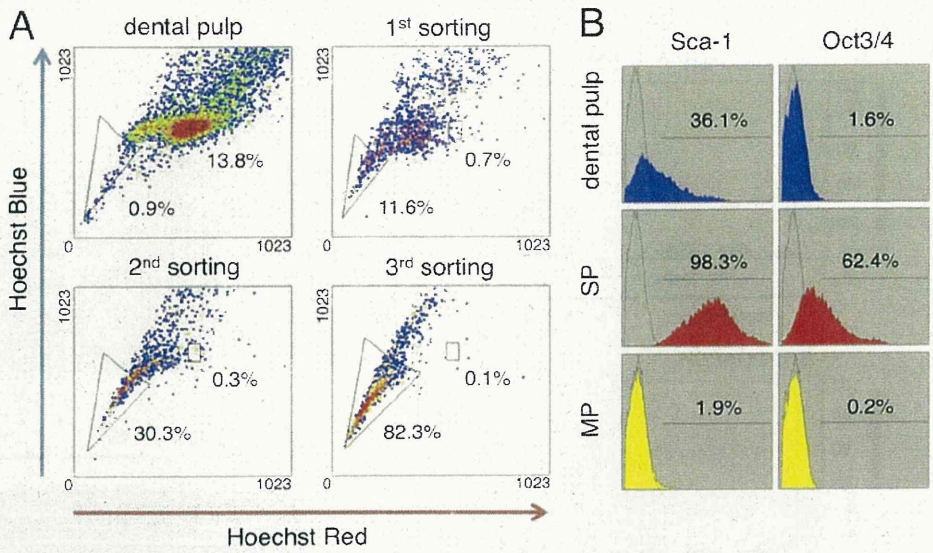

FIGURE 1. **Isolation of SP cells from mDP cell line.** *A*, flow cytometry analysis of SP cells. mDP cells made up ~0.9% of the total cell population with a relatively lower level of Hoechst 33342 fluorescence (SP cells), while 13.8% of the population was maintained as MP cells. Using repeated cell sorting, the SP cell population was enriched by 11.6% at the first sorting, 30.3% at the second sorting, and 82.3% at the third sorting. *B*, expression of the stem cell markers Sca-1 and Oct3/4 in dental pulp, SP, and MP cells.

because of the limited numbers of cells available. We enriched an SP cell population and established an SP cell line using a cell sorting technique. mDP cells were obtained from mouse incisor tooth germs and immortalized, as previously described (22). The cells were then stained with Hoechst dye and sorted to enrich the SP cell fraction. Cell sorting was repeated three times and SP cells were enriched from about 0.9% to 82.3% in the gated area (Fig. 1*A*). This SP cell line showed high expression levels of the stem cell markers Sca-1 and Oct3/4 when compared with the majority population (MP) cells, which was comprised of a greater number dental papilla cells in various differentiation stages (Fig. 1*B*).

Because the SP cells expressed a set of stem cell markers, we examined their multipotency. Using an odontoblast differentiation medium containing BMP2 or BMP4, the SP cells were induced to express DSPP, a marker of odontoblasts, whereas the expression of the undifferentiated cell marker Bcrp1 was decreased (Fig. 2*A*). In osteoblast differentiation medium, the SP cells showed increased levels of ALP and von Kossa staining, as well as expressions of the osteoblast marker genes osteocalcin, osteonectin, and Runx2, whereas the MP cells showed no induction of expression of those genes (Fig. 2, *B* and *C*). When SP cells were cultured in differentiation medium for adipogenesis or neurogenesis, they were Oil-Red-O positive or showed neurite outgrowths, along with high levels of adipogenic expression and protein expressions of neurogenic markers, such as *PPARγ* and Neurofilament-M, respectively (supplemental Fig. S1, *A*--*D*). These results suggest that the SP cell line established in this study has a high level of multipotency.

