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Mesenchymal Stem Cells Increase Collagen Infiltration and Improve Wound Healing Response to Porous Titanium Percutaneous Implants

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Abstract

Epidermal downgrowth, commonly associated with long-term percutaneous implants, weakens the skin-implant seal and greatly increases the vulnerability of the site to infection. To improve the skin attachment and early tissue integration with porous metal percutaneous implants, we evaluated the effect of bone marrow-derived mesenchymal stem cells (BMMSCs) to provide wound healing cues and vascularization to the dermal and epidermal tissues in establishing a barrier with the implant. Two porous metal percutaneous implants, one treated with BMMSCs and one untreated, were placed subdermally on the dorsum of Lewis rats. Implants were evaluated at 0, 3, 7, 28, and 56 days after implantation. Histological analyses evaluated cellular infiltrates, vascularization, quantity and quality of tissue ingrowth, epidermal downgrowth, and fibrous encapsulation. The amount of collagen infiltrating the porous coating was significantly greater for the BMMSC-treated implants at 3 and 28 days post implantation compared to untreated implants. There was an early influx and resolution of cellular inflammatory infiltrates in the treated implants compared to the untreated, though not statistically significant. Vascularization increased over time in both treated and untreated implants, with no statistical significance. Epidermal downgrowth was minimally observed in all implants with or without the BMMSC treatment. Our results suggest that BMMSCs can influence an early and rapid resolution of acute and chronic inflammation in wound healing, and can stimulate early collagen deposition and granulation tissue associated with later stages of wound repair. These findings provide evidence that BMMSCs can stimulate a more rapid and improved barrier between the skin and porous metal percutaneous implant.

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Conflict of Interest

All authors confirm that there is no potential conflict of interest including employment, stock ownership, consultancies, honoraria, paid expert testimony, and patent applications/registrations influencing this work. The authors are solely responsible for the content and writing of this manuscript.

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Keywords

Porous titanium; *in vivo*; mesenchymal stem cells; percutaneous implant

Introduction

Bone-anchored hearing aids, dental implants, and osseointegrated percutaneous prosthetics are clinically used metal percutaneous devices that are implanted for the lifetime of the individual, and all require an impassable attachment between the skin and the device. When the skin and soft tissue attachment to the implant breaks down or weakens, the susceptibility to infection substantially increases and places the entire device at risk of failure, leading to potential tissue morbidity and device removal.

Previously, we demonstrated with a rabbit model that percutaneous implants with a commercially pure titanium porous coating have a 7-fold decreased risk of infection compared to percutaneous implants with smooth polished titanium surfaces [1]. We observed that in a majority of the implants, the epidermis had migrated internally along the percutaneous component, creating a sinus tract that was filled with keratin and degenerative neutrophils. In fact, several other groups have demonstrated this same result, which is commonly referred to as epidermal downgrowth, and when accompanied by a sinus tract, marsupialization [2–5]. It is thought that epidermal downgrowth/marsupialization is one of the leading factors attributed to the implant site's risk of infection [2–4, 6–8].

The explanation for this phenomenon is not entirely clear, though possible explanations could include insufficient wound healing signaling cues and cell contact inhibition in the epidermal and dermal tissues [9–11], along with insufficient vascularization of the dermal tissues in providing nutrients for the restoration of the epidermal and dermal barrier [2, 3, 12]. It is also thought that implant motion in the subcutaneous tissue space creates an unstable environment for epidermal attachment to the implant [3, 4, 13]. To address these and other potential mechanisms, there is a need to investigate biological strategies that can interact with the dynamic nature of the dermal and epidermal tissues such that the epidermal downgrowth is prevented, and tissue attachment to the percutaneous implant is maximized.

One such strategy is that of mesenchymal stem cell (MSC) therapy, which is currently being exploited for treatment of several clinical conditions due to their pivotal role in tissue repair and regeneration. Adult MSCs reside in several niches within the body, though for most clinical applications, bone marrow-derived mesenchymal stem cells (BMMSCs) and adipose-derived mesenchymal stem cells (ASCs) are used [14]. Several wound healing studies through the years have published very promising results regarding the ability of MSCs to accelerate wound healing [15–18], increase vascularization [16–18], increase cellularity [16–18], and increase collagen content and wound strength [15, 18, 19]. It is suggested that MSCs produce these effects through two suggested mechanisms: (1) through paracrine signaling mechanisms, releasing soluble signaling factors, including epidermal growth factor (EGF), keratinocyte growth factor (KGF), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor- α (VEGF- α), angiopoietin-1 (Ang-1), macrophage inflammatory protein (MIP-1a and MIP-1b), platelet-derived growth factor (PDGF-BB), fibroblast growth factor (FGF), among others [20–22]; and/or (2) through differentiation into resident cells [16, 23].

With regards to the ability of MSCs to influence healing around biomaterials, Prichard et al. showed that when ASCs are attached to a biomaterial and implanted in subcutaneous tissues, the ASCs are able to attenuate the foreign body response (FBR) and increase the microvascular density adjacent to the implant surface [24]. Additional studies that have

evaluated scaffolds or implants seeded with MSCs have also reported similar effects of increased vasculature and increased rates of healing within the MSC-treated scaffolds [25, 26].

