Functional odontoblastic-like cells derived from human iPSCs

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The induced pluripotent stem cells (iPSCs) have an intrinsic capability for indefinite self-renewal, large-scale expansion and can differentiate into all types of cells. Here, we tested the potential of iPSC from dental pulp stem cells (DPSCs) to differentiate into functional odontoblasts. DPSC were reprogrammed into iPSCs via electroporation of reprogramming factors OCT4, SOX2, KLF4, LIN28 and L-MYC. The iPSCs presented overexpression of the reprogramming genes and high protein expressions of alkaline phosphatase, OCT4 and TRA-1-60 in vitro and generated tissues from three germ layers in vivo. Dentin discs with PLLA scaffolds containing iPSCs were implanted subcutaneously into immunodeficient mice. After 28 days from implantation, the iPSCs generated a pulp-like tissue with the presence of tubular dentin in vivo. The differentiation potential after long-term expansion was assessed in vitro. iPSCs and DPSCs of passage 4 and 14 were treated with either odontogenic medium or extract of bioactive cement for 28 days. Regardless of the passage tested, iPSCs expressed putative markers of odontoblastic differentiation and kept the same mineralization potential while DPSC P14 failed to do the same. Analysis of these data, collectively, demonstrates that human iPSCs can be a source to derive human odontoblast for dental pulp research and test bioactivity of materials.

Keywords: Dentinogenesis, Pulp Biology, Biomaterial(s), Cell differentiation, Odontoblast(s), Tissue Engineering

Introduction

Immortalized cells lines are often used to test biological properties of materials. Nonetheless, the cells may differ genetically and phenotypically from their tissue of origin, present altered cytomorphology or lose specific markers that influence their responsiveness to stimuli (Hynds and Giangreco 2013; Kaur and Dufour 2014). Alternatively, primary cells are not modified and keep many of the specific biological properties. Nonetheless, they have a short lifespan in culture before becoming senescent (Vertrees et al. 2008).

In dentistry, there is a trend to adopt mesenchymal stem cells (MSCs) from oral sources to promote tissue regeneration or test biomaterials. Indeed, dental pulp stem cells (DPSCs) is one of the most used models since several dental biomaterials may pose risk or stimulate the dental pulp (Collado-Gonzalez et al. 2016; Rosa et al. 2013). Unfortunately, MSCs undergo irreversible proliferation-arrested state and present a decline in the differentiation capability after long-term *in vitro* culture and aging (Ren et al. 2016; Stolzing et al. 2008). For instance, MSCs isolated from the umbilical cord and from the dental pulp present lower potential for adipogenic and osteogenic differentiation in passage (P) 10 compared to P6 (Ren et al. 2016). Likewise, DPSCs lose their odontogenic differentiation potential and mineralization capacity during senescence (Mehrazarin et al. 2011). Consequently, there is an increase in the variability of the biological properties and decrease in the usability of cellular stocks as passage number progresses (Calles et al. 2006; Stolzing et al. 2008).

Induced pluripotent stem cells (iPSCs) can overcome some of these limitations. They can be generated by the overexpression of a set of genes through several methods (Rosa et al. 2014; Takahashi and Yamanaka 2006). The iPSCs have high self-renewal capability, allow a large-scale expansion and can differentiate into cells from the three germ layers (Takahashi and Yamanaka 2006). iPSCs have been extensively used for drug screening and disease modelling (Ebert et al. 2012). Moreover, iPSC-based and -derived cells present high sensitivity to apoptotic induction and are useful to characterize the effects of materials and substrates (Dzhoyashvili et al. 2015; Takayama et al. 2013).

iPSCs have been reprogrammed from cells from several oral sources (Egusa et al. 2010; Hynes et al. 2015; Miyoshi et al. 2010; Yan et al. 2010). These iPSCs have the potential to regenerate cementum and periodontal ligament *in vivo* when combined with a silk scaffold and enamel matrix derivatives (Duan et al. 2011; Wen et al. 2012). Interestingly, iPSCs from mouse embryonic fibroblast mixed with mesenchymal cells placed in close contact with the epithelial cells were capable to form bone-like structures and, in some cases, dentin-like and dental pulp-like structures *in vivo* (Wen et al. 2012). Despite of these exciting results, the ability of iPSCs from human origin to differentiate into functional odontoblasts *in vivo* remain unknown.

The derivation of functional odontoblasts remain as one of the most challenging issues in dental research. This is governed by several signalling molecules and growth factors such as BMP-4, which

plays crucial roles regulating tooth initiation, morphogenesis and shape development (Aberg et al. 1997; Vainio et al. 1993). Notably, BMP-4 has the potential to induce odontoblastic differentiation of pluripotent stem cells *in vitro*. For instance, embryonic stem cells-derived MSCs differentiated into odontoblast-like cells under the stimulation of FGF-8 and BMP-4 *in vitro* (Kidwai et al. 2014). Also, mouse embryonic stem cells cultured in a collagen type-I scaffold combined BMP-4 presented high gene expression of DSPP and high alkaline phosphatase activity (Kawai et al. 2014). Similar approach has shown that embryonic bodies from mouse iPSC treated with BMP-4 presented high gene expression of DSPP and DMP-1 *in vitro* (Ozeki et al. 2013). Hence, BMP-4 may be an interesting alternative to induce the odontoblastic differentiation of human iPSCs.

The objective of this study was to evaluate whether human iPSCs have the potential to differentiate in odontoblasts *in vivo*. In addition, we have evaluated the effects of long-term expansion on the odontoblastic differentiation potential and mineralization capability of both iPSCs and DPSCs. The hypotheses tested were: i) iPSCs can differentiate into functional odontoblast *in vivo* and ii) there will be no decrease in the odontogenic differentiation and mineralization capability of iPSCs after long term expansion compared to DPSCs *in vitro*.

Material and methods

Ethics compliance

The use of severe immunocompromised mice (SCID), human DPSCs and teeth were approved by NUS relevant committees (IACUC 2013-05239, IBC 2014-00762, IRB, 2094/2013). Animal experimentation followed the ARRIVE guidelines.

Cell culture, iPSC generation and characterization

The DPSCs were cultured under basal growth culture medium [Dulbecco's modified Eagle's medium (Invitrogen, USA), supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin (PS, Invitrogen)]. DPSCs were characterized for the expression of CD90, CD105, CD73 and CD34 by fluorescence-activated cell sorting analysis (BD Fortessa, BD Biosciences, Germany). The DPSCs were characterized for alkaline phosphatase (ALP, Alkaline Phosphatase Live Stain, LifeTechnologies, USA), TRA-1-60 and OCT-4 (1:500, Abcam, USA).

