Second-harmonic generation microscopy for assessment of mesenchymal stem cell-seeded acellular dermal matrix in wound-healing

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ABSTRACT

Direct intra-skin injection of mesenchymal stem cells (MSCs) and the use of biomaterial scaffolds for grafts are both promising approaches of skin wound repair, however they still cannot generate skin that completely resembles the natural skin structures. In this study, we combined these two approaches by using acellular dermal matrix (ADM) recellularized with MSCs to repair cutaneous wounds in a murine model and two-photon fluorescence (TPF) microscopy and second-harmonic generation (SHG) microscopy to assess the effects of this therapy on wound healing. Bone marrow-derived mesenchymal stem cells (BM-MSCs) were tagged with GFP and seeded into ADM (ADM-MSC) via MSC and ADM co-culture. ADM-MSC, ADM or saline was applied to murine excisional skin wounds and wound-healing was evaluated by histological examination on days 7, 14, 21 and TFP microscopy on days 1, 3, 5 and 21 post-treatment. ADM-MSC promoted healing significantly more than treatment with ADM or saline alone, as it led to substantial neovascularization and complete skin appendage regeneration. Furthermore, the SHG microscopic imaging technique proved to be a useful tool for monitoring changes in the collagen network at the wound site during the healing process and assessing the effects of different therapies.

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1. Introduction

Full-thickness skin wounds can result from a variety of injuries, such as acute trauma, chronic ulcers, and extensive burns, and can cause numerous physiological and functional problems. The ideal treatment should provide a coverage that supports the repair of skin structure and restoration of skin function. Towards this goal, many studies have sought to develop biomaterials that not only resemble the natural skin tissue architecture, but also facilitate cell colonization [1].

Acellular dermal matrices (ADMs) are the native extracellular matrices which are derived from human or animal skin by removing the epidermis and all dermal cells. ADMs are widely used for skin reconstruction and surgical applications (burn injuries, ulcers and post trauma surgery) [2] as they can be rapidly integrated into the wound tissues with favorable biocompatibility [3]. In the applications of ADMs for different wound healing, ADMs usually act as a scaffold for host cellular infiltration and undergo progressive remodeling to form functional tissue without immunogenic response [4]. Collagen proteins are the main component of the acellular scaffolds and other minor extracellular matrix (ECM) proteins such as fibronectin, laminin and vimentin are also included [2].

Another strategy for improving skin regeneration in cutaneous wounds is to use stem cell-based therapies. Recent studies have shown that mesenchymal stem cells (MSCs) have great regenerative potential, and their multipotentiality and the ability to proliferate in vitro for long periods of time [5] are also advantageous for use in therapy. The mechanisms of MSCs enhancing tissue repair are complex, as MSCs can participate in all three phases (inflammation, proliferation and remodeling) of skin regeneration [6,7].
Many studies have suggested that MSCs can function as vascular pericytes [8] and contribute to tissue repair by secreting factors that can stimulate the proliferation and differentiation of endogenous progenitors, decrease inflammatory reactions and promote angiogenesis [9,10]. Others reported that MSCs cultured in vitro on dermal equivalents acquired the phenotype and function of myofibroblasts, leading to ECM remodeling and matrix contraction [11–13]. MSC-seeded ADM is considered to be a promising strategy for accelerating wound healing [7,14], but it has not been extensively tested experimentally.

