Introduction

Mesenchymal stem cells (MSCs) have been isolated from various tissues, such as bone marrow and adipose tissues, and known to have specific surface markers and pluripotency.\(^1,2\) Similarly, mesenchymal cells isolated from iliac/cancellous bone can be induced to differentiate into cartilage and adipose tissues and have MSC-like properties as well as properties of bone progenitor cells.\(^3\) Mesenchymal cells with MSC-like properties can also be isolated from the jawbone,\(^4\) and their clinical applications have been reported in the field of craniomaxillofacial surgery.\(^5,6\) In the craniomaxillofacial region, the neural crest stem cells (NCSCs) play an essential role during the embryonic stage. After the neural crest disappears as the developmental stage progresses, only mesenchymal tissue remains with characteristics of neural crest, and this has attracted attention as the fourth

Original Contribution

Differences in pluripotency of jawbone- and iliac bone-derived mesenchymal cells

Kazuno Moriyama,\(^1\) Yasuharu Yamazaki,\(^1\) Yoshika Sugimoto,\(^1\) Takayuki Sugimoto,\(^1\) Kenichi Kumazawa,\(^1\) Kyoko Baba,\(^2\) Yumiko Sone,\(^1\) Akira Takeda\(^1\)

\(^1\)Department of Plastic and Aesthetic Surgery, Kitasato University School of Medicine
\(^2\)Department of Plastic Surgery, Kitasato University Medical Center

Objective: We evaluate osteogenic differentiation, adipogenic differentiation and chondrogenic differentiation of the jawbone- and iliac bone-derived mesenchymal cells.

Methods: Human bone-derived mesenchymal cells from: iliac, maxillary, mandibular, and alveolar bone samples were classified into 4 groups with induction to osteogenic, adipogenic, or chondrogenic differentiation (differentiation induction groups), and without induction (a non-differentiation induction group). Osteogenic differentiation was qualitatively and quantitatively evaluated by the determination of calcium (Ca) concentrations and alizarin red S staining, adipocyte differentiation was qualitatively analyzed by oil red O staining, and chondrogenic differentiation was qualitatively and quantitatively determined by hematoxylin and eosin staining, toluidine blue staining, and using real-time reverse transcription polymerase chain reaction (real time RT-PCR).

Results: Regarding the induction of osteogenic differentiation, Ca levels in the differentiation induction group started increasing 2−3 weeks after induction, and cells in this group were stained with alizarin red S; however, no significant differences were observed in the calcification level using the non-repeated measure one-way ANOVA and Kruskal-Wallis H-test. Regarding the induction of adipogenic differentiation, cells in the induced group were successfully stained with oil red O. The lipid droplet formation ability was categorized into the following 3 groups, with the highest ability in iliac bone-derived cells followed in the descending order by mandibular bone-then maxillary bone-derived cells. No adipogenic differentiation was observed for the induced alveolar bone-derived cells. Regarding the levels of chondrogenesis measured using real time RT-PCR, cells from different tissues were categorized into 3 groups: the level of chondrogenesis was the highest for iliac bone-derived cells, followed in the descending order by alveolar bone-derived cells, maxillary and mandibular bone-derived cells. However, 2 groups of cells were found in alveolar bone-derived cells, i.e., cells that underwent chondrogenic induction and cells that did not.

Conclusion: Differences were observed in the differentiation induction between jawbone- and iliac bone-derived mesenchymal cells depending on from where the cells were harvested.

