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# Stem cells derived from burned skin - The future of burn care

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## ARTICLE INFO

## ABSTRACT

*Background:* Thermal injuries affect millions of adults and children worldwide and are associated with high morbidity and mortality. The key determinant for the survival of burns is rapid wound healing. Large wounds exceed intrinsic wound-healing capacities, and the currently available coverage materials are insufficient due to lack of cellularity, availability or immunological rejection.

*Methods:* Using the surgically debrided tissue, we isolated viable cells from burned skin. The isolated cells cultured in tissue culture dishes and characterized.

*Findings:* We report here that debrided burned skin, which is routinely excised from patients and otherwise considered medical waste and unconsciously discarded, contains viable, undamaged cells which show characteristics of mesenchymal skin stem cells. Those cells can be extracted, characterized, expanded, and incorporated into created epidermal-dermal substitutes to promote wound healing in immune-compromised mice and Yorkshire pigs without adverse side effects.

*Interpretation:* These findings are of paramount importance and provide an ideal cell source for autologous skin regeneration. Furthermore, this study highlights that skin contains progenitor cells resistant to thermal stress.

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## 1. Introduction

Thermal injuries are one of the most devastating and lethal traumata a patient can incur, outranking the combined incidence of HIV and tuberculosis with over 11 million people requiring medical attention and 265,000 fatalities per annum [1,2]. During the recent decades, many advances have improved the outcomes of burn patients. However, the single most important factor in determining survival or death of a burn patient is wound coverage and wound healing [3,4]. The current standard of care generally requires burn wounds excision within 72 hours post-burn injury. This early excision is not only critical for survival but also reduces the source of inflammatory stimuli and attenuates detrimental systemic reactions including hypermetabolic responses [5,6], as well as pathologic local responses, e.g., keloid formation or hypertrophic scarring [1,7–11]. Autologous skin grafting is considered the gold standard for wound coverage after the removal of the burned skin [12–15]. However, harvesting autologous skin is invasive, creates a new wound in a healthy skin area, and it bears the risk for wound complications such as pain, scarring, and delayed healing [1,10,11,16–18]. In addition, the larger the burn, the less healthy skin remains for autologous skin grafting that is limiting its availability for grafts. These complications are indicative of the need for alternative wound coverage materials [13,14,19,20]. However, to date, no such

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## Research in context

### Evidence before this study

Advances in resuscitation, wound healing, pulmonary support, and infection have improved post-burn outcomes, but a severe burn is still associated with significant morbidity and mortality. While autografting is the surgical gold standard for this patients, lack of intact skin prohibits a successful and complete autografting when there is a large burn size.

### Added value of this study

We isolated cells from a dermal component of discarded burned skin. This tissue is usually considered "waste tissue." The isolated cells are viable and show characteristics of human mesenchymal stem cells (MSCs). Formation of biomaterial sheets using these cells and application of them onto excisional wounds of immune-incompetent mice as well as porcine models demonstrate that the Bd- MSCs facilitate healing and decrease healing time. Bd-MSCs presents numerous advantages when compared to other sources of MSCs. It does not raise the ethical issues that act as an obstacle to embryonic or cadaverous stem cell extraction. Almost no patient refuses to donate these discarded tissues. Moreover, cell isolation from the burned skin is a non-invasive procedure for the patient since removing burned skin is part of the routine standard of care for burned patients. Furthermore, since they are the patient's own stem cells, the chance of immunological reaction and rejection is substantially low. These findings are of paramount importance to the burned patients since quick; permanent wound closure is essential in the management of burned patients.

### Implications of all the available evidence

New advances in tissue engineering using composite scaffold fabricated from natural and synthetic biomaterials together with advances in 3D bioprinting provide the promising 3D microenvironment for manufacturing skin substitutes using patients' own burned BD-MSCs.

feasible coverage material exists [21–24]. Current materials are either ineffective, cause immunologic rejections, take too long to produce sufficient cell numbers, are too expensive, or are acellular [13,24].

The skin contains stem cells in a very complex structure with complex function [25,26]. While an array of stem cells have been described for the epithelial layer of the skin, little is known about dermal reconstitution during skin healing [27–32]. It is reported that fibroblast-like cells from surrounding intact cells as well as recruited mesenchymal progenitor cells are contributing to the reconstitution of dermis [33–36]. Moreover, recent data is showing that myeloid lineage cells may directly convert into the fibroblast-like cells and contribute in dermis reconstitution [37,38]. After injury, these cells migrate towards the wound bed and form a new tissue, considered granulation tissue, which is essential for wound healing since it fills the wound gap and provides a scaffold for epithelialization [29,32] and neovascularization [33,39]. Nevertheless, the final phenotype of cells in granulation tissue milieu is mesenchymal and that suggests mesenchymal stem cell therapy as a promising treatment for the management of deficient or complex wounds. Various comparative preclinical studies using different animal models of skin healing support this notion [30,32,40–46]. However, none of the current stem cell sources (e.g., bone-marrow, adipose and umbilical cord) have yet to be clinically relevant due to inherent limitations such as lack of

availability, ethical concerns, need for invasive extraction methods and the risk of immunologic rejection for allogenic sources. Moreover, the identity of skin fibroblast-like cells is unique, yet heterogenous [47]. This raises the possibility that skin mesenchymal cells might be the ideal cells for dermis reconstitution in compare with other sources of mesenchymal stem cells.

Here, we report that severely burned skin (full-thickness burn, third-degree burn, damage extended to the epidermis and entire dermis), which is routinely excised and discarded and considered as medical waste - contains viable MSC (burn-derived mesenchymal stem cells, BD-MSCs) that can be used for skin regeneration and wound healing. We extracted, characterized, and expanded those cells in vitro, incorporated them into an established wound coverage material, routinely used in the clinic. We then applied the cellular coverage material onto excisional wounds in immune-incompetent mice as well as immune-competent pigs. Our data demonstrate that BD- MSCs facilitates healing and decreases healing time. The thermally injured skin provides an ideal source of mesenchymal stem cells for regenerative medicine and is of paramount importance to burn patients.

## 2. Materials and methods

### 2.1. Tissue sampling

After surgical prep and cleaning, the surgically debrided tissue was added to the sterile containers, wrapped carefully and transferred to the research laboratory for further analysis.

### 2.2. Cell extraction and tissue culture

Burned derived-MSCs were extracted using two different methods that both were able to successfully isolate viable mesenchymal stem cells out of burned tissue. We used either an enzymatic cell extraction method, for which burned skin was homogenized and incubated in collagenase I (Supplement Text 1) or a conventional extraction method: First we washed the tissue in PBS with 1% and 2% Ab/Am. Afterward, we cut the tissue into 1 cm wide squares and placed in DMEM growth medium made up of High Glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% Ab/Am. Before placing, a few scratches were made on the dermal surface of the skin using a surgical scalpel. That scratches enhanced the number of isolated cells. The medium was changed every 2–3 days. When cells began to grow out from the tissue, the tissue was removed, and adherent cells continued to grow.