Thus far most of the research on MSC activity and collagen remodeling during the wound healing process has been done in vitro or in biopsied animal tissues, due to a lack of feasible means to directly monitor the dynamic interaction between MSCs and the ECM remodeling in wound site during the healing process. Using nonlinear microscopic optical imaging techniques may resolve this issue since cell activities can be traced and collagen structure can be monitored intravitaly [15,16]. Two-photon fluorescence (TPF) and second-harmonic generation (SHG) are two well-known nonlinear imaging methods that are commonly used in tissue imaging experiments. In TPF microscope framework, only the intrinsic and do not require extra extrinsic dyes, thus SHG microscopic imaging is co-localized with ECM, which can be excited by simultaneous (within the focal plane) near-infrared femtosecond pulse light is adopted, these nonlinear optical phenomena that have been utilized in microscopic imaging of tissues, namely the TPF microscopy and SHG microscopy, respectively. TPF and SHG rely on different nonlinear light-matter interaction mechanisms, but can usually be taken together to generate combined images in a same optical microscope imaging system [17,18]. In TPF microscope framework, only fluorophores within the focal plane can be excited by simultaneous (within 10–16 s) absorption of two photons. This restricts the fluorescence emission to the focal plane, thus greatly reducing out-of-plane photobleaching, phototoxicity and ultimately achieving intrinsic three-dimensional resolution. In SHG microscopy, as an intense input laser light passes through non-centrosymmetric molecules, it induces a second-order nonlinear polarization in those molecules. As a result, an emission light with exactly twice the frequency (half the wavelength) of input is generated. SHG signals are tissue-intrinsic and do not require extra extrinsic dyes, thus SHG microscopy fully preserves the normal function of biological systems. The fibrous collagen type I in skin tissue has been proved to be the source of strong intrinsic SHG signals [17,19]. Both TPF and SHG have the ability to construct 3D images of specimens, and since the near-infrared femtosecond pulse light is adopted, these nonlinear microscopic imaging methods have gained the ability of imaging thick tissues while eliciting minimal damages on them. Naturally, TPF microscopy and SHG microscopy have been widely used for visualization of cells, tissues and organs [18,20,21].

In the current study, ADM was seeded with MSCs and was then used to treat full-thickness skin defects. The wound healing process was examined with TPF and SHG imaging to dynamically trace the activities of MSCs and collagen remodeling. The results indicate that MSCs seeded on ADM scaffold as a wound bed sheet can promote the dermal structural regeneration, angiogenesis, re-epithelialization and collagen remodeling, and ultimately accelerate the healing of murine cutaneous wounds.

2. Materials and methods

2.1. Preparation of ADM scaffolds

Skin from five-week-old male BALB/c mice was used to prepare ADM scaffolds. The mice were euthanized via cervical dislocation. The dorsal hair was shaved and pieces of 3 × 5 mm² full-thickness skin were harvested. The skin tissues were treated with 0.25% Dispase (Aoboking, Beijing, China) at 4 °C for 48 h to separate the epidermis and then immersed in PBS supplemented with 0.3% Triton- X-100, 0.25% sodium deoxycholate and 0.02% EDTA under continuous oscillation, at 37 °C for 48 h in order to remove the cellular components from the matrix. The decellularized scaffolds were then treated with a fat digestion solution of chloroform and methanol (v/v = 1:1) for 1–2 h and washed three times in PBS. Finally, the scaffolds were sterilized in 70% ethanol for 30 min and washed three times with sterile PBS. The ADM scaffolds were stored in DMEM medium at 4 °C till use.

2.2. SEM (scanning electron microscope) imaging of ADM

Decellularized ADM scaffolds were fixed in 2.5% glutaraldehyde at 4 °C for at least 24 h. Scaffolds were then dehydrated by immersion in an ascending ethanol series (30%, 50%, 75%, 90%, and 100%), treated twice with isoamylacetate, critical point dried and sputter-coated with a 30 nm gold layer. Samples were imaged using a scanning electron microscope (Zeiss Ultra 55, Carl Zeiss, Jena, Germany) in order to show the collagen organization and construction of the ADM scaffolds.

2.3. Seeding of MSCs on ADM

For cell culture, pieces of ADM scaffolds (6 mm in diameter) were cultured for two weeks and the culture media were replaced every two days. For transplantation onto animal, these scaffolds were cultured for 1 day after seeded with MSCs.

2.4. Imaging instrument and methods

Nonlinear optical imaging, the TPF and SHG microscopic imaging was co-achieved in a two-photon system by using a commercial LSM 710 NLO confocal microscopy system (Zeiss, Jena, Germany) equipped with a femtosecond Ti:Sapphire lasers (Chameleon Vision II, Coherent, Santa Clara, CA, USA), which provides a pulse light with tunable excitation wavelengths from 680 nm to 1080 nm. SHG imaging of collagen fiber was obtained by excitation at 820 nm, while TPF imaging of MSCs was done by excitation at 850 nm. The immunofluorescent images of CD-31, α-SMA and K10 were achieved in a single-photon system by using the commercial LSM 710 NLO confocal microscopy (Zeiss, Jena, Germany) with the LASOS HeNe gas laser operated at 543 nm. The nuclei were imaged by excitation of DAPI either in two-photon system at 720 nm or single-photon system with the Coherent cube 405-FP solid-state laser operated at 405 nm depending on the convenience of non-transition of imaging system in a same image frame. The laser light was in horizontal polarization direction and the optical signals were collected in the backward geometry.

