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14. ABSTRACT

Following full thickness burn injuries, the subcutaneous adipose tissue often suffers severe damage. Even when the hypodermal fat layer is not fully involved, surgical intervention usually results in removal of the hypodermis down to muscle fascia. Although this avoids the complications of inadvertently leaving necrotic foci in the wound bed, it negates the benefit of retaining viable hypodermal adipose tissue and microvasculature. Grafting onto fat has been shown to reduce wound contraction, especially in extremity burns located near joints, resulting in better range of motion and improved sensation. This indicates that grafts onto fat may heal better and have improved innervation. In addition, the removal of the hypodermal tissue results in loss of vasculature and poor graft take, leading to wound contraction and scarring. The purpose of this study was to evaluate application of micro-fragmented adipose tissue (Lipogems) as a hypodermal skin substitute using hydrogels in a porcine full-thickness wound model. We hypothesize that early reconstruction of hypodermis using purified Lipogems and hydrogels will improve angiogenesis, healing, and scar appearance.

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1. INTRODUCTION:

Subject: Burn wound care is a major socio-economic problem in the United States (**1-3**). If not appropriately treated, burn wounds may result in long-term healing complications, such as contraction, and scarring, which negatively impact the quality of life of an individual. The current standard of care to treat deep partial thickness and full thickness burns involves excision to viable wound bed followed by early coverage with a split thickness skin graft. Even when the hypodermal fat layer is not fully involved, surgical intervention usually results in removal of the hypodermis down to muscle fascia to avoid the complications of inadvertently leaving necrotic foci in the wound bed. Understanding the importance of hypodermis in the development of scarring has great importance in achieving optimal functional recovery following thermal injuries. Moreover, currently available skin substitutes do not include the adipose tissue layer in their constructs. A recent clinical study shows that skin grafting on subcutaneous fat to have a substantial influence on reduction of repeated reconstructive surgeries and long term scarring which may reduce serious deformity complications (**4**). Therefore our effort in this project focuses on reconstruction/replacing subcutaneous adipose tissue after a full-thickness skin injury. Recently, a point-of-care adipose tissue processing technology (Lipogems) has been developed that is a sterile closed system in which lipoaspirate (adipose tissue collected during liposuction procedure) is treated only by mechanical processes and filtering methods without any enzymatic digestion. For clinical use, Lipogems can be isolated at the 'bed-side' in the surgical suite. The Lipogems essentially contains mesenchymal stem cells, pericytes, and endothelial cells in a cluster within the collagenous extracellular matrix (**5-7**). Moreover, the advantage of Lipogems is currently an FDA approved device and the resulting processed tissue can be used for immediate clinical use. In order to optimize the use of Lipogems we have taken a systematic approach to evaluate processed adipose tissue *in vitro* and then *in vivo*.

Purpose: The major goal of this project is to reconstruct full-thickness wound with microfragmented adipose tissue (Lipogems). In order to assess the feasibility, and efficacy of using Lipogems to treat full thickness skin loss, we have used a porcine full-thickness wound model. Wherein, following excision, adipose layer is spared to prepare hydrogel-Lipogems formulations and used along with autologous meshed split thickness skin graft (mSTSG) to cover the full-thickness wounds.

Scope: We aim at providing a cost-effective, bedside treatment option to regenerate full-thickness wounds with better long term healing. To accomplish this goal, we propose to deliver Lipogems using platelet free plasma (PFP) hydrogels. Lipogems will retain the essential cell populations necessary for regeneration, and when delivered via hydrogel matrix will improve healing of full-thickness wounds with better scar outcomes. The Lipogems-hydrogel treatment will allow cells within the Lipogems to recapitulate the 3-D microenvironment and act as a conducive medium to foster better graft take and wound healing outcomes.

2. KEYWORDS:

Porcine Excision Wound, Skin Graft, Adipose Tissue, Lipogems, Hydrogels

ACCOMPLISHMENTS: The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?
List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Major Goals
1. Optimization and characterization of Lipogems and hydrogel based formulations.
2. Screen and evaluate optimized Lipogems-hydrogel based formulations in a 30 cm^2 porcine full-thickness acute excision wound model for improved graft take, vascularization, and wound healing.
3. Evaluate the optimal Lipogems-hydrogel formulation using a 30 cm^2 full-thickness porcine burn wound model to assess healing and scarring (anticipated start date: January 2019).

Specific Aim 1: Optimization and characterization of Lipogems and hydrogel based formulations	**Timeline (Months)**	**Status**
Major Task 1: Human Lipogems-hydrogel based formulation	**Y1**	Delayed start: Y1Q4
Subtask 1: Lipogems-PEGylated PFP hydrogel formulation	9-12	Completed:Y2Q2 Y1Q4-Y2Q2
Subtask 2: Lipogems-PEGylated fibrin hydrogel formulation	9-12	
Subtask 3: Lipogems-collagen hydrogel formulation	9-12	
Subtask 3: *In vitro* characterization Lipogems-hydrogel formulations	12-15	
Milestone(s): *1. Human and porcine adipose tissue Lipogems preparation protocol established.* *2. In vitro Lipogems and Lipogems-hydrogels characterization completed.* *3. Written and submitted 1st manuscript (Pending, manuscript in-preparation).*		
Specific Aim 2: Screening and evaluation of Lipogems in full-thickness porcine wound model	**Y2**	Start: Y2Q1 Completed:Y2Q4
Major Task 1: Full-thickness excision wound model (30cm^2 wounds)	7-24	Y2Q1-Y2Q4
Subtask 1: Autologous adipose tissue isolation	13-24	
Subtask 2: Surgical procedure	7-24	
Major Task 2: Treatment of full-thickness wounds with Lipogems and Lipogems-hydrogels	10-24	
Major Task 3: End point measurements	13-24	
Subtask 1: Photo-Documentation of Healing	13-24	
Subtask 2: Assessment of graft take and healing	13-24	
Major Task 4: Data Analysis	22-30	Pending
Milestone(s) Achieved: *1. Treatment protocol established to evaluate Lipogems and Lipogems-hydrogel formulation.* *2. Effective dose of Lipogems and Lipogems-hydrogels identified.* 3. *Efficiency of Lipogems and Lipogems-hydrogel formulation evaluated.*		

What was accomplished under these goals?

1. MAJOR ACTIVITIES:

- **Optimization and characterization of Human Lipogems and hydrogel based formulations** *(completed Y1 task).*
- **Screening and evaluation of Lipogems in full-thickness porcine wound model**

2. SPECIFIC OBJECTIVES

- Optimized method to isolate Lipogems (human and porcine) of desirable size range was established.
- Optimized volume fraction of human Lipogems to hydrogel (using PEG-PFP, PEG-fibrin and Collagen hydrogels) that allowed cells from the Lipogems to migrate and proliferate within the hydrogel microenvironment.
- Treatment protocol established to evaluate Lipogems and Lipogems-hydrogel formulation in a porcine full thickness wound healing model.
- Effective dose of Lipogems and Lipogems-hydrogels identified.
- Efficiency of Lipogems and Lipogems-hydrogel formulation evaluated *in vivo*.

3. KEY OUTCOME (*for the current reporting period-Year 2*)

METHODS:

a. Isolation of Lipogems from porcine and human adipose tissue: Lipogems from porcine and human subcutaneous adipose tissue was isolated using the kit supplied by LIPOGEMS, USA. The isolation process involves mechanical shearing of the adipose sample without addition of any enzymes. Briefly, emulsion of fragmented adipose tissue forms within the barrel and the saline flow washes the tissue, under gravitational force until saline barrel clears and allowing the processed adipose tissue (Lipogems) to float which are then extruded through the grey filter and collected for further experiments (**Figure 1**). The cluster of Lipogems were collected in a separate sterile conical tube and used further for preparing Lipogems-hydrogel (collagen, FPEG and PEG-PFP) formulations. Lipogems isolated from both human and porcine sources were cryopreserved using standard cryoprotectant media (Dulbecco's Minimal Essential Media (DMEM) containing 10% dimethyl sulfoxide and fetal bovine serum.

b. Preparation of Hydrogels:

***Collagen Hydrogel*:** Collagen hydrogels were created by initiating fibril formation in type 1 collagen (900 µl, 5 mg/mL of rat tail tendon collagen; Travigen) by adjusting the pH to 6.8–7.0 using 100 µl of 10x Dulbecco's phosphate buffered saline (PBS) and 23 µl of 1N sodium hydroxide (NaOH). The fibrillated collagen was added to a 6-well cell culture insert and incubated for 30-40 min at 37°C to allow complete gelation of collagen.

***PEGylated Fibrin hydrogel (FPEG)*:** PEGylated fibrin hydrogel was prepared by mixing 250 µl of succinimidyl glutarate modified polyethylene glycol (SG-PEG-SG) (8 mg/ml in tris buffered saline; TBS) with 500 µl of fibrinogen stock (40 mg/ml in TBS) incubated for 20 min in a 5% CO_2 humidified incubator at 37°C. After incubation, the PEGylated fibrinogen wase cross-linked with 1 ml of thrombin stock (25 U/ml in 40 mM of calcium chloride ($CaCl_2$) at a

final concentration of 10 U/mL in a cell-culture insert (6-well format), the mixture was incubated in a 5% CO_2 humidified incubator at 37°C for 10 min to allow for complete gelation. ***PEGylated platelet free plasma (PEG-PFP) hydrogel*:** PEG-PFP hydrogels were prepared from PFP isolated from both human and porcine whole blood. Briefly, PEG (8mg/ml) was mixed with a PFP at a 1:20 v/v ratio. This mixture was then incubated for 10 minutes in a 5% CO_2 humidified incubator at 37°C. Gelation of the PEG-PFP liquid mixture was then initiated using human thrombin (10U/ml of PEG-PFP) and incubated for 20 minutes in a 5% CO_2 humidified incubator at 37°C to obtain PEGylated PFP hydrogels.

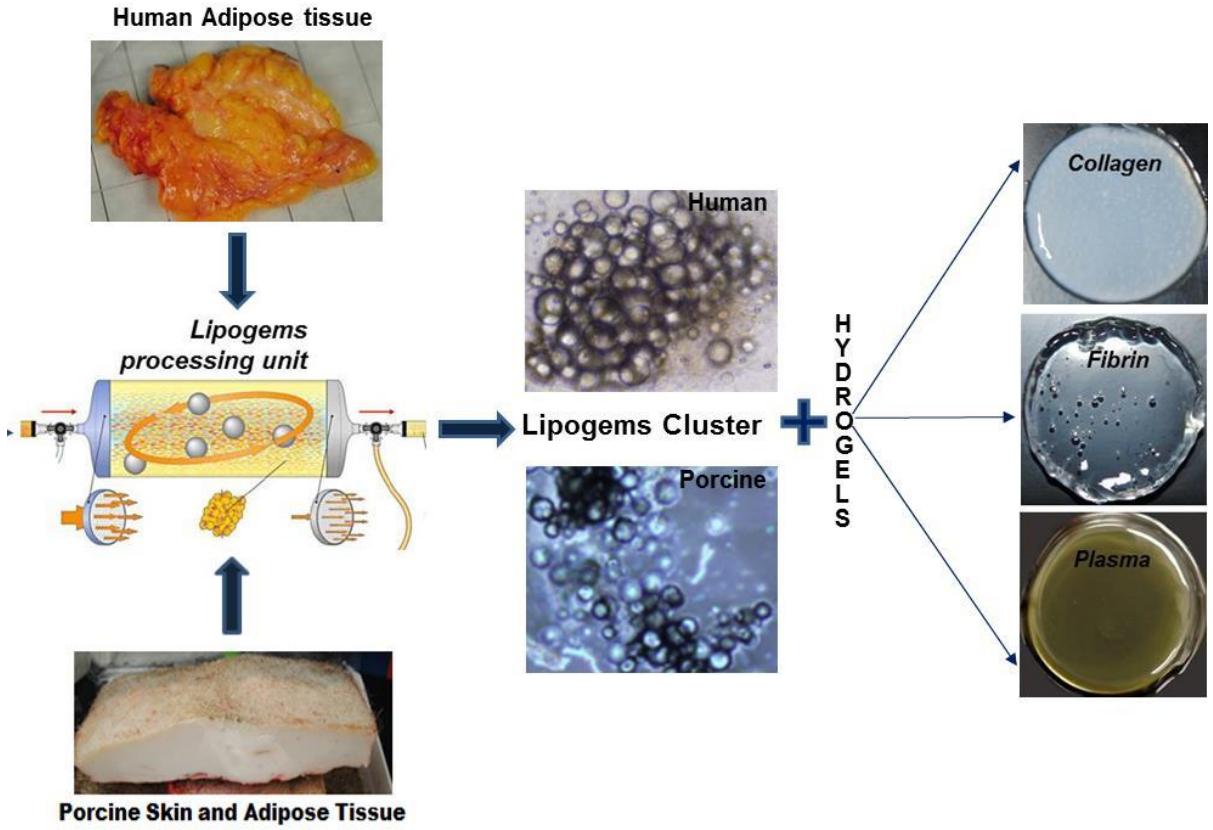


Figure 1: Schematic representation of Lipogems isolation process from porcine and human adipose tissue and formulation of Lipogems-hydrogels

c. Hydrogel-Lipogems formulations: Different hydrogels (collagen, FPEG and PEG-PFP) were prepared by mixing human Lipogems clusters of different volumes (50 µl, 100 µl and 250 µl/ml of PEGylated PGP hydrogel). Briefly, Lipogems cluster of different concentrations were mixed with collagen, FPEG and PEG-PFP solution in a 12-well format cell culture insert and gelled as mentioned above. Lipogems incorporated hydrogels (1.1 cm^2 surface; 12mm diameter and ~ 0.5 to 0.7 cm thick) were maintained in MesenPRO RS™growth media for 10 days and photomicrographs were recorded.

Note: Best performing hydrogel, *PEG-PFP hydrogel*, was selected by observing the cell sprouting over 12 days and further used for the *in vivo* analysis.

d. Full-thickness excision wound model: The animal studies were carried out with ACURO (MB150163) and IACUC approval, and has been conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations, and the principles of the Guide for the Care and Use of Laboratory Animals. Prior to excision and treatment, 100 ml of blood was obtained and used for preparing PEG-PFP hydrogels. Full thickness skin loss was performed using a surgical blade down to fat/fascia, with appropriate pain control and anesthetic protocols. The area excised was approximately a 28-36 cm^2 dermal piece from the central dorsum of the pig. A total of 50 6 cm diameter wounds (10 per animal; N=5 pigs) were created. Of 10 wounds, 9 were excised to fascia and treated while one wound was carefully excised to only remove the epidermis and dermis, leaving the subcutaneous fat layer intact. All wound edges and two equally large growth control areas were tattooed. The experimental timeline, collection of tissue biopsies, and end point measurements are shown in **Figure 2**.

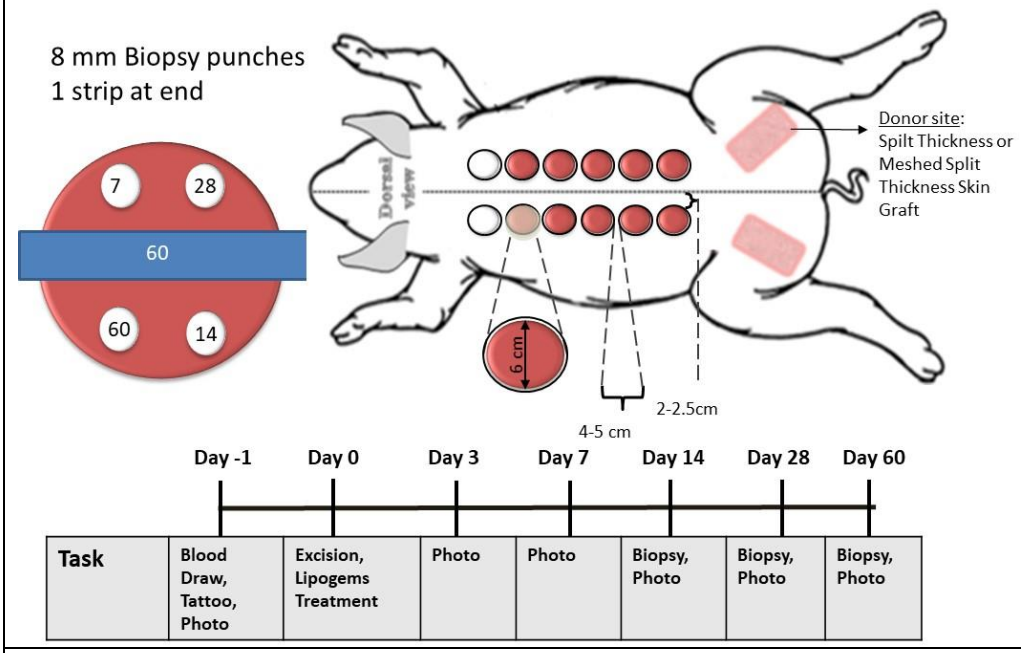


Figure 2: Schematic diagram of experimental wounds on the dorsum, time-line for treatment and assessment protocol, and biopsy procedure of pigs are represented above.

e. *In vivo* efficiency of porcine Lipogems and PEG-PFP-Lipogems: PEG-PFP hydrogels-Lipogems were prepared by mixing the Lipogems extracted from the surgically excised adipose tissue with hydrogel solution prior to gelation. Briefly, PEG-PFP hydrogels were prepared from PFP isolated from porcine blood. PEG-PFP with Lipogems clusters of different volumes (1 ml and 2 mlper 8mL of PEG-PFP hydrogel) were mixed with PEG-PFP solution in a 6 cm diameter sterile aluminum molds and gelled, the wounds were randomized (n=8/pig) and treated with the PEG-PFP hydrogels ± Lipogems of different concentration represented in **Figure 3A**. After application PEG-PFP hydrogels ± Lipogems (**Figure 3B**), all the wounds were covered with a split-thickness skin graft (STSG) that was harvested from the hind legs using a Zimmer pneumatic dermatome (Zimmer Inc, Warsaw, IN) set at the thickness of 12 thousandths of an inch (0.012") and meshed using a skin mesher at 1.5:1 ratio to get a mSTSG (**Figure 3C**). One of the wounds up to fat served as positive control and the other excised till fascia served as a negative control. Each wound was then covered with sterile antimicrobial Telfa wrapped gauze

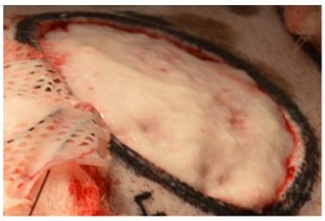
and overlaid with plain gauze. Additional dressing using tape, Ace bandages, stockinette and/or cloth jacket were applied. Punch biopsies (8 mm) were be taken on post treatment days with a strip through the middle also collected on the day of euthanasia (day 60).

Treatment Groups

A

	Groups
1	No treatment control (excised to fascia)
2	No treatment control (excised to fat)
3	PEG-PFP hydrogel
4	Lipogems 1mL
5	Lipogems 2 mL
6	Lipogems 1mL+PEG-PFP hydrogel
7	Lipogems 2mL+PEG-PFP hydrogel

B



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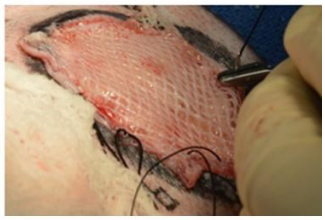


Figure 3: Experimental groups used (Table A), Lipogems processed from excised porcine adipose tissue and applied on the wounds (B) and covered with meshed split thickness autograft (C).

f. End point measurements:

Histology: The wound samples were fixed in 10% neutral buffered formalin, blocked in paraffin wax and 5µm section were cut and stained using Masson's Trichrome stain (MTS). Light microscopic images were taken using a Leica microscope (DMI 3000, Buffalo Grove).

Measurement of healing rate: Wound contraction were measured using pictures taken at different day pre and post treatment by Silhouette star (Aranz Medical), a non-contact device. Effect of Lipogems formulations on wound healing were calculated with measurements of the wound size compared to their original size. The unwounded growth control areas were used to normalize the wound size.

Measurement of blood perfusion: The blood flow microcirculation on the surface of the wound bed was measured using a commercially available Laser Speckle Imaging (LSI) system (moorFLPI-1, Moor Instruments). The blood flow perfusion of the entire wound was measured at high spatial and temporal resolution using a standardized setup. Briefly, laser speckle lens was aimed vertically and exactly perpendicular to the wound surface, the device focus and zoom dials were adjusted according to manufacturer's recommendations to achieve optimal image resolution in the field of view. After optimal adjustment, the wound along with tattoo was captured. High-resolution speckle images were acquired using a charge-coupled device camera (CCD). Perfusion data analysis expressed in laser speckle perfusion units (LSPU) was performed offline using the moorFLPI analysis software tool.

