LNGFR⁺THY-1⁺ Human Pluripotent Stem Cell-derived Neural Crest-Like Cells Have the Potential to Develop Into Mesenchymal Stem Cells

Takehito Ouchi ^{a,b}, Satoru Morikawa ^{a,b}, Shinsuke Shibata ^b, Kimiko Fukuda ^c, Hironobu Okuno ^b, Takumi Fujimura ^{b,d}, Tatsuo Kuroda ^d, Manabu Ohyama ^{e,f}, Wado Akamatsu ^{b,g}, Taneaki Nakagawa ^a, and Hideyuki Okano ^{b,*}

^a Department of Dentistry and Oral Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

^b Department of Physiology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

^c Department of Biological Science, Tokyo Metropolitan University, Hachioji-shi, Tokyo, Japan

^d Department of Pediatric Surgery, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

^e Department of Dermatology, Kyorin University School of Medicine, Mitaka-shi, Tokyo, Japan

^f Department of Dermatology, Keio University School of Medicine, Shinjuku-ku, Tokyo, Japan

^g Center for Genomic and Regenerative Medicine, Juntendo University School of Medicine, Bunkyo-ku, Tokyo, Japan

***Corresponding author:**

Hideyuki Okano, Department of Physiology, Keio University School of Medicine, 35

Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan

Tel: +81-3-5363-3747; Fax: +81-3-3357-5445; E-mail: hidokano@a2.keio.jp

Abbreviations: Mesenchymal stem cells (MSCs), Neural crest cells (NCCs), Human embryonic stem cells (ESCs), Human induced pluripotent stem cells (iPSCs), Neural Crest-Like Cells (NCLCs), LNGFR⁺THY-1⁺ neural crest-like cells (LT-NCLCs)

Abstract word count: 235

Total word count (Introduction to Discussion): 3599

Number of figures: 8

Number of references: 52

ABSTRACT

Mesenchymal stem cells (MSCs) are defined as non-hematopoietic, plastic-adherent, self-renewing cells that are capable of tri-lineage differentiation into bone, cartilage or fat *in vitro*. Thus, MSCs are promising candidates for cell-based medicine. However, classifications of MSCs have been defined retrospectively; moreover, this conventional criterion may be inaccurate due to contamination with other hematopoietic lineage cells. Human MSCs can be enriched by selection for LNGFR and THY-1, and this population may be analogous to murine PDGFR α ⁺Sca-1⁺ cells, which are developmentally derived from neural crest cells (NCCs). Murine NCCs were labeled by fluorescence, which provided definitive proof of neural crest lineage, however, technical considerations prevent the use of a similar approach to determine the origin of human LNGFR⁺THY-1⁺ MSCs. To further clarify the origin of human MSCs, human embryonic stem cells (ESCs) and human induced pluripotent stem cells (iPSCs) were used in this study. Under culture conditions required for the induction of neural crest cells, human ESCs and iPSCs-derived cells highly expressed LNGFR and THY-1. These LNGFR⁺THY-1⁺ neural crest-like cells, designated as LT-NCLCs, showed a strong potential to differentiate into both mesenchymal and neural crest lineages. LT-NCLCs proliferated to form colonies and actively migrated in response to serum concentration. Furthermore, we transplanted LT-NCLCs into chick embryos, and traced their potential for survival, migration and differentiation in the host environment. These results suggest that LNGFR⁺THY-1⁺ cells identified following NCLC induction from ESCs/iPSCs shared similar potentials with multipotent MSCs.

Keywords: Mesenchymal stem cells, Neural crest cells, LNGFR, THY-1

1. Introduction

Mesenchymal stem cells (MSCs) were first reported in bone marrow (Friedenstein et al., 1974), and were eventually found to be distinct from hematopoietic stem cells (Koide et al., 2007). MSCs are defined as plastic-adherent cells with the capacity for self-renewal, which differentiate into multiple mature cell lineages. MSCs are capable of differentiation into bone, cartilage, and fat (Pittenger et al., 1999). MSCs have been identified not only in bone marrow but also in other adult somatic tissues, umbilical cord (Forraz and McGuckin, 2011), and fat tissues (Zuk et al., 2001).

Recently, MSCs have been successfully applied to regenerative medicine (Caplan, 2007; Murphy et al., 2013; Patel et al., 2013) for cell therapy for damaged tissues (Stappenbeck and Miyoshi, 2009) including spinal cord injury, epidermolysis bullosa, acute liver failure and cirrhosis and myocardial infarction (Sakai et al., 2012; Tamai et al., 2011; Volarevic et al., 2014; Wollert and Drexler, 2005). MSCs have also been reported as capable of contributing the repair of sites of injury and supplying trophic support (Caplan and Correa, 2011) as well as for immune modulation (Akiyama et al., 2012; Shi et al., 2011). Thus, MSCs are promising candidates for cell transplantation therapy in humans. A significant drawback of MSCs is that they can only be identified retrospectively following culture of bone marrow and selection for adherent cells. MSCs are then further defined by their potential for differentiation. Indeed, prospective analysis of MSCs is hampered due to contamination with other precursor cells and hematopoietic lineage cells in bone marrow culture. Therefore, the true origin and derivation of MSCs have yet to be completely elucidated (Mabuchi et al., 2013; Morikawa et al., 2009a).

In murine stem cell research, transgenic cell lines are often utilized to visualize cellular origin and subsequent differentiation. It was previously demonstrated that PDGFR α ⁺Sca-1⁺ purified mouse MSCs originate from neural crest cells during development (Morikawa et al., 2009b). P0-Cre/Floxed-EGFP mice and Wnt-Cre/Floxed-EGFP mice were used to identify the cells derived from neural crest (Morikawa et al., 2009b; Nagoshi et al., 2008). These murine neural crest-derived cells could be detected in adult bone marrow, where they maintained a multipotent capacity. Additional studies further demonstrated that neural crest lineage cells developed into multipotent MSCs (Isern et al., 2014; Morikawa et al., 2009b; Nagoshi et al., 2008; Neirinckx et al., 2013; Takashima et al., 2007).

