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Functional Odontoblastic-Like Cells Derived from Human iPSCs

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Abstract

The induced pluripotent stem cells (iPSCs) have an intrinsic capability for indefinite self-renewal and large-scale expansion and can differentiate into all types of cells. Here, we tested the potential of iPSCs from dental pulp stem cells (DPSCs) to differentiate into functional odontoblasts. DPSCs were reprogrammed into iPSCs via electroporation of reprogramming factors *OCT-4, 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 3 germ layers in vivo. Dentin discs with poly-L-lactic acid scaffolds containing iPSCs were implanted subcutaneously into immunodeficient mice. After 28 d 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 passages 4 and 14 were treated with either odontogenic medium or extract of bioactive cement for 28 d. 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 odontoblasts 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 (Kaur and Dufour 2012; Hynds and Giangreco 2013). Alternatively, primary cells are not modified, and they keep many of the specific biological properties. Nonetheless, they have a short life span 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) are one of the most used models, since several dental biomaterials may pose risk or stimulate the dental pulp (Rosa et al. 2013; Collado-Gonzalez et al. 2016). Unfortunately, MSCs undergo irreversible proliferation-arrested state and present a decline in the differentiation capability after long-term culture and aging (Stolzing et al. 2008; Ren et al. 2016). For instance, MSCs isolated from the umbilical cord and from the dental pulp present lower potential for adipogenic and osteogenic differentiation in passage 10 (P10) as compared with 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 a 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 (Takahashi and Yamanaka 2006; Rosa et al. 2014). The iPSCs have high self-renewal capability, allow a large-scale expansion, and can differentiate into cells from the 3 germ layers (Takahashi and Yamanaka 2006). iPSCs have been extensively used for drug screening and disease modeling (Ebert et al. 2012). Moreover, iPSC-based and iPSC-derived cells present high sensitivity to apoptotic induction and are useful to characterize the effects of materials and substrates (Takayama et al. 2013; Dzhoyashvili et al. 2015).

iPSCs have been reprogrammed from cells from several oral sources (Egusa et al. 2010; Miyoshi et al. 2010; Yan et al. 2010; Hynes et al. 2015). These iPSCs have the potential to

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A supplemental appendix to this article is available online.

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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, when mixed with mesenchymal cells placed in close contact with the epithelial cells, were capable to form bone-like structures and, in some cases, dentin- and dental pulp–like structures in vivo (Wen et al. 2012). Despite these exciting results, the ability of iPSCs from human origin to differentiate into functional odontoblasts in vivo remains unknown.

The derivation of functional odontoblasts persists as one of the most challenging issues in dental research. This is governed by several signaling molecules and growth factors, such as BMP-4, which plays crucial roles regulating tooth initiation, morphogenesis, and shape development (Vainio et al. 1993; Aberg et al. 1997). Notably, BMP-4 has the potential to induce odontoblastic differentiation of pluripotent stem cells in vitro. For instance, embryonic stem cell–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 with BMP-4 presented high gene expression of DSPP and high alkaline phosphatase activity (Kawai et al. 2014). A similar approach has shown that embryonic bodies from mouse iPSCs 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 into odontoblasts in vivo. In addition, we evaluated the effects of long-term expansion on the odontoblastic differentiation potential and mineralization capability of iPSCs and DPSCs. The hypotheses tested were as follows: 1) iPSCs can differentiate into functional odontoblasts in vivo, and 2) there will be no decrease in the odontogenic differentiation and mineralization capability of iPSCs after long-term expansion as compared with DPSCs in vitro.

Material and Methods

Ethics Compliance

The use of severe combined immunodeficiency (SCID) mice and human DPSCs and teeth were approved by NUS Institutional Animal Care and Use Committee, 2013-05239; NUS Institutional Biosafety Committee, 2014-00762; and NUS Institutional Review Board, 2094/2013. Animal experimentation followed the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Cell Culture and iPSC Generation and Characterization