Melanin and Erythema Content: Pigmentation and vascularity of wounds treated with Lipogems±PEG-PFP groups and the normal skin site was measured over 60 days using the DermaLab Combo device (Cortex Technology, Denmark). To measure the pigmentation and vascularity, the color probe was place on the wound surface over the clear front and illuminated by the white LED lights. Spectrophotometry readings were recorded at 550±30 nm and 660

nm±60nm for hemoglobin and melanin, respectively. Pigmentation and erythema was determined using the melanin and hemoglobin values, respectively.

RESULTS:

a. Human Lipogems-cell sprouting within hydrogels:

The characteristics of cells sprouting out from human Lipogems clusters (50, 100 and 200 µl/ml of hydrogels) into three different hydrogels, collagen; FPEG and PEG-PFP were observed for 12 days. Regardless of hydrogel type, formulation with 50 µl/ml exhibited more cell sprouted over time. With increasing volume of Lipogems, less cell sprouts were observed. Collagen hydrogel showed fewer sprouts that the FPEG and PEG-PFP hydrogels. Specifically, collagen hydrogels with 100 and 200 µl/ml Lipogems showed minimal amount of cell growth (**Figure 4a**). Cells within FPEG hydrogels embedded with 50 and 100 µl/ml Lipogems exhibited elongated structure by day 8 and connected to form tubular structure by 12 days. In FPEG hydrogels with 200 µl/ml Lipogems, very few tubular connections were observed (**Figure 4b**). PEG-PFP hydrogels with 50 and 100 µl/ml Lipogems showed higher cell sprout density by day 8, in comparison to collagen and FPEG hydrogels. Although PEG-PFP hydrogels did not show cell sprouting until day 8, a significant number of cells were observed by day 12 (**Figure 4c**). Overall, PEG-PFP hydrogel with 50 and 100 µl/ml Lipogems allowed Lipogems to form robust cell network formation.

Following these results we down-selected PFP-PFP hydrogel to deliver Lipogems to treat full thickness excision wounds. After the completion of last quarter (Y2Q3), we observed Lipogems 1 ml and 2 ml per hydrogels were best performing with no graft loss, therefore we selected these formulation for further full thickness wound experiments.

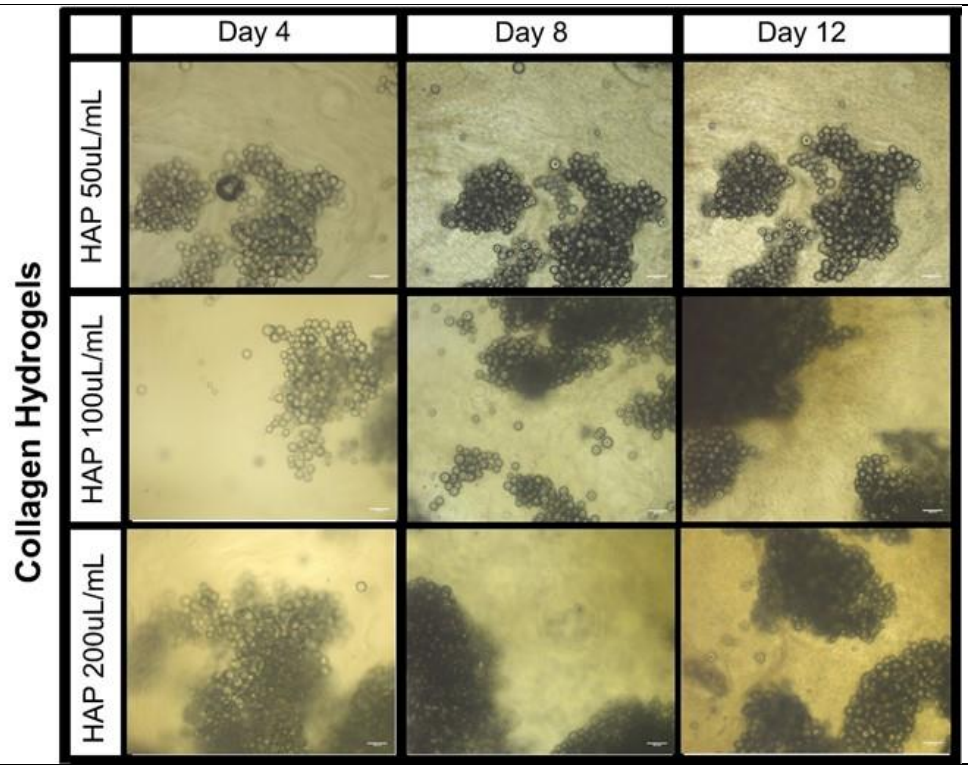


Figure 4a: Light micrographs of cells sprouting within the collagen hydrogel embedded with different concentrations of Lipogems. Images original magnification: ×40.

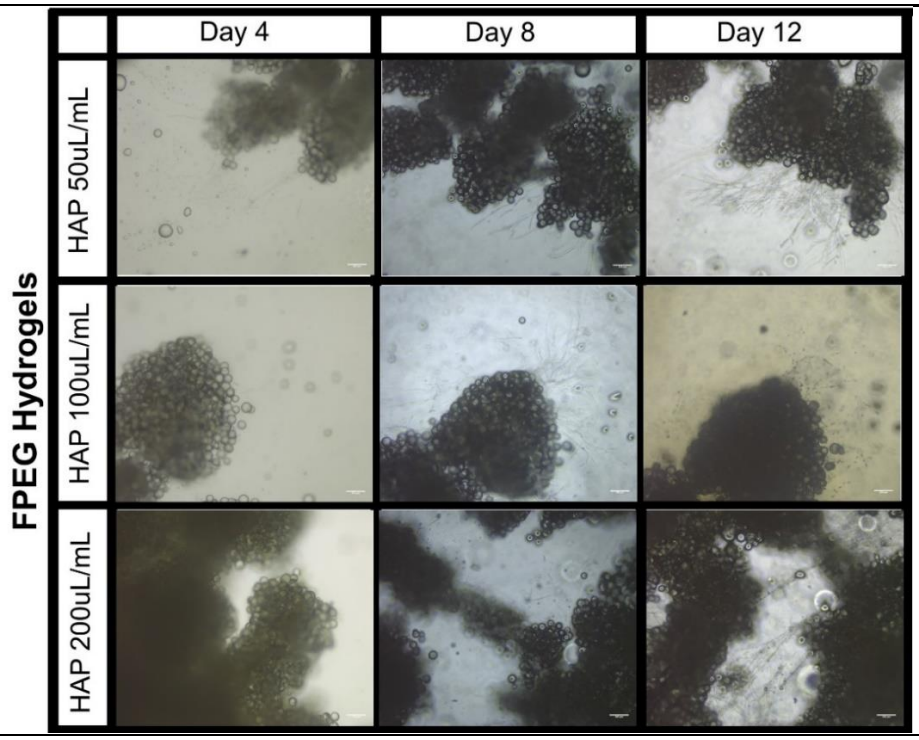


Figure 4b: Light micrographs of cells sprouting within the FPEG hydrogel embedded with different concentrations of Lipogems. Images original magnification: ×40.

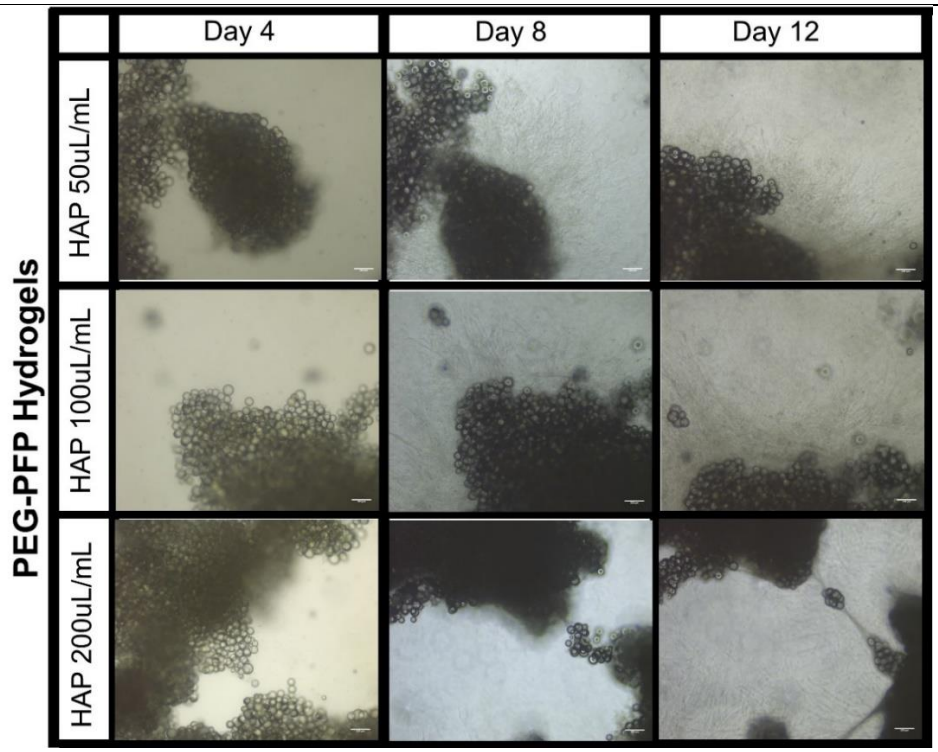


Figure 4c: Light micrographs of cells sprouting within the PEG-PFP hydrogel embedded with different concentrations of Lipogems. Images original magnification: ×40.

b. Effect of Lipogems+hydrogel formulation on full thickness wounds

Wound contraction

The overall contraction of wounds (day 60) treated with Lipogems±PEG-PFP hydrogels were significantly higher in comparison to the wound with intact subcutaneous adipose layer covered with mSTSG. However, the no difference were found in rate of contraction in comparison to wounds excised to fascia and covered with mSTSG (**Figure 5**). A closer analysis indicated contraction rate observed in wounds treated with Lipogems alone, were slightly lower than the graft on to fascia. However, use of Lipogems showed significantly less contraction than the hydrogel treated group.

Note: We did not observe a significant difference in contraction between wound treated with 1 ml or 2 ml Lipogems±hydrogels. Therefore, we combined groups of different Lipogems concentrations with or without hydrogel into a single group analysis. Overall, the 'n' value per group increased.

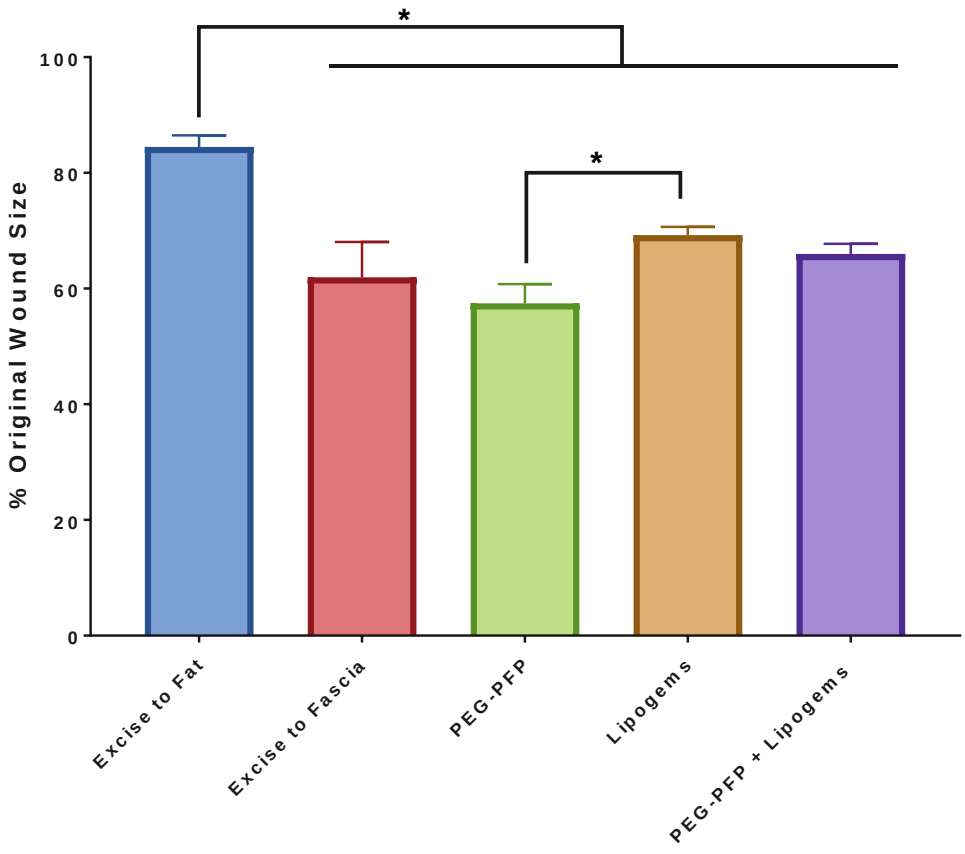


Figure 5: Percentage wound contraction observed for a period of 60 days (N=5).

Perfusion:

With the use of LSI we were able to capture the dynamics of blood perfusion over the course of healing. The flux was recorded at all the time points mentioned in figure 2. A characteristic panel

of LSI produced live images are shown in **figure 6A**. The image color represent the blood flux distribution in a wound; low flux seen as blue, medium flux values are seen as green and high flux values are seen as orange/yellow and red. On day 7, all the wounds excised up to fascia exhibited high blood perfusion, regardless of the treatment groups. Wound excised up to fat and treated with mSTSG showed the least flux. On day 14, most of the wounds excised up to fascia and treated with Lipogems ± hydrogels started to resolve showing lower perfusion, whereas wounds treated with PEG-PFP hydrogel and mSTSG showed more visible flux, indicated by the presence of wide spread yellow –orange color pattern. Post day 14, all the wounds resolved with similar flux pattern. We further quantitated the fold change in blood perfusion for each region of interest within a wound. **Figure 6B** shows perfusion to be higher in wounds that were excised up to fascia and treated with Lipogems and PEG-PFP in comparison to wounds that had intact fat layer and treated with mSTSG. Of note, wound excised to fascia but treated with Lipogems +PEG-PFP hydrogel were not significant from wounds excised to fat and treated with mSTSG, indicating the positive effect of PEG-PFP+Lipogems on wound perfusion.

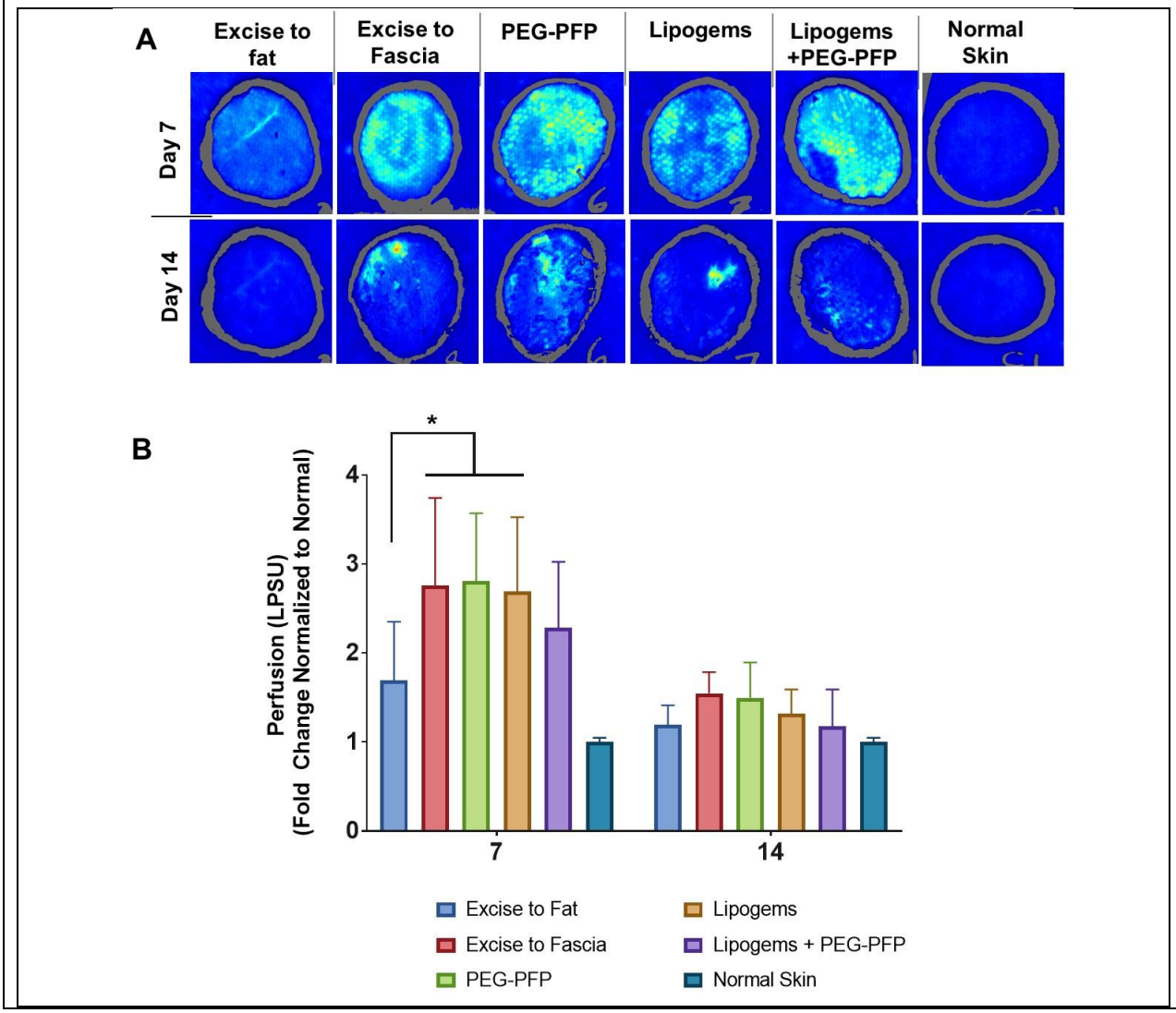
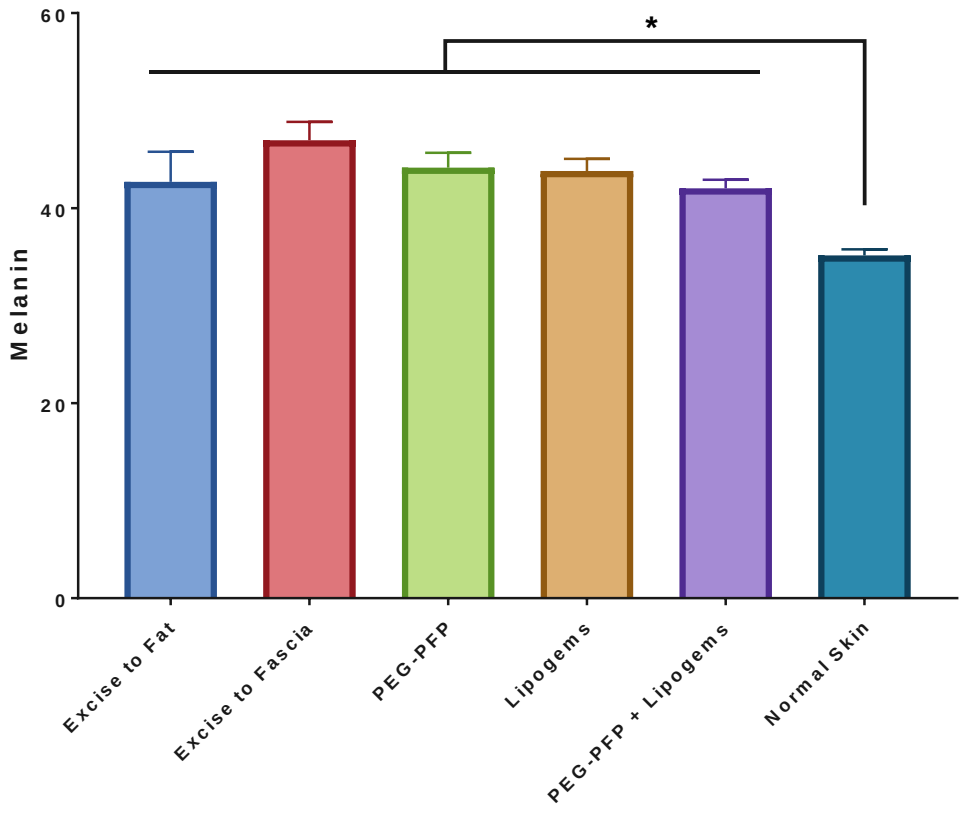


Figure 6: Laser speckle images of wound on day 7 and day 14 (A). The flux data with laser speckle perfusion unit showing the change in dynamics of wound perfusions from day 7 to day 14 (B).