The DPSCs (P4, $1x10^6$ input cells) were reprogrammed using episomal iPSC reprogramming vectors (Epi5™ Episomal iPSC Reprogramming Kit, Thermo Fischer Scientific, USA) that contains an oriP/EBNA-1 backbone for delivering the reprogramming Yamanaka factors (OCT-4, SOX-2, KLF-4, L-MYC, and LIN28). The oriP/EBNA-1 mediates the import and retention of vector DNA into the nuclei.

The nucleofection of the reprogramming vectors was performed via electroporation using the Neon Transfection System (Thermo Fischer Scientific, USA). The parameters used were: 1650 V, three pulses of 10 ms with a total of 3 μg of reprogramming plasmids as described in the Appendix.

The reprogramming allowed the successful generation of six colonies. All the iPSCs colonies were characterized for ALP (LifeTechnologies), TRA-1-60 and OCT-4 (1:500, Abcam) via with fluorescence microscopy (FV1000, Olympus Optical, Japan). For the teratoma formation assay, $3x10^5$ iPSCs were mixed with 50 μl of Matrigel (BD Matrigel™, BD Biosciences, USA) and injected subcutaneously into the dorsum of 5- to 7-week-old female SCID mice (n=4, CB-17 SCID; InVivos, Singapore). After 9 weeks, the tumours were retrieved, fixed and stained with hematoxylin and eosin.

Genetic expression of reprogramming factors and episomal vector components (oriP and EBNA-1) were analyzed in both DPSCs and iPSCs of P4 by quantitative real-time QPCR as previously described (Xie et al. 2016). Primer sequences are shown in the Appendix. Genome-wide screening for gross chromosomal abnormalities was carried out for both DPSCs (P4) and iPSCs (P4 and P14) as described in the Appendix.

Odontoblastic differentiation *in vitro* and *in vivo*

First, we checked the effects of BMP-4 on the expression of odontogenic genes in iPSCs. For this, $1x10^5$ iPSCs (P4) were seeded in matrigel-coated 6-well plates and cultured with StemMACSTM-brew XF culture media (Miltenyi Biotec, USA). The clone was transferred into 12-well suspension plate (Greiner Bio-One GmbH, Germany) and maintained for seven days with embryonic body (EB) culture media (AggreWell™ EB Formation Medium, Stemcell Technologies, Canada). The EBs were moved into tissue culture 6-well plates and treated with differentiation media [DMEM (Invitrogen) supplemented with 100 nM dexamethasone (Sigma–Aldrich, USA), 5mM b-glycerophosphate (Sigma–Aldrich), 50 lg/ml ascorbate phosphate (Sigma–Aldrich), 10% FBS (Invitrogen)] supplemented with different concentrations of BMP-4 (25, 50 or 100 ng/ml). iPSCs cultured with basal culture medium were used as control. The expressions of MSX-1, MEPE, DSPP and DMP-1 were evaluated after 10 days by QPCR (CFX Connect™ Real-Time PCR Detection System, Bio-Rad). For all the genetic studies, we prepared three independent samples which were used for three PCR reactions.

The odontogenic potential was confirmed *in vivo*. For this, the iPSCs (P4) were treated with the differentiation medium described above supplemented with 100 ng/ml of BMP-4 (odontogenic medium) for 10 days. After, $6x10^5$ cells were seeded in poly-L-lactic acid (PLLA, Boehringer Ingelheim, Germany) scaffolds cast within the pulp chamber of human third molars as described in the Appendix. Six samples were transplanted subcutaneously into the dorsum of SCID mice. After 28 days, specimens were retrieved and fixed with 4% formaldehyde solution in phosphate-buffered saline for 24 h at 4°C, demineralized (Decalcifier II, Surgipath, USA) for 4 h at room temperature and stained with hematoxylin and eosin (H&E). DPSCs (P4) and scaffolds devoid of cells were the controls.

Immunohistochemical analysis of the tissues formed was performed with anti-DMP-1 (1:1000, Abcam) and anti-human mitochondria antibodies (1:500, Abcam, USA). Negative controls were tissue sections stained with an isotype-matched non-specific IgG antibody (Rosa et al. 2013).

Thereafter, we assessed the ability of iPSCs to undergo odontogenic differentiation after long-term expansion *in vitro*. For which, we treated both DPSCs and iPSCs (P4 and P14) with the odontogenic medium for 10 days. The gene expressions of MEPE, DSPP and DMP-1 were evaluated after 10 days by QPCR. The mineralization potential was assessed by exposing the cells to extracts of a bioactive cement (Biodentine, Septodont, France). We selected this calcium-silicate based cement due to its ability to induce cells to secrete mineralized matrix and to promote hard tissue regeneration *in vitro* and *in vivo*. The cement was mixed per manufacturer's instructions and loaded into moulds (10 mm x 1 mm). Following the initial setting (12 min), the sample was placed in the incubator for 24 h (37°C, 95% humidity). Thereafter, the sample was immersed in 10 mL of DMEM and incubated for 24 h (37°C, 95% humidity). Next, the disc was removed, the eluent was filtered (0.22 µm pore size) and used to treat the cells. After 10 days, the amount of calcium per DNA cells was quantified as described in the Appendix.

Statistical analysis

Statistical analysis for qPCR was performed with two-way ANOVA with and multiple comparisons were performed using Tukey's post hoc test. Normality test was performed with Shapiro-Wilk test. Kruskal-Wallis was used for alizarin red S staining. A pre-set significance level of 5% was set for all the tests (SPSS 22.0, IBM, USA).

Results

Prior to the reprogramming, we checked the expression of mesenchymal stem cells markers in the DPSCs. Approximately 99% of the DPSC were positive for CD90, CD105 and CD73 and negative for CD34. The DPSCs presented spindle-shaped fibroblastoid morphology (Fig. 1A) and positive protein expression for ALP, TRA-1-60 and OCT-4 (Appendix). After the electroporation of the vectors, the DPSCs formed six colonies. Notably, the colony used in this study presented high nucleus-to-cytoplasm ratio (Fig. 1B) and positive expression of the pluripotency markers TRA-1-60 and OCT-4 (Fig. 1C and 1D) and high endogenous ALP (Fig. 1E) *in vitro*. We also checked the expression of the pluripotency-related genes in that colony after the electroporation. There were significant increases of all reprogramming genes and no detectable expression of episomal vector components oriP and EBNA-1 (Fig. 1F) comparing to DPSCs ($p < 0.05$). The pluripotency was confirmed *in vivo* by the teratoma formation assay. We have injected $3x10^5$ iPSCs into the subcutaneous space of SCID mice and the tumours were retrieved after nine weeks. The histological analysis showed teratomas with neuroepithelial-like tissues (ectoderm), cartilage (mesoderm) and secretory tubule-like structures

(endoderm) from the three germ layers (Fig. 1G to 1I). Finally, we characterized the karyograms to check whether the reprogramming could induce karyotipic abnormalities. The karyotype of iPSCs was as normal as the one from the parental DPSCs (Fig 1J and K) and no abnormalities were detected in iPSCs after long-term expansion (P14 in Fig. 1L). Altogether, we successfully reprogrammed DPSCs into iPSCs via the electroporation of episomal vectors.

