Prevascularized Stem Cell Sheet for Full-Thickness Skin Wound Repair

Daniel Radke, Lei Chen, Shaohai Qi, and Feng Zhao

1 Introduction

There are approximately 15,000–20,000 hospitalizations per year for acute burn injuries in the USA, as recorded by the American Burn Association [1]. Burn injuries are one of the leading causes of full-thickness skin wounds, for which the gold standard of treatment remains transplantation of an autologous full-thickness graft (FTG) or skin flap [2]. Alternative approaches and skin substitutes have been developed for burn treatment, but autologous FTG demonstrates better immunological acceptance and better match skin color and texture. Hence, it has been preferred as a safe and economically viable grafting method. However, the limited supply of donor skin and unavoidable donor-site injury restrict their ability to treat extensive and severe wounds.

One promising alternative is the application of an autologous split-thickness skin graft (STSG), in which only the epidermis and a portion of the dermis are harvested rather than the full thickness. STSG still requires re-epithelialization and ongoing wound therapy until the donor site is repaired, but these donor sites may be harvested repeatedly to resurface large wounds. STSG can be used under unfavorable conditions where FTG would fail, such as a recipient's wound having moderate infection or less vasculature [3]. However, STSG is more fragile than FTG and can contract significantly during the healing process, leading to poor cosmetic outcome, physical disability, and reduced pliability [4]. These disadvantages may be overcome by combining STSG with engineered dermal substitutes, but the insufficient blood supply at early stages of the transplantation causes these grafts to experience relatively long hypoxic and ischemic periods after surgery and suffer from degeneration and necrosis [5]. While bioengineered products such as natural substitutes—human allograft, Oasis wound matrix®; synthetic substitutes—Biobrane™; and permanent skin substitutes—Epicel®, and Integra® have been commercially available for years, major problems still exist with material sources, manufacturing techniques, material compatibility, and therapeutic effects associated with these skin grafts [6].

Cell-based therapies, especially those using stem cells, for improving the survival and therapeutic effect of skin grafts have emerged as a new approach in recent years [7]. Mesenchymal stem cells (MSCs) exhibit excellent potential for accelerating wound healing due to their self-renewal ability, secretion of paracrine factors, and ability to differentiate into different cell lin-

D. Radke · F. Zhao (✉)
Department of Biomedical Engineering, Michigan Technological University, Houghton, MI, USA
e-mail: dlradke@mtu.edu; fengzhao@mtu.edu

L. Chen · S. Qi
Department of Burns, The First Affiliated Hospital of Sun Yat-Sen University, Guangzhou, China
e-mail: chenleigzburns@163.com; qishaohaigzburns@163.com

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eages [8]. Numerous studies reported positive results when utilizing MSCs for various wound regeneration applications [9–12]. Local delivery of rat adipose-derived MSCs to an excisional wound healing model showed enhanced epithelialization and granulation tissue deposition [13]. Human MSCs (hMSCs) grafted in impaired healing diabetic mice significantly improved healing by recruiting large amounts of host mouse MSCs to the wound bed, producing factors such as vascular endothelial growth factor (VEGF) and platelet-derived growth factor receptor-α (PDGFR-α), which can stimulate angiogenesis [14]. Compared to traditional cell delivery strategies, such as cell injection or spraying, cell sheet engineering techniques enhance the cell delivery efficacy to injured tissue [15]. The cell sheet preserves cell-cell junctions, extracellular matrix (ECM), and cell-matrix connections, thus restricting the cells to the wound bed [16]. The rat adipose-derived MSC sheet demonstrated beneficial effects in diabetic wound healing when combined with artificial skin, where the cells accelerated wound healing directly by functioning as pericytes and indirectly through the secretion of paracrine signaling factors [17].

The primary factor that influences the quality of a transplanted STSG is the sufficient supply of blood to the skin graft [18]. During the first 48 h of transplantation the graft is engorged by plasmatic fluid, and a poorly vascularized bed hinders plasmatic diffusion [18]. In addition, newly formed blood vessels in the wound bed can deliver oxygen, nutrients, and essential growth factors to hypoxic and ischemic STSGs at early stages after placement [19]. A cell sheet with preformed microvessels may enhance angiogenesis as well as further improve tissue function by supporting cell survival and accelerating the integration of the graft with host tissues [20, 21].