Long-term healing outcome:

DermaLab Combo was used to assess the melanin (pigmentation) and erythema (redness or vascularity) at day 60. Five individual regions of interest within a wound and the normal skin area were measured. **Figure 7A** shows the melanin content measure on terminal day (D60). The melanin content within all the wounds were significantly higher to the normal skin, indicating the wound remodeling is still in progress. However, there was no difference in pigment content between the treatment group, regardless of the treatment type, the wounds with intact fat and covered with mSTSG.

Similar to melanin, erythema was measured using the vascularity measure probe to assess the erythema within the wound on day 60. **Figure 7B** shows the erythema measured on terminal day (D60). The erythema in the wounds excised to fascia and covered with mSTSG were significantly higher than the normal skin. All of the treatment groups other than Lipogems+PEG-PFP did not show a significant erythema value, indicating synergistic treatment with hydrogel and Lipogems to reduce wound erythema. In addition there was not significant difference in erythema content in Lipogems+PEG-PFP treated wounds to wounds excised to fat and covered with mSTSG and also the normal skin.



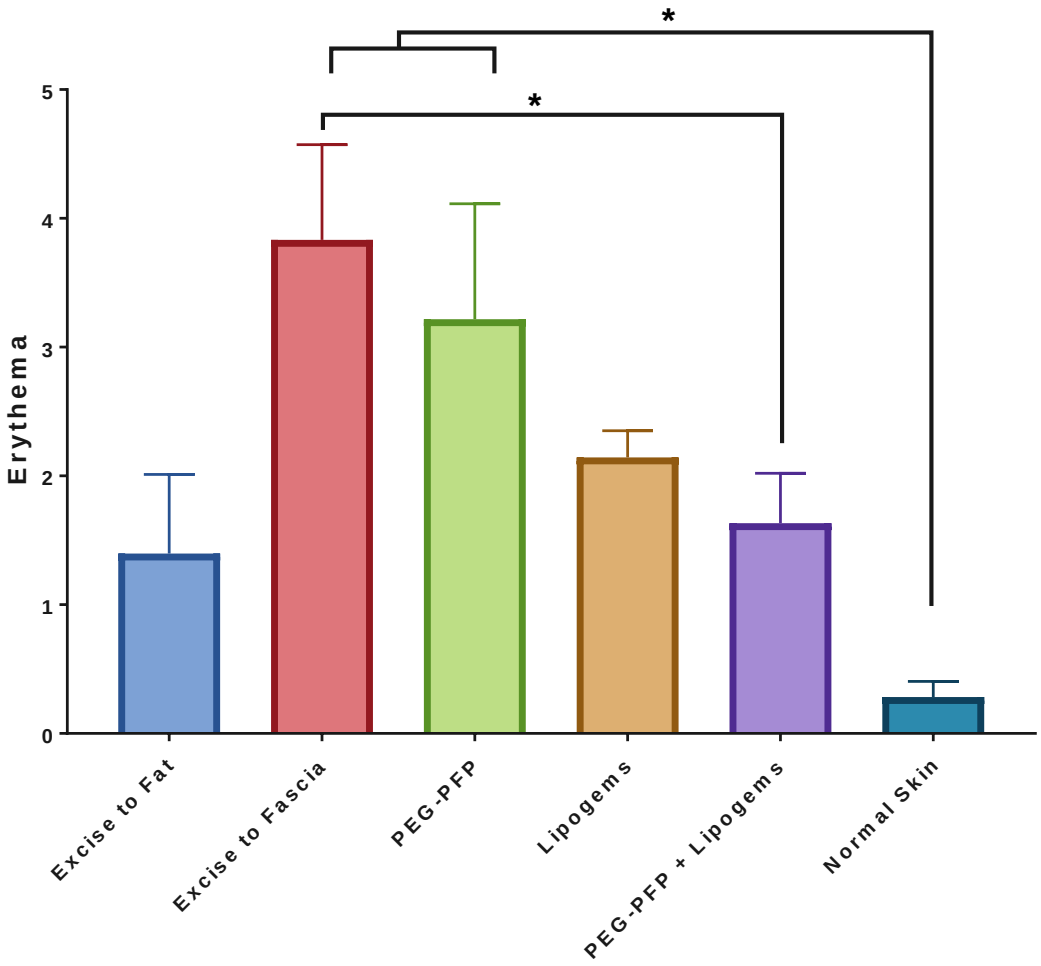


Figure 7: Pigmentation and vascularity of wounds treated with Lipogems±PEG-PFP hydrogels measured using DermaLab Combo. Graph shows values of melanin (A) and erythema (B) to represent pigmentation and vascularity respectively.

Histology:

The MTS histological images showed presence of thicker granulation tissue on day 28 than the wound with intact subcutaneous adipose layer covered with mSTSG, indicating active remodeling. It is interesting to note the wounds excised to fascia and treated with mSTSG were still showing areas with discontinuous epidermis. On day 60, all the treatment groups and the wound with mSTSG alone exhibited complete closure (**Figure 8**). Still, the dermal layer were thicker in treatment groups (Lipogems±PEG-PFP hydrogels) in comparison to the wound with intact subcutaneous adipose layer covered with mSTSG. Overall, there were no graft losses in the groups treated with hydrogel alone and hydrogel+ 1 ml and 2 ml Lipogems (the histological panel show representative images captured with 2 ml Lipogems±PEG-PFP hydrogel).

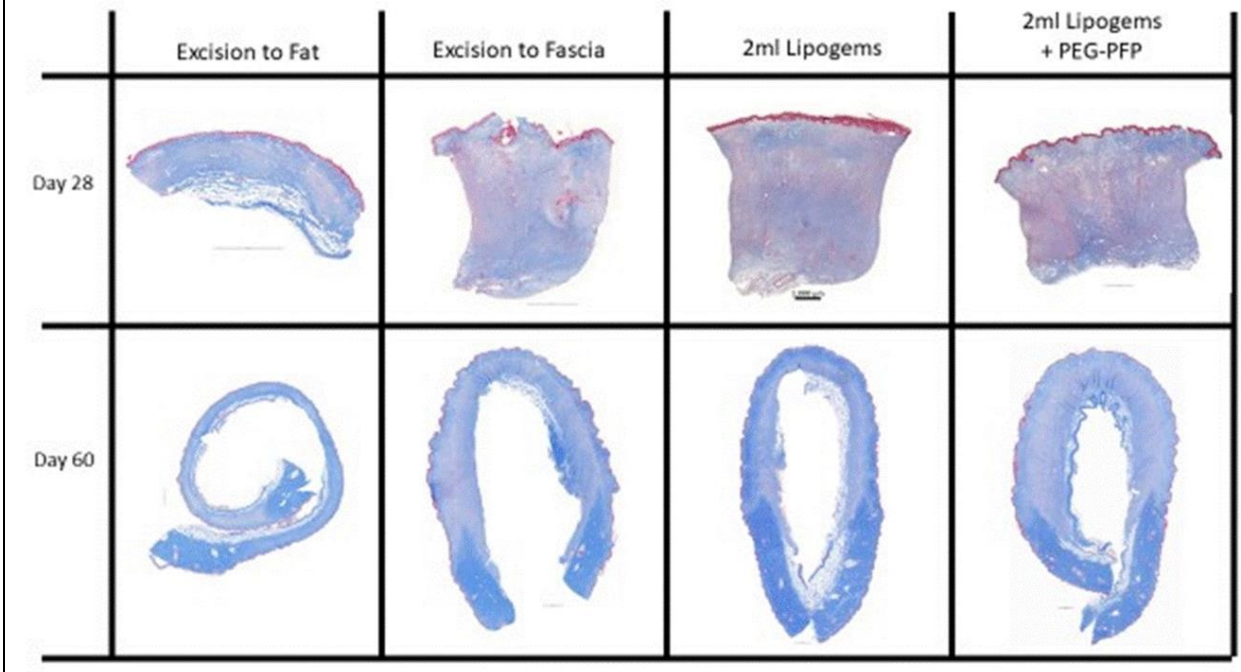


Figure 8: MTS stained sections of tissue biopsies collected from wound treated with 2 ml Lipogems±PEG-PFP hydrogels

DISCUSSION

Adipose tissue reconstruction/replacement in burn victims is less established because of a variety of reasons. Some of the factors include, survival of a thick skin graft, variability in preparation of lipoaspirate, and lack of standardized method of delivering isolated adipose tissue. However, the benefits of using lipoaspirate in treating post-burn hypertrophic scars has been established and reiterates beneficial characteristics of adipose tissue in cases of burn wound treatment (**8**). Therefore, the goal of this project is to establish a protocol to deliver minimally manipulated adipose tissue to the wound site and aid the regenerative healing process. In this approach, we have demonstrated that adipose tissue could be extracted with discarded/excised skin using Lipogems, a sterile closed loop mechanical device without use of any exogenous enzymes.

In this reporting period, Lipogems from human adipose tissue was isolated using the kit provided by the manufacturer. Lipogems formulations with different hydrogels were prepared and tested *in vitro*. Results shows PEG-PFP hydrogels to provide a more conducive microenvironment for the cells to grow out of Lipogems cluster which were able to proliferate and differentiate. The human adipose tissue clusters harbors adipose tissue mesenchyme is the stem cells, called 'adipose-derived stem cells' (ASCs) are responsible for tubular network formation. Further, ASCs been shown to be the important cell type for tissue regeneration (**9, 10**). Regardless of species type ASCs are the functional element in forming vascular network and we have observed similar results, *in vitro*, and documented in our earlier reports. Lipogems clusters were embedded in different hydrogels (collagen, fibrin and plasma) and investigated *in vitro*. Of all the hydrogels used collagen medium had very few cell grown out from the Lipogems cluster, suggesting Lipogems to favor hydrogels with fibrin frame-work corroborating to our finding that PEG-PFP hydrogel promotes human ASCs to form tubular like networks (*completion of pending year 1 specific aim*).

Based on the *in vitro* results, we identified Lipogems cluster volume and the best performing hydrogel to deliver the Lipogems. For the *in vivo* studies, three different dose of Lipogems were selected and delivered using PEG-PFP hydrogels. The primary reason for this approach is optimizing a formulation and establishing an *in vivo* treatment protocol. In particular porcine model, which are considered closest surrogate to human skin wound healing, still have different hypodermis with a dense fibrous architecture. We optimized isolation of Lipogems from porcine adipose tissue from the excised skin and combined with autologous PFP to treat full thickness excision wounds. In the previous reporting period (Y2Q3) three different Lipogems doses were tested (N=3);1 ml, 2 ml and 3 ml Lipogems±PEG-PFP hydrogel formulations and observed no graft loss in formulations with 1 ml and 2 ml Lipogems. Therefore, hydrogels with 1mL and 2mL of Lipogems were used for further experiments. In this reporting period, two pigs with full-thickness excision wounds were treated with 1 ml and 2 ml Lipogems±PEG-PFP hydrogels. There were no graft failure, and the applied Lipogems integrated within the wound with minimal rejection, consistent with the previous treatments. Wound contraction measurement of wound treated with Lipogems (1 ml, and 2 ml) ±PEG-PFP hydrogel formulations did not significantly impact reduction in contraction, though not significant, however there was positive trend in the contraction with Lipogems+2 ml hydrogel formulation.

The quality and long term effect of Lipogems±PEG-PFP on the wound healing was monitored using non-invasive techniques. Early assessment (Day 7) all the wounds excised up to fascia shows high blood perfusion, regardless of the treatment groups. This may be attributed to high capillary plexus formed during this initial phase of healing (**11, 12**). However wound excised to fascia but treated with Lipogems +PEG-PFP hydrogel were not significant from wounds excised to fat and treated with mSTSG, indicating the positive effect of PEG-PFP+Lipogmes on wound perfusion. Two weeks post treatment, blood flow measurement of all the wounds, regardless of treatment group, regressed and did not show any significant difference. These results indicate early blood flow flux may be an indicator of how well a wound may progress to complete healing, i.e., regression of perfusion comparable to wound with intact fat layer may proceed with normal progression.

Finally, we performed an objective measurement of long-term healing outcome; viz., pigmentation and erythema. Clinically, severe wounds, like burns, are susceptible to either hyper or hypopigmentation and increased erythema (**13**). Therefore, it is important to assess these parameters to demonstrate the effect of treatments used in long-term healing outcome. Data generated from the DermaLab probes showed significantly higher melanin content within all the wounds in comparison to the normal skin, indicating the wound are hyperpigmented and are still in remodeling phase. However, the erythema values were lower in the wounds treated with Lipogems+PEG-PFP, which did not show a significant increase in comparison wounds excised to fat and covered with mSTSG and also the normal skin. These findings suggest the application of PEG-PFP+Lipogems may have a positive effect on long-term scar outcome. Therefore, it is worthy to conduct further investigation using a more severe wound model like deep-partial or full thickness burn wound model. Moreover, a recent study shows burn wound in a Red Duroc porcine wound model with pronounced erythema, pigmentation and contraction, in comparison to scars from excision wounds (**14**). Our next step is to use porcine burn wound model to assess the effect of plasma hydrogels and Lipogems on long-term healing. Overall, in this current reporting period we accomplished the goals to establish Lipogems isolation protocol, optimized Lipogems volume and identified the most conducive hydrogel platform to treat full-thickness wounds. In addition, we have established an objective/quantitative non-invasive post-injury measurement of long-term

wound healing parameters. This will be a useful tool to eliminate inter-observer subjective measure variabilities of scar assessment.

Key outcomes (Year 2):

- Prepared human Lipogems-hydrogels – collagen, PEG-PFP and FPEG and identified most favorable hydrogel media for the cells to sprout from the Lipogems cluster. We observed Lipogems to favor PEG-PFP hydrogel than the FPEG and collagen hydrogels.
- Autologous Lipogems can be isolated and used as a bed-side treatment option full thickness wounds for treatment of full thickness skin wounds and formulated with autologous platelet free blood plasma hydrogels.
- The Lipogems±PEG-PFG hydrogel can be successfully delivered to the excision wound without graft loss. The Lipogems formulation exhibited contraction trending positively towards positive control, i.e., wound with intact subcutaneous adipose layer covered with mSTSG.
- The Lipogems+PEG-PFP treatment showed positive effect on long-term healing outcome, specifically erythema.

4. DEVELOPMENTS

One of our research goals is to reduce the number of revision surgeries that a patient would undergo after a severe burn. The current standard of care is to utilize the patient's own skin, which imparts a donor site injury. Therefore, it is equally important to achieve desirable outcome with stringent use of autograft. Aligning to clinical practice, in cases where there is limited availability of donor skin, we are currently pursuing using mSTSG with higher mesh ratio (e.g., 4:1) and use plasma hydrogel as a tissue sparing adjunct. The D28 photograph images of full-thickness burn wounds treated with 4:1 mSTSG + PEG-PFP hydrogel showed less adhesion of allograft (cadaver skin) in comparison to wounds treated only with allograft (**Figure 9**). The study is on-going and our next step will be supplement the widely meshed STSG with Lipogems±PEG-PFP hydrogel and assess the long-term healing outcome.

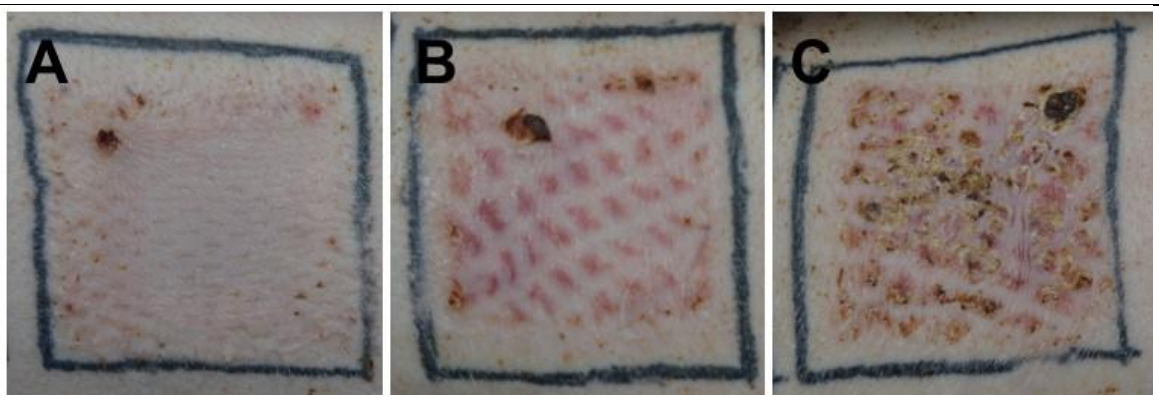


Figure 9: Digital image of full-thickness burn wounds treated with mSTSG 3:1 ratio (A), 4:1 mSTSG +PEG-PFP hydrogel+allograft (B) and 4:1 mSTSG+Allograft (C).

5. GOALS NOT MET

In vitro studies on Lipogems processing and hydrogel + Lipogems preparation characterization manuscript is under preparation.
Anticipated submission date to peer-reviewed Journal: End of next quarter (Y3Q1).

What opportunities for training and professional development has the project provided?
If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state "Nothing to Report."

1. We were able to train two technical support staffs, Mr. David Larson and Mr. Sergio Garcia in Lipogems isolation technique. It is a valuable platform technology that has promising application potentials in treating burn wounds. Moreover, it is an FDA approved device currently in use for performing reconstructive surgery, e.g., breast reconstruction.
2. PI of this project (Dr. Natesan) and staff scientist (Dr. Stone) were able to self-train and develop formulations combining Lipogems and biomaterials in-house, perform pre-clinical studies.

How were the results disseminated to communities of interest?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?
If this is the final report, state "Nothing to Report."

In the next quarter,

1. We will start screening, Lipogems and Lipogems-hydrogel in a full-thickness porcine burn wound model. Animal protocol is submitted for IACUC review and approval. Once approved we will submit to seek ACURO approval to conduct the study.
2. We will establish treatment procedure, and evaluate wound healing progression and document relevant end points, which includes wound contraction measurement, non-invasive analysis and histological assessment.

4. IMPACT: Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

The observation made from this study that stem cells can be non-enzymatically sourced from adipose tissue and used in 3-D hydrogel scaffold to derive similar function of an isolated ASC population, has provided a new, and less-manipulative method to use stem cells for further wound

healing application. The results may be more favorable, because of fewer hurdles for future FDA approval.

What was the impact on other disciplines?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

- Isolation and storage of adipose derived stem cells involved several different steps, time and labor. Lipogems can impact the process of stem cell storage and use, since the clusters can be easily isolated by a service provider (e.g., research nurse), stored and retrieved and still possess similar potentials of enzymatically isolated stem cells.
- The entire process of Lipogems isolation can be carried out in a clinical suite, therefore requiring sophisticated laboratory space and equipment to isolate stem cells.
- Use of Lipogems may improve functional recovery and cosmesis of burn wound patients.

What was the impact on technology transfer?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

LIPOGEMS, USA, has an established CRADA with USAISR, wherein they provide Lipogems isolation kits. Upon mutual agreement and interest, Lipogems-hydrogel technology can be transferred in future to the company for further product development and approval.

What was the impact on society beyond science and technology?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report in the year

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:

Changes in approach and reasons for change

Nothing to Report in the year

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report in the year

Changes that had a significant impact on expenditures

There are no significant deviations, unexpected outcomes, or changes in approved protocols

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Significant changes in use or care of human subjects

Nothing to report

Significant changes in use or care of vertebrate animals.

Nothing to report

Significant changes in use of biohazards and/or select agents

[Nothing to report

6. **PRODUCTS:** List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- **Publications, conference papers, and presentations**
  Report only the major publication(s) resulting from the work under this award.

  Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

None during the reporting period

  Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: Author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

1. Shanmugasundaram Natesan, Randolph Stone II, Rodney K Chan, Robert J Christy; Editor: Xiao-Dong Chen Chapter 8 - Mesenchymal Stem Cell–Based Therapies for Repair and Regeneration of Skin Wounds A Roadmap to Non-Hematopoietic Stem Cell-based Therapeutics, From the Bench to the Clinic; 2019, Pages 173-222, *Academic Press*.

  Other publications, conference papers, and presentations.