Using this previously established work with MSCs, the goal of this study was to evaluate the ability of BMMSCs to stimulate and influence an improved skin and soft tissue integration with porous titanium percutaneous implants, hypothesizing that *BMMSC-treated implants will have a more rapid and robust tissue integration compared to untreated implants*. We tested our hypothesis by evaluating five outcomes important in assessing successful wound healing and tissue response to percutaneous implants: (1) inflammatory cellular infiltrates, (2) neovascularization, (3) quality and quantity of tissue integration, (4) epidermal downgrowth, and (5) fibrous encapsulation. Through histological evaluations of these five outcomes, we were able to assess the influence the BMMSC treatment had on *stimulating a more rapid and robust tissue integration* with the percutaneous implant.

Materials and Methods

Ethical Statement

All animal studies were performed according to the *Guide for the Care and Use of Laboratory Animals* [27] and all protocols were approved by the University of Utah Institutional Animal Care and Use Committee (IACUC).

Study Design

The Lewis rat served as the animal model and BMMSC source. Lewis rats are commonly used for wound healing studies, and since they are a syngeneic species, this allowed transfer of BMMSCs from one rat to another rat without concern for an immunogenic response [28]. The study consisted of 25 animals that were randomly assigned to five groups based on the experimental time point: 0 day (n=3), 3 days (n=6), 7 days (n=6), 28 days (n=5), and 56 days (n=5). Each animal received two implants: (1) treated with 6×10^6 MSCs and (2) untreated (control). Using a simple, computerized randomization procedure, the two implants were randomly assigned placement on the rat dorsum to accommodate for any placement-specific biases.

Implant Fabrication

The percutaneous implant consisted of a Ti6Al4V substrate fabricated by the School of Medicine Machine Shop (University of Utah, Salt Lake City, UT, USA). The percutaneous portion of the implant was cylindrical with a 5mm diameter. At 3mm from the implant top, the implant surface gradually sloped outward to a final subcutaneous base diameter of 17mm. The implant height was 12mm (Fig. 1). These substrates received a commercially pure titanium porous coating (P^2 Thortex, Inc., Portland, OR, USA) that was 1mm thick on the substrate. The porous coating had a ~55% porosity that was previously determined using microCT (Xradia MicroXCT system), and had an average pore size of ~360 μ m that was previously determined using scanning electron microscopy (SEM, Hitachi S3000-N).

Endotoxin Testing, Passivation, and Sterilization

All porous titanium percutaneous implants were passivated according to ASTM F86 standards. Briefly, the implants were sonicated in distilled water, then in acetone (Sigma-Aldrich, St. Louis, MO, USA), followed by another distilled water wash before being soaked in 49% nitric acid (Macron Chemicals, Center Valley, PA, USA) for 2 hours. They were then sonicated in distilled water and allowed to air-dry overnight.

Prior to each experiment all implants were sterilized as routinely performed using an autoclave (NAPCO 8000-DSE, Winchester, VA, USA).

All implants were tested for endotoxin before and after sterilization using the LAL QCL-1000® Assay (Lonza, Walkersville, MD, USA), according to manufacturer's directions. Endotoxin levels were found to be below detection level (< 0.05 EU/ml).

Bone Marrow-Derived Mesenchymal Stem Cell (BMMSC) Culture and Scale-Up

The BMMSCs were derived from a 4-month old male Lewis rat and were purchased from Texas A&M University System Health Science Center. The BMMSCs were cultured in complete growth medium, consisting of MEM α with L-glutamine (Gibco-Invitrogen, Carlsbad, CA, USA), 20% FBS (Premium select, Atlanta Biologicals, Lawrenceville, GA, USA), 2% L-glutamine (200mM, Gibco-Invitrogen, Carlsbad, CA, USA), and 1% antibiotic/antimycotic (Gibco-Invitrogen, Carlsbad, CA, USA). The cells were seeded at 100 cells/cm² density, cultured in T-75 tissue culture flasks (Falcon, BD Biosciences, Bedford, MA, USA), and passaged at 80% confluency with 0.25% Trypsin/EDTA (Gibco-Invitrogen, Carlsbad, CA, USA).

To scale-up the number of BMMSCs needed for the *in vivo* transplantations, the BMMSCs were seeded at a density of 1000 cells/cm² and cultured in HYPERFlask™ Cell Culture Vessels (Corning Inc., Lowell, MA, USA). Passage 8 BMMSCs were then cryopreserved in aliquots of 9×10^6 cells until *in vivo* transplantation. All implants were treated with one single lot of P.8 BMMSCs.

