

Therapeutic effects of platelet derived growth factor overexpressed-mesenchymal stromal cells and sheets in canine skin wound healing model

Namyul Kim¹, Kyeong Uk Choi¹, Eunbee Lee¹, Seoyun Lee¹, Jiwon Oh¹, Woo keyoung Kim¹, Sang-Ho Woo^{1,2}, Dae-Yong Kim^{1,2}, Wan-Hee Kim¹ and Oh-kyeong Kweon¹

¹BK21 PLUS Program for Creative Veterinary Science Research, Research Institute for Veterinary Science, College of Veterinary Medicine and ²Department of Veterinary Pathology and Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, Seoul, Korea

Summary. Adipose-derived mesenchymal stromal cells (Ad-MSCs) have excellent potential for skin wound repair. Moreover, platelet-derived growth factor (PDGF) has strong wound healing properties. The purpose of the present study was to compare the healing effects of PDGF-overexpressing canine allogeneic Ad-MSCs (PDGF-MSCs) and their cell sheets (PDGF-CSs) as compared to unexpressed Ad-MSCs (U-MSCs) and their cell sheets (UCSs) in a cutaneous wound healing model induced upon dogs. In *in vitro* study, the expression of immunomodulatory and growth factors was assessed by qRT-PCR. In *in vivo* study, cells and sheets were transplanted into a square-shaped full-thickness (1.5×1.5 cm) skin defect model created in 12 dogs. After 5 and 10 days, wounds were harvested and evaluated macroscopically and histopathologically. The qRT-PCR results showed that the PDGF-B gene was significantly upregulated ($p<0.05$) in PDGF-CS and PDGF-MSCs groups. Upon gross analysis of the wound, all stromal cells and their sheet groups showed accelerated ($p<0.05$) cutaneous wound healing compared to the negative control groups. As compared to U-MSCs and UCSs, the PDGF-MSCs showed significant epithelization on days 5 and 10 of healing, whereas PDGF-CSs showed improved epithelization only on day 10. In the granulation tissue analysis, PDGF-CSs and UCSs

promoted more formation ($p<0.05$) of upper granulation tissue, collagen, and activated fibroblasts than PDGF-MSCs, and U-MSCs. Especially, the PDGF-CSs presented the highest formation and maturation of granulation tissue among all groups. All considered, PDGF overexpressed stromal cells or cells sheets can improve cutaneous wound healing in a canine model.

Key words: Platelet-derived growth factor, Adipose-derived mesenchymal stromal cell sheets, Wound healing, Dog

Introduction

Wound healing is a complex process that requires a well-orchestrated sequence, including hemostasis, cell migration, angiogenesis, extracellular matrix deposition and remodeling (Hu et al., 2014). Skin regeneration after extensive, full-thickness wounding caused by burns, abrasions, or diabetic ulcers is still challenging. To accelerate skin regeneration, many tissue-engineering techniques, such as biomaterials, biocompatible scaffolds, and stem cells, have been investigated (Hu et al., 2014; Dehkordi et al., 2019).

Tissue engineering has emerged as a promising strategy for wound healing. In particular, mesenchymal stem cells (MSCs) have excellent potential for therapeutic use in skin repair (Isakson et al., 2015). Adipose-derived mesenchymal stem cells, a type of MSCs, exhibit various advantageous properties: a large number of autologous cells that can easily be harvested, the highest proliferation and differentiation potential

Offprint requests to: Oh-Kyeong Kweon, BK21 PLUS Program for Creative Veterinary Science Research, Research Institute for Veterinary Science, College of Veterinary Medicine, Seoul National University, 1 Gwanak-ro, Gwanak-gu, Seoul 08826, Korea. e-mail: ohkweon@snu.ac.kr

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(Kern et al., 2006). Various approaches for cell delivery have been attempted to improve wound healing, such as intradermal injection, cell sheets, and 3D collagen gel scaffold (Kim et al., 2011; Cerqueira et al., 2013; Martinello et al., 2018). Intradermal injections around wound margins with stem cell suspensions are a useful and commonly reported technique for wound healing (Isakson et al., 2015). Cell sheet technology also has been a useful method for cell transplantation in regenerative medicine, mainly because high-density cell sheet constructs have the ability to increase cell residence time and cell survival rate and do not destroy scaffolds at transplantation sites. Such attributes may make cell sheet technology beneficial over scaffold-based approaches or cell suspensions (Matsuda et al., 2007).

Platelet-derived growth factor (PDGF) is one of the growth factors that play a key role in each stage of wound healing. PDGF has been proven to proliferate granulation tissue, increase angiogenesis, and stimulate wound healing (Barrientos et al., 2008). Effects of topical application of exogenous PDGF for accelerating tissue repair have been demonstrated in animal model and human clinical studies (Embil et al., 2000; Robson et al., 2005; Judith et al., 2010). Human recombinant rh-PDGF-BB (Becaplermin) has been the first drug of its kind to be approved by the U.S. FDA (Food and Drug Administration) for use in diabetic foot ulcers in 1997. In addition to that, it has significant wound healing effects in a Phase IV clinical trial (Robson et al., 2005).

To increase the maintenance of large amounts of PDGF within the wound bed, several PDGF-gene delivery methods have been attempted, such as those employing adenovirus or lentivirus in wounds (Liechty et al., 1999; Man et al., 2005). Nevertheless, the use of lentivirus-based PDGF-overexpressed stromal cells or cell sheet therapy is not yet to be reported. Therefore, we hypothesized that PDGF-overexpressing Ad-MSCs (PDGF-MSCs) or their cell sheets (PDGF-CS) could improve the wound healing capacities over Ad-MSCs. This acceleration in wound healing can be due to the large amount of PDGF secreted by PDGF-MSCs or PDGF-CS within the wound bed. Moreover, we presumed that the wound healing response could vary depending on the cell delivery system used, such as sheets or cell suspensions. Henceforth, the purpose of this study was to compare the therapeutic effect of PDGF-CSs, PDGF-MSCs, unexpressed Ad-MSC sheets (UCSs) and unexpressed Ad-MSCs (U-MSCs) in a cutaneous wound model in dogs.

Materials and methods

Isolation and culture of canine Ad-MSCs

All experimental procedures on animals in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University in the Republic of Korea (SNU-180403-8). Allogeneic canine

Ad-MSCs were isolated according to a previously described procedure (Ryu et al., 2009). Briefly, gluteal subcutaneous adipose tissue was collected from 4 healthy male dogs aged 2 years under aseptic conditions. The tissue was washed with Dulbecco's phosphate-buffered saline (DPBS; Gibco, Grand Island, NY, USA), minced, and then digested with 1 mg/ml collagenase type I (Sigma-Aldrich, USA) at 37°C for 1 hour with intermittent shaking. The suspension was washed with DPBS and then centrifuged at 980×g for 10 minutes. Stromal vascular fraction pellets were suspended with DPBS and filtered using a 100-μm nylon mesh. The samples were incubated with low-glucose Dulbecco's modified Eagle's medium (DMEM; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; HyClone) in a humidified atmosphere at 37°C with 5% CO₂. The residual non-adherent red blood cells and unattached cells were removed by washing with DPBS after 24 hours and fresh culture media was introduced. The culture medium was replaced every 48 hours until confluence. Cells were subcultured up to the third passage. Further experimentation was carried out with the third passage of cells.

Lentiviral packaging, transfection and culture of PDGF-MSCs

The canine PDGF-B gene was cloned with reference to the gene database in Pubmed. Lentiviral vector and pPACK Packaging Plasmid Mix (System Biosciences, USA) were used for lentiviral packaging. According to the manufacturer's guidelines, the PDGF-B specific primer set was inserted into a pCDH-EF1-MCS-pA-PGK-copGFP-T2A-Puro vector using the restriction enzymes EcoRI and BamHI (System Biosciences, USA). HEK293T cells (Thermo Scientific) were seeded in a 100-mm petri dish with 10% FBS, 1% penicillin/streptomycin in DMEM at 37°C, and 5% CO₂. The following day, a lentiviral packaging mix (System Biosciences) and lentiviral transgene vectors were added to the cells for transfection. The cells were cultured for 48 hours at 37°C, 5% CO₂, and the medium was changed after 15 hours. After a 48 hours incubation period, a green fluorescent protein (GFP)-labeled PDGF-B-expressing virus particles were collected from culture media. When AD-MSCs at passage 1 reached 50-60% confluence in the 100-mm culture dish, the PDGF-expressing virus particles were transduced into the cells. At 90% confluence, transduced Ad-MSCs were selected using puromycin (3 μm/ml, Thermo Fisher Scientific, USA), and the culture medium was replaced after 2 days. Transduced Ad-MSCs were subcultured up to the third passage. The third passage of transduced Ad-MSCs was used for experiments.

Cell sheet preparation: UCSs, PDGF-CSs

UCSs and PDGF-CSs were produced as previously described using the cell sheet fabrication method

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(Cerqueira et al., 2013). Ad-MSCs and PDGF-MSCs were plated at a density of 1×10^4 cells/cm² in a 100-mm culture dish and cultured in growth medium supplemented with 82 µg/ml L-ascorbic acid 2-phosphate (Sigma, Germany) for 10 days. When cell sheets reached 80-90% confluence, they were retrieved by mechanical peeling with a cell scraper (SPL life science, Republic of Korea) and used for experiments.