1. Randolph Stone II, David Larson, John Wall, Nicole L Wrice, Kyle Florell, Robert Christy, Shanmugasundaram Natesan*; Treatment of Full Thickness Wounds with Microfragmented Adipose Tissue and Plasma-Based Hydrogels Improves Healing Outcome; International Society for Burn Injuries, New Delhi, 03 DEC 2018, Poster; acknowledgement of federal support under this award: Yes

- **Website(s) or other Internet site(s)**

None during the reporting period

- **Technologies or techniques**
  Identify technologies or techniques that resulted from the research activities. In addition to a description of the technologies or techniques, describe how they will be shared.

1. Lipogems-hydrogel technology has potentials to be transferred in future. Pre-clinical data in following year will enable us to generate results to evaluate the efficacy of the formulation.
2. The current indications for Lipogems use is in reconstructive surgeries. Dialogues with clinicians may be initiated to use this technology to isolated adipose tissue fragments for burn wound healing application.

- **Inventions, patent applications, and/or licenses**

None during the reporting period

- **Other Products**

Biospecimen: Human Lipogems were isolated and cryopreserved. This can be used for future in vitro experiment purposes.

Model: A porcine model to reconstruct subcutaneous hypodermis is established.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: ***Shanmugasundaram Natesan****, PhD*
Project Role: *PI*
Researcher Identifier (e.g. ORCID ID): orcid.org/0000-0003-4213-3111
Nearest person month worked: *5*
Contribution to Project: *Dr. Natesan was responsible for overall experimental design, involved in optimizing the process of isolating Lipogems from porcine adipose tissue and oversee progress of this proposal. He conducted animal studies and data analysis. Also, Dr. Natesan was responsible for the submission of animal and IRB protocols for ACRUO and HRPO approval*

Name: ***Randolph Stone II****, PhD*
Project Role: *Postdoctoral research associate*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *8*
Contribution to Project: *Dr. Stone was key in optimizing the size range of Lipogems clusters from porcine adipose tissue. He conducted in vitro studies using Lipogems-PEGylated PFP hydrogel formulations. Dr. Stone conducted animal surgeries and data analysis*

Name: ***Mr. David Larson****, MS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *8*
Contribution to Project: *Mr. Larson isolated helped in Lipogems isolation, in vitro culture, and performed animal surgieries.*

Name: ***Mr. Sergio Garcia****, BS*
Project Role: *Research Technician*
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: *4*
Contribution to Project: *Mr. Garcia performed animal studies, maintenance of medical records and every day animal care.*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report

What other organizations were involved as partners?

LIPOGEMS, USA, in-kind provided Lipogems isolation kits

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS

REFERENCES:

1. http://ameriburn.org/who-we-are/media/burn-incidence-fact-sheet/; ABA burn incidence fact sheet.
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9. APPENDICES: Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

APPENDIX 1: ABSTRACT ACCEPTED FOR PRESENTATION

Treatment of Full Thickness Wounds with Microfragmented Adipose Tissue and Plasma-Based Hydrogels Improves Healing Outcome

Randolph Stone II, David Larson, John Wall, Nicole L Wrice, Kyle Florell, Robert Christy, Shanmugasundaram Natesan
US Army Institute of Surgical Research, JBSA Fort Sam Houston, TX

Background: Full thickness skin wounds, such as third degree burns, often suffers severe damage including the subcutaneous adipose tissue. Most of the surgical interventions involves autograft or allograft application and does not replace adipose tissue; often leading to wound contraction and scarring. We hypothesize that early reconstruction of hypodermis using purified microfragmented adipose tissue called "Lipogems" and blood plasma-based hydrogels will improve angiogenesis, epithelialization, healing, and scar appearance. In this study we have used porcine full-thickness excision wounds as a screening model to test our hypothesis.

Methods: Lipogems were obtained using a point-of-care non-enzymatic mechanical shearing method. Lipogems-plasma hydrogels were prepared by suspending Lipogems (10-25% v/v) in a polyethylene glycol (PEG)-platelet free plasma (PEG-PFP) liquid mixture followed by cross-linking with thrombin. Morphology of cells sprouting from Lipogems within the PEG-PFP hydrogel was observed for 2 weeks, *in vitro*, using light microscopy. Frozen sections of the Lipogems-gel were cut and stained using alpha smooth muscle actin (αSMA) antibody to observe the cytoskeletal morphology. *In viv*o studies were carried out using porcine full-thickness 6 cm diameter excisional wound model, created on the dorsum of three anesthetized Yorkshire pigs. The efficiency of porcine Lipogems-PEG-PFP hydrogels to improve healing outcomes, such as, contraction, and scarring, was then assessed using paraffin sections stained with Masson's trichrome stain.

Results: Light microscopic images of cells growing out from Lipogems within the PEG-PFP hydrogel formed tubular networks, similar to adipose-derived stem cells and immunofluorescence staining with αSMA confirmed the observation of cells sprouts within the hydrogel are involved in formation of tubular network-like structures. Porcine full-thickness excision wounds treated with Lipogems-PEG-PFP hydrogels as an adjunct with meshed split-thickness skin graft (mSTSG) showed successful graft take assessed on day 7. Histological images analyzed on days 14, 28, 60 and 90 days showed the wounds treated with Lipogems±PEGylated PFP hydrogels started to exhibit a decreasing trend in contraction by day 90.

Conclusions: Results indicate that mechanically processed adipose tissue retains viability and essential factors necessary for active healing to take place. Further, Lipogems-PEG-PFP treatment could reduce scarring and avoid the necessity of multiple revision surgeries.

Applicability of Research to Practice: The Lipogems-PEG-PFP hydrogels described in this study provides a 'point-of-care' treatment option to mitigate scarring that may be translated to burn wounds as well for burn wounds. The entire process of Lipogems isolation and hydrogel preparation can be carried out in a clinical suite; therefore, requiring less sophisticated laboratory space and equipment to isolate and deliver stem cells.

Theme: Burn Reconstruction, Scar Management and Rehabilitation.

<u>APPENDIX 2</u>: Book Chapter

Shanmugasundaram Natesan, Randolph Stone II, Rodney K Chan, Robert J Christy; Editor: Xiao-Dong Chen Chapter 8 - Mesenchymal Stem Cell–Based Therapies for Repair and Regeneration of Skin Wounds A Roadmap to Non-Hematopoietic Stem Cell-basesd Therapeutics, From the Bench to the Clinic; 2019, Pages 173-222, *Academic Press*.

PDF Copy of Book Chapter Attached

Chapter 8

Mesenchymal Stem Cell–Based Therapies for Repair and Regeneration of Skin Wounds

Shanmugasundaram Natesan[1], **Randolph Stone II** [1], **Rodney K. Chan**[2,3], **Robert J. Christy**[1]

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1. INTRODUCTION

1.1 Wound Healing

Wounds are generally classified into two main categories, acute and chronic. Acute wounds are the result of some form of trauma, accidental or surgery-induced, or burn. In the US alone, over 50 million trauma, 50 million surgical procedures, and 1 million burn-related operations are performed every year [1]. These wounds heal over time and follow what is considered a normal wound healing cascade of events consisting of hemostasis, inflammation, cell proliferation, and remodeling [2]. A wound that fails to follow this normal process and doesn't heal in an expected amount of time (generally 4–6 weeks) is termed a "chronic" wound. These wounds remain unhealed for a variety of reasons: infection, prolonged inflammation characterized by infiltration of neutrophils, high levels of inflammatory cytokines [e.g., interleukins (IL-1β and IL-6), tumor necrosis factor alpha (TNF-α)], and/or an imbalance in granulation tissue matrix remodeling, due to increased levels of matrix metalloproteinases (MMPs) [3]. Most chronic wounds are categorized as venous, pressure, diabetic foot, or arterial insufficiency ulcers and affect about 6.5 million people in the US [4]. The incidence of these wounds is increasing due to increased life expectancy, combined with the growing number of people with comorbidities.

A Roadmap to Nonhematopoietic Stem Cell-Based Therapeutics. https://doi.org/10.1016/B978-0-12-811920-4.00008-2

1.2 Physiological Cascade of Wound Healing

The wound healing cascade is a dynamic sequence of overlapping events involving chemical signals, extracellular matrix (ECM) molecules, and a wide range of cell types. Immediately after injury, platelets accumulate at the site of injury and a fibrin clot forms via the coagulation cascade. Thrombin induces platelet degranulation, which leads to the release of growth factors [e.g., platelet-derived growth factor (PDGF-α/β)], transforming growth factors (TGF-α/β), and epidermal growth factor (EGF)]. Inflammation, the second phase of wound healing, occurs 1−5 days after injury and involves the recruitment of inflammatory cells into the wound. Neutrophils are the first responders followed by macrophages. Neutrophils help to decontaminate the wound through phagocytosis of bacteria and foreign bodies. Macrophages are essential for the initiation and propagation of new tissue formation at the wound site and facilitate the transition to the proliferative phase. The third phase, which begins between day 3 and 12, reestablishes the integrity of the epidermis and dermis at the wound site. This is accomplished by the formation of granulation tissue as the immune cells resolve and fibroblasts and other cells begin to proliferate to form the ECM and vasculature. By day 21, the final phase has begun with remodeling of the collagen [type III (provisional) to type I (predominant collagen type in normal skin) collagen] to complete the wound healing process which restores ∼80% of the original skin strength [5].

1.3 Aberrant Wound Healing

During wound healing, fibroblasts restore mechanical stability of the skin by producing ECM molecules (e.g., collagen, fibronectin, elastin, and glycoproteins) [6]. Prolonged inflammation, delayed wound closure, and infection are factors that can lead to aberrant wound healing, resulting in contracture and scarring [7]. Hypertrophic scars (HTS) and keloids involve the dysregulation of a wide array of cellular and molecular factors in both the epidermal and dermal layers which result in wounds that heal with excessive deposition of ECM proteins [8]. Keloids and HTS exhibit upregulation of markers such as cyclooxygenase (COX), connective tissue growth factor, TGF-β, alpha smooth muscle actin (α-SMA), TNF-α, and several proinflammatory interleukins (IL-1α, IL-1β, and IL-6) [7]. The major difference between HTS and keloid is that HTS are confined to the border of the original wound, while keloids grow outside of this boundary [9] and have increased deposition of hyalinized collagen and blood vessels within the dermis.

1.4 Standard of Care

Over the last two decades, several professional societies (Wound Healing Society, International Society of Burn Injuries, American Burn Association) have recognized the need to develop practical guidelines that establish a

standard of care for achieving the best treatment in a cost-effective manner [2,10]. In this chapter, we will provide an overview of the standard of care for wounds with the caveat that each wound type (trauma, surgical, burn, or chronic) has its own nuances and specific strategies. A general overall plan carefully considers the following: (1) Patient assessment, (2) Stabilization if necessary to include inhalation injury and resuscitation, (3) Surgical management to close the wound, (4) Infection prevention and control, (5) Pain management, (6) Wound care, (7) Nutrition, (8) Rehabilitation (physical therapy and exercise), and (9) Scar management [10].

The clinician needs to assess the wound, as well as the patient, to determine what type of standard of care needs to be implemented. An initial acute wound may become chronic if the patient is unable to follow instructions, has underlying comorbidities [human immunodeficiency virus (HIV), obesity, diabetes, high blood pressure, high cholesterol, smoker, drug use, alcoholism], or other issues that compromise the healing environment. A few such issues may include limited resources, limited healthcare access, poor nutrition, age, or even an occupation that requires an individual to work in the heat or on their feet all day. Some patients will present with wounds (trauma and burns) that need to be evaluated quickly and a plan immediately implemented to save their life. During this time, stabilization, airway management, breathing and ventilation, fluid management, and circulation and cardiac status are issues that must be monitored. Closing the wound can be a simple procedure (sutures or staples for a cut) to extremely complex [skin grafting for large total body surface area (TBSA) burns]. As part of the plan to close the wound, the wound bed must be properly prepared so that normal healing can proceed. Debridement of any nonviable tissue and bacteria would be performed down to the level of a viable wound bed to help increase granulation tissue formation. Infection is a major problem with any type of wound with contributing risk factors such as a large wound area, deep wounds, anatomical locations that are easier to get infected (groin), presence of necrotic tissue, reduced tissue perfusion, systemic factors, malnutrition, comorbidities listed above, and even current medications (immunosuppressants). It is generally thought that the reason chronic wounds "stall" is due to high bacterial load which inhibits progression through the normal wound healing cycle. Debridement of the wound bed is one of the most reliable methods for removing bacteria from wounds [11]. Without proper infection control, no healing can proceed and these wounds will typically develop into chronic wounds.

The presence of pain is an indicator of injury. All surgical interventions are accompanied by some form of pain, whether it is acute or chronic, and appropriate measures must be taken to provide adequate pain control. Wound care encompasses how the wounds are dressed and cleansed during the wound healing process [10]. There are literally thousands of different products available for dressing wounds and a number of characteristics must be considered when choosing the right dressing because there is not one that is

suitable for all types of wounds. These variables include the following: ability to maintain a moist wound environment, nonadherent, no or minimal pain for the patient, cost-effective, sterile, high absorption properties (depends on amount of wound exudate from minimal to highly exuding), impermeable to microorganisms, antimicrobial, safe to use, requires infrequent dressing changes, and allows proper gas exchange [10]. No studies to date have definitively shown that a certain wound cleansing agent is more efficacious in preventing infections; however, simply irrigating the wound with tap water has been correlated with decreased bacterial counts [10].

The process of wound repair requires a great deal of energy to rebuild the structural components of the skin and not delay healing. Additional nutritional requirements (proteins, fats, carbohydrates, vitamins, and minerals) may be necessary to fill this need, depending on the severity of the wound (e.g., large TBSA burns or other wounds that require a long hospital stay). Specifically, protein levels must be carefully monitored to ascertain if the body is catabolizing itself when sufficient quantities of protein are not available [1]. Promising studies have tested the addition of certain amino acids, such as arginine and glutamine, to enhance wound healing [12]. Additional protein supplementation can help stop muscle wasting during long hospital stays but increased exercise is also required. Physical and occupational therapists are utilized as early as possible to limit these types of issues. The overall plan is to allow wounds to heal in the most optimized fashion; however, some wounds will eventually result in an unwanted scar. Currently, we cannot predict which patient or wound will heal with scar tissue. While not always possible, the clinician can create suture lines parallel to tension lines to decrease overabundant scar formation [13]. New interventions are being tested to treat existing scars [e.g., the use of lasers (both fractional CO_2 and pulsed dye)] [14].

2. MESENCHYMAL STEM CELLS

Mesenchymal stem cells (MSCs), as the name suggests, are “mesenchyme or middle” cells within the stroma of all adult organs. MSCs are ubiquitous and can be found in a wide array of tissue sources. They are the building blocks that aggregate and form specific tissue structures such as skin, bone, or muscle [15]. Most importantly, MSCs are the body’s own drug store and possess the unique ability to “Repair and Restore” tissue function in the event of an injury. MSCs were first identified and isolated from bone marrow stroma and express surface markers different from hematopoietic stem cells (HSCs). Only in the last decade have MSCs emerged as a valuable cell-based therapeutic option for wound healing. Growing research on the “stem cell niche,” a specific microenvironment containing specific discrete and dynamic domains that maintain stem cells in an undifferentiated and self-renewable state, has led to the understanding that most tissues contain a resident population of stem cells

for maintaining tissue homeostasis [16,17]. Currently, MSCs can be isolated from skin, adipose tissue, umbilical cord, cord blood, amniotic membrane and fluid, Wharton jelly, dental pulp, synovial membrane, trabecular bone, periosteum, and muscle. Table 8.1 lists these sources of MSCs and information on cell surface markers and degree of multipotency.

2.1 Definition and Source

Over the past decade many laboratories have developed methods for isolating and characterizing MSCs from a variety of sources. Over the years, different names and surface markers have been used to identify MSCs; however, in 2013 the International Society of Cellular Therapy (ISCT) proposed guidelines for defining MSCs. According to these guidelines, "MSCs are a population of stromal cells capable of self-renewal and also propagate a defined set of mature differentiated progeny committed towards a specific cell type" [18]. MSCs are a heterogeneous stem cell population, and exhibit the following characteristic features: (1) adherence to cell culture plastic under routine culture conditions, (2) multipotent differentiation potential (i.e., the capacity to differentiate into multiple cell lineages, such as osteoblasts, chondroblasts, and adipocytes), and (3) expression of several specific cell surface markers [CD105 (cluster of differentiation 105), CD73, and CD90] but not others [CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA (human leukocyte antigen) class II surface markers] [19]. Unlike embryonic stem cells (ESCs), which are pluripotent and capable of differentiating into all cell types of the body, MSCs are limited to differentiation to cell types of their tissue of origin. However, due to ethical and legal constraints in the human use of ESCs, MSCs are preferred for regenerative treatments [20].

2.2 MSCs in Skin Injury

Normal skin function is maintained by a constant self-renewal of cells within the different layers of the skin. However, following an acute injury, there is a significant loss of skin structure, including cellular elements, and MSCs play an important role in the repair process by functioning in the following broad categories:

1. *Homeostasis*: MSCs residing within the skin maintain tissue health.
2. *Homing*: Circulating stem cells acquire signals from the host injury site and migrate to the wound site.
3. *Paracrine signaling*: MSCs at the wound site interact with local tissue by secreting factors to initiate the repair process.
4. *Cellular interaction and repair*: MSCs transdifferentiate to skin-specific cell types and interact with host cells to regenerate lost tissue.
5. *Restore*: Soluble factors and cells interact and communicate with MSCs to remodel the wound bed to return skin to normal function.

TABLE 8.1 Representative Tissue Sources of MSCs With Demonstrated Cell Surface Markers and Multipotency

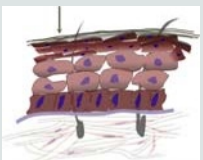
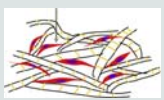
Tissue Source	Mesenchymal Stem Cells	Markers	Multipotency
Epidermis and hair follicle 	1. **Basal epithelial stem cells**: Also called as inter follicular epidermal stem cells, resides in the epidermal region between the orifices of hair follicles. 2. **Hair follicle stem cells**: Progenitor cells of hair follicle; includes, dermal papillae (DP) stem cells, bulge cells and sebaceous gland (SG) stem cells.	1. Integrin-α6, Integrin-β1, CD71 and ΔNp63. 2. Sox9, Tcf3, K5, K14, and p63.	1. Keratinocytes and stratum corneum, melanocytes and neurocytes. 2. Sebocytes, suprabasal keratinocytes, melanocytes, hair follicle, dermal papilla, neurons, glial cells, sweat gland epithelial cells and adipocytes.
Dermis 	1. **Skin-derived precursor cells (SKP cells)**: A cultured adult stem cell from the dermis that generates both neural and mesodermal progeny. 2. **Dermal mesenchymal stem cells (dermal MSCs)**: Undifferentiated population of fibroblast-like stromal cells present within the dermis.	1. Nestin, Sox-2, Sox-9, Pax3, Vimentin and Fibronectin. 2. CD90, CD29, CD44, CD13, CD59, Fibronectin, and Vimentin.	1. Neurons, glia, Schwann cells, smooth muscle cells, adipocytes, osteoblasts, chondrocytes. 2. Adipocytes, osteoblasts, chondrocytes, neurons.

TABLE 8.1 Representative Tissue Sources of MSCs With Demonstrated Cell Surface Markers and Multipotency—cont'd

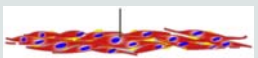
Tissue Source	Mesenchymal Stem Cells	Markers	Multipotency
Bone marrow 	**Bone marrow mesenchymal stem cells (BMSCs)**: Heterogeneous population of stromal cells present within the bone marrow space.	CD13, CD29, CD44+, CD49e, CD73, CD90, CD105, CD146, CD166, SSEA4+, CD49f.	Osteocytes, chondrocytes, adipocytes, hepatocytes, cardiomyocytes, pancreatic cells and neuronal cells.
Adipose tissue 	**Adipose-derived stem cells (ASCs)**: Heterogeneous population of stromal cells present within the perivascular spaces of adipose tissue.	CD29, CD44, CD71, CD73, CD90, and CD105.	Osteocytes, chondrocytes, adipocytes, neuronal cells, epithelial cells.
Skeletal muscle 	**Muscle-derived stem cells (MDSCs)**: A population of progenitor cells present in quiescent state within the skeletal muscle. Also, contains satellite cells, which are often considered as muscle progenitor cells.	CD34, CD56 and CD144.	Adipocytes, osteocytes, chondrocytes, myoblasts, endothelial cells.