Several cell markers have been reported to identify murine MSCs. Some reports suggested that leptin receptor and Nestin mark murine bone marrow MSCs (Ding et al., 2012; Isern et al., 2014; Mendez-Ferrer et al., 2010; Zhou et al., 2014). Another group reported that Gremlin-1 is an effective surface marker to track MSCs in both developing and adult skeletal tissues (Worthley et al., 2015). Thus, MSCs have very important roles not only in normal development but also in maintenance of adult tissues. Other transgenic studies further revealed the unique features of murine MSCs (Matsuzaki et al., 2014).

Our group previously identified human MSC markers (i.e., LNGFR and THY-1), which can be used to avoid *in vitro* contamination and perform prospective identification (Aomatsu et al., 2014; Mabuchi et al., 2013; Ogata et al., 2015; Yasui et al., 2016). LNGFR⁺THY-1⁺ human MSCs isolated from bone marrow, decidua, and fat tissues exhibited higher potentials for mesenchymal differentiation as well as for specific functional properties compared to conventional adherent-cultured MSCs (Mabuchi et al., 2013). However, the

developmental origin of this population has yet to be elucidated. To further characterize these purified human MSCs, we focused on their origins by using human pluripotent stem cells (ESCs / iPSCs) in this study.

In 2007, the discovery of human iPSCs provided a new perspective on developmental biology and regenerative medicine (Takahashi et al., 2007). Use of human iPSCs, similar to ESCs, enables us to study pluripotent cells without destroying human fertilized eggs. Use of iPSCs can overcome ethical concerns and may demonstrate the derivation of the specific cell type that mimics normal developmental fate *in vitro and in vivo*. In this study, NCLCs that originated from human pluripotent stem cells shared characteristics of purified MSCs, and closely resemble results from our previous reports that describe MSCs derived from NCCs (Fukuta et al., 2014; Morikawa et al., 2009b).

2. Material and methods

2.1. Ethical approval

This study was performed in accordance with ethics protocols approved by the Keio University Ethics Committee (20080016 and 20140005).

2.2. Human ESC and iPSC preparation and culture

Human ESC cell line H1, (Thomson et al., 1998), human iPSC cell line 201B7 (Takahashi et al., 2007), and iPSC cell line WD39 (Imaizumi et al., 2012) were cultured in Matrigel coated 6-well plates in mTeSR-1 medium (BD Biosciences) under feeder-free conditions, and passaged using protease and collagenase (Accutase; Innovative Cell Technologies).

mTeSR-1 was changed daily. Differentiated cells from human ESC and iPSC colonies were removed during the daily medium change.

2.3. Induction of NCLCs

NCLCs were induced according to previously described methods (Bajpai et al., 2010). Briefly, human ESCs / iPSCs were cultured under feeder-free culture conditions in 6-well adherent culture dishes. These cells had clear margins and obvious contrast between nucleus and cytoplasm. The human ESC / iPSC colonies were removed by collagenase. Neural crest medium consisted of 1:1 ratio of DMEM/F12 (GIBCO) and Neurobasal Media (GIBCO) with 0.5% of x100 GlutaMAX (GIBCO), 1% Gem21 Neuroplex (GEMINI BIO-PRODUCTS), 0.5% N2 Supplement (GIBCO), 20 ng ml⁻¹ of hbFGF (REPRO TECH), 20 ng ml⁻¹ of hEGF (REPRO TECH), 20 ng ml⁻¹ insulin (Nacalai Tesque), and 0.5% penicillin/streptomycin. After one day (day 1), dead cells were removed and the medium changed. On day 4, spheres were collected and the other attached cells were discarded. Spheres were placed on 100 mm adherent culture plates (BD Falcon). On day 9 or 10, cells that migrated from spheres were defined as NCLCs.

2.4. Cell analysis and sorting

NCLCs-derived from human pluripotent stem cells were mechanically detached and immunostained using the following mouse monoclonal antibodies: anti-human LNGFR antibody (ME20.4-1.H4; Miltenyi Biotech) and anti-human THY-1 antibody (5E10; BD Bioscience). The concentration of the antibodies was adjusted to 1:500/1.0 × 10⁷ cells.

After sorting for the LNGFR⁺THY-1⁺ fraction, markers for neural crest cells, hematopoietic lineage cells, and epithelial cells were analyzed. To determine neural crest cells, anti-HNK-1 (anti-CD57) antibody (NK-1; Chemicon) was used. To determine the hematopoietic-negative status, cells were analyzed for lymphocyte, monocyte, eosinophil, and neutrophil markers using the Lineage Cocktail kit (anti-CD3, CD14, CD16, CD19, CD20 and CD56 antibodies against SK7, MfP9, 3G8, SJ25C1, L27 and NCAM16.2 respectively; BD Bioscience).

PECAM-1 (CD31) antibody (WM59; BD Bioscience) was used for platelet-negative selection. EpCAM (CD326) antibody (caa7-9G8; Miltenyi Biotech) was used for the determination of epithelial cells. The concentration of the antibodies was adjusted to 1:100, and added to each sample ($1.0 - 5.0 \times 10^5$ cells). Dead cells were detected by PI (propidium iodide; Sigma). Analysis and sorting were performed by flow cytometry (Aria III; BD Biosciences). Flowjo software (Tree Star) was used for data analysis.

2.5. Mesenchymal lineage induction

A mesenchymal tri-lineage differentiation assay was performed as described previously (Houlihan et al., 2012). Sorted LNGFR⁺THY-1⁺ cells were incubated in each induction medium provided in the human MSC adipogenic differentiation bulletKit™ (LONZA), osteogenic differentiation bulletKit™ (LONZA), and chondrogenic differentiation bulletKit™ (LONZA). For chondrogenic induction, TGF-β3 (LONZA) (10 ng ml^{-1}) and BMP-6 (R&D Systems) (500 ng ml^{-1}) were added. Oil Red O staining, Alizarin Red staining, and Toluidine Blue staining were performed following previously described protocols (Houlihan et al.,

2012).

2.6. Induction of neural crest lineage

Neural crest lineage induction was carried out following the previously described methods with slight modifications (Menendez et al., 2013). Briefly, LNGFR⁺THY-1⁺ cells (1.0×10^5 /cm²) were cultured in neural crest medium. After adhesion, the neural crest medium was changed to differentiation medium (DMEM/F12 (GIBCO), 1% N2 supplement (GIBCO), 2% B27 (GIBCO), and penicillin/streptomycin).