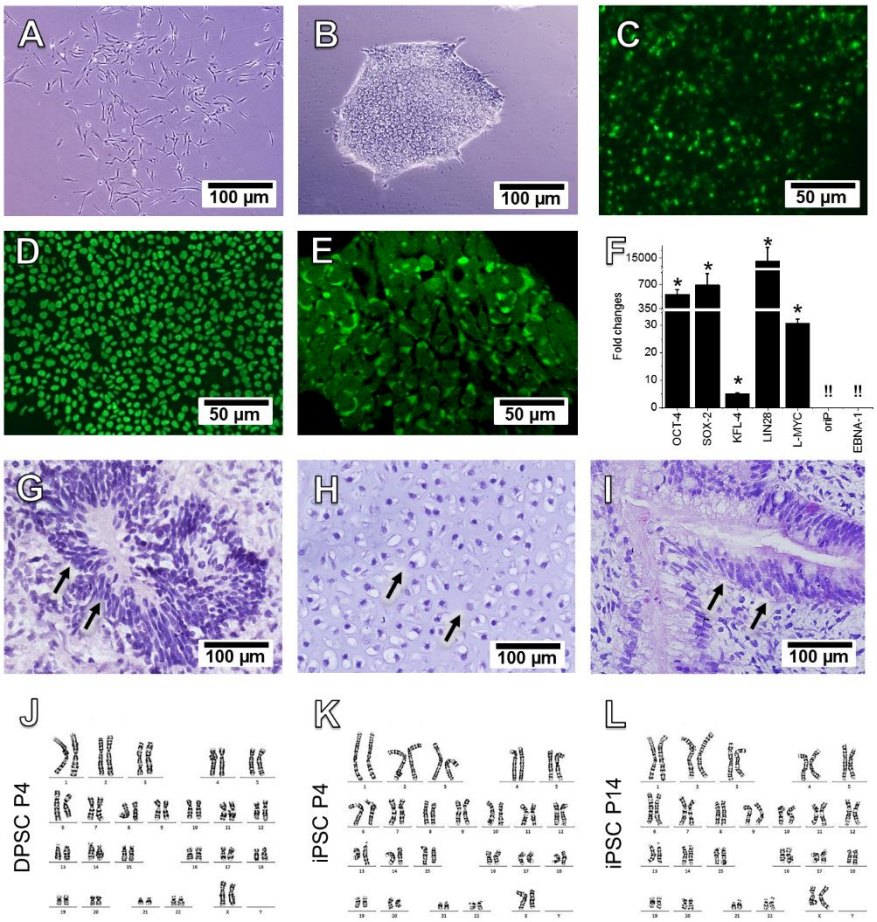

Figure 1. Phase contrast microscopy revealed that dental pulp stem cells (DPSCs) presented spindle-shaped fibroblastoid morphology (**A**). After the electroporation, the cells assembled into colonies with a high nucleus:cytoplasm ratio (**B**). Immunofluorescence analysis of the colony used in this study showed positive protein expression of the traditional markers for pluripotency (TRA-1-60, OCT-4) and high alkaline phosphatase activity (in green; **C**–**E**, respectively). The gene expressions of reprogramming genes in the cells from the colony were significantly higher (**P* < 0.05) as compared with the DPSCs used in the reprogramming (**F**). The episomal vector components were not detected in the cells from the colony (!! in panel F). Error bars indicate standard deviations. Cells from the colony were implanted in mice to test the pluripotency in vivo. After 9 wk from implantation, the teratomas formed presented cells and tissues derived from the 3 germ layers, confirming the generation of bona fide pluripotent stem cells. The arrows indicate the neuroepithelial-like tissues (**G**; endoderm), cartilage (**H**; mesoderm), and secretory tubule-like structures (**I**; ectoderm) present in the teratomas. The karyograms showed that no karyotypic abnormalities were detected in the DPSCs used for the reprogramming (**J**) or pluripotent stem cells after short- and long-term expansion (**K** and **L**). iPSC, induced pluripotent stem cell; P, passage.

After, we evaluated the effects of BMP-4 concentration in the expression of putative markers for odontoblastic differentiation of iPSC *in vitro*. Regardless of the concentration of BMP-4 used, the odontogenic medium increased significantly the expression of all genes tested compared to the iPSC

maintained in basal growth medium (Fig. 2, $p < 0.05$). Interestingly, 100 ng/ml of BMP-4 resulted in significantly higher expression of MEPE, DMP-1 and DSPP comparing to the other concentrations tested. Hence, we selected this formulation for the subsequent investigations. The odontogenic potential was further evaluated *in vitro* with cells from colony #6 that also presented high expression of these markers when treated with odontogenic medium supplemented with 100 ng/ml of BMP-4 compared to the untreated control (Appendix Fig. 3, $p < 0.05$)

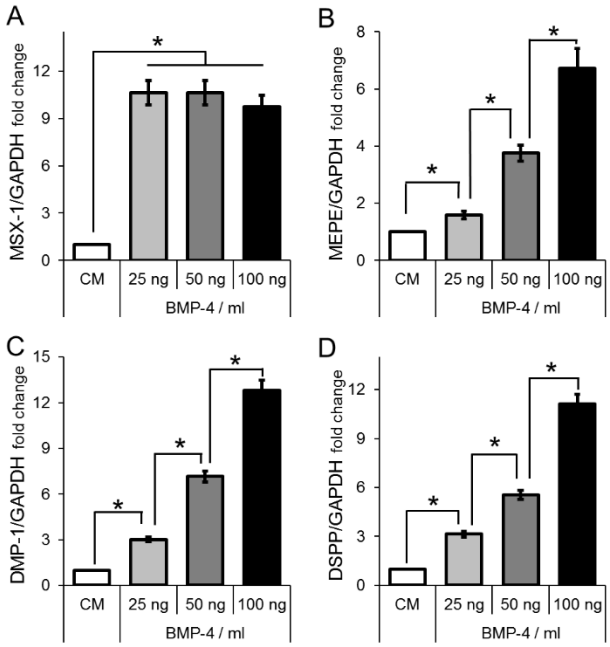

Figure 2. Effects of BMP-4 in the odontogenic differentiation of induced pluripotent stem cells (iPSCs) in vitro. iPSCs were treated with odontogenic medium with different concentrations of BMP-4 for 10 d. The quantitative real-time polymerase chain reaction analysis showed that iPSCs exposed to odontogenic medium with BMP-4 presented significantly higher gene expression of putative markers of odontoblastic differentiation as compared with the untreated control (CM). Except for *MSX-1*, there was a dose-dependent effect for BMP-4, with 100 ng/ mL resulting in significant higher expression of the markers studied as compared with the other concentrations tested and with control (*P <0.05). Error bars indicate standard deviations.