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

The DPSCs (P4, 1×10^6 input cells) were reprogrammed with episomal iPSC reprogramming vectors (Epi5 Episomal iPSC Reprogramming Kit; Thermo Fischer Scientific) that contained an oriP/EBNA-1 backbone for delivering the reprogramming factors (*OCT4, SOX2, KLF4, L-MYC, 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 with the Neon Transfection System (Thermo Fischer Scientific). The parameters were as follows: 1,650 V and 3 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 6 colonies. All the iPSCs colonies were characterized for ALP, TRA-1-60, and OCT-4 via fluorescence microscopy (FV1000; Olympus Optical). For the teratoma formation assay, 3×10^5 iPSCs were mixed with 50 µL of Matrigel (BD Matrigel; BD Biosciences) and injected subcutaneously into the dorsum of 5- to 7-wk-old female SCID mice (n = 4, CB-17 SCID; InVivos). After 9 wk, the tumors 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 DPSCs and iPSCs of P4 by quantitative real-time polymerase chain reaction (qPCR) as previously described (Xie et al. 2017). Primer sequences are shown in the Appendix. Genome-wide screening for gross chromosomal abnormalities was carried out for 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, 1×10^5 iPSCs (P4) were seeded in Matrigel-coated 6-well plates and cultured with StemMACSTM-brew XF culture media (Miltenyi Biotec). The clone was transferred into a 12-well suspension plate (Greiner Bio-One GmbH) and maintained for 7 d with embryonic body culture media (AggreWell EB Formation Medium; Stemcell Technologies). The EBs were moved into tissue culture 6-well plates and treated with differentiation media—DMEM (Invitrogen) supplemented with 100nM dexamethasone (Sigma-Aldrich), 5mM b-glycerophosphate (Sigma-Aldrich), 50 µg/mL of ascorbate phosphate (Sigma-Aldrich), and 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 d by qPCR (CFX Connect Real-Time PCR Detection System; Bio-Rad). For all the genetic studies, we prepared 3 independent samples, which were used for 3 qPCR reactions.

The odontogenic potential was confirmed in vivo. For this, the iPSCs (P4) were treated with the differentiation medium described earlier, supplemented with 100 ng/mL of BMP-4 (odontogenic medium) for 10 d. Afterward, 6×10^5 cells were seeded in poly-L-lactic acid (Boehringer Ingelheim) 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 d, specimens were retrieved and fixed with 4% formaldehyde solution in phosphate-buffered saline for 24 h at 4 °C, demineralized (Decalcifier II; Surgipath) for 4 h at room temperature, and stained with hematoxylin and eosin. DPSCs (P4) and scaffolds devoid of cells were the controls. Immunohistochemical analysis of the tissues formed was performed with anti-DMP-1 (1:1,000; Abcam) and anti-human mitochondria antibodies (1:500; Abcam). Negative controls were tissue sections stained with an isotype-matched nonspecific 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 d. The gene expressions of *MEPE, DSPP*, and *DMP-1* were evaluated after 10 d by qPCR. The mineralization potential was assessed by exposing the cells to extracts of a bioactive cement (Biodentine; Septodont). 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 the manufacturer's instructions and loaded into moulds (10 × 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, and the eluent was filtered (0.22-µm pore size) and used to treat the cells. After 10 d, the amount of calcium per DNA was quantified as described in the Appendix.