Cells extracted and cultured from both methods were treated the same. Upon reaching 80% confluency, cells were resuspended in 0.05% trypsin-EDTA for a total of one or two passages, depending on the sample. Cells were kept frozen in liquid nitrogen until the beginning of the experiment.

### 2.3. Flow cytometry assay

Flow cytometry was performed on BD-MSCs using markers for MSCs (Negative markers: CD34, CD45, and positive markers: CD73, and CD105) by using a BD LSR II Flow Cytometer. Live cells were selected and the CD34-/CD45- population, was gated for CD73+/CD105+. Antibodies used were CD34 FITC (Invitrogen), CD45 APC/cy7 (Biolegend), CD73 PE (eBioscience) and CD105 APC (eBioscience).

### 2.4. Colony forming unit-assay

In 6-well plates BD-MSCs and passage 8 of normal human fibroblasts were seeded in duplicates at three different cell concentrations (100, 500 and 1000 cells/100 mm). Cells were cultured for two weeks with one change of growth medium. They were stained with 0.5% Crystal Violet (Sigma) in methanol for 15 min at room temperature. They were

washed twice with PBS then imaged for quantification. The number of colonies and the number of colonies larger than 3 mm in diameter were counted.

### 2.5. In vitro differentiation

Adipogenic differentiation: Cells were seeded in 24 well plates with a 6000 cells/well concentration. Adipogenic cells were cultured in low glucose DMEM supplemented with 10% FBS, 1% Ab/Am, 1 mM of sodium pyruvate, 0.1 mM of ascorbic acid-2-phosphate, 1% insulin-transferrin-selenium, 100 nM of dexamethasone and 10 ng/mL of TGF-β3. Control fibroblasts and burn derived MSCs were grown in low glucose DMEM growth medium Cells were placed in an incubator at 37 °C in 5% $CO_2$ for 14 days. The medium was changed twice weekly.

Osteogenic differentiation: Cells were seeded in 24 well plates with a 6000 cells/well concentration. Osteogenic cells were cultured in low glucose DMEM supplemented with 10% FBS, 1% Ab/Am, 0.05 mM ascorbic acid-2-phosphate, 10 mM β-glycerophosphate and 100 nM dexamethasone. Control cells were cultured in DMEM growth medium for fibroblast and burn derived MSCs. Cells were placed in an incubator at 37 °C in 5% $CO_2$ for 21 days. The medium was changed twice weekly.

Chondrogenic differentiation: Cells were seeded at a density of 200,000 cells per 15 ml falcon tube. Chondrogenic pellets were covered with 0.5 mL of low glucose DMEM supplemented with 10% FBS, 1% Ab/Am, 1 mM of 3-isobutyl-1-methylxanthine, 10 μg/mL of insulin, 60 μM of indomethacin and 1 μM of dexamethasone. Control fibroblast and burn derived MSC pellets were covered with 0.5 mL of DMEM growth medium. Cells were placed in an incubator at 37 °C in 5% $CO_2$ for 35 days. The medium was changed three times weekly, being careful not to disrupt cell pellet. After 35 days of chondrogenic differentiation, cell pellets were removed from the 15 mL falcon tubes and placed in 10% formalin for 24 h then placed in 70% ethanol for an additional 24 h. Aggregates were afterward embedded in paraffin, cut into 5 μm slices and placed on microscope slides.

### 2.6. Differentiation staining

Oil Red O staining: After two weeks of adipogenic differentiation, the medium was removed, and wells were rinsed with PBS. Cells were then fixed in 10% formalin for 30 min, rinsed with distilled water and stained with Oil Red O for 5 min (Sigma-Aldrich). Following multiple rinses with water, cells were stained with hematoxylin (Sigma). Intracytoplasmic lipid droplets appear in red and nuclei in dark blue.

Alizarin red staining: After three weeks of osteogenic differentiation, the medium was removed, and wells were rinsed with PBS. Cells were then fixed in 10% formalin for 30 min, rinsed with distilled water and stained with Alizarin red (Sigma-Aldrich) in the dark for 45 min. Cells were washed with distilled water prior to imaging. Calcium deposits appear in red.

Alcian Blue Staining: For chondrogenic samples, the paraffin-embedded slides were deparaffinized with citrosol and rehydrated through graded ethanol to water. Slides were incubated in 1% alcian blue 3GX (Santa Cruz Biotechnology) in 3% acetic acid in water for 30 min at RT. The stain was washed with tap water then distilled water then counterstained with 0.1% nuclear fast red (Santa Cruz Biotechnology). Slides were washed for 1 min in tap water then dehydrated through increasing grades of ethanol, cleared in citrosol and mounted with the xylene-based mounting medium.

Immunofluorescent adipogenic cell culture staining: Samples were then fixed in 4% paraformaldehyde, permeabilized with 0.25% Triton X-100 and incubated with anti-human rabbit perilipin antibody (Cell Signalling). Samples were afterward incubated with a secondary anti-rabbit biotinylated antibody then DyLight 649 streptavidin (Vector Labs).

### 2.7. Control group, scaffold

Our used control is the current gold standard in burn care, a meshed acellular bilayer scaffold consisting of bovine collagen with a removable silicon layer (Integra©), introduced in 1980.

### 2.8. In vivo experiments - mice

Ten 6–8 week-old nude mice (Jackson Laboratories) were used in this experiment. This experiment was reviewed by the ethics committee and approved (AUP #: 15-503). Five mice randomly allocated to the control group and five in the treatment group. All mice were placed under isoflurane anesthetic and received two 6 mm full-thickness punch wounds on their mid back. Each wound was surrounded with a silicone ring (sutured tightly) to prevent wound healing through skin contraction. Control wounds received 100 μl of Matrigel only, and treatment wounds received the same volume of Matrigel containing 110,000 BD-MSCs/wound. Matrigel was of high concentration and was applied dropwise in liquid form and then allowed to gel. The wound bed and silicone ring were covered with Tegaderm® transparent film dressing which adhered to the surrounding intact skin. Mice were monitored for twelve days on a daily basis then sacrificed. Mice were injected with 33.3 mg/kg BrdU in PBS (5.5% DMSO) 8 h prior to sacrifice to detect cell proliferation.

Skin histology: After 12 days, the scar area with surrounding normal skin was removed, fixed in 10% formalin and embedded in paraffin. Briefly, we fixed the tissue specimens in 10% buffered formalin overnight at room temperature, preserved in 70% ethanol and embedded in paraffin. Specimens were cut into 5 μm sections. A serial section of the scar or healing wound was performed. The largest wound diameter or central wound section was chosen for trichrome staining and the adjacent sections were used for other Immunohistochemistry stainings.

Trichrome staining: Paraffin-embedded slides were deparaffinized through citrosol and rehydrated through grades of ethanol for staining. Staining solutions were from Electron Microscopy Sciences unless otherwise stated. Samples were kept for one hour in Bouin's solution at 56 °C then consecutively stained in Weigert's Iron Hematoxylin Working Solution (Sigma-Aldrich), Biebrich scarlet-acid Fuchsin solution, phosphomolybdic-phosphotungstic acid solution, and aniline blue solution. Collagen appears in blue, nuclei in black and muscle, cytoplasm and keratin in red. Slides were observed by light microscope LeicaDM 2000 LED. The average wound size was calculated by measuring the length of the wound bed which is evident by a lack of dark blue collagen staining and thicker keratinization at the edges. The keratinocyte thickness was calculated by measuring the thickness of the red keratinocyte layer at the middle of the wound.

