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Mesenchymal stromal cells promote the proliferation of basal stem cells and efficient epithelization in organotypic models of wound healing

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Abstract

Adipose derived mesenchymal stromal cells (ADSCs) represent a fascinating tool in the scenario of wound healing and regenerative medicine. Recent data already demonstrated that ADSCs could exert a stimulatory action on epithelial cells through secretion of soluble factors. The aim of the present study was to assess how ADSCs guide wound re-epithelization in vitro in the presence of keratinocytes. We used an organotypic model of wound healing and we seeded keratinocytes on a ADSC-induced dermal matrix. Conventional hematoxylin–eosin stain and immunohistochemistry staining for Ki67, p63 and pan-keratins were performed at different timepoints. Histological sections of organotypic cultures showed complete coverage of the ADSC-induced matrix by keratinocytes. Proliferation of basal stem cells was found to be the main mechanism responsible for epithelization of the dermis. In conclusion, ADSC do not only stimulate dermal regeneration through collagen deposition but also promote epithelization.

Highlights

- Mesenchymal stromal cells (MSCs) are widely used in regenerative medicine and wound healing.
- MSCs do not only stimulate dermal regeneration through collagen deposition but also promote epithelization.
- MSCs directly target the basal stem cell niche and promote its proliferation, migration and subsequent differentiation.

KEYWORDS
ADSC, epithelization, MSC, stem cell, wound heling

1 | BACKGROUND

Chronic wounds are today a major problem in public health (de Leon et al., 2016; Green et al., 2014). Despite the recent advances in the field of wound healing, current strategies are still not completely satisfactory because of long healing time, relatively high rates of recurrence and poor quality of the newly formed tissue (Bhardwaj et al., 2017). New therapeutic approaches for chronic wounds are urgently needed

and new cell-based strategies have been developed in the last decades (Brett et al., 2017). Mesenchymal stromal cells (MSCs) represent a fascinating tool in tissue engineering for their regenerative and immunomodulatory properties (Samsonraj et al., 2017). MSCs are a subset of multipotent cells present in tissues of mesenchymal origin (Gronthos et al., 2003; Selich et al., 2019; Trubiani et al., 2019). MSCs are characterized by the presence of specific surface markers (Dominici et al., 2006; Viswanathan et al., 2019; Yin et al., 2019) and regenerative properties (Andrzejewska et al., 2019; Han et al., 2017; Pittenger et al., 1999). MSCs interact with both lymphocytes and dendritic cells, modulating cytokine secretion (Börger et al., 2017; Paganelli et al., 2021; Tomic et al., 2011; Zhao et al., 2010; Zhao et al., 2016). Adipose-derived MSCs (ADSCs), in particular, are a subset of MSCs of interest for clinical purposes not only for their relative abundance in the human body but also for the safety of the isolation procedure (Samsonraj et al., 2017; Zuk et al., 2002).

Several works already reported on the efficacy of ADSC-based therapies in wound healing, both in in vivo and in in vitro models (Huayllani et al., 2020). However, only few studies clarify the mechanisms underlying this action and most of them are focused on ADSC-mediated effects on fibroblasts and collagen production (Kim et al., 2007; Paganelli et al., 2020). ADSCs have also been shown to exert a stimulatory action on keratinocytes (Paganelli et al., 2019). The pro-epithelizing properties of MSCs seem to be mainly mediated by the secretion of soluble factors (Kim et al., 2018). Several works hypothesized that ADSCs could directly differentiate toward a keratinocyte phenotype (Dos Santos et al., 2019). Anyway, to what extent this is likely to happen in the presence of epithelial precursors remains unclear, since basal and hair follicle stem cells seem to be the main responsible for re-epithelization in the physiological wound healing process (Yang et al., 2020).

The aim of the present study was to assess how ADSCs guide wound re-epithelization in vitro in the presence of keratinocytes. In particular, our goal was to check basal stem keratinocyte proliferation was responsible for wound closure in the presence of ADSCs. For this purpose, we used an organotypic model of wound healing with keratinocytes being seeded on an ADSC-induced dermal scaffold.