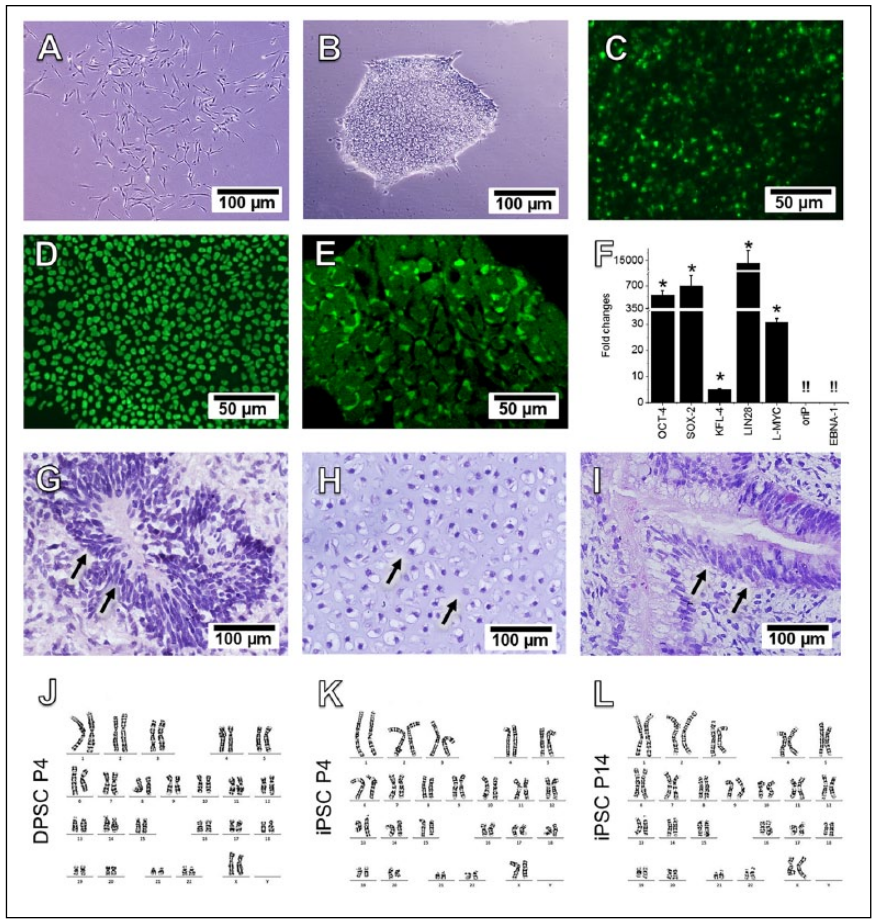

Figure 1. Phase contrast microscopy revealed that dental pulp stem cells (DPSCs) presented spindle-shaped fibroblastoid morphology (**A**). After the electroporation, the cells assembled into colonies with a high nucleus:cytoplasm ratio (**B**). Immunofluorescence analysis of the colony used in this 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.

Statistical Analysis

Statistical analysis for qPCR was performed with 2-way analysis of variance, and multiple comparisons were performed with Tukey's post hoc test. Normality test was performed with Shapiro-Wilk test. Kruskal-Wallis was used for alizarin red S staining. A preset significance level of 5% was set for all the tests (SPSS 22.0; IBM).

Results

Prior to the reprogramming, we checked the expression of MSC markers in the DPSCs. Approximately 99% of the DPSCs 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

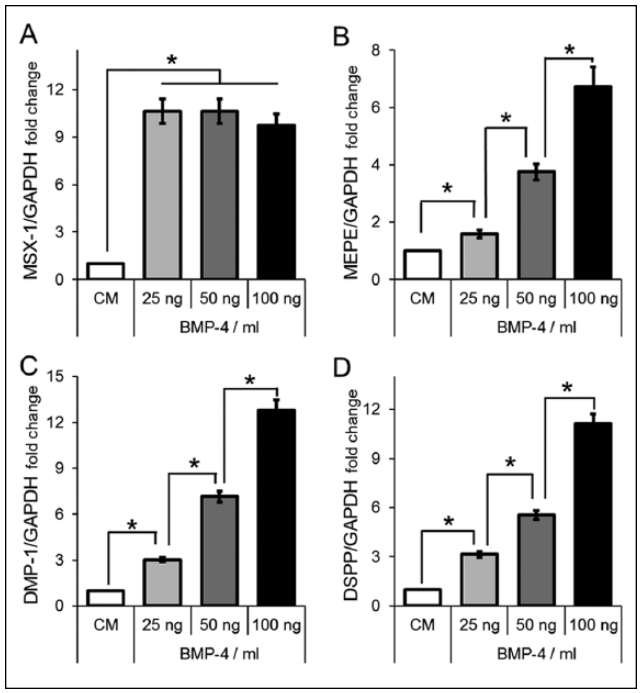

Figure 2. Effects of BMP-4 in the odontogenic differentiation of induced pluripotent stem cells (iPSCs) in vitro. iPSCs were treated with odontogenic medium with different concentrations of BMP-4 for 10 d. The quantitative real-time polymerase chain reaction analysis showed that iPSCs exposed to odontogenic 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.