2.5. Cell proliferation assay

In the co-cultured ADM scaffolds, MSC proliferation was quantitatively assessed by measuring the fluorescence intensity of GFP using ImageJ (NIH) software in 3D mode [22]. After seeding onto the ADM scaffolds, most of the MSCs attached to the plates and ADM scaffolds after 6 h of incubation. On days 1, 4, 7, and 14, the ADM scaffolds were gently washed to remove non-adherent and dead cells and then fixed in 10% formalin prior to imaging. Images were taken by TPF and SHG microscopy and recorded in 512 × 512 pixels with Plan-Neofluor 20X objectives (NA 0.8). For each sample (n = 5 ADMs), 3 random fields were chosen within the ADM scaffold and a stack of focal planes were taken from these 3 fields in the cellularized area using a 5 μm step size. Fluorescent intensity of every field was analyzed using ImageJ (NIH) with the “3D object counter” plug-in. Each experiment was repeated three times. The proliferation indexes were based on the fluorescence intensity values expressed relative to that of 14 day co-cultured ADM.

2.6. Wound model and ADM-MSC implantation

Eight-week-old BALB/c male mice were used. The dorsal skin of each mouse was shaved and sterilized with 70% ethanol and iodine prior to surgery. Animals were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg, Fuchen, Tianjin, China). Once the mice were anesthetized, a 7 mm biopsy punch was used to make an incision on the dorsum, and then the circular region of tissue was grabbed and pulled with forceps and excised with scissors to form a full-thickness wound. The wounded mice were divided into three groups, to be treated with ADM-MSC, ADM only or saline as control. After implanting ADM-MSC, ADM or saline, Comfeel transparent dressings (Coloplast, Beijing, China) were used to cover the wound sites to prevent catching, biting and infection.

2.7. Histological and immunofluorescent analysis

Wound samples were harvested on designated days post-surgery. Samples were fixed in 10% formalin at room temperature for at least 24 h, embedded in paraffin and sectioned in 5 μm increments. Sections were made through the center of the wound and perpendicular to the skin surface. The sections were deparaffinized, rehydrated, washed in distilled H2O and stained with hematoxylin and eosin (H&E). For immunofluorescent labeling, sections were washed in PBS, blocked with 1% bovine serum albumin (pH = 6.0) and heated in a steamer for 10 min, and then blocked by incubation with Peroxide Block and normal goat serum (Beyotime, Shanghai, China). Samples were then incubated overnight with primary antibodies against CD31, α-SMA and K10 (1:1000, 1:1000, 1:200, respectively, Millipore Chemicon, Billerica, MA, USA) at 4 °C. After washing, rhodamine-labeled secondary antibody (1:50, KPL, Gaithersburg,
MD, USA) was applied for 2 h. All sections were stained by DAPI (Sigma) for 15 min to visualize the nuclei. Sections were imaged by single-photon system and analyzed. Three random fields of each section (n = 5) were chosen, and mature vessels containing erythrocytes were counted and measured using Image J (HIN) in order to evaluate the density and diameter of blood vessels in the wound areas.

2.8. Intravital imaging of ADM-MSC explants

After the mice were anesthetized and wound area sterilized (n = 3), 5 random regions were chosen within the wound area and were collected on days 1, 3, 5 and 7. These tissue samples were immersed in 0.9% SPSS (stroke-physiological saline solution) and then imaged by TPF and SHG microscopy using the stack scan mode. The imaging system sequentially scanned the samples and a stack of focal planes were taken along the z axis using a 2 μm step size. The consequent planes were then composited into a three-dimensional image on a computer screen. All procedures were finished within 30 min to ensure that the chosen tissue samples were alive.

2.9. Skin maturity quantification

Skin structure was assessed on day 21 post-treatment using H&E-stained histologic sections, according to previously published methods [23,24]. Specific criteria were used to assess each wound for dermal differentiation, epithelial maturation, and skin appendage regeneration.

