



## Original Article

## Cytological character of mini pig mesenchymal stromal cells from various tissues and the attempt of cell sheet formation



Nobuyuki Kaibuchi<sup>a, b</sup>, Takanori Iwata<sup>a, b, \*\*</sup>, Satoru Onizuka<sup>a, b</sup>, Kosei Yano<sup>b</sup>, Masayuki Yamato<sup>b</sup>, Teruo Okano<sup>b, \*</sup>, Tomohiro Ando<sup>a</sup>

<sup>a</sup> Department of Oral and Maxillofacial Surgery, Tokyo Women's Medical University School of Medicine, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

<sup>b</sup> Institute of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University (TWMU), 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan

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## ABSTRACT

**Introduction:** Large animal experiments are important for translational research in regenerative medicine. Recently, mini pigs have been used in large animal studies and surgical training. The use of multipotent mesenchymal stromal cell (MSC) sheets for the treatment of many diseases is increasing. The purpose of the present study was to establish optimal methods for generating mini pig MSC sheets from various tissues and to compare the properties of MSCs in these sheets.

**Methods:** MSCs were isolated from the bone marrow, adipose, periodontal ligament, gingiva, or periosteum of mini pigs. The proliferation, markers, and mRNA expression of these MSCs were examined. Colony-forming and differentiation assays were performed. MSCs were seeded onto temperature-responsive culture dishes to develop MSC sheets.

**Results:** MSCs derived from bone marrow (BMSCs), adipose (ASCs), periodontal ligament (PDLCS), gingiva (GMSCs), and periosteum (PSCs) were positive for MSC-related markers. BMSCs and PSCs showed increased proliferation compared with other MSCs. The osteogenic potential of PDLCS and the adipogenic potential of PSCs were the highest among these MSCs. The expression levels of COL1A1 and COL3A1 in BMSCs and PSCs were significantly higher than those in other MSCs. The expression levels of FGF2, VEGFA, ICAM-1, and TIE-1 in GMSCs were significantly higher than those in other MSCs. PSCs showed the highest levels of TGF- $\beta$ 1 and ANG-1 expression among all MSC types. We succeeded in developing MSC sheets from BMSCs, ASCs, and PSCs.

**Conclusions:** We developed methods to generate MSC sheets from various tissues of mini pigs, and these methods are useful to pursue regenerative translational research using mini pigs.

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## 1. Introduction

Large animal experiments are an unavoidable step in translational research for regenerative medicine. Dogs, cats, and

monkeys have commonly been used in large animal studies. However, the disadvantages of the use of these animals include their consideration as companions and low reproduction rates. By contrast, pigs are commonly used as a food source and for surgical training [1,2]. In particular, it is easy to handle mini pigs because their body sizes are relatively small. Moreover, the size of the organs and faces of mini pigs are similar to those of humans [3]. Thus, there are many advantages to using mini pigs in various studies [4,5].

“Cell sheet engineering” has been reported as an effective approach for corneal dysfunction [6], myocardial infarction [7], esophageal ulcerations [8], diabetic ulcers [9], periodontitis [10], and osteonecrosis of the jaw [11]. Moreover, multipotent

\* Corresponding author.

\*\*Corresponding author. Institute of Advanced Biomedical Engineering and Science, Department of Oral and Maxillofacial Surgery, Tokyo Women's Medical University, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162-8666, Japan. Fax: +81 3 3359 6046.

E-mail addresses: [iwata.takanori@twmu.ac.jp](mailto:iwata.takanori@twmu.ac.jp) (T. Iwata), [tokano@twmu.ac.jp](mailto:tokano@twmu.ac.jp) (T. Okano).

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mesenchymal stromal cells (MSCs)<sup>1</sup> stimulate angiogenesis via the secretion of angiogenic factors, including vascular endothelial growth factor (VEGF)<sup>2</sup> and hepatocyte growth factor (HGF),<sup>3</sup> and stabilize new blood vessels by differentiating into pericytes [12]. In addition, many animal model studies and clinical trials have reported that MSCs have immunomodulatory and anti-inflammatory activities [13,14]. We previously reported that MSC sheets promote angiogenesis and wound healing by secreting VEGF and HGF [9,11]. Because of these MSC activities, the use of MSC sheets can be expected to increase in the treatment of many diseases.

The purpose of the present study was to establish optimal methods for creating MSC sheets derived from various tissues of mini pigs, including bone marrow, adipose tissue, periodontal ligaments, gingiva, and periosteum, for use in large animal studies in translational research. Furthermore, we compared the properties of these MSCs and observed that gingiva can be the best MSC source to promote angiogenesis, and the periosteum can be the best MSC source for bone regeneration.