The ability of iPSCs to differentiate into functional odontoblasts was further confirmed *in vivo*. Cells were seeded in degradable scaffolds and transplanted subcutaneously into the dorsum of mice for 28 days. The histological analysis revealed that the iPSCs formed a pulp-like tissue within the pulp chamber containing structures resembling tubular dentin (arrows in Fig. 3A). o confirm whether the human iPSCs acquired a mature odontoblast phenotype *in vivo*, we checked the expression of DMP-1 by immunohistochemistry. Notably, cells neighbouring the dentin-like structures present within the soft tissue presented positive expression for DMP-1 similar to the one observed in the tissue obtained with DPSCs (arrows in Fig. 3C and 3D). Immunostaining with antibody specific for human mitochondria confirmed that the tissues formed were populated by human cells (Fig 3E, 3F and Appendix Fig. 4).

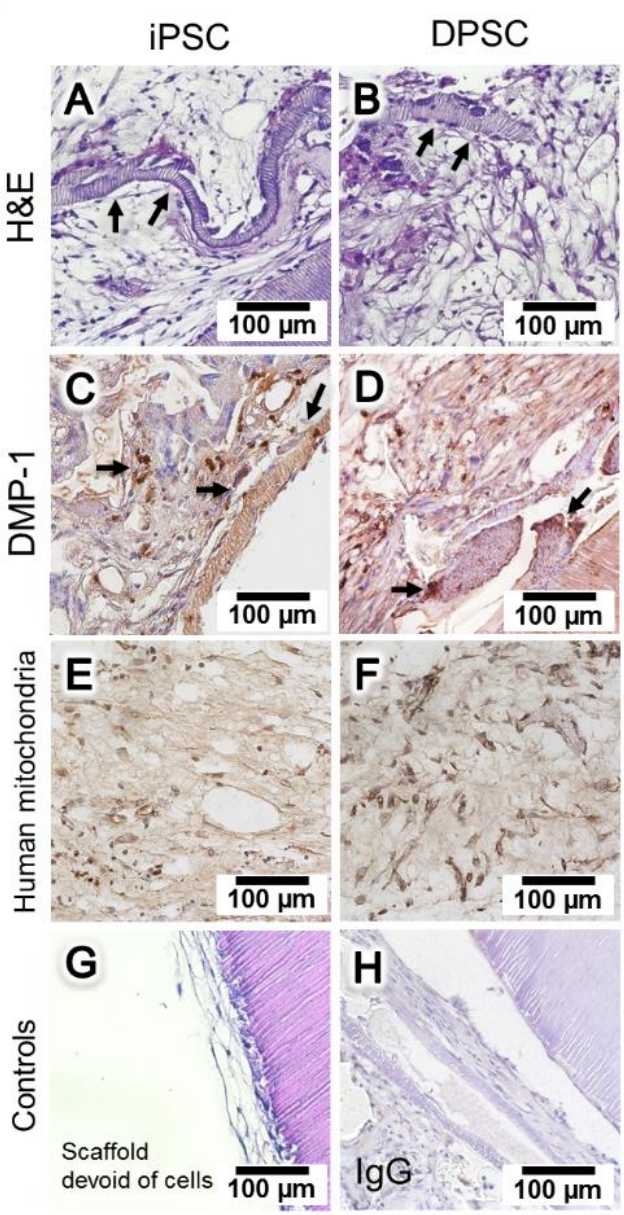

Figure 3. Induced pluripotent stem cell (iPSC)–derived odontoblast-like cells in vivo. iPSCs and dental pulp stem cells (DPSCs) were seeded in scaffolds cast in the pulp chamber of 1-mm-thick dentin slices and implanted in mice for 28 d. Slides were processed for histologic analysis and observed under optical microscope (40×). The hematoxylin and eosin (H&E) staining showed that iPSCs and DPSCs both formed a pulp-like tissue with the presence of dentin-like tubular structures within loose connective tissues (arrows in **A** and **B**). Immunohistochemical analysis confirmed the positive expression of DMP-1 in cells neighboring the mineralized structures present in the dental pulp–like tissue formed by iPSCs and DPSCs (arrows in **C** and **D**). Immunohistochemistry for human mitochondria confirmed that the tissues formed were populated with human cells (**E** and **F**). Controls were scaffolds devoid of cells (**G**) or tissue sections stained with an isotype-matched irrelevant antibody (**H**).

Finally, we checked the ability of iPSCs and DPSCs to undergo odontoblastic differentiation after short and long-term expansion (P4 and P14) *in vitro*. Cells were treated with the odontogenic medium for ten days and the expressions of putative markers for odontoblastic differentiation were evaluated by PCR (Fig. 4A to 4C). For P4, both DPSCs and iPSCs presented significant increases in the expression of all the genes tested when treated with the odontogenic medium ($p < 0.05$). However,

after long-term expansion (P14), the odontogenic medium increased the expression of all genes in iPSCs but only MEPE in DPSCs ($p < 0.05$). The mineralization potential was evaluated by treating the cells with the extract of a bioactive cement for ten days (Fig 4D). The iPSCs-derived odontoblastic-like cells deposited similar amount of Ca *per* ng of DNA for both passages tested. Notably, there was a decrease of ~40% in the calcium deposition of DPSCs P14 compared to P4.