Key words: mesenchymal cell, differentiation induction, osteogenesis, adipogenesis, chondrogenesis
type of mesenchymal tissue. These mesenchymal cells are found in the maxillofacial region as well as in the entire body, including some MSCs in the bone marrow of the limbs. NCSCs derived from MSCs capable of differentiating into neurocytes and glial cells of the autonomic nervous system as well as smooth muscle, bone, cartilage, adipose, melanocytes, and some hormone-producing cells. However, it remains unknown whether NCSCs and MSCs are the same stem cells because NCSCs are defined based on the developmental origin, whereas MSCs are defined based on their cellular properties. Furthermore, mesenchymal cells derived from different tissues may differ in biological activity because of differences in their developmental origin; however, the details of this biological mechanism remain unknown. For example, the iliac bone tissue has undergone endochondral ossification, whereas jawbone tissue has undergone intramembranous ossification. Therefore, as a biological characterization study of mesenchymal cells from the iliac and jawbones, we investigated qualitative and quantitative differences in cellular kinetics after the induction of osteogenic, adipogenic, and chondrogenic differentiation.

Materials and Methods
This study was approved by the Ethical Board of Kitasato University (B12-101), and written informed consent was obtained from all the patients prior to participation in the study. The materials used were surplus tissue samples from patients who had undergone surgery at the Department of Plastic and Aesthetic Surgery of Kitasato University Hospital; 4 types of samples: the iliac, maxillary, mandibular, and alveolar bones (from the maxillary bone) were included. The patients’ ages ranged from 5 to 33 (mean, 17) years (Table 1).

Culturing human bone marrow-derived mesenchymal cells
Surplus bone fragments that would normally have been discarded postoperatively were used. The samples were transferred from the operating room to the culture room at room temperature so as not to allow them to dry, immediately subjected to the experiment, washed with phosphate buffer (PBS-); Wako Pure Chemical, Osaka), and then cultured in α-minimum essential medium (α-MEM; Life Technologies, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, MO, USA), 1 ng/ml β-FGF (R&D Systems, MN, USA), penicillin 100 units/ml, and streptomycin 100 μg/ml (Pen Strep; Life Technologies, CA, USA; α-MEM culture medium) at 37°C in a 25 cm² flask (Sumitomo Bakelite, Tokyo) in a 5% CO₂ incubator. The outgrown cells were subcultured, and cells from the first-passage were suspended in Cell Banker solution (Nippon Zenyaku Kogyo, Fukushima) and cryopreserved at -80°C.

Table 1. A total of 16 specimens were included (7 from males and 9 from females). Donors’ mean age 17 years, range 5–33. Four specimens each were harvested from the iliac, maxilla, mandible, and alveolar bones (derived from the maxilla).

<table>
<thead>
<tr>
<th>No</th>
<th>Age, y</th>
<th>Sex</th>
<th>Donor site</th>
<th>Diagnosis</th>
<th>Cryopreservation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>M</td>
<td>Iliac crest</td>
<td>Lt CLP</td>
<td>2 years and 9 months</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>F</td>
<td>Iliac crest</td>
<td>Bil CLA</td>
<td>2 years and 10 months</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>M</td>
<td>Iliac crest</td>
<td>Lt CLP</td>
<td>2 years and 8 months</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>F</td>
<td>Iliac crest</td>
<td>Lt CLA</td>
<td>3 years and 2 months</td>
</tr>
<tr>
<td>5</td>
<td>22</td>
<td>M</td>
<td>Maxilla</td>
<td>jaw deformity</td>
<td>1 year and 11 months</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>M</td>
<td>Maxilla</td>
<td>jaw deformity</td>
<td>2 years and 6 months</td>
</tr>
<tr>
<td>7</td>
<td>20</td>
<td>F</td>
<td>Maxilla</td>
<td>micrognathia</td>
<td>1 year and 11 months</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>F</td>
<td>Maxilla</td>
<td>jaw deformity</td>
<td>2 years and 1 month</td>
</tr>
<tr>
<td>9</td>
<td>22</td>
<td>M</td>
<td>Mandible</td>
<td>jaw deformity</td>
<td>1 year and 11 months</td>
</tr>
<tr>
<td>10</td>
<td>24</td>
<td>F</td>
<td>Mandible</td>
<td>jaw deformity</td>
<td>1 year and 9 months</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
<td>M</td>
<td>Mandible</td>
<td>jaw deformity</td>
<td>2 years and 4 months</td>
</tr>
<tr>
<td>12</td>
<td>33</td>
<td>M</td>
<td>Mandible</td>
<td>jaw deformity</td>
<td>2 years and 10 months</td>
</tr>
<tr>
<td>13</td>
<td>20</td>
<td>F</td>
<td>Alveolar bone</td>
<td>Lt CLP</td>
<td>2 years and 5 months</td>
</tr>
<tr>
<td>14</td>
<td>9</td>
<td>F</td>
<td>Alveolar bone</td>
<td>Lt CLA</td>
<td>2 years and 11 months</td>
</tr>
<tr>
<td>15</td>
<td>7</td>
<td>F</td>
<td>Alveolar bone</td>
<td>impacted tooth</td>
<td>2 years and 11 months</td>
</tr>
<tr>
<td>16</td>
<td>16</td>
<td>F</td>
<td>Alveolar bone</td>
<td>jaw deformity</td>
<td>2 years and 10 months</td>
</tr>
</tbody>
</table>