Characterization of BMMSCs

To verify a consistent multilineage differentiation potential, passages 6–9 of BMMSCs were differentiated into adipogenic and osteogenic lineages over a 3-week period using a commercial kit according to manufacturer's directions (StemPro®, Invitrogen, Carlsbad, CA, USA). To confirm differentiation, Oil Red O (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the lipid droplets of the adipogenic cultures, and Alizarin Red S (Sigma-Aldrich, St. Louis, MO, USA) was used to stain the calcium deposits of the osteogenic cultures. Dermal fibroblasts (CRL-1414, ATCC, Manassas, VA, USA) and epidermal cells (CCL-68, ATCC, Manassas, VA, USA) were used as controls.

To confirm the immunophenotype of the BMMSCs, the cells were stained for a panel of cell surface markers, according to Harting et al. [29] and Dominici et al. [30]. The BMMSCs (passages 6–8) were stained with the following fluorescent-conjugated antibodies: CD90-PerCP/Cy5.5 (BioLegend, San Diego, CA, USA), CD29-FITC (LifeSpan BioSciences, Seattle, WA, USA), CD45-APC/Cy7 (BioLegend, San Diego, CA, USA), CD34-PE/Cy7 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CD79 α -PE (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and CD11b-AF647 (AbD Serotec, Raleigh, NC, USA). Isotype controls included the following: APC Mouse IgG1, κ (BioLegend, San Diego, CA, USA), FITC Armenian Hamster IgG (BioLegend, San Diego, CA, USA), FITC Mouse IgG2a, κ (BioLegend, San Diego, CA, USA), and PE Mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Flow cytometry was performed on a FACSCanto-II Analyzer (Becton-Dickinson, San Jose, CA, USA) with appropriate compensation using BD CompBead Plus Particles (BD Biosciences, San Diego, CA, USA), and data were analyzed using FACSDiva software (Becton-Dickinson, San Jose, CA, USA). Results are expressed as a percent of the total cells gated, which are calculated by subtracting the percent gated of non-labeled cells from the percent gated of labeled cells.

Seeding of BMSCs on Porous Coated Percutaneous Implants

The day prior to surgery, an aliquot of cells was thawed and recovered in complete growth medium. Before surgery, the cells were detached with 0.25% Trypsin/EDTA (Gibco-Invitrogen, Carlsbad, CA, USA), and 6×10^6 cells were suspended in 100 μ l of MEM α (Gibco-Invitrogen, Carlsbad, CA, USA). The BMSC suspension was carefully added in 10 μ l droplets onto the porous coated implant. The treated implant was incubated at 37°C with 5–10% CO₂ for 1–2 hours, then carefully transported to the surgery suite, where transplantation occurred within 4–6 hours after cell seeding. Our prior *in vitro* validation studies showed that maximal cell adherence and maximal cell viability can be achieved if cells were seeded in MEM α and delivered within a 4–12 hour time frame after cell seeding. The implants that were untreated (control) were submersed in sterile MEM α (Gibco-Invitrogen, Carlsbad, CA, USA) prior to being inserted in the tissue.

Animal Surgeries

Male Lewis rats (n=25, ~170 g and ~6 weeks old), were obtained (Harlan Laboratories, Livermore, CA, USA), and their health was monitored for a week after arrival to ensure fitness of use for surgical procedures. Prior to surgery, animals were housed in groups of three, and after surgery, animals were individually housed (Thoren Caging Systems, Inc., Hazleton, PA, USA). The average room temperature was 71°C with 33% relative humidity, and a 12hr on/12hr off light cycle. Animals were fed a standard laboratory diet and water *ad libitum*.

All surgeries were performed under sterile conditions with aseptic technique. Animals were induced with 3–5% Isoflurane (VetOne, Meridian, ID, USA) via inhalation and maintained at 1–3% during operation. Animals were monitored throughout surgical procedures, specifically heart rate, respiratory rate, blink reflex, skin color, temperature, and percent Isoflurane setting. The dorsum of the rat was close-shaved, then animal was positioned on the surgical table. A routine surgical scrub was performed on the dorsum, consisting of alternating scrubs of Povidone-Iodine Solution (Purdue Products L.P., Stamford, CT, USA) and 70% ethyl alcohol, finished with a final scrub of chlorhexidine (CareFusion, San Diego, CA, USA) [31]. A 4-cm incision was made diagonally across the dorsum and two subcutaneous pockets were created with blunt dissection. The anterior subcutaneous pocket was 2.5cm lateral to the spine on the right side of the animal, just posterior to the scapula. The posterior subcutaneous pocket was 2.5cm lateral to the spine on the left side of the animal, just anterior to the ilium. Using a 4.0mm biopsy punch (Robbins Instruments, Chatham, NJ, USA), a hole was placed through each subcutaneous pocket, being 2.5cm from the central incision and a 5-cm distance between the two implants. The porous titanium percutaneous implants were then carefully inserted into the subcutaneous pockets and the percutaneous components were inserted through the holes in the skin.