I. Dermal differentiation grading was defined based on following 4-grade criteria: 1, thin, dense, and monotonous fibrosis; 2, thicker but still dense and monotonous fibrosis; 3, two layers but not completely discreet; 4, two discreet layers with superficial fibrosis and loose alveolar tissue within the deep layer;

II. Epithelial maturation grading used the following criteria: 1, thin and no reticulation; 2, occasional reticulation; 3, moderate reticulation; 4, thick with complex reticulation;

III. Skin appendage regeneration grading was defined according to the following criteria: 1, no developing glandular structure; 2, minimal developing glandular structure; 3, considerable developing glandular structure; 4, mature glandular structure and developing hair follicles.

2.10. Statistical analysis

All data are represented as means ± standard deviation (SD) with at least three independent replicates. Difference between groups was analyzed by two-tailed Student’s t-test. A p value <0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of mouse ADM

The average thickness of ADMs after the treatment of deep-in-the-wound layer and decellularization was measured to be 0.58 ± 0.17 mm (n = 5). SHG microscopy and SEM images revealed the shape, orientation, density of collagen fibers and the spacious structure characteristics of ADM scaffolds. DAPI-staining nuclei were not found in ADM sections (Fig. 1).

3.2. Proliferation of MSCs on ADM scaffolds in vitro

Fig. 2A and B shows the different morphological characteristics of MSCs grown on different surfaces. MSCs grown on ADM had a more elongated and spindle-like shape (Fig. 2B) than those cultured in a dish (Fig. 2A); this may have been caused by the rough surface of the ADM. These EGFP-labeled MSCs exhibited bright green fluorescence under the fluorescent microscope (Fig. 2A and B).

The proliferation of MSCs in scaffolds was monitored using two-photon system. The distribution and amount of MSCs were measured on different co-culture days after these MSC-seeded scaffolds were fixed and stained directly with DAPI. MSCs continued to proliferate in the scaffolds and MSCs gradually invaded deeper layers of scaffolds. In Fig. 2C, the MSC nuclei in deeper layers appeared out of focus (white arrows) compared to those on the surface of ADM. To determine the spreading distance, the ADM-MSC scaffolds (n = 5) were scanned for GFP fluorescence in 0.5 μm steps, and the depth from the surface to the first plane in which fluorescence from MSCs disappeared was defined as the distance of cell spreading. On days 1, 7 and 14, these distances reached 4.61 ± 1.27 μm, 15.94 ± 3.18 μm, and 32.06 ± 2.30 μm respectively (Fig. 2E). Fig. 2D and F shows the MSC proliferation in ADM scaffolds on days 1, 4, 7 and 14, with proliferation indexes of 0.18 ± 0.07%, 0.32 ± 0.04%, 0.45 ± 0.11% and 100 ± 0.09%, respectively. The proliferation indexes were based on the fluorescence intensity values expressed relative to that of 14 day co-cultured ADM.

Fig. 1. Characterization of ADM scaffolds. (A) The appearance of ADM after decellularization treatment in a 30 mm culture dish. (B) Imaging of ADM section with DAPI-staining reveals the absence of cell components in ADM. (C) SHG imaging of ADM in vitro provides a new means to visualize collagen fibers. (D) SEM images of ADM show shape, orientation, and structure characteristics of collagen fibers in ADM. (Bottom) Papillary dermis side photograph shows arrangement of collagen fibers in ADM; (Top) Epidermis side photograph displays a huge cavity in the scaffold caused by removal of hair follicles via the decellularization process; (Fibers) Papillary dermis side photograph exhibits the more subtle structure of collagen fibers at high magnification. Scale bar, 100 μm (B and C), 10 μm (D-Bottom and D-Top), 1 μm (D-Fibers).
3.3. MSC-ADM promotes wound healing in vivo

Mice were euthanized on days 7, 14 and 21 post-treatment, for clinical observation and histological analysis. The wound healing process was significantly accelerated in the ADM-MSC and ADM groups compared with the control group (Fig. 3A and B). In the control group, even on day 21, the wounds were still not fully closed and were covered with eschar in some areas. In addition, the regenerated skin in the control group was translucent and thin, with no skin appendage regeneration. In ADM group, the borders between grafts and wounds were distinguishable and skin appendage had started to regenerate in forming skin. In the ADM-MSC group, the peripheral region of the wound appeared to have integrated into the surrounding native skin tissue and there was complete appendage regeneration.