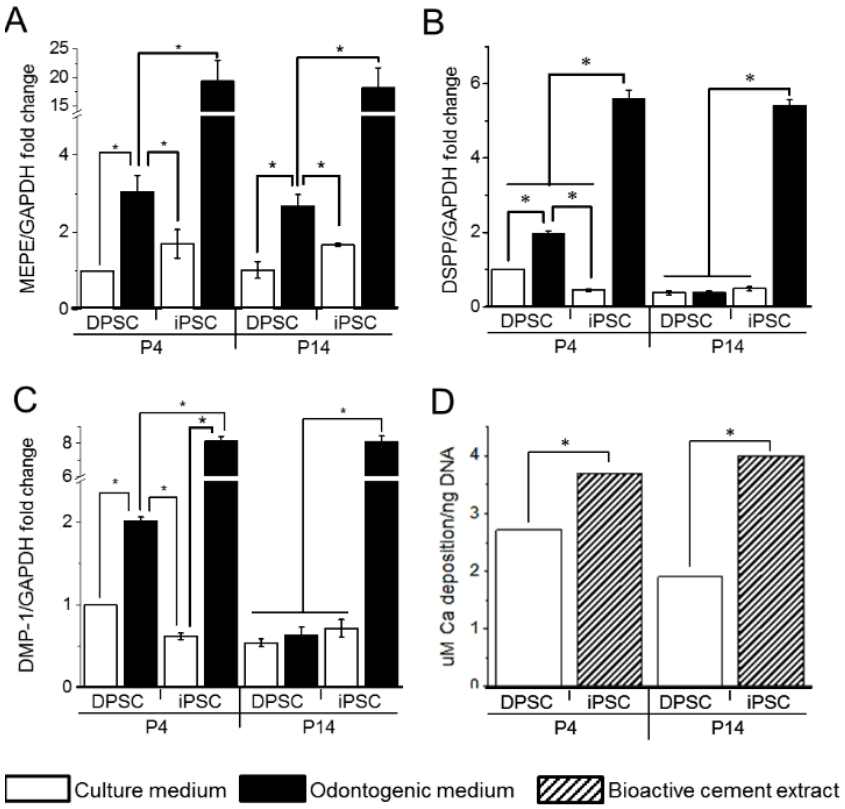

Figure 4. Odontoblastic differentiation and mineralization potential after short- and long-term expansion in vitro: passages 4 and 14 (P4 and P14), respectively. For P4, dental pulp stem cells (DPSCs) and induced pluripotent stem cells (iPSCs) presented a significant increase in the expression of the odontoblastic-related genes when treated with the odontogenic medium with 100 ng of BMP-4 for 10 d as compared with the cells kept with basal culture medium. For P14, there were significant increases of all genes tested for iPSCs, whereas DPSCs experienced a significant increase for *MEPE* only. There was a decrease in the amount of calcium deposited by DPSCs from P4 to P14 when treated with the extract of a bioactive cement. The calcium depositions for iPSCs were similar and higher than DPSCs regardless of the passage tested (*$P < 0.05$). Error bars indicate standard deviations.

Discussion

Animal and human iPSCs have been differentiated into several cell types such as ameloblasts, neurons, osteoprogenitor and neural crest–like cells (Cai et al. 2013; Duan et al. 2011; Otsu et al. 2012; Wen et al. 2012; Zou et al. 2012). Despite of these remarkable achievements, the ability of human iPSCs to differentiate into functional odontoblasts *in vivo* has not been reported. Hence, our first hypothesis was to test whether iPSCs could be reprogrammed from human DPSCs and further be differentiated into odontoblasts *in vitro* and *in vivo*.

First, we characterized the cells before and after the electroporation. They presented high expression of reprogramming genes and markers commonly used to confirm the pluripotent character (Fig. 1C to 1F). The iPSCs formed teratomas *in vivo* consisting of differentiated derivatives of all three primary germ layers (Fig. 1F to 1I) confirming their pluripotency. The electroporation of episomal vectors is a laborious reprogramming technique with low reprogramming efficiency. We obtained only 6 colonies out of 10^6 input DPSCs similar to what was previously observed for human fibroblasts (Yu et al. 2009). Despite of these disadvantages, this technique exempt the use of viral vectors and the oriP/EBNA-1-based episomal vector presents a wide range of host cells including the human ones (Son et al. 2016).

Animal-derived odontoblast lineages have been established from murines and porcine (Iwata et al. 2007; MacDougall et al. 1998). Yet, the derivation and maintenance of functional human odontoblasts remain as one of the most challenging issues in dental research. Notably, the presence of 100 ng/ml of BMP-4 in the differentiation media increased significantly the expression of the putative markers for odontoblastic differentiation in the iPSCs comparing to the other concentrations tested or control (Fig. 2). Similar increase in the expression of these markers were observed in dental stem cells undergoing odontoblastic differentiation *in vitro* and *in vivo* (Bento et al. 2012; Rosa et al. 2013). Most importantly, the iPSCs seeded in the scaffolds and implanted in the SCID mice have generated a pulp-like tissue similar to the one obtained with DPSCs *in vivo*. Notably, there was the formation of dentin-like tubular structures within the pulp tissue generated (arrows in Fig. 3A and 3B). Immunohistochemistry for DMP-1 showed positive expression of this marker in cells surrounding the dentin-like structures confirming that the cells acquired an odontoblast phenotype *in vivo* (Fig. 3C and 3D). Finally, the positive expression of human mitochondria confirmed that tissues engineered were populated with human cells (Fig. 3E, 3F and Appendix Fig. 4). Collectively, our data confirms the hypothesis that iPSCs can be obtained from human DPSCs and differentiate into functional odontoblasts *in vivo*.

Long-term expansion of cells can cause alterations in their biology that result in irreversible proliferation-arrested state and decline in the differentiation capability (Izadpanah et al. 2008; Ren et al. 2016). Hence, our second hypothesis was to test whether the iPSCs could retain their odontogenic and mineralization potential after long-term expansion. We have observed that DPSCs P14 treated with the odontogenic medium experienced a marginal increase in the expression of MEPE whereas DSPP and DMP-1 remained similar to the untreated DPSCs (Fig. 4A to 4C). However, iPSCs of both passages experienced significant increases of the putative markers that are highly expressed during odontogenic differentiation (Bento et al. 2012; Rosa et al. 2013).

Finally, we evaluated the mineralization potential of the differentiated iPSCs when treated with the extract of a bioactive cement that induces mineralization in DPSCs (Collado-Gonzalez et al. 2016). For DPSCs, there was a decrease in the amount of calcium deposition comparing P4 and P14 (from 2.6

to 1.7 µM of Ca/ng DNA) denoting a plausible disruption of the biological activity and differentiation potential of DPSC after *in vitro* expansion. Notably, the odontoblastic-like cells derived from iPSCs deposited approximately 3.8 µM of calcium/ng DNA even after long-term expansion (Fig. 4D). This ability can be correlated with the high expression of odontoblast-related genes observed in iPSC cells after short and long-term expansion (Fig. 4A to 4C). These findings confirm the second hypothesis and reassure the potential of these cells as promising alternatives to test bioactivity of biomaterials.

Despite of the exciting results, this research presents its own limitations. For instance, BMP-4 alone does not reflect the complex system of proteins that are finely orchestrated during the odontoblastic differentiation. In addition, there is the need to fully characterize the iPSC-derived odontoblasts and compare their genetic and protein profiles with naturally occurring odontoblasts. Notwithstanding the *in vivo* production of tubular mineralized structures observed here, their formation within the soft tissue could cause extensive calcification of the regenerated dental pulp. This must be considered in future research aiming the use of iPSC-derived odontoblast-like cells for pulp regeneration. Finally, future studies shall unveil whether the mechanisms involved in the odontoblastic differentiation of iPSCs are similar to those observed in adult stem cells. Still, the findings presented here confirm that human iPSCs can be a source for the derivation of odontoblast-like cells.