Expressions of Runx2 and DSPP in SP Cells Cultured with SF2 Cells—We analyzed epithelial and mesenchymal stem cell interactions by culturing SP cells with rat dental epithelial SF2 cells that had been engineered to express a GFP-myc-HA tag on the cell membrane surface. This allowed us to distinguish between SP and SF2 cell types (supplemental Fig. S2). SP cells were cultured with or without SF2 cells for 48 h, and total RNA was isolated from the mixed cell cultures (Fig. 3*A*). The expressions of Runx2 and DSPP were increased in SP cells that had been cultured with SF2 cells, as compared with those cultured without SF2 cells (Fig. 3*B*). Because Runx2 and DSPP are expressed by both odontoblasts and ameloblasts, co-cultured SP and SF2 cells were separated into individual cell populations using the anti-HA antibody, which specifically recognizes SF2 cells (Fig. 3*C*). We found a dramatic increase in the expression level of Runx2 in SF2 cells as compared with SP cells (Fig. 3*D*). No epithelial marker was detected in SP cells co-cultured with SF2 cells, suggesting that the SP cells had differentiated into odontoblasts (data not shown). Runx2 is expressed in enamel matrix-secreting ameloblasts, but not in the pre-secretion stage of ameloblasts (25). Our results suggest that the SF2 cells had fully differentiated into enamel matrix-secreting ameloblasts by co-culturing with SP cells. The expression of DSPP was up-regulated in both cell types. However, in MP cells, which are fully differentiated dental papilla cells, no expression of Runx2 or DSPP was induced by co-culturing with SF2 cells (data not shown). These results indicate that epithelial and mesenchymal stem cell interactions promote individual differential states in SF2 and SP cells.

Involvement of Exogenous Factors from Dental Epithelium in DSPP Expression of SP Cells—We attempted to identify the factors in dental epithelial cells involved in SP cell differentiation by treating SF2 cells with 4% PFA to inhibit extracellular signaling, including the effects of growth factors (Fig. 4*A*). Ammonia treatment, through a process known as denudation, removes all cell components except the extracellular matrices and is often used for three-dimensional matrix cell culture experiments (26). DSPP expression in SP cells was partially inhibited by PFA treatment, while they retained the extracellular matrix network. This result suggests that the extracellular environment including extracellular matrices, growth factors, and cell-cell