Collagen structures in the dermis of regenerated skin in three groups at day 21 were visualized by combined TPF/SHG imaging. Fig. 3C shows that the underlying network structure of collagen fibers in ADM-MSC group was well organized and morphologically closer to normal dermal tissue. The collagen content was lower and fibers more scattered in the ADM and control groups 21 days after surgery indicating that the healing dynamics were accelerated in the ADM-MSC group compared to the ADM and control groups.

3.4. Evaluation of angiogenesis response

The angiogenic response, an important step in healing process for severe injuries, was also analyzed. Immunohistological staining of wound sections was done for the endothelial protein CD31 (Fig. 4A). Early vascular networks and a large number of endothelial cells were present in and around the wounds treated with ADM-MSC, but were not observed in wounds treated with only ADM or saline.

Alpha-smooth muscle actin (α-SMA) staining of wound sections of different treatments on day 14 identified vasculature stabilized with smooth muscle cells. A significant increase in vascular networks layered with smooth muscle cells was also seen within the wounds treated with ADM-MSC (Fig. 4B). There was only a small amount of vascular vessels and smooth muscle cells found in wounds treated with ADM or saline. Blood vessel density was significantly higher in wounds treated with ADM-MSC (337 ± 50/mm²) vs. wounds treated with ADM (121 ± 24/mm²) or saline only (49 ± 24/mm²). Moreover, vessel diameter was larger in the healing
tissues of the ADM-MSC group compared with the ADM and control groups (24.54 ± 7.21 μm, 15.54 ± 2.13 μm, 8.79 ± 1.64 μm) (Fig. 4D). These data indicate that ADM-MSC promoted the recruitment of endothelial cells to the wound area and enhanced vessel growth in healing wounds.

3.5. Evaluation of skin re-epithelialization

Sections of the wounds were stained with the epithelial protein cytokeratin 10 (K-10) to evaluate re-epithelialization. Layered epithelial structures were observed in wound areas of mice in the ADM-MSC group at 14 days post-surgery, however these were not observed in the ADM group mice. In the control group mice, the wounds were still covered with eschar (Fig. 5A, C and E). Matured epithelial structures were observed in mice in the ADM-MSC group 21 days post-surgery. In contrast, the epithelial layers of ADM and control group mice were thinner and the K-10 positive cells were observed in random positions (Fig. 5B, D, and F). These results suggest that ADM-MSC contributed to re-epithelialization and appendage regeneration of skin wounds.

3.6. Intravital imaging of ADM-MSC graft

The activity of MSCs seeded in ADM grafts and the collagen in regenerated tissue in the wound environment were analyzed within 1 week in order to further investigate the impact of ADM-MSC in skin wound healing. The imaging process was conducted as outlined in Fig. 6A. Intravital imaging of the ADM-MSC grafts (Fig. 6B) revealed that the MSCs seeded in the ADM migrated from the scaffolds (the layer at a depth 120 μm) to the regenerated tissue in the bottom (the layer at a depth 20 μm). The numbers of live MSCs within different areas were determined by GFP fluorescence intensity. As shown in Fig. 6C, the number of cells gradually decreased in the 120 μm layer and no cells were observed after 5 days. In the 20 μm layer, the cell number increased. The cell number in the middle and border areas was also analyzed at 5 and 7 days. The numbers of cells in these two areas were approximately the same on day 5 (Fig. 6D). However, on day 7 there were more cells in the border areas than in the middle areas. The results above indicate that, after being grafted to the wounds, the MSCs moved to the regenerated tissue from the ADM-MSC scaffolds and they survived in the regenerated tissue for at least one week, especially in the periphery of lesions.

On day 1 after the treatment, degradation of ADM scaffolds (120 μm layer) was observed in both the ADM-MSC and ADM groups compared to an ungrafted ADM scaffold (Fig. 6E). Also, the degradation of collagen fibers was more significant in the ADM-MSC group than in the ADM group. By day 3, the scaffolds in the ADM group tended to be degraded more than on day 1 and the ADM scaffolds were hardly detectable in the ADM-MSC group.