2.7. Colony forming unit fibroblast (CFU-F) assay

LNGFR⁺THY-1⁺ cells (5.0×10^3) were plated on a 100 mm dish. The medium was composed of DMEM (SIGMA), 20% FBS (GIBCO), 20 ng ml⁻¹ hbFGF (SIGMA), 10 mM HEPES, and 1% penicillin/streptomycin. After 14 days of culture, colonies were identified as those containing >50 cells.

2.8. Transwell migration assay

LNGFR⁺THY-1⁺ cells (1.5×10^4) were plated on a cell culture insert (8 µm pore size). FBS (1%) was added to the upper chamber and 20% to the lower chamber. After 24 hours, cells were counted in the lower chamber.

2.9 Cell Transplantation and cell tracing in vivo

We transplanted and tracked human iPSC(WD39)-derived LNGFR⁺THY-1⁺ cells in chicken

embryo. LNGFR⁺THY-1⁺ cells were sorted and maintained as spheroids in 6-well ultra-low adherent culture plate (CORNING). LNGFR⁺THY-1⁺ cells were adjusted to 1.0×10^4 / well, and the medium was composed of DMEM (SIGMA), 10% FBS (GIBCO), 1% L-glutamine (Nacalai Tesque), and 1% penicillin/streptomycin. We transfected ffLuc (Hara-Miyauchi et al., 2012), a fluorescence protein (modified Venus) fused with a luminescence protein (Luciferase2) by lentivirus to visualize LNGFR⁺THY-1⁺ cells. Fertilized chicken eggs were incubated at 38°C to control for the appropriate Hamburger–Hamilton (H.H.) stage (Hamburger and Hamilton, 1951). One aggregation of the LNGFR⁺THY-1⁺ cells was transplanted into the future somite region of the chick embryo at H. H. stages 9–12. LNGFR⁺THY-1⁺ cells were treated with a luciferase substrate, D-(–)-2-(6'-hydroxy-2'-benzothiazolyl) thiazone-4-carboxylic acid (D-luciferin) (150µg/ml) (Summit Pharmaceuticals International Corporation), and bioluminescence signals were measured using an In Vivo Imaging System (IVIS)-spectrum and CCD optical macroscopic imaging system (Caliper). Specimens were isolated from fertilized eggs and fixed with 4% PFA, cryoprotected with 15 and 30% sucrose, and sectioned with a cryostat for immunohistochemistry.

2.10. Immunofluorescence

Human LNGFR⁺THY-1⁺ cells were transferred to 8-well glass chambers and maintained in neural crest medium. Cells were fixed with 4% paraformaldehyde (PFA) and washed with 0.1M PBS. After one hour incubation with blocking buffer (Nacalai Tesque) at room temperature, the following primary antibodies were applied: mouse monoclonal anti-human

Ki-67 (MM1; Leica, 1:200), goat polyclonal anti-human SOX10 (R&D Systems, 1:200), rabbit monoclonal anti-human AP2 α (C83E10; Cell Signaling Technology, 1:100), rabbit polyclonal anti-human NESTIN (Eurofins Genomics, 1:200), rabbit polyclonal anti-human NANOG (ReproCELL, 1:200), and mouse monoclonal anti-human OCT3/4 (C-10; Santa Cruz, 1:200).

Neurogenic induced cells were stained with mouse monoclonal anti-human S100 β (beta subunit) (SH-B4; Sigma, 1:1000), mouse monoclonal TUJ1 (anti-human beta-Tubulin isotype III) (SDL.3D10; Sigma, 1:400), mouse monoclonal anti-human SMA (1A4; Sigma, 1:400), and rabbit polyclonal anti-rat Peripherin (Chemicon, 1:200). Tissues sections were stained with goat polyclonal anti-GFP (ROCKLAND, 1:200), mouse monoclonal anti-human CD49d (9F10; BioLegend, 1:200), mouse monoclonal anti-human PCNA (PC10; Merck Millipore, 1:200), rabbit polyclonal anti-human PAX9 (Sigma, 1:200). Secondary antibodies conjugated with Alexa Fluor 555 were used to detect TUJ1, S100 β , Ki-67, PCNA, CD49d and PAX9. Alexa Fluor 488 conjugated secondary antibodies were used for the others. Immunofluorescence staining was analyzed using a fluorescence microscope (BZ9000) (Keyence) and LSM700 (Carl Zeiss).

2.11. Statistical analysis

All results were presented as averages $\pm$ SEM (means of the standard error). All data were representative of at least three independent experiments, and statistical significance was determined with Kruskal–Wallis and Steel-Dwass tests using SPSS 22 and JMP 11.

3. Results

3.1. Generation of $LNGFR^+THY-1^+$ cells from human ESCs and iPSCs

To obtain a sufficient number of cells *in vitro*, human pluripotent stem cells were used (Fig. 1A). Cell differentiation was induced following a previously described protocol for neural crest induction (Bajpai et al., 2010). On day 4, seeded cells formed floating spheres, and only these floating spheres were used for the next adherent culture in new dishes. Cells were attached on day 6 or 7, and spindle-shaped cells migrated out of the sphere. On day 9 or 10, a larger number of cells was observed due to cell proliferation during neural crest induction (Fig. 1B).

Fig. 1. Induction of cells derived from human pluripotent stem cells (ESCs / iPSCs).

(A) Culture conditions used for induction (B) Cells derived from human pluripotent stem cells generated spheres until day 4. Spheres were harvested and placed into a new culture dish, and cells subsequently attached and spontaneously migrated by ~day 7. Induced cells exhibited a typical spindle shape following 9–10 days of incubation. Scale bars: 100 μ m.

Induced cells showed spindle-shaped morphology, which is typical for somatic MSCs (Fig. 2A). To confirm purification of human MSCs, the expression of LNGFR and THY-1, which are highly expressed in MSCs, was analyzed using flow cytometry (Fig. 2B). The efficiency of LNGFR⁺THY-1⁺ cell induction from each human pluripotent stem cell line was as follows: ESC line H1: 35.76 ± 1.2% (N = 5); iPSC line 201B7: 14.98 ± 2.3 (N = 5) and iPSC line WD39: 22.82 ± 2.8 (N = 7) (Fig. 2C).