Among all cell types used for angiogenesis and neovascularization, the role of endothelial cells (ECs) has been studied extensively. ECs are able to initiate postnatal neovascularization and express a series of growth factors and cytokines including platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-1 (IL-1), IL-5, and IL-6, which are beneficial for wound repair [22]. When co-cultured with hMSCs, the formation of a microvessel network is promoted due to the supporting and stabilizing functions of hMSCs [23]. Cross talk between ECs and hMSCs upregulates the expression of angiogenic genes such as von Willebrand factor (vWf), platelet/endothelial cell adhesion molecule-1 (PECAM-1), and cadherin 5 [24]. Furthermore, EC/hMSC cross talk has been shown to improve angiogenesis via synergistic effects [25]. Therefore, an hMSC cell sheet (HCS) co-cultured with ECs may facilitate neovasculature formation. It is anticipated that the prevascularized hMSC sheet (PHCS) will improve skin graft survival when combined with a STSG for full-thickness skin wound repair.

2 Technique

For cell sheet culture, the passage of three to five hMSCs were seeded on cover glasses coated with 20 μg/mL collagen I, and cultured under a hypoxic condition (2% O_2) for 4 weeks to obtain HCS. The HCS with a diameter of 2 cm was harvested by gently peeling the cell layers off the cover glass. To fabricate PHCS, ECs were seeded on top of hMSC sheets and cultured under a normoxic condition (20% O_2) for 1 week to obtain PHCS in endothelial cell growth medium (Fig. 1) [26].

Due to the importance of the immune system in wound healing [27, 28], an immune competent rat model was chosen for the purpose of simulating healing processes in human wound repair [29]. To begin with, Sprague-Dawley (SD) rats were anesthetized using gas anesthesia. The back of the rat was shaved and sterilized using alcohol wipes. A small part of SD rat's dorsum was cut off, shaping a round full-thickness wound with a diameter of 20 mm. The STSG was made by the removal of panniculus carnosus as well as deep partial dermis from the skin excision (Fig. 2) [26]. For the purpose of secretion drainage, fenestrations with equal spacing were created on STSG with a sharp-tipped scalpel. Three layers of cell sheets were grafted on the wound and then covered by STSG. The STSG transplantation

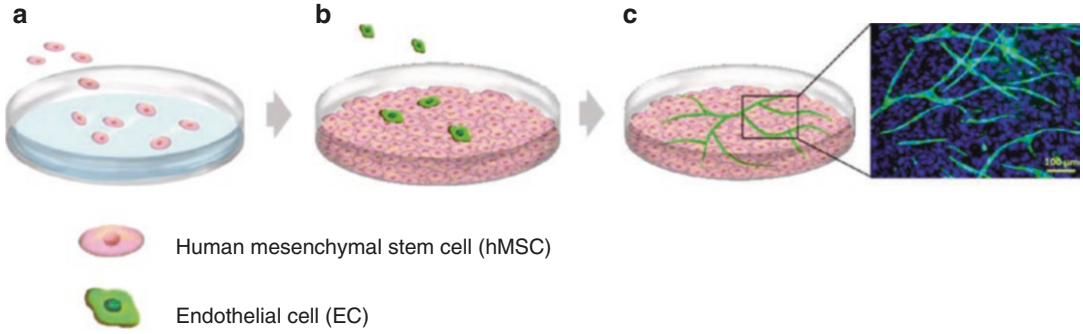

Fig. 1 Fabrication of PHCS via hypoxic hMSC culture (**a**), normoxic seeding of ECs on mature HCS (**b**), and development of microvasculature visualized with immunofluorescence (**c**)

