

## Short Communication

# An Easy Methodology for Generation of MSC-Like Cells from iPSCs using Human Gingival Tissue Collected during Oral Implant Operation

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- MSC like cells (MSLCs)

## Abstract

Human induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) have considerable potential in regenerative medicine. Moreover, there are cell banks that collect stem cells for HLA-matched medical applications.

In this study, using episomal plasmids vectors, we generated iPSCs that were derived from human gingival fibroblasts (HGF). The gingival tissues were collected as medical waste after dental implant operation. Transfected cells were cultured for six days in DMEM containing 10% FBS. Cells were subsequently transferred onto SNL76/7 feeder cells in human embryonic stem cell medium (DMEM/F-12 containing 20% KSR, 2 mM L-glutamine, 1% NEAA, 0.1 mM 2-ME, and 5 ng/mL bFGF). For passaging, iPSC colonies were detached with a CTK solution [70% PBS, 20% KSR and the other 10% mixture solution (2.5 µg/mL trypsin, 1 mg/mL collagenase IV and 1 mM CaCl<sub>2</sub>/PBS)] and split at a 1:4 ratio every 5 or 6 days. iPSCs expressed pluripotent stem cell-specific markers, such as ALP, OCT3/4, NANOG, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 as determined by histochemistry, immunochemistry and gene expression analysis. iPSCs were successfully differentiated into MSC-like cells (MSLCs) that were evaluated by flow cytometry for human mesenchymal markers such as CD44, CD73, CD90, CD105, CD34 and CD45, and their trilineage differentiation ability.

In this report, we introduce a practical way to obtain MSLCs. Our culture methodologies demonstrate that iPSCs could be a promising source of readily accessible stem cells to investigate the potential of MSLCs for future clinical applications.

## ABBREVIATIONS

**ESCs:** Human Embryonic Stem Cells; **iPSCs:** Human Induced Pluripotent Stem Cells; **MSCs:** Mesenchymal Stem Cells; **MSLCs:** Mesenchymal Stem Cell-Like Cells (MSLCs); **KSR:** Knockout-Serum Replacement (Life technology, 10828-028); **bFGF:** Human Recombinant Basic Fibroblast Growth Factor (PeproTech, 100-18B). **NOTE:** Reconstitute 50 µg of bFGF in 5 mL of 0.22 µm filtered water. Aliquot and store at -20°C; **CTK:** 2.5 µg/mL trypsin (Life technology, 27250018), 1 mg/mL collagenase IV (Life technology, 17104-019), 20% KSR, 1 mM CaCl<sub>2</sub> (Wako, 039-00475)/PBS, 70% PBS (Life technology, 14190-250); **GFRM:** Growth Factor Reduced Matrigel (BD, 354230); **VTN-N:** Vitronectin-N (Life

technology, A14700); **Gelatin-coated dishes:** To make 0.1% gelatin solution, dissolve 1.0 g of gelatin powder (Sigma, G1890-100G) in 1000 mL of distilled water after autoclave sterilized. Add enough 0.1% gelatin solution to cover the entire area of the culture dish bottom; **Human ESCs/iPSCs medium:** To make 550mL, mix 423 mL DMEM/F12 (Life technology, D6421), 110 mL KSR, 5.5 mL 200 mM L-glutamine (Nakarai, 16948-04), 5.5 mL NEAA (Life technology, 11140-076) and 5 mL pen/strep (Nacarai, 26253-84). As ready to use, add final concentration 15 mM 2-ME (Life technology, 21985-023) and 5 ng/mL bFGF (Repro CELL, RCHEOT003) into the medium before use. **MSLCs**

**differentiation medium:** Start with 450 mL DMEM low glucose medium and add 50 mL MSC grade FBS (Gibco, 12662029) and supplement with bFGF at 10 ng/mL; **HGF culture medium:** DMEM containing 10% FBS (MP Biomedical, Inc, 2916754), Store at 4°C up to two weeks.