CLP, cleft lip and palate; CLA, cleft lip and alveolus; Rt, right; Lt: left; Bil, bilateral
first-passage cells were cryopreserved for up to 5 years. The cells were thawed, cultured, and subcultured, and the second-passage cells were then used for the experiments.

**Osteogenic differentiation induction**
Cells (1 × 10^5/well) were seeded in a 6-well plate (Sumitomo Bakelite, Tokyo) and cultured at 37°C in a 5% CO₂ incubator. Subconfluent cells were subjected to differentiation induction using α-MEM supplemented with 10% FBS, penicillin 100 units/ml and streptomycin 100 μg/ml, 0.1 μM dexamethasone (Sigma-Aldrich), 10 mM β-glycerophosphate (Calbiochem, CA, USA), and 0.05 mM ascorbic acid (Wako Pure Chemical, Osaka; osteogenic differentiation induction medium). The osteogenic differentiation potency was evaluated after 6 weeks of differentiation induction. The non-induction group of cells was similarly cultured in the α-MEM culture medium for 6 weeks. The media were replaced with respective fresh preparations every 3 days (Figure 1).

**Adipogenic differentiation induction**
Cells were seeded in the same manner as described in the osteogenic differentiation induction section and cultured in the osteogenic differentiation induction medium for 3 weeks. The cells were then subjected to differentiation induction using α-MEM supplemented with 10% FBS, penicillin 100 units/ml and streptomycin 100 μg/ml, 1 μM dexamethasone, 0.01 mg/ml insulin (Wako Pure Chemical, Osaka), 0.2 mM indomethacin (Wako Pure Chemical), and 0.5 mM isobutylmethylxanthine (Sigma-Aldrich; adipogenic differentiation induction medium). The adipogenic differentiation was assessed after 3 weeks of differentiation induction. The non-induction group of cells was similarly cultured in α-MEM culture medium for up to 6 weeks. The media were replaced with respective fresh preparations every 3 days (Figure 1).

**Chondrogenic differentiation induction**
The induction of differentiation in chondrocytes was performed according to the pellet culture method. The second-passage cells (5.0 × 10^5) were transferred to a 15-ml polypropylene tube and centrifuged at 1,000 rpm for 5 minutes at 20°C to obtain a pellet. After at least 24 hours, the cells were subjected to differentiation induction using a chondrogenic differentiation induction medium (PromoCell MSC Adipogenic Differentiation Media). The chondrogenic differentiation potency was evaluated after 3 weeks of differentiation induction treatment. The media were replaced with respective fresh preparations every 3 days (Figure 1).

**Evaluation of differentiation potency**
1. Osteogenic differentiation
After washing the cell surface twice with PBS(-), the cells were fixed with 100% ethanol. The cells were then stained with 1.3% Alizarin red S solution (Wako Pure Chemical) for 2 min, washed with distilled water, and allowed to dry.