Gene expression for identification of PDGF overexpression using qRT-PCR

Total RNA was isolated using a Hybrid-RTM RNA extraction kit (GeneAll Biotechnology, Republic of Korea) and the RNA concentration was measured with an Epoch microplate spectrophotometer (BioTek Instruments, Inc., USA). Complementary DNA (cDNA) was synthesized using a PrimeScript II First-strand cDNA Synthesis Kit (Takara Bio Inc., Japan) according to the manufacturer's protocol. The qRT-PCR chain reaction was performed using a StepOnePlus™ Real-Time PCR system (Thermo Fisher Scientific, USA) with SYBR® Premix Ex Taq™ (Takara Bio Inc., Japan). Untreated Ad-MSCs were used as an extrinsic control, while glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene. PCR data were analyzed using the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001). Primer sequences used for target genes are listed in Table 1.

Transplantation into full-thickness excisional wound models

Animals

Twelve healthy adult beagle dogs (2-3 years old, 10.1±1.8 kg) were used for this experiment. All dogs were maintained in accordance with the animal care

guidelines of the Institute of Laboratory Animal Resources at Seoul National University. All animals were considered to be healthy based on physical examination, complete blood count, and serum chemistry. Animals were caged individually under a standard environment.

Anesthesia

Animals were pre-medicated with medetomidine (5 µm/kg, IV), tramadol (4 mg/kg, IV) and cefazolin (22 mg/kg, IV). Anesthesia was induced with alfaxalone (2 mg/kg, IV) and maintained with isoflurane 1.5% with oxygen. Lactated Ringer's solution was administered via IV at 10 mg/kg/h. Heart rate, blood pressure, respiratory rate, rectal temperature, end-tidal CO₂, pulse oximetry and spirometer were checked with an anesthetic monitoring system (GE Healthcare, USA) throughout anesthesia. Each animal was positioned in sternal recumbency, and the dorsal area from the cranial part of the thorax to the middle part of the abdomen was clipped and prepared aseptically.

Wound creation and transplantation of stromal cells and their sheets

All animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University in the Republic of Korea (SNU-180403-8) and all surgical procedures were performed concerning international animal welfare rules. Six full-thickness wounds were created with a #15 scalpel blade on either side of the dorsal region. Each wound size was 1.5×1.5 cm, and each wound on a side was separated by 3 cm as not to interrupt other wounds during healing (Fig. 1). Wounds were randomly assigned into six groups: PDGF-CS, UCS, PDGF-MSCs, U-MSCs and control groups for sheets (Control-S) and

Table 1. Primers for quantitative real-time polymerase chain reaction.

Gene	Primer sequence (5'-3')	
	Forward	Reverse
PDGF-B	CCGAGGAGCTCTACGAGATG	AACTCTCCAGCTCGTCTCCA
VEGF	CTATGGCAGGAGGAGAGCAC	GCTGCAGGAAACTCATCTCC
COX-2	ACCGGCCATTATCCTAATCC	TCCGAGTTCTCCTGGCTTTA
IL-6	TTTTCTGCCAGTGCCTCTTT	GGCTACTGCTTTCCTACCC
IL-10	CCTGGGAGAGAAGCTCAAGA	TGTTCTCCAGCACGTTTCAG
TNF-α	ACCACACTCTTCTGCCTGCT	TGGAGCTGACAGACAACCAG
KGF	AGCCCTGAGCGACATACAAG	TGCCACTATTCCAAGTCCA
EGF	TGTGTCTGGCTCTGAATGGC	AAGACATCCCTGGCGTTAC
IGF-1	AAATCAGCAGTCTTCCAACCCA	ACACGAACTGAAGAGCGTCC
FGF-2	TGCCTACCTAGATGCTGGACA	GAGCTTTGGCCGTATTTCCATT
GAPDH	CATTGCCCTCAATGACCACT	TCCTTGGAGGCCATGTAGAC

PDGF-B, platelet-derived growth factor B; VEGF, vascular endothelial growth factor; COX-2, cyclooxygenase-2; IL-6, interleukin-6; IL-10, interleukin-10; TNF-α, tumor necrosis factor-α; KGF, keratinocyte growth factor; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; IGF-1, insulin-like growth factor-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cells (Control-M). In PDGF-CS or UCS groups, the allogeneic cell sheets (5×10^6 cells) were carefully placed to cover the wound beds entirely. In PDGF-MSC or U-MSC groups, allogeneic cells (5×10^6 cells) were injected with saline solution intradermally around the wound area. In the Control-S groups, wounds were not treated with anything. In the Control-M groups, 1 ml saline was injected in the same manner as described for cell groups. Wounds were covered with a sterile adhesive dressing (Hiper-pore®, WON Biogen, Republic of Korea) and cotton rolls (Daehan medical, Republic of Korea) were applied in multiple layers with crossing over the shoulders and between forelimbs to prevent the bandage from slipping caudally. Lastly, an elastic bandage (Daehan medical, Republic of Korea) as a tertiary layer was applied over the cotton roll layers.

Evaluation of wound healing

Gross analysis

Digital photographs of wounds were taken at days 5 and 10. Percentages of epithelialization, contraction, and total wound healing were measured as previously described (Bohling et al., 2004) using Image J software (Wayne Rasband, NIH, USA). Briefly, the outermost margin area of the wound was considered as the total wound area (A), the area within the outer margin of the unhealed wound was the open wound area (B) and the area between these two margins was the area of epithelialization (C). Within these areas, the percent of

epithelialization, contraction and total wound healing were calculated as follows.

$$\% \text{ epithelialization} = \frac{\text{Area of epithelium}}{\text{Total wound area}} \times 100$$

$$\% \text{ contraction} = 100 - \frac{\text{Total wound area}}{\text{Original wound area}} \times 100$$

$$\% \text{ total wound healing} = 100 - \frac{\text{Open wound area}}{\text{Original wound area}} \times 100$$

Histopathological Analysis

Skin samples, including wounded and adjacent normal skin, were harvested at days 5 and 10, fixed in 10% formalin for 24 hours, and embedded in paraffin. H&E and Masson's trichrome staining were conducted using routine protocols. All samples were scanned by a microscope slide scanner (Pannoramic® SCAN II, 3D HISTECH Ltd., Hungary) and analyzed in a blinded fashion using a slide image analysis program (CaseViewer™, 3D HISTECH Ltd., Hungary).

Thickness and shape of neo-epidermis. On H&E-stained samples, the shape and thickness of the neo-epidermis overlying the wound edge were evaluated based on the a previously described method (Larouche et al., 2011). Briefly, 10 parts of randomly selected neo-epidermis were measured in each tissue section and compared among groups at days 5 and 10, respectively.

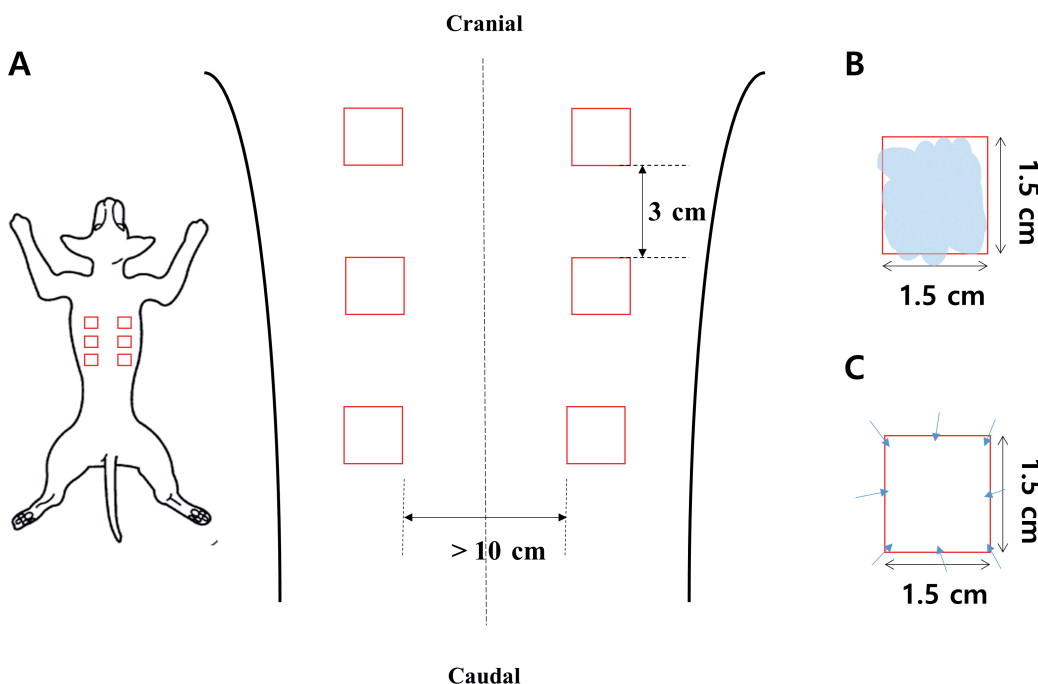


Fig. 1. Wound creation and transplantation of a stem cell sheet and a stem cell suspension into the wound. **A.** The layout of full-thickness wounds created on the dorsal region in the dog. The wounds were made bilaterally and the size of the wounds was 1.5×1.5 cm (square). Wounds were 3 cm away from each other. **B.** In sheet groups (PDGF-CS and UCS), a cell sheet was placed to cover the entire wound area and in cell groups (PDGF-MSCs and U-MSCs) (**C.**) a cell suspension was injected intradermally at eight injection sites around the wound edge.

Quantification and maturation of granulation tissue and blood vessels density. The quantification and the maturation of the newly formed granulation tissue were evaluated using Image J software (Wayne Rasband, NIH, USA) followed by H&E staining. For quantification, we measured the size of the granulation tissue in experimental groups as compared to control groups. Moreover, the maturation of granulation tissue was calculated by the histological score index (HS index, HS/mm²). The histological score index is an average of the histological score per granulation tissue, based on a histological score (HS) that ranges from 1 to 12 (Table 2) (Jacobi et al., 2002). Granulation tissues at day 10 were divided into upper and lower layers and then evaluated; upper granulation tissue is rich in inflammatory cells and vessels and lower granulation tissue is rich in collagens and connective tissues. The calculation of the HS index was performed as follows.