Fig. 2. Detection of LNGFR and THY-1 expression by flow cytometry. (A)

LNGFR⁺THY-1⁺ cells derived from the ESC line (H1) and iPSC lines (201B7 and WD39). (B) Induction efficiency of LNGFR⁺THY-1⁺ cells determined by flow cytometry. (C) Human ESCs (H1) showed the highest induction efficiency among these three groups. p values calculated for each of the cell lines were: H1-201B7 (0.0326), H1-WD39 (0.0159) and 201B7-WD39 (0.2355). Scale Bars: 100 μ m, *p < 0.05.

3.2. Characterization of LNGFR⁺THY-1⁺ cells

To investigate the characteristics of LNGFR⁺THY-1⁺ cells, specific marker expression was analyzed *in vitro*. Some LNGFR⁺THY-1⁺ cells were positive for the immature and mature neural crest cell markers AP2 α , SOX10, and NESTIN and HNK-1 (Fig. 3A) Of note, LNGFR⁺THY-1⁺ cells were completely negative for the pluripotency markers NANOG and OCT3/4 (Fig. 3C). They were almost negative for hematopoietic lineage markers and were also negative for the epithelial marker CD326 (Fig. 3D). These findings suggest that the purified population of LNGFR⁺THY-1⁺ cells demonstrated neural crest-like cell differentiation. These cells are designated as LT-NCLCs (LNGFR⁺THY-1⁺ neural crest-like cells) in this report.

Fig. 3. Cell type-specific marker analysis of human iPSC-derived LNGFR⁺THY-1⁺ cells.

(A, B) Human iPSC (WD39) derived LNGFR⁺THY-1⁺ cells expressed NCC markers (AP2 α , SOX10, NESTIN and HNK-1) as determined by immunocytochemistry (A) and by flow cytometry (B). (C) LNGFR⁺THY-1⁺ cells were negative for the pluripotent stem cell markers, NANOG and OCT3/4, by immunocytochemical analysis. (D) LNGFR⁺THY-1⁺ cells expressed neither hematopoietic cell markers nor the epithelial cell surface marker (CD326) by flow cytometric analysis. Scale Bars: 50 μ m.

3.3. Potential of LT-NCLCs to differentiate into mesenchymal and neural crest lineages

One of the defining characteristics of somatic LNGFR⁺THY-1⁺ MSCs is their capacity for tri-lineage differentiation into osteocytes, chondrocytes, or adipocytes (Mabuchi et al., 2013). Therefore, tri-lineage evaluation of LT-NCLCs was performed *in vitro* to confirm whether they maintained MSC potential. The capacity of LT-NCLCs for osteogenic differentiation *in vitro* was evaluated using osteo-induction media. After 2 weeks of osteo-induction culture, mineralization of matrix was assessed based on the presence of Alizarin Red-positive mineralized nodules (left panel in Fig. 4A). Chondrogenic lineage differentiation from LT-NCLCs was also examined. LT-NCLCs were cultured in three-dimensional cell aggregates in chondrogenic differentiation medium, and they formed pellets stained by Toluidine Blue (middle of Fig. 4A). The capacity for LT-NCLC adipogenic differentiation *in vitro* was investigated by culture in adipogenic media, which demonstrated that LNGFR⁺THY-1⁺ cells could differentiate into Oil Red O-positive lipid-containing adipocytes (right in Fig. 4A). Therefore, the LNGFR⁺THY-1⁺ cells generated from human pluripotent cell lines exhibited the typical characteristics of MSCs as well as those of somatic cells.

Next, purified LT-NCLCs were tested for their potential to differentiate into neural crest lineage cells (Fig. 4B). Neuronal differentiation was observed following incubation with neurogenic differentiation medium. The cells expressed both beta-Tubulin isotype III and Peripherin following 5–7 days of culture; which implied differentiation into peripheral neurons. The cells were also positive for S100 and SMA, which indicated the differentiation

of LT-NCLCs into glial cells and smooth muscle cells, respectively (Fig. 4B). Therefore, LT-NCLCs demonstrated typical features of both NCCs and MSCs; similar to previous reports (Morikawa et al., 2009b).

Fig. 4. Mesenchymal and neural crest lineage differentiation. (A) LT-NCLCs maintained the ability to differentiate into mesenchymal lineages (osteocytes, chondrocytes, and adipocytes) as determined by staining with Alizarin Red, Toluidine Blue, and Oil Red O, respectively. (B) LT-NCLCs can differentiate into neural crest lineages (neurons, glial cells, and smooth muscle cells). This was determined by the result of immunocytochemistry using antibodies specific for β III-Tubulin, Peripherin for identifying neurons, with S100 for glial cells, and SMA for smooth muscle cells. Scale bars: 100 μ m.

3.4. Proliferation and migration capacity of LT-NCLCs

The capacity for self-renewal is one of the most important characteristics of stem cells. To investigate this potential in LT-NCLCs, we performed immunohistochemistry and colony forming assays. Immunocytochemical evaluation on LT-NCLCs indicated that some differentiated cells expressed Ki-67, a marker for G1, S, G2 and M phase, which suggests active cell division (Fig. 5A). A previous report indicated that one out of six somatic $\text{LNGFR}^+\text{THY-1}^+$ cells showed colony-forming activity (Mabuchi et al., 2013). Results from CFU-F assays performed using LT-NCLCs in this study demonstrated an even lower colony forming efficiency (Fig. 5B).

The capacity for migration is also a typical feature of mesenchymal and neural crest lineage stem cells, especially during the embryonic period. To investigate the migration potential of LT-NCLCs, we performed a trans-well migration assay using LT-NCLCs derived from the three human cell lines. In this assay, LT-NCLCs from all three lines showed active migration in response to high concentration of FBS (Fig. 5C). The human ESC line H1-derived LT-NCLCs had the highest migration among the three groups, while the human iPSC (line 201B7)-derived LT-NCLCs demonstrated the lowest migration capacity (Fig. 5C). These results indicated that purified LT-NCLCs, which express NCC markers, maintained potential for active expansion and for migration.

A **B**

Colony Forming Unit Assay (Number of cells to form a colony)	
ESC (H1)	23.6 ± 1.5
iPSC (201B7)	44.9 ± 2.6
iPSC (WD39)	26.8 ± 1.6

C

Frequencies of migration (%)	
ESC (H1)	8.4 ± 0.010
iPSC (201B7)	5.4 ± 0.005
iPSC (WD39)	6.9 ± 0.005

Fig. 5. Colony forming unit (CFU-F) and trans-well migration assays. (A) Sorted LT-NCLCs were cultured in MSC medium, and their expression of cell proliferation markers was evaluated. LT-NCLCs labeled with cell proliferation marker Ki-67 by immunocytochemistry indicated active cell division. Scale bars: 100 μ m. (B) Efficacy of colony forming units (N = 3, from each group) is indicated in (B). Human ESC (H1) derived LT-NCLCs showed the highest capacity for colony formation. (C) Human ESC (H1) derived LT-NCLCs had the highest migration activity (N = 3, from each group).