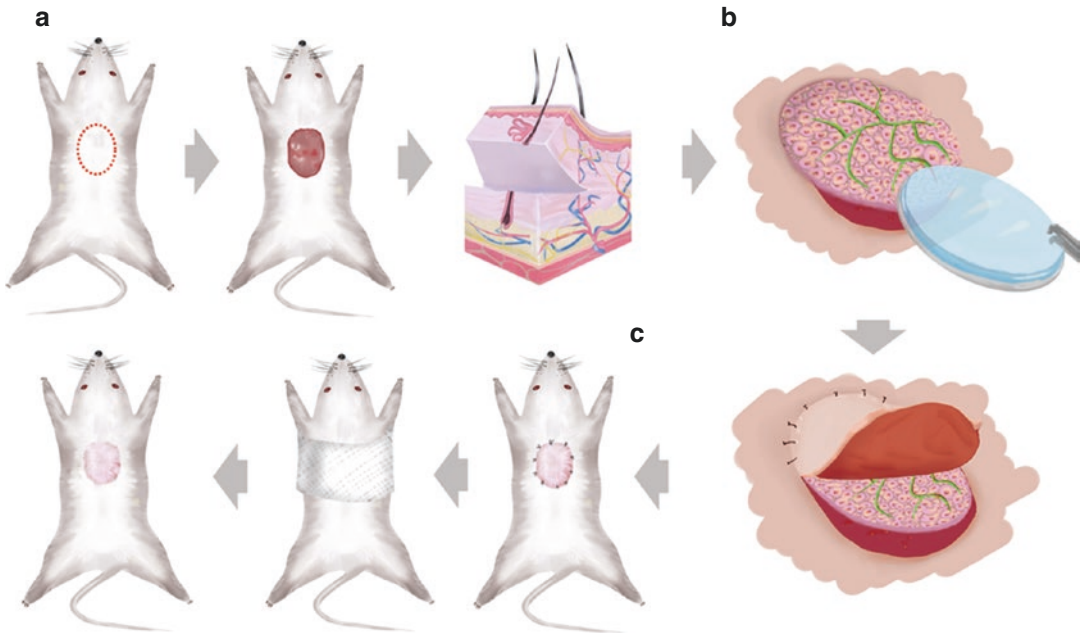

Fig. 2 Illustration of procedure including defect generation (**a**), PHCS grafting (**b**), and wound healing (**c**)

without cell sheets was served as control. The grafts as well as the adjacent wound margin were sutured with interrupted stitches, and then secured with a padded bolster (proper pressure provision and scratch prevention). The bolsters were changed on postoperative day 3 and removed on day 7. The implant contraction was observed over 28 days post-surgery. The relative size change of grafts was evaluated by gravitational planimetry and expressed as a percentage of the remaining skin graft size to its original wound size.

3 Discussion

The survival of skin grafts after implantation relies mainly on nutrient diffusion from the wound bed at the early stage of implantation [30, 31]. Engineered PHCSs transplanted in combination with an autologous STSG were found to promote vascularization and healing in a full-thickness wound model. The PHCS-STSG implants displayed no hemorrhage or necrosis after 3 days, preserved most skin appendages and connective tissues, and exhibited a controlled and

resolved inflammatory response by 14 days without incurring the fibrosis associated with prolonged immune response [32]. This resulted in significantly less contraction than STSG alone and morphology closer to the surrounding normal skin, indicating better performance in a sequence of overlapping wound-healing events including inflammatory response, neovascularization, proliferation, collagen deposition, and remodeling over the course of 28 days [33].

Hair follicles, skin glands, and dermal collagen are important components for reconstitution of skin structure and function. The number of skin appendages, especially sebaceous glands, significantly decreases in STSG transplantations. Simultaneously, collagen fibers degrade or undergo necrosis with slight hemorrhage. These phenomena are probably caused by surgical operation and insufficient blood supply [34]. Histological analysis after 28-day in vivo trials showed that PHCS significantly improved STSG performance in repairing full-thickness skin wounds, better maintaining the graft structure and components (hair follicles and sebaceous glands) from the beginning and remodeling collagen to integrate with host tissue without the occurrence of necrosis, hemorrhage, or fibrosis (Fig. 3) [26].

It has been highlighted that the optimal composition of skin substitute scaffolds should mimic that of normal skin in order to enhance clinical effectiveness [35]. Both HCS and PHCS contain large amounts of ECM molecules including collagen I, collagen III, collagen IV, elastin, fibronectin, and laminin [36]. The presence of these preexisting ECM components can aid in accelerating the regeneration of dermal tissue. Results from the in vivo evaluation suggest that the embedded cell sheets, especially the PHCS, may have also mitigated cellular processes associated with graft fibrosis and contraction.

