outcomes measured. Previous research has attempted to correlate the histological appearance of wounded tissues with clinical outcomes or scarring (Kleinbeck *et al.*, 2009; Faucher *et al.*, 2010; Kleinbeck *et al.*, 2010; Beausang *et al.*, 1998). In general, restoration of the microscopic architecture of the upper portion of the wound bed (epidermis and papillary dermis) was observed in scars with better cosmetic results. Of particular importance was the re-establishment of rete ridges of the epidermis that projected into the papillary dermis. Decreased depth and density of rete ridges is often seen in hypertrophic scars and poor healing (Kleinbeck *et al.*, 2009; Beausang *et al.*, 1998).

The current study demonstrated the presence of male DNA in female wounded and unwounded tissues at days 0, 7 and 10, although a decline in amplification was observed as essentially no signal by day 14. It is unknown whether this diminishing signal was due to migration of the MSCs from the local wound environment, engulfment of the MSCs by macrophages undergoing phagocytosis as part of the normal healing process, or other mechanisms of cell processing and tissue regeneration. This study suggests evidence for persistence of MSCs in the local tissue bed over one week following cell administration.

The use of culture expanded autologous BM-MSCs has been reported to be promising for the treatment of acute surgical and chronic non-healing wounds (Falanga *et al.*, 2007; Yoshikawa *et al.*, 2008) when tested in non-randomized, small scale clinical trials. The use of adipose cells has rapidly increased in the clinical arena as well (Gimble *et al.*, 2012). However, to further explore MSCs in larger clinical trials as a treatment modality for complex cutaneous wounds involves addressing several important questions. For example, the exact fate of locally and systemically delivered MSCs has not been clearly established and many pre-clinical and clinical studies have shown variations in engraftment of MSCs in a variety of tissues (Laurila *et al.*, 2009). Indeed, the question as to whether engraftment of cells is required for clinical efficacy is strongly debated (Prockop, 2009). Our large animal model could be used for tracking cells that are labeled prior to local injection, a task that is otherwise not clinically feasible.

5. Conclusions

We shown the safety and feasibility of allogeneic stem cell injection in a clinically-relevant animal model for treating cutaneous wounds. We isolated and characterized BM- and AT-derived MSCs based on consensus criteria from Gottingen Minipigs. (Dominici *et al.*, 2006). We also showed that *in vivo* local injection of allogeneic MSCs into the wound bed was safe and potentially efficacious in promoting wound repair. The model developed in the current

study could be used to address many basic and translational research questions associated with cell-based therapies for wound healing, soft tissue remodeling and tissue regeneration.

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Figure 1.

Wound model/surgical procedure methodology. A) Representative wound grid photograph. Partial thickness cutaneous wounds were created in strips shown and separated by epidermal autograft. Each 2×2 cm square was intradermally injected with either saline, BM- , or AT-MSCs (1×10^6 cells / mL) based on a random location scheme. B) Dressings included (L-R) polymer (sIPN-not part of this study), fibrin glue/gauze, autologous skin graft, Xeroform, and additional polymer (sIPN-not part of this study)

Figure 2.

A) MSC characterization from BM and AT via differentiation potential and surface marker expression. A) Representative microscopic image views of undifferentiated BM- and AT-derived MSCs (left column), as well as differentiated along adipogenic (middle column) and osteogenic lineages (right column). Oil Red O staining shows lipid vacuoles stained red while Alizarin Red S staining shows deposits of calcium crystals stained orange to brown. B) Representative fluorescent activated cell-sorting analysis of BM- and AT-derived MSCs for different cell surface markers

Figure 3.

Representative photographs illustrating macroscopic wound healing of tissues 7 and 21 days after wounding and treatment. Blinded assessment modeled after the Vancouver Scar Scale included visual inspection for vascularity, pigmentation, evidence of infection and rejection, as well as palpation for height, contracture and pliability. There was no evidence of rejection or abnormal pathology associated with allogeneic MSC injection such as desquamation, bullous reaction and vascular thrombosis. The rate of epithelialization was grossly similar for MSC-treated and saline control wounds dressed with bismuth gauze (Xeroform) and fibrin sealant (Tisseel); all wounds were healed by day 21 and all treatments trended toward improved cosmesis

Figure 4.