TABLE 8.1 Representative Tissue Sources of MSCs With Demonstrated Cell Surface Markers and Multipotency—cont'd

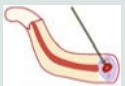
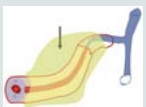
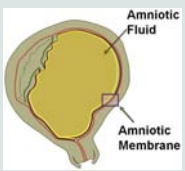
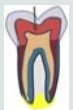
Tissue Source	Mesenchymal Stem Cells	Surface Markers	Multipotency
Umbilical Cord 	**Umbilical cord blood–derived MSCs (UCB-MSCs)**: A heterogeneous population of fibroblast-like stromal cells present within the umbilical cord blood.	CD13, CD29, CD44, CD73, CD90, CD105, αSMA, CD49e and Src Homology (SH) proteins: SH2, SH3, SH4.	Osteocytes, chondrocytes, adipocytes, fibroblasts, neuronal cells.
Wharton Jelly 	**Wharton jelly–derived MSCs (WJ-MSCs)**: A population of stromal cells within a jelly-like substance inside the umbilical cord, called Wharton jelly.	CD13, CD29, CD29b, CD44, CD49e, CD54, CD73, CD90, CD105, CD166, HLA-I, HLA-G6, Nanog, zinc finger protein 42, SOX-2, Oct-4.	Adipocyte, osteoblast, chondrocyte, neuronal cells, endothelial cells.
Placental 	**Amniotic fluid–derived stem cells (AFSCs)**: The population of cells in fluid within the placental sac.	CD29, CD44, CD73, CD90, CD105, CD 117, Oct-4, Nanog, stage-specific embryonic antigen (SSEA)-4, HLA-G6.	Adipocytes, chondrocytes, osteocytes, hepatocytes, neural cell, cardiomyocytes.
	Amniotic membrane–derived stem cells (AMSCs): The amnion is the innermost extraembryonic membrane that surrounds the fetus. It has a population of stem cells in the epithelial and chorion surfaces.	CD90, CD105, CD44, CD73, CD29, HLA-G6, SSEA-4, Nanog.	Osteocytes, chondrocytes, adipocytes, neuronal cells, epithelial cells.

TABLE 8.1 Representative Tissue Sources of MSCs With Demonstrated Cell Surface Markers and Multipotency—cont'd

Tissue Source	Mesenchymal Stem Cells	Surface Markers	Multipotency
The periodontium, (tooth) 	**Dental stem cells (DSCs)**: Dental stem cells (DSCs) are MSC-like populations with self-renewal capacity. There are five distinct population of DSCs; dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle precursor cells (DFPCs).	STRO-1, CD29, CD44, CD73, CD90, CD105, CD146, CD166, CD271, Oct-4, Sox2, Nanog, Nestin, SSEA-4, and Stro-1.	Osteocytes, chondrocytes, adipocytes, neuronal cells, hepatocytes, skeletal and cardiomyocytes, and endothelial cells.
Periosteum 	**Periosteum-derived progenitor cells (PDCs)**: Periosteum is a thin vascular membrane that covers the external surface of bone and contains a rich source of multipotent cells (PDCs).	CD73, CD90, CD105, CD146 and CD166.	Chondrocytes, osteoblasts, and adipocytes.

Following tissue injury, quiescent stem cells in the skin become mobilized and play a major role in the renewal of basal and suprabasal keratinocytes and hair follicles to maintain barrier functions. Several elegant studies have shown that MSCs regulate the function of a plethora of immune cells: T lymphocytes (T cells), B lymphocytes (B cells), natural killer cells (NK-cells), and dendritic cells (DCs) [21–23]. MSCs cocultured with DCs and T cells secreted increased amounts of the antiinflammatory cytokines IL-4 and IL-10 and decreased production of antiinflammatory cytokines TNF-α and interferon gamma [24]. More than just influencing the secretory function of immune cells, MSCs have been shown to modulate the phenotype of tissue macrophages by a process called "MSC educated macrophages" [25]. Consistent with in vitro studies, MSCs were shown to effectively suppress the T cell response in vivo [23]. The immunomodulatory role of MSCs is central to their ability to polarize macrophages to a reparative phenotype (M2) switching the tissue injury to a healing phase [26]. Intravenously administered MSCs accumulate at the wound site and colocalize with pan-cytokeratin in epithelia, CD31 (endothelial cell marker), and α-SMA (myofibroblast and pericyte marker) in the granulating dermal wound bed, and improve overall healing [27]. Intradermally administered MSCs also improve wound closure by significantly elevating the tissue levels of stem cell proliferation and transmigration factor Wnt3 (wingless integrated family 3A), PDGF receptor alpha (PDGFR-α), and vascular endothelial growth factor (VEGF) [26]. In addition, implanted MSCs stimulated the recruitment of endogenous (i.e., host) stem cells to the wound site [28]. Although the number of engrafted MSCs decreased over the course of healing, repair initiated by grafted MSCs continued, suggesting that MSCs play an active therapeutic role in promoting tissue repair and regeneration.

3. SKIN—A RESERVOIR OF MSCS

Skin is a complex organ and is composed of a number of cellular and extracellular components. Although there is a significant amount of cross-talk between the different elements, we have simplified the context by compartmentalizing them into epidermal, dermal, and hypodermal (including subcutaneous adipose) layers (Fig. 8.1).

3.1 Epidermis-Derived Stem Cells

3.1.1 Basal Epithelial Stem Cells

An epithelial stratum is composed of several cell layers of keratinocytes which undergo continuous shedding and renewal to maintain barrier function. In the interfollicular region (i.e., the epidermal region between the orifices of hair follicles) resides a population of undifferentiated cells called "epithelial stem cells" (epithelial SCs) or interfollicular epidermal stem cells. Epithelial SCs

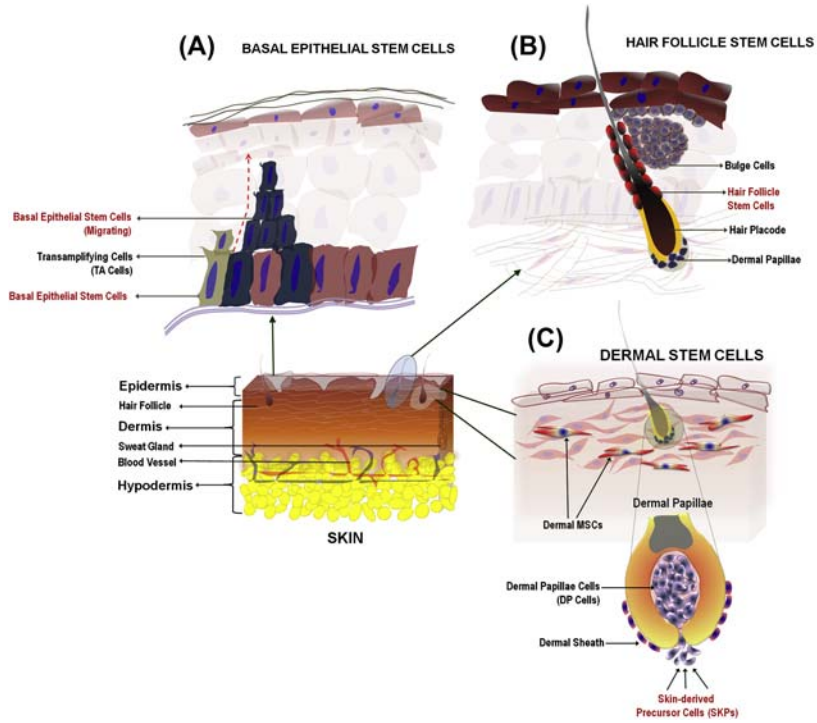


FIGURE 8.1 **Rich sources of mesenchymal stem cells in skin.** Stem cells have been isolated from basal epithelia or the interfollicular region (A); hair follicles (B); and dermal papillae (C).

are surrounded by a rich milieu of basement membrane protein. To maintain skin homeostasis, epithelial SCs divide, delaminate from the basement membrane, migrate upwards, and differentiate into keratin expressing (K1-14) epithelial cells (Fig. 8.1A) [29]. However, not all undifferentiated cells are epithelial SCs, that is, they do not undergo symmetrical cell division. Some of the cells divide several times, then exit the basal layer and move toward the suprabasal layer, and get terminally differentiated. These cells are called as transit-amplifying cells (TACs).

Intriguingly, epithelial SC division and differentiation is highly influenced by the basement membrane constituents, such as growth factors (EGF and TGF-β), structural proteins (laminin 5, integrin $\alpha_6\beta_4$, and $\alpha_3\beta_4$), and ECM protein (collagen IV) [17]. Therefore, following a physiological insult (e.g., superficial burn or excisional wound) epidermal regeneration occurs spontaneously from the wound margins inward resulting in migration and proliferation of basal keratinocytes. During this process, the keratinocytes secrete soluble growth factors along with basement membrane proteins and in coordination with underlying papillary dermal fibroblasts regenerate the epidermal layer [30]. Large wounds, such as a high TBSA burn, often require an epithelial substitute. Currently,

cultured epithelial autografts (CEA) are widely used to treat such wounds [31]. However, due to cell expansion time requirements posed by CEA, there is a need to find alternate sources. Although much epithelial stem cell research is still in its infancy, harnessing its potential to regenerate epidermis following an injury holds promise.

3.1.2 Hair Follicle Stem Cells

Hair is an appendage of skin, and plays a crucial role in thermoregulation, as well as cosmesis. Hair follicles originate from mesenchymal components called dermal papillae (DP) and undergo a perpetual cycle of growth, regression, and rest. Like epidermal cells, a follicle continuously sheds and renews following a three-phase cycle [32]. During this process, stem cells present at different anatomical locations along the axis of the hair shaft ensure maintenance of epidermal homeostasis, hair regeneration, and repair of epidermis after injury. Hair follicle stem cells (HFSCs) originate from the DP, bulge region, and sebaceous glands (SG) (Fig. 8.1B). DP is generated through a temporal series of epithelial−mesenchymal interactions that result in the formation of the hair placode. DPs are sites of underlying mesenchymal cell condensation and are the source of HFSCs [33]. DP cells are often identified by the expression of Sox-2 (sex determining region Y-box 2), a transcription factor that is essential for maintaining self-renewal. In addition, DP cells have been shown to generate neodermis in composite skin that results in increased graft survival in vivo [34]. Another population of stem cells that contribute to wound repair are bulge cells which are identified by their expression of transcription factor 3 and after activation follow the Wnt3 signaling pathway to restore homeostasis [35]. Following a skin injury, bulge cells assume an epithelial-like phenotype, rapidly generate TACs, get recruited to the epidermis, and migrate radially to the center of the wound resulting in acute wound repair [36]. Originating from the bulge cells are SG precursor cells, which participate in epidermal renewal, as well as new hair formation [37]. Although, all three progenitor cells (DP, bulge, and SG cells) originate from different locations, they express K5, K14, and p63 that guide them to differentiate along specific lineages. There are increasing numbers of preclinical animal studies showing that HFSCs accelerate excisional wound healing by significantly increasing the reepithelialization and regeneration of dermal structures [35]. Further, HFSCs from a patient's own skin can be sourced, isolated, and expanded to treat their skin injuries. A recent clinical study provides evidence that punch-type grafts containing intact scalp hair follicles improve healing of chronic venous ulcers after transplantation into the base of wounds. While there is no evidence that stem cells were actually transplanted, this novel treatment protocol opens up more possibilities for treating chronic wounds [38].

3.2 Dermal-Derived Stem Cells

Stem cells have also been found in the dermis. Although less studied than epithelial SCs, investigators have identified three self-renewing populations of cells in the dermal connective tissue matrix: neural crest stem cells, MSC-like dermal stem cells, and hematopoietic cells [39].

The dermal-derived stem cells are collectively called "skin-derived precursor cells" (SKPs) and generate neural and mesodermal progeny (Fig. 8.1C). Neural precursors from the skin can be cultured from dissociated dermis as SKP clusters or spheres and proliferate to form multiple spheroids. Cells within the SKP spheres express neural (nestin, Sox-2, and Sox-9) and mesenchymal markers (vimentin and fibronectin). Unlike MSCs, neural crest cells do not differentiate to the epithelial phenotype; instead they readily differentiate to mesodermal cells expressing neuronal markers (βIII tubulin and neurofilament-M) [16]. However, investigators have been successful at stimulating them to differentiate into adipocytes, osteoblasts, and chondrocytes [40]. SKPs have been proposed to be stem cells contained within the follicular DP niche. However, a recent finding has shown the existence of a group of SKPs of nonfollicular origin present in dermal capillaries that are responsible for the generation of neural crest–derived progeny such as Merkel cells, Schwann cells, and melanoblasts. Therefore, for cell transplant therapies, SKPs are an attractive alternative option to Schwann cells [41].

A second subpopulation of stem cells within the dermis consists of MSC-like cells. Dermal MSCs phenotypically resemble MSCs derived from bone marrow and adipose tissue and express stem cell specific markers (CD90, CD29, CD44, CD13, CD59, fibronectin, and vimentin). Dermal MSCs can be isolated by standard enzymatic digestion methods using collagenase and can be differentiated to osteogenic, adipogenic, and chondrogenic lineages [42]. While the therapeutic usefulness of dermal MSCs is still to be explored, a recent study demonstrated that dermal stem cells, derived from a granulating wound bed, were activated following injury and exhibited multilineage differentiation capacity [43]. In addition, dermal MSCs possess immunosuppressive potential and inhibited $CD8^+$ T lymphocyte homing to the wound bed [44].

A third subpopulation of stem cells within the dermis consists of hematopoietic cells. While it is beyond the scope of this chapter to elaborate on the role of dermal HSCs, it is worthwhile to mention that dermal-derived HSCs are the endogenous source of stem cells that positively affect wound healing [45]. $CD45^+$ cells within the DP secrete granulocyte colony stimulating factor to initiate proliferation and differentiation of mature granulocytes and neutrophils and help recruit HSCs from the systemic circulation [46]. Collectively, dermis offers a new source of MSCs that can be easily isolated and culture expanded for wound healing applications. Still, this source

of MSCs is far from clinical application, due to lack of standardized cell isolation and expansion protocols, and proof of efficacy through preclinical animal studies.

4. OTHER NONSKIN RESERVOIRS OF MSCS

The unique ability of MSCs to adapt to austere microenvironmental conditions, like chronic wounds and severe burns, has led many investigators to explore a variety of cell sources other than skin. The following section will focus on those MSCs that have been shown to be effective in wound repair and regeneration.

4.1 Bone Marrow–Derived Stem Cells

Bone marrow–derived MSCs (BMSCs) are one of the most successfully investigated stem cells for wound healing purposes. The versatility of BMSCs extends beyond wound healing and ranges from treatment of acute injuries to pathological diseases. Bone defects, diabetes, graft versus host disease, cardiovascular diseases, autoimmune diseases, neurological pathology, liver diseases, and kidney disease represent the variety of diseases that have been studied and are addressed elsewhere in this volume.

4.1.1 Source, Isolation, and Characterization

Marrow found in spongy bone is a source of adherent and nonadherent cell populations. The nonadherent cells are comprised of HSCs which generate peripheral blood cells. The adherent population consists of stromal cells which exhibit fibroblast morphology and give rise to mesodermal cell types like osteoblasts and chondrocytes. It was not until 1991 that stromal cells were proposed to be mesenchymal "stem" cells of bone marrow origin and henceforth BMSCs [15]. In clinical practice, BMSCs are isolated from iliac crest bone marrow aspirates, cultured from whole blood, or separated using the Ficoll density gradient method. Pure clones of BMSCs are then isolated from colonies that adhere to plastic cell culture surfaces. The efficiency of the isolate is then determined by the ability of these cells to form fibroblast-colony forming units (F-CFUs) [15]. Finally, BMSCs are identified by their hallmark characteristic expression of specific cell-surface markers (CD105, CD73, and CD90) and trilineage differentiation capacity following the ISCT guidelines.

4.1.2 Immunomodulatory Properties of BMSCs

As mentioned earlier in Section 2.2, BMSCs respond to local and systemic inflammatory cytokines and suppress both B cells and T cells. BMSCs modulate inflammation by the following mechanisms: (1) interaction with host immunogenic cells resulting in a phenotypic switch to antiinflammatory cells, (2) stimulation of host immune cells to secrete cytokines that negate

inflammation, and (3) secretion of antiinflammatory proteins. The first response to local wound inflammation is endogenous recruitment of progenitor cells to initiate the repair process. BMSCs influence macrophages to release IL-10 and TGF-β, switching them to an antiinflammatory phenotype. These mediators stimulate the proliferation of fibroblasts and suppress B and T cell proliferation [21,47]. The antiinflammatory properties of BMSCs are mediated by inhibiting the differentiation of monocytes into antigen-presenting immunogenic dendritic cells, thereby tipping the differentiation to IL-10 producing cell types [48]. A feedback loop exists between BMSCs and local macrophages. BMSCs secrete the antiinflammatory protein TNF-α stimulated gene/protein 6 (TSG-6), which reduces nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) signaling in resident macrophages. Interestingly, the elevated levels of NF-κB (in response to tissue injury) stimulate BMSCs to express prostaglandin E_2 (PGE_2), a principal mediator of host inflammation. PGE_2 in turn binds to the macrophages and changes their phenotype from proinflammatory (M1 macrophage) to antiinflammatory (M2 macrophage) while stimulating M2 macrophages to secrete IL-10 [49]. Collectively, growing evidence from recent research indicates that BMSCs have significant potential to favorably influence inflammation, as well as other aspects of skin injury.

4.1.3 BMSCs in Acute Wound Care

BMSCs are well-known for their ability to enhance the healing of acute wounds by promoting angiogenesis, inducing collagen deposition, and enhancing reepithelialization. BMSCs secrete a number of soluble factors that affect the recruitment of specific cell types to the wound including keratinocytes, fibroblasts, endothelial cells, and macrophages. Soluble "trophic factors," secreted by BMSCs, are responsible for a wide range of effects on all phases of healing from the inflammatory phase to complete closure and scarring. Some of the key factors are: (1) immunomodulatory molecules (IL-10, PGE_2, and indoleamine 2,3-dioxygenase), (2) antiapoptotic factors [VEGF, insulin growth factor 1 (IGF-1), TGF-β, and hepatocyte growth factor (HGF)], (3) angiogenic factors (basic fibroblast growth factor (bFGF), VEGF, IL-6, and monocyte chemoattractant protein 1), (4) chemoattractants, and (5) antiscarring factors (bFGF and HGF) [50]. During healing, a wound bed transforms from a transient field of protein-rich matrix filled with a naïve capillary plexus to mature granulation tissue with patent microvessels. Stabilization of newly formed blood vessels is vital for maintenance of tissue viability. On the other hand, stability of blood vessels is dependent on interactive forces exerted by the surrounding matrix molecules. In a nutshell, vessel stability involves highly coordinated interactions between several cellular and noncellular components. Among the cells involved, endothelial and perivascular cells interact with extracellular molecules (α-SMA, fibronectin, and integrin) to regulate and

maintain vessel integrity. Therefore, whatever the nature of the initial insult, there exists an intrinsic depot of quiescent progenitor cells that are first responders that initiate the repair process. It was not until 2008 when Crisan et al. identified and sorted perivascular cells to homogeneity from diverse human organs and showed them to yield BMSCs in culture. Further, the study also provided evidence that blood vessel walls (lining the luminal surface) housed BMSCs which could be activated when needed to migrate to the site of injury to restore tissue function and homeostasis [51].

To this end, a substantial body of evidence is available which describes how endogenous BMSCs respond to tissue injury. Still, the challenge ahead is to harness the therapeutic benefits of culture-expanded BMSC's where self-repair is not possible. In a mouse excision wound model, green fluorescent protein (GFP)-labeled BMSCs administered intravenously were found at the wound site as early as 2 days post injury and appeared as dendritic-like cells. While most of the cells were likely inflammatory in nature, GFP^+ cells were also found in dermal, epidermal, and follicular regions providing evidence that engraftment of BMSCs is a functional event [52]. When BMSCs were administered in proximity to the wound, a qualitative wound closure was observed, such as superior rete ridge formation along with major dermoepidermal junction and higher granulation tissue content. The quality of skin regeneration was attributed to trophic factors (VEGF, IGF, EGF, and HGF) secreted by BMSCs [53]. While BMSCs have been successfully used in many preclinical wound healing models and shown to positively influence healing, the majority of the research findings pivot around their immunomodulatory role. Some studies show BMSCs participate in the healing process beyond inflammation. BMSCs injected near the site of a wound were found to accumulate at the wound site in response to secondary lymphoid tissue chemokines secreted by host keratinocytes and differentiated into multiple skin cell types including keratinocytes, endothelial cells, pericytes, and monocytes [54]. In another study, murine burn wounds were treated with injected BMSCs and found to have positive effects on wound healing. In these wounds, over half of the fibroblast population and a small population of keratinocytes were found to be bone marrow-—derived, while a significant percentage of BMSCs were engrafted into the dermal matrix [27]. Further, BMSCs have been found to restore wound tensile strength in aged mice, and this effect was dependent on host macrophage activity [55]. Collectively, evidence from preclinical experiments demonstrates BMSCs have positive effects on wound healing.