Conclusion

Human DPSCs can be successfully reprogrammed into iPSCs via electroporation. The iPSCs generated a pulp-like tissue with functional odontoblasts capable to produce tubular dentin-like structures *in vivo*. Contrarily from DPSCs, the iPSCs maintained the odontogenic and mineralization potential after long-term expansion. Hence, the odontoblastic-like cells derived from human iPSCs have the potential to diminish the problems related to the biological variability differentiation potential of DPSCs after expansion *in vitro*. Finally, the derivation of odontoblasts from iPSCs open new opportunities to improve biomaterial testing and modelling disorders of tooth development.

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Conflict of Interest

The authors declare no competing financial interest.

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Functional odontoblastic-like cells derived from human iPSCs

APPENDIX

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Materials and Methods

Reprogramming method

The nucleofection of the reprogramming vectors was performed using the Neon Transfection System (Thermo Fischer Scientific, USA). The parameters used were: 1650 V, three pulses of 10 ms with a total of 3 µg of reprogramming plasmids. Immediately after the electroporation, the cell suspension was poured into warm medium in a 6-well plate pre-coated with gelatin (37 °C, 5% CO_2). After 24 h, the culture medium was changed to Fibroblast Medium (DMEM, 10% FBS, 2mM GlutaMax 1%, Invitrogen) supplemented with 0.5 µg/ml of puromycin for 5 days (Invitrogen). On day 5, mouse embryonic fibroblasts (SNL 76/7, Sigma-Aldrich, USA) were seeded on a gelatin-coated plate ($5x10^5$ cells per well of a 6-well plate). On Day 6, the transfected DPSCs ($2x10^4$) were seeded onto the wells with pre-seeded SNL feeder cells with 2 ml of hES Medium (Knockout DMEM/F12 containing 20% knockout serum replacement, 2mM glutamine, 0.1 mM nonessential amino acids, 0.1 mM 2-mercaptoethanol, 10 ng/ml bFGF, and 50 µg/ml of PS, Invitrogen) for ten days. On Day 16, the medium was replaced by hES/PSGro Medium (StemRD, USA) for five days. On day 21 the iPSC-like colonies were picked and transferred to 6-well plates.

Karyotype analyses

Six karyograms were analysed per group. Briefly, cells were incubated overnight with 20µl of BrdU and 20µl of diluted colcemid (Thermo Fisher Scientific). After, cells were detached (1X Trypsin-EDTA, Invitrogen), the solution was spun down (1500 rpm, 10 min) and the supernatant removed. Five ml of hypotonic solution was added to the pellet and incubated in waterbath (37°C, 20 min). One ml of fixative (3:1 methanol/glacial acetic acid) was mixed to the culture and spun down (1500 rpm, 10 min). The supernatant was removed and 5 ml of fixative was added to the pellet and kept at -20°C overnight. Slides were prepared and placed on a hotplate at 56°C for 1 hour. After banding, the slides were scanned and analysed using Ikaros System (MetaSystems Group, Inc., USA).

Dentin slice and scaffold preparation

Extracted non-carious human third molars were collected at the National University of Singapore Dental Clinic with the donors' informed consent and Institutional Review Board approval (NUS 2094/2013). The teeth were kept in 0.5% Chloramine T solution (Sigma-Aldrich, USA) for no longer than 4 weeks. Teeth were mounted on a precision sectioning cutter (Isomet Low Speed Saw, Buehler, USA) and two parallel transverse cuts were performed at the cervical region (MK-303 Professional; MK Diamond Products Inc., Torrance, CA) under cooling with sterile phosphate-buffered saline (Invitrogen, USA). As a result, one 1.0-mm thick tooth slice was obtained per tooth that were disinfected with alcohol 70% (w/v) for 5 min and kept in distilled water.

To cast the scaffold, 5% (w/v) solution of poly-L-lactic acid (PLLA, mol wt 85,000-160,000, Sigma-Aldrich) was dissolved in chloroform overnight. The pulp cavities were filled with NaCl (250µm - 425µm) and the PLLA solution was dropped on the pulp cavity. The solution was then kept for 24 h in a dry cabinet for the chloroform to evaporate and the NaCl was removed by soaking the whole set in four times in 10 ml of distilled water for 6 h each.

Thereafter, $6x10^5$ cells (DPSCs or iPSCs) were resuspended in seeded 10 µl of DMEM and gently deposited onto the scaffold surface. The samples (dentin slice/scaffold/cells) were stored in an incubator (37 °C, 5% CO2) for 30 min and subsequently implanted in the subcutaneous pockets created in the dorsum of 5- to 7-week-old female SCID mice.

Primer sequences

The primer sequences used in this study are presented in Table 1.

Appendix Table 1. Primers sequences

Primer	Sequence
OCT-4 Forward	GGCCAGTGACTGCGATTAAAC
OCT-4 Reverse	CCTTCGAGTGTGCTTTAGCAT
SOX-2 Forward	GCCGAGTGGAAACTTTTGTCG
SOX-2 Reverse	GGCAGCGTGTACTTATCCTTCT
LIN28A Forward	AGCGCAGATCAAAAGGAGACA
LIN28A Reverse	CCTCTCGAAAGTAGGTTGGCT
L-MYC Forward	CTGCGGGGAGGATTTCTACC
L-MYC Reverse	CATGCAGTCACGGCGTATGAT
KLF-4 Forward	CGGACATCAACGACGTGAG
KLF-4 Reverse	GACGCCTTCAGCACGAACT
oriP Forward	TTCCACGAGGGTAGTGAACC
oriP Reverse	TCGGGGGTGTTAGAGACAAC
EBNA-1 Forward	ATCGTCAAAGCTGCACACAG
EBNA-1 Reverse	CCCAGGAGTCCCAGTAGTCA
MSX1 Forward	ACACAAGACGAACCGTAAGCC
MSX1 Reverse	CACATGGGCCGTGTAGAGTC
DMP-1 Forward	CTCCGAGTTGGACGATGAGG
DMP-1 Reverse	TCATGCCTGCACTGTTCATTC
DSPP Forward	TGGCGATGCAGGTCACAAT
DSPP Reverse	CCATTCCCACTAGGACTCCCA
MEPE Forward	GGCCAGTGACTGCGATTAAAC

