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ORIGINAL PAPER

Comparison of the characteristics of mesenchymal stem-like cells derived by integration-free induced pluripotent stem cells in different single-cell culture media under feeder-free conditions

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Abstract

Generating mesenchymal stem-like cells (MSLCs) from induced pluripotent stem cells (iPSCs) can be a practical method for obtaining the sufficient cells for autologous tissue engineering. Single-cell culturing in specific medium and non-feeder cells is an alternative and promising strategy to overcome problems of embryo culture; however, little is known about how different culture media affect the proliferation and differentiation of MSLCs. We first derived MSLCs from iPSCs with non-integrating episomal plasmid vectors (hereafter 409B2 cells) using three different cell culture media, including single-cell culture medium in feeder-free condition: mTeSR1, DEF-CS500, or StemFit AK02N. The morphology of all MSLCs was completely altered to a fibroblastic morphology after four passages. Surface antigens CD29, CD44, CD73, CD90, but not CD34 and CD45, were expressed in all passages. RUNX2 was expressed in MSLCs cultured in all three feeder-free media, while SOX9 and PPARγ were expressed in MSLCs cultured in only DEF-CS500. MSLCs derived from DEF-CS500, which is a single-cell culture medium, grew at a slightly faster rate than those cultured in other media and expressed early-stage genes for tri-lineage differentiation. Taken together, these findings provide valuable information for generating MSLCs using single-cell culture methods.

Keywords Mesenchymal stem-like cells · Feeder-free conditions · Induced pluripotent stem cells · Human skin

Introduction

Mesenchymal stem cells (MSCs) are adherent fibroblast-like cells derived from the bone marrow [1]. Human MSCs are multipotent and have previously been differentiated into several mesodermal tissues, including the bone, adipose tissue, and cartilage in vitro [2]. MSC have generated a great amount of enthusiasm over the past decade as a novel therapeutic paradigm for a variety of diseases [3].

Successful reprogramming of human and mouse somatic cells into induced pluripotent stem cells (iPSCs) via ectopic overexpression of pluripotency-associated transcription factors is considered a major scientific breakthrough [4–8]. Similar to embryonic stem cells (ESCs) [9–11], human iPSCs can proliferate indefinitely while simultaneously retaining pluripotency, and they can differentiate into all cell types. iPSCs have been previously generated from dermal fibroblasts [6], peripheral blood cells [12], dental pulp cells [13], gingival fibroblasts [14], periodontal ligaments [15], oral mucosal cells [16], and mesenchymal stromal cells [17].

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Recently, mesenchymal stem-like cells (MSLCs) were successfully generated from ESCs/iPSCs [18–21]. MSLCs derived from iPSCs are expected to be a promising new strategy of stem cell transplantation, displaying the advantages of both iPSCs and MSCs. Hynes et al. [18] demonstrated that in vivo subcutaneous implantation of the iPSC–MSLCs generated through retroviral transduction into NOD/SCID mice exhibited the capacity to form mature mineralized structures which were histologically similar to mature bone. However, genomic integration of transgenes increases the risk of tumorigenesis and mortality in chimeric and progeny mice developed from iPSCs [22]. Okita et al. [23] suggested the generation of 409B2 iPSCs derived from human skin, using episomal plasmid vectors. The iPSCs were used for age-related macular degeneration treatment and were generated through episomal plasmid vectors from skin fibroblasts obtained from patients [24].Thus, MSLCs derived from integration-free human iPSCs were required for clinical applications.

In general, feeder cells and serum-containing medium have been used in conventional culture systems for hESCs and hiPSCs [6, 11]. Murine and human-derived feeder cells are widely used to culture hESCs and hiPSCs; however, these cells have been deemed unsuitable for stem cell culture in some cases [25–27]. Fetal bovine serum (FBS)-containing medium is normally used to culture feeder cells. Reduction or complete obliteration of serum- and animal-derived products is required to meet clinical application standards. We previously cultured human gingival fibroblast (HGF)-derived iPSCs in a feeder-free condition for 3 days and successfully derived MSLCs from HGF–iPSCs via induction of direct differentiation for 4 weeks [28]. Recently, improved chemically defined single-cell culture media showed excellent performance at feeder-free condition [29]. Single-cell culturing approach with specific medium and non-feeder cells is the alternative and promising strategy to overcome problems of embryo culture method [29]. However, little is known about how these media affect the cell proliferation and differentiation characteristics of MSLCs.