Immunohistochemistry: Skin wound slides were deparaffinized and rehydrated for staining and incubated in antigen decloaker (Biocare) in a pressure cooker at 110 °C for 4 min for antigen retrieval. Samples were blocked with 3% $H_2O_2$ for 10 min before antibody incubation for 1 h. Ki67 and BrdU antibodies (Cell Signaling) were used for detection of these proteins, as well as CD31 and CD11b in the porcine model. DNA was denatured with HCl to allow access of the BrdU antigen, thus detecting proliferating cells in the wound area. Antibodies were visualized through HRP polymer detection kits (Biocare) followed by betazoid DAB chromogen kit (Biocare). Slides were counterstained with hematoxylin, dehydrated and mounted with xylene-based mounting medium. Slides were observed by light microscope LeicaDM 2000 LED. Positive cells were then quantified in the dermal area of the wound bed and their density was evaluated. Error bars show the 95% confidence interval and statistical analysis was done using the Student's t-test.

### 2.9. In vivo experiments - swine

Four male Yorkshire pigs (Colwell Farms, Canada) with a weight of 20–30 kg were used. This experiment was reviewed by the ethics

committee and approved (AUP #: 16-600). We compared wound healing in a 5 x 5 cm created full-thickness burn, excising the burned skin wounds, treated with either acellular Integra© ($n = 4$) or with Integra© containing 5.000, 200.000 or 400.000 BD-MSCs/cm$^2$ (each wound, n = 4). Surgical procedures and wound care was adapted from [48]. Supplement Fig. 4 shows a schematic of the experiment and the different wound care modalities used. In this pilot study, we did not have location-matched controls.

BD-MSC incorporation in a control scaffold: Cultured BD-MSCs were trypsinized, counted and resuspended in standard cell culture medium (see above). On a sterile cell culture dish, cells were applied with a 16-channel pipette on top of the bovine collagen of Integra©, with the silicone side facing down. After cell application, scaffolds were incubated at 37 degrees Celsius at 5% CO for 15–20 min, before completely submerging them in medium and stored in a tissue incubator for 12 h. The cell culture medium was washed off for 30 min on a Rocker. Cellularized skin scaffolds were stored in PBS on ice for a maximum of 2 h until application onto the wounds.

Assessment of epithelialization: Photographs of the wounds together with a scale bar were taken intraoperatively with each dressing change. Epithelialization was measured from day 10 (=removal of the silicone layer of Integra©) to day 23 (=first complete closure of a wound by epithelialization). We measured from the hairline to the visible epithelialization line. Measurements were done in Image J Version 1.51 for MAC.

Epithelialized Area: We measured total wound area and non-epithelialized area. The epithelialized wound area was calculated as follows: total wound area - non-epithelialized area.

Epithelialization Speed: To take into account that due to different wound contractions and different mechanical strains all wounds had a slightly different size, we calculated the epithelialization speed between day 10 and day 23 as follows: epithelialized area at day 23/days after removal of silicone.

Vascularization: We measured the neovascularization of absolute vessels, on immunohistological stained microscopy slides under the microscope, using the marker anti-CD31-antibody. Each sample was assessed on three different microscopy sections with the calculated mean, always using the same depth of the measuring point.

Inflammation: In the same way the inflammation was assessed using the anti-CD11b-antibody marker specific for monocytes, macrophages, and granulocytes, with the following counting on each histological sample three times in the same depth from the epidermis into the dermis.

### 2.10. Statistical analysis and graphical representation

Statistical analysis was done via Prism GraphPad Version 5.0a for Mac and Microsoft Excel 2016 for Mac. Two groups were compared with an unpaired t-test, more than two groups with a one-way ANOVA with a post-hoc Tukey test. A p-value < .05 was considered statistically significant.

All graphs are made with Prism GraphPad Version 5.0a for Mac and display mean ± SEM.

## 3. Results

### 3.1. Discarded, severely burned skin contains viable mesenchymal stem cells

We used excised human skin from patients that suffered at least a third-degree burn and evaluated skin of five patients with either scald or flame burns (Supplement Fig. 1). We extracted cells (Protocol in Supplement Text 1) and plated, regardless of the etiology of the burn trauma. 24 h after skin excision and cell extraction, an average number of 16,140 ± 5416 viable cells per cm$^2$ burned skin was attached to the plastic surface of the cell culture flask (Supplement Fig. 1). Using the non-enzymatic method, the cut tissue (~1 cm$^2$) placed in DMEM growth medium. We observed cell outgrowth from the skin into the tissue culture dish regardless of the fact whether they are from the corner of discarded burn skin or the center of discarded skin.

Flow cytometry analysis of freshly isolated live cells showed that the majority of isolated cells are positive CD73 and CD105 while negative for CD34 and CD45, characteristics of mesenchymal stem cells. These cells form colonies in a colony-forming assay (Fig. 1) and could differentiate into the three lineages of mesenchymal cell progeny (adipogenic, chondrogenic, osteogenic differentiation), confirming their multipotent capacity (Fig. 2). Since the viable cells extracted from burned skin meet the definition criteria for mesenchymal stem cells [49], we refer those cells as burn-derived mesenchymal stem cells (BD-MSCs).

### 3.2. BD-MSCs are safe to use

To address the major safety concerns of cell therapy, we assessed the tumor formation potential of BD-MSCs in vitro and in vivo. BD-MSCs did not show any tumorigenic potential in soft agarose cultures nor when injected subcutaneously into immune-incompetent mice within 20d compared to tumorigenic cells (Fig. 3). Moreover, we observed no adverse side effects - neither systemically nor locally in the 30d porcine experiment.

### 3.3. BD-MSCs enhance wound healing in mice

Confirming the pluripotency and safety of BD-MSc, we evaluated the reparative and regenerative potential of these cells in mice. Ten immunodeficient athymic mice were subjected to 4 mm excisional punch biopsy (1 per side, equidistant from the spine) and randomly divided into two group of treatment and control. BD-MSCs embedded in Matrigel to assure cell adhesion to the wound bed, while the control mice only received Matrigel (Fig. 4A). We observed mice over 12 days to allow all wounds to fully close. Mice treated with BD-MSCs visibly displayed faster healing (Fig. 4B). Histological examination of the healed wounds at day 12 post-wounding showed a significantly smaller wound size (Fig. 4C–E), as well as a thinner keratinocyte layer (Fig. 4C, D, F) in the BD-MSC group.

We further evaluated the effect of BD-MSC treatment on wound healing by evaluating the granulation tissue formation and the state of cell proliferation (Fig. 5). Granulation tissue formation and proliferation activity usually pick up at 7 days post wounding in this wound model. At day 12, while the control animals still showed high proliferation activity, mice received BD-MSCs have passed the pick of proliferation phase and showed a lower proliferation profile - characteristic of late proliferation phase and early remodeling phase.