Abnormal keratinocyte differentiation and their abnormal cytokine secretion are two factors demonstrated to contribute to tissue fibrosis and contraction by activating fibroblasts [37–39]. The epidermis of PHCS-STSG-implanted rats maintained its original appearance, and epidermal ulcers were soon covered with new, thin epidermis within 3 days in HCS-STSG rats, while the group with STSG alone showed less recovery. The thick-

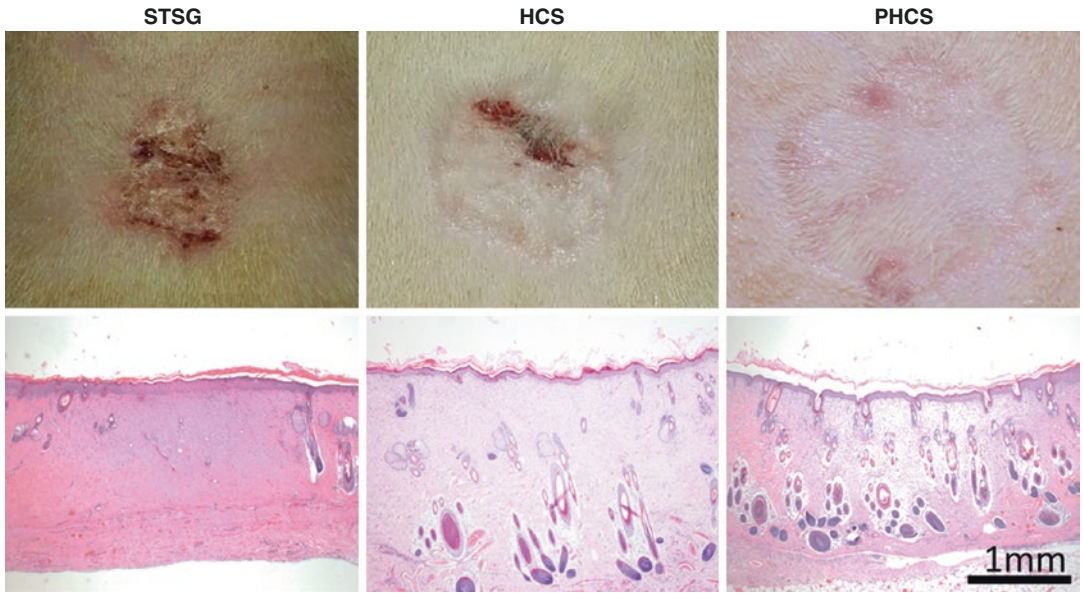

Fig. 3 Results of 28-day in vivo evaluation of capacity for full-thickness wound repair in immune-competent Sprague-Dawley rats of split-thickness skin graft alone (STSG), STSG with hMSC cell sheet (HCS), and STSG with prevascularized hMSC cell sheet (PHCS)

ness of the neoepidermis in the STSG group increased significantly over time due to epidermal hyperplasia, a common response of dermal wounds characterized by overdevelopment of the epithelial cell layer. In contrast, HCS and PHCS epidermis maintained a below-trend change and quickly returned to their normal morphology. Since the epidermis is the outmost layer of skin, a quick recovery of this layer can provide a barrier to infection from environmental pathogens and maintain water homeostasis inside the skin. However, excess epidermal hyperplasia results in dermal fibrosis and interferes with normal skin function [40]. Interestingly, it was found that the therapeutic result of different grafts (STSG alone, STSG with HSC, or STSG with PHSC) correlated with the growth factor levels in the preceding cell sheets, suggesting that the paracrine activity may dominate the healing process in the full-thickness wound model. PHCS were found to contain about 2.6 times more angiopoietin 1, a growth factor recognized in promoting vessel stabilization and tightness, compared to HCS.

Conclusion

STSG transplants are frequently used when simple nonsurgical wound care is not applicable; however STSG commonly has problems with graft necrosis due to inadequate nutrient supply at the early stages of wound healing, resulting in the formation of scar tissue that does not function the same as normal skin [33, 41, 42]. These problems can be alleviated through the use of hMSCs to promote early vascularization and mediate the wound-healing process. An in vivo evaluation with immune-competent animals demonstrated that the use of prevascularized stem cell sheets enhanced STSG by greatly reducing skin contraction during healing, preserving skin appendages, increasing the number and area of microvessels, mitigating inflammatory reactions, and resulting in a morphology that more closely resembled normal skin. Both HCS and PHCS combined with STSG exhibited markedly enhanced therapeutic value in the rat model and may be useful for facilitating the healing of full-thickness wounds in humans.

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