The present study aimed to derive MSLCs from 409B2 iPSCs using three different cell culture media, including single-cell culturing medium at feeder-free condition and assess gene expression profiles in the early stage for trilineage differentiation.

Materials and methods

Cell culture

409B2 cells were provided by RIKEN BRC (Tsukuba, Japan) through the Project for Realization of Regenerative Medicine and the National Bio-Resource Project of the MEXT, Japan. 409B2 cells were maintained in ESC culture medium at 37 °C and 5% CO_2. These cells were cultured on SL10 feeder cells (ReproCELL, Kanagawa, Japan). The ESC medium comprised DMEM-F12 medium (SIGMA, St. Louis, MO, USA) supplemented with 20% KnockOut Serum Replacement (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (Nacalai Tesque, Kyoto, Japan), 1% non-essential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), and 5 ng/mL basic FGF (ReproCELL). For routine passaging, iPSCs were detached using CTK solution (2.5 mg/mL Trypsin, 1 mg/mL Collagenase IV, 20% KSR, 1 mM $CaCl_2$/PBS, and 70% PBS) and sub-cultured at a split ratio of 1:3 every 4–5 days.

Derivation of MSLCs

409B2 cells were cultured in mTeSR1 (Stemcell Technologies, Vancouver, BC, Canada) and two single-cell culture media, which are DEF-CS500 medium (Takara Bio Europe AB Göteborg, Sweden) and StemFit AK02N (AJINOMOTO, Tokyo, Japan), on growth factor-reduced Matrigel (GFRM; BD Biosciences, San Jose, CA, USA) without SL10 feeder cells for 3 days. The medium was replaced with low-glucose DMEM (Gibco), containing 10% FBS and 10 ng/mL basic FGF (ReproCELL), and changed every 2 days. Cells were passaged with 0.025% trypsin (Life Technologies, Grand island, NY, USA) after 2 weeks and seeded on a gelatin-coated plate. After 4 passages, cell morphology was altered to a fibroblastic form. Cells were passaged every 3–5 days at a split ratio of 1:3 (Fig. 1).

Determination of the population of MSCs and undifferentiated cells

We assessed the expression of MSC markers CD73 (344006; BioLegend) and CD90 (12-0909-42; Thermo Fisher Scientific Inc., Waltham, MA, USA) and undifferentiated cell markers SSEA-4 (560796; BD Biosciences) and TRA-1-60 (560193; BD Biosciences). MSLCs and iPSCs expressed MSC markers and undifferentiated cell markers, respectively. Thus, we identified the portion MSCs and undifferentiated cells serving as residual iPSCs during the derivation of MSLCs from iPSCs.

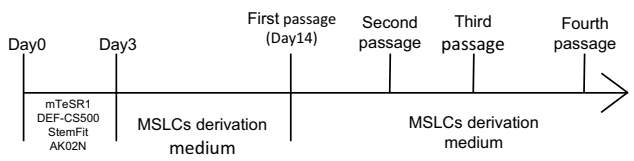

Fig. 1 Schematic representation of the derivation of mesenchymal stem-like cells

Flow cytometry analysis of MSLCs

A single-cell suspension (2×10^5) was obtained upon treatment with 0.025% trypsin (Life Technologies). Cell surface antigens were stained in phosphate buffered saline (PBS) with 2% Newborn Calf Serum (Bovogen Biologicals, Essendon, VIC, Australia). The cell suspension was incubated with specific antibodies for 30 min at 4 °C. Murine anti-human antibodies were used at the recommended concentrations. We assessed the following markers of human MSLCs: CD29 (303007; BioLegend, San Diego, CA, USA), CD44 (400112; BioLegend), CD73, CD90, CD105 (323206; BioLegend), CD34 (343510; BioLegend), CD45 (304008; BioLegend), SSEA-4, and TRA-1-60. The stained cells were analyzed using FACSVerse (BD Biosciences), and the resulting data were analyzed using the FlowJo software (BD Biosciences).