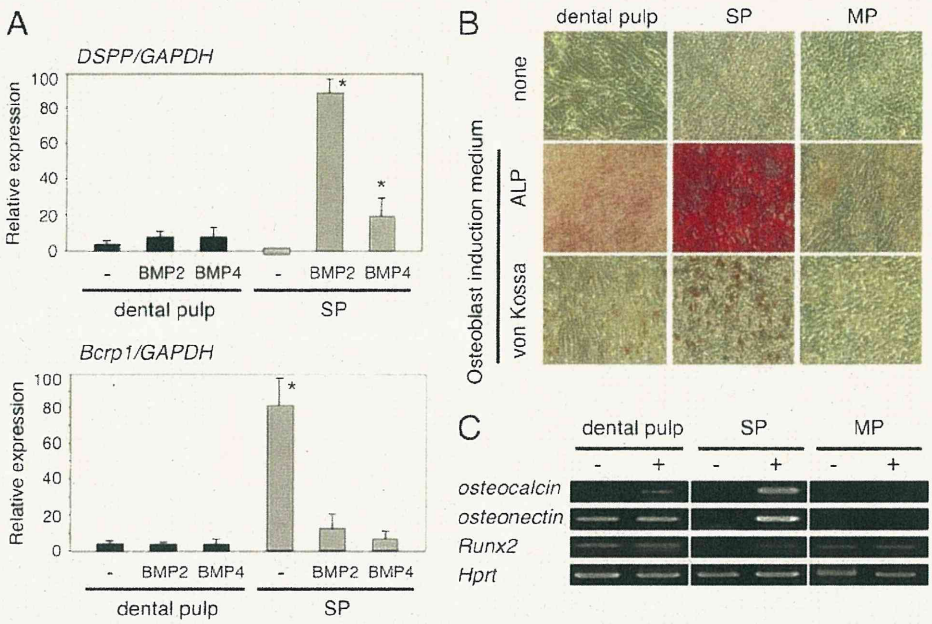

FIGURE 2. **Odontoblast and osteoblast differentiation in SP cells.** *A*, differentiation of SP cells to odontoblasts. Expression of the odontoblast marker DSPP and the undifferentiated mesenchymal marker Bcrp1 in dental pulp (*black bar*) and SP cells (*gray bar*) cultured with or without BMP2 or BMP4. *B*, differentiation of SP cells to osteoblasts in osteoblast induction medium (*Osteogenic cond.*). ALP and von Kossa staining of dental pulp, SP, and MP cells. *C*, expressions of osteoblast markers in dental pulp, SP, and MP cells cultured in regular (−) or osteoblast induction medium (+). *, $p < 0.05$.

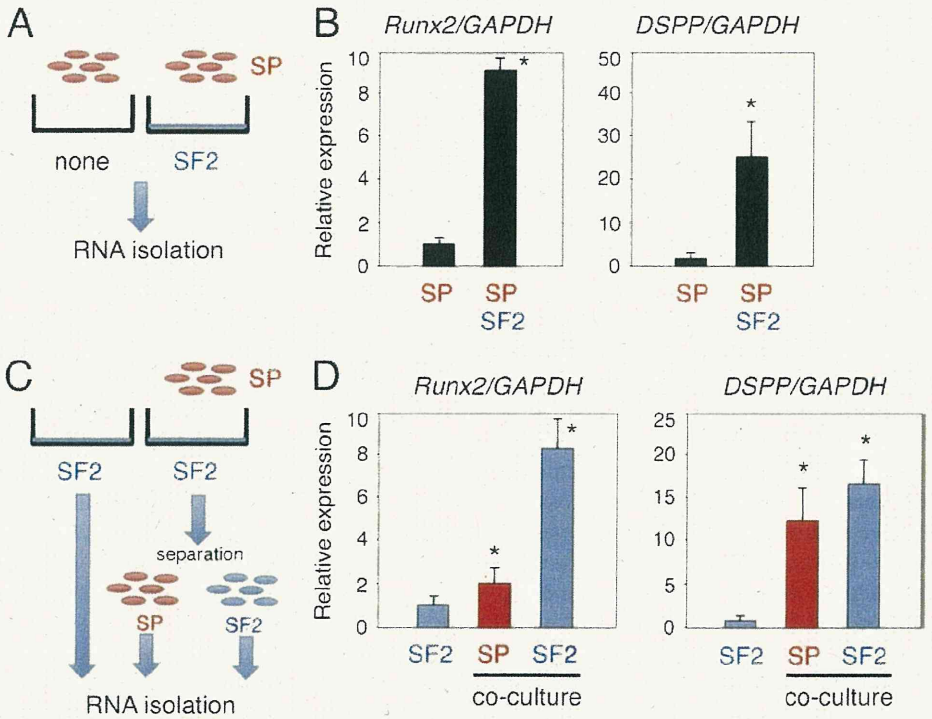

FIGURE 3. ***In vitro* epithelial-mesenchymal interaction system using dental epithelial cells (*SF2*) and dental mesenchymal stem cells (*SP*) to promote odontogenic cell differentiation.** *A* and *C*, schematic diagram of the co-culture system. *B*, comparisons of *Runx2* and *DSPP* gene expressions between the SP monolayer culture and SP and SF2 cell co-culture system. *C*, total RNA samples were separately prepared from SP and SF2 cells, using the anti-HA antibody. *D*, expressions of Runx2 and DSPP in co-cultured SF2 (*blue*) and SP (*red*) cells. The expression level of GAPDH was used an internal control. *, $p < 0.05$.

interaction produced by SF2 cells contributes to odontoblast induction. Denuded SF2 cells were also incapable of inducing DSPP expression in SP cells (Fig. 4*B*). Odontoblast induction of SP cells was observed in co-cultures with living SF2 cells, indicating that some types of soluble secreted molecules and matrices from SF2 cells are required to induce SP cells to undergo odontogenic differentiation.