We also stained the BD-MSC treated wounds at day 12 with HLA class 1ABC antibody (Supplement Fig. 2). Our results show that BD-MSCs were still present in the newly formed tissue after complete wound closure and epithelialization. These data show that topical BD-MSC treatment accelerated wound healing, reduced scar formation and did not lead to adverse side effects in mice.

### 3.4. BD-MSCs enhance wound healing in pigs

After determination of the safety and positive effect of BD-MSC treatment in mice, we used a porcine wound-model with 5 × 5 cm excisional wounds. Porcine wounds resemble human wound healing, the closest next to primate-models [50,51].

For higher clinical relevance, we first incorporated BD-MSCs in the meshed bilayer Integra© an FDA-approved, synthetic wound coverage material scaffold considered as gold-standard in burn care [15]. BD-MSCs could be incorporated easily in a straightforward protocol, by pipetting them onto the bovine collagen layer which was later placed directly onto the wound bed (Supplement Fig. 3). The incorporated BD-MSCs displayed complete attachment to the material and a healthy cell morphology 12 h after pipetting.
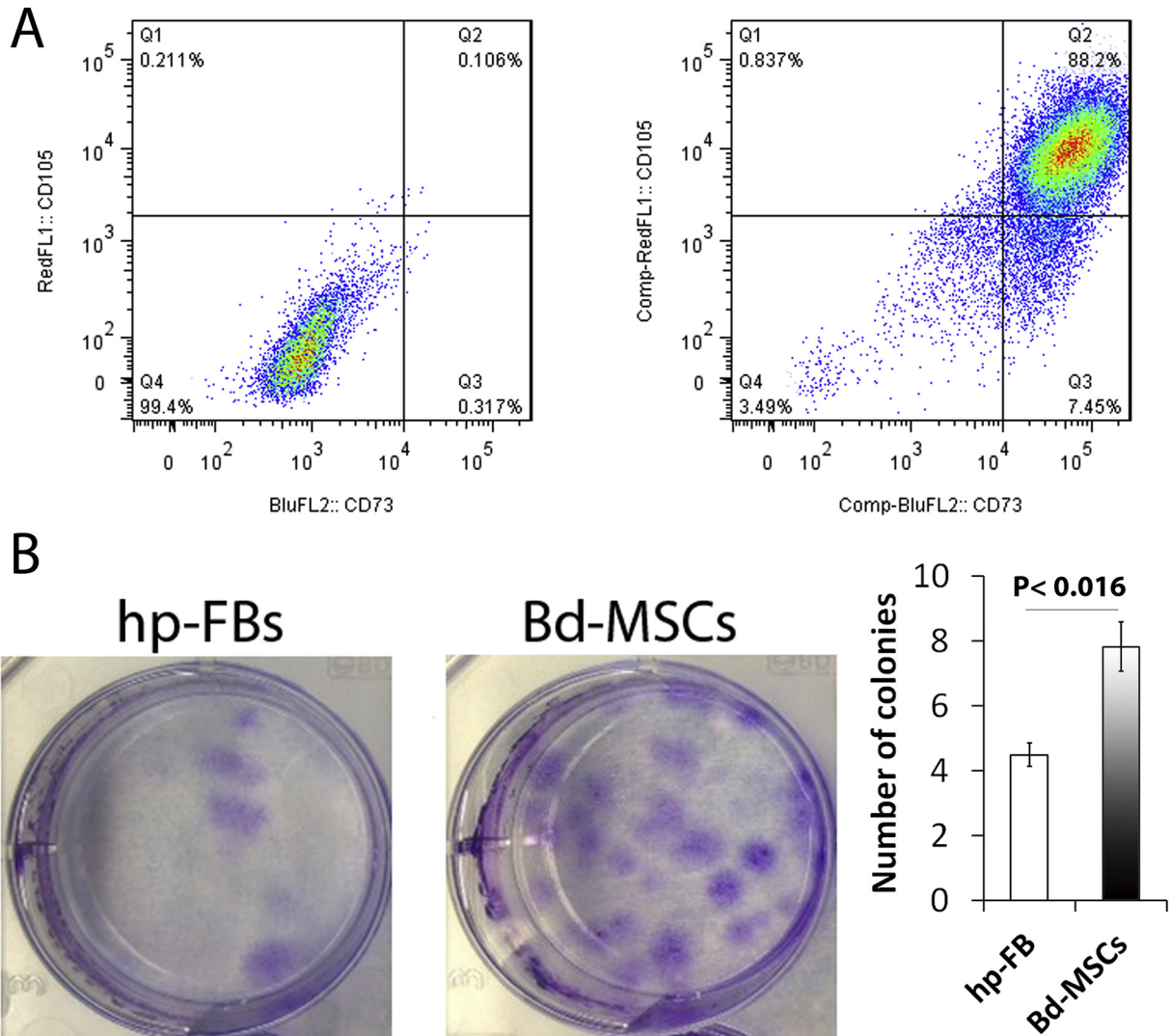
**Fig. 1.** Burn derived dermal cells show characteristics of mesenchymal progenitor cells. Flow cytometry of isolated cells from discarded burned skin is showing that majority of these cells (88%) are positive for mesenchymal markers CD73 and CD105. The left panel is unstained while the right panel is a representative of stained cells. Note that CD34+/CD45+ cells were gated out before analyzing. (B) Colony forming assay shows a significant increase in the number of colonies formed in burn derived cells (BD-MSCs) compared with that formed from high passage fibroblasts (FBs). Graphics show MEAN ± SEM.

We placed the scaffold containing BD-MSC on the $5 \times 5$ cm full-thickness excisional wounds ($n = 4$ for each group) on the back of Yorkshire pigs ($n = 4$) and compared with acellular, conventional Integra© following an established wound care protocol [48] (Supplement Fig. 4).

BD-MSC treatment of excisional wounds in the porcine model showed the pro-wound healing properties of BD-MSCs obtained in the previous murine study without any adverse side effects. Although a robust phenotype was not observed, continuous evaluation of the wounds showed that wounds treated with BD-MSC exhibit an accelerated epithelialization time and a larger re-epithelialized area (Fig. 6). We started to assess the extent of re-epithelialisation after the silicone (bi-) layer of Integra© peeled off (day 10). Beside enhanced re-epithelialization, we observed more number of blood vessels in the dermal component of reconstituted skin, another characteristic of enhanced skin healing (Fig. 7). No safety concern or adverse effect could be detected in wounds treated with BD-MSCs.

## 4. Discussion

We show for the first time that full-thickness burned skin that is usually discarded to avoid further morbidities contains viable mesenchymal skin stem cells. These cells are a readily available source of skin stem cells and can be extracted, expanded in vitro, and used as an adjunctive to existing wound coverage materials in a straightforward and a cost-effective incorporation process. Furthermore, these findings represent a potential new skin stem cell therapy source originating from the injured organ itself. Unfortunately, the current standard of care suggests discarding this invaluable source of stem cells as a medical waste while this unexploited commodity might change a wound care paradigm. We demonstrated the wound healing potential of BD-MSCs in two different animal models mice and swine without any adverse side effects. This is an exciting discovery for stem cell research as well as the burn community since this will change the way we manage and practice stem cell therapy in burns and potentially complex wound care in the future.