In vitro differentiation of MSLCs

We assessed the early-stage differentiation of osteoblasts, chondroblasts, and adipocytes. Osteogenic differentiation was performed using StemPro Osteogenesis Differentiation Kit (Gibco). Chondrogenic differentiation was performed using StemPro Chondrogenesis Differentiation Kit (Gibco). Adipogenic differentiation was performed using StemPro Adipogenesis Differentiation Kit (Gibco). These kits contained all the reagents required for inducing MSLCs to be committed to osteogenesis, chondrogenesis and adipogenesis. MSLCs cultured without a differentiation supplement constituted the control group. Osteogenic, chondrogenic, and adipogenic differentiation was evaluated after 3 and 7 days, 3 and 7 days, and 7 and 14 days, respectively.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated using QIAcube (QIAGEN, Limburg, Netherlands) in accordance with the manufacturer's protocol, and single-stranded cDNA was synthesized using Transcriptor Universal cDNA Master (Roche Diagnostics, Mannheim, Germany). mRNA levels were analyzed via quantitative real-time PCR using a TaqMan Gene Expression Assay (RUNX2, Hs01047973_m1; SOX9, Hs01001343_g1; PPARγ, Hs01115513_m1; Applied Biosystems) on a StepOnePlus PCR system (Applied Biosystems). The PCR cycle conditions were as follows: 2 min at 50 °C, 20 s at 95 °C, and 40 cycles of 1 s at 95 °C and 20 s at 60 °C. *GAPDH* was co-amplified as an internal standard (human GAPDH endogenous control; Applied Biosystems), relative to which, gene expression was quantified, using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

All experiments were conducted in quadruple and repeated at least 3 times. All data were expressed as the mean and standard deviation. Differences were evaluated by analysis of variance with Student's *t* test (Statcel 4, OMS Publisher, Tokorozawa, Japan). Differences were considered significant at $p < 0.05$.

Results

Derivation of MSLCs

We investigated whether MSLCs can be derived from 409B2 cells. As shown in Fig. 2, iPSCs displayed ES cell-like morphology on day 1 when cultured in mTeSR1 medium or StemFit AK02N medium on GFRM, respectively. In DEF-CS500, iPSCs expanded homogeneously. MSLC morphology was not changed between mTeSR1, DEF-CS500 and StemFit AK02N. As shown in Fig. 2, light microscopy images indicate the differences in cell morphology between the starting iPSC populations and the three MSLCs derived from different iPSC populations on day 21 when cultured mTeSR1 medium, DEF-CS500 medium, or StemFit AK02N medium on GFRM, respectively.

Determination of the population of MSCs and undifferentiated cells

We determined the portion of MSCs (expressing CD73 and CD90) and undifferentiated cells (expressing SSEA-4 and TRA-1-60) during the derivation of MSLCs from iPSCs (Fig. 3). On day 1 or 7, the percentage of undifferentiated cells cultured in all media was higher than that of the MSCs. On day 14, the percentage of MSCs cultured in DEF-CS500 medium was significantly higher than the percentage of undifferentiated cells ($p < 0.05$). On day 21 (approximately 4 passages), the proportion of MSCs cultured in all media was more than 90%.

Characteristics of MSLCs

We evaluated the characteristics of MSLCs via flow cytometry analysis (Fig. 4). MSLCs expressed MSC markers CD29, CD44, CD73, and CD90, but not CD34, CD45, SSEA-4, and TRA-1-60. CD34 and CD45 are endothelial and hematopoietic cell markers; SSEA-4 and TRA-1-60 are undifferentiated cell markers. Since MSLCs did not express CD34, CD45, SSEA-4, and TRA-1-60, we used them to distinguish MSLCs from endothelial or hematopoietic cells and undifferentiated cells. No difference of characteristics of MSLCs was detected on day 21 when cultured on mTeSR1