2 | EXPERIMENTAL DESIGN

2.1 | Cell isolation and organotypic cultures

In line with previously published protocols, cells were obtained using both enzymatic and mechanical digestion of fat tissue (Paganelli et al., 2019). Once isolated, the stromal-vascular fraction (SVF) was plated into 75 cm^2 flasks in culture medium enriched with 10 ng/ml basic fibroblast growth factor (bFGF; Sigma-Aldrich, USA), defined as stromal medium. When primary cultures reached 70%–80% confluence, cells were re-plated into 75 cm^2 culture flasks for at least 4–5 passages to obtain an ADSC-enriched population (see Supporting Information 1 and 2). Afterwards, cells were plated in culture medium enriched with 250 μM Ascorbic acid (defined as extracellular-matrix inducing medium) in a 24-well plate (15.000 cells/cm2). Fresh ECM-inducing medium was replaced every 2–4 days for a variable period ranging from 3 to 5 weeks. After this period, the matrix was collected in a Transwell insert (Costar, USA).

Keratinocytes were cultured in keratinocyte growth medium, composed of Dulbecco's Modified Eagle's Medium/Ham's F12 Medium (DMEM/F12, 3:1) (Seromed-Biochrom KG, Berlin, Germany) containing insulin (5 μg/ml, Sigma), transferrin (5 μg/ml, Sigma), triiodothyronine (2 nM, Sigma), hydrocortisone (0.4 μg/ml, Sigma), adenine (180 mM, Sigma), mouse epidermal growth factors (EGF, 10 ng/ml, Sigma) 10% fetal calf serum (FCS, Serumed Biochrom) and 1.8 mM calcium.

ADSC-derived matrices were placed on polycarbonate membranes (0.4 μm pore size, Corning, USA) in transwell permeable insert. Then, keratinocytes were seeded (3 x 10^4 for each well), leaving the insert at the air-liquid interface. Keratinocyte growth medium (KGM) was left at the bottom of the well and changed every 2 days.

2.2 | Immunohistochemistry

ADSC-keratinocyte co-cultures underwent conventional histochemical staining. Cell cultures were harvested, fixed in formalin and paraffin embedded. Several 4 μm-thick sections were obtained using a microtome (Leitz, Germany), stained with conventional hematoxylin and eosin (HE), Periodic Acid Schiff (PAS), C. Masson's Trichrome (MT) and evaluated at the microscope (Axioskope 40, Zeiss, Germany). Images were captured by a digital camera (Nikon Corp., Tokyo, Japan).

Several 4 μm-thick sections from previously formalin fixed and paraffin embedded organotypic-culture samples at day 7 and 14 of co-culture were obtained using a microtome (Leitz, Germany). After deparaffinization, rehydration and antigen retrieval, slides were put in citrate buffer pH 6 at 98°C for 30 min, and then washed in PBS. Endogenous peroxidase was neutralized through a 5-min incubation with H2O2 0.3% and subsequent washing in PBS. Goat serum was used to block non-specific interactions. Afterwards, slides were incubated with anti-p63 and anti-Pan Keratin (AE1/AE3/PCK26) Primary Antibody (Ventana, Tucson, Arizona) for 30 min at 37°C. After another cycle of incubation with secondary-antibodies, 3,3′-diaminobenzidine (DAB, Ventana, Tucson, Arizona) revealed the presence of the antigens (p63 and keratin). Sections were then washed in PBS and counterstained with Hematoxylin. The same procedure was also performed on sections at day 2 and 4 for Ki67 IHC (immunohistochemical) staining, using anti-Ki67 primary antibodies (Ventana, Tucson, Arizona).

3 | RESULTS

Histological sections of organotypic cultures showed complete coverage of the ADSC-induced matrix by keratinocytes starting very early after co-culturing (Figure 1A).

After only 2 days of culture, the presence of ki67-positive keratinocytes confirmed active proliferation of epithelial cells on the

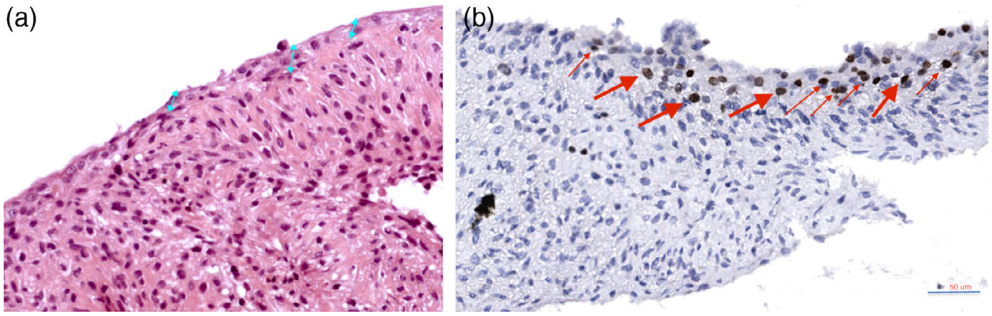

FIGURE 1 Epithelization in organotypic models of wound healing. (a) Hematoxylin and eosin (HE) stain performed at day 2 of co-culturing; the epidermal layer is indicated with blue arrows. (b) Immunohistochemistry performed after only 2 days of culture: The presence of ki67-positive cells (black nuclei, indicated with red arrows) reveals and confirms active proliferation of keratinocytes