BD-MSCs presents with numerous advantages when compared to other sources of MSCs in the current wound healing treatment. They do not raise the ethical issues that represent an obstacle to embryonic or cadaverous stem cell extraction. Almost no patient refuses to donate these discarded tissues making such therapies universally applicable. Furthermore, cell isolation from burned skin is a non-invasive with no added risk to the patient since debridement of affected burned tissues
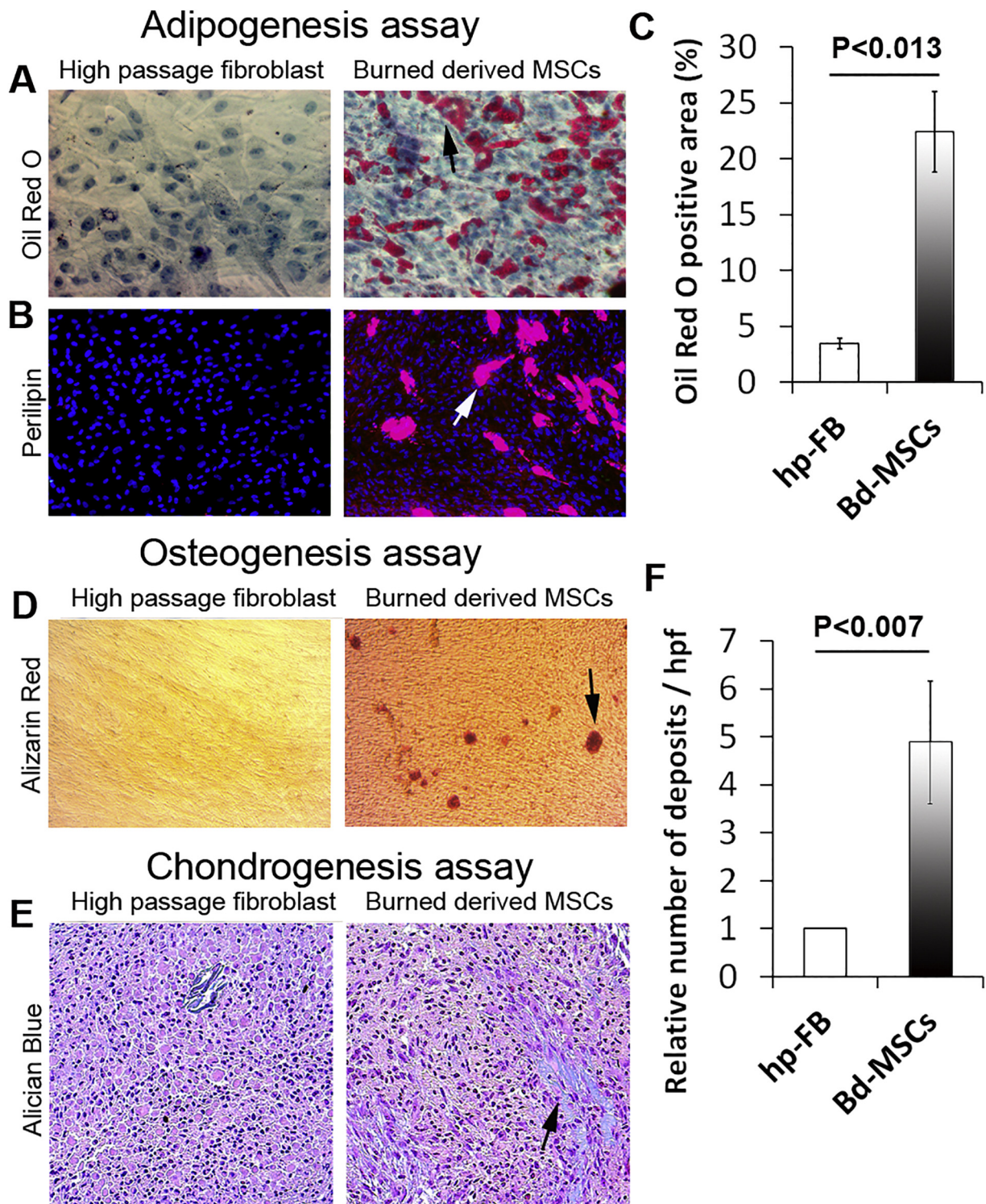
**Fig. 2.** Burn derived dermal cells differentiate into the three lineages of mesenchymal stem cell progeny. (A-B) Oil red O and Perilipin staining show enhanced adipogenesis (fat deposits marked with an arrow) in BB-MSCs compared with high passage fibroblast. (C) Quantification of area stained positive for Oil Red O. (D) Alizarin Red staining shows an increased number of calcium-rich deposit formation (arrow) in BD-MSCs compared with the number of deposits which formed from high passage fibroblasts (FBs). (E) Alcian Blue staining shows enhanced the number and bigger area of alcianophilic regions (arrow) in BD-MSCs cultured in chondrogenic media. (F) Quantification of number of deposits in D. Graphics show MEAN ± SEM.