Once both implants were placed, the central incision was closed with an interrupted vertical mattress suture using 4-0 Vicryl (Ethicon®, Johnson & Johnson, Somerville, NJ, USA). Upon anesthesia recovery and physical mobility, animals were returned to their cages and administered Buprenorphine (Hospira, Lake Forest, IL, USA), 0.05 mg/kg, subcutaneous, for analgesia, and as necessary twice per day following 72 hours post surgery. Animals were given Rimadyl wafers (Rodent MD's™, 2 mg/tablet, Bio-Serv®, Frenchtown, NJ, USA) for continued pain-relief and water *ad libitum* for 24–72 hours following surgical procedure. Note that there is evidence that non-steroidal anti-inflammatory drugs (NSAIDs) may delay wound healing [32]. All animals received the Rimadyl wafers, though at a low dosage and for a short period (~24–48hrs). Any potential effect of the NSAID wafers on the healing capacity would equally be attributed to both treated and untreated implants. Once animals were no longer showing signs of pain, they were returned to their standard laboratory diet.

Animals were observed daily during the first week after surgery, and every other day thereafter until sacrifice. Signs of clinical infection of the implant, any changes to the implant, and overall animal health and well-being were assessed.

Implant Harvest and Histology Processing

The animals were euthanized via CO₂ asphyxiation. The implant specimens along with generous tissue margins were carefully excised from the dorsum and fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA, USA). The specimens were then dehydrated through ascending grades of ethyl alcohol (Tissue Tek Vacuum Infiltration Process, Miles, Scientific, USA), and embedded in methyl methacrylate (MMA) according to routine laboratory procedures [33]. Upon polymerization, transverse sections (~1mm thick) were cut using a water-cooled, high-speed, lapidary slab saw with a diamond-edged cutting blade (Lortone, Inc., Mukilteo, WA, USA; MK Diamond Products, Inc., Torrence, CA, USA). These sections were ground to 150µm thickness and polished to an optical finish using a variable-speed grinding wheel (Buehler Inc., Lake Bluff, IL, USA).

The sections were stained with hematoxylin and eosin (H&E) or Multiple Stain Solution (MSS, Polysciences, Inc., Warrington, PA, USA). For H&E staining, the slides were placed in Mayer's Hematoxylin (Electron Microscopy Sciences, Hatfield, PA, USA) at 50–55°C for 2–3 hours, then washed in running tap water for 10 minutes. Slides were placed in Eosin Y-Phloxine (Richard Allan Scientific, Kalamazoo, MI, USA) with Glacial Acetic Acid (Fisher Scientific, Pittsburgh, PA, USA) Solution (3:1) for 10–30 minutes, then rinsed in 100% ethyl alcohol (Fisher Scientific, Pittsburgh, PA, USA). The slides stained with MSS were placed in acid-alcohol (1% hydrochloric acid; 70% ethyl alcohol) for 5–10 minutes, then rinsed in distilled water. The MSS was added drop-wise on the slide to completely cover the section, incubated at 50–55°C on a slide warmer for 8–10 minutes, then gently rinsed in running tap water.

Histology Analyses

Slides were interpreted using a light microscope (Optiphot-2, Nikon, Japan; BX41, Olympus, Center Valley, PA, USA). Images were captured (Retiga 1300, QImaging, Surrey, BC, Canada) and measurements were made using Bioquant Nova Prime software (version 6.9.10 MR, Bioquant Image Analysis, Nashville, TN, USA).

All histology slides were de-identified by one author (KJC), and blindly interpreted and analyzed by two authors (DI and LDM). Thirteen 1mm² boxes were analyzed around the implant (Fig 2). A Mertz Graticle was used to standardize the location and the 1mm² box area for cell counting, tissue volume fill, and overall interpretation and analysis. Five outcomes were analyzed (Table 1): cellular infiltrates, neovascularization, quality and rate of tissue ingrowth, epidermal downgrowth, and fibrous encapsulation.

Statistical Analysis

All data are presented either as means ± standard error of the mean (SEM), or means ± standard deviation (SD). The data obtained within each group were analyzed using a Paired t-Test ($p < 0.05$, two-tailed, 95% CI) (SPSS vs.11.5, Armonk, NY, USA), meaning implants within each animal were paired. To test for significance across time between the measured outcomes, a multiple comparison procedure was performed using the Benjamini-Hochberg test ($p < 0.05$, two-tailed, 95% CI) (Stata/IC 10.1, College Station, TX, USA). We report Benjamini-Hochberg adjusted p values, which maintains the false discovery rate (FDR) at the nominal alpha 0.05 level [34]. Controlling for multiplicity in the standard fashion, such as with the Bonferroni procedure, that controls the family-wise error rate (FWER), is not justified, while control for the FDR provides the correct control for multiplicity [34–36].

Results

Characterization of BMMSCs

The BMMSCs were successfully differentiated into the adipogenic and osteogenic lineages, as seen by the formation and staining of lipid droplets and calcified extracellular matrix deposits (Fig. 3). Differentiation was not observed in the control BMMSCs that did not receive the differentiation medium. Further, differentiation was not observed in the dermal fibroblasts and epidermal cells that were cultured in the differentiation media (data not shown). The cell surface markers were detected in consistent proportions on the BMMSC populations, showing greater than 90% positive for CD90 and CD29, and less than or around 10% positive for CD45, CD34, CD11b, and CD79α (Table 2).