The number of blood vessels was quantified at days 5 and 10 in H&E-stained samples. In 10 randomly selected 100× magnification fields, all visible blood vessels within were counted, and the mean number of vessels per field was calculated. The number of vessels was normalized to the tissue area.

Collagen abundance and organization. In Masson's trichrome-stained slides, collagen abundance and organization were evaluated by modifying the scale suggested by Farghali et al (2017). The scoring was modified as follows: 0-2, no to rare collagen or disorganized collagen fibers in granulation tissues; 3-5, mild collagen deposition that comprised 25-50% of granulation tissues and randomly orientated collagen fibers; 6-8, moderate collagen deposition that comprised 50-75% of granulation tissues, and well-oriented collagen fibers. 9-11, abundant total collagen that comprised most of the granulation tissues and well-organized fibers. Parallel-oriented wavy collagen fibers with well-stained blue color were considered to be more organized than randomly disorientated and inconsistent blue-colored collagen fibers.

Immunohistochemical analysis

To identify keratinocytes proliferation in the

neoepidermis and activated fibroblasts in the granulation tissues, immunohistochemistry was performed with primary antibodies against Ki67 (rabbit polyclonal IgG, ab155580, 1:1000, Abcam Cambridge, MA) as a proliferation marker and fibroblast activation protein, alpha (FAP) (rabbit polyclonal IgG, ab53066, 1:100, Abcam Cambridge, MA) as reactive fibroblasts marker. Briefly, 5 µm frozen paraffin sections were deparaffinized and rehydrated, and the heat-induced antigen retrieval method was conducted using a pressure cooker in 10mM citrate buffer solution. Endogenous enzymes were inactivated with 0.3% H₂O₂ for 30 min and nonspecific antibody binding was blocked with 2.5% normal serum blocking solution (S-1012, Vector labs, CA). The sections were incubated overnight at 4°C with primary antibodies, then incubated with anti-rabbit IgG biotinylated secondary antibody (ImmPRESS™ HRP reagent kit, Vector labs, CA) for 30 min at room temperature, followed by 3,3'-diaminobenzidine (DAB; Vector labs) for 2 min. Lastly, counterstain was performed with hematoxylin and sections were mounted and examined under a light microscope.

The number of Ki67-positive cells in the neoepidermis was counted in 10 randomly selected 400× magnification fields, and the mean number of expressed cells per field was calculated. Nuclei stained to brown or claybank was considered a positive cell. For quantification of FAP, 10 random fields per granulation tissue at 200× magnification were chosen, and a semiquantitative scale of 1 to 8 was used. The rating scale is described as follows: 1-2, rare to minimum fibroblasts stained in granulation tissue; 3-4, mild and irregular pattern of activated fibroblasts; 5-6, moderate and well-oriented fibroblasts over granulation tissue; 7-8, abundant, dense and compact organized fibroblasts all over the granulation tissue.

Statistical analysis

The values are presented as the means±standard deviation (SD). All statistical analyses were performed using the IBM SPSS statistics 23.0 software (Chicago, IL, USA). Data analysis was carried out by the Kruskal-Wallis test with Mann-Whitney's post hoc test. A p value of less than 0.05 was considered statistically significant.

Table 2. Histological scoring system.

Scores	Granulation tissue
1-3	None to minimal cell accumulation, no granulation tissue or epithelial travel
4-6	Thin, immature granulation, dominated by inflammatory cells with a few fibroblasts, capillaries, or collagen deposition, and minimal epithelial migration
7-9	Moderately thick granulation tissue, dominant inflammatory cells, more fibroblasts and collagen deposition, extensive neovascularization, and minimal to moderate migrating epithelium
10-12	Thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition, and epithelium partially to completely covering the wound

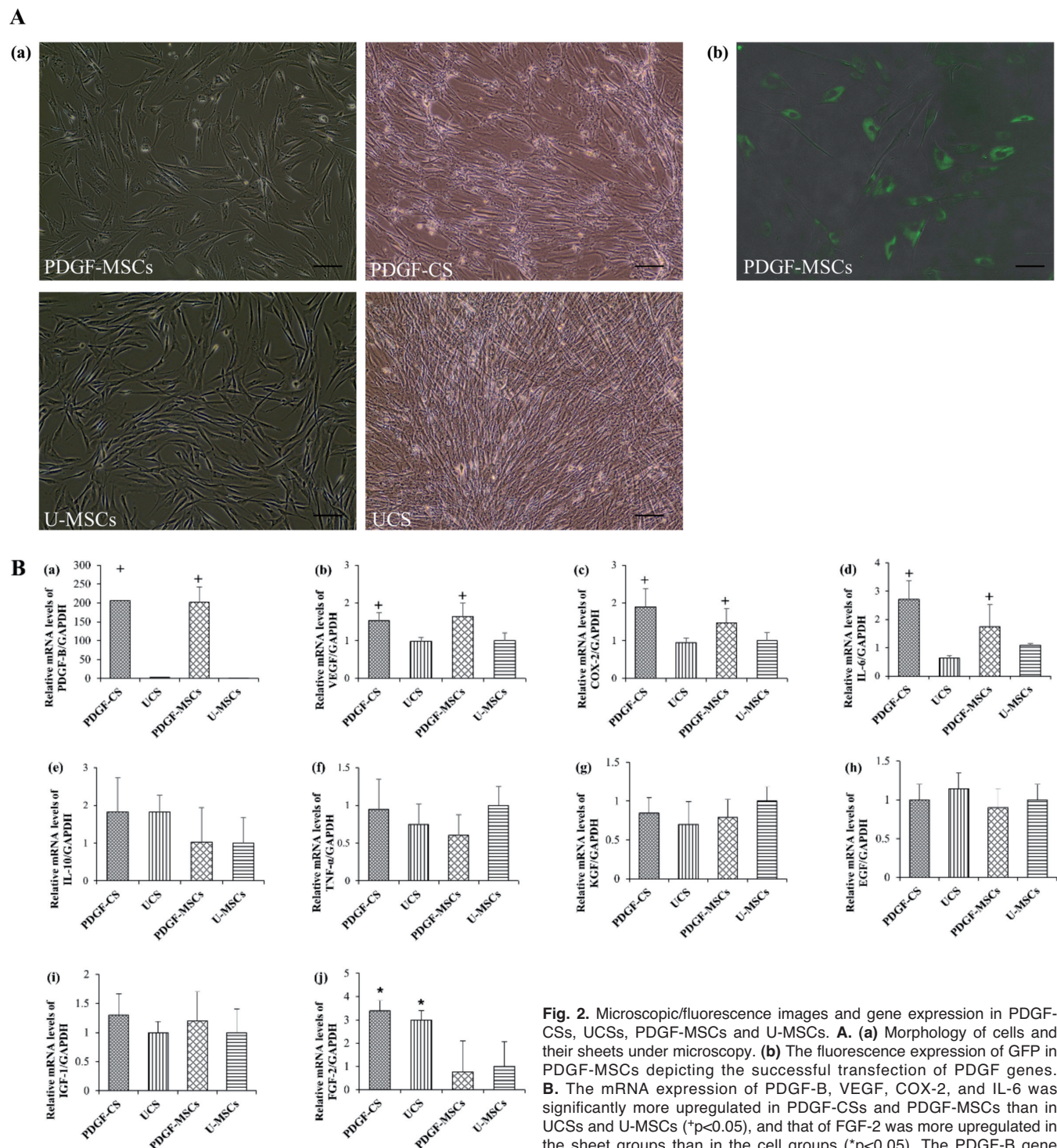


Fig. 2. Microscopic/fluorescence images and gene expression in PDGF-CSs, UCSs, PDGF-MSCs and U-MSCs. **A.** (a) Morphology of cells and their sheets under microscopy. (b) The fluorescence expression of GFP in PDGF-MSCs depicting the successful transfection of PDGF genes. **B.** The mRNA expression of PDGF-B, VEGF, COX-2, and IL-6 was significantly more upregulated in PDGF-CSs and PDGF-MSCs than in UCSs and U-MSCs (* $p < 0.05$), and that of FGF-2 was more upregulated in the sheet groups than in the cell groups (* $p < 0.05$). The PDGF-B gene

upregulation was the most significant among genes (* $p < 0.05$) and for IL-10 and TNF- α gene expression, no statistically significant difference was observed among groups. Also, the genes of epidermis markers, KGF, EGF, and IGF-1, were not significantly expressed in any groups. * $p < 0.05$ versus unexpressed groups (UCS, U-MSCs), * $p < 0.05$ versus cell groups. Scale bars: A(a), 200 μm ; A(b), 100 μm .