## INTRODUCTION

Human embryonic stem cells (ESCs) / human induced pluripotent stem cells (iPSCs)-derived cells and mesenchymal stem cells (MSCs) have been used for the treatment of several diseases. Recently, MSCs have been used for the treatment of scleroderma or neurodegenerative disease [1,2]. ESCs/ iPSC-derived retinal pigment epithelium (RPE) cells have been used for clinical treatment of dry/wet type age-related macular degeneration (AMD) [3-5]. The safety, quality control used for iPSC-derived cell products may have differed depending on the reprogramming reagents, cell sources and differentiation methods. As for the cell sources used to generate iPSCs, a variety of cell types or tissues have been reported, including dermal fibroblasts [6], peripheral blood [7], cord blood [8], gingival fibroblasts [9], apical papilla and oral mucosa [10]. Among these somatic cells, fibroblasts from gingival tissues have a distinct advantage for iPSCs generation. These tissues are considered biomedical waste generated in the process of dental treatments such as periodontal surgery and dental implants [9]. Thus, it is not necessary to recruit donors for cell collection. In addition, gingival wounds are reported to heal relatively quickly compared to skin wounds [11]. iPSCs-derived MSC-like cells (MSLCs) reportedly exhibit greater proliferation than primary cultures of bone marrow-derived MSCs [12] and they might lack tumorigenic potential, making them safer for implantation into humans.

In this report, we assessed the generation of iPSCs from human gingival fibroblasts (HGF), and we describe an easy and robust method for differentiating MSLCs from HGF-iPSCs in the absence of sorting process. We explain each protocol step, focusing on problems we have encountered as stated in the notes. Specifically, they are induced to differentiate into MSLCs simply by using a single cytokine, i.e., bFGF. Thus, they can be induced to differentiate at a lower cost than other culture methods requiring multiple cytokines. In the course of this study, we compared ECM type and BSA grade for their impact on MSLC generation.

## MATERIALS AND METHODS

Here, we describe an easy method for generating MSLCs from iPSCs. We explain each protocol step, focusing on problems we have encountered as stated in the notes. Moreover, we explain the optimal culture conditions in the Results and Discussion sections.

**Step 1: Collection of HGFs from gingival tissue and generation of iPSCs from HGFs (HGF-iPSCs)**

- 1-1. We collected gingival connective tissues that were discarded during dental implant surgery.
- 1-2. Human gingiva connective tissues were cut into 1 x 1 mm pieces and placed on 35-mm tissue culture dishes in a safety cabinet.

- 1-3. The tissue culture dishes were placed under the glass slide. We checked the adherence of the gingiva connective tissue on culture dish.
- 1-4. 2.5 mL HGF culture medium was put in a well per 6-well culture dish.
- 1-5. The tissue culture dishes were incubated at 37°C, in 5% CO<sub>2</sub> for 1 week. The medium was replaced every 3 days.
- 1-6. When HGFs had migrated out of the tissue and proliferated, the tissue pieces and glass slide were removed. When the cells reached sub-confluence, they were dissociated with 0.25% trypsin and transferred to 60-mm tissue culture dishes (passage 1).
- 1-7. HGFs were regularly passage at a 1:3 ratio every 3 or 4 days.
- 1-8. 2.0x10<sup>5</sup> HGFs were frozen for storage using Cell Banker 1. (NIPPON ZENYAKU KOGYO CO., LTD.)
- 1-9. Put the cryogenic vial in a freezing container and place in a -80°C refrigerator for one day. Place the cryogenic vial in the gas phase of a liquid nitrogen tank or in a -150°C refrigerator for long-term storage.
- 1-10. Take out a frozen vial of HGFs from the liquid nitrogen tank and put the vial into a 37°C water bath until most (but not all) of the liquid has thawed.
- 1-11. Wipe the vial with 70% ethanol and then transfer the cell suspension to a 15 mL tube and add 9 mL HGF medium slowly and mix the cell suspension well.
- 1-12. Centrifuge at 400 G for 5 min at 4°C, and then aspirate the supernatant. Re-suspend the cell pellets in 2 mL fresh HGF medium.
- 1-13. They were cultured (2 x10<sup>5</sup> cells) for 3 days in a well of a 6-well plate with 2 mL of HGF medium
- 1-14. After six passages, 6 × 10<sup>5</sup> HGFs were transfected by episomal plasmids. Thus, pCXLE-hOCT3/4-shp53-F, pCXLE-hSK, and pCXLE-hUL were mixed at 1 µg each (Add gene, Cambridge, MA, USA) using program DT-130 of Amaxa™ 4D-Nucleofector™ (Lonza, Basel, Switzerland).
- 1-15. Infected cells (6 × 10<sup>5</sup> HGF) were cultured with HGF medium for 7 days. After 7 days, these cells were passage on 100-mm MMC-SNL feeder layer dishes with HGF medium. NOTE: MMC-SNL was prepared as previously described [13].
- 1-16. The following day, the culture medium was replaced with human ESCs/iPSCs medium, and the medium was replaced every day.
- 1-17. Approximately 25 days after transduction, some of the colonies were mechanically picked up while observing with a microscope and transferred to a 24-well plate containing MMC-SNL feeder layers.
- 1-18. After several passages, these ESCs-like colonies were evaluated for characterization and generation of cell stocks.