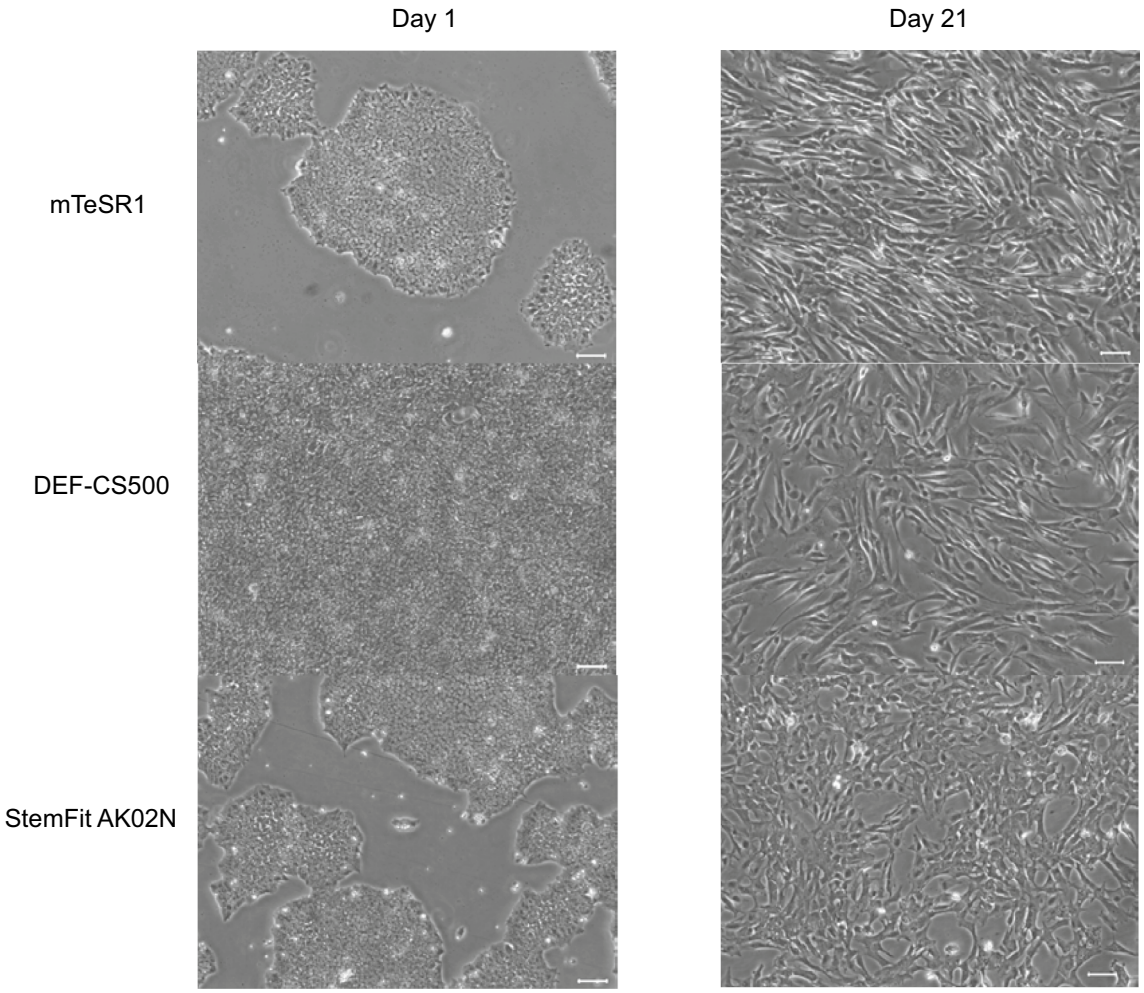

Fig. 2 Morphology of induced pluripotent stem cells on day 1 and mesenchymal stem-like cells on day 21. Cells in mTeSR1 medium, DEF-CS500 medium, and StemFit AK02N medium

medium, DEF-CS500 medium, or StemFit AK02N medium on GFRM.

Gene expression analysis

RUNX2 is an early-stage marker of osteoblast differentiation. *RUNX2* expression in MSLCs derived in all feeder-free media in the osteogenesis differentiation group was significantly higher than that of MSLCs in the control group on day 3 ($p < 0.05$). No significant difference in *RUNX2* expression in MSLCs cultured in mTeSR1 was observed between the osteogenesis differentiation and control groups on day 7 ($p > 0.05$) (Fig. 5a, b). SOX9 is early-stage marker of chondroblast cell differentiation. On days 3 and 7, SOX9 expression in the control group was significantly lower than that in MSLCs cultured in DEF-CS500 ($p < 0.05$) (Fig. 6). *PPARγ* is an early-stage marker of adipocyte differentiation. *PPARγ* expression of MSLCs cultured in DEF-CS500 medium only displayed significantly higher expression in the adipogenesis differentiation group than in the control group ($p < 0.05$) during the study period (Fig. 7).