Results

Morphology and gene markers expression of cells and their sheets

Upon gross examination, PDGF-CS and UCS

appeared as a condensed contiguous layer, unlike PDGF-MSCs and U-MSCs. Upon microscopic examination, PDGF-MSCs and U-MSCs had similar morphology; in addition, PDGF-CS and UCS also exhibited alike morphology. (Fig. 2A(a)). Through GFP expression under fluorescence microscopy, it was

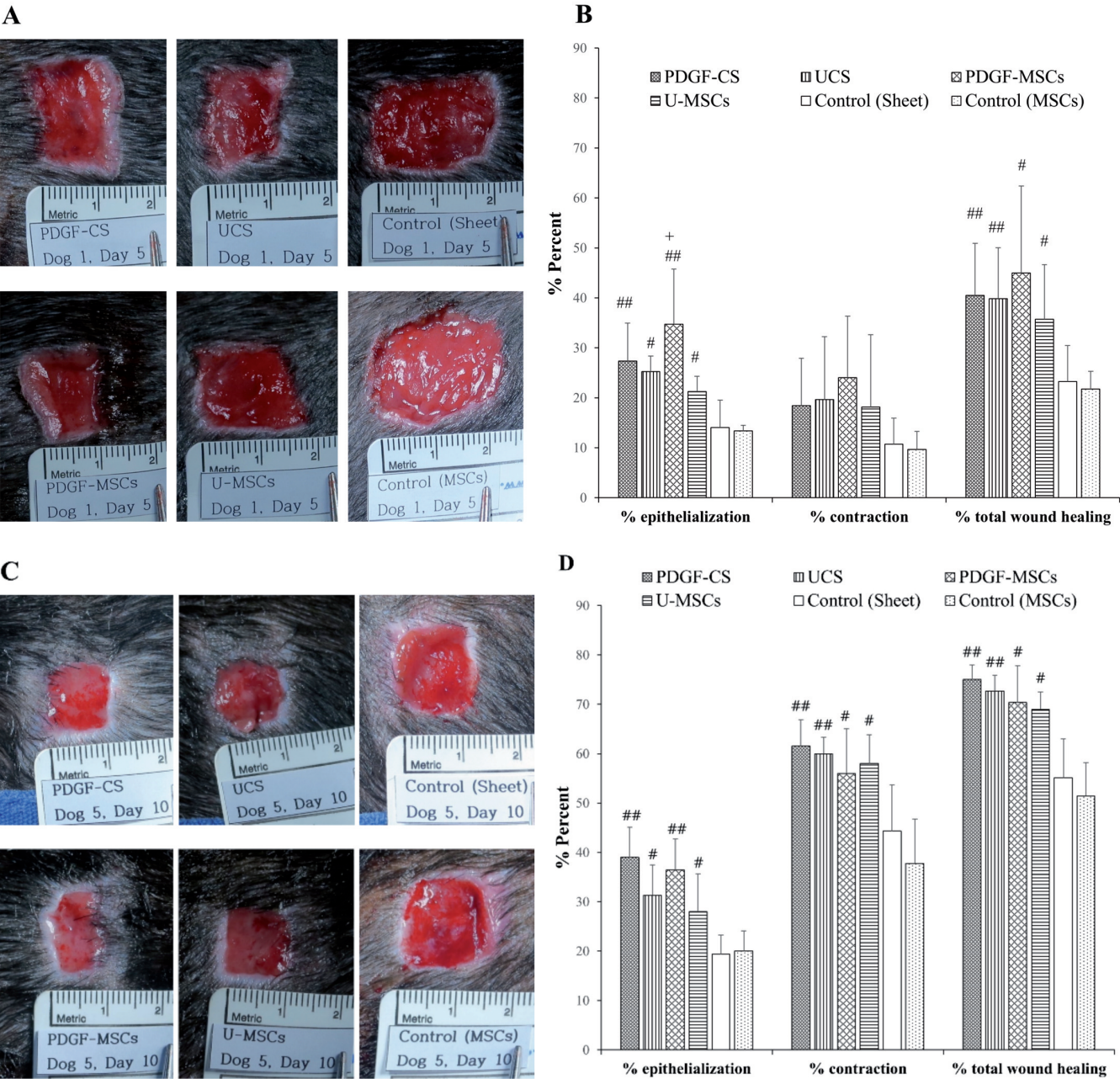


Fig. 3. Gross images of wound healing and evaluation of epithelialization, contraction, and total wound healing rates at days 5 and 10. Representative macroscopic images of experimental groups and control groups at days 5 (**A**) and 10 (**C**). The PDGF-MSCs group showed wider and thicker epithelialization than other groups at day 5 (* $p < 0.05$) (**A**). Percent of epithelialization, contraction, and total wound healing was calculated using photographs of wounds at days 5 (**B**) and 10 (**D**); # $p < 0.05$ and ## $p < 0.01$ versus the control groups, * $p < 0.05$ versus unexpressed groups (UCS, U-MSCs).

confirmed that PDGF-MSCs were successfully transfected with PDGF-B (Fig. 2A(b)). Of the gene markers, PDGF-B was upregulated by approximately 200-fold in PDGF-CSs and PDGF-MSCs, which was the most remarkable upregulation among genes (Fig. 2B(a)). The VEGF gene, a vascular-related marker, was approximately 1.5-fold upregulated in PDGF-CS and PDGF-MSCs ($^+p<0.05$) (Fig. 2B(b)) and COX-2 and IL-6, inflammatory markers, were also approximately 2-fold upregulated in those groups ($^+p<0.05$) (Fig. 2B(c),(d)). The FGF-2 gene was more upregulated in the sheet groups ($^*p<0.05$) (Fig. 2B(j)). Epidermis markers, KGF, EGF, and IGF-1, were not significantly expressed in any of the groups (Fig. 2B(g),(h),(i)). IL-10 and TNF- α also were not noticeably expressed among the groups (Fig. 2B(e),(f)).

Gross findings in wounds

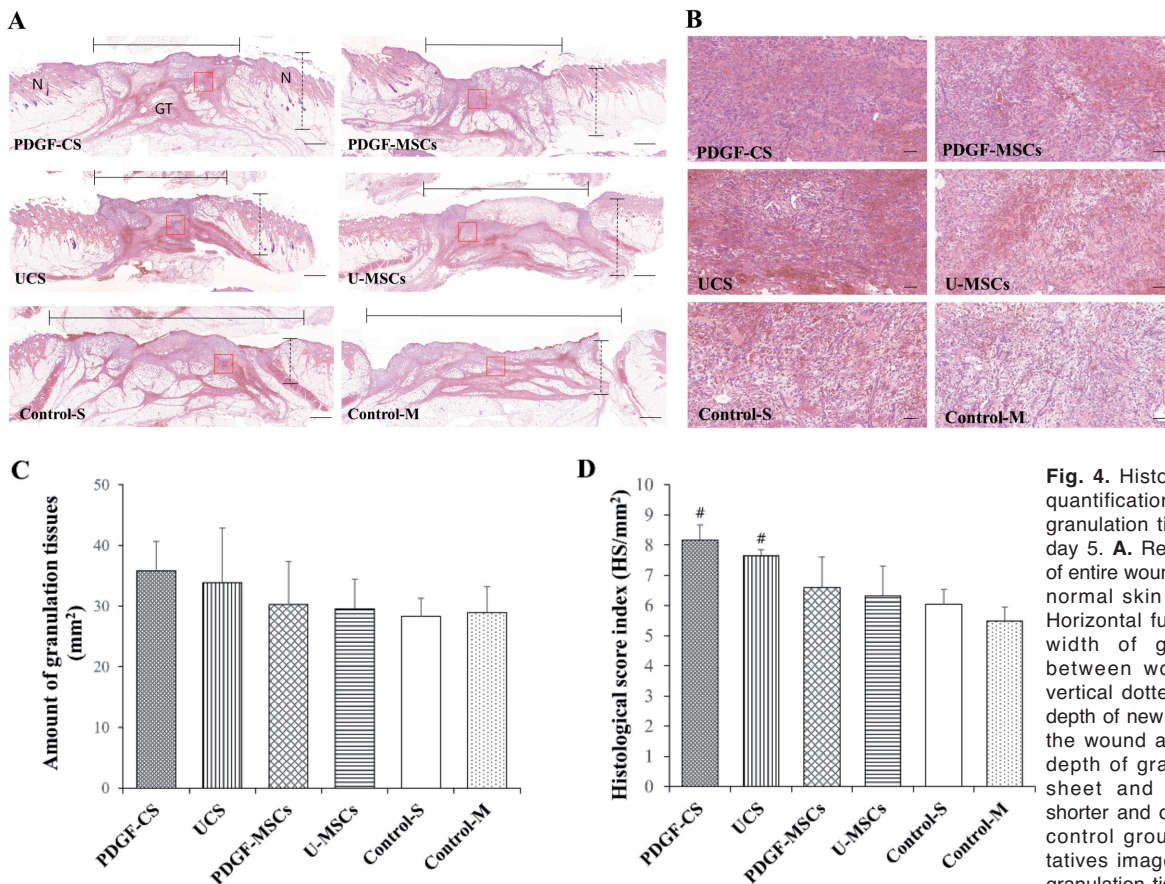
Percent epithelialization

On day 5, the mean percent epithelialization in all

stromal cell and sheet groups was significantly higher than in the control groups ($^{\#}p<0.05$, $^{\#\#}p<0.01$). The PDGF-MSCs group showed a higher epithelialization rate as compared to unexpressed groups like UCS and U-MSCs groups ($^+p<0.05$) (Fig. 3B). As shown in the day 5 gross images, the PDGF-MSCs groups presented a broader and thicker epithelialization than other groups (Fig. 3A). At day 10, a similar pattern was identified; all stromal cell and sheet groups showed higher epithelialization than control groups ($^{\#}p<0.05$, $^{\#\#}p<0.01$) and PDGF-CS and PDGF-MSCs groups tended to have higher rates than UCS and U-MSCs groups, although this was not significant (Fig. 3C,D).

Percent contraction

Mean percent contraction on day 5 for stromal cell groups (PDGF-MSCs, U-MSCs) and sheet groups (PDGF-CS, UCS) seemed to be higher than that for all control groups, but not statistically different (Fig. 3B). At day 10, stromal cell groups ($^{\#}p<0.05$) and sheet groups ($^{\#\#}p<0.01$) had a significantly higher contraction



A). C. Representation of the amount of granulation tissue and histological score index in wound area (D). The histological score index of PDGF-CS and UCS groups was significantly higher than that of control groups ($^{\#}p<0.05$). GT, granulation tissue; N, normal skin; $^{\#}p<0.05$ versus the control groups. Scale bars: A, 1000 μ m; B, 50 μ m.