Clinical Observations

There were no surgical infections or complications. All animals healed uneventfully and successfully made it to the experimental end point. There were no symptoms of clinical infection and no implants were lost in the study.

Histological Observations and Histopathology Interpretations

Day 0 Animals (n=3/3)—The implants within the “day 0” animals were *in situ* approximately 30 minutes before being harvested and fixed. Tissue surrounding the implant was healthy, and was observed to not be integrating with the porous surface. The only cells observed in the porous coating were macrophages and red blood cells, both on the ventral and caudal side of the implant (Fig. 4). Interestingly, all of the treated implants demonstrated a macrophage infiltration in the porous coating, while only one of the three untreated implants displayed this infiltration. Further, there was an absence of polymorphonuclear leukocytes within the porous surface of all implants.

Day 3 Animals (n=6/6)—At time of sacrifice, most “day 3” implants, including treated and untreated, still had a fibrin clot formation at the skin/implant interface (Fig. 5). The treated implants had a significantly increased ($p = 0.05$) infiltration of collagen matrix and a significantly decreased ($p < 0.05$) presence of fibrin/serum in porous coating compared to the untreated implants (Fig. 6). The treated implants had higher macrophage and lymphocyte counts, while the untreated implants had higher PMN counts (Figs. 7 and 8). There was very little vasculature seen within the pores of both implants (Fig. 7). The epidermis in both treated and untreated implants appeared to be attaching to the porous surface. As the fibrous capsule was poorly defined at this early time point, no measurements are reported.

Day 7 Animals (n=6/6)—By one week, the fibrin clot had resolved at the skin/implant interface for both treated and untreated implants. For the treated implants, the epidermis was integrating and the dermal tissue was beginning to fill pores, with majority of tissue being an immature collagenous infiltration with little fibrin/serum. Granulomatous inflammation was present, with an increased fibroblast infiltration and collagen deposition compared to the untreated implants (Fig. 7). The overall tissue response reflected a chronic inflammation phase of wound healing. Vasculature in the treated implants was still minimal. Scattered red blood cells and remnants of hemorrhage were present, indicative of implant motion within the tissue space.

As for the untreated implants, there was no epidermal downgrowth and the epidermis was integrating into the porous coating in most implants. Dermal tissue was beginning to integrate in some implants, though not all, and was commonly a fibrin/serum stromal tissue, with occasional immature collagen infiltration (Fig. 7). The untreated implants presented with fewer inflammatory cells compared to the treated implants, though no statistical

significance ($p = 0.09$) was found (Fig. 8). Vasculature was minimally present, and mostly seen outside the pores. A few implants had remnants of a hemorrhage, suggesting motion in the tissue space.

Fibrous capsule measurements are not reported for treated and untreated implants as there was an absence of a structured and continuous encapsulation.

Day 28 Animals (n=5/5)—At four weeks, the skin attachment had stabilized at the skin/implant interface for treated and untreated implants (Fig. 5). For the treated implants, the epidermis had thoroughly migrated into the porous coating, with vascularized tissue observed in the higher pores above where epidermis was integrating (Fig. 9). A fibrovascular tissue was integrating into pores with significantly more ($p < 0.05$) collagen compared to the untreated (Fig. 6). Cellular infiltrates had decreased since 7 days, with granulation tissue present in and around pores (Fig. 8). Hemosiderin was seen in the tissue of some implants which is indicative of bruising that probably resulted from implant movement.

For the untreated implants, most of the tissue integrating was a granulomatous inflammatory tissue with little fibrovascular tissue, evident of later chronic inflammatory response to early granulation tissue formation. Epithelium was integrating with all implants, though slight downgrowth was observed in two implants; however, similar to the treated implants, there was vascularized tissue in pores above the epidermal integration (Fig. 9). Cellular infiltrates increased from the 7-day time point, demonstrating higher inflammatory cell counts, with fewer fibroblast infiltrates in contrast to the treated implants (Fig. 8).

Fibrous capsule thickness was similar between the treated and untreated implants ($50.7 \pm \text{SD}10.5 \mu\text{m}$ and $58.7 \pm \text{SD}11.5 \mu\text{m}$, respectively), with vascularization observed in capsule.

Day 56 Animals (n=5/5)—At 8 weeks, the skin was very settled around the implant for both treated and untreated. For the treated implants, epidermal and dermal integration was consistent in all implants, including vascularized tissue in pores of post above where epidermis was integrating, similar to that seen at 28 days though with more mature tissue. A minimal inflammatory response was observed, with granulation tissue present and evidence of tissue reorganization. A mature collagen filled the pores, with a slight decrease in cellular infiltrates compared to 28 day implants, and a slight increase in vascularization (Figs. 6 and 8). The metal surface was lined with flat macrophages, indicating a foreign body reaction.