is part of routine and therapeutic practice. Consequently, the greater the body surface affected by burn injury the increased need for stem cells to regenerate and replace the lost skin and concomitantly, the higher the amount of excised skin is that can be potentially used to extract the BD-MSCs. Moreover, since they are patient's own skin stem cells, the chance of immunological reaction and rejection is negligible. This autologous availability marks a stark difference to adipose tissue, bone marrow MSC, umbilical cord stem cells in terms of harvesting and extraction, which lead to discomfort, pain and a long recovery time as well as fear and ethnically concern, despite an advantageous promotion of wound healing [9,14,19,30,32]. Moreover, through lineage tracing and transplantation assays, it is shown that mesenchymal cells within
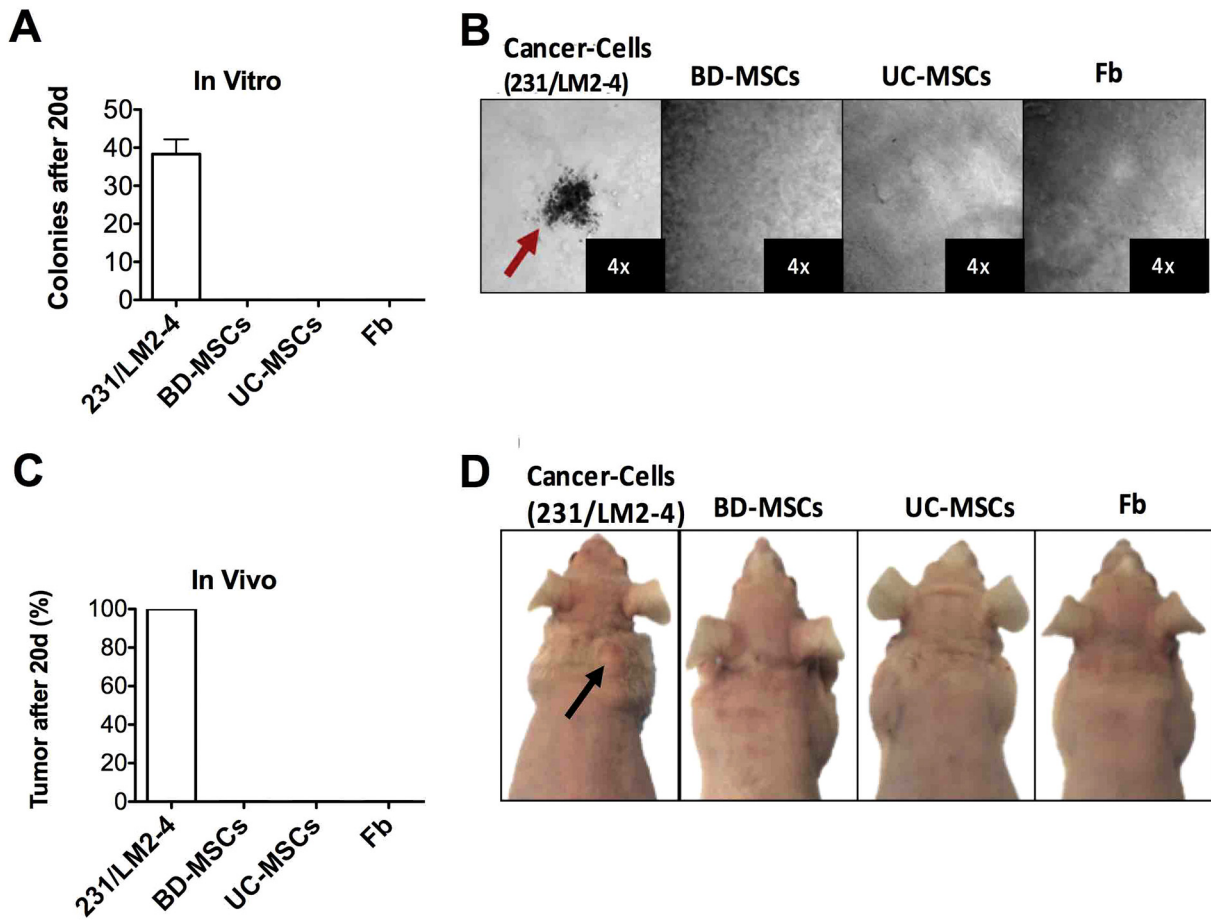
**Fig. 3.** Burn derived dermal cells are non-tumorigenic. (A, B) BD-MSCs did not cause In vitro tumor formation in soft agarose after 20 d (arrow: tumor colony in the cancer cell group). (C, D) No tumor formation was observed in the BD-MSC group within 20 d in vivo after subcutaneous injection in athymic mice (arrow: visible tumor in the cancer cell group).

the skin are diverse and heterogeneous, each has a different susceptibility [37,39,47,52–56]. That raise the possibility that the ideal source of stem cells for regenerating dermis should arise from the dermis, likely a distinct population of cell within these cell type, and that questions the suitability of using other sources in regenerating dermis, considering their versatile developmental lineage. During the last decade, there has been an exponential increase of potential skin stem cell sources in pre- and clinical studies reported in literature [27,29–32]. However, to date, we still lack an ideal source for the extraction and use of those cells in regenerative medicine despite the surge of research in the field. Although MSCs are present in the intact human dermis, lack of healthy donor site limits feasibility of using these cells for large burns. The autologous source of burned tissue overcomes this lack of availability, demonstrating macroscopically better homogenous wound healing compared with the current gold-standard in burn care, as well as less scarring, increased vascularization and decreased inflammation.

MSCs are generally considered to be safe. However, there are studies reporting that MSC treatment can lead to adverse effects such as pneumonia and the facilitation of cancer [57,58]. BD-MSCs derived from the patient's own skin and can be re-transplanted after a sufficient expansion in vitro. Since donor cells come from the patient themselves and additionally we did not see any adverse effects or tumor formation in vitro or in vivo the risks are maximally mitigated making BD-MSCs an elegant source for stem cell therapy. These BD-MSCs can be integrated easily and cost-effectively in an already widely used and FDA-approved wound coverage material or any other scaffold for many clinical purposes. We also explored the incorporation

of BD-MSCs in a variety of other wound dressings with different application techniques. BD-MSCs have consistently shown good integration without cell-loss or reduction of proliferation. We were able to extract skin-derived stem cells and integrate them efficiently into skin substitutes that can be placed in the same location where the extracted stem cells come from. This gives hope that we are one step further towards the end-goal of cell therapy in skin regeneration - the creation of an off-shelf skin that only needs two components: a skin scaffold and stem cells [59,60]. Moreover, the isolated cells will be applied to the same location of extraction, and this increases the chance of successful grafting, considering the nonhomogeneity of fibroblast-like cells in a different part of the skin. Whether this mesenchymal cells can be used as a source for epithelial cells of the skin, needs further research [30,32,61–63]

The murine study showed accelerated wound healing and a significant reduction in scarring
indicated by the reduced size of granulation tissue and a reduced thickness of the keratinocyte layer. This is promising considering that burn patients not only suffer from delayed wound closure but also develop excessive scarring. The debilitating contracture is one of the major contributors to a reduced quality of life in burn survivors [11,59,64]