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rate than control groups (Fig. 3D). However, no significant difference was observed among experimental groups at days 5 and 10 (Fig. 3A-D).

Percent total wound healing

At both days 5 and 10, the total wound healing rate

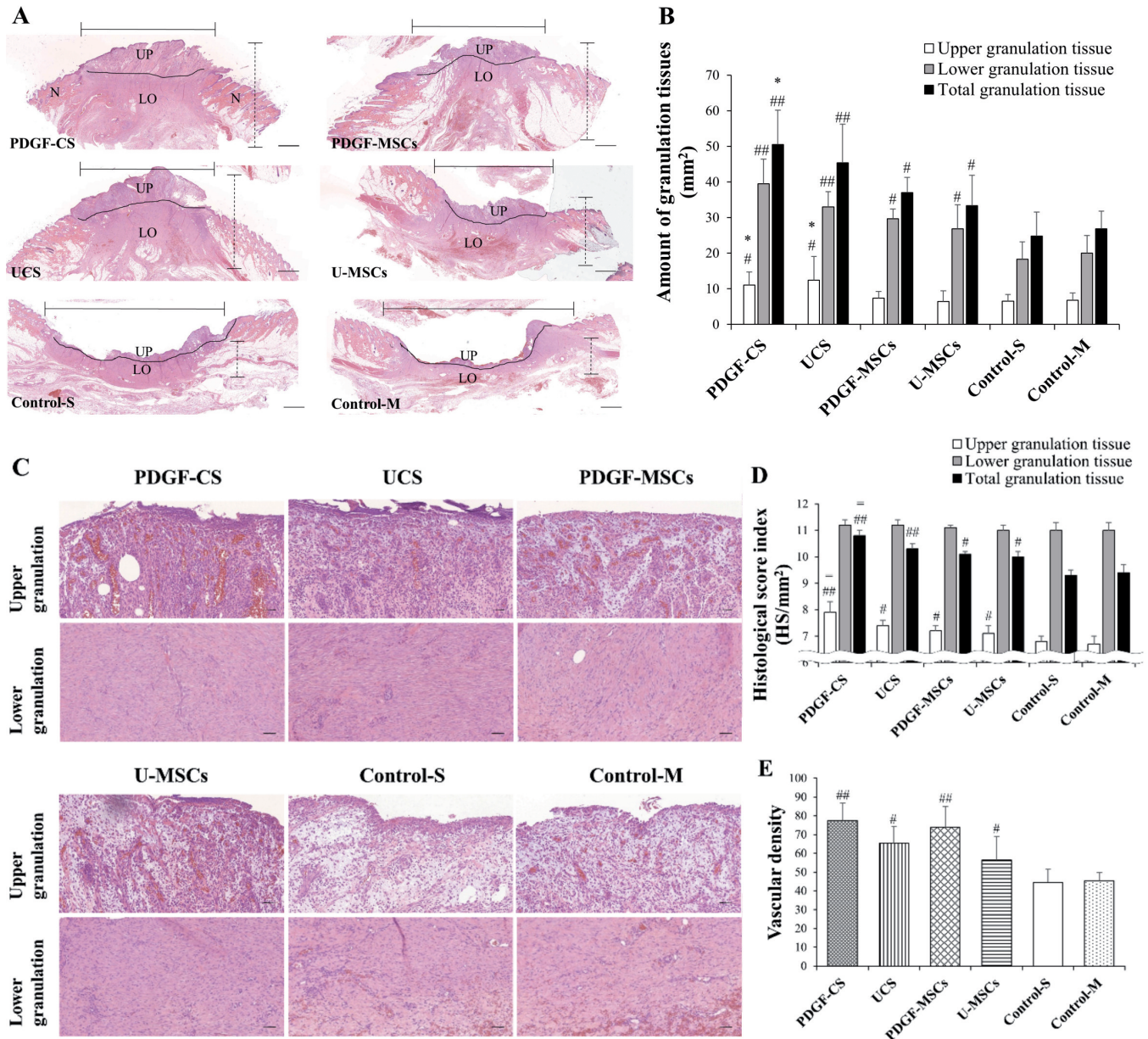


Fig. 5. Histological analysis for quantification and maturation of granulation tissues in wounds at day 10. **A.** Representative image of entire wound area with adjacent normal skin with H&E stain. Horizontal straight lines above tissues indicate the width of granulation tissue and vertical dotted lines indicate the depth of new granulation tissues in the wound area. Upper and lower granulation tissues are distinguished by a curved line. **B.** Representation of the amount of upper, lower, and total granulation tissues. The total granulation tissues in the sheet ($^{##}p<0.01$) and cell groups ($^{#}p<0.05$) were significantly more organized than in control groups. Among experimental groups, the PDGF-CS groups presented the largest granulation tissue size ($^{*}p<0.05$). **C.** Representative image of magnifying the upper granulation tissue and lower granulation tissue with H&E stain. Representation of histological score index (**D**) and vascular density (**E**) in wound area. The histological score of total granulation tissues in sheets ($^{##}p<0.01$) and cell groups ($^{#}p<0.05$) was higher than in control groups and PDGF-CS had the highest histological score on upper granulation tissue and total granulation tissue among groups ($^{##}p<0.01$, $^{*}p<0.05$) (**D**). UP, upper granulation tissue; LO, lower granulation tissue; N, normal skin; $^{#}p<0.05$, $^{##}p<0.01$ versus the control groups, $^{*}p<0.05$ versus cell groups, $^{=}p<0.05$ versus the rest of the groups. Scale bars: A, 1000 μ m; C(upper), 100 μ m; C(lower), 50 μ m.

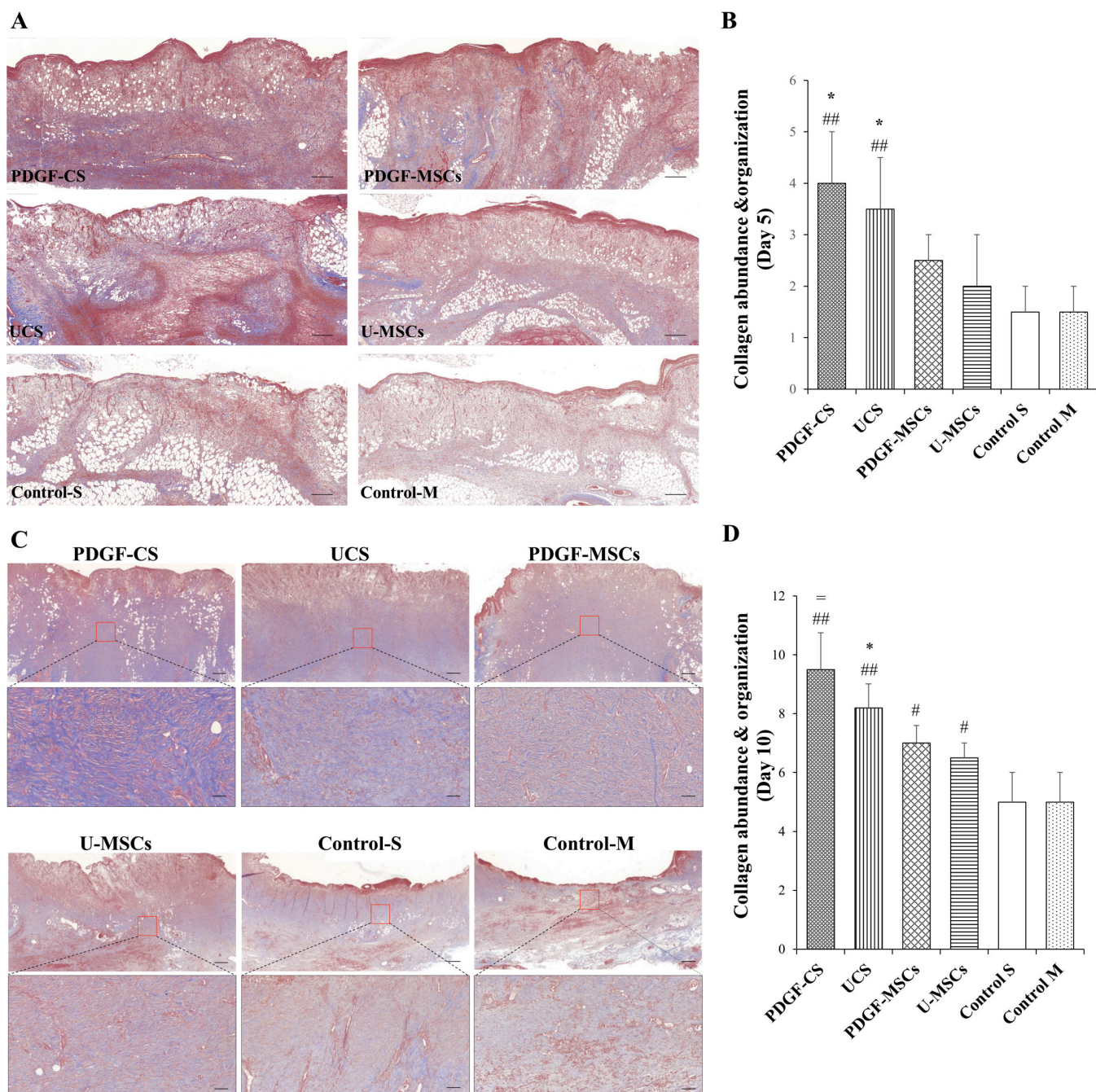


Fig. 6. Collagen deposition and organization in wounds. **A.** Representative images of collagen deposition in wounds at day 5 (Masson's trichrome). Collagen in sheet groups ($^{##}p<0.01$, $^{*}p<0.05$) significantly increased when compared to both control and cell groups. **B.** Quantitative data of collagen abundance and organization at day 5. **C.** Representative images of collagen deposition in wounds at day 10 (Masson's trichrome) and inset high magnification images indicated collagen density and collagen fibers orientation. All sheet ($^{##}p<0.01$) and cell groups ($^{*}p<0.05$) had significantly more collagen infiltration than in control groups and PDGF-CS groups ($^{*}p<0.05$) showed significant collagen deposition and much denser and thicker fibers in wounds than the rest of the groups. **D.** Quantitative data of collagen abundance and organization at day 10. $^{#}p<0.05$, $^{##}p<0.01$ versus the control groups, $^{*}p<0.05$ versus cell groups, $^{*}p<0.05$ versus the rest of the groups. Scale bars: A, C(main), 500 μm ; C(inset), 50 μm .