## 2. Methods

### 2.1. Animals

Three mini pigs (10-month-old females) were obtained from the Laboratory Animal Research Station, Nippon Institute for Biological Science (NIBS, Tokyo, Japan) and used in the present study. All experimental protocols were approved by the animal welfare committee of Tokyo Women's Medical University.

### 2.2. Isolation and culture of MSCs from various tissues

MSCs were isolated from bone marrow, adipose, periodontal ligament, gingiva, and periosteum as previously described [9–11]. For example, bone marrow cells were collected from femurs, centrifuged at  $400 \times g$  for 5 min and subsequently cultured in complete medium ( $\alpha$ -MEM GlutaMAX [Invitrogen, Carlsbad, CA, USA] supplemented with 20% fetal bovine serum [FBS, Moregate Biotech, Bulimba, Australia] and 1% penicillin/streptomycin [Sigma–Aldrich, St Louis, MO, USA]) at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. Four days after seeding, the floating cells were removed after washing with phosphate-buffered saline (PBS, Life Technologies, Carlsbad, CA, USA), and the culture medium was replaced with fresh medium. After four days, the entire medium was replaced, and the adherent proliferating cells (bone marrow-derived mesenchymal stromal cells [BMSCs])<sup>4</sup> were subcultured using 0.25% trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA, Life Technologies) every three days.

The cells from solid tissues were isolated in the following manner. Subcutaneous fat was collected from the abdomens and enzymatically digested with PBS containing 0.1% type A collagenase (Roche Diagnostics, Indianapolis, IN, USA) under shaking for 1 h at 37 °C. The stromal-vascular fraction (SVF)<sup>5</sup> was collected after centrifugation at  $700 \times g$  for 5 min at room temperature. Mandibular premolars were extracted, and periosteum and gingiva were obtained from the lower jaw. The extracted teeth and periosteum were rinsed five times for 3 min each with  $\alpha$ -MEM GlutaMAX containing 1% penicillin/streptomycin. The periodontal ligaments were separated from the surface of the mid-third portion

of the extracted root, and the periosteum was dispersed with  $\alpha$ -MEM GlutaMAX containing 1% penicillin/streptomycin, 0.8 PZ-U/mL collagenase type I (Serva Electrophoresis, Heidelberg, Germany) and 1200 PU/mL dispase (Sanko Junyaku, Tokyo, Japan) under shaking for 45 min at 37 °C. The gingiva was sterilized three times with povidone-iodine and  $\alpha$ -MEM GlutaMAX containing 1% penicillin/streptomycin. The sterilized gingiva was cut into small pieces and treated with 1200 PU/mL dispase for 2 h at 37 °C. The gingival connective tissues were separated from the epithelium using forceps and minced using surgical micro scissors. The connective tissues were treated with 0.8 PZ-U/mL collagenase type 1 under shaking for 45 min at 37 °C.

Single cell suspensions of SVF, periodontal ligaments, periosteum, and gingiva were passed through a 70-mm strainer (Falcon, BD Labware, Franklin Lakes, NJ, USA) and cultured in complete medium in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> at 37 °C. After 24 h, the floating cells were removed, and the medium was replaced with fresh medium. The adherent cells (adipose-derived MSCs [ASCs]),<sup>6</sup> periodontal ligament-derived MSCs (PDLs),<sup>7</sup> periosteum-derived MSCs (PSCs),<sup>8</sup> and gingiva-derived MSCs (GMSCs)<sup>9</sup> were subcultured using trypsin-EDTA every three days until passage five (Fig. 1). For long-term culture, these MSCs were seeded onto 100-mm culture dishes at a density of 3000 cells/dish every seven days for 105 days.

### 2.3. Flow cytometry assay

MSCs isolated from each tissue at passage five were suspended in 500  $\mu$ L of PBS supplemented with 2% FBS and 10  $\mu$ g/mL of each specific antibody. To detect the surface markers, an Alexa Fluor-coupled antibody against CD29 (561496, BD Biosciences, Franklin Lakes, NJ, USA), fluorescein isothiocyanate (FITC)-coupled antibody against CD44 (ab19622, Abcam, Cambridge, UK), phycoerythrin (PE)-coupled antibody against CD90 (561970, BD Biosciences), and PE-coupled antibody against CD105 (ab69772, Abcam) were used. For the isotype control, Alexa Fluor-coupled mouse IgG1 $\kappa$  (55771, BD Biosciences), FITC-coupled rat IgG2b $\kappa$  (ab136125, Abcam), PE-coupled mouse IgG1 $\kappa$  (550618, BD Biosciences), and PE-coupled mouse IgG2a $\kappa$  (ab103534, Abcam) were substituted for the primary antibodies. PE-coupled goat IgG2a $\kappa$  (ab130787, Abcam) was used for the secondary antibody. After incubation for 30 min on ice, the MSCs were washed with PBS supplemented with 2% FBS and suspended in 500  $\mu$ L of PBS supplemented with 2% FBS. The cell fluorescence was measured using a flow cytometer (Gallios, Beckman Coulter, Brea, CA, USA).