MEPE Reverse	CCTTCGAGTGTGCTTTAGCAT

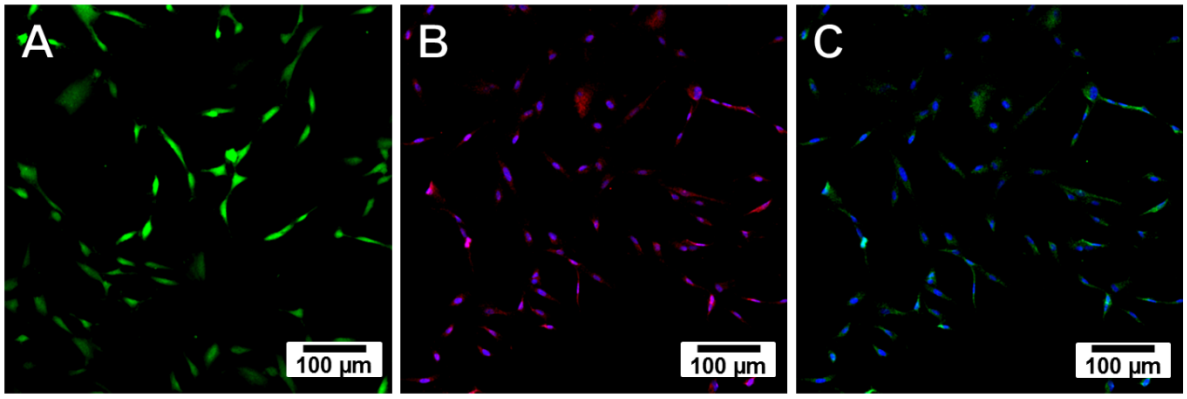
Alizarin red S staining

Cells were washed in phosphate-buffered saline (Invitrogen) and fixed with 4% paraformaldehyde at room temperature for 20 min. After washing the fixative with deionized water, cells were stained with 40 mM ARS (pH 4.2, Sigma–Aldrich, USA) per well. The plates were incubated at room temperature for 30 min, washed four times with distilled water and cells were inspected using a phase contrast microscope (Olympus IX70 microscope, Japan). Following, 200 µL of 10% acetic acid (v/v) was added to each well and incubated at room temperature for 30 min. Cells were scraped from the plate, transferred to a microcentrifuge tube and vortexed for 30 s. The solution was heated to 85°C for 10 min and transferred to ice for 5 min. The slurry was then centrifuged (20,000 g / 15min) and 500 µL of the supernatant transferred to a new microcentrifuge tube. Finally, 200 µL of 10% ammonium hydroxide (v/v) was added to the solution. The absorbance of aliquots (100 µL) of the supernatant was measured using a microplate reader (Multiskan GO, ThermoScientific, USA) at a wavelength of 405 nm. The amount of calcium was normalised against to genomic DNA content obtained with DNAzol (Invitrogen) and measured using a spectrophotometer (NanoDrop ND-1000 Spectrophotometer, ThermoScientific).

Results

DPSCs characterization

Before the reprogramming, the protein expression of the DPSCs used to generate the iPSCs were characterized for markers of pluripotency. The DPSCs presented positive expression for ALP, TRA-1-60 and OCT-4 (Fig. Appendix 1A to C). DPSCs are known to express embryonic stem cell markers Oct-4, Nanog, SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81 (Kerkis et al. 2006).



Appendix Fig. 1. Characterization of markers for pluripotency of DPSCs. The fluorescences were observed under confocal microscope. Cells presented positive expression of ALP (green in A), TRA-1-60 (purple in B) and OCT-4 (green in C). Nuclei were stained with DAPI (blue in B and C).

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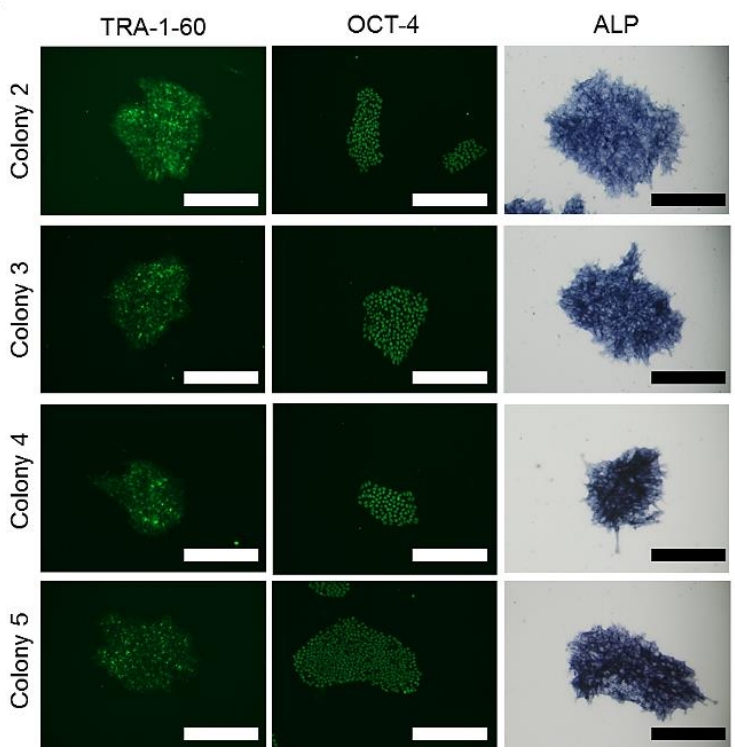
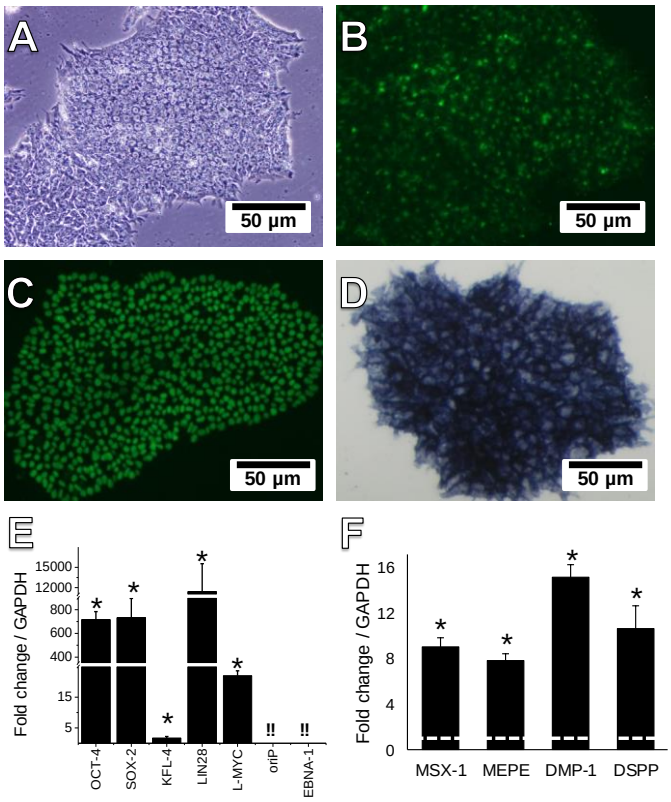
Characterization of markers for pluripotency and odontogenic potential

The reprogramming allowed us to obtain six distinct colonies. The cells from the other five colonies obtained presented positive protein expression of TRA-1-60, OCT-4, and high alkaline phosphatase activity (Appendix Fig. 2 and 3). Further characterizations have shown that cells from colony #6 presented significantly high expressions of reprogramming genes (* = $p<0.05$) comparing to the DPSCs (Appendix Fig. 3E). Thereafter, the cells from colony #6 were treated with the odontogenic medium supplemented with 100 ng of BMP-4 for ten days and the gene expression of putative markers for odontogenic differentiation assessed by RT-PCR. There were significantly increases (* = $p<0.05$) in the gene expression of all the markers analysed comparing to the untreated control (dashed line in Appendix Fig. 3F). In summary, the cells from the colony #6 presented positive protein expression of classical markers for pluripotency and increased gene expression of the reprogramming factors. Moreover, when treated with the odontogenic medium, the cells from colony #6 presented similar odontogenic potential as observed for colony #1 used in the main manuscript.