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of stromal cell groups ($^{##}p<0.05$) and sheet groups ($^{##}p<0.01$) were significantly higher than that of the control groups (Fig. 3B,D). No statistically significant difference regarding total wound healing was observed among experimental groups at days 5 and 10 (Fig. 3B,D).

Granulation of tissue formation and maturation in wounds

The absolute amount of newly formed granulation tissue was not significantly different among groups at day 5 (Fig. 4C). However, the shape of granulation tissue among groups was significantly different. The width and depth of granulation tissues in sheet and cell groups were shorter and deeper than those in control groups (Fig. 4A). The granulation tissue of sheet and cell groups seemed to be denser and more compact, especially PDGF-CS and UCS groups. The histological score index of PDGF-CS and UCS groups was significantly higher than that of control groups ($^{#}p<0.05$) (Fig. 4B,D). PDGF-MSCs and U-MSCs groups also showed higher infiltration of inflammatory cells and fibroblasts than control groups, but this was not

statistically significant.

At day 10, the size and depth of granulation tissues of PDGF-CS and UCS groups were more prominent (Fig. 5A). The amount of upper granulation tissue in PDGF-CS and UCS groups ($^{#}p<0.05$, $^{*}p<0.05$) was significantly greater than that in cells and control groups. Lower granulation tissues sizes in sheet ($^{##}p<0.01$) and cell groups ($^{#}p<0.05$) were significantly greater than in control groups, and the total granulation tissues in the sheet ($^{##}p<0.01$) and cell groups ($^{#}p<0.05$) were significantly more organized than in control groups (Fig. 5A,B). Among experimental groups, the PDGF-CS groups presented the largest granulation tissue size. When compared to cell groups, significantly greater tissue sizes were observed ($^{*}p<0.05$) (Fig. 5B).

In the assessment of tissue maturation at day 10 using the HS index, upper granulation tissues in sheet and cell groups showed a higher density of fibroblasts, blood vessels and collagen deposition than in control groups ($^{#}p<0.05$, $^{##}p<0.01$) (Fig. 5C,D). Particularly the upper granulation of PDGF-CS groups showed the most monocytes infiltration, the large diameter of blood vessels and the highest density of fibroblasts, and collagen that smoothly connected to lower granulation

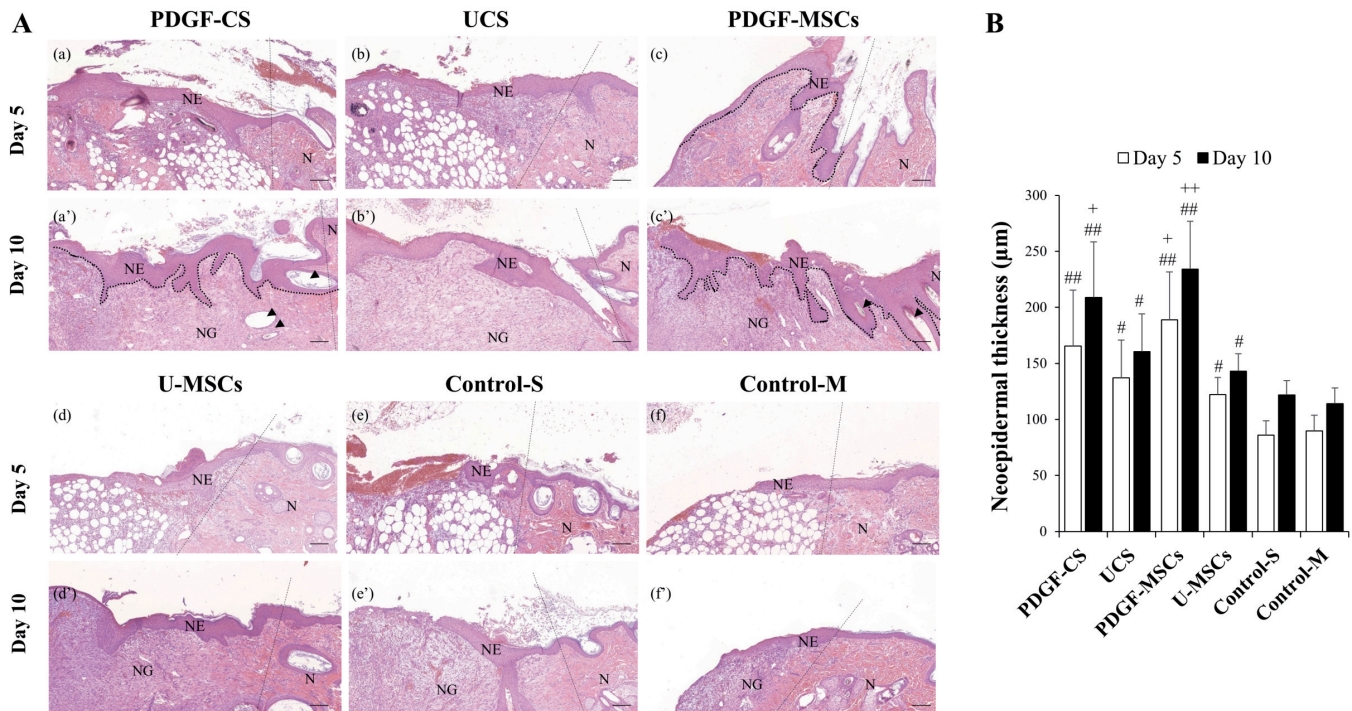


Fig. 7. Neopidermis shape and thickness measurement. **A.** Representative images of epidermis regeneration at day 5 (a-f) and day 10 (a'-f') stained with H&E evidencing the difference in the shape and thickness of the neopidermis among groups. Rete ridges-like structures (outlined by the dashed line) were identified in the neopidermis of PDGF-MSCs groups (c) at day 5 and PDGF-CS (a') and PDGF-MSCs (c') groups at day 10. The regeneration of the skin appendages such as hair follicle (arrow head) was identified in the neopidermis and neogranulation of PDGF-CS (a') and PDGF-MSCs (c') groups at day 10. A straight dotted line indicates the border between a wound and normal skin. The PDGF-MSCs groups showed the thickest neopidermis among groups at both days 5 and 10. **B.** Quantitative data of neopidermis thickness at days 5 and 10. NE, neopidermis; NG, neogranulation; N, normal skin; $^{#}p<0.05$, $^{##}p<0.01$ versus the control groups, $^{*}p<0.05$, $^{++}p<0.01$ versus unexpressed groups (UCS, U-MSCs). Scale bars: 200 μm.

tissues ($\bar{p}<0.05$). Lower granulation tissues presented high maturation of tissue conditions dominated by extensive collagens, fibroblasts, and no PMNL in all groups (Fig. 5C,D). Overall, the quality of total granulation tissues in sheets ($^{##}p<0.01$) and cell groups ($^{\#}p<0.05$) was higher than in control groups and PDGF-CS had the highest histological score on upper granulation tissue and total granulation tissue among groups ($^{##}p<0.01$, $\bar{p}<0.05$) (Fig. 5C,D).

Vascular density

On day 5, the density of blood vessels in the granulation tissue was not significantly different among groups. On day 10, the number of blood vessels increased and a significant difference was found. PDGF-CS, PDGF-MSCs, UCS groups ($^{##}p<0.01$) and U-MSCs group ($^{\#}p<0.05$) showed more blood vessels in the wound tissues than the control groups (Fig. 5C,E). Despite no statistical difference among experimental groups, PDGF-CS and PDGF-MSCs groups presented a tendency to have large diameter vessels, as well as the most and the second most number of blood vessels among the groups (Fig. 5C,E).

Collagen deposition

At day 5, collagen deposition in wounds of sheet groups ($^{##}p<0.01$, $^*p<0.05$) significantly increased when compared to both control and cell groups (Fig. 6A,B). Collagen in the wounds of the control groups was rarely found and wounds in the cell groups showed more collagen fibers than in control groups, but this was not significant (Fig. 6A,B). At day 10, wounds in all sheet ($^{##}p<0.01$) and cell groups ($^{\#}p<0.05$) had significantly more collagen infiltration than in control groups and PDGF-CS groups ($\bar{p}<0.05$) showed significant collagen deposition with much denser and thicker fibers in wounds than the rest of the groups (Fig. 6C,D).