For the untreated implants, there was good epithelial integration with the pores. A mild inflammatory response was observed along with a fibrovascular tissue. Foreign body response was beginning as evidenced by the metal surface lined with macrophages and foreign body giant cells (Fig. 10). The untreated implants did have higher counts of foreign body giant cells compared to the treated implants, though no statistical significance ($p = 0.56$) was found (Fig. 8). There was a higher influx of cellular infiltrates compared to the treated implants and the 28-day untreated implants, though not statistically significant ($p = 0.08$) (Fig. 8).

The fibrous capsule thickness was slightly higher for the untreated implants compared to the treated implants, being $69.6 \mu\text{m}$ ($\pm \text{SD} 20.1 \mu\text{m}$), and $61.6 \mu\text{m}$ ($\pm \text{SD} 21.0 \mu\text{m}$), respectively. No statistical significance was determined ($p = 0.53$).

Discussion

Preventing epidermal downgrowth and improving the epidermal and dermal integration with porous metal percutaneous implants is of paramount importance for long-term functionality and sustainability. This long-term seal is critical for eliminating the risk of infection development at the skin-implant interface.

We have shown that implants treated with MSCs have an accelerated production of a collagen matrix into the porous coating compared to untreated implants. Further, this was mirrored by the fact that fibrin/serum was significantly decreased over time in the treated implants compared to the untreated. We have also shown that MSCs stimulated an accelerated and short-lived acute inflammatory wound healing response that transitioned into a chronic wound healing response, as evidenced by the early influx and resolution of inflammatory cellular infiltrates, much earlier than that observed with the untreated implants. Our data suggest that the foreign body response was also slightly decreased by evidence of fewer FBGCs and a thinner fibrous encapsulation; however, the differences between treated and untreated were not large enough to produce statistical significance. Unlike many previous studies, we did not see a significant difference in neovascularization between the treated and untreated implants. We also were not able to show significant differences or any trends regarding the epidermal downgrowth phenomenon between the treated and untreated implants as there was minimal downgrowth overall in both groups.

It is known that in normal wound healing conditions, BMMSCs play a fundamental role in collagen type I and III production [37]. Previous studies confirm our results in that cutaneous wounds treated with MSCs resulted in an increased rate of collagen synthesis and greater formation of granulation tissue compared to untreated wounds [15, 18, 19]. These studies have further demonstrated that with an increase in collagen synthesis this results in an increase in wound strength [15]. Though we didn't measure the tissue pull-out force, it is possible that when MSCs are seeded on porous coated percutaneous implants, a stronger integration potentially could result between the biomaterial surface and the tissue. Future studies investigating the pull-out force and other parameters measuring the strength of attachment are warranted to positively confirm this MSC-effect.

With regards to the increase in cellular infiltrates, it has been shown that BMMSC conditioned medium recruits CD4/80+ and CD68 macrophages to the wound site at 7 and 14 days after application [20]. Similarly, others have shown BMMSCs to increase the cumulative cellular infiltrates in treated wounds at 7 days and 14 days post- transplantation [16, 17]. These previous studies confirm our results, and together they reflect that BMMSCs play a fundamental role in recruiting macrophages and other inflammatory cellular infiltrates to the wound site to begin tissue repair. The macrophage infiltration in the "day 0" implants was an interesting finding. In light of the above results, possible explanations regarding the early and more prominent recruitment of macrophages is through a response to the presence of the transplanted cells, or a migratory response to the chemokine release from the BMMSCs. Chen et al. demonstrated both *in vitro* and *in vivo* that BMMSCs secrete high levels of MIP-1 and monocyte chemoattractant protein (MCP-5), both of which are important in the recruitment of macrophages [20]. Additionally, they also showed increased secretion of RANTES from BMMSCs compared to the secretion profile from dermal fibroblasts [20].

In addition to the early recruitment of inflammatory cells, it has also been demonstrated that MSCs attenuate the foreign body response [24, 38]. Our results weakly corroborate this previous data in that the fibrous capsule thickness was decreased with treated implants and FBGCs were not as prevalent. Differences in results could be attributed to the materials

being investigated in that the previous work evaluated polyurethane materials, whereby we were investigating titanium, and studies have shown the FBR to vary depending on the material properties [39]. In addition, the FBR varies with respect to surface texturing [40], and since we were investigating a porous surface compared to the smooth surface in the Prichard et al. study, this is yet another factor that may have influenced different results. Possible mechanisms involved in the MSC-attenuated FBR may be due to the early resolution of inflammation, specifically macrophages, since macrophages are crucial in development of fibrosis and formation of FBGCs. Given that there are few studies showing interplay between MSCs and FBR progression, further work is needed to provide more convincing results elucidating possible cellular and signaling mechanisms.

One main impetus of this study was to prevent epidermal downgrowth with MSC treatments, by influence of increased vascularization and/or increased wound healing cues. With regards to vascularization, our results did not coincide with previous evidence that MSCs increase neovascularization in wounds [16, 17] and around implanted biomaterials [24]. Similar to our results, McFarlin and colleagues demonstrated an absence of significant differences in neovascularization between MSC treated and untreated wounds [15]. It is not entirely clear why the MSC treatment did not significantly increase neovascularization. One possible suggestion is that with the early resolution of the acute inflammatory stage of wound healing and the accelerated infiltration of granulation tissue, it is possible there was an early resolution of angiogenesis and thus early disintegration of blood vessels [41]. Though we did not see an overall decrease in numbers of blood vessel formation, we did see the production rate of neovascularization decrease over the eight week period.