2. Calcium quantification
After washing the cell surface thrice with PBS(-), 0.5 N HCl was used to extract Ca, and quantification was determined once every week by the Arsenazo-III method.

---

**Figure 1.** Protocol for differentiation induction and methods for the analysis of differentiation
Pluripotency of jaw and iliac bone-derived MCs

Figure 2. Ca-producing Capability

No significant differences were observed using the non-repeated measure ANOVA and Kruskal-Wallis H-test ($P = 0.053$)

Figure 3. Osteogenesis (alizarin red S staining) and adipogenesis (oil red O staining)

A. Cells in the differentiation induction group were stained with alizarin red S.

B. Differentiation induction groups of I-MCs, Mx-MCs, and Md-MCs were stained with oil red O, and staining intensities differed depending on the harvesting site. A-MCs in the differentiation induction group were not stained.
using the Espa Ca Kit (Nipro, Osaka). Mann-Whitney’s U test was used for statistical analysis.

3. Adipogenic differentiation
After washing the cell surface twice with PBS(-), the cells were fixed with 10% formalin. The cells were then pretreated with 60% isopropanol, stained with Oil Red O solution (Sigma-Aldrich), and washed with 60% isopropanol and PBS(-).

4. Chondrogenic differentiation
Chondrocyte mass was washed twice with PBS, gelatinized with iPCell (GenoStaff, Tokyo), and fixed with 4% PFA (Wako Pure Chemical). After paraffin embedding, thin slices were cut and stained with hematoxylin and eosin (H&E) and toluidine blue staining (Wako Pure Chemical, Osaka).

5. Quantitative RT-PCR
After washing chondrocyte mass with PBS(-), RiboPure Kit (Thermo Fisher Scientific, MA, USA) was used to extract total RNA. cDNA was synthesized via reverse transcription reactions using the Quantitect Reverse Transcription Kit (QIAGEN, Hilden, Germany) at 42°C for 30 minutes and 95°C for 3 minutes. The CFX96 Real-Time System (Bio-Rad, CA, USA) was used for quantitative PCR. Reaction conditions used were: cDNA (2 μl), 2 μl distilled water, 5 μl TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, MA, USA), 1 μl TaqMan Gene Expression Assay primer (Thermo Fisher Scientific, MA, USA), and 1 μl TaqMan Gene Expression Assay probe (Thermo Fisher Scientific, MA, USA). The CFX96 Real-Time System (Bio-Rad, CA, USA) was used for quantitative PCR. Reaction conditions used were: cDNA (2 μl), 2 μl distilled water, 5 μl TaqMan Gene Expression Master Mix (Thermo Fisher Scientific, MA, USA), 1 μl TaqMan Gene Expression Assay primer (Thermo Fisher Scientific, MA, USA), and 1 μl TaqMan Gene Expression Assay probe (Thermo Fisher Scientific, MA, USA).

Figure 4. Staining results
Scientific, MA, USA); 50°C for 2 minutes, 95°C for 10 minutes, and (95°C for 15 seconds and 60°C for 60 seconds) × 40 cycles. The TaqMan Gene Expression Assay primers used were aggrecan (ACAN, assay ID Hs00153936_m1) and TATA-binding protein (TBP, assay ID Hs00427621_m1). The amounts of ACAN produced were normalized with the amount of TBP used as a housekeeping gene.

Results

Osteogenic potency

The Ca level, measured as an index of calcification in the osteogenic differentiation induction medium, was detectable after 2−3 weeks of culture. Ca production increased over time in the differentiation induction group, whereas the Ca level remained undetectable in the non-differentiation induction group. No significant differences in the Ca concentration were observed (Figure 2). Similarly, no significant differences were observed using the non-repeated measure one-way ANOVA and Kruskal-Wallis H-test (P = 0.053). Alizarin red S staining, which indicates the presence of Ca from cells that were induced to differentiate into osteoblasts, was confirmed in the differentiation induction group. The staining intensity differed depending on the harvesting site (Figure 3A).