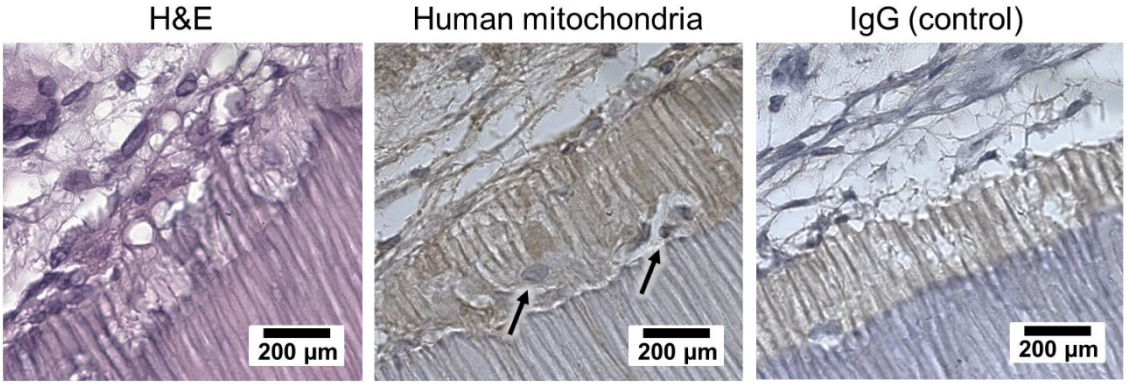
Appendix Fig. 2. Colonies #2 to #5 presented positive protein expression of classical markers of pluripotency TRA-1-60, OCT-4, and high alkaline phosphatase activity (scale bar = 75 µm)



Appendix Fig. 3. Characterization and odontogenic potential of colony #6. Phase contrast microscopy revealed that after the electroporation the DPSC have reorganized into a colony (A). The immunofluorescence characterization showed positive protein expression of TRA-1-60 (B), OCT-4 (C), and high alkaline phosphatase activity (D). The expressions of reprogramming genes were significantly higher (* = $p<0.05$) comparing to the original DPSCs. The components of episomal vectors components were not detected (!! in panel E). The cells from this colony were treated with odontogenic medium with 100 ng of BMP-4 for ten days. The RT-PCR analysis showed significantly increases in the gene expression of markers of odontoblastic differentiation comparing to the untreated control (dashed line, * = $p<0.05$ in panel F).

Dental pulp tissue engineered with iPSCs: human mitochondria expression

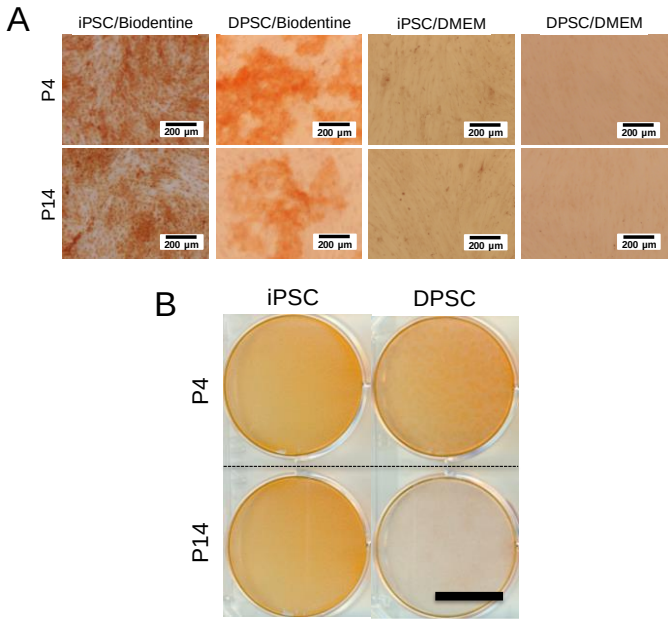
The human-specific mitochondrial antibody is used to confirm the origin of tissues engineered with human cells in animal models. The high magnification images (100 X) in Appendix Fig. 4 show that both the soft tissue and cells layering the newly formed dentin-like structure presented positive expression for the human-specific mitochondrial antibody. In addition, it can be observed a difference in the palisade aspect of the newly formed dentin-like tissue comparing to the dentin from the tooth slice. Likewise, previous publication has shown differences in the dentin deposited by cells derived from adult human stem cells compared to odontoblasts *in vivo* (Sakai et al. 2010).



Appendix Fig. 4. The immunohistochemical analysis of the tissues obtained with the iPSCs revealed that both the cells from the soft tissue and those layering the dentin presented positive expression for human-specific mitochondrial antibody confirming that they were derived from the human cells implanted. Interestingly, in some areas, there were few cells trapped within the newly formed dentin (arrows).

Mineralization potential after short and long-term expansion *in vitro*.

Mesenchymal stem cells present a decline in the differentiation capability after long-term expansion in vitro (Izadpanah et al. 2008; Stolzing et al. 2008). Conversely, iPSCs have high self-renewal capability that allows for large-scale expansion. (Rosa et al. 2014; Takahashi and Yamanaka 2006). Hence, we have assessed the mineralization potential of both iPSC (colony #1) and DPSC after short (P4) and long-term expansion (P14). Cells were seeded in 24-well plates and treated with an extract of a bioactive cement (Biodentine) for ten days and stained with alizarin red S. The mineral deposition was qualitatively assessed by optical microscopy available in Appendix Fig 5. Notably, both iPSCs P4 and P14 presented similar mineralization treated with Biodentine. For DPSCs, there was a decrease of the amount of mineralized content in P14 compared to P4.



Appendix Fig. 5. Mineralization potential after short and long-term expansion *in vitro*. iPSCs (colony #1) and DPSCs of both P4 and P14 were treated with extract of Biodentine for 10 days and

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stained with alizarin red S and imaged. The qualitative analysis (A and B) showed that iPSCs presented similar mineralization patterns for both passages while there was a decrease in the mineral content for DPSC P14 compared to P4. Cells treated with basal culture medium (DMEM) were used as controls (scale bar in B = 1.3 cm).

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