Next, we screened the factors secreted from SF2 cells that promote odontogenic cell differentiation from epithelial and mesenchymal cells using cell culture chambers, which allowed

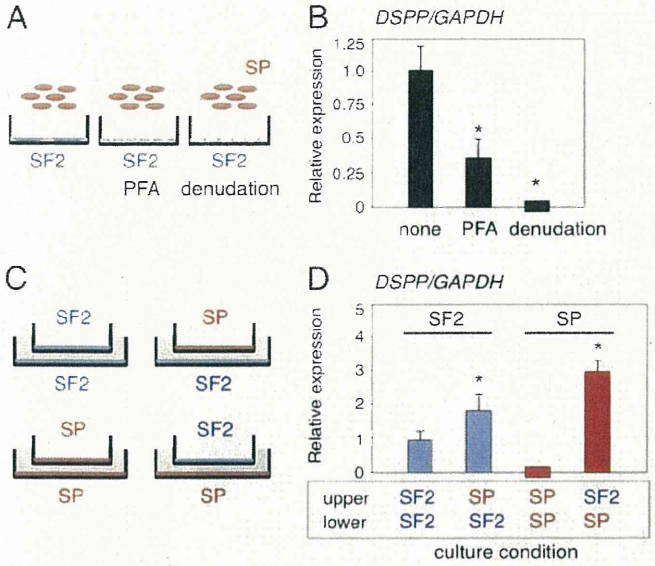

FIGURE 4. **Co-culture conditions for screening of odontogenic cell differentiation using *in vitro* cell-cell interaction system.** *A*, SP cells were cultured on SF2 cells in monolayers, then fixed with 4% paraformaldehyde (*PFA*) or treated with ammonia (denudation). *B*, DSPP expression in SP cells co-cultured under different conditions. *C*, four sets of co-culture conditions using cell chambers were analyzed. *D*, DSPP expression in SF2 cells (*blue*) and SP cells (*red*) cultured in lower dishes, with co-culture partner cells in the upper chambers. The expression level of GAPDH was used an internal control. *, $p < 0.05$.

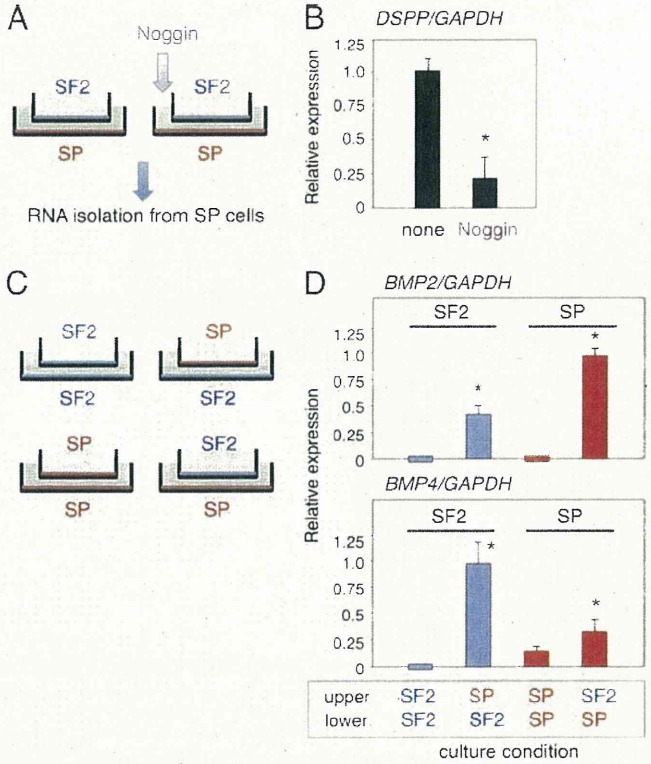

FIGURE 5. ***In vitro* epithelial-mesenchymal interaction system shows that crosstalk BMP signaling is essential for odontogenic cell differentiation.** *A*, total RNA was isolated from SP cells co-cultured with SF2 cells in the presence or absence of Noggin recombinant protein. *B*, DSPP expression in SP cells co-cultured with SF2 cells after blocking BMP signaling. *C*, four sets of culture conditions using cell chambers were analyzed. *D*, BMP2 and BMP4 expressions in SF2 (*blue*) and SP (*red*) cells, with co-culture partner cells in the upper chambers. *, $p < 0.05$.