Discussion

Integration-free human iPSCs have been generated previously, using several methods [30–36]. Human skin-derived iPSCs 409B2 are generated via suppression of p53 and nontransforming L-Myc with episomal plasmid vectors [23]. In this study, we reported the derivation of MSLCs from 409B2 in three different feeder-free condition media. MSLCs expressed MSC markers and not undifferentiated cell markers after 28 days and expressed early-stage markers during tri-lineage differentiation.

In the present study, we followed the method of Umezaki et al. and used trypsinization to generate MSLCs from iPSCs induced via feeder-free differentiation with GFRM. We previously reported [28] the derivation of MSLCs on GFRM,

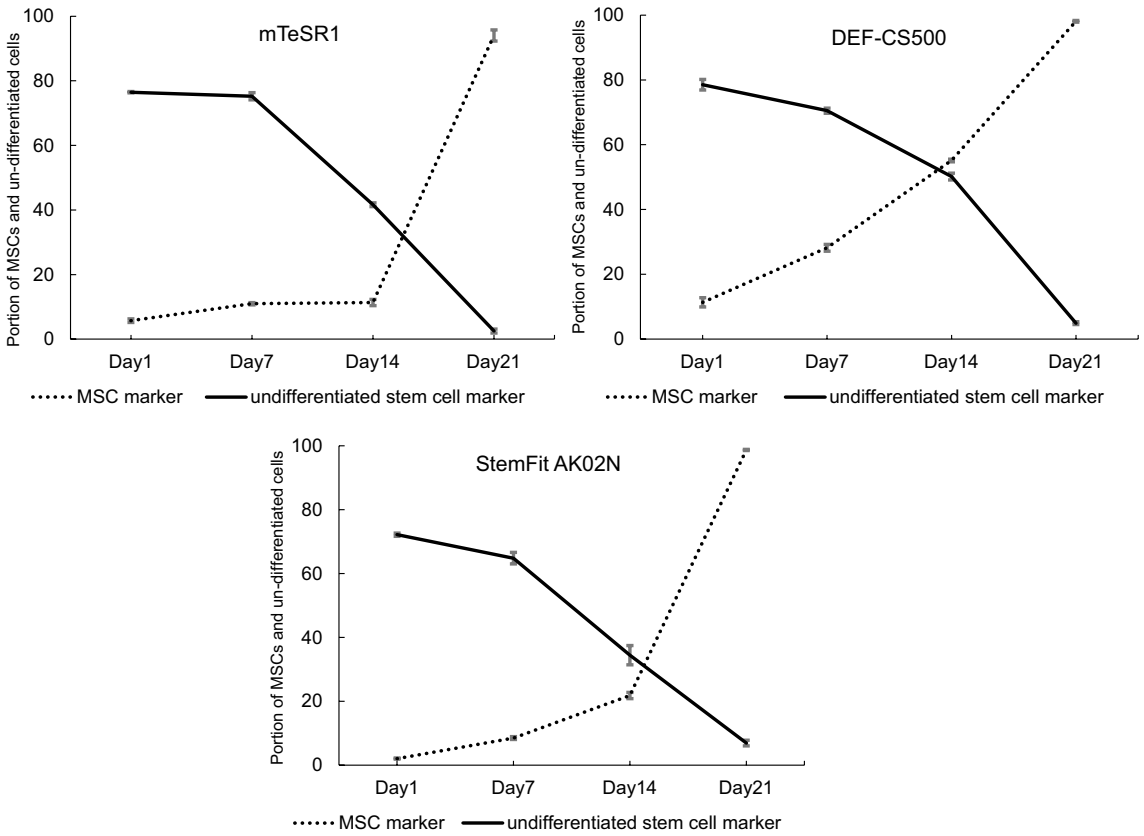

Fig. 3 Schematic representation of the derivation of mesenchymal stem-like cells in mTeSR1 medium, DEF-CS500 medium, and StemFit AK02N medium

which occurred more rapidly than that on the feeder layer or VTN-N. We selected mTeSR1, DEF-CS500, or StemFit AK02N medium, and developed to culture iPSCs in feeder-free condition media. mTeSR1 and DEF-CS500 are not xeno-free media; however, AK02N is a xeno-free medium. iPSCs were seeded as cell aggregates in mTeSR1 medium and as single cells in DEF-CS500 and StemFit AK02N media. MSLCs cultured in DEF-CS500 medium expanded homogeneously and grew slightly faster than those in other media. The reason for the difference in growth rate is unclear; however, culturing of iPSCs as single cells and using a non-xeno-free medium may be effective in deriving MSLCs.