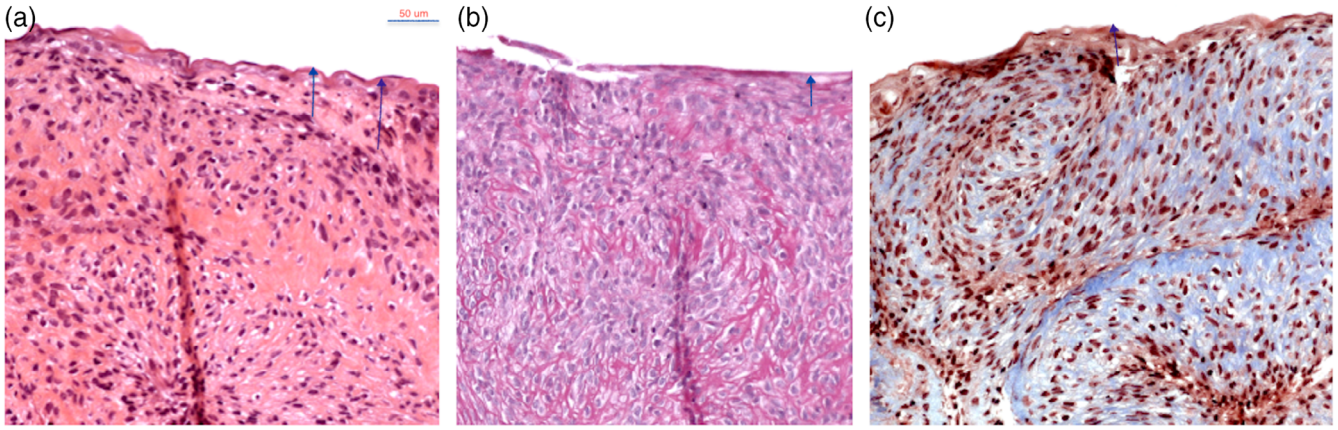

FIGURE 2 Histological sections of organotypic cultures at day 7, showing complete coverage of the ADSC-induced matrix by keratinocytes (dark blue arrows indicate epithelial coverage). Cells were stained with conventional (A) hematoxylin and eosin (HE), (B) periodic acid Schiff (PAS), C. Masson's Trichrome (MT). In particular, PAS stains for the detection of proteoglycans (purple-magenta color), while MT reveals collagen fibers (green/blue color; nuclei are colored in brown)