3.5. The *in vivo* potential of LT-NCLCs

To investigate the migration and differentiation potential of human iPSC(WD39)-derived LT-NCLCs *in vivo*, we transplanted these cells and tracked their behavior in chicken embryo. LT-NCLCs were labeled with the fluorescence and luminescence fusion protein ffLuc (Hara-Miyauchi et al., 2012) (Fig. 6A). LT-NCLCs formed the cell aggregation for 24 hours in the ultra-low cluster culture plate and were transplanted into the future somite region (Fig. 6B). Bioluminescence signal from survived LT-NCLCs was detected with the application of D-Luciferin using IVIS spectra for macroscopic imaging (Fig. 6C). Our results indicate that the transplanted LT-NCLCs survived in the host chicken embryonic environment.

Two days after transplantation, active migration of the human LT-NCLCs in sclerotome region was observed with the fluorescence localization originated from the lentiviral infection of ffLuc (Fig. 7A). To evaluate the differentiation potential using immunohistochemistry, frozen sections were prepared from the transplanted tissue. We analyzed the proliferation and differentiation capacity by immunohistochemistry. Venus-positive human LT-NCLCs, migrated in the subcutaneous space two day after transplantation, highly expressed CD49d, which imply the neural crest lineage differentiation (Fig. 7B). Transplanted human LT-NCLCs also weakly expressed PCNA (Proliferative Cell Nuclear Antigen), suggesting the ability for proliferation while migration in the chicken embryo (Fig. 7C). We evaluated these cells using lineage-specific markers of sclerotome development (PAX9) as well as MSC and NCC differentiation; however we detected only

limited potential of differentiation at 1–2 days post transplantation.

In summary, human LT-NCLCs transplanted in the chicken embryo demonstrated some potential to proliferate and migrate *in vivo* adapted to the recipient environment, but limited potential to differentiate up to two days post transplant.

Fig. 6. Transplantation of human LT-NCLCs into fertilized chicken embryo. (A) Lentiviral reporter construct with fluorescence protein fused with Luciferase (ffLuc) under the control of EF promoter. (B) Sorted human LT-NCLCs formed the cell aggregation, and were transplanted into chicken future somite region (arrow). (C) Transplanted-living cells were detected by bioluminescent with In Vivo Imaging System (IVIS) 2 days post operation. Scale bars: (B) 500 μ m (C) 1 mm.

A

B

C

D

Fig. 7. Immunohistochemical evaluation of transplanted human LT-NCLCs *in vivo*. (A)

At the second days post operation, human LT-NCLCs labeled with fluorescence showed the active migration to the sclerotome region from the original area (arrow). (B) With immunohistochemistry, human LT-NCLCs, detected in the subcutaneous area, demonstrated the high level of CD49d expression, suggesting the neural crest lineage differentiation. (C) Transplanted human LT-NCLCs also expressed PCNA, a proliferation marker, at one day post operation (arrowheads). (D) The expression of progenitor markers, including PAX9, was not detected in the human LT-NCLCs but detected in the surrounding the host cells in sclerotome region. Scale bars: (A) 500µm, (B) – (D) 20µm.

4. Discussion

Historically, MSCs have been defined based on their potentials to adhere to a plastic plate in the presence of serum and to differentiate into mesenchymal lineages. The same definition can be applied to several other tissue specific MSCs. MSCs have the potential to differentiate into neuronal lineage tissues (Arthur et al., 2008; Kohyama et al., 2001; Sanchez-Ramos et al., 2000) and they may be used to repair damaged tissues that originated from neural crest (Wollert and Drexler, 2005). This implies the MSCs and NCCs may share common features. In fact, some NCCs may have the potential to differentiate into mesenchymal lineages (Hagiwara et al., 2014), and MSCs have been isolated from several neural crest derived tissues (Janebodin et al., 2011; Wong et al., 2006) that maintain the features of their neural crest lineage. Therefore, some MSCs might originate from NCCs and can migrate into neural crest derived tissues while maintaining the potential of MSCs in adult tissues (Fig. 8).

Fig. 8. Schematic of proposed cell lineage differentiation. Neural crest derivatives (green colored cells) are delaminated from dorsal neural tube and migrate to specific

locations over the course of development. In this study, LNGFR⁺THY-1⁺ cells were detected after neural crest induction of cells derived from human ESCs and iPSCs. Previous *in vivo* studies demonstrated that somatic MSCs, (LNGFR⁺THY-1⁺ cells), were detected in various adult tissues. Therefore, it is possible to speculate that LNGFR⁺THY-1⁺ cells are found in neural crest derivatives in embryo, and that they migrate to form neural crest-derived tissue, where they maintain the potential of both MSCs and NCCs.

In a previous study, our group identified pure populations of murine MSCs (Morikawa et al., 2009a) and also human MSCs (Mabuchi et al., 2013). In the mouse, purified MSCs partially originated from the neural crest (Morikawa et al., 2009b). Other research groups have also demonstrated a contribution of mouse MSCs to normal development in embryonic stages and also in the adult stage (Worthley et al., 2015). Tracking of fluorescently labeled cells during development of mice has proven to be exceedingly useful in understanding the origin of different cell lineages. Unfortunately, labeling and tracking of specific lineage cells in humans is not feasible due to ethical concerns.

In human bone marrow, LNGFR and THY-1 are specifically and highly expressed on the cell surface of MSCs (Mabuchi et al., 2013). This LNGFR⁺THY-1⁺ cell population has been isolated from several types of tissues, and it demonstrates the typical characteristics of MSCs (Aomatsu et al., 2014; Mabuchi et al., 2013; Ogata et al., 2015; Yasui et al., 2016). Focusing on the LNGFR⁺THY-1⁺ cell fraction within the mesenchymal and neural crest lineages thus represented a meaningful approach to clarify the relationship between MSCs and NCCs.