Per the International Society for Cellular Therapy (ISCT), MSCs express the surface markers CD73, CD90, and CD105 ($\geq 90\%$), and not the hematopoietic markers CD14, CD34, CD45, CD19, and HLA-DR ($\leq 2\%$), and they are multipotent [37]. In the present study, MSLCs expressed CD29, CD44, CD73, and CD90, but not CD34 and CD45. Only CD105 expression differed from that of MSCs derived from the bone marrow. We previously reported that HGF-iPSCs express CD105. Differences in cell isolation from the gingiva and the skin would have influenced this result.

RUNX2, SOX9, and *PPARγ* are some of the most prominent differentiation factors of osteoblasts, chondroblasts, and adipocytes, respectively [38–40]. CD105-expressing human MSCs are multipotent and can induce osteoblasts in vitro and osteogenesis in vivo [24]. *RUNX2* was expressed in MSLCs not expressing CD105 cultured in all three feeder-free media. *SOX9* and *PPARγ* were expressed in MSLCs cultured in only DEF-CS500 medium. Details regarding the constituents of this medium have not been reported and variable culture conditions (i.e., the number and timing of replacement of culture) may change these results. However, BoresterÖm et al. [41] established chondrocyte-derived iPSC and fibroblast-derived iPSC lines, and compared their capacities for differentiation into cartilage. Thereafter, DEF-CS500 medium and GFRM have been used for adaptation of iPSCs to feeder-free expansion. However, the findings of the present study that the characteristics of MSLCs can be changed depending on the type of growth media used may provide important insight into the development of MSLCs.