### 2.4. Colony-forming assay

MSCs isolated from each tissue at passage five were seeded onto 100-mm culture dishes at a density of 1000 cells/dish and cultured in complete medium. Seven days after seeding, the MSCs were stained with 0.5% crystal violet (Kanto Chemical, Tokyo, Japan) in methanol for 5 min and washed twice with distilled water. Subsequently, colonies larger than 2 mm in diameter were counted.

### 2.5. Differentiation assay

To examine osteogenesis, MSCs at passage five were seeded onto 100-mm culture dish at a density of 1000 cells/dish and cultured for 7 day as previously described [15]. The medium was replaced with osteoinductive medium, comprising complete medium

<sup>1</sup> MSCs, multipotent mesenchymal stromal cells.

<sup>2</sup> VEGF, vascular endothelial growth factor.

<sup>3</sup> HGF, hepatocyte growth factor.

<sup>4</sup> BMSCs, MSCs derived from bone marrow.

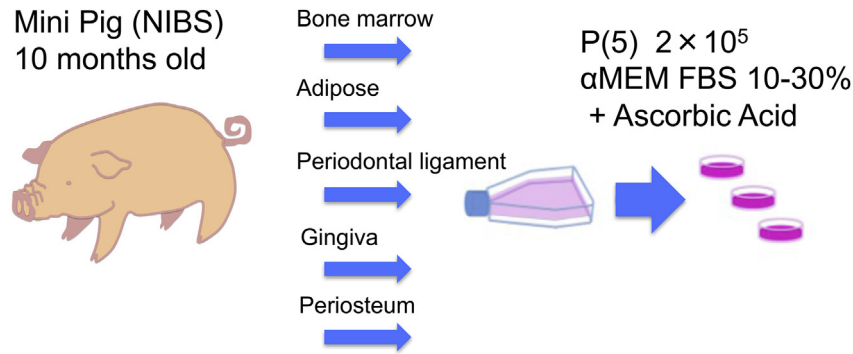
<sup>5</sup> SVF, stromal-vascular fraction.

<sup>6</sup> ASCs, MSCs derived from adipose.

<sup>7</sup> PDLs, MSCs derived from periodontal ligament.

<sup>8</sup> GMSCs, MSCs derived from gingiva.

<sup>9</sup> PSCs, MSCs derived from periosteum.



**Fig. 1.** Multipotent mesenchymal stromal cells (MSCs) were isolated from bone marrow, adipose, periodontal ligament, gingival, and periosteum tissue. MSCs isolated from each tissue at passage five were seeded onto temperature-responsive culture dishes at a cell density of  $2 \times 10^5$  cells/dish.

supplemented with 82  $\mu\text{g}/\text{mL}$  L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industry, Osaka, Japan), 10 mM  $\beta$ -glycerophosphate (Sigma–Aldrich), and 10 nM dexamethasone (Dexart, Fuji Pharma, Tokyo, Japan), for an additional 14 days. The MSCs were fixed with 4% paraformaldehyde and stained with 1% alizarin red S solution (Wako), and the alizarin red S-positive colonies were counted. To examine adipogenesis, MSCs at passage five were seeded onto 100-mm culture dish at a density of 1000 cells/dish and cultured for seven days, as previously described [15]. The medium was replaced with adipogenic medium, comprising complete medium supplemented with 100 nM dexamethasone, 0.5 mM isobutyl-1-methylxanthine (Sigma–Aldrich), and 50 mM indomethacin (Wako), for an additional 14 days. The MSCs were fixed with 4% paraformaldehyde and stained with fresh Oil Red O solution (Wako), and the Oil Red O-positive colonies were counted.