In the present study, NCLCs were induced from human pluripotent stem cells. These cells partially expressed LNGFR and THY-1 (Fig. 1 and 2). It was reported that LNGFR is an efficient MSC cell surface marker (Alvarez-Viejo et al., 2013; Quirici et al., 2002), which is not expressed in hematopoietic lineage cells (Quirici et al., 2002). Developmentally, murine purified MSCs are partially originated from NCCs (Morikawa et al., 2009b). Together, this evidence suggested that LNGFR is a common marker for both MSCs and NCCs in human. To reveal this common feature of human NCCs and MSCs *in vitro*, neural crest cell induction was carried out using human pluripotent stem cells. LNGFR⁺THY-1⁺ cells isolated from NCLCs, designated as LT-NCLCs, demonstrated the expression of immature and mature NCC markers (AP2 α , SOX10, and NESTIN) and were distinct from hematopoietic and epithelial lineages (Fig. 3). LT-NCLCs can differentiate into mesenchymal cells including osteogenic, chondrogenic and adipogenic cells, and they can also differentiate into neural crest derived cells including peripheral neurons, glial cells, and smooth muscle cells *in vitro* (Fig. 4). In addition, LT-NCLCs demonstrated active motility, a common feature of NCCs and MSCs (Fig. 5). Thus, data generated from LT-NCLCs indicated that LNGFR⁺THY-1⁺ cells derived from human pluripotent stem cell lines share similar characteristics with both NCCs and MSCs. The *in vivo* study shown in figures 6 and 7 showed that grafted human LT-NCLCs survived with the potential for proliferation and migration. Human LT-NCLCs survived in host environment and exhibited the potential to migrate, expressing the neural crest cells and mobilization marker CD49d (Lee et al., 2010; Mabuchi et al., 2013) as shown in Fig.7B. Transplanted human LT-NCLCs identified in chicken sclerotome region did not express PAX9, whereas the surrounding cells actively

expressed PAX9, which is normally expressed in this developmental chicken tissue (LeClair et al., 1999; Rodrigo et al., 2003). Sclerotome is the embryonic origin of skeletal tissues and recent reports have shown some overlap in the properties of MSCs and SSCs (Chan et al., 2015; Morikawa et al., 2016; Worthley et al., 2015). The present study shows that purified human pluripotent stem cell-derived MSCs, in terms of LNGFR⁺THY-1⁺ cells, have multipotent differentiation ability *in vitro*, and migration and environmental adaptation capacity *in vivo*. These findings have potential for future applications in stem cell biology and cell transplantation therapies in human.

Acknowledgements

We sincerely thank Drs. Kunimichi Niibe, Yo Mabuchi, Makoto Hosoya, Sadafumi Suzuki, Yu Yamaguchi, Yuka Yamada, and colleagues in the Okano laboratory for their help. We thank Douglas Sipp for critical reading of the manuscript. This work was supported by JSPS KAKENHI grants numbered 24592965, 15K11221 (T. N.) and 25463226 (S. M.), by the Practical Research for Innovative Cancer Control from Japan Agency for Medical Research and development (AMED) to S.S., and by the Program for Intractable Disease Research Utilizing Disease-specific iPS Cells from the Japan Science and Technology Agency (JST) and the Ministry of Education, Science, Sports and Culture (MEXT) to H.O.

Conflicts of interest

H.O. is a paid Scientific Advisor Board of San Bio Co. Ltd. Other authors have no conflict of interests to declare.

References

- Akiyama, K., Chen, C., Wang, D., Xu, X., Qu, C., Yamaza, T., Cai, T., Chen, W., Sun, L., Shi, S., 2012. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 10, 544-555.
- Alvarez-Viejo, M., Menendez-Menendez, Y., Blanco-Gelaz, M.A., Ferrero-Gutierrez, A., Fernandez-Rodriguez, M.A., Gala, J., Otero-Hernandez, J., 2013. Quantifying mesenchymal stem cells in the mononuclear cell fraction of bone marrow samples obtained for cell therapy. *Transplant. Proc.* 45, 434-439.
- Aomatsu, E., Takahashi, N., Sawada, S., Okubo, N., Hasegawa, T., Taira, M., Miura, H., Ishisaki, A., Chosa, N., 2014. Novel SCRG1/BST1 axis regulates self-renewal, migration, and osteogenic differentiation potential in mesenchymal stem cells. *Sci. Rep.* 4, 3652.
- Arthur, A., Rychkov, G., Shi, S., Koblar, S.A., Gronthos, S., 2008. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. *Stem Cells* 26, 1787-1795.
- Bajpai, R., Chen, D.A., Rada-Iglesias, A., Zhang, J., Xiong, Y., Helms, J., Chang, C.P., Zhao, Y., Swigut, T., Wysocka, J., 2010. CHD7 cooperates with PBAF to control multipotent neural crest formation. *Nature* 463, 958-962.
- Caplan, A.I., 2007. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol.* 213, 341-347.
- Caplan, A.I., Correa, D., 2011. The MSC: an injury drugstore. *Cell Stem Cell* 9, 11-15.
- Chan, C.K., Seo, E.Y., Chen, J.Y., Lo, D., McArdle, A., Sinha, R., Tevlin, R., Seita, J., Vincent-Tompkins, J., Wearda, T., Lu, W.J., Senarath-Yapa, K., Chung, M.T., Marecic, O., Tran, M., Yan, K.S., Upton, R., Walmsley, G.G., Lee, A.S., Sahoo, D., Kuo, C.J., Weissman, I.L., Longaker, M.T., 2015. Identification and specification of the mouse skeletal stem cell. *Cell* 160, 285-298.
- Ding, L., Saunders, T.L., Enikolopov, G., Morrison, S.J., 2012. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 481, 457-462.
- Forraz, N., McGuckin, C.P., 2011. The umbilical cord: a rich and ethical stem cell source to advance regenerative medicine. *Cell Prolif.* 44 Suppl 1, 60-69.
- Friedenstein, A.J., Deriglasova, U.F., Kulagina, N.N., Panasuk, A.F., Rudakowa, S.F., Luria, E.A., Ruadkow, I.A., 1974. Precursors for fibroblasts in different populations of

hematopoietic cells as detected by the in vitro colony assay method. *Exp. Hematol.* 2, 83-92.