Adipogenic potency

Oil red O staining, which indicates the presence of lipid droplets, was observed in the differentiation induction group, whereas no staining was observed in the non-differentiation induction group. The staining intensity, determined by the area of stained cells per field of view, was the highest for iliac bone-derived mesenchymal cells (I-MCs) followed in the descending order by mandibular bone-derived mesenchymal cells (Md-MCs) and maxillary bone-derived mesenchymal cells (Mx-MCs). Alveolar bone-derived mesenchymal cells (A-MCs) did not undergo adipogenic differentiation induction (Figure 3B).

Chondrogenic potency

Cells in the differentiation induction group formed masses

Figure 5. Relative expression levels of ACAN varied depending on the harvesting site.
in the tube; and those cell masses gradually increased in size, formed a globular shape, and showed increased elasticity. The cell masses in the non-induction group, however, were fragile; and their size remained unchanged. Histological staging of differentiation using thin-sliced samples of the cell masses revealed an abundant extracellular matrix inside the cell mass and the outermost layer forming a capsule of a perichondrium-like fibrous layer in the induction group (Figure 4A). Staining with toluidine blue in the induction group showed metachromasia due to mucopolysaccharide secretion of all cells, and chondrocyte-like cells were observed for I-MCs (Figure 4B). Relative gene expression level analysis based on real time RT-PCR revealed differences between cells that underwent chondrogenic induction and cells that did not and were higher in the following order: I-MCs ≥ A-MCs > Mx-MCs = Md-MCs (Figure 5).

Discussion
Stem cells have the self-renewal ability and pluripotency and are classified into two different types: embryonic stem cells and somatic stem cells. Tissues primarily comprise parenchymal cells, which are responsible for the primary function of the tissue, and mesenchymal cells, which support parenchymal cells. Hematopoietic stem cells found in the bone marrow and MSCs are the most well-known mesenchymal cells. Human MSCs are found as a rare cell population in the bone marrow accounting for 0.001%—0.01% of nucleated cells. The percentage of stem cells existing in developing organs may be higher than those in mature organs; however, human organs during infancy or early childhood have not been adequately studied, most likely, due to ethics. Specimens used in the present study were surplus tissues harvested intraoperatively from four different sites in relatively young individuals, 5 to 22 years of age, and considered suitable for the evaluation of differentiation potencies. Those tissues if not used for research would otherwise have been discarded.

The osteogenic differentiation induction experiment showed no differences in induction between cells harvested from different sites. However, Crespi et al. reported that, in the dental and oral surgery field, bone grafting with grafts from the maxillofacial region produced better clinical outcomes than did bone grafting with iliac bone grafts. Moreover, intramembranous ossification and endochondral ossification require different cytokines and growth factors. The usefulness of bone grafts selected based on the developmental origin has been a subject of discussion. Furthermore, MSCs found in the femur and iliac bones have a lower level of osteogenic differentiation potential as the donor’s age increases. Although the time of surgery and patient’s age should be considered, the jawbone appears to be a better harvesting site for autologous bone grafts when an equivalent amount of bone is required in the dental and oral surgery field.