the factors to be secreted into cell culture medium (Fig. 4*C*). Heterologous combinations of SF2 and SP cells were important for promotion of DSPP expression in both types of cells. We found that co-cultures consisting of SF2 cells in the upper chamber and SP cells in the lower chamber were most effective for stimulation of DSPP gene expression in SP cells (Fig. 4*D*). These results suggest that secreted factors are important for induction of DSPP expression in SP cells co-cultured with dental epithelial cells.

Regulation of DSPP Expression in SP Cells via BMP2-BMP4 Crosstalk—The involvement of several different types of growth factors has been reported in epithelial-mesenchymal interactions, for example, BMPs were shown to promote dental mesenchymal cell differentiation (27). We examined the potential involvement of BMPs in SP cell differentiation by adding soluble Noggin, which antagonizes BMP activity, to cell chamber cultures that contained SP cells in the lower chambers (Fig. 5*A*). The presence of Noggin in culture medium resulted in down-regulation of the expression of DSPP in SP cells as compared with the control cells (Fig. 5*B*). Therefore, BMPs are required for induction of DSPP expression in SP cells co-cultured with dental epithelial cells. In tooth germ development, BMP4 is involved in epithelial-mesenchymal interaction, and also regulates the mesenchymal expression of Msx1 and Msx2, which are important for tooth development, whereas BMP2 promotes dental mesenchymal differentiation (27). However, details regarding crosstalk between BMP2 and BMP4 in dental epithelial and mesenchymal stem cell interactions have not been elucidated. We sought to clarify the role of BMPs in these interactions by examining the expressions of BMP2 and BMP4 in SF2 and SP cells using a separated chamber assay (Fig. 5*C*). The expression of BMP2 was higher in SP cells than SF2 cells under the heterologous combination culture condition, whereas BMP2 was not detected in homologous cultures (Fig. 5*D*). In contrast, the expression of BMP4 was higher in SF2 cells than in SP cells in the heterologous combinations (Fig. 5*D*). Taken together, these results suggest that the interactions between dental epithelium and dental mesenchymal stem cells induce BMP4 and BMP2, which, in turn, promote odontogenic cell differentiation via paracrine and autocrine signaling.

Optimization of Co-culture Conditions for Differentiation of iPS Cells into Ambn-expressing Dental Epithelial Cells—Following interaction with SF2 cells, SP cells differentiated into DSPP expressing cells, but not ameloblasts (Figs. 3, 4, and 5). This may be because SP cells are mesenchymal stem cells and committed to differentiate into mesenchyme lineage cell types. Therefore, we used mouse iPS cells to examine whether these cells can be differentiated into ameloblasts when cultured with SF2 cells. However, SF2 cells did not effectively promote their differentiation (data not shown), which may be due to the necessity of factors from differentiated dental epithelial cells for differentiation of iPS cells into ameloblasts. To test this possibility, we subcloned 25 different SF2 cell lines and examined the expression levels of the Ambn gene. Of these lines, the SF2-24 cell line expressed Ambn at the highest level (supplemental Fig. S3*A*). Dental epithelium SF2-24 cells grew tightly together in a