Another explanation to the limited epidermal downgrowth, between both treated and untreated implants, could be related to the implant geometry, specifically the gradual sloping surface. This sloping surface may have provided a slight tension to the skin, specifically to the underlying dermal tissue, which potentially could have stimulated keratinocyte, fibroblast, and myofibroblast proliferation and migration [42, 43]. We eliminated right angles in our implant design as, to our knowledge, there are no biological right angles to which there is native tissue integration. Further, it has been shown in *in vivo* models that when a device with a right angle is implanted subcutaneously, a dead space is typically formed and filled with inflammatory cells, as can be seen in both subcutaneous [13, 44] and percutaneous applications [3, 4, 45–47]. On the other hand, *in vitro* studies have demonstrated that cells can integrate and produce a collagen matrix within various geometries, including right angles [48]. The effect of the implant geometry should be looked into further, especially since our previous results, which used a percutaneous implant with right perpendicular angles, demonstrated epidermal downgrowth in nearly all of the porous metal percutaneous implants [1].

With that said, in addition to the implant geometry being a potential contributing factor to the differences observed in epidermal downgrowth from our previous work, another potential factor could be the animal model. In the present study, a rat animal model was utilized; whereas, in our previous work, a rabbit model was investigated. The decision to change animal models was based on several reasons, with one being the need to have an autologous transplantation of MSCs, which could be achieved using a syngeneic rat species. Even though both rats and rabbits wound heal by contraction mechanisms, each animal possesses a unique physiology and anatomy that may potentially contribute to the differences observed in epidermal downgrowth between the two studies.

To address the theory that motion or movement of the percutaneous implant can inhibit epidermal attachment, thus contributing to the downward migration [3, 4, 13], we observed histological signs of movement in the subcutaneous space (e.g. remnants of hemorrhage and

hemosiderin). However, these histological observations were not accompanied by epidermal downgrowth. Further work evaluating implant geometry, as stated above, and wound healing signaling cues important in epidermal migration might provide more insight as to possible reasons why the epidermis may or may not form a stable attachment with the implant.

Though we have demonstrated some encouraging results in this study, there are some limitations to be kept in mind. First, an inability to accurately know the number of viable MSCs delivered to the tissue. During the cell seeding process, the cells were seeded with equal distribution throughout the entire porous coating on the dorsal portion of the implant. When the implant-cell construct was placed in the animal, all the cells that were in the uppermost portion of the post most likely died. Thus, the total cell number that was delivered was most likely less than what was estimated. Second, and related to the first, is that any shearing force between the tissue and implant during *in situ* implantation may have pulled off some of the cells on the implant surface. This could possibly be accommodated for by modifying the titanium surface with adhesion proteins such as collagen, fibronectin, or laminin that may ultimately increase the strength of attachment between the MSCs and the implant surface. Third, though we placed the implants as far apart as possible (5cm), we cannot confirm that the cells did not migrate to the untreated implant; however, this implant arrangement allowed us to eliminate animal variability in directly comparing treated and untreated implants. Lastly, limitations in the rat animal model greatly limit the human clinical translation since not only is the skin structure different between rats and humans, but also rats heal by contraction as opposed to epithelialization, which is the predominate skin healing mechanism in humans.

We have demonstrated that porous titanium percutaneous implants treated with MSCs accelerate tissue integration into the implant and accelerate the wound healing response and tissue reorganization in the pores. While MSCs are known to increase rate of healing in cutaneous wounds, we have now presented results that suggest that MSCs can increase rate of healing and integration to porous metal percutaneous implants. With the current use of long-term percutaneous implants in the clinic and the various problems associated with skin integration, this study presents encouraging data that could further be explored to improve the functionality and longevity of these clinically used percutaneous devices.

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Fig. 1. (A) Porous metal percutaneous implant used in study. (B) Scanning electron microscopy image (SEM) of titanium porous coating having $\sim 360\mu\text{m}$ pore size and $\sim 55\%$ porosity (magnification: $50\times$; accelerating voltage: 20.0kV). Scale bar is 1mm in length.

Fig. 2. Histology analysis template displaying the 13, 1mm² boxes around the implant surface. This is a cartoon graphic of the titanium substrate, thus the boxes are positioned over the 1mm porous coating.

Fig. 3. Differentiation of P.8 BMMSCs. (A) Control cells in complete growth medium (4× magnification). (B) Adipogenic differentiation and Oil Red O staining of lipid droplets (10× magnification). (C) Osteogenic differentiation and Alizarin Red S staining of calcium deposits (10× magnification).

Fig. 4.