In our study, we could detect human BD-MSCs in the scar/wound tissue of mice after complete wound closure. However, it is unclear if those cells were still in their MSC-state or if they could integrate and maybe further differentiate and therefore fully regenerate the skin. Most studies did not find a long-term survival of foreign MSCs in wounds which is mainly attributed to the harsh wound environment [65] and
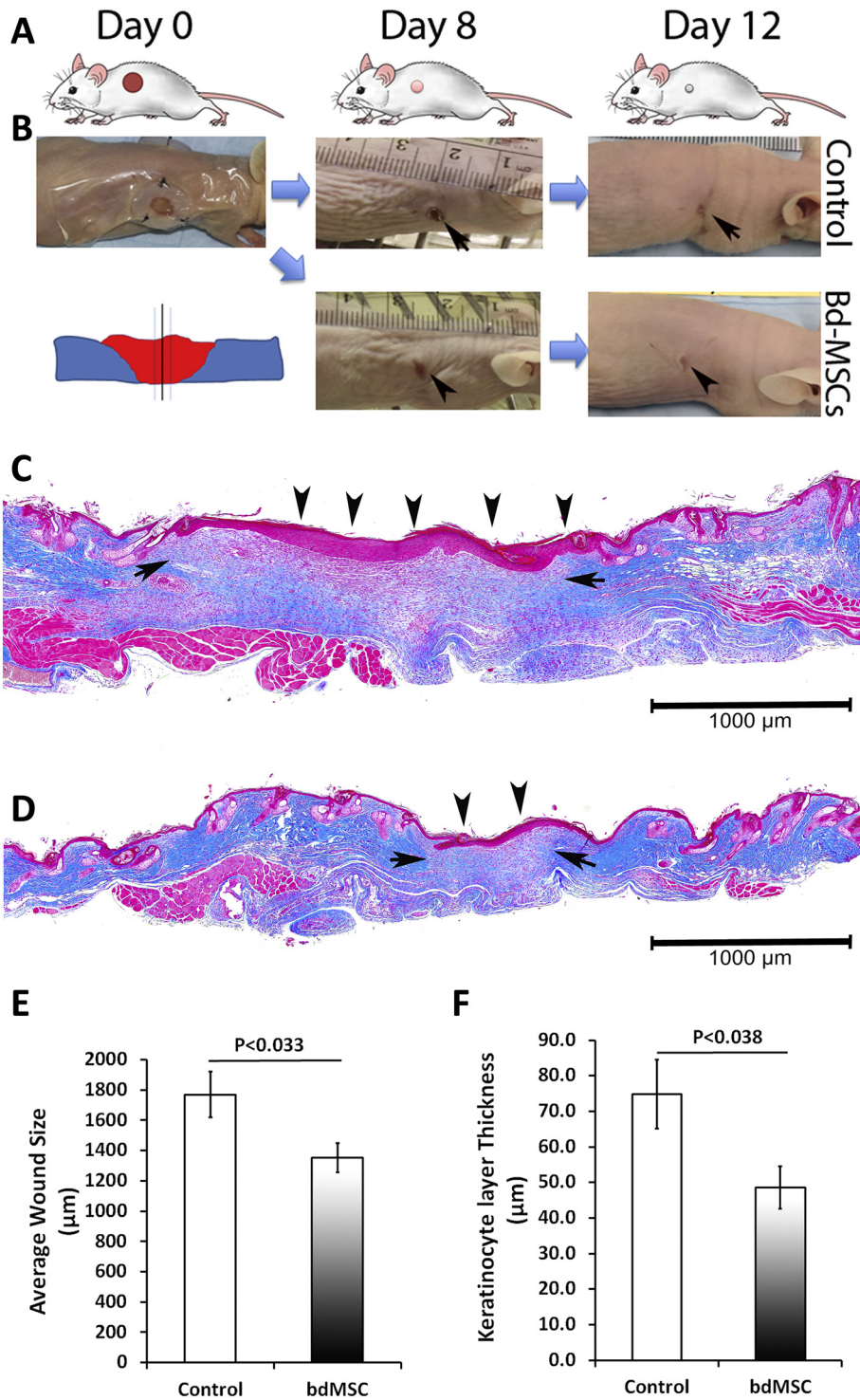
**Fig. 4.** Burn derived MSCs enhance skin healing. Schematic of in vivo animal experiment. (B) Time course measurement of wounded skin shows faster healing in excisional biopsies which were treated with BD-MSCs compared with control group. Note the arrows in days 8 and 12 post biopsy. (C–D) Trichrome staining of healed skin 12 days post wounding shows smaller scar size and thinner keratinocyte layer in the wounds exposed to BD-MSCs. Arrows show the border of normal skin with the healing bed and arrowheads mark the newly formed keratinocyte layer. (E) Quantification of wound size. (F) Quantification of keratinocyte layer thickness. Graphics show MEAN ± SEM.

differentiation potential. Further studies need to answer those important questions.

Our porcine study showed an increased epithelialization speed and area, confirming the acceleration of wound healing by BD-MSC treatment. Furthermore, a significantly higher number of new blood vessels have been observed in wounds treated with BD-MSC. More study is underway to evaluate whether the enhanced neovascularisation is the result of general enhanced wound healing or there is a direct effect of BD-MSCs on neovascularisation.

Beside the translational advantages of BD-MSCs, it is interesting that a group of cells in the dermis with stemness characteristics resist the heat after burn. It is possible that stress response in dermal mesenchymal stem cells make them resistant to this stress. Burn is accompanied by hypermetabolism that is characterized by a considerable increase
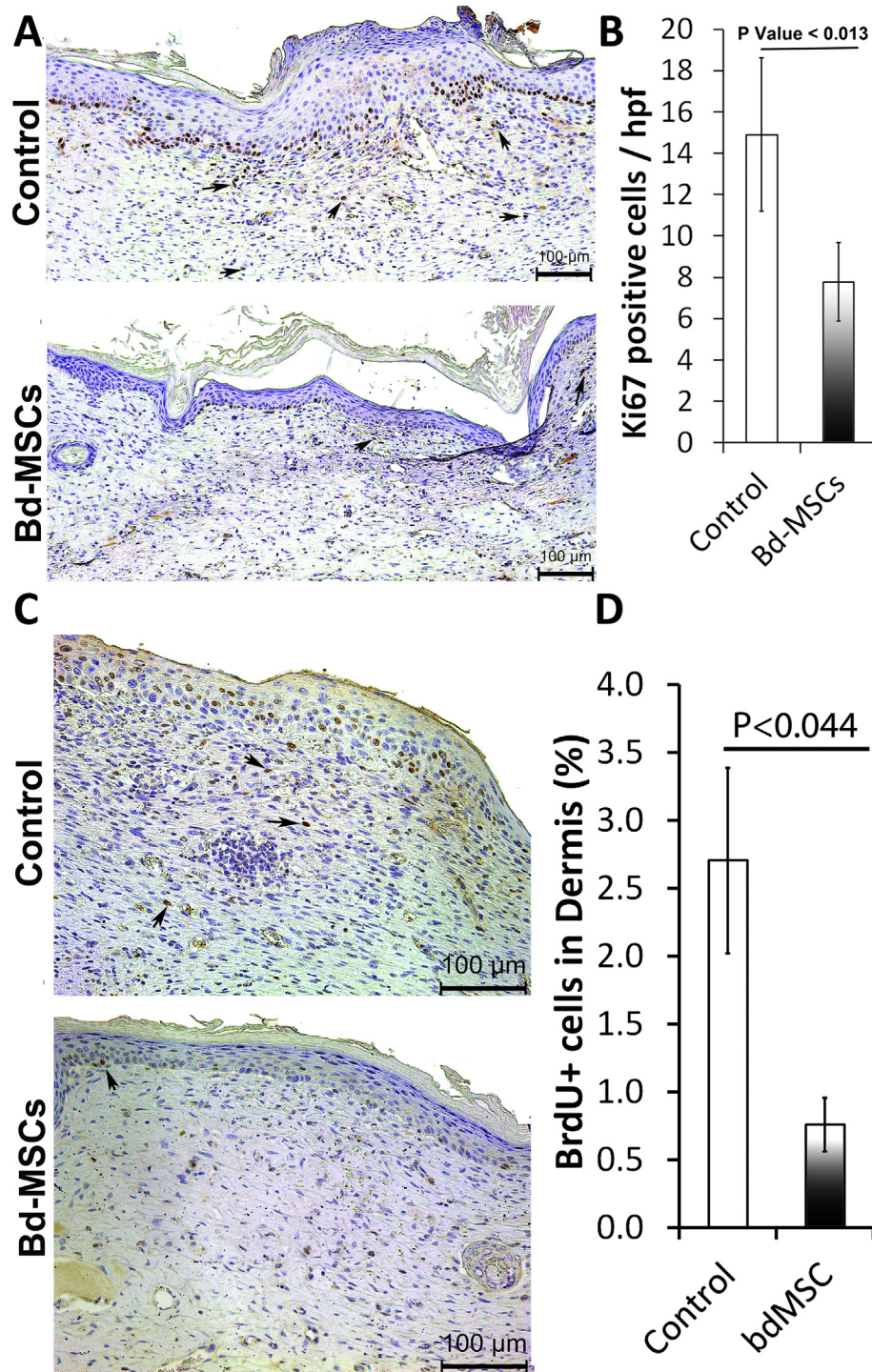
**Fig. 5.** Burn derived MSCs shorten the proliferative phase of skin healing. (A) Ki67 staining of healed skin 12 days post wounding shows significantly less Ki67+ cells in the healing bed of wounds treated with BD-MSCs. Arrows show Ki67+ cells. (B) Quantification of the number of Ki67 positive cells. (C) BrdU incorporation of healed skin 12 days post wounding shows significantly less BrdU incorporation in the healing bed of wounds treated with BD-MSCs. Arrows show cells which are incorporated with BrdU. (D) Quantification of the number of BrdU-positive cells. Graphics show MEAN ± SEM.