#### 2.6. Isolation of RNA and real-time polymerase chain reaction (PCR)

Total RNA was isolated using a QIAshredder and an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Subsequently, the cDNA was synthesized using the Superscript VILO cDNA Synthesis Kit (Invitrogen). Quantitative real-time PCR (StepOnePlus System, Applied Biosystems, Foster City, CA, USA) was performed using primers and probes (TaqMan Gene Expression Assays, Applied Biosystems) specific for collagen 1A1 (COL1A1)<sup>10</sup> (Ss03373340\_m1), collagen 3A1 (COL3A1)<sup>11</sup> (Ss04323790\_m1), VEGFA (Ss03393990), fibroblast growth factor 2 (FGF2)<sup>12</sup> (Ss03375809\_u1), transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1)<sup>13</sup> (Ss03382325\_u1), angiopoietin-1 (ANG-1)<sup>14</sup> (Ss03380513), intercellular adhesion molecule-1 (ICAM-1)<sup>15</sup> (Ss033923), tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1 (TIE-1)<sup>16</sup> (Ss03373354), and  $\beta$ -actin (Ss03376081\_u1). The mean fold-changes in gene expression relative to  $\beta$ -actin were calculated using the  $\Delta\Delta\text{CT}$  method at each time point.

#### 2.7. Preparation of MSC sheets

MSCs isolated from each tissue at passage five were seeded onto temperature-responsive culture dishes (35-mm diameter, UpCell, Cell

Seed, Tokyo, Japan) at a density of  $2 \times 10^5$  cells/dish. The MSCs were cultured in  $\alpha$ -MEM GlutaMAX (Invitrogen) with 10, 20, or 30% FBS (Moregate Biotech) and 1% penicillin/streptomycin (Sigma–Aldrich) with 82  $\mu\text{g}/\text{mL}$  ascorbic acid (Wako) for 5–9 days. The temperature of the culture dishes was reduced to room temperature, and subsequently the medium was removed to produce MSC sheets (Fig. 1).

#### 2.8. Histological analysis

The cell sheets were harvested, fixed with 4% paraformaldehyde, and embedded in paraffin. Next, the sections (5- $\mu\text{m}$  thickness) were cut using a microtome (Model 2255, Leica Microsystems, Wetzlar, Germany), and the sections were stained with hematoxylin and eosin. The thicknesses of the cell sheets from each tissue were measured in triplicate.

#### 2.9. Statistics

The mean differences were analyzed using one-way analysis of variance, followed by Tukey's multiple range test using JMP Pro 11.0.0 (SAS, Cary, NC, USA). A p-value of less than 0.05 was considered significant.

### 3. Results

#### 3.1. Cell proliferation and MSC markers

The cumulative number of MSCs from each tissue was counted at 105 days. BMSCs and PSCs showed increased proliferation compared to the MSCs from other tissues (Fig. 2A). Flow cytometric analysis revealed that BMSCs, ASCs, PDLCS, GMSCs, and PSCs were positive for the MSC-related markers to CD29, 44, and 90, and negative for the endothelial cell marker to CD31. There were no significant differences in the presence of MSC markers in the different tissues (Fig. 2B).

#### 3.2. Colony-forming assay and differentiation assay

In the colony-forming assay, the number of GMSCs and PSCs was significantly higher than the number of MSCs from other tissues (Fig. 3A). The MSCs from each tissue cultured with osteoinductive medium for 21 days showed alizarin red S-positive calcium deposits. The osteogenic potential of PDLCS was the highest of all cells from all tissues. ASCs and GMSCs showed little differentiation into osteoblasts (Fig. 3B). Each type of MSC cultured with adipogenic medium for 21 days had oil red O-positive lipid droplets. The adipogenic potential of PSCs was the highest among cells from all tissue types. BMSCs, ASCs, and GMSCs showed little

<sup>10</sup> COL1A1, collagen 1A1.

<sup>11</sup> COL3A1, collagen 3A1.