As shown in Fig. 6F and 1 day after grafting in the ADM-MSC group, the collagen network appeared in the regeneration bottom layer and was only detected in the ADM group 5 days after treatment (Data was not shown). On day 7, the regenerated collagen network was close to that in normal skin tissue in morphology. Fig. 6E revealed that the regeneration of collagen networks occurred earlier and more completely in the ADM-MSC group compared to the ADM group.
3.7. Evaluation of skin regeneration

Regenerated skin structure was analyzed by dermal differentiation, epithelial maturation and skin appendage regeneration \((n = 10)\). The scores of these groups imply that ADM-MSC promoted significant skin maturation. The ADM-MSC wounds had defined dermal structures and mature epithelial structures with sebaceous glands and hair follicles. As shown in Fig. 7A, the histological scores of the ADM-MSC group at 21 days were significantly higher than both the ADM and the control groups \((p < 0.05)\). Furthermore, when the treatment was extended to 35 days, ADM-MSC promoted complete skin regeneration and integration into normal skin, with no wound contraction. However, visible scars and contraction of lesions were found in both ADM and control groups at 35 days and the regenerated skin was thin and frail in the control group (Fig. 7B).

4. Discussion

Various strategies, such as epidermal replacement, engineered dermal constructs and engineered skin substitutes, have been developed to use as skin replacements for wound management [25]. ADM is a dermal substitute that retains the native structure of dermis. Complete removal of cellular components reduces the immunogenicity of allogeneic and xenogeneic biologic scaffolds, and may facilitate tissue remodeling [26]. Alternatively, MSCs have been an attractive cell source for regenerative medicine for treatment of skin injuries. Successfully using MSCs for skin regeneration requires a sufficient quantity of MSCs and effective delivery of the MSCs to the wound site. Theoretically, ADM can serve as a scaffold for the delivery of MSCs to the target wound, while the MSCs promote retention and neovascularization of the scaffold [27].

In this study, we demonstrated that MSCs could attach \textit{in vitro} to ADM prepared from male BALB/c mice. Previous research has established that burns exhibit minimal scar formation if they are primarily healed in less than 21 days whereas, if healing is incomplete at 21 days, satisfactory regeneration is unlikely [28]. In the current study, at 21 days after treatment, wound closure and re-epithelialization were enhanced and vascular density around the wounds were higher in the ADM-MSC group compared with the ADM and control groups. Furthermore, full-thickness excisional wounds were improved and repaired with allogeneic ADM graft.
treatment and the improvement and repair was even more significant, and included complete skin appendage regeneration, when wounds were treated with MSC-seeded ADM grafts. Another consideration for wound repair is the formation of scars, which are caused by deposition of excess ECM by fibroblasts in the wound bed. These structures carry a variety of undesirable consequences, including unsightly appearance on the skin. In addition, scars lack much of the normal makeup of the skin, such as follicles and nerve endings and also do not retain the normal tensile strength of undamaged skin [29]. Excess ECM synthesis by fibroblasts is caused by insufficient dermal components in the wound bed. The utilization of ADM scaffolds can provide a sufficient amount of dermal tissue to reduce scar formation and contraction in wound healing. MSC-based cell therapy, in combination with matrix scaffolds, has become a potential strategy to improve wound healing outcomes [30]. A similar study was done by Anlun M. et al., in which the transplantation of an ADM-MSC scaffold contributed to the healing process of cartilage defects in a monkey model [31].

Revascularization of the wound bed is a crucial stage during the normal wound healing process. New vessels form and develop in the granulation tissue to supply oxygen and nutrients to the wound area. Angiogenesis is a complex process that is controlled by the balance of proangiogenic and antiangiogenic factors [32]. MSCs play an important role in this process and endogenous MSCs are mobilized and recruited to the wound bed [33]. In the current study, both the exogenous MSCs and endogenous endothelial cells were recruited to the regenerated tissue in the ADM-MSC group at 7 days post-treatment. Also, the density of stable and functional vessels was higher in the newly formed dermal layers in ADM-MSC-treated wounds after 14 days, suggesting that exogenous MSCs can promote angiogenesis. Relative studies have demonstrated that mesenchymal cell type is necessary to establish a mature vascular network either in vitro [34] or in vivo [35]. Other research has suggested that the improved MSC-mediated angiogenesis is partially related to the release of proangiogenic factors [36], such as VEGF-α and Ang-1, which play key roles in angiogenesis by stimulating endothelial cell proliferation, migration, and