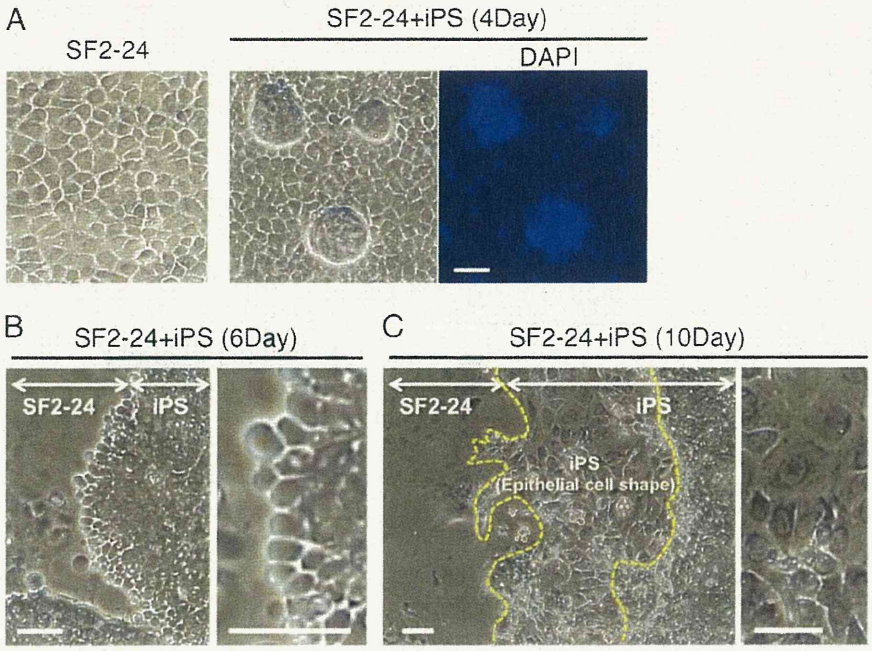

FIGURE 6. **Epithelial cell shapes of iPS cells after co-culturing with SF2-24 cells.** *A*, phase micrographs of monolayer SF2-24 cells and iPS cells cultured with SF2-24 feeder cells for 4 days, followed by DAPI staining. *B* and *C*, low and high magnification phase micrographs of iPS cells on MMC-treated SF2-24 feeder cells after 6 (*6Day*) and 10 days (*10Day*). Enlarged image shows a part of the iPS cells with epithelial cell shapes. *C*, epithelial cell cluster formed by iPS cell-derived epithelial cells (area within *yellow dashed line*). Bar, 50 mm.

square or cuboidal shape (Fig. 6*A*), and expressed Ambn and cytokeratin-14 (CK14), but not the reprogrammed factors Sox2, Klf4, and Oct3/4 (supplemental Fig. S3*B*). On the other hand, iPS cells formed colonies that expressed Nanog promoter-driven GFP (data not shown) as well as Klf4, Sox2, Oct3/4, and Nanog, but not Ambn or CK14 (supplemental Fig. S3*B*).

We also examined the effects of differentiation by co-culturing iPS cells with MMC-treated non-proliferating SF2-24 feeder cells (Fig. 6*A*). The shape of the co-cultured iPS cells was clearly rounded along the boundary of the clusters after 6 days (Fig. 6*B*). These cells had migrated and formed what appeared to be epithelium after 10 days (area surrounded by *yellow dashed line*, Fig. 6*C*).

The differentiation of iPS cells was then determined by RT-PCR analysis. First, we examined the specificity of mouse Ambn locked nucleic acid (LNA) primer sets (supplemental Fig. S4). A mouse Ambn LNA primer set specifically detected the mouse Ambn gene, but not the rat Ambn gene (supplemental Fig. S4*A*). Using this primer set, Ambn expression was not detected in mouse iPS cells or MEFs (supplemental Fig. S4*B*). Next, we examined co-culture conditions for the differentiation of iPS cells into dental epithelium (Fig. 7*A*). iPS cells co-cultured with MMC-treated SF2-24 cells showed a high expression of the mouse Ambn gene, while those co-cultured with PFA-treated or non-treated SF2-24 cells did not (Fig. 7*B*). SF2-24 feeder cells expressed rat Ambn when co-cultured with iPS cells, while that expression was reduced at 10 days (Fig. 7*C*).