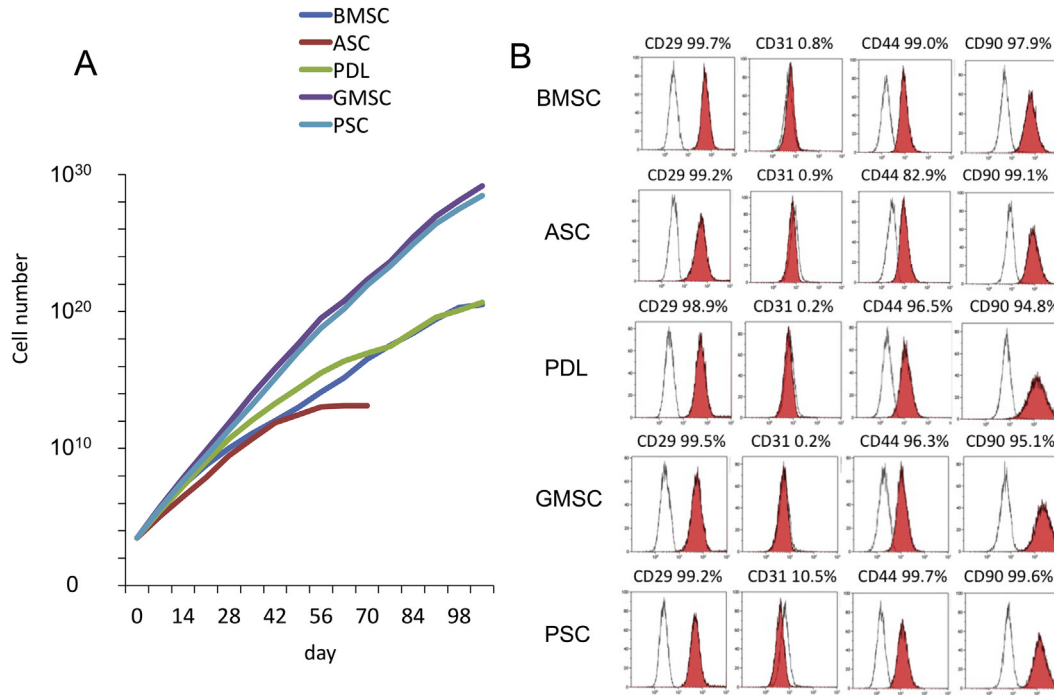
<sup>12</sup> FGF2, fibroblast growth factor 2.

<sup>13</sup> TGF- $\beta$ 1, transforming growth factor- $\beta$ 1.

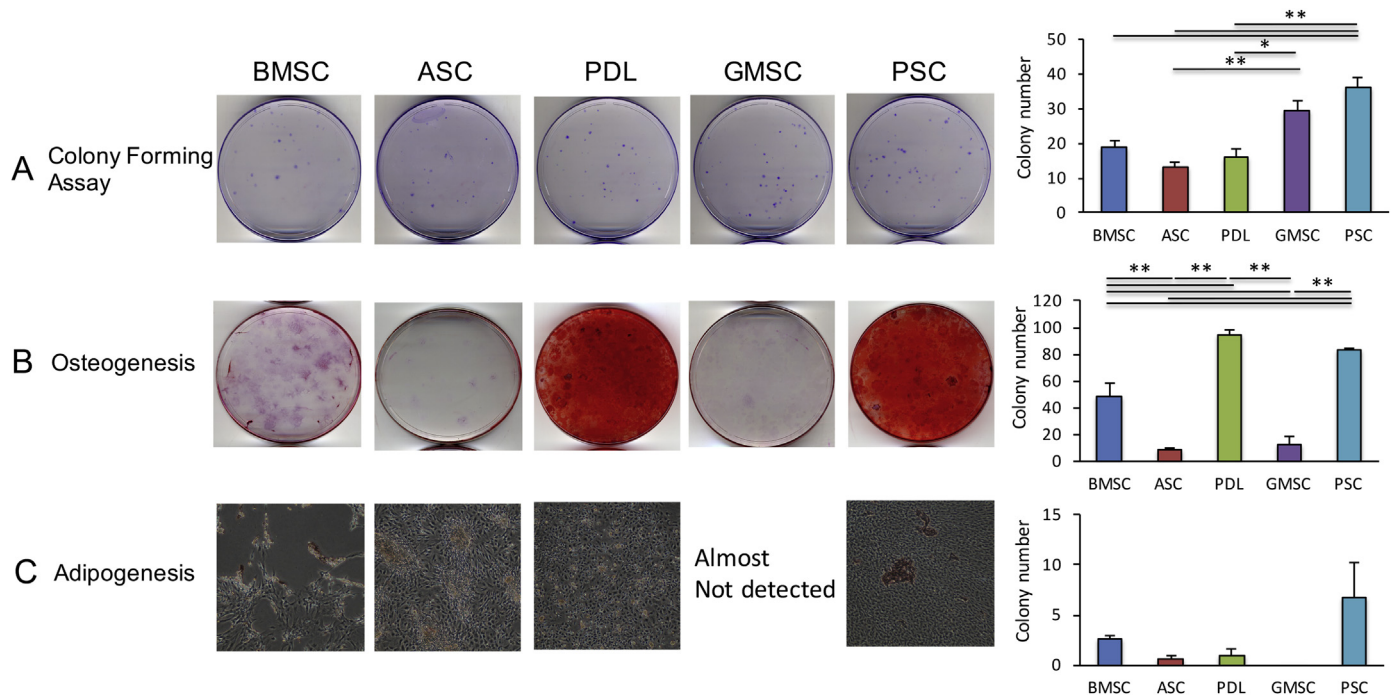
<sup>14</sup> ANG-1, angiopoietin-1.