Tissue and cellular infiltrates of “day 0” implant. (A) Skin and underlying soft tissue interfacing with porous coating (black) of percutaneous component on implant (scale bar is 1mm; 4× original magnification; H&E). (B) Macrophage infiltration, along with red blood cells, into porous coating (black) (scale bar is 100μm; 20× original magnification; H&E).

Fig. 5. Porous titanium percutaneous implants at 3 days and 28 days post-transplantation. (A and B) Implants at 3 days with residual blood clot at skin-implant interface on both treated (A) and untreated (B) implants. (C and D) Implants at 28 days showing the skin more settled around the post and appearing healthy around treated (C) and untreated (D) implants.

Fig. 6.

Tissue infiltration throughout 56 days. (A) Percent infiltration of fibrin/serum into the porous coating of treated and untreated implants. At 3 days, treated implants had significantly less fibrin/serum compared to untreated implants ($*p < 0.05$). (B) Percent infiltration of collagen matrix into the porous coating of treated and untreated implants. Treated implants had significantly more collagen at 3 days ($*p = 0.05$) and at 28 days ($\# p < 0.05$) compared to untreated implants. Data are represented as means + SEM, $n=5-6$.

Fig. 7.

Tissue reactions to treated and untreated implants at 3 and 7 days. (A) Treated implant (I) from a 3-day animal showing increased cellular infiltration (black arrow), increased collagen matrix, and decreased fibrin/serum within porous coating (P) compared to untreated implant (scale bar is 100 μ m; 10 $\times$ original magnification; H&E). (B) Untreated implant (I) from a 3-day animal showing increased fibrin/serum infiltration in porous coating, with little cellular infiltration (black arrow) and little collagen matrix deposition (10 $\times$ original magnification; H&E). (C) Granulomatous inflammation tissue infiltrating (white arrow) and surrounding a treated implant (I) from a 7-day animal. Tissue contained many macrophages, fibroblasts, collagen matrix, and vasculature (scale bar is 1mm; 4 $\times$ original magnification; H&E). (D) Very few fibroblasts infiltrating (white arrow) porous coating of untreated implant (I) from a 7-day animal. Early collagen matrix deposition with fewer inflammatory cell infiltrates (4 $\times$ original magnification; H&E).

Fig. 8.

Cellular infiltrates and neovascularization over the 56-day period. (A) Cellular infiltrates over the 56-day period in treated and untreated implants. The total number of cells peaked at 7 days for the treated and thereafter slowly decreased. The cell numbers increased throughout time for the untreated implants, peaking at 56 days. (B) The individual cells comprising the cellular infiltrates over 56 days between the treated and untreated implants. Notice the trend in macrophages as they peak at 7 days for treated implants and then slowly decrease; however, for the untreated implants they substantially increase throughout 56 days. (C) Neovascularization between the treated and untreated implants throughout the 56-day period. Data are represented as means + SEM, n=5–6.

Fig. 9.

Epidermal attachment with treated and untreated implants at 28 days. (A and B) Epidermal integration (blue arrows) with porous coating (black) on percutaneous component of **treated** implant. There was vascularized (white arrows), viable tissue in pores above where epidermis appeared to be integrating (scale bar is 100 μ m; 10 $\times$ original magnification; H&E). (C and D) Epidermal integration (blue arrow) with porous coating (black) on percutaneous component of **untreated** implant. Note viable tissue with blood vessels (white arrows) in pores above where epidermis appeared to be integrating (10 $\times$ original magnification; H&E).

Fig. 10. Untreated implant at 56 days demonstrating increased inflammatory cell influx in porous coating. Foreign body giant cells (white arrows) and macrophages (black arrow) lining implant surface in untreated implant (scale bar is 100 μ m; 10 $\times$ original magnification; H&E).

Table 1

Histology outcomes and procedures for analysis and interpretations. Refer to Figure 2 for implant locations. Each box was 1mm² and covered porous coating. Polymorphonuclear leukocytes (PMNs); foreign body giant cells (FBGCs).

Outcomes	Locations	Analysis
Cellular Infiltrates (PMNs, Lymphocytes, Plasma Cells, Macrophages, FBGCs)	Boxes 1–13	Each cell type was counted at 200× magnification
Neovascularization	Boxes 1–13	Number of blood vessels ($7\mu\text{m}$) were counted at 200× magnification
Tissue Ingrowth	Boxes 1–13	Determined % fill of collagen and % fill of fibrin/serum using 100× magnification
Epidermal Downgrowth	~Boxes 1 and 13; 3 measurements taken per side	Measured distance (μm) between leading edge of epidermis and starting location of downgrowth at 200× magnification
Fibrous Capsule	Boxes 2–12; 3 measurements taken per box	Measured distance (μm) of fibrous capsule thickness at 200× magnification

Table 2

Cell surface marker expression determined by flow cytometry on P.8 BMMSCs. Data is represented as percent of the gated population.

Cell Surface Marker	Percent Positive	Percent Negative
CD29	100	0
CD90	100	0
CD34	3.3	96.7
CD45	0.2	99.8
CD11b	1.1	98.9
CD79a	13.8	86.9