**Step 2: Establishment of MSLCs from HGF-iPSCs.**

- 1-1. For generation of MSLC, HGF-iPSCs were cultured with mTeSR1 medium (STEMCELL technology, ST-05850) on GFRM-coated dishes without feeders for 3 days. NOTE: GFRM coated dishes: Enough 100uL GFRM solution to cover the entire area of the culture 6-well plate bottom. Store at 4°C up to one days.
- 1-2. After three days in human ESCs/iPSCs culture on feeder-free conditions, the culture medium was replaced with MSLC medium every two days for 2 weeks.
- 1-3. During the early stage (passages 1 to 3), differentiated cells were passage with 0.025% trypsin (Life Technology, R001100) and transferred in the same medium on a gelatin-coated Biocoat dish (Corning™ BioCoat™). The cell transfers were carried out every 3–5 days at a split ratio of 1:1 (passage1) or 1:2 (passage 2-3).
- 1-4. After passaging four times, the cell morphology changed to a fibroblastic shape (Figure 2). We investigated the effects of coating agents, bFGF activity and FBS grades in medium for cell differentiation and cell behavior.
- 1-5. Passaging was carried out every 3–5 days at a split ratio of 1:3 using 0.025% trypsin.

For optimization of MSLCs culture conditions, cells were cultured in ReproFF2 (Repro CELL, RCHEMD006) medium and TeSR1 medium on VTN-N-coated culture dishes at the initial stage of differentiation and cultured under human ESCs/iPSCs medium using feeder cells

**Step 3: Freezing of MSLCs**

- 1-1. When MSLCs reached 70 - 80% confluence in 100-mm dishes, frozen stocks were prepared. Towards that end, the culture medium was replaced with 10 mL MSLC differentiation medium.
- 1-2. The cells were dissociated with 0.025% trypsin solution. Next, MSLC differentiation medium was added to inactivate the trypsin.
- 1-3. Cells were suspended in 500 μL of Cell Banker 1 (Nippon Zenyaku Kogyo Co., Ltd) solution by pipetting gently several times.

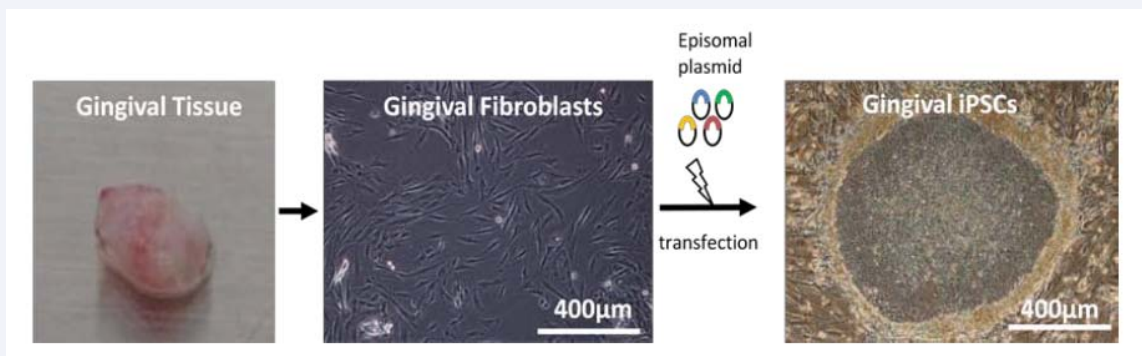
NOTE: We have confirmed that the cells can be frozen even StemCell Banker 3. Moreover, SremCell Banker 3 solution can be expect clinical use. (MF system: 225MF40001) (Nippon Zenyaku Kogyo Co., Ltd)