<sup>15</sup> ICAM-1, intercellular adhesion molecule-1.

<sup>16</sup> TIE-1, tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains 1.



**Fig. 2.** The cumulative number of multipotent mesenchymal stromal cells (MSCs) from each tissue type was counted at 105 days. Flow cytometric analysis revealed that bone marrow-derived MSCs, adipose-derived MSCs, periodontal ligament-derived MSCs, gingiva-derived MSCs, and periosteum-derived MSCs were positive for MSC-related markers identified using CD29, 44, and 90 antibodies and negative for the endothelial cell marker using the CD31 antibody.



**Fig. 3.** In the colony-forming assay, gingiva-derived multipotent mesenchymal stromal cells (MSCs) and periosteum-derived MSCs showed significantly increased colony formation compared to the MSCs from other tissues. The MSCs from each tissue type were cultured with osteoinductive medium for 21 days and analyzed for alizarin red S-positive calcium deposits. The MSCs from each tissue type were cultured with adipogenic medium for 21 days and analyzed for Oil Red O-positive lipid droplets.

differentiation into adipocytes. Particularly, few adipocytes were observed in the GMSC culture dishes. However, there was no significant difference in results of the adipogenesis assay based on tissue type (Fig. 3C).

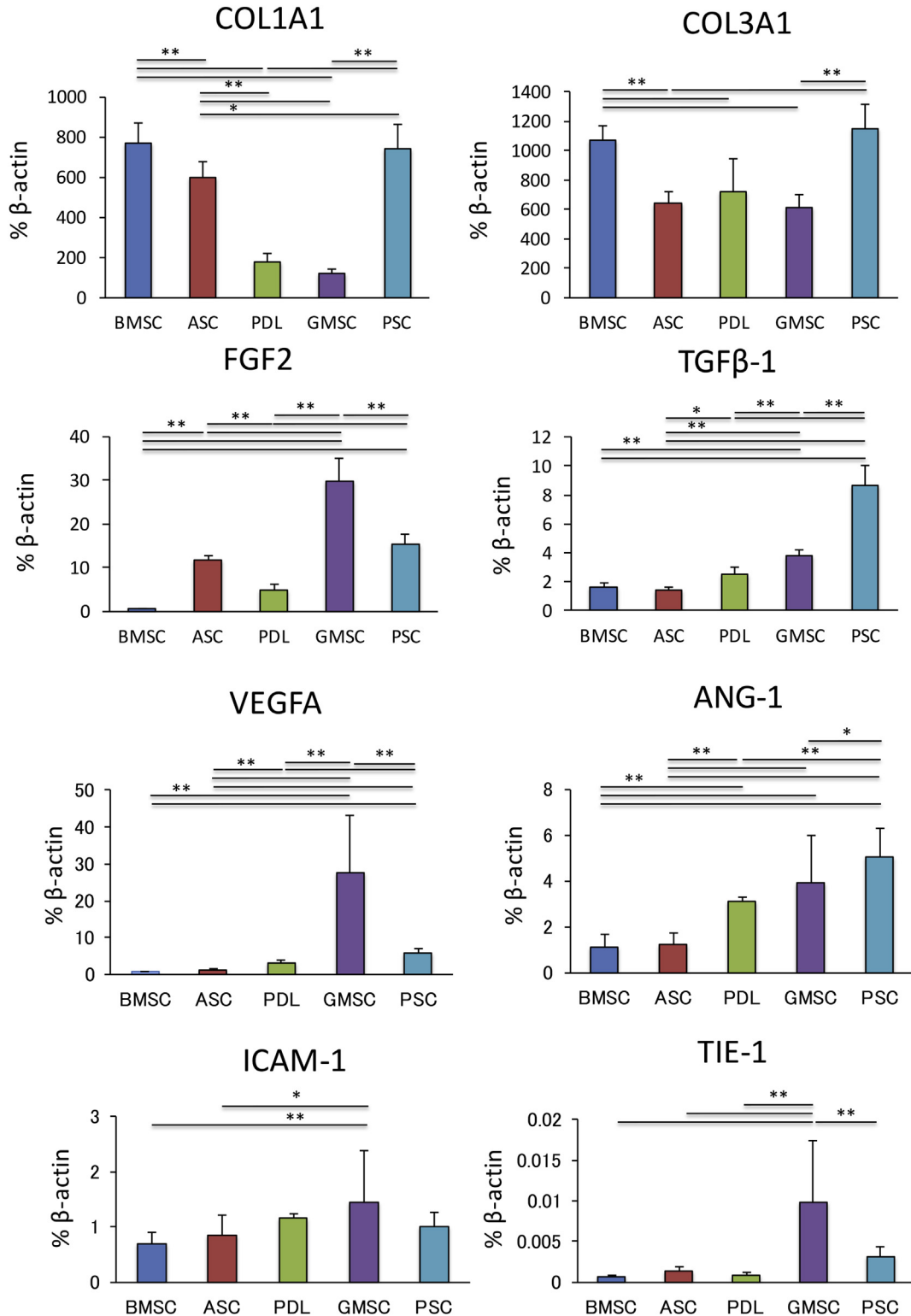
### 3.3. mRNA expression levels

The mRNA expression levels of some genes were evaluated in MSCs from each tissue. The expression levels of COL1A1 and

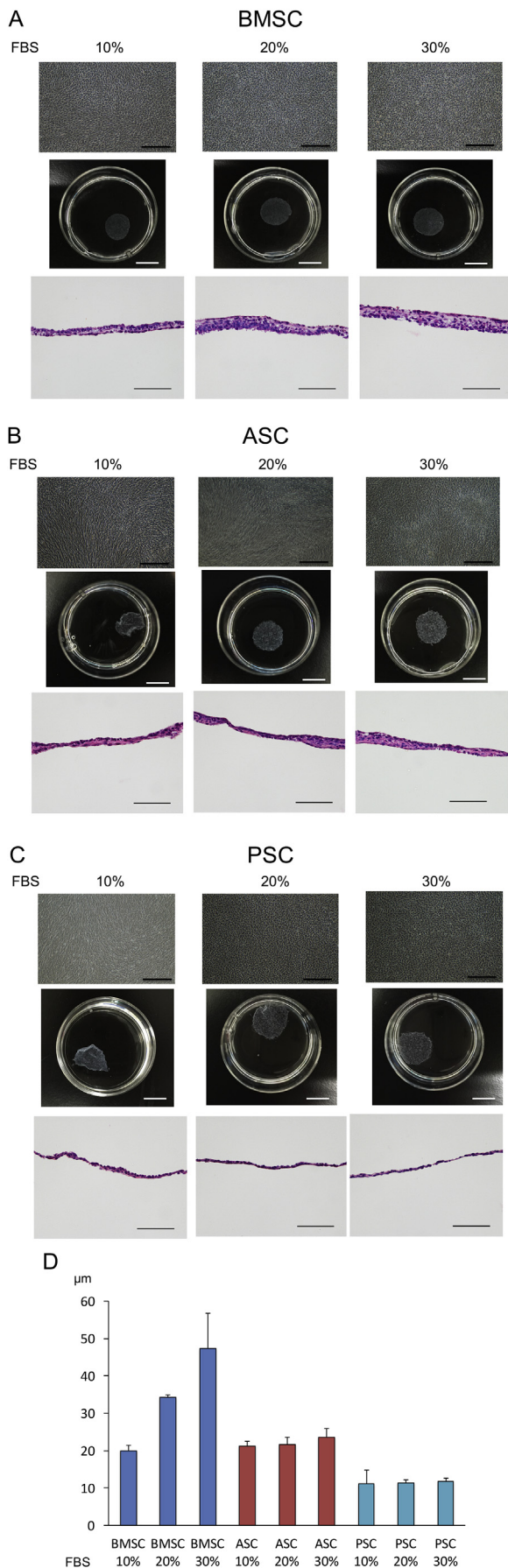
COL1A1 in BMSCs and PSCs were significantly higher than those in MSCs from other tissues. The expression levels of FGF2, VEGFA, ICAM-1, and TIE-1 in GMSCs were significantly higher than those in MSCs from other tissues. PSC showed the highest levels of TGF-β1 and ANG-1 expression of MSCs from all tissue types.

### 3.4. The development of MSC sheets from each tissue

We generated MSC sheets from each tissue using temperature-responsive culture dishes and various serum concentrations. Although we succeeded in developing MSC sheets from BMSCs,



**Fig. 4.** The expression levels of COL1A1 and COL3A1 in bone marrow-derived multipotent mesenchymal stromal cells (MSCs) and periosteum-derived MSCs were significantly higher than those in MSCs from other tissues. The expression levels of FGF2, VEGFA, ICAM-1, and TIE-1 in gingiva-derived MSCs were significantly higher than those in MSCs from other tissues. Periosteum-derived MSCs showed the highest levels of TGF-β1 and ANG-1 expression of MSCs from all tissue types.