Temporal effect of MSCs on macroscopic wound healing. The treatment sites were compared based on vascularity, pigmentation, pliability and height. The wounds covered with petrolatum gauze are shown in Panel A whereas wounds covered with fibrin glue are shown in Panel B. Data was reported as the average wound score ($\pm$ standard deviation) based on blinded observation ($n = 2$ per treatment per time point). All wounds were healed by day 21 and all treatments trended toward improved cosmesis as indicated by lower scar scores. Wounds covered with Xeroform revealed differences in scar assessment among the BM-MSC, AT-MSC and saline treatments at postoperative day 7; however, there was no statistical significance by day 21 among the treatment groups (BMMSCs, 1 ± 0.5 ; AT-MSCs, 0.5 ± 0.5 ; saline control, 1 ± 0). Wounds covered with fibrin sealant were nearly identical at $\pm$ postoperative day 7 and displayed significant variability in cosmetic outcomes as healing progressed, with both MSC-treated groups showing improved scar scores at day 21 compared to controls (BM-MSCs, 1 ± 0 ; AT-MSCs, 0.5 ± 0.5 ; saline control, 3 ± 0.8 , $p < 0.01$)

Figure 5.

Representative histologic photomicrograph of tissues 7 and 21 days after wounding and treatment. Tissues are aligned so that approximately the top third of each photo contains dead or necrotic tissue such as stratum corneum. Unwounded tissue treated with saline, AT- or BM-MSCs was included as additional control. Hematoxylin and eosin stains at 4x magnification, scale bar represents 0.1 mm. The unwounded skin shows a tightly packed, dark-stained, relatively thin epidermis. Beneath this layer is the dermis, comprised of supportive and connective tissue. Remodelled and granulation tissues were most evident in the Autograft group since the horizontal gray line (x) where the partial thickness graft was replaced on the wounded skin. Similarly, this remodelling zone was observed in the dermal-epidermal junction (arrow). The healing epidermis has an undulating appearance with projections of the epidermis into the underlying dermis, known as rete ridges or pegs (double arrow). At day 7, all treatments showed varying degrees of epidermal healing and thickness, which persisted over time as remodeling occurred. As the epidermis thinned and matured, the rete ridges were retained. By day 21 in all groups, the dermis more closely resembled that of unwounded skin in collagen organization compared to the initial remodelling zone of granulation tissue.

Figure 6.

Epidermal thickness of wounds treated with MSC injection (AT-, BM- and saline control) and covered with various dressings examined histologically. The wounds covered with petrolatum gauze are shown in Panel A whereas the wounds covered with fibrin glue are shown in Panel B. Data was reported as the average of 10 total viewing regions in biopsies from two separate pigs $\pm$ standard deviation. Statistical significance is indicated by *. The qualitative trends observed microscopically were again demonstrated. Epidermal thickness was observed in all treatment groups at day 7. Over time, the epidermis thinned similarly in all wounds covered with Xeroform, regardless of injection type (Fig. 6A). Alternatively, the wounds covered with fibrin glue demonstrated restoration of rete ridges (a hallmark of healing) with significantly greater epidermal thickness in the MSC-treated groups compared to saline controls (Fig. 6B). There were no differences between AT- and BM-MSC treated wounds in the fibrin glue/non-adherent gauze dressing group

Figure 7.

Male DNA content was assessed in female tissues via PCR amplification of a 377 bp segment from chromosome Y. Gel electrophoresis is shown for samples injected with AT-MSCs at time of wounding, day 0 and harvested at day 7. Unwounded, untreated male and female tissues were used as positive and negative controls. [L = ladder; 1) positive control (male tissue); 2) negative control (untreated female tissue); 3) female tissue with male BM-MSCs at time of injection (day 0); 4) Xeroform treatment, female tissue with male BM-MSCs (day 7); 5) fibrin treatment, female tissue with male BM-MSCs (day 7); 6) Xeroform treatment, female tissue with male AT-MSCs (day 7); 7) fibrin treatment, female tissue with male AT-MSCs (day 7)]