Fukuta, M., Nakai, Y., Kirino, K., Nakagawa, M., Sekiguchi, K., Nagata, S., Matsumoto, Y., Yamamoto, T., Umeda, K., Heike, T., Okumura, N., Koizumi, N., Sato, T., Nakahata, T., Saito, M., Otsuka, T., Kinoshita, S., Ueno, M., Ikeya, M., Toguchida, J., 2014. Derivation of mesenchymal stromal cells from pluripotent stem cells through a neural crest lineage using small molecule compounds with defined media. *PLoS One* 9, e112291.

Hagiwara, K., Obayashi, T., Sakayori, N., Yamanishi, E., Hayashi, R., Osumi, N., Nakazawa, T., Nishida, K., 2014. Molecular and cellular features of murine craniofacial and trunk neural crest cells as stem cell-like cells. *PLoS One* 9, e84072.

Hamburger, V., Hamilton, H.L., 1951. A series of normal stages in the development of the chick embryo. *J. Morphol.* 88, 49-92.

Hara-Miyauchi, C., Tsuji, O., Hanyu, A., Okada, S., Yasuda, A., Fukano, T., Akazawa, C., Nakamura, M., Imamura, T., Matsuzaki, Y., Okano, H.J., Miyawaki, A., Okano, H., 2012. Bioluminescent system for dynamic imaging of cell and animal behavior. *Biochem. Biophys. Res. Commun.* 419, 188-193.

Houlihan, D.D., Mabuchi, Y., Morikawa, S., Niibe, K., Araki, D., Suzuki, S., Okano, H., Matsuzaki, Y., 2012. Isolation of mouse mesenchymal stem cells on the basis of expression of Sca-1 and PDGFR- α . *Nat. Protoc.* 7, 2103-2111.

Imaizumi, Y., Okada, Y., Akamatsu, W., Koike, M., Kuzumaki, N., Hayakawa, H., Nihira, T., Kobayashi, T., Ohyama, M., Sato, S., Takanashi, M., Funayama, M., Hirayama, A., Soga, T., Hishiki, T., Suematsu, M., Yagi, T., Ito, D., Kosakai, A., Hayashi, K., Shouji, M., Nakanishi, A., Suzuki, N., Mizuno, Y., Mizushima, N., Amagai, M., Uchiyama, Y., Mochizuki, H., Hattori, N., Okano, H., 2012. Mitochondrial dysfunction associated with increased oxidative stress and alpha-synuclein accumulation in PARK2 iPSC-derived neurons and postmortem brain tissue. *Mol. Brain* 5, 35.

Isern, J., Garcia-Garcia, A., Martin, A.M., Arranz, L., Martin-Perez, D., Torroja, C., Sanchez-Cabo, F., Mendez-Ferrer, S., 2014. The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *eLife* 3, e03696.

Janebodin, K., Horst, O.V., Ieronimakis, N., Balasundaram, G., Reesukumal, K., Pratumvinit, B., Reyes, M., 2011. Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. *PLoS One* 6, e27526.

Kohyama, J., Abe, H., Shimazaki, T., Koizumi, A., Nakashima, K., Gojo, S., Taga, T., Okano, H., Hata, J., Umezawa, A., 2001. Brain from bone: efficient "meta-differentiation" of marrow

stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation* 68, 235-244.

Koide, Y., Morikawa, S., Mabuchi, Y., Muguruma, Y., Hiratsu, E., Hasegawa, K., Kobayashi, M., Ando, K., Kinjo, K., Okano, H., Matsuzaki, Y., 2007. Two distinct stem cell lineages in murine bone marrow. *Stem Cells* 25, 1213-1221.

LeClair, E.E., Bonfiglio, L., Tuan, R.S., 1999. Expression of the paired-box genes Pax-1 and Pax-9 in limb skeleton development. *Dev. Dyn.* 214, 101-115.

Lee, G., Chambers, S.M., Tomishima, M.J., Studer, L., 2010. Derivation of neural crest cells from human pluripotent stem cells. *Nat. Protoc.* 5, 688-701.

Mabuchi, Y., Morikawa, S., Harada, S., Niibe, K., Suzuki, S., Renault-Mihara, F., Houlihan, D.D., Akazawa, C., Okano, H., Matsuzaki, Y., 2013. LNGFR(+)THY-1(+)VCAM-1(hi+) cells reveal functionally distinct subpopulations in mesenchymal stem cells. *Stem Cell Reports* 1, 152-165.

Matsuzaki, Y., Mabuchi, Y., Okano, H., 2014. Leptin receptor makes its mark on MSCs. *Cell Stem Cell* 15, 112-114.

Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., Macarthur, B.D., Lira, S.A., Scadden, D.T., Ma'ayan, A., Enikolopov, G.N., Frenette, P.S., 2010. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 466, 829-834.

Menendez, L., Kulik, M.J., Page, A.T., Park, S.S., Lauderdale, J.D., Cunningham, M.L., Dalton, S., 2013. Directed differentiation of human pluripotent cells to neural crest stem cells. *Nat. Protoc.* 8, 203-212.

Morikawa, S., Mabuchi, Y., Kubota, Y., Nagai, Y., Niibe, K., Hiratsu, E., Suzuki, S., Miyauchi-Hara, C., Nagoshi, N., Sunabori, T., Shimmura, S., Miyawaki, A., Nakagawa, T., Suda, T., Okano, H., Matsuzaki, Y., 2009a. Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J. Exp. Med.* 206, 2483-2496.

Morikawa, S., Mabuchi, Y., Niibe, K., Suzuki, S., Nagoshi, N., Sunabori, T., Shimmura, S., Nagai, Y., Nakagawa, T., Okano, H., Matsuzaki, Y., 2009b. Development of mesenchymal stem cells partially originate from the neural crest. *Biochem. Biophys. Res. Commun.* 379, 1114-1119.

Morikawa, S., Ouchi, T., Shibata, S., Fujimura, T., Kawana, H., Okano, H., Nakagawa, T., 2016. Applications of Mesenchymal Stem Cells and Neural Crest Cells in Craniofacial Skeletal Research. *Stem Cells International* 2016, 1-8.

Murphy, M.B., Moncivais, K., Caplan, A.I., 2013. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp. Mol. Med.* 45, e54.

Nagoshi, N., Shibata, S., Kubota, Y., Nakamura, M., Nagai, Y., Satoh, E., Morikawa, S., Okada, Y., Mabuchi, Y., Katoh, H., Okada, S., Fukuda, K., Suda, T., Matsuzaki, Y., Toyama, Y., Okano, H., 2008. Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. *Cell Stem Cell* 2, 392-403.