ASCs, and PSCs, PDLCs and GMSCs could not be harvested as a sheet (Fig. 5A–C). Whereas the culture period for BMSC sheets was five days, that for ASC and PSC sheets was nine days. In terms of thickness, PSC sheets were thinner than BMSC or ASC sheets. Moreover, the thickness of BMSC sheets was dependent on the FBS concentration (Fig. 5D).

#### 4. Discussion

MSCs have been reported as effective and useful cell sources for regenerative therapies [9,11,13,14]. As MSCs can be isolated from various mesenchymal tissues, differences in the abilities of MSCs from different tissues have been reported [16,17]. Furthermore, there are also differences depending on the species that serves as the MSC source. Thus, many factors should be considered when performing animal studies using MSCs.

The proliferation of human BMSCs and synovium- and periosteum-derived cells is retained in culture, even at passage 10; however, proliferation of human ASCs and muscle-derived cells is lost at passage 4–7 [16]. As shown in Fig. 2A, the proliferation of mini pig ASCs was lost before 105 days, consistent with the results of the human MSC study.

The increased osteogenic potential of human BMSCs, PSCs, and synovium-derived cells and increased adipogenic potential of human ASCs and synovium-derived cells were observed in an in vitro study [16]. Another study showed that the calcification potentials of rat PSCs and muscle-derived cells were increased, and the Oil Red O positive colony rate provided higher adipogenic potential in ASCs and synovium-derived cells than those in other tissue types [17]. The results of the present study showed that the osteogenic potential of mini pig BMSCs, PDLCs, and PSCs was higher than that of MSCs from other tissues, and the adipogenesis of mini pig MSCs, except PSCs, was minimal. This result may reflect differences in the effects of human adipoinductive and pig adipoinductive media. To establish appropriate adipoinductive media for pig experiments, further studies are needed.

MSCs secrete trophic factors, including FGF, VEGF, and platelet-derived growth factor [18]. In the present study, the expression levels of various genes in each MSC source tissue type were determined. The preferred MSC source tissue is dependent on gene expression. The results showed that PSCs exhibit higher levels of COL1A1, COL3A1, TGF- $\beta$ 1, and FGF2 mRNA expression than other tissue-derived MSCs. Based on these results, PSCs may be preferred for bone regeneration over MSCs from other tissues. The expression of the genes for angiogenesis in GMSCs was higher than that in other MSCs. Thus, GMSCs may be effective for wound healing or the treatment of ischemic diseases. A previous in vitro experiment showed significantly higher VEGF expression levels in osteogenic-differentiated human ASCs compared to BMSCs [19]. In a study using a murine ischemic disease model, ASC administration resulted in the remarkable attenuation of ischemic damage and significantly higher levels of HGF and ANG-1 expression in ischemic brain tissue compared with BMSCs [20]. In a previous micro pig study, the expression of TGF $\beta$ 1/2 in ASCs was not significantly different from that of BMSCs and dermal skin-derived MSCs [21]. Although many studies using ASCs for wound healing or ischemic

**Fig. 5.** We developed multipotent mesenchymal stromal cell (MSC) sheets of bone marrow-derived MSCs (BMSCs), adipose-derived MSCs (ASCs), and periosteum-derived MSCs (PSCs). Whereas the culture period for BMSC sheets was five days, the culture period for ASC or PSC sheets was nine days. In terms of thickness, PSC sheets were thinner than BMSC or ASC sheets. Moreover, BMSC sheets become thicker with increasing fetal bovine serum concentration. Scale bar: Morphological image: 10 mm; Macro image: 10 mm; Histological image: 100  $\mu$ m.

diseases have been reported, GMSCs may be more effective than ASCs based on the results of the present mini pig study.

We showed that BMSCs, ASCs, and PSCs could be harvested in sheets using temperature-responsive culture dishes. However, PDLs and GMSCs could not be harvested as sheets under the culture conditions used in the present study. This finding may reflect the fact that these MSCs did not develop in multiple layers, although they were cultured over a long period. The culture conditions may need optimization to generate MSC sheets from these cells. In addition, we showed that BMSC sheets are thick enough to transplant easily without scaffolds.

## 5. Conclusion

We developed MSC sheets from cells derived from bone marrow, adipose, and periosteum tissue in mini pigs, which may be useful for animal studies.

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