The adipogenic differentiation induction experiment revealed that I-MCs had a higher level of adipogenic differentiation potential. In the spinal cord, the red marrow, which is the center of hematopoiesis since puberty, starts to rapidly decrease at around 60 years of age, and the fatty marrow then starts to occupy the greater parts of the bones resulting that more red marrow is found in the vertebral bodies, iliac, and sternum. Thus, Mx-MCs and Md-MCs are less responsive to adipogenic differentiation induction than are I-MCs, which likely reflects the differences in the developmental routes. This result also suggests that jawbone-derived MCs are useful as a graft option for jawbone reconstruction. However, trunk bones remain to represent the first choice for the construction of an extensive bone defect because of the limited amount of jawbone that can be harvested. We previously reported that human mesenchymal cells from the iliac cancellous bone remain to be osteogenically potent, pluripotent, and safe after long-term cryopreservation. In addition, the viability of cryopreserved human iliac cancellous bone-derived mesenchymal cells has often been reported. Therefore, we expect that the use of these cells can reduce the burden on the bone harvesting site in clinical cases requiring multiple bone grafts.

In the chondrogenic differentiation induction experiment, the real time RT-PCR results revealed that some A-MC preparations were induced to differentiate into chondrocytes, and the other A-MC preparations were not. The former preparations were from 16- and 20-year-old donors, and the latter preparations were from 7- and 9-year-old donors. Various stem cells with high proliferative and differentiation potentials are found in the odontogenic tissue derived from the neural crest, including dental pulp stem cells (DPSCs) found in the dental pulp in adults, stem cells from human exfoliated deciduous teeth (SHED) found in the pulp of exfoliated primary teeth, and periodontal ligament stem cells (PDLSCs) identified from the periodontal ligament. Bone marrow-derived MSCs are superior to SHED and DPSCs in terms of the chondrogenic differentiation potency, and genes relevant to chondrogenic differentiation are expressed at high levels. In the presence of TGF-β (transforming growth factor beta 1),
the chondrogenic potential of PDLSCs is higher than that of bone marrow-derived MSCs.\textsuperscript{25} Therefore, the observed difference in the chondrogenic potential may likely be attributable to differences in donor age (e.g., school-age donors before exfoliation of primary teeth vs. adolescence-age donors), rather than various stem cells present in the alveolar bone differing in viability.

Future perspectives
Primarily, jawbone-derived MSCs undergo intramembranous ossification, whereas iliac bone-derived MSCs undergo endochondral ossification. Such developmental differences are likely to underlie their different biological activities. Therefore, a more suitable donor site should be selected for reconstruction with bone grafts in clinical practice, based on an adequate understanding of these properties. However, given age-dependent differences in differentiation induction and the limited graft availability at donor sites, future clinical development of regenerative medicine technology in this field is paramount.

Periosteum-derived stem/progeny cells (PSCs), which can differentiate into bone, are found in the periosteum.\textsuperscript{26} The inner layer of the periosteum containing abundant vascular components is responsible for bone growth via intramembranous ossification,\textsuperscript{26} and MSCs are also present in the same region. PSCs have a higher level of proliferative capacity than do MSCs, and differentiate into bone, cartilage, adipose tissue, and muscle fiber,\textsuperscript{27} and show excellent bone formation in the presence of a growth factor.\textsuperscript{23} In the cortical bone grafted to the mandible, bone remodeling is not maintained, and bone resorption occurs when the external surface of the cortical bone graft is not covered with the periosteum and exemplifies the importance of the periosteum for bone tissue maintenance.\textsuperscript{28} Basic research regarding the relationship between grafted bone tissue and the periosteum is required to perform bone transplantation in clinical practice. Also, future studies warrant research on differences in neurogenic differentiation induction depending on the harvesting site of because there are reports on differentiation induction of mesenchymal cells into neurocytes.

We demonstrate the differences in osteogenic, adipogenic, and chondrogenic differentiation potentials between jawbone- and iliac bone-derived MCs. Functional differentiation via different developmental routes were considered to underlie differences in differentiation potency depending on the site of cell harvesting.

Acknowledgements
This work received Grant-in-Aid for Scientific Research(C) (17K11852) from the Ministry of Education, Culture, Sports, Science and Technology, Japan. We thank the staff of the Kitasato University Hospital Department of Clinical Laboratory, especially Yumiko Sone for assisting in the technical aspects of the research.

Conflicts of Interest: None

References