of the vectors, the DPSCs formed 6 colonies. Notably, the colony used in this study presented a high nucleus:cytoplasm ratio (Fig. 1B), positive expression of the pluripotency markers TRA-1-60 and OCT-4 (Fig. 1C, D), 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) when compared with DPSCs ($P < 0.05$). The pluripotency was confirmed in vivo by the teratoma formation assay. We injected 3×10^5 iPSCs into the subcutaneous space of SCID mice, and the tumors were retrieved after 9 wk. The histologic analysis showed teratomas with neuroepithelial-like tissues (ectoderm), cartilage (mesoderm), and secretory tubule-like structures (endoderm) from the 3 germ layers (Fig. 1G–I). Finally, we characterized the karyograms to check whether the reprogramming could induce karyotypic abnormalities. The karyotype of iPSCs was as normal as the one from the parental DPSCs (Fig. 1J, 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.

Afterward, we evaluated the effects of BMP-4 concentration on the expression of putative markers for odontoblastic differentiation of iPSC in vitro. Regardless of the concentration of BMP-4 used, the odontogenic medium significantly increased the expression of all genes tested as compared with 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* when compared with 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, which also presented high expression of these markers when treated with odontogenic medium supplemented with 100 ng/mL of BMP-4 as compared with the untreated control (Appendix Fig. 3; $P < 0.05$).

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 d. The histologic analysis revealed that the iPSCs formed a pulp-like tissue within the pulp chamber containing structures resembling tubular dentin (arrows in Fig. 3A). To confirm whether the human iPSCs acquired a mature odontoblast phenotype in vivo, we checked the expression of DMP-1 by immunohistochemistry. Notably, cells neighboring the dentin-like structures 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, D). Immunostaining with antibody specific for human mitochondria confirmed that the tissues formed were populated by human cells (Fig. 3E, F; Appendix Fig. 4).

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 10 d, and the expressions of putative markers for odontoblastic differentiation were evaluated by qPCR (Fig. 4A–C). For P4, 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 10 d (Fig. 4D). The iPSC-derived odontoblastic-like cells deposited similar amount of calcium per nanogram (Ca/ng) of DNA for both passages tested. Notably, there was a decrease of ~40% in the calcium deposition of DPSCs in P14 as compared with P4.

Discussion

Animal and human iPSCs have been differentiated into several cell types, such as ameloblasts, neurons, and osteoprogenitor and neural crest–like cells (Duan et al. 2011; Otsu et al. 2012; Wen et al. 2012; Zou et al. 2012; Cai et al. 2013). Despite 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 be further differentiated into odontoblasts in vitro and in vivo.