- 1-4. 500 μL of the 1.x10<sup>6</sup> cells per mL cells suspension were transferred to freezing stock 2.0 mL vials.
- 1-5. Put the cryogenic vial in a freezing container and place in a -80°C refrigerator for one day. The following day, the vials were placed in vapor phase of liquid nitrogen.

Step 1 to 4 can generate MSLCs from iPSCs, starting with a piece of human gingival tissue derived during an implant treatment. iPSCs were examined for growth rate, immune staining, RT-PCR, karyotypic analysis and the ability for differentiation *in vitro* and *in vivo*.

**Characterizations of iPSCs and MSLCs**

The cultured cells were fixed with a 4% PFA solution for 30 mins at 4°C. Cells were then rinsed with PBS. We assessed the alkaline phosphates activity of cultured iPSCs with a Vector Blue® Alkaline Phosphatase Substrate kit (Vector, SK5300) according to the manufacturer's instructions. Immunostaining of cultured cells was carried out as described previously online [13]. We examined the stained cells using an inverted confocal microscope. RT-PCR was conducted as described previously [13] and the G-band method was used for karyotypic analysis. After EB formation *in vitro*, we could show characteristic regional expression of embryonic markers specific for different cellular lineages in differentiated iPSCs. Flow cytometric analysis was used for single cell surface antigen staining using iPSCs or MSLCs that were obtained after treatment with Accutase (Innovative Cell Technologies, Inc.) or 0.025% trypsin. Single cells were suspended in PBS with 2% human serum albumin (Mitsubishi-Tanabe Pharmaceuticals, Osaka, Japan). The cell suspension was incubated with specific antibodies for 30 min at 4°C. The stained cells were analyzed with a FACS Aria II (BD). MSLCs were assessed using a set of antibodies, including those targeting human mesenchymal markers (CD44, CD73, CD90 and CD105) (BD Stem flow™ hMSC Analysis Kit, Cat. No 562530) and negative markers (CD34 and CD45) (BD, 341071). The pluripotent ability of MSLCs was determined with antibodies against SSEA-3 and TRA1-60. Moreover, we performed analysis for trilineage differentiation of MSLCs *in vitro* as previously described by Umezaki [12].



**Figure 1** Generation of iPSCs from HGF using gingival tissue and episomal plasmid.

**Table 1:** Characterization of generated iPSCs.

Cell morphology	ES cell-like (Dish-like form)
Growth rate	4-6 days / passage
ALP staining	Positive
Pluripotency markers (Immunochemical staining: Oct3/4, Nanog, SSEA-3, SSEA-4, TRA1-60, TRA1-81)	Positive
qRT-PCR ( <i>POU5F1, NANOG, SOX2, KLF4, REX1, C-MYC, TERT, DPPA5</i> )	Positive
Karyotype (G-band)	Normal
In vitro differentiation assay (Embryoid Body Formation) (Immunochemical staining: AFP, a-SMA, beta-tubulin )	Positive Positive
In vivo differentiation assay (Teratoma formation)	Positive

## RESULTS AND DISCUSSION

### Generation of iPSCs from HGFs

We examined the success of the transfection conducted with Amaxa 4D and found that over 90% of the cells were positive at the following day. Twenty-five days after transfection, we picked ES-like colonies from culture plates (Figure 1). We evaluated four of the colonies for pluripotency-related gene expression by quantitative RT-PCR (*POU5F1, NANOG, SOX2, KLF4, REX1, CMYC, TERT* and *DPPA5*). All genes were expressed at levels similar to those found in human ESCs. In addition, the established HGF-iPSCs lines were assessed for ALP activity and OCT3/4, NANOG, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 surface cell markers (Table 1). Additional characterization included assessment of growth curves, G-band staining in karyotypic analysis and the ability to differentiate *in vitro* / *in vivo*. A summary of all the data is shown in Table 1.