The interrelationship between BMSCs and the vasculature is another area of intense research that is relevant to wound repair. In general, blood vessels form by two distinct mechanisms: (1) angiogenesis: the sprouting of existing vessels or intussusceptive angiogenesis and (2) vasculogenesis: de novo blood vessel formation from endothelial precursors or angioblasts [56]. BMSCs contribute to the process of blood vessel formation by mobilizing to the

provisional wound matrix, resulting in the generation of de novo blood vessel—the central dogma of "vasculogenesis." Not to detract from the importance of angiogenesis, an extremely crucial step in wound repair, a number of studies have consistently demonstrated that exogenously delivered BMSCs help stabilize blood vessel formation. In a recent study, treatment with BMSCs resulted in higher vessel density and thus enhanced local blood supply within a burn wound [57]. A plausible mechanism of vessel stabilization can be attributed to PDGF-β secreted by remnant host pericytes, which in turn stimulate BMSCs to be recruited to the site of damaged blood vessels and facilitate pericyte−endothelial cell interactions. Pericytes further release angiopoietin 1 (Ang-1) causing the formation of new tubular networks by interacting with its tyrosine kinase receptor, Tie-2 [58]. Although these studies provide mechanistic evidence, the individual contributions of BMSCs, resident in the perivascular region of host vasculature, and those administered exogenously for treatment are difficult to discern.

4.1.4 BMSCs Alleviate Hypertrophic Scars

Following wound closure, skin undergoes constant remodeling to attain near normal architecture. Excessive production of collagen due to fibroblast overproliferation causes the wound to heal with an HTS. Current therapeutic approaches like surgical excision, compression bandage, and corticosteroid injections produce less than desirable results, and often require revision surgery. A recent long-term follow-up study showed that BMSCs reduced scar formation and had better healing outcomes [27]. Since inflammation plays a major role in the scar formation process [7], BMSCs are logical candidates for modulating the inflammatory response through the M1 to M2 switch in macrophage phenotype which results in the production of antiinflammatory (IL-10 and PGE_2) and antifibrotic (HGF, bFGF, and VEGF) factors [50]. Fibroblasts respond to HGF and downregulate TGF-β1 and collagen (I and III) expression. In addition, HGF, along with PGE_2, decreases myofibroblastic differentiation [59]. Therefore, the role of BMSCs in reducing HTS can be considered a constitutive paracrine effect of antiinflammatory and anti-ibrotic factors.

4.1.5 Role of BMSCs in Diabetic Wound Managements

Diabetic wounds are physiologically different from normal skin wounds and present a dynamically different microenvironment that demands an appropriate therapeutic intervention. In this context, stem cells are sensitive to wound milieu and respond to microenvironmental stimuli. Much of the research reported in the literature has evaluated BMSCs in an acute wound setting, while there are relatively few studies carried out using BMSCs to treat chronic wounds. Diabetic foot ulcers (DFUs) are one such complex clinical condition which if left untreated can lead to amputation [60]. BMSCs are a

potential therapy for this condition because they exhibit low expression of major histocompatibility complex 1 (MHC-I), possess high self-tolerance capacity (i.e., tolerance to self-antigens), and most importantly, are distributed throughout peripheral blood vessels. Due to the immunomodulatory properties of BMSCs, they are arguably one of the better options for treating immunocompromised DFUs. Their natural ability to induce regulatory T cells (protects the wound from cytotoxic T cells) and reduce NK-cell proliferation makes BMSCs an excellent option for treating DFUs [61]. BMSCs implanted subcutaneously in a diabetic rat with an excision wound were able to mobilize and home to ischemic sites of the wounded tissues. The implanted BMSCs promoted granulation tissue formation and reepithelialization. Faster reepithelialization was triggered through increased keratinocyte proliferation and secretion of growth factors (EGF and IGF), in addition to MMP2 [62]. BMSCs also significantly enhanced angiogenesis by increasing the expression of VEGF at the wound site [63]. Unlike acute wounds, chronic wounds often show poor collagen remodeling due to an imbalance in matrix production-to-degradation ratios. A recent investigation indicated BMSCs improved wound-breaking strength with significant increases in collagen levels [64]. Apart from BMSCs directly influencing healing, they also alter metabolic status to accelerate healing. Excision wounds of diabetic mice treated with BMSCs showed depressed metabolic indices for glucose and lipids, while increasing insulin levels. This change in metabolic status resulted in higher wound closure rates [65].

Regardless of wound type, BMSCs primarily influence the healing process by modulating inflammation and stabilizing the vascular network within the wound bed. Every wound microenvironment is unique and has different levels of cytokines. It is fascinating that BMSCs are able to adapt to a particular wound condition and respond in a manner that restores homeostasis and wound healing. It is this property of BMSCs that has put them in the forefront of therapeutic tools for treating various types of skin injuries.

4.2 Adipose-Derived Stem Cells

When researchers started to explore MSCs for their therapeutic potential, multiple sources of adult stem cells were discovered. In 2001, Zuk et al. were the first to report that lipoaspirates, previously considered waste tissue from patients undergoing elective cosmetic liposuction surgery, contain a reservoir of multipotent adult progenitor cells [66]. The discovery of progenitor cells in adipose tissue was somewhat expected since this tissue is derived from the mesenchyme of embryoid bodies.

4.2.1 Stromal Vascular Fraction and ASCs

Lipoaspirates can be easily obtained from patients undergoing elective liposuction procedures and a stromal vascular fraction (SVF), containing a

collection of nonexpanded, heterogeneous cells, isolated after enzymatic digestion of the tissue. In this procedure, a hollow blunt-tipped cannula is introduced into the subcutaneous space through a small incision. By gentle to-and-fro movement through the adipose tissue, the fat layer is mechanically disrupted and simultaneously extracted via suction through the cannula. The raw lipoaspirate is then digested using collagenase, washed with buffer, and the cells pelleted ("processed lipoaspirate"). The cell pellet is then suspended in cell culture media and plated in a tissue culture flask. The cells that attach to the flask are designated as "adipose tissue-derived stromal/stem cells" or "adipose-derived stromal/stem cells" (ASCs). Since 2001, research on ASCs has exploded from the cellular and molecular level to clinical trials.

Worldwide, many research groups have adopted similar protocols, with subtle modifications, for isolating stem cells and given these different populations of cells various names. Therefore, to arrive at a standard definition, the International Federation for Adipose Therapeutics and Science (IFATS) reached a consensus and adopted the term "adipose-derived stem cells" [67]. Following the initial work of identifying ASCs within processed lipoaspirate, Zuk et al. extensively characterized ASCs and identified them by expression of a panel of specific cell-surface markers (i.e.,: CD29, CD44, CD71, CD90, and CD105) and their ability to differentiate to adipogenic, chondrogenic, osteogenic, myogenic, and neurogenic cell lineages [68]. Due to differences in markers used to characterize ASCs, the ISCT defined ASCs as cultured cells that (1) express CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31, (2) differentiate into adipogenic, chondrogenic, and osteogenic lineages, and (3) adhere to cell culture plastic surfaces and form colony forming unit-fibroblast (CFU−F). Finally, the committee recommends distinguishing them from BMSCs by their expression of CD36 and lack of CD106 expression [18].

4.2.2 Use of Stromal Vascular Fraction to Promote Wound Healing

SVF is a mixture of cells, containing fibroblasts, circulating hematopoietic cells, pericytes, endothelial cells, MSCs, and preadipocytes [18], derived from enzymatically digested adipose tissue. Since MSCs share a number of markers with other connective tissue cell types (e.g., CD90 expressed by both fibroblasts and MSCs), it is difficult to confidently define the percentages of individual cell types within an SVF preparation. This heterogeneity in cell composition has limited research efforts to explore the potential benefits of SVF for wound healing purposes. Nevertheless, there are several studies that have shown that SVF has favorable wound healing properties. For example, deep partial- and full-thickness burn wounds show an increase in VEGF levels at the wound site and reduced inflammatory response when treated with SVF. In addition, SVF-treated wounds were well vascularized and granulated [69]. As mentioned above, the contribution of specific cell types in SVF towards

wound healing is complicated by the heterogeneity of the cell population. Recent microarray analysis suggests that SVF cells express high levels of genes responsible for epithelial development (e.g., EGF, chemokine receptors CXCR1, CCR2, and CCR3) may contribute toward enhanced wound closure [70]. Further, immune-selected $CD248^{+}$ cells in SVF were identified to be proangiogenic and found to be responsible for enhancing wound healing by promoting increased vessel growth within the wound bed [71]. With more in-depth studies, it may be possible to dissect out the beneficial effects of individual factors or cell populations within SVF. However, it should be emphasized that the current use of SVF is restricted to autologous applications with limited numbers of stem cells available for treatment purposes.

4.2.3 ASCs: Origin and Role in Tissue Homeostasis

While SVF holds enormous promise, stem cell–based therapies require reproducibility and homogeneous cell populations. Henceforth, ASCs gained importance for clinical use since clones of homogenous populations could be obtained in sizeable numbers. Another reason ASCs became an attractive option over other MSCs because they could be easily isolated and are relatively abundant. Lipoaspirates of adipose tissue, compared with bone marrow, contain more than 100 to 1000 times the number of multipotent cells per cubic centimeter, making adipose tissue an attractive source of stem cells for regenerative therapies [72].

Following the discovery that blood vessel walls harbor MSCs [51], a paradigm shift transpired in identifying ASCs around the microvessels of adipose tissue. Later in the same year, two seminal studies provided proof for a perivascular origin of ASCs [73,74]. Most ASCs were $CD34^{+}$, resided in a periendothelial location, and functioned to stabilize host blood vessels (Fig. 8.2). Collectively, both studies concurred that ASCs expressed both stem cell–specific surface markers (CD90 and CD105) and pericytic markers [neuroglial factor 2 (NG2), PDGFR-α/CD140a and PDGFR-β/CD140b]. While there was some disparity in the markers selected to define the immunophenotype of ASCs (CD10, CD13, STRO-1, CD146, 3G5), there were many surface markers shared in common (CD73, CD90, and CD105) that complied with the ISCT recommendations. In addition, culture expanded ASCs exhibited multilineage differentiation (adipogenic, osteogenic, and chondrogenic), confirming that the perivascular stromal cells are “true” stem cell populations [73,74].

For many years, HSCs and BMSCs were thought to be the major stem cell types that were able to home to sites of injury and initiate the self-repair process. Voog and Jones elegantly described that every tissue has a “niche” that is responsible for maintaining stem cell populations in their undifferentiated state until activated to restore homeostasis [75]. In this context, human skin constantly self-renews to maintain its barrier functions

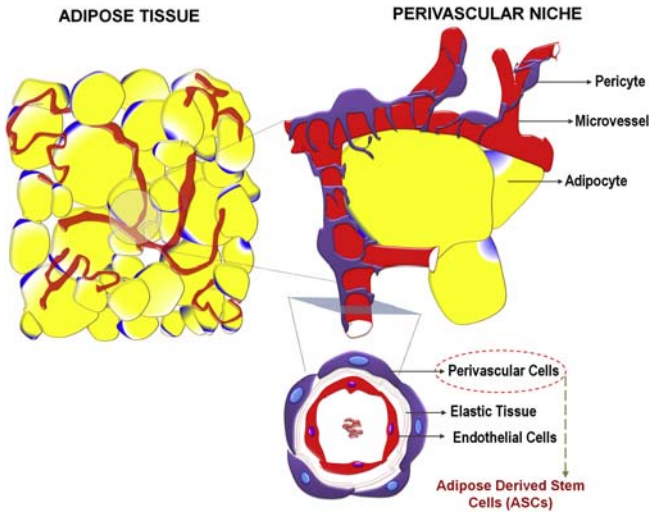


FIGURE 8.2 **Regions containing stem cells in adipose tissues.** Schematic showing the location of perivascular cells that gives rise to adipose stem cells in adipose tissues.

through stem cells resident in the epidermis and associated adnexa. Sox-9 (follicular-derived), keratin 15 (K15) (bulge-derived), and LRG6^{+} upper isthmus stem cells were discovered to control the self-renewal of skin [37]. A recent study has shown that intradermal adipose, unrelated to subcutaneous adipose tissue, regulates the activation of follicular stem cells via PDGFR signaling [76]. Further, these intradermal adipocytes dynamically promote epithelial stem cell activity and subsequently regulate the skin microenvironment. Paralleling these observations, our studies identified PDGFR-β^{+} cells that are localized to vascular beds in subcutaneous adipose tissue and are part of the perivascular niche and give rise to subcutaneous ASCs. In addition, ASCs in this study were isolated from discarded human burn tissues, providing evidence that resident stem cells in the hypodermis are preserved during and after severe thermal injury [77]. The location of the ASCs in the perivascular space (the interface between endothelium and adipocytes) and their ability to both support vascular structures and generate adipocytes suggests that ASCs may play an important role in the maintenance of skin homeostasis [78]. Still, it is unclear if these local stem cells in subcutaneous adipose tissue are the first responders to any skin injuries. Therefore, future studies will need to unravel the regulatory machinery responsible for the transmigration of tissue-resident ASCs in response to skin injury.

4.2.4 Immunomodulatory Role of ASCs in Wound Healing

ASCs exist in an undifferentiated state within the stroma of adipose tissue until there is an injury (external stimulus) that induces the secretion of cytokines to

initiate tissue repair. Analysis of the secretome profile of ASCs reveals that they express an array of proinflammatory factors: IL-6, IL-8, IL-7, IL-11, and TNF-α [79]. The mechanism of ASC induction has been attributed to the activation of downstream signaling through toll-like receptors (TLR-1, TLR-2, TLR-3, TLR-4, TLR-5, TLR-6, and TLR-9) causing the secretion of increased cytokine levels [80]. It is well documented that TLRs play a major role in triggering the innate immune response when encountering unfavorable conditions, for example, infection, hypoxia, and inflammation. Upon exposure to austere conditions, ASCs respond to the local stimulus and secrete proinflammatory cytokines; for example, TNF-α, which is also secreted by host macrophages and monocytes, stimulates ASC migration to the wound site. In addition, TNF-α also potentiates ASCs to secrete IL-6, IL-8, and other paracrine factors to enhance the healing process [81].

Another key immunosuppressive soluble factor secreted by ASCs is PGE_2. This mediator is composed of lipids and regulates a number of functions related to inflammation. As described earlier (Section 4.1.2), PGE_2 influences macrophages to switch from the M1 to M2 phenotype causing them to secrete the antiinflammatory cytokine IL-10. In addition, PGE_2 has an inhibitory influence on the maturation of dendritic cells and a direct effect on the proliferation and cytokine production of T lymphocytes [82]. The secretion of PGE_2 by ASCs is increased in the presence of dendritic cells leading to resolution of the inflammatory phase [83]. Evidence has consistently shown that ASCs and BMSCs share some common mechanisms for suppressing inflammation. However, when secretory functions are compared, ASCs have more potent effects on immune cells than the BMSCs [84]. In addition, ASCs have been hypothesized to be immune-privileged due to their lack of MHC II cell surface antigen [85]. For these reasons, ASCs are considered more suitable for clinical applications for treatment of a variety of diseases.

4.2.5 ASCs and Acute Wound Care

Following tissue injury, ASCs migrate out of their perivascular niche towards the site of injury to initiate the repair process. Activation of ASCs in response to injury causes them to lose their "stemness" and assume a differentiated phenotype. This phenomenon was elegantly demonstrated in human adipose tissue, wherein the activation of stromal cells was dependent on their proximity to injury. All cells in adipose tissue near injury were α-SMA^+. In contrast, stromal cells more distant from the injury continued to express CD34 [86]. Based on this observation, it is logical to assume that cell culture-expanded ASCs, implanted into an injury site, will relinquish their "natural stem cell state" for a more functional phenotype consistent with the local wound microenvironment. To date, the fate of ASCs in a healing wound is unknown but studies have demonstrated that ASCs survive for several days post implantation [87]. In our studies, we were able to detect ASCs in the bed

of an excisional wound in a rodent model 12 days post implantation. Further, ASCs localized in the wound bed provided evidence that the implanted cells were playing a functional role in the healing process [77].

ASCs respond to injury by secreting many different growth factors (e.g., IGF, HGF, and VEGF) that are well-known effectors of angiogenesis and tissue stroma regeneration [79]. ASCs injected around a wound site accelerate healing through antioxidant and cell-stimulatory effects, mainly mediating the activation of dermal fibroblasts and keratinocytes [88]. Consistent with the above study, ASCs secrete several key ECM proteins (i.e., collagens and fibronectin) which facilitate host cell infiltration and proliferation. Further, ASCs significantly reduce wound size and accelerate reepithelialization from the periphery of the wound [89]. ASCs that had been labeled with GFP and implanted in a mouse wound model were localized to the suprabasal layer of the epidermis and expressed K5 and K14, providing evidence that the implanted cells transdifferentiated into host cells. The same study also showed that ASCs enhanced neovascularization by differentiating into endothelial cells and secreting VEGF. Most interestingly, ASCs fused with host epithelial cells. In addition, female ASCs that had been transplanted into an irradiated male recipient were observed to fuse with host keratinocytes [81]. While only 0.1% of the cells were found to fuse with host cells, the above study provides proof that ASCs are involved in paracrine interactions and, most importantly, integrate into the wound.

Successful wound healing depends on the development and functional recovery of blood vessels. Since ASCs normally reside juxtaposed to vessel walls, it is intuitively compelling that ASCs are likely to be involved in the formation of supporting perivascular structures. During vasculogenesis, formation of the perivascular network precedes endothelial cell recruitment. In this process, pericytes found distant to the vascular advancing front were PDGFR-β^+ and NG2$^+$, whereas cells close to the tip region only expressed PDGFR-β. This switch in phenotype is unique to pericytes and reaffirms that ASCs are responsible for stabilizing newly formed blood vessels and restoring vascular patency [73]. Like endogenous populations, culture expanded ASCs implanted in a wound site home to the perivascular space of nearby host blood vessels [77]. In addition, pericytes release Ang-1 which results in the formation of new tubular networks through interactions with the endothelial cell Tie-2 receptor [58]. Recently, it was reported that Ang-1 and Tie-2 levels increase during the latter stages of wound healing, leading to blood vessel stabilization [90]. Therefore, the observed increase in Ang-1 expression by ASCs strongly suggests a role for these cells in wound revascularization.

Changes in local TGF-β levels initiate the migration of PDGFR-β^+ ASCs into the wound site. Once in the wound, TGF-β stimulates the transdifferentiation of ASCs into α-SMA$^+$ myofibroblasts that display collagen contraction in vitro [91]. Consistent with these observations, rabbit excision wounds treated with

ASCs has increased formation of granulation tissue. Further, the engrafted ASCs proliferate within the wound, exhibit an activated fibroblast phenotype, an increase in the number of endothelial cells, and recruited macrophages to the wound site [92]. SDF-1 (stromal cell-derived factor 1) is an important chemokine that plays a central role in tissue repair and angiogenesis and exerts its biological function by binding to two G-protein−coupled receptors: CXCR4 and CXCR7 [93]. SDF-1 is found in wound fluid and is responsible for ASC migration, as well as other in vitro and in vivo activities [94]. In vivo, transmigration and homing of ASCs has been evaluated in a parabiosis mouse wound model, where syngeneic mice were attached to each other laterally at their flanks. In this model, ASCs were delivered through a pullulan hydrogel and shown to increase the recruitment of circulating BMSCs via the SDF-1/CXCR4 pathway. In addition, locally applied ASCs initiated neovascularization of the wound bed [95].

Next, large animal preclinical models will be necessary to validate these observations and assess the safety and efficacy of ASCs. Local delivery of allogeneic ASCs to porcine partial-thickness excision wounds produced faster epithelial regeneration, thickening by day 7 and subsequent thinning, and the formation of rete-ridges. This investigation also tested the persistence of stem cells by applying ASCs of the opposite sex to the wound. Male ASCs were detected in wounds on a female pig 10 days post treatment. Although the fate of the stem cells was not determined, this study holds promise for the use of allogeneic stem cells in stimulating wound healing [96]. Taken together, ASCs appear to be one of the most promising candidates for wound healing applications.