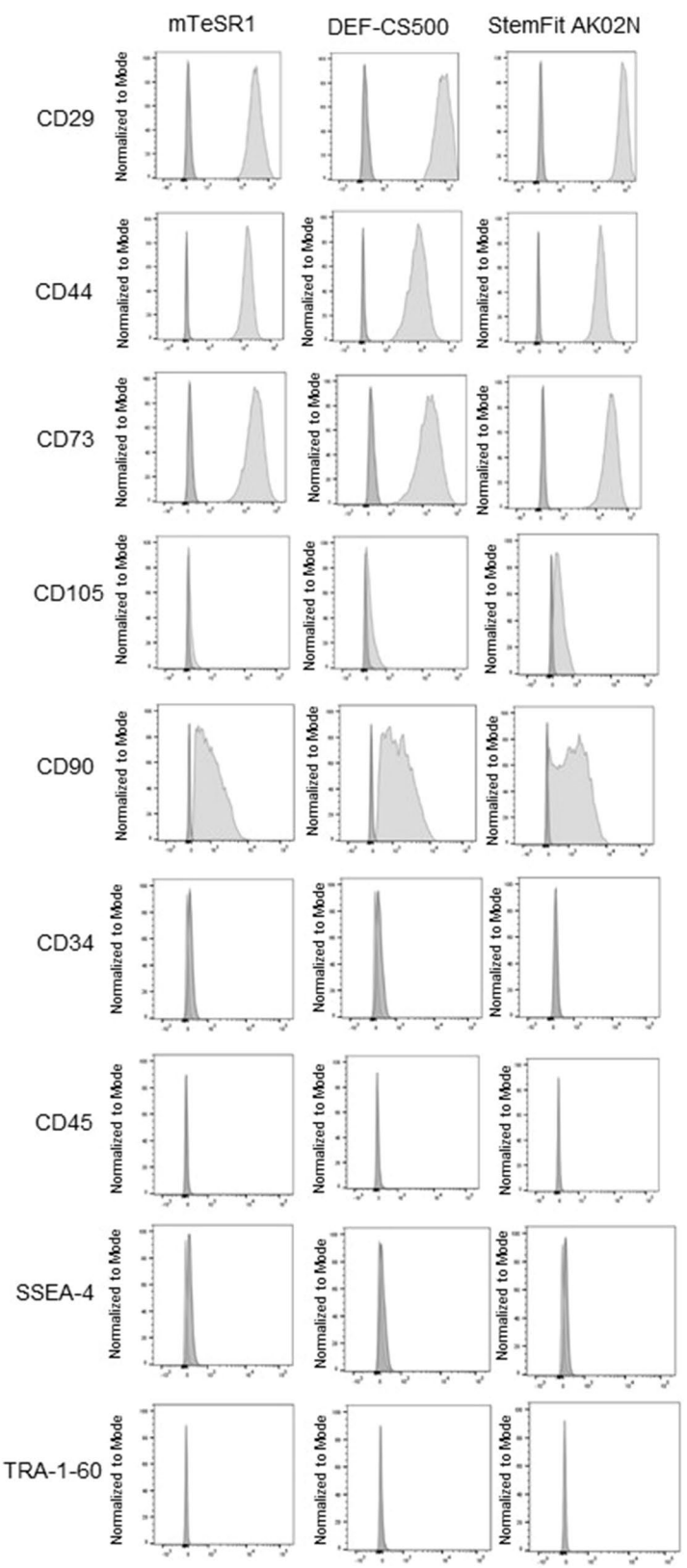

Fig. 4 Flow cytometry analysis of mesenchymal stem cell-related surface markers (CD29, CD44, CD73, CD90, and CD105), endothelial and hematopoietic cell markers (CD34 and CD45), and undifferentiated cell markers (SSEA-4 and TRA1-60) on mesenchymal stem-like cells cultured in mTeSR1, DEF-CS500 and StemFit AK02N medium

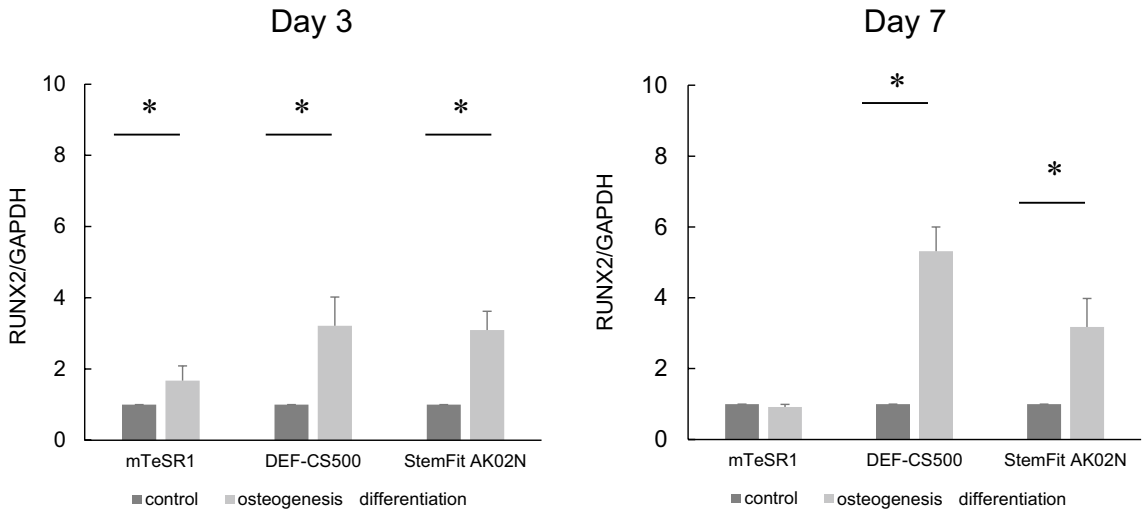

Fig. 5 *Runx2* expression on day 3 and day 7. Data are expressed as the mean and standard deviation values. *$p < 0.05$