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**Fig. 5.** Effects of ADM-MSC on re-epithelialization. In control, ADM and ADM-MSC groups, confocal observation of wound sections, immunostained with K-10, are shown on day 7 (A, C and E) and day 14 (B, D and F) after treatment. These results revealed that ADM-MSC contributed to re-epithelialization. Nuclei were stained with DAPI. Scale bar, 50 μm.

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**Fig. 6.** The activity of MSCs and ECM in the wound sections. (A) Diagram of the intravital imaging procedure of ADM-MSC grafts. (a) The wound model was made and the ADM-MSC graft was implanted. (b) Random wound areas were chosen and collected in border and middle areas. (c) Tissue samples were scanned from the regenerated bottom layer to the scaffold layer along the z axis. (B) TPF/SHG 3D scanning images of living wound tissue harvested from ADM-MSC groups, 1, 3, 5, and 7 days after treatment. (C) Quantification of cell numbers on a 20 μm layer and a 120 μm layer of each selected section based on (B). Values are relative to the fluorescent intensities of the MSCs on 20 μm layer after 7 days of treatment. (D) The percentages of MSCs in middle and border areas at 5 and 7 days. (E) Degradation of ADM scaffolds in the junction areas in 2 groups on days 1 and 3 after treatment. (F) Regeneration of the collagen network in newly formed tissue. The results proved that, after implantation, the MSCs tended to move to the newly formed tissue from the ADM-MSC scaffolds. The degradation and regeneration of the ECM around the junction area occurred earlier in ADM-MSC group compared to ADM group. Red-SHG of collagen, Green-GFP of MSCs. Scale bar, 100 μm (E), 50 μm (F). Values expressed as means ± SD.
organization into tubules. In our model, further experiments are needed to determine whether the exogenous MSCs promoted angiogenesis by secreting these proangiogenic factors or by differentiating into pericytes to facilitate vessel maturation.

Dermal tissue undergoes dynamic changes during wound healing. These changes have been extensively investigated via histological methods in fixed skin biopsy specimens. However, such ex vivo assessment methods have often limited utility since only
thin tissue slices stained with extrinsic dyes can be analyzed. The development of methods that detect intrinsic signals in minimally processed tissues will greatly improve our understanding of the skin conditions and diseases [37,38]. Recently, the collagen-sensitive SHG microscopic imaging technique has been widely used for ECM imaging in the biomedicine field, even for the in vivo assessment of collagen structure changes during human skin aging [39,40]. This method provides unique imaging characteristics, such as high image contrast, high spatial resolution, optical 3D sectioning, minimal invasiveness, deep penetration and no interference from background light. Most importantly, the tissue can be completely sectioned, minimal invasiveness, deep penetration and no interference from background light. Most importantly, the tissue can be complete skin regeneration. Values shown are means ± SD. *p < 0.05, **p < 0.01.

Fig. 7. Evaluation of regenerated skin tissue. (A) Histological analysis scores of wounds in terms of dermal differentiation and epithelial and skin appendage regeneration according to the description in the Materials section. The histological scores of the ADM-MSC group were much higher at 21 days. (B) Photos of wound healing in the 3 groups, 5 weeks after grafting treatment, indicated that ADM-MSC contributed to complete skin regeneration. Values shown are means ± SD. *p < 0.05, **p < 0.01.

In conclusion, the current study clearly demonstrated that MSC-seeded ADM scaffolds promoted significant neovascularization, ECM remodeling and complete skin regeneration and have great potential as a unique device for superior clinical treatment of full-thickness skin wounds. Furthermore, the nonlinear optical imaging technique used in this study provided an additional method for assessment of wound healing.

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References


5. Conclusion

In conclusion, the current study clearly demonstrated that MSC-seeded ADM scaffolds, although further experiments are needed to confirm this connection.

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.03.011.