4.2.6 ASCs Influence Chronic Wound Healing

Although the wound *milieu* of acute and chronic wounds is different, standard clinical practice for treating them is similar. Chronic wounds differ in their severity due to the deleterious events associated with the healing process: prolonged inflammation, excessive matrix degradation, vascular insufficiency, and other existing comorbidities. The functional dynamic range of ASCs extends beyond their ability to treat acute wounds and has potential for treating chronic wound as well. Due to their proinflammatory secretome, treatment of diabetic wounds with ASCs show significant increases in the level of important healing factors such as EGF and VEGF [97]. A recent gene expression analysis of ASCs cultured as monolayer or cellular aggregates showed key putative markers that are positive regulators of wound healing such as growth factors (TGF-β, FGF, IGF, KGF, HGF, VEGF) and ECM proteins (collagen VI, fibronectin, Tenascin C, MMP2, and MMP14). Further, this study also provided evidence for accelerated healing of diabetic wounds treated with ASC aggregates mainly through paracrine effects of secreted factors [98]. When ASCs were applied directly to a diabetic wound, minimal effects on granulation tissue formation, reepithelialization, and capillary density were

observed. However, when autologous ASCs were delivered in a collagen carrier healing was enhanced by regenerating the granulation and epithelial tissues. These studies provide evidence suggesting that the austere conditions found in diabetic wounds require an appropriate carrier for delivery of ASCs [99].

Much recent research has focused on the angiogenic potential of ASCs. Even ASCs from diabetic mice produce significant levels of VEGF and improve the neovascularization of ischemic tissue [100]. A well-known effect of ASCs is their ability to secrete SDF-1 and promote vasculogenesis. Genetically modified ASCs overexpressing SDF-1 promoted diabetic wound healing by secreting proangiogenic factors and differentiating into endothelial-like cells [101]. Activation of $CXCR4^+$ and $CXCR7^+$ by SDF-1 is central to ASC migration to sites of injury. Increasing evidence suggests a critical role for SDF-1 in cell apoptosis and stem cell survival [102].

ASCs have been shown to be beneficial in treating other chronic wounds such as pressure ulcers. Though the etiology differs, the healing of pressure ulcers is impaired due to an exacerbated inflammatory phase, delayed granulation, and wound reepithelialization. A recent study provides preclinical evidence that pressure ulcers treated with ASCs contain reduced levels of inflammatory cells and improved recovery of subcutaneous adipose tissue, suggesting ASC benefits extend beyond their antiinflammatory and angiogenic potentials [103]. Collectively, ASCs are immune-privileged, secrete beneficial trophic factors, and positively modulate the local wound environment by inducing neovascularization, as well as enhancing matrix reorganization.

4.2.7 Therapeutic Benefits of ASCs in Scar Alleviation and Reconstructive Surgeries

The cellular and molecular changes responsible for the formation of hypertrophic scar are complex. Experimental evidence has shown that the mediators of scar formation are mostly endogenous proinflammatory cytokines, which dynamically change during the course of healing [104]. A challenge for clinicians is to determine if a wound will heal normally or with a scar. Management of scars is governed by standard clinical practice, that is, surgical revision protocols, compression therapy, corticosteroid therapy, radiotherapy, and laser therapy [7]. With increasing evidence accumulating on the immunomodulatory effects and antiinflammatory cytokines secreted by ASCs [79,80], it is likely that their use in wound healing will have benefits and suppress the exaggerated inflammatory status. A recent in vitro study provided evidence showing that ASCs cocultured with hypertrophic scar fibroblasts significantly decreased the production of collagen I and III, fibronectin, TGF-β, IL-6, IL-8, and α-SMA [105]. Corroborating these results, Zhang et al. elegantly demonstrated that intralesion injection of ASCs reduced scar elevation index and transcript levels of α-SMA and collagen I [106]. The mechanism by which ASCs

decrease collagen deposition and scar formation was through downregulation of p38 protein levels [107].

In clinical practice, minced adipose tissue or lipoaspirate is frequently used to reconstruct hypodermis [108]. Adipose tissue–treated wounds show enhanced expression of proliferation cell nuclear antigen (PCNA) and Factor VIII-related antigen, which indicate that active cell differentiation and proliferation are present [109]. Recently, we have shown that skin grafting on a wound with intact fat tissue heals with less scarring and contracture in a porcine full-thickness wound model [110]. The underlying subcutaneous tissue directly influences the organization of the collagen matrix, which was shown through decreased expression of α-SMA, and the wounds healed with less fibrotic scarring. These research findings define a role for ASCs, in the adipose tissue niche, in decreased scar formation. Therefore, investigations are underway to create a more standardized method of wound reconstruction using ASCs as a therapeutic tool to prevent scar formation.

4.3 Placental-Derived Stem Cells

Human umbilical cord and amnion are typically discarded after child birth. Fortuitously, this tissue is a rich source of MSCs which can be isolated from umbilical cord blood (UCB), Wharton jelly (WJ), a gelatinous substance within the cord, the outer amniotic membrane (AM), and amniotic fluid (AF) (Fig. 8.3).

4.3.1 Umbilical Cord Blood–Derived MSCs (UCB-MSCs)

UCB is a desirable source of MSCs because of its low cost and ease of collection using noninvasive methods. Similar to bone marrow, UCB contains HSCs as well as MSCs. UCB-MSCs are isolated as a population of mononuclear cells, using standard Ficoll separation method, followed by culture on tissue culture plates where the cells adhere, exhibit a fibroblast-like morphology, and satisfy the ISCT requirements for an MSC population. Typically, they are positive for a panel of stem cell surface markers (CD44, CD73, CD90, CD105) and able to differentiate into osteogenic, adipogenic, and chondrogenic cell lineages [111]. UCB-MSCs are less immunogenic and display low alloreactivity to lymphocytes. Recently, the immunosuppressive properties of BMSCs, ASCs, and UCB-MSCs were evaluated and UCB-MSCs were found to significantly reduce the macrophage secreted proinflammatory cytokines IL-1α, IL-6, and IL-8 [112].

Research results to date have suggested that UCB-MSCs improve acute wound healing by transdifferentiating into epithelial keratinocytes. 5-bromodeoxyuridine-labeled UCB-MSCs have been localized to the epithelia of full-thickness excision wounds in Balb/C mice where they were also found to express K19 [113]. Supporting these findings, fluorescently labeled UCB-

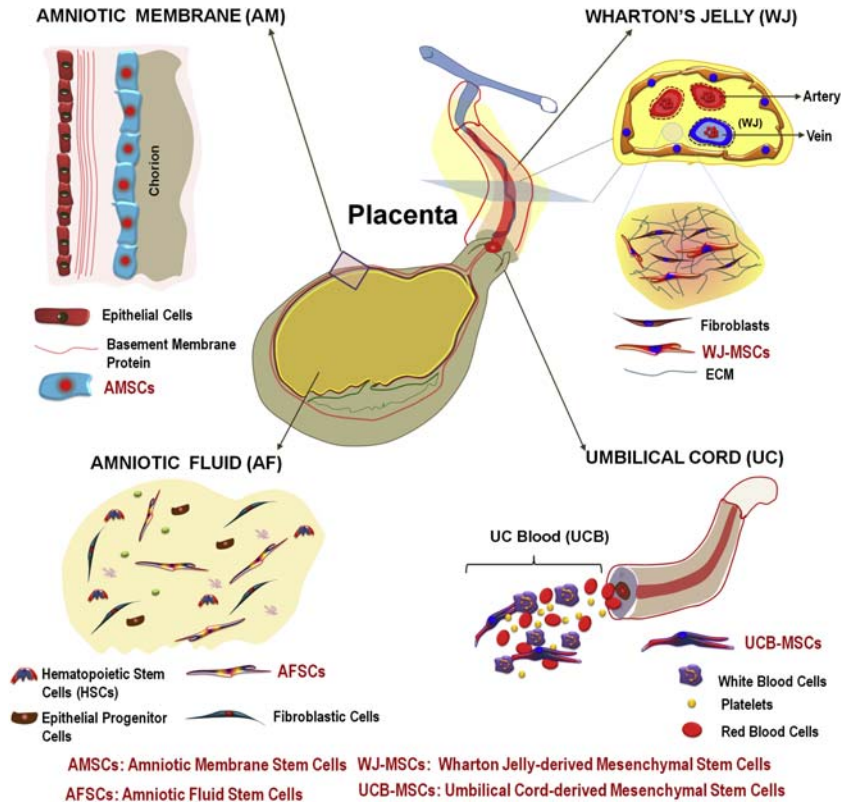


FIGURE 8.3 **Mesenchymal stem cells in human placenta.** Schematic showing the major fetal sources of mesenchymal stem cell populations: amniotic membrane and fluid, Wharton jelly, and umbilical cord blood. *AFSCs*, amniotic fluid stem cells; *AMSCs*, amniotic membrane stem cells; *UCB-MSCs*, umbilical cord—derived mesenchymal stem cell; *WJ-MSCs*; Wharton jelly—derived mesenchymal stem cell.

MSCs were found within the epithelia of healing wounds and the grafted cells expressed K8 and K10 [114]. Migration of UCB-MSCs to the epithelia followed MMP-12-dependent degradation of collagen V and fibronectin activated by extracellular signal regulated kinase and NF-κB [6]. It is worth remembering that elevated levels of NF-κB from the host inflammatory response to tissue injury, stimulates the secretion of PGE_2 by MSCs. Collectively, UCB-MSCs have a strong influence on wound reepithelialization and an antiinflammatory response via the NF-κB signaling pathway. In diabetic wounds, UCB-MSCs have positive effects on wound healing by stimulating the expression of VEGF within the wound site, which increases the number of newly formed blood vessels [8]. Due to variability between donors and low yield, UCB-MSCs require extensive characterization and standardization before they can be used for therapeutic applications.

4.3.2 Wharton Jelly–Derived MSCs (WJ-MSCs)

In 1656, Thomas Wharton identified and described a jelly-like substance inside umbilical cords that consisted of a collagenous matrix rich in glycosaminoglycans and glycoproteins. Subsequently this substance became routinely referred to as "Wharton jelly." Within this matrix a population of MSCs was identified and isolated and found to express stem cell surface markers and was capable of multilineage differentiation as defined by ISCT [115]. WJ-MSCs have been shown to be hypoimmunogenic and express HLA-G6, a marker of immune tolerance during pregnancy [116]. WJ-MSCs do not stimulate T cells due to a lack of the CD86 surface antigen [117]. A unique property of WJ-MSCs is that they display some of the characteristics of ESCs, such as expression of octamer-binding transcription factor 4 (Oct-4), Sox-2, zinc finger protein 42, and Nanog, suggesting that they possess the ability to be culture expanded to large numbers without losing their stemness [118].

Human WJ-MSCs have been reported to improve burn wound healing [119]. Treated wounds exhibit a significant decrease in the levels of IL-1, IL-6, and TNF-α. In addition, VEGF levels and cutaneous wound microcirculation were higher in treated animals. More interestingly, antiinflammatory cytokines IL-10 and TSG-6 were found to be significantly increased. These effects were mediated through the expression of COX-1/2 and by production of PGE_2 [6], which appears to be a common pathway for BMSCs and ASCs to elicit antiinflammatory responses through a feedback of PGE_2 binding to the host macrophages and converting them to a profibrotic phenotype [120]. In contrast, WJ-MSCs have been reported to stimulate higher secretion of matrix-degrading enzymes, MMP-1 and urokinase, without any long-term benefits on healing [8]. Although there are mixed reports, there is translation potential for these cells due to their low immunogenicity and ease of isolation.

4.3.3 Amniotic Fluid and Membrane-Derived MSCs

Amnion consists of an outer thin membrane, the amniotic membrane (AM), and amniotic fluid (AF) within the sac (Fig. 8.3). The identification of hematopoietic progenitor cells, within a heterogeneous population of cells in human AF was first reported by Torcielli et al. in 1993 [121]. Subsequently, In't Anker et al. identified the presence of multipotent MSCs in AF (=AFSCs) that displayed an expression pattern similar to BMSCs and exhibited multilineage differentiation potential as recommended by the ISCT [122]. In addition, AFSCs expressed Oct-4 and CD117, a receptor protein present on human ESCs, indicating that they shared the characteristic of pluripotency with ESCs. More interestingly, AFSCs are capable of undergoing 250 population doublings while retaining their normal karyotype, which makes them particularly attractive for regenerative medicine applications [123].

Secretome analysis has shown that AFSCs produce a number of growth factors and cytokines involved in wound healing, including IL-6, IL-8, TGF-β, VEGF, and EGF which play key roles in vascularization and remodeling [124]. In addition, AFSCs inhibit T cell proliferation, based on a lack of CD3-mediated stimulation of peripheral blood mononuclear cells. In vivo, subcutaneously implanted AFSCs have been shown to stimulate paracrine recruitment of endothelial and multipotent mesenchymal progenitors [125]. In addition, full-thickness wounds treated with AFSCs promoted angiogenesis and epithelial cell regeneration through upregulation of TGF-β and fibronectin expression [126]. Recently, it was shown that AFSCs accelerate wound healing by suppressing the local inflammatory response and not by secretion of repair-related factors. Further, AFSCs have been shown to transdifferentiate into cytokeratin producing cells (K5, K10, K14) in vitro, and when applied topically over full-thickness wounds in Balb/C mice localize to the epidermis, providing direct evidence for their participation in wound repair [127]. In addition to these favorable properties, AFSCs offer the advantages of low immunogenicity and lack of tumorigenicity; however, there are no established standards for their use in the clinic.

Human AM consists of an inner epithelial layer, intermediate basement membrane layer, and an outer layer which contains MSCs (Fig. 8.3). Currently, AM is used in clinical practice as a biological wound dressing and for various other tissue engineering applications. AM has also been explored as a source of stem cells [128]. Like other MSCs, AMSCs attach to plastic culture surfaces, are spindle-shaped in culture, capable of forming F-CFUs, express MSC cell surface markers (CD90, CD105, CD44, CD73, and CD29), and exhibit multilineage differentiation potential [129]. AMSCs are an immune-privileged cell type, block the maturation of monocytes to antigen presenting dendritic cells, and express HLA-G6 leading to the induction of immunosuppressive regulatory T cells [130]. The wound healing potential of AMSCs has been attributed to the plethora of cytokines found in the amnion, such as TGF-β2, tissue inhibitors of MMPs (TIMP-1,2), VEGF, angiogenin, and PDGF [131]. Uberti et al. demonstrated that amnion-derived cellular cytokines induced macrophage, keratinocyte, and fibroblast migration in vitro and accelerated epithelialization of partial-thickness burns in guinea pigs [132]. Furthermore, these studies provided evidence that amnion-derived cytokines improved both acute and chronic wounds in rat models and enhanced healing of chronic wounds in a porcine diabetic wound model [133]. However, there is little evidence to show that AMSCs, themselves, participate in wound healing, although a recent study has shown that placental MSCs enhanced angiogenesis in a diabetic rat model. In that study, the placental MSCs promoted angiogenesis by inducing the production of proangiogenic molecules such as VEGF, HGF, TGF-β, and IGF [134]. Although many of the published preclinical studies with AMSCs have evaluated their potential in degenerative processes induced by inflammatory and fibrotic mechanisms (e.g., Parkinson disease, stroke, spinal

injury, pulmonary and liver fibrosis) [135], AMSCs hold enormous potential for skin and wound healing applications.

The quest to identify different sources of MSCs has led to the identification of several different anatomical locations from which MSCs can be harvested. This includes dental pulp, muscle, urine, and synovium. Emerging evidence leans strongly to the hypothesis "every tissue has inherent populations of stem cells programmed to restore tissue homeostasis and function." Furthermore, to harness the benefits of MSCs it is extremely important to deliver them to the desired site using an appropriate delivery vehicle. Numerous biological and synthetic tissue engineered scaffolds are currently under investigation and development, which may improve the performance of MSCs for enhancing tissue regeneration capacity by providing an ideal microenvironment.

5. CLINICAL EVALUATION OF STEM CELL–BASED THERAPIES

The final challenge for developing the full therapeutic potential of MSCs involves their successful translation to the clinic to treat human diseases that currently lack effective therapies or where therapies are inadequate. The breadth of clinical conditions being considered for MSC therapy is wide and includes: bone/cartilage disorders, cardiovascular diseases, gastrointestinal diseases, pulmonary disease, skin-related pathologies, cancer and neuromuscular and autoimmune disorders. Recently, a comprehensive mapping of stem cell–based clinical trials registered at www.clinicaltrials.gov was presented as phylogenetic-like tree visualizations and chord diagrams. Data mining and knowledge discovery revealed that of the 5788 trials screened, more than half were testing/evaluating the performance of allogeneic MSCs. The most targeted pathologies were cardiovascular and nervous system diseases. The use of MSCs in treating musculoskeletal conditions, immune system diseases, wounds, and injuries have significantly increased since 2009. Of the various types of MSCs, the use of ASCs compared with BMSCs in clinical trials has increased [136]. Most of the wounds and injury-related clinical trials are in phase I/II stages. Corroborating this finding, our database search (Table 8.2; www.clinicaltrials.gov) found only 25 trials related to wound healing (search criteria includes: *wounds and stem cells*; *burn and stem cells*; *wounds and injuries + stem cells*; *ulcers + diabetes and stem cells*; *MSC and wounds*; *MSCs and ulcer*; *MSCs + wounds and injures*; *search results date*; *July, 2017*). Most of the trials are related to chronic wound applications (diabetic foot ulcers, venous ulcers, and pressure ulcers) and only 7 trials are active for burn injury interventions. Of note, the reported studies recruited deep partial-thickness or third-degree burn wound patients and treated them with allogeneic ASCs delivered through hydrogel sheets or platelet-poor plasma fibrin hydrogels. The primary outcome measure is to determine the degree of healing when a skin flap is applied following ASC treatment (study # 3183622

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT03211793	Mesenchymal stromal cells as treatment for digital ulcers in systemic sclerosis	Recruiting	• Systemic sclerosis • Digital ulcer	• Drug: mesenchymal stromal cells • Other: placebo	Interventional • Phase 1 • Phase 2
NCT03183622	A follow-up study to evaluate the safety of ALLO-ASC-DFU in ALLO-ASC-BI-101 Clinical trials	Active, not recruiting	• Burn	• Biological: ALLO-ASC-DFU	Observational
NCT03167138	Micro-fragmented adipose tissue (Lipogems) injection for chronic shoulder pain in persons with spinal cord injury	Recruiting	• Shoulder pain • Shoulder impingement syndrome • Rotator cuff impingement syndrome • Rotator cuff tendinitis • Rotator cuff syndrome of shoulder and allied disorders • Spinal cord injuries	• Biological: autologous micro-fragmented adipose tissue • Device: lipogems system	Interventional early phase 1
NCT03113747	Allogeneic ADSCs and platelet-poor plasma fibrin hydrogel to treat the patients with burn wounds (ADSCs-BWs)	Recruiting	• Second- or third-degree burns	• Biological: ALLO-ASCs	Interventional • Phase 1 • Phase 2

Continued

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications—cont'd

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT02961699	Healing chronic venous stasis wounds with autologous cell therapy	Not yet recruiting	• Wound, non penetrating	• Device: transpose RT system • Other: debridement/ dressing of wound	Interventional early phase 1
NCT02948023	Stem cells therapy for corneal blindness	Recruiting	• Corneal injuries • Corneal burns • Corneal scars and opacities	• Biological: ex-vivo cultivated limbal stem cell pool	Interventional phase 1
NCT02831075	A clinical study using adipose-derived stem cells for diabetic foot	Recruiting	• Peripheral vascular disease • Ischemia • Diabetic foot	• Biological: adipose-derived stem cell • Biological: saline	Interventional phase 1
NCT02796079	A clinical study using autologous bone marrow stem cell for diabetes-related vascular complications	Recruiting	• Peripheral vascular disease • Ischemia • Diabetic foot	• Biological: mesenchymal stem cells • Biological: saline	Interventional phase 1
NCT02790957	Plerixafor in diabetic wound healing	Recruiting	• Diabetes • Wounds • Critical limb ischemia	• Drug: Plerixafor • Drug: placebo	Interventional phase 2

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications—cont'd

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT02785198	Passive training as a treatment for diabetic foot ulcers	Recruiting	• Diabetic foot ulcers	• Device: passive knee extensor machine	Interventional
NCT02742844	Clinical trial to investigate efficacy and safety of the IMP in patients with nonhealing wounds originating from ulcers	Recruiting	• Skin ulcer venous stasis chronic	• Biological: APZ2 application	Interventional • Phase 1 • Phase 2
NCT02672280	Safety and exploratory efficacy study of collagen membrane with mesenchymal stem cells in the treatment of skin defects	Not yet recruiting	• Wounds • Diabetic foot ulcers • Burns	• Device: medical collagen membrane with MSC • Device: medical collagen membrane	Interventional • Phase 1 • Phase 2
NCT02669199	MSCs source of sweat gland cells of large area skin injury patients transplant of the wound	Completed	• MSCs	• Biological: MSCs	Interventional phase 1
NCT02619877	Clinical study to evaluate safety and efficacy of ALLO-ASC-DFU in patients with diabetic foot ulcers	Recruiting	• Diabetic foot ulcer	• Biological: ALLO-ASC-DFU • Procedure: standard therapy	Interventional phase 2