Neirinckx, V., Coste, C., Rogister, B., Wislet-Gendebien, S., 2013. Concise review: adult mesenchymal stem cells, adult neural crest stem cells, and therapy of neurological pathologies: a state of play. *Stem Cells Transl Med* 2, 284-296.

Ogata, Y., Mabuchi, Y., Yoshida, M., Suto, E.G., Suzuki, N., Muneta, T., Sekiya, I., Akazawa, C., 2015. Purified Human Synovium Mesenchymal Stem Cells as a Good Resource for Cartilage Regeneration. *PLoS One* 10, e0129096.

Patel, D.M., Shah, J., Srivastava, A.S., 2013. Therapeutic potential of mesenchymal stem cells in regenerative medicine. *Stem Cells Int* 2013, 496218.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., Marshak, D.R., 1999. Multilineage potential of adult human mesenchymal stem cells. *Science* 284, 143-147.

Quirici, N., Soligo, D., Bossolasco, P., Servida, F., Lumini, C., Deliliers, G.L., 2002. Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp. Hematol.* 30, 783-791.

Rodrigo, I., Hill, R.E., Balling, R., Munsterberg, A., Imai, K., 2003. Pax1 and Pax9 activate Bapx1 to induce chondrogenic differentiation in the sclerotome. *Development* 130, 473-482.

Sakai, K., Yamamoto, A., Matsubara, K., Nakamura, S., Naruse, M., Yamagata, M., Sakamoto, K., Tauchi, R., Wakao, N., Imagama, S., Hibi, H., Kadomatsu, K., Ishiguro, N., Ueda, M., 2012. Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. *J. Clin. Invest.* 122, 80-90.

Sanchez-Ramos, J., Song, S., Cardozo-Pelaez, F., Hazzi, C., Stedeford, T., Willing, A., Freeman, T.B., Saporta, S., Janssen, W., Patel, N., Cooper, D.R., Sanberg, P.R., 2000. Adult bone marrow stromal cells differentiate into neural cells in vitro. *Exp. Neurol.* 164, 247-256.

Shi, C., Jia, T., Mendez-Ferrer, S., Hohl, T.M., Serbina, N.V., Lipuma, L., Leiner, I., Li, M.O., Frenette, P.S., Pamer, E.G., 2011. Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands. *Immunity*

34, 590-601.

Stappenbeck, T.S., Miyoshi, H., 2009. The role of stromal stem cells in tissue regeneration and wound repair. *Science* 324, 1666-1669.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131, 861-872.

Takashima, Y., Era, T., Nakao, K., Kondo, S., Kasuga, M., Smith, A.G., Nishikawa, S., 2007. Neuroepithelial cells supply an initial transient wave of MSC differentiation. *Cell* 129, 1377-1388.

Tamai, K., Yamazaki, T., Chino, T., Ishii, M., Otsuru, S., Kikuchi, Y., Inuma, S., Saga, K., Nimura, K., Shimbo, T., Umegaki, N., Katayama, I., Miyazaki, J., Takeda, J., McGrath, J.A., Uitto, J., Kaneda, Y., 2011. PDGFR α -positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. *Proc. Natl. Acad. Sci. U. S. A.* 108, 6609-6614.

Thomson, J.A., Itskovitz-Eldor, J., Shapiro, S.S., Waknitz, M.A., Swiergiel, J.J., Marshall, V.S., Jones, J.M., 1998. Embryonic stem cell lines derived from human blastocysts. *Science* 282, 1145-1147.

Volarevic, V., Nurkovic, J., Arsenijevic, N., Stojkovic, M., 2014. Concise review: Therapeutic potential of mesenchymal stem cells for the treatment of acute liver failure and cirrhosis. *Stem Cells* 32, 2818-2823.

Wollert, K.C., Drexler, H., 2005. Mesenchymal stem cells for myocardial infarction: promises and pitfalls. *Circulation* 112, 151-153.

Wong, C.E., Paratore, C., Dours-Zimmermann, M.T., Rochat, A., Pietri, T., Suter, U., Zimmermann, D.R., Dufour, S., Thiery, J.P., Meijer, D., Beermann, F., Barrandon, Y., Sommer, L., 2006. Neural crest-derived cells with stem cell features can be traced back to multiple lineages in the adult skin. *J. Cell Biol.* 175, 1005-1015.

Worthley, D.L., Churchill, M., Compton, J.T., Taylor, Y., Rao, M., Si, Y., Levin, D., Schwartz, M.G., Uygur, A., Hayakawa, Y., Gross, S., Renz, B.W., Setlik, W., Martinez, A.N., Chen, X., Nizami, S., Lee, H.G., Kang, H.P., Caldwell, J.M., Asfaha, S., Westphalen, C.B., Graham, T., Jin, G., Nagar, K., Wang, H., Kheirbek, M.A., Kolhe, A., Carpenter, J., Glaire, M., Nair, A., Renders, S., Manieri, N., Muthupalani, S., Fox, J.G., Reichert, M., Giraud, A.S., Schwabe, R.F., Pradere, J.P., Walton, K., Prakash, A., Gumucio, D., Rustgi, A.K., Stappenbeck, T.S., Friedman, R.A., Gershon, M.D., Sims, P., Grikscheit, T., Lee, F.Y., Karsenty, G., Mukherjee,

S., Wang, T.C., 2015. Gremlin 1 identifies a skeletal stem cell with bone, cartilage, and reticular stromal potential. *Cell* 160, 269-284.

Yasui, T., Mabuchi, Y., Toriumi, H., Ebine, T., Niibe, K., Houlihan, D.D., Morikawa, S., Onizawa, K., Kawana, H., Akazawa, C., Suzuki, N., Nakagawa, T., Okano, H., Matsuzaki, Y., 2016. Purified Human Dental Pulp Stem Cells Promote Osteogenic Regeneration. *J. Dent. Res.* 95, 206-214.

Zhou, B.O., Yue, R., Murphy, M.M., Peyer, J.G., Morrison, S.J., 2014. Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* 15, 154-168.

Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, J.W., Katz, A.J., Benhaim, P., Lorenz, H.P., Hedrick, M.H., 2001. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng.* 7, 211-228.