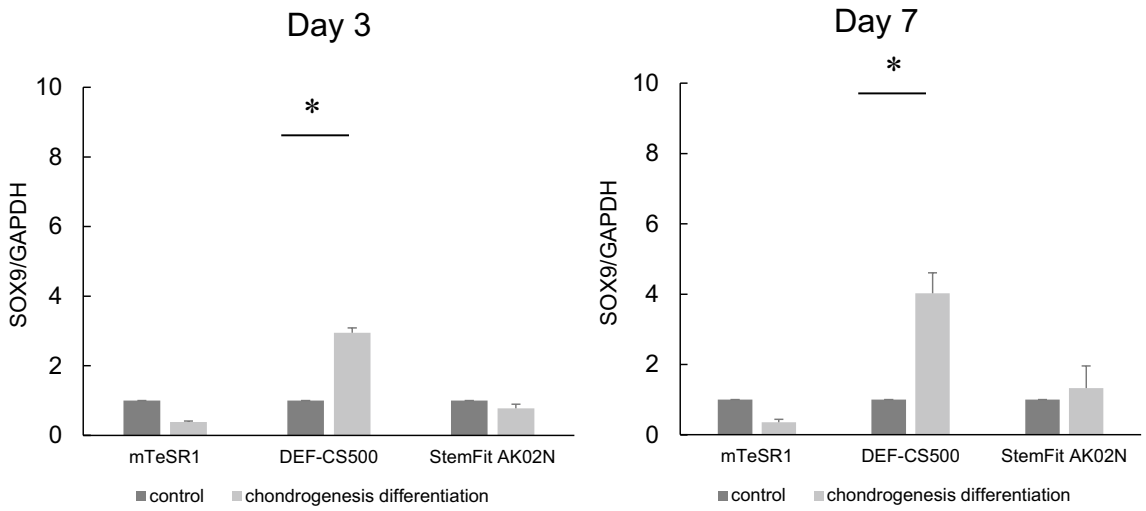

Fig. 6 *Sox9* expression on day 3 and day 7. Data are expressed as the mean and standard deviation values. *$p < 0.05$

Conclusion

In the present study, we successfully derived MSLCs from 409B2 cells, using all three feeder-free condition media and GFRM. MSLCs cultured in DEF-CS500 medium, which is a single-cell culture media, displayed slightly faster derivation than those cultured in other media and induced early-stage gene expression during tri-lineage differentiation. In future, in vitro studies are needed to investigate protein expression during tri-lineage differentiation of MSLCs. Bone and cartilage tissue engineering is widely required in the oral and maxillofacial aspects of dentistry and MSLCs obtained in this study, derived from integration-free iPSCs, are expected to have potential applications in autologous cell therapy.

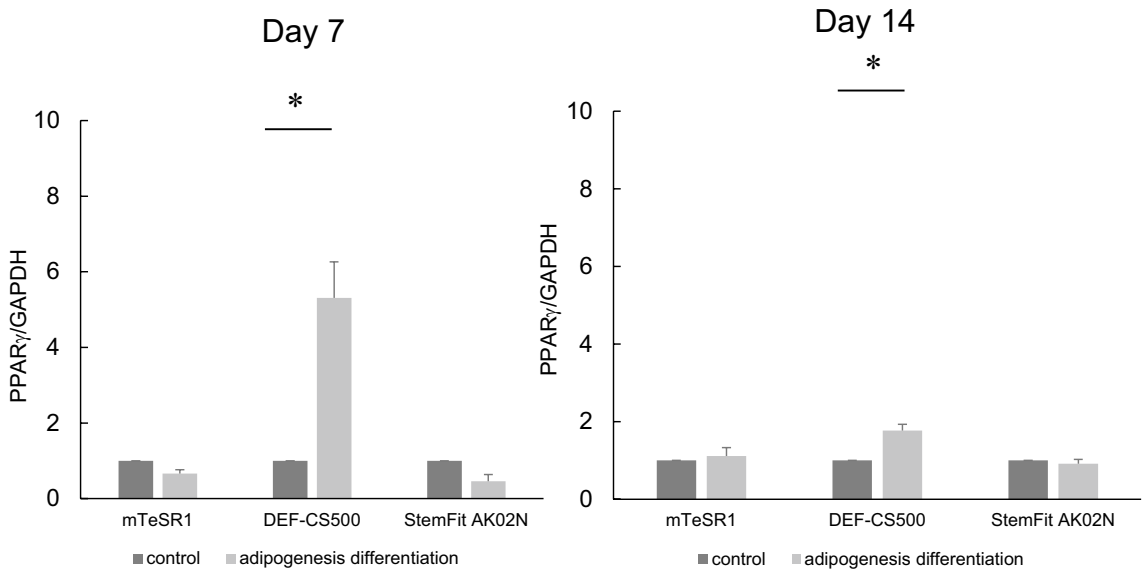

Fig. 7 *PPARγ* expression on day 7 and day 14. Data are expressed as the mean and standard deviation values. $*p < 0.05$

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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