Interestingly, expressions of the stem cell markers Sox2, Oct3/4, Nanog, Fgf4, and Gdf3 were not changed throughout the co-culture period, because of the existence of undifferentiated iPS cells (Fig. 7*C*), while those of the endodermal markers Cdx2 and Gata6 were also not increased. Furthermore, the mesodermal marker Brachyury was highly expressed in iPS cells, because of technical contamination resulting RNA extraction from MEFs used for maintenance of the iPS cells, and then gradually decreased over time. We also observed increased expressions of the mouse ameloblast markers Ambn and Enamelin (Enam), as well as the epithelial markers CK14 and p63, in iPS cells after 7 and 10 days (Fig. 7*C*). Furthermore, the expression of epiprofin/Sp6, a transcription factor highly expressed in dental epithelium (28), was increased in those cells (supplemental Fig. S5). A similar expression pattern was observed in co-cultured iPS cells separated from SF2-24 cells using the anti-HA antibody (data not shown).

Differentiation of iPS Cells into Ambn-expressing Dental Epithelial Cells—We then examined the protein expression of Ambn in iPS cells using immunostaining. Approximately 95% of the epithelial-like cells were positive for Ambn (Fig. 8*A*), while the immunofluorescence intensity of Ambn was stronger in iPS cells than in SF2-24 cells (Fig. 8*B*). Therefore, mouse iPS cells differentiated into dental epithelium, but not into endodermal or mesodermal cells.

We attempted to identify the factors involved in differentiation of iPS cells into dental epithelium by culturing with MEFs in medium conditioned by SF2-24 cell cultures (Fig. 9*A*). Culturing with SF2-24 condition medium induced the expression of Ambn in iPS cells, indicating an involvement of soluble factors including growth factors, and extracellular matrices derived from SF2-24 cells (Fig. 9*B*). Next, we examined the effect of Ambn on differentiation of iPS cells into dental epithelial cells. Expression vectors for the full-length (AB1), C-terminal (AB2), and N-terminal (AB3) half of Ambn (Fig. 9*C*) were separately transfected into Ambn low-expressing cells (SF2-7), then conditioned media from those cells or recombinant Ambn