Epithelialization

From the histological observation of H&E images, it was clear that the shape and the thickness of the neoepidermis were different among groups. First, PDGF groups like PDGF-CS and PDGF-MSCs groups ($^{##}p<0.01$) and unexpressed groups like UCS and U-MSCs groups ($^{\#}p<0.05$) showed a longer and thicker neoepidermis than that in control groups both at days 5

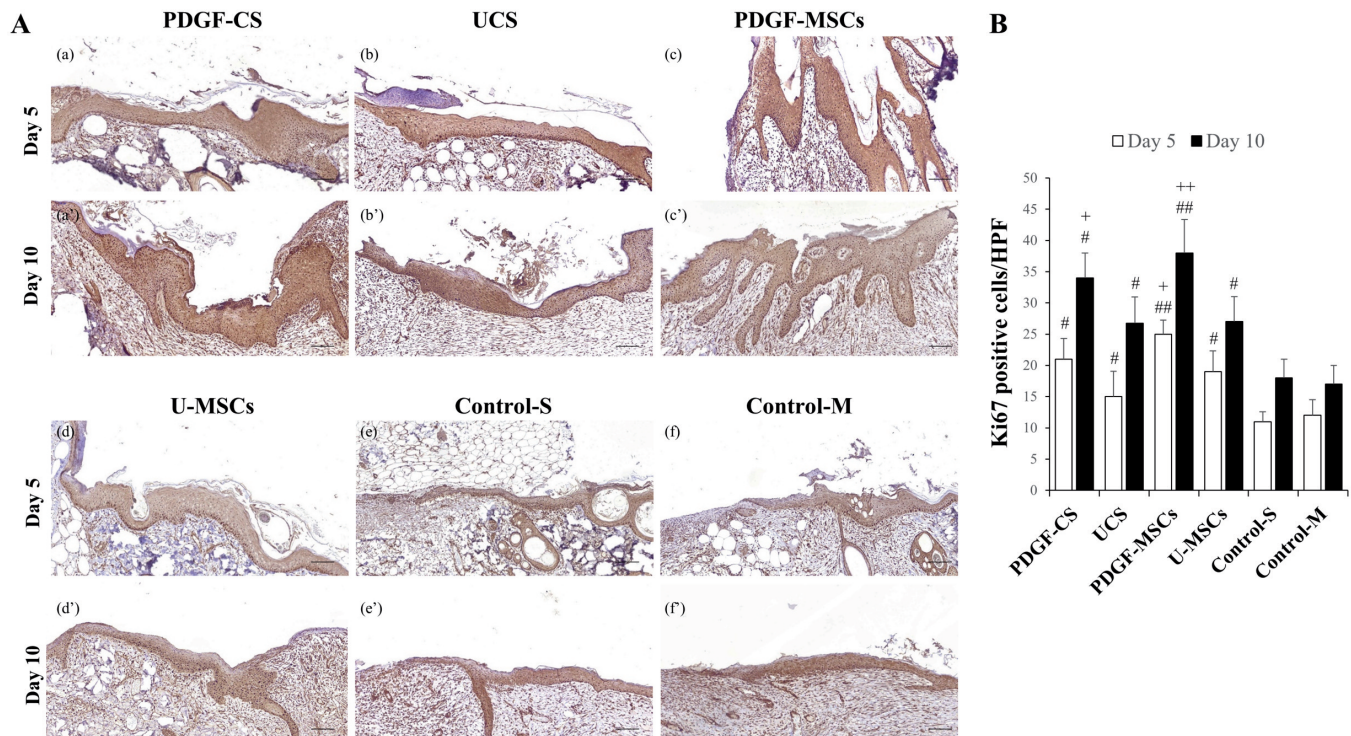


Fig. 8. Keratinocyte proliferation (Ki67) in the neoepidermis. **A.** Immunohistochemical analysis of the expression of Ki67 in the epidermis at day 5 (a-f) and day 10 (a'-f') evidencing the different pattern and number of its expression in neoepidermis among groups. **B.** Quantitative data of Ki67-positive cells of the neoepidermis in the high power field (400 $\times$) at days 5 and 10. PDGF-CS and PDGF-MSCs groups presented significantly more Ki67 expression when compared to unexpressed groups such as UCS, U-MSCs groups ($^*p<0.05$, $^{++}p<0.01$). The PDGF-MSCs groups showed the greatest number of expression cells among experimental groups at days 5 and 10. $^{\#}p<0.05$, $^{##}p<0.01$ versus the control groups, $^*p<0.05$, $^{++}p<0.01$ versus unexpressed groups (UCS, U-MSCs). Scale bars: 100 μ m.

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and 10 (Fig 7A,B). While the epidermis in the control groups was hypertrophied and slightly migrated over the granulation tissue adjacent to the normal tissue (Fig. 7A(e),(e'),(f),(f')), the epidermis in the experimental groups was greatly hypertrophied, actively migrated, and invaded over the granulation tissue (Fig. 7A(a)(a')-(d)(d')) at days 5 and 10. Among the experimental groups, only PDGF-MSCs groups at day 5 and PDGF-MSCs and PDGF-CS groups at day 10 presented prominent rete ridges-like structures (Fig. 7A; dashed lines under neoepidermis) and skin appendages like hair follicles frequently (Fig. 7A; arrowhead). Moreover, those PDGF groups showed a significant difference regarding the shape and thickness of neoepidermis when compared to unexpressed groups ($^+p<0.05$, $^{++}p<0.01$) (Fig. 7A(a'),(c),(c'),B). The PDGF-MSCs groups

showed the thickest neoepidermis among groups on both days 5 and 10.

Keratinocyte proliferation

On day 5, in the control groups, Ki67 expression was identified mostly in basal layers (Fig. 8A(e),(f)), while for experimental groups, its expression was found in both basal and suprabasal layers (Fig. 8A(a)-(d)) and especially in PDGF-MSCs groups, the expression was distinct (Fig. 8A(c)). Moreover, on day 10, its expression was located within most of the basal cells and several suprabasal cells in the control groups (Fig. 8A(e'),(f')), while in experimental groups, the expression was found throughout the basal and suprabasal layers (Fig. 8A(a)-(d)). Rete ridge-like structures in the PDGF-MSCs

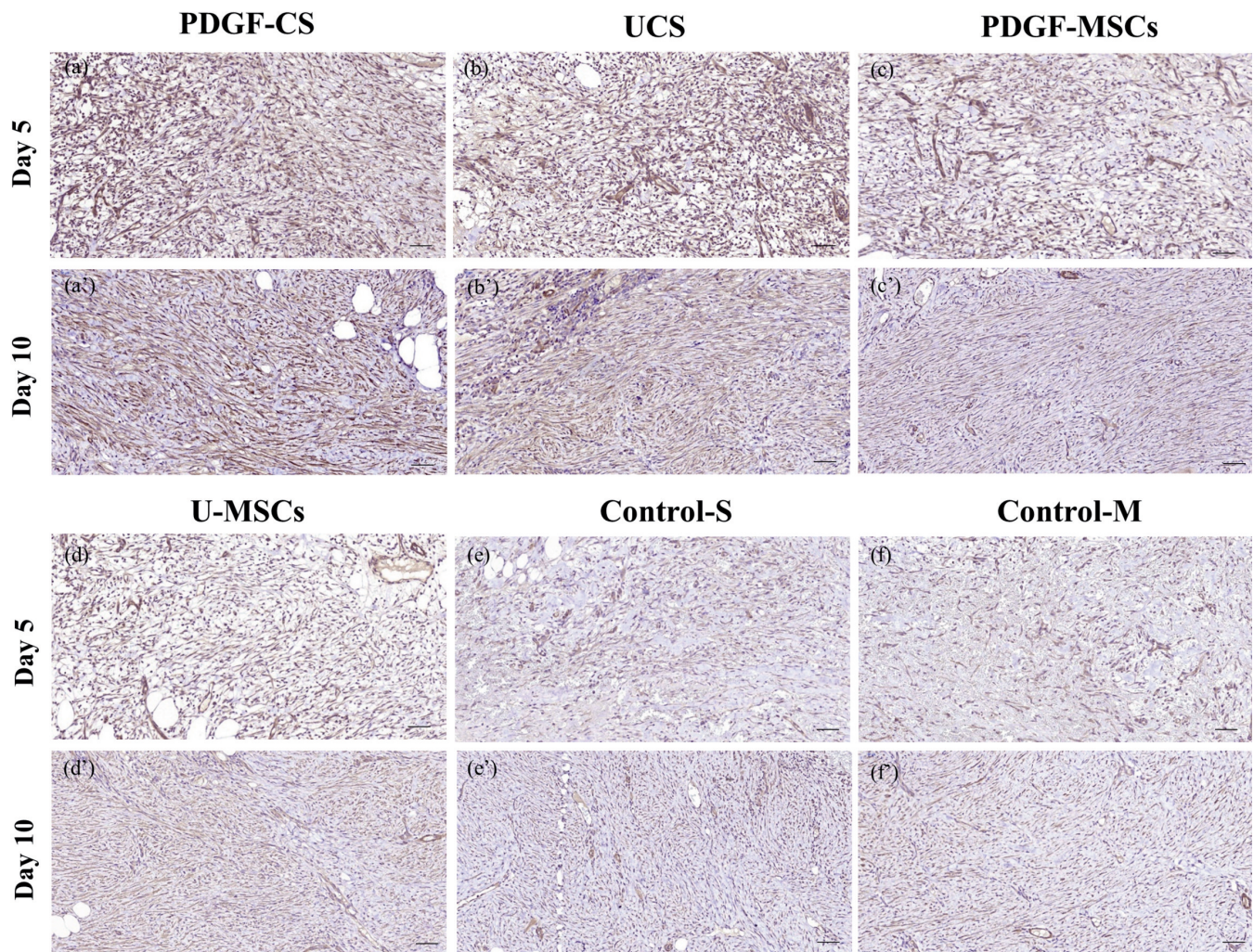


Fig. 9. Activated stromal fibroblasts in granulation tissues (fibroblast activation protein alpha (FAP)). FAP were similarly presented as collagen depositions. Immunohistochemical analysis of the expression of FAP in granulation tissues at day 5 (a-f) and day 10 (a'-f') evidencing the different density and pattern of its expression among groups. Scale bars: 50 μ m.

groups showed the most prominent Ki67 expression throughout the layers among groups (Fig. 8A(c')).