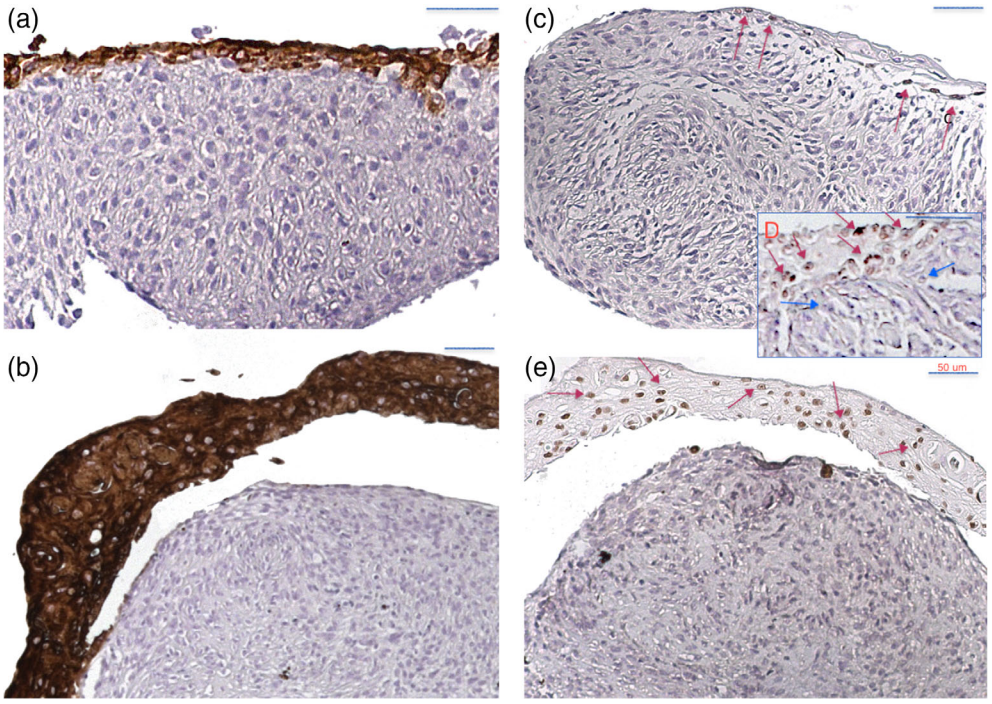

FIGURE 3 Immunohisto chemical stains for cytokeratins and p63 on organotypic cultures of ADSCs and human keratinocytes. (A and B) IHC stain for Pan-keratin (in brown) and (C–E) p63 (brown nuclei, red arrows) performed after 2 (A–C) and 14 (B–E) days. Panel D shows at higher magnification p63 positive nuclei (red arrows) and normal hematoxylin-stained purple nuclei (blue arrows) after 10 days of co-culturing

surface of the ADSC-based scaffolding material, supporting the pro-epithelizing properties of ADSCs (Figure 1B).

In line with previously obtained data (Paganelli et al., 2019), epithelization occurred in few days and the dermal and epidermal portions could be easily distinguished even with conventional histology colorations after only 1 week of co-culturing (Figure 2).

Pan-keratin staining confirmed the epithelial nature of the proliferating keratinocytes at the air-liquid interface (Figure 3A–C).

In particular, the positivity for p63 in the epidermis highlighted the presence of basal stem cells. In fact, while Ki67 indicates active cellular proliferation in general, p63 is a reliable keratinocyte stem cell marker (Figure 3C,D).

These findings confirmed re-epithelization in the presence of ADSCs to occur through active proliferation of basal stem cells.

4 | DISCUSSION AND CONCLUSIONS

In this study keratinocytes were grown on an ADSC-induced matrix, maintained at an air–liquid interface, with the aim of reproducing an in vitro model of ADSC-induced wound healing (Collawn et al., 2012).

ADSCs have already been demonstrated to be capable of secreting collagen, fibronectin and other components of the extracellular matrix (Paganelli et al., 2019) that can act as integrin receptors and promote cell motility in the provisional matrix (DiPersio et al., 2016; Widgerow, 2013). Therefore, not only ADSC synthetize themselves their scaffolding material, but are also potentially capable of giving rise to proto-tissues. Moreover, ADSC-induced dermal scaffolds have already been demonstrated to be efficiently seeded with human keratinocytes, with concomitant deposition of collagen IV at the dermal-epidermal junction and basal membrane formation (Paganelli et al., 2019).

ADSC have been proven to accelerate healing, mainly through the production of soluble factors (Collawn et al., 2016; Luo, Yi, Liang, et al, 2019). Not only ADSCs promote fibroblast proliferation, collagen secretion and wound bed vascularization, but also modulate keratinocytes through the secretion of growth factors and cytokines, such as HGF, FGF-1, G-CSF, GM-CSF, IL-6, VEGF, and TGF-β3 (Barrientos et al., 2008; Barrientos et al., 2014; English et al., 2009; Kim et al., 2009; Moon et al., 2012). ADSC secretome is currently thought to promote cell migration and proliferation in the skin through activating Wnt/β-catenin signaling during the wound healing process (He et al., 2020; Ma et al., 2019). Moreover, ADSC conditioned medium possess anti-oxidative properties, as also shown by several studies demonstrating its protective effects in UVB-induced skin aging (Li et al., 2019). Given the importance of secretory molecules produced by MSCs, soluble factors and extracellular vesicles from MSC conditioned medium are currently under investigation as cell-free therapeutic strategies for cutaneous wound healing (Casado-Díaz et al., 2020; Wu et al., 2018).

Our data successfully demonstrate that ADSC not only stimulate dermal regeneration through collagen deposition but also promote epithelization. Moreover, the presented in vitro model of ADSC-mediated wound healing clearly shows that epithelization of ADSC-induced matrix occurs through migration and proliferation of basal stem cells, as underlined by IHC staining.

The present paper gives new insights in the mechanisms underlying the use of MSCs in wound healing, showing that MSC stimulate epithelization directly targeting the basal stem cell niche and promoting its proliferation and subsequent differentiation.

We confirm the key role of ADSCs as a possible cell-based strategy in regenerative medicine. However, despite the promising data achieved in this setting, further research aimed at comparing alternative strategies in terms of both safety and efficacy is needed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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