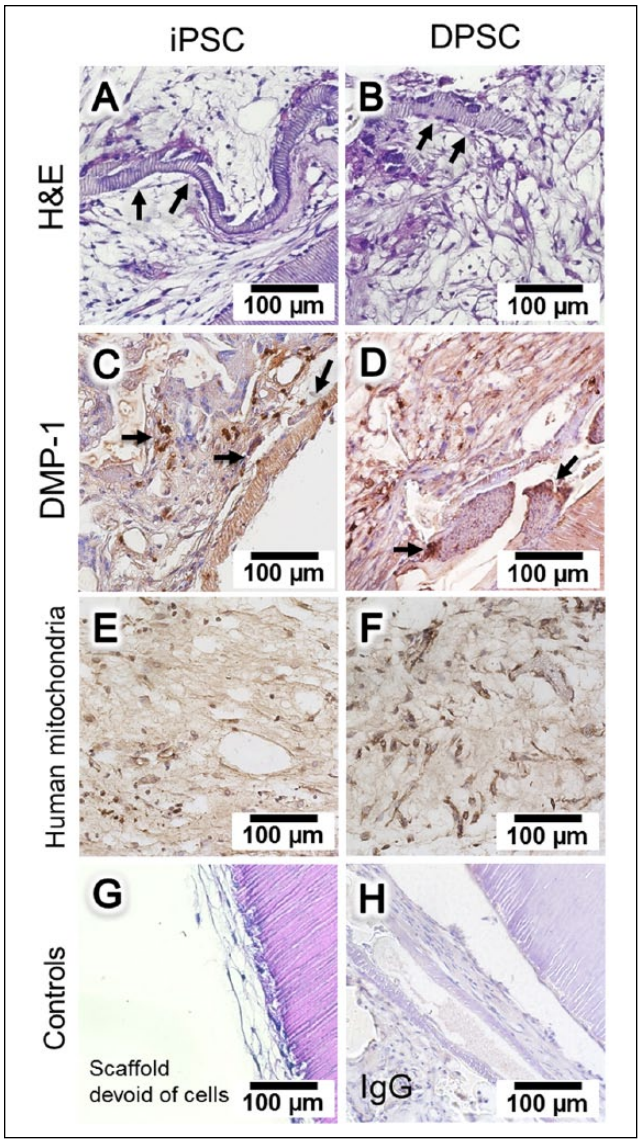

Figure 3. Induced pluripotent stem cell (iPSC)–derived odontoblast-like cells in vivo. iPSCs and dental pulp stem cells (DPSCs) were seeded in scaffolds cast in the pulp chamber of 1-mm-thick dentin slices and implanted in mice for 28 d. Slides were processed for histologic analysis and observed under 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**).

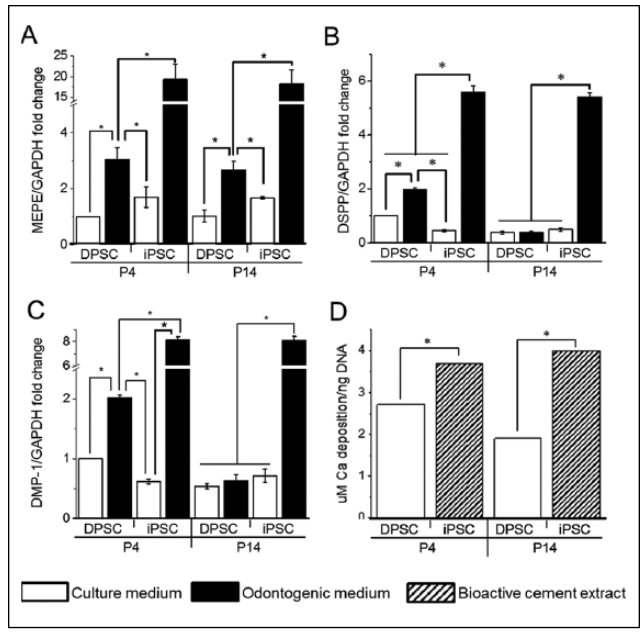

Figure 4. Odontoblastic differentiation and mineralization potential after short- and long-term expansion in vitro: passages 4 and 14 (P4 and P14), respectively. For P4, dental pulp stem cells (DPSCs) and induced pluripotent stem cells (iPSCs) presented a significant increase in the expression of the 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.

First, we characterized the cells after the electroporation. They presented high expression of reprogramming genes and markers commonly used to confirm the pluripotent character (Fig. 1C–F). The iPSCs formed teratomas in vivo consisting of differentiated derivatives of all 3 primary germ layers (Fig. 1F–I), 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 these disadvantages, this technique exempt the use of viral vectors, and the oriP/EBNA-1-based episomal vector presents a range of host cells, including the human ones (Son et al. 2016).

Animal-derived odontoblast lineages have been established from murine and porcine models (MacDougall et al. 1998; Iwata et al. 2007). Yet, the derivation and maintenance of functional human odontoblasts remain one of the most challenging issues in dental research. Notably, the presence of 100 ng/mL of BMP-4 in the differentiation media significantly increased the expression of the putative markers for odontoblastic differentiation in the iPSCs as compared with the other concentrations tested or control (Fig. 2). Similar increases 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 important, the iPSCs seeded in the scaffolds and implanted in the SCID mice 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, B). 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, D). Finally, the positive expression of human mitochondria confirmed that the tissues engineered were populated with human cells (Fig. 3E, F; Appendix Fig. 4). Collectively, our data confirm 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 an irreversible proliferation-arrested state and a decline in their 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 observed that P14 DPSCs 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–C). 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 between P4 and P14 (from 2.6 to 1.7 μM of Ca/ng of 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 Ca/ng of DNA even after long-term expansion (Fig. 4D). This ability can be correlated with the high expression of odontoblast-related genes observed in iPSCs after short- and long-term expansion (Fig. 4A–C). These findings confirm the second hypothesis and reassure the potential of these cells as promising alternatives to test bioactivity of biomaterials.