The number of Ki67 expressing cells in the neoepidermis also differed among groups (Fig. 8B). All experimental groups had more expression cells than the control groups at days 5 and 10 ($^{\#}p<0.05$, $^{\#\#}p<0.01$). At day 5, only the PDGF-MSCs groups and at day 10, PDGF-CS and PDGF-MSCs groups presented significantly more Ki67 expression when compared to unexpressed groups such as UCS, U-MSCs groups ($^{+}p<0.05$, $^{++}p<0.01$). The PDGF-MSCs groups showed the greatest number of expression cells among experimental groups at days 5 and 10 (Fig. 8B).

Activated stromal fibrosis

Activated stromal fibroblasts were similarly presented as collagen depositions. At day 5, PDGF-CS (5.0 ± 0.7) and UCS (4.5 ± 0.8) groups indicated significantly higher expression than control-S (2.5 ± 0.5) and control-M (2.25 ± 0.4) groups ($^{\#}p<0.05$) and the PDGF-CS group (5.0 ± 0.7) showed the most FAP expression (Fig. 9(a),(b),(e),(f)). At day 10, PDGF-CS groups (7.25 ± 0.4) (Fig. 9(a')) had the most well-oriented, abundant and densely expressed fibroblasts among groups and showed more significantly expressed stains than the rest of the groups ($^{\#\#}p<0.01$, $^{\#}p<0.05$) (Fig. 9(a')-(f')). The UCS (6.75 ± 0.4) groups indicated more FAP expression than cell groups ($^{\#\#}p<0.01$, $^{*}p<0.05$). PDGF-MSCs (5.25 ± 0.4) (Fig. 9(c')) and U-MSCs (5.5 ± 0.5) (Fig. 9(d')) groups presented significant expression compared to control-S (3.5 ± 0.5) (Fig. 9(e')) and control-M (3.75 ± 0.4) (Fig. 10(f')) ($^{\#}p<0.05$). No statistical difference between PDGF-MSCs and U-MSCs was observed at days 5 and 10.

Discussion

In the present study, our results showed that all experimental groups, PDGF-CSs, UCSs, PDGF-MSCs, and U-MSCs, accelerated cutaneous wound healing. Among them, PDGF groups, especially PDGF-MSCs, significantly increased epithelialization histologically. PDGF-CSs and UCSs promoted the formation of upper granulation tissue, collagen, and activated fibroblasts compared to PDGF-MSCs, and U-MSCs. Especially, PDGF-CSs presented the highest formation and maturation of granulation tissue among all groups.

MSCs have been used to improve wound healing under impaired or normal condition (Isakson et al., 2015). Among paracrine factors, PDGF, a potent growth factor that is FDA approved for wound healing, has been proven to enhance the proliferation of fibroblasts and epithelialization, and thus accelerate wound healing (Barrientos et al., 2008). We potentiate the usefulness of Ad-MSCs by transfecting with PDGF genes and upregulating PDGF expression by approximately 200-fold. In addition, VEGF, vascular-related factor and COX-2 and IL-6, inflammatory factors, were slightly

upregulated.

Regarding re-epithelialization, previous *in vivo* studies demonstrated that PDGF accelerated re-epithelialization in the wounds of many species through topical application, adenovirus-mediated expression, and gene transfer therapy (Liechty et al., 1999; Judith et al., 2010). In this study, our result also showed that there was an increase in re-epithelialization in dogs administered PDGF overexpressing cells and sheets as compared to those administered others. Feasible reasons were that PDGF upregulated the production of insulin-like growth factor-1 (IGF-1), which increased keratinocyte motility (Rabhi-Sabile et al., 1996) and PDGF activated and stimulated macrophages and fibroblasts to secrete keratinocyte-stimulating substances, like keratinocyte growth factor (KGF) (Pierce et al., 1991). Given that epidermis related factors, such as KGF, EGF, and IGF-1, were not significantly expressed in PDGF-CS and PDGF-MSCs, a great amount of PDGF expression might play a primary role in keratinocyte proliferation and re-epithelialization. One study reported that the effect of PDGF on epithelialization was due to activated keratinocytes in wound margins reacting directly to PDGF (Pierce et al., 1991).

In the present study, significantly thicker neoepidermis and more keratinocyte proliferation in the PDGF-MSCs group were observed earlier as compared to those in the PDGF-CS group. While cell sheet transplantation yielded more cell survival compared to cell injection into the wound (Takeuchi, 2016), the cells in cell sheet remain mainly on wound bed, not the margin in which the keratinocytes are located. It could, thus, be suggested that intradermal injection of PDGF-MSCs around the wound may induce a higher number of PDGF-MSCs to proliferate the keratinocytes of the epidermis while PDGF-CS just contacts wound margins. Further studies will be needed for cell differentiation of MSCs in wound.

Wound contraction was related to the locomotion of proliferating fibroblasts into wound beds and the differentiation of fibroblasts into myofibroblasts (Darby et al., 1990). Myofibroblasts predominate within the wound and increase early wound closure (Richey et al., 1989). Fibroblasts make collagen to reinforce the wound as myofibroblasts contract (Stadelmann et al., 1998). MSCs increased wound contraction and reduced wound healing time with significantly higher FGF levels in the wound (Uysal et al., 2014) and PDGF was known to play a role in contraction by enhancing the proliferation of fibroblasts, inducing the myofibroblast phenotype, and stimulating fibroblasts to construct collagen matrices (Barrientos et al., 2014). As shown in previous studies, our results also presented that In the gross analysis, significant wound contraction was present in experimental groups, compared to control groups on day 10, although the PDGF groups showed no significant wound contraction compared to unexpressed groups. Histologically, the width and depth of granulation tissue

observed by H&E staining were narrower and deeper, respectively, in the experimental groups. While in control groups, the shape of granulation tissue was wide and shallow, which might suggest that experimental groups had more contraction of granulation tissues.

Among the experimental groups, the PDGF-CS group showed the most granulation tissue formation and had higher histological scores than the UCS groups on day 10. In addition, collagens and activated fibroblasts were more abundant, dense and well organized in PDGF-CS groups than in UCS groups. Previous studies have shown that PDGF stimulates mitogenicity and chemotaxis of fibroblasts, macrophages, and smooth muscle cells to wound areas (Heldin and Westermark, 1999) and also increases macrophage-mediated tissue debridement and granulation tissue formation (Uutela et al., 2004). Although the exact mechanism is yet to be elucidated, the wound healing effect of exogenous PDGF has been demonstrated in other acute and chronic wound models (Gowda et al., 2015; Cheng et al., 2007). In previous *in vivo* studies, PDGF was shown to improve the proliferation of fibroblasts, and, thus the production of ECM (Cheng et al., 2007; Lin et al., 2006). In addition, it was shown to stimulate fibroblasts to contract collagen matrices (Rhee and Grinnell, 2006) and enhance granulation tissue formation (Cheng et al., 2007). As in previously reported studies, presumably overexpressed PDGF seems to have an effect on the wound beds for granulation tissue formation and maturation in this study.

The sheet groups presented significant maturation of granulation tissue on day 5 and accelerated improvement in the quantity and quality of both the upper and lower granulation tissue by day 10, while the cell groups only showed the maturation of granulation tissues at day 10. The difference in granulation tissue response for cell sheets and cells may be attributable to the cell delivery system. Previous studies have reported that the general approach for cell delivery has been the direct injection of single-cell suspensions around the wound bed (Nie et al., 2011; Rodriguez et al., 2015); however, using this approach, a few of the transplanted cells persist, migrate and participate in wound healing of wound (Suh et al., 2005). Also, a recent study showed that within 2 weeks after intradermal injection around wounds, the number of MSCs significantly decreased in the cutaneous wound bed (Wu et al., 2007). This was presumably because the residence time of MSCs in the wound bed was reduced (Wu et al., 2007) or the proper microenvironment was needed for MSCs survival and transdifferentiation (Yew et al., 2011). An innovative alternative to the limitation of cell injection is cell sheet engineering. Cell sheets are a scaffold-free approach in that cell-to-cell junctions are preserved via cell junction proteins and cells form a contiguous cell sheet along with their deposited extracellular matrix (Matsuda et al., 2007). Thereby, cell residence time, engraftment, and cell survival rates increased (Lin et al., 2013), overcoming one of the major problems with the injection of cell suspensions. One

study reported that Ad-MSCs were detected in the wound bed at 21 days after applying cell sheets on the wound (McLaughlin and Marra, 2013). As shown in previously reported studies, along with in this study, long residences of MSCs of cell sheets in wounds could produce different response by increasing the migration of fibroblasts and neo-vascularization in the wound bed.

This is the first study to report the characteristics and therapeutic effects of PDGF-overexpressed MSCs and their sheets in comparison to MSCs and their sheets in dog skin. Further studies will be required to examine the therapeutic effects of PDGF-overexpressed MSCs and sheets in impaired skin conditions, such as diabetic ulcers and burns in dogs. Furthermore, in the future, this technique could be applied to treat various skin conditions in humans.

In conclusion, PDGF-overexpressed stromal cells or cells sheets can improve cutaneous wound healing in dogs. PDGF-CS improved the most granulation tissue formation and maturation, and PDGF-MSCs accelerated epithelialization the most.

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