Continued

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications—cont'd

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT02619851	A clinical trial to evaluate the safety and efficacy of ALLO-ASC-DFU for second deep degree burn injury subjects	Not yet recruiting	• Burn injury	• Biological: ALLO-ASC- DFU • Device: conventional therapy	Interventional Phase 2
NCT02590042	Safety of adipose-derived stem cell stromal vascular fraction	Not yet recruiting	• Abnormally healing wounds • Scars • Soft tissue defects	• Biological: ADSC-SVF-002	Interventional phase 1
NCT02577731	Hematopoietic stem cell dysfunction in the elderly after severe injury	Recruiting	• Trauma injury	• Other: bone marrow collection • Other: muscle tissue collection • Other: blood collection • Other: clinical data collection	Interventional
NCT02394873	A study to evaluate the safety of ALLO-ASC-DFU in the subjects with deep second-degree burn wound	Completed	• Burn	• Biological: ALLO-ASC-DFU	Interventional phase 1
NCT02375802	Adipose-derived stromal cells (ASC's) and pressure ulcers	Recruiting	• Pressure ulcer	• Biological: adipose-derived stromal cells • Drug: placebo	Interventional phase 1

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications—cont'd

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT02304588	Stem cell therapy for patients with vascular occlusive diseases such as diabetic foot	Recruiting	• Diabetic foot • Lower limb ischemia	• Biological: mesenchymal stem cells	Interventional phase 1
NCT02104713	Stem cell therapy to improve burn wound healing	Recruiting	• Skin burn degree second	• Biological: allogeneic (MSC's) application to the burn wounds	Interventional phase 1
NCT02092870	Adipose derived regenerative cellular therapy of chronic wounds	Unknown status	• Diabetic foot • Venous ulcer • Pressure ulcer	• Drug: adipose derived stem cells	Interventional phase 2
NCT01572376	Autologous bone marrow stem cells in pressure ulcer treatment	Completed	• Type IV pressure ulcers • Chronic wounds • Spinal cord injury	• Procedure: infusion of autologous bone marrow stem cells after wound debridement.	Interventional • Phase 1 • Phase 2
NCT01443689	Allogeneic stem cell therapy in patients with acute burn	Unknown status	• Burns	• Biological: human umbilical cord mesenchymal stem cells • Biological: human cord blood mononuclear cells and human umbilical cord mesenchymal stem cells • Drug: conventional therapy	Interventional • Phase 1 • Phase 2

Continued

TABLE 8.2 Clinical Studies Evaluating the Use of MSCs in Skin Wound Healing Applications—cont'd

Clinicaltrials.gov					
Clinical Trial Number	**Clinical Trial Title**	**Recruitment**	**Conditions**	**Interventions**	**Characteristics**
NCT01065337	Induced wound healing by application of expanded bone marrow stem cells in diabetic patients with critical limb ischemia	Completed	• Diabetic foot	• Biological: tissue repair cells (TRC) • Biological: bone marrov stem cells (BMC)	Interventional phase 2
NCT00815217	The role of lipoaspirate injection in the treatment of diabetic lower extremity wounds and venous stasis ulcers	Unknown status	• Diabetic wounds • Venous stasis wounds	• Procedure: injection of lipoaspirate • Other: control	Interventional
NCT00710411	Inflammatory response after muscle and skeleton trauma	Completed	• Multiple trauma		Observational
NCT00535548	Hematopoietic stem cell therapy in chronic wounds using a pressure sore model	Unknown status	• Chronic Wounds • Pressure sores • Hematopoietic stem cells • Wound healing	• Biological: stem cell therapy	Interventional phase 1

The data were retrieved from the www.clinicaltrial.org database.

and 3113747, Table 8.2). Some of the current ongoing clinical trials involve the use of allogeneic ASCs in phase 1/2 multicenter clinical trials. In these studies (studies # 2679851 and 2394853; Table 8.2), human subjects sustaining deep second-degree burn wounds $\geq 100\,cm^2$ were treated with allogeneic ASCs and followed for up to 3 years. The primary end point of the studies is rate of wound closure and reepithelialization. ASCs are anticipated to improve healing through paracrine mechanisms, that is, by secretion of VEGF and HGF. Also, they are expected to control inflammation and increase reepithelialization. A similar phenomenon was explained by Feisst et al. as bystander effect, that is, the transplanted stem cells are clinically effective due to their modulation of the host environment through secretion of cytokines, which in turn elicits paracrine effects on host tissue [137].

In contrast to burn wounds, which have been typically treated with ASCs, chronic wounds have been treated with BMSCs. In 2003, a proof-of-principle study was conducted to demonstrate the efficacy of BMSCs in treating chronic wounds. Treatment was either applied directly to the wound (2−4 mL bone marrow aspirate; not injected) or injected into the periphery (1−3 mL aspirate) of the wound in patients. Five days post transplantation, the wounds received up to three additional treatments with cultured BMSCs ($\sim 1 \times 10^6$−1×10^7 cells/intervention). Biopsy specimens showed that the BMSCs had engrafted based on the appearance of immature hematopoietic cells. Overall, clinical and histologic evidence showed that healing was proceeding with reduced scarring [138]. In a second study, 20 patients were treated with culture expanded BMSCs. Two patients had severe burns and the other 18 had intractable dermatopathologies (skin ulcers) of the lower leg or foot. Wounds were treated with BMSCs seeded onto an artificial dermis (Pelnac). After 5 days of treatment, histologic examination of skin biopsy specimens showed that the BMSC/Pelnac composite graft promoted formation of fibrous tissue containing a mild infiltration of inflammatory cells. Further, immunohistochemical staining revealed the presence of $CD34^+$ cells within the wound bed in addition to newly formed vascular endothelia. In all patients, BMSC transplantation facilitated the wound healing process [139]. Some of the clinical trials that are currently underway and treating chronic wounds (e.g., diabetic foot ulcers, pressure ulcers) with BMSCs are listed in Table 8.2 (study # NCT02796079). Most of the studies are either in phase 1/2. A similar search result has been recently published by Li et al. [140]. In a prospective randomized clinical study, diabetic wounds were treated with topically applied and locally injected autologous BMSCs or whole blood (control). The healing rate of patients receiving the different treatments was compared and documented for 3 months. At 2 weeks, there was a reduction in average wound area of 17.4% in the BMSC-treated group compared to 4.84% in control group; whereas at 12 weeks, the difference in average wound area reduction was no longer statistically significant. However, the proportion of wounds

considered healed was higher in the treated group (40%) compared to the control group (29.2%) [141].

Finally, clinical studies have demonstrated clear effects of MSC treatment on fibrosis. Twenty patients suffering from progressive lesions after radiation therapy were treated with autologous lipoaspirate and then monitored for 33 months. Injection of lipoaspirate reduced fibrosis and resulted in the formation of mature preadipocytes after 1 month and multilocular adipocytes at 2 months. After 1 year, patients treated with lipoaspirate had reduced scarring and wound biopsies showed a well-formed microcirculation and mature adipose tissue [142]. By use of concepts demonstrated to be effective at alleviating hypertrophic scar formation in reconstructive surgery, minced adipose tissue or lipoaspirate have been used to reconstruct hypodermis [108]. Although not presently being evaluated in clinical trials, ASCs may have significant clinical potential in plastic surgery [143]. Regardless of pathophysiology, MSCs broadly exert their therapeutic benefits in wound healing through: (1) Immunomodulation via secretion of antiinflammatory cytokines, (2) Direct cellular interaction, (3) Transdifferentiation to tissue-specific cell types, and (4) Stimulating recovery of injured cells in the late reparative phase.

6. MANUFACTURING AND FDA REQUIREMENTS

The most important consideration to keep in mind when preparing MSCs as therapeutic agents is "how will manufacturing of stem cells under current good manufacturing practices (cGMP) affect their healing potential and safety?" For this reason, the Food and Drug Administration (FDA) has a Center for Biological Evaluation and Research (CBER), which regulates human cells, tissues, and cellular and tissue-based products (HCT/P) intended for implantation, transplantation, infusion or transfer into a human recipient, including HSCs. HCT/P should meet the following criteria:

1. HCT/P are minimally manipulated.
2. HCT/P are intended for homologous use only.
3. Manufacture of HCT/P does not involve the combination of the cells or tissues in a manner that raises new clinical safety concerns.
4. "The HCT/P does not either have a systemic effect and is not dependent upon the metabolic activity of living cells for its primary function or vice-versa, only if it is for autologous use, allogeneic use in a first-degree or second-degree blood relative; or for reproductive use" [144].

Use of stem cells for skin regeneration should follow the homologous category, that is, recipient cells or tissues that are identical to the donor cells or tissues and perform one or more of the same basic functions in the recipient as the cells or tissues performed in the donor. An advantage of MSCs is that they perform the primary functions of restoring homeostasis involving immune modulation, revascularization, and remodeling. MSCs perform the same basic

function(s) even when not used in the same anatomic location as in the donor [145].

For successful clinical translation, standard operating procedures should be adopted to generate MSCs under cGMP. GMP compliant production methods for different types of MSCs will follow general protocols that include details related to the production facility itself, cell culture infrastructure, defined media and/or xeno-free culture conditions, cell expansion, cryopreservation, long-term storage conditions, and quality control procedures [146]. Important variables related to isolating the cells that must be considered include tissue processing techniques, reagents used to process the tissue, and purification steps. Safety testing is also critical, including assays for potential microbial, fungal, endotoxin, mycoplasma, and viral contamination [147]. Following isolation, established protocols must be in place for obtaining clonogenic pure populations of MSCs, in vitro confirmation of surface marker expression, assessment of aberrations in copy number (karyotyping), mitochondrial DNA sequencing, and gene promoter methylation patterns. Lastly, and of upmost importance, MSCs long-term maintenance of stemness and their method of delivery are evaluated preclinically using suitable animal models [148]. A recent analysis provides comprehensive data suitable for comparing manufacturing technologies and current target clinical indications for cell-based therapies. The analysis indicates MSC-based clinical trials are increasing more than any other cell type. While phase 4 clinical applications of MSCs are few in number, advancements in automated techniques and standardization of MSC manufacturing protocols indicate that the clinical translation of various MSC types for skin wound healing purposes will be forthcoming in the near future.

Another important FDA guideline includes the manufacture of stem cells under current good tissue practices (CGTP) by facilities that perform this phase of the production process under contract, agreement, or other arrangement for another HCT/P establishment. The core CGTP requirements include: facilities, environmental controls, equipment, supplies and reagents, recovery, processing and process controls, storage, receipts, predistribution shipment and distribution of HCT/P, and donor eligibility determinations, screening and testing [149]. Intertwined with manufacturing intricacies are the bioethical complications, posing more hurdles for the clinical translation of MSCs. With the implementation of the World Health Organization (WHO) mandate "WHO Guiding Principles on Transplantation," it is expected that a global consensus on standard manufacturing protocols will be achieved and universally adopted. To that end, a consortium has been established that includes governmental agencies (National Institutes of Health and FDA), foundations (California Institute of Regenerative Medicine), and international societies (ISCT and the International Society for Stem Cell Research) responsible for promulgating rules for the conduct of clinical translation studies [150]. With internationally endorsed standards, it is expected that future clinical trials will be performed

with well-characterized cells under standardized conditions and provide results that can be confirmed.

7. FUTURE PROSPECTS

The use of adult MSCs as therapeutic agents in wound healing and regeneration continues to gain attention. Recent studies have broadened our understanding of the complex cellular interactions that take place between MSCs and the host and opened up new avenues for successfully treating previously intractable wounds. More challenging and important for successful translation in the future is the selection of the right type of MSC and delivery vehicle for different wound environments/pathologies. In the majority of ongoing clinical trials, MSCs are either applied directly or injected. In the future, new delivery devices (e.g., hydrogels, nano-/microparticles, nanofibers, ECMs, spheroids, and synthetic scaffolds) and precisely tuned biomaterials will deliver MSCs in the right microenvironment or niche so that they can perform at their maximum capacity. Lastly, it is essential to fully understand the regulatory path so that the translation of MSCs will proceed with seamless FDA approval. Although stem cells are classified as biologics, their path to clinical approval is cumbersome. MSC therapeutic benefits greatly rely on their secretome and this growing understanding has led researchers to focus on isolating small molecules secreted by MSCs, typically exosomes and micro RNAs, and use them as therapeutics. If successful, these small molecules would pave the way for novel clinical interventions capable of replacing MSCs, while still reaping similar outcomes, with less regulatory hurdles.

GLOSSARY

FDA The Food and Drug Administration (FDA or USFDA) is a federal agency of the United States Department of Health and Human Services.

Mesenchymal stem cells Mesenchymal stem cells (MSCs) are multipotent stromal cells that can differentiate into a variety of cell types, including osteoblasts (bone cells), chondrocytes (cartilage cells), myocytes (muscle cells) and adipocytes (fat cells).

Regeneration Renewal or restoration of a body, bodily part, after injury or as a normal process.

Skin injury These are wounds that go through the skin to the fat tissue. Caused by a sharp object, scrapes, abrasions, scratches and burns.

Wound healing A natural restorative response to tissue injury involving a complex cascade of cellular events that generates resurfacing, reconstitution, and restoration of the tensile strength of injured skin.

LIST OF ACRONYMS AND ABBREVIATIONS

α-SMA Alpha smooth muscle actin
AF Amniotic fluid
AFSCs Amniotic fluid stem cells
AM Amniotic membrane

Ang-1 Angiopoietin 1
ASCs Adipose tissue–derived stem cells
B cells B lymphocytes
bFGF Basic fibroblast growth factor
BMSCs Bone marrow–derived stem cells
CBER Center for Biological Evaluation and Research
CD Cluster of differentiation
CEA Cultured epithelial autografts
cGMP Current good manufacturing practice
CGTP Current good tissue practice
COX Cyclooxygenase
CXCR/CCR Chemokine receptors
DC Dendritic cells
DFUs Diabetic foot ulcers
DP Dermal papillae
ECM Extracellular matrix
EGF Epidermal growth factor
ESCs Embryonic stem cells
F-CFU Fibroblast-colony forming units
FDA Food and Drug Administration
GFP Green fluorescent protein
HCT/P Human cells, tissues, and cellular and tissue-based products
HFSCs Hair follicle stem cells
HIV Human immunodeficiency virus
HLA Human leukocyte antigen
HSCs Hematopoietic stem cells
HTS Hypertrophic scars
IFATS International Federation for Adipose Therapeutics and Science
IGF Insulin growth factor
IL Interleukin
ISCT International Society of Cellular Therapy
K1–K19 Cytokeratin 1–19
M1 Proinflammatory macrophage
M2 Antiinflammatory macrophage
MHC Major histocompatibility complex
MMPs Matrix metalloproteinases
MSCs Mesenchymal stem cells
NF-κB Nuclear factor kappa-light-chain-enhancer of activated B-cells
NG2 Neuroglial factor 2
NK-cells Natural killer cells
Oct Octamer-binding transcription factor
PDGF Platelet-derived growth factor
PDGFR Platelet-derived growth factor receptor
PGE_2 Prostaglandin E_2
SC Stem cells
SDF-1 Stromal cell–derived factor 1
SG Sebaceous glands
SKPs Skin-derived precursor cells
Sox Sex determining region Y-box
SVF Stromal vascular fraction

TACs Transit-amplifying cells
TBSA Total body surface area
T cells T lymphocytes
TGF Transforming growth factors
Tie-2 Tyrosine kinase receptor for Ang-1
TIMP Tissue inhibitors of MMPs
TLR Toll-like receptors
TNF Tumor necrosis factor
TSG TNF-α stimulated gene/protein
UCB Umbilical cord blood
UCB-MSCs Umbilical cord blood−derived MSCs
VEGF Vascular endothelial growth factor
WHO World Health Organization
WJ Wharton jelly
WJ-MSCs Wharton jelly−derived MSCs
Wnt3 Wingless integrated family 3A

DISCLAIMER

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Micro-fragmented Adipose Tissue and Blood Plasma-based Hydrogels for Treatment of Combat Associated Burn Injuries

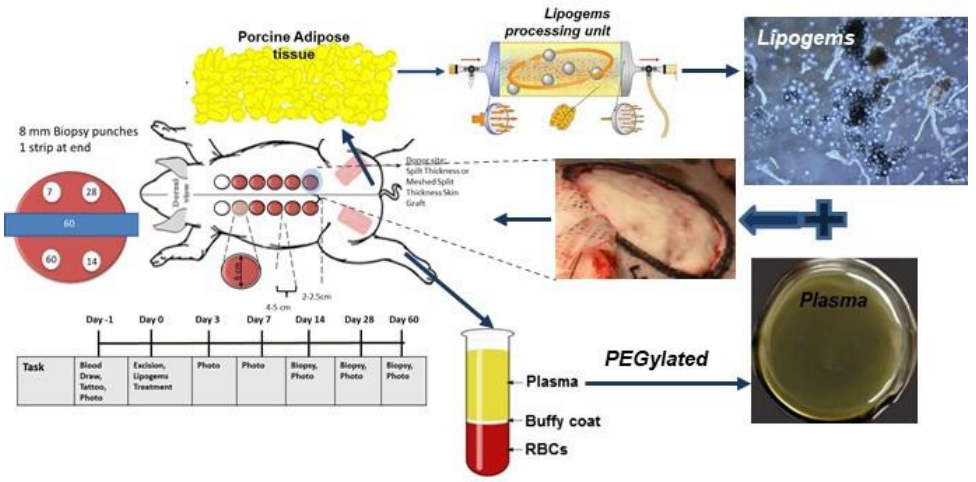
MB150163
W81XWH-16-2-0063
PIs: Shanmugasundaram Natesan Org: Metis Foundation | USAISR Award Amount: $741,986

Study Specific Research Aims

- Optimize and characterize micro-fragmented adipose tissue (Lipogems) – hydrogel formulations *in vitro*.
- Screen and evaluate optimized Lipogems-hydrogel based formulations in a 30 cm^2 porcine full-thickness acute excision wound model for improved graft take, vascularization, and wound healing.
- Evaluate the optimal Lipogems-hydrogel formulation using a 30 cm^2 full-thickness porcine burn wound model to assess healing and scarring.

Approach

We propose to improve acute burn wound healing using purified micro-fragmented adipose tissue and hydrogel based formulations. The proposed approach provides burn surgeons an active platform for a successful grafting protocol that will improve burn wound repair through enhanced wound vascularization, graft take, and reduced scarring.



Accomplishment: Autologous Lipogems can be isolated and used as a bed-side treatment option full thickness wounds.

Timeline and Cost

Activities	FY 17	FY 18	FY 19
Optimization and characterization of Lipogems and hydrogel based formulations			
Screening and evaluation of Lipogems in full-thickness (30cm^2 wounds)porcine wound model			
Evaluation of optimized Lipogems-plasma hydrogel formulations in a full-thickness burn wound model (30cm^2 wounds).			
Estimated Budget ($K)	$199k	$233k	$308
Actual Budget ($K)			

Updated: 30-October-2018

Goals/Milestones:

CY17 Goal – Optimization and characterization of Lipogems and hydrogel based formulations.

☑ Optimize and characterize Lipogems-hydrogels formulations.

CY18 Goals – Screening and evaluation of Lipogems in full thickness porcine excision wound model

☑ Establish excision wound animal protocol to evaluate Lipogems and Lipogems-hydrogel formulation.

☑ Identify effective dose of Lipogems and Lipogems-hydrogels.

☑ Determine efficacy of cryopreserved vs. freshly isolated Lipogems .

CY19 Goal – Evaluate the effect of Lipogems on full-thickness burn wounds

☐ Determine feasibility of isolating Lipogems from debrided hypodermis.

☐ Efficacy of debrided fat Lipogems vs. freshly isolated/cryopreserved Lipogems in a full thickness burn wound.

☐ Efficacy of optimized Lipogems and Lipogems-hydrogels dose on graft take and healing.

☐ Determine efficacy of optimized Lipogems and Lipogems-hydrogels dose on contraction and scarring.

Budget Expenditure to Date

Projected Expenditure: $156,402

Actual Expenditure: $180,643.74