Despite 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. In contrast to the 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 opens new opportunities to improve biomaterial testing and modeling disorders of tooth development.

Author Contributions

H. Xie, contributed to design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; N. Dubey, contributed to design, data acquisition, and analysis, drafted and critically revised the manuscript; W. Shim, T. Cao, contributed to design, data analysis, and interpretation, drafted and critically revised the manuscript; C.J.A. Ramachandra, contributed to conception, design, data acquisition, and analysis, drafted and critically revised the manuscript; K.S. Min, V. Rosa, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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References

Aberg T, Wozney J, Thesleff I. 1997. Expression patterns of bone morphogenetic proteins (BMPs) in the developing mouse tooth suggest roles in morphogenesis and cell differentiation. Dev Dyn. 210(4):383–396.

Bento LW, Zhang Z, Imai A, Nor F, Dong Z, Shi S, Araujo FB, Nor JE. 2012. Endothelial differentiation of SHED requires MEK1/ERK signaling. J Dent Res. 92(1):51–57.

Cai J, Zhang Y, Liu P, Chen S, Wu X, Sun Y, Li A, Huang K, Luo R, Wang L, et al. 2013. Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. Cell Regen (Lond). 2(1):6.

Calles K, Svensson I, Lindskog E, Haggstrom L. 2006. Effects of conditioned medium factors and passage number on Sf9 cell physiology and productivity. Biotechnol Prog. 22(2):394–400.

Collado-Gonzalez M, Garcia-Bernal D, Onate-Sanchez RE, Ortolani-Seltenerich PS, Lozano A, Forner L, Llena C, Rodriguez-Lozano FJ. 2016. Biocompatibility of three new calcium silicate-based endodontic sealers on human periodontal ligament stem cells. Int Endod J. 50(9):875–884.

Duan X, Tu Q, Zhang J, Ye J, Sommer C, Mostoslavsky G, Kaplan D, Yang P, Chen J. 2011. Application of induced pluripotent stem (IPS) cells in periodontal tissue regeneration. J Cell Physiol. 226(1):150–157.

Dzhoyashvili NA, Shen S, Rochev YA. 2015. Natural and synthetic materials for self-renewal, long-term maintenance, and differentiation of induced pluripotent stem cells. Adv Healthc Mater. 4(16):2342–2359.

Ebert AD, Liang P, Wu JC. 2012. Induced pluripotent stem cells as a disease modeling and drug screening platform. J Cardiovasc Pharmacol. 60(4):408–416.

Egusa H, Okita K, Kayashima H, Yu G, Fukuyasu S, Saeki M, Matsumoto T, Yamanaka S, Yatani H. 2010. Gingival fibroblasts as a promising source of induced pluripotent stem cells. Plos One. 5(9):e12743.

Hynds RE, Giangreco A. 2013. Concise review: the relevance of human stem cell-derived organoid models for epithelial translational medicine. Stem Cells. 31(3):417–422.

Hynes K, Menichanin D, Bright R, Ivanovski S, Hutmacher DW, Gronthos S, Bartold PM. 2015. Induced pluripotent stem cells: a new frontier for stem cells in dentistry. J Dent Res. 94(11):1508–1515.

Iwata T, Yamakoshi Y, Simmer JP, Ishikawa I, Hu JC. 2007. Establishment of porcine pulp-derived cell lines and expression of recombinant dentin sialoprotein and recombinant dentin matrix protein-1. Eur J Oral Sci. 115(1):48–56.

Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J, Bunnell BA. 2008. Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. Cancer Res. 68(11):4229–4238.

Kaur G, Dufour JM. 2012. Cell lines: valuable tools or useless artifacts. Spermatogenesis. 2(1):1–5.

Kawai R, Ozeki N, Yamaguchi H, Tanaka T, Nakata K, Mogi M, Nakamura H. 2014. Mouse ES cells have a potential to differentiate into odontoblast-like cells using hanging drop method. Oral Dis. 20(4):395–403.

Kidwai FK, Movahednia MM, Iqbal K, Jokhun DS, Cao T, Fawzy AS. 2014. Human embryonic stem cell differentiation into odontoblastic lineage: an in vitro study. Int Endod J. 47(4):346–355.