in resting energy expenditure and substantial whole-body catabolism, all associated with mitochondrial alteration [66]. Whether the mitochondrial changes in the dermal stem cells selectively protect them is the subject of a new line of research and needs further research to verify. Nonetheless, recent studies indicated that mitochondrial dynamics is associated with self-renewal and resistance to differentiation in some stem cells [67,68].

Burn-derived stem cells are a promising new source of skin stem cells for regenerative medicine and burn-wound management. Without being limited by the common obstacles of stem cell therapies and its availability, this autologous method will revolutionize the way we treat burn patients and potentially patients with complex wounds.

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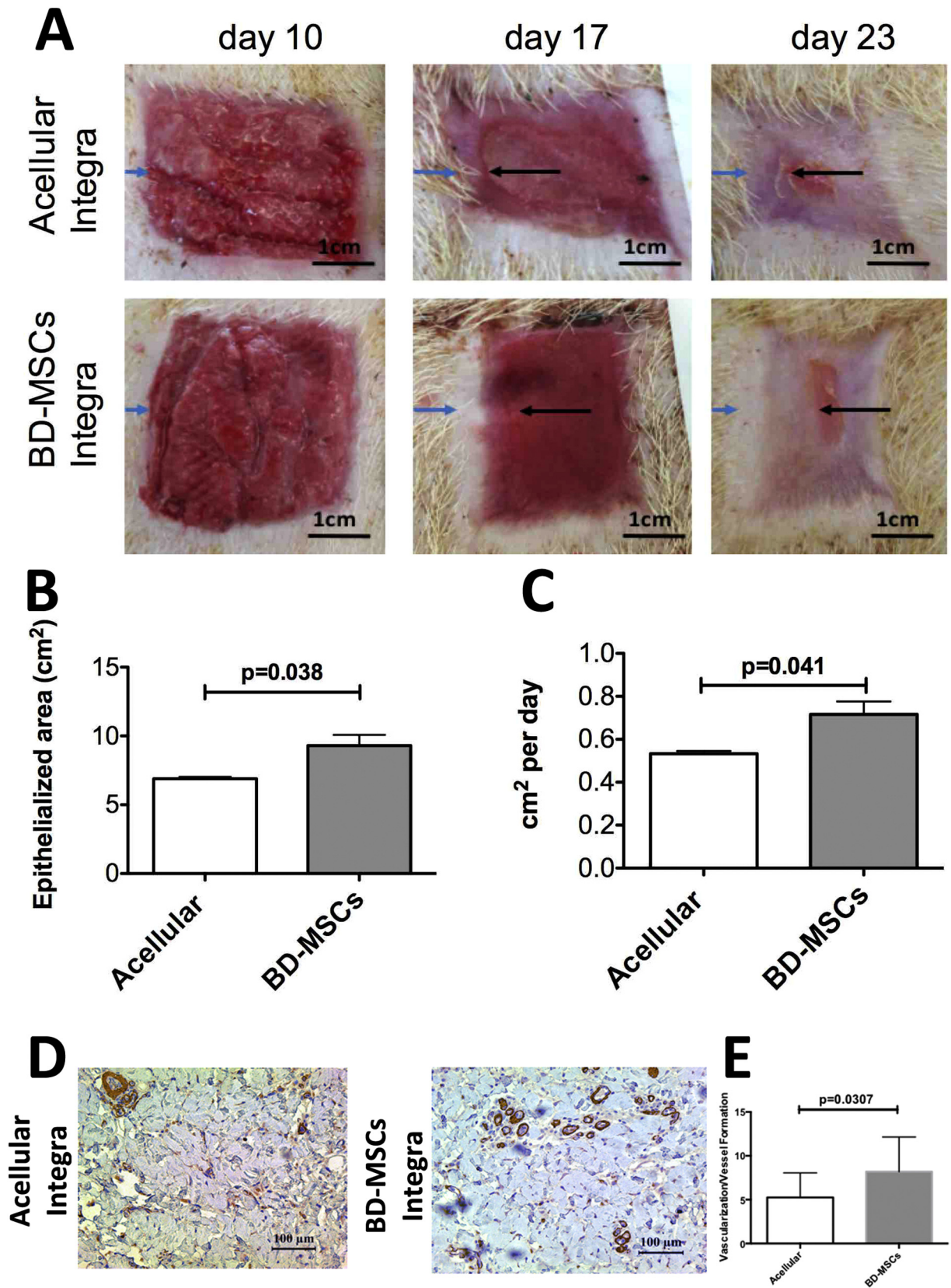
Fig. 6. BD-MSCs improved re-epithelialization and neovascularization in procine wounds. (A) The total epithelialized area after 23 days was higher in BD-MSC treated wounds. (B) The overall epithelialization speed (cm²/day) assessed between day 10 and 23 was higher in the BD-MSC group. (C) Representative wound images of the two groups on day 10, day 17, and day 23 (Blue arrow: start of wound edge; Black arrow: epithelialization boarder, red arrow: scar, contracture), with macroscopically more homogenous wound healing in the BD-MSC group. Graphics show MEAN ± SEM. (D, E) CD31 stained healed skin 28 days post wounding shows significantly more blood vessels in the granulation tissue of BD-MSC group. (E) Quantification of the number of CD31 positive vessels. (Graphics show MEAN ± SEM.

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## Authors contributions

**AA-N:** Isolation and characterization of the cells, study design and conduction of the study in-vitro and in mice and guidance for all experiments. Analyzing results, writing and editing the manuscript. **RD:** Study design, Porcine experiments, contribution in the statistical analysis of the porcine experiment and the writing of this manuscript. **GE:** Contribution in the writing of manuscript, histologic data analysis, porcine experiments. **AD:** Study design, execution of the porcine and mice experiment. **AP:** Study design, execution of the porcine experiment. **CB:** Study design and execution of the mice experiments. **MGJ:** study design, the clinical part, guidance for all experiments, data analysis, writing and editing of the manuscript.

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