### Effects of culture supplements necessary for MSLCs differentiation induction

During cultivation for differentiation of MSLCs, iPSCs morphology gradually changed. After four passages, MSLCs were established from iPSCs (Figure 2). To optimize the MSLCs' culture environment, we examined the coating on the dishes. We found that cell adhesion, cell growth and differentiation of MSLCs were best on gelatin-coated dishes after four passages. On the other hand, without gelatin, differentiation was weak at the P1 stage of differentiation, and cell attachment was lacking at the P2 stage. MSC-grade FBS promoted strong cell adhesion and cell growth during MSLCs differentiation induction compared to conventional FBS. The addition of bFGF notably improved cell adhesion and cell growth, and it promoted cell differentiation as shown by changes in cell morphology (Table 2). After four passages, we analyzed the cells by flow cytometry. Flow cytometry confirmed that CD44, CD73, CD90 and CD105 were highly expressed and CD34 and CD45 were absent from the human MSLCs used for the differentiating experiments. Next, we examined trilineage differentiation *in vitro*. During the induction of differentiation, the medium was changed every three days, and the differentiation was evaluated after 21 days by alizarin red staining, oil red staining, and toluidine blue staining. The trilineage differentiation confirmed that the MSLCs were capable of osteogenic differentiation, adipogenic differentiation and chondrogenic differentiation [14].

### Efficient differentiation of MSLCs from human iPSCs depended on culture conditions

We examined differentiation from iPSCs with and without feeder layers. iPSCs culture conditions used (1) mTeSR-1 medium on human recombinant vitronectin (VTN-N), (2) mTeSR-1 medium on GFRM and (3) ReproFF2 medium on VTN-N. We found that growth or differentiation varied with the culture environment. MSLCs differentiation was optimal with mTeSR1 medium on GFRM-coated dishes. Other culture conditions did not support differentiation into MSLCs (Table 3). These results suggested the differentiate capacity of iPSCs was sensitive to cell culture conditions. GFRM is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in extracellular matrix proteins including laminin (a major component), collagen IV, heparin sulfate proteoglycans, fibronectin, vitronectin and entactin / nidogen. These protein complexes likely promote differentiation better than vitronectin alone. Unfortunately, because Matrigel cannot be used clinically due to its sarcoma derived materials, select of other ECM or drug administration of a tumor suppressor may be required. And, it was also described in detail the method of cell manufacturing, that the SOP (Standard Operating Procedure) is very important in cell therapy.

## CONCLUSIONS

We assessed the generation of iPSCs using readily accessible cells that were transfected by episomal plasmid vectors. The starting material consisted of human gingival fibroblasts obtained from biomedical waste generated during dental treatments. The iPSCs differentiated into MSLCs using a no-sort method. In this study, we identified effective differentiated culture conditions permitting easy and effective differentiation of iPSCs into MSLCs. Critical parameters included the grade of FBS, presence of bFGF and coated gelatin plates for expansion and passage of MSLCs. The MSLCs demonstrated their capacity for trilineage differentiation into osteogenic, adipogenic, and chondrogenic cells. Our results demonstrated that gingival iPSCs and differentiated MSLCs could be useful for future clinical applications. Finally, the determination of the quality standard of FBS grade such reagent is an important Step towards clinical research.