MacDougall M, Selden JK, Nydegger JR, Carnes DL. 1998. Immortalized mouse odontoblast cell line MO6-G3 application for in vitro biocompatibility testing. Am J Dent. 11(Spec No.):S11–S16.

Mehrazarin S, Oh JE, Chung CL, Chen W, Kim RH, Shi S, Park N-H, Kang MK. 2011. Impaired odontogenic differentiation of senescent dental mesenchymal stem cells is associated with loss of Bmi-1 expression. J Endod. 37(5):662–666.

Miyoshi K, Tsuji D, Kudoh K, Satomura K, Muto T, Itoh K, Noma T. 2010. Generation of human induced pluripotent stem cells from oral mucosa. J Biosci Bioeng. 110(3):345–350.

Otsu K, Kishigami R, Oikawa-Sasaki A, Fukumoto S, Yamada A, Fujiwara N, Ishizeki K, Harada H. 2012. Differentiation of induced pluripotent stem cells into dental mesenchymal cells. Stem Cells Dev. 21(7):1156–1164.

Ozeki N, Mogi M, Kawai R, Yamaguchi H, Hiyama T, Nakata K, Nakamura H. 2013. Mouse-induced pluripotent stem cells differentiate into odontoblast-like cells with induction of altered adhesive and migratory phenotype of integrin. Plos One. 8(11):e80026.

Ren H, Sang Y, Zhang F, Liu Z, Qi N, Chen Y. 2016. Comparative analysis of human mesenchymal stem cells from umbilical cord, dental pulp, and menstrual blood as sources for cell therapy. Stem Cells Int. 2016:3516574.

Rosa V, Toh WS, Cao T, Shim W. 2014. Inducing pluripotency for disease modeling, drug development and craniofacial applications. Expert Opin Biol Ther. 14(9):1233–1240.

Rosa V, Zhang Z, Grande RH, Nor JE. 2013. Dental pulp tissue engineering in full-length human root canals. J Dent Res. 92(11):970–975.

Son M-Y, Lee M-O, Jeon H, Seol B, Kim JH, Chang J-S, Cho YS. 2016. Generation and characterization of integration-free induced pluripotent stem cells from patients with autoimmune disease. Exp Mol Med. 48:e232.

Stolzing A, Jones E, McGonagle D, Scutt A. 2008. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. Mech Ageing Dev. 129(3):163–173.

Takahashi K, Yamanaka S. 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 126(4):663–676.

Takayama K, Kawabata K, Nagamoto Y, Kishimoto K, Tashiro K, Sakurai F, Tachibana M, Kanda K, Hayakawa T, Furue MK, et al. 2013. 3d spheroid culture of hESC/hiPSC-derived hepatocyte-like cells for drug toxicity testing. Biomaterials. 34(7):1781–1789.

Vainio S, Karavanova I, Jowett A, Thesleff I. 1993. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell. 75(1):45–58.

Vertrees RA, Goodwin T, Jordan JM, Zwischenberger JB. 2008. Tissue culture models. In: Zander DS, Popper HH, Jagirdar J, Haque AK, Cagle PT, Barrios R, editors. Molecular pathology of lung diseases. Molecular Pathology Library, vol 1. New York (NY): Springer. p. 150–165.

Wen Y, Wang F, Zhang W, Li Y, Yu M, Nan X, Chen L, Yue W, Xu X, Pei X. 2012. Application of induced pluripotent stem cells in generation of a tissue-engineered tooth-like structure. Tissue Eng Part A. 18(15–16):1677–1685.

Xie H, Chua M, Islam I, Bentini R, Cao T, Viana-Gomes JC, Castro Neto AH, Rosa V. 2017. CVD-grown monolayer graphene induces osteogenic but not odontoblastic differentiation of dental pulp stem cells. Dent Mater. 33(1):e13–e21.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. 2010. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem Cells Dev. 19(4):469–480.

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA. 2009. Human induced pluripotent stem cells free of vector and transgene sequences. Science. 324(5928):797–801.

Zou X-Y, Yang H-Y, Yu Z, Tan X-B, Yan X, Huang GTJ. 2012. Establishment of transgene-free induced pluripotent stem cells reprogrammed from human stem cells of apical papilla for neural differentiation. Stem Cell Res Ther. 3(5):43.