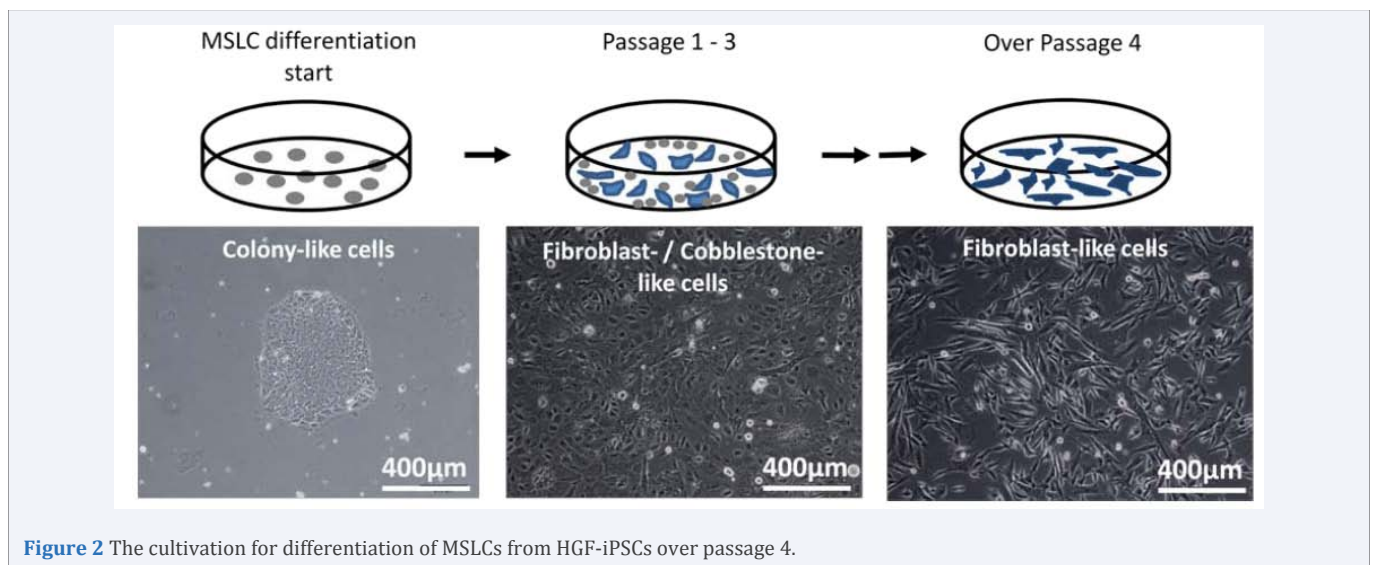
## ACKNOWLEDGEMENTS

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**Table 2:** Effects of culture supplements necessary for induction of MSC differentiation.

	Passage number	P1		P2		P3		P4	
	Cell behavior state	Cell adhesion	Cell growth	Cell adhesion	Cell growth	Cell adhesion	Cell growth	Cell adhesion	Cell growth
Coating plate (with MSC-grade FBS and bFGF)	With gelatin	+++	+++	+++	+++	+++	+++	+++	+++
	Without gelatin	+	+	-	-	-	-	-	-
FBS-grade (with gelatin and bFGF)	FBS	++	+	++	+	++	-	-	-
	MSC-grade FBS	+++	+++	+++	+++	+++	+++	+++	+++
bFGF activity (with gelatin and MSC-grade FBS)	With bFGF	+++	+++	+++	+++	+++	+++	+++	+++
	Morphology	Fibroblast- / Cobblestone-like Morphology		Fibroblast- / Cobblestone-like Morphology		Fibroblast- / Cobblestone-like Morphology		Fibroblast-like Morphology	
	Without bFGF	+++	+++	+++	+++	++	++	++	++
	Morphology	No change		No change		No change		No change	



**Figure 2** The cultivation for differentiation of MSLCs from HGF-iPSCs over passage 4.

**Table 3:** Efficiency of MSLC differentiation from human iPSCs was associated with culture conditions.

iPSCs maintenance conditions		Human ESCs /iPSCs medium on Feeder (SNL)	ReproFF2 medium	mTeSR1 medium	mTeSR1 medium
	Coating ECM		Vitronectin-N (VTN-N)	Vitronectin-N (VTN-N)	Growth Factor Reduced Matrigel (GFRM)
MSLC differentiation		Impossible	Impossible	Impossible	Possible

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