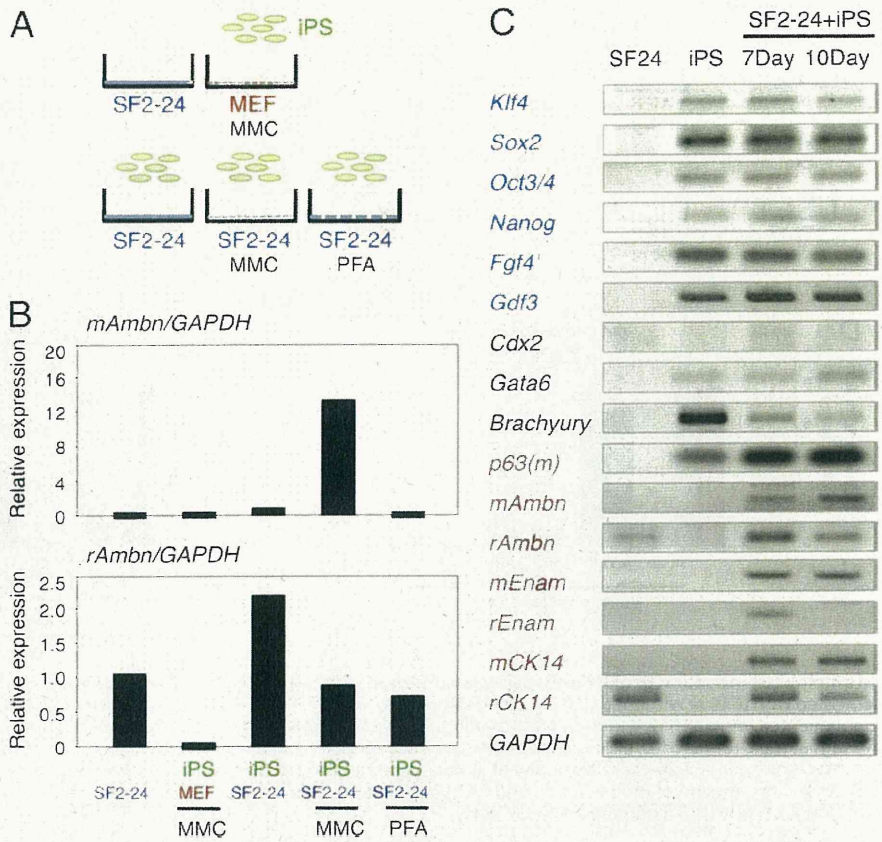

FIGURE 7. **Effects of culture conditions on ameloblast induction of iPS cells.** *A*, iPS cells were co-cultured with SF2-24 cells, MMC-treated (*MMC*) MEFs, MMC-treated SF2-24 cells or PFA-treated SF2-24 cells. *B*, Ambn expression in mouse iPS (*upper panel*) and rat-derived SF2-24 (*bottom panel*) cells in different co-culture conditions for 10 days. *C*, time course analysis of gene expressions of stem cell (*blue*), endo/mesoderm (*black*), and ameloblast (*red*) markers in iPS cells co-cultured with SF2-24 cells for 7 (*7Day*) and 10 days (*10Day*).

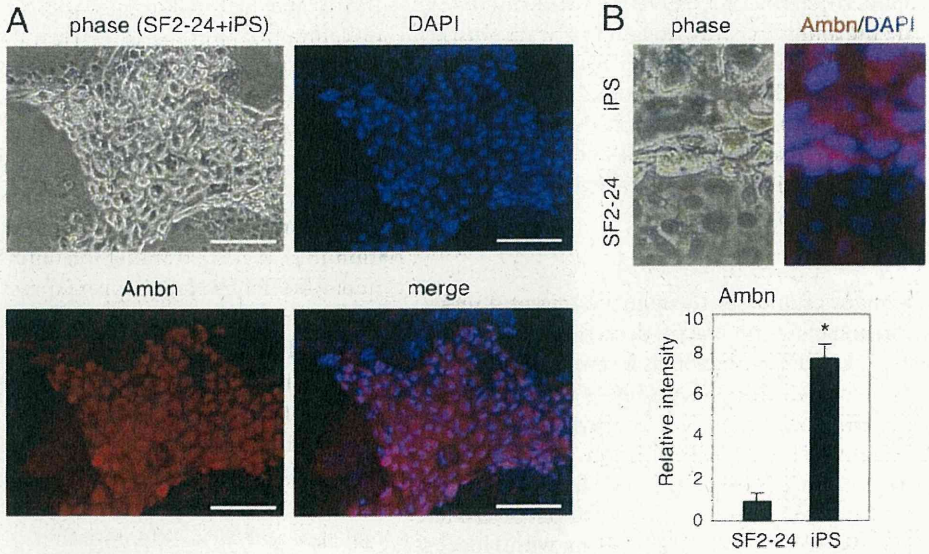

FIGURE 8. **Expression of Ambn, an ameloblast specific protein, in iPS cells co-cultured with SF2-24 cells.** *A*, phase micrographs of iPS cell colonies cultured with mitomycin C-treated SF2-24 cells. Hoechst staining (*blue*), Ambn staining (*red*), and merged images. *B*, high magnifications of phase and merged images in *A*. *Bottom panel*, relative expression levels of Ambn protein in SF2-24 and iPS cells cultured in ameloblast induction system. *, $p < 0.05$; Bar, 100 mm.

proteins (AB1, -2, or -3) were added to cultures of iPS cells. Conditioned media from SF2-24 cells and full-length AMBN-expressing cells, but not from other transfectants or recombinant Ambn proteins, induced Ambn expression in iPS cells (Fig. 9*D*), indicating that Ambn may be necessary for differentiation of iPS cells into dental epithelium. Previously, we showed that neurotrophic factor NT-4 is important for the differentiation of ameloblasts (29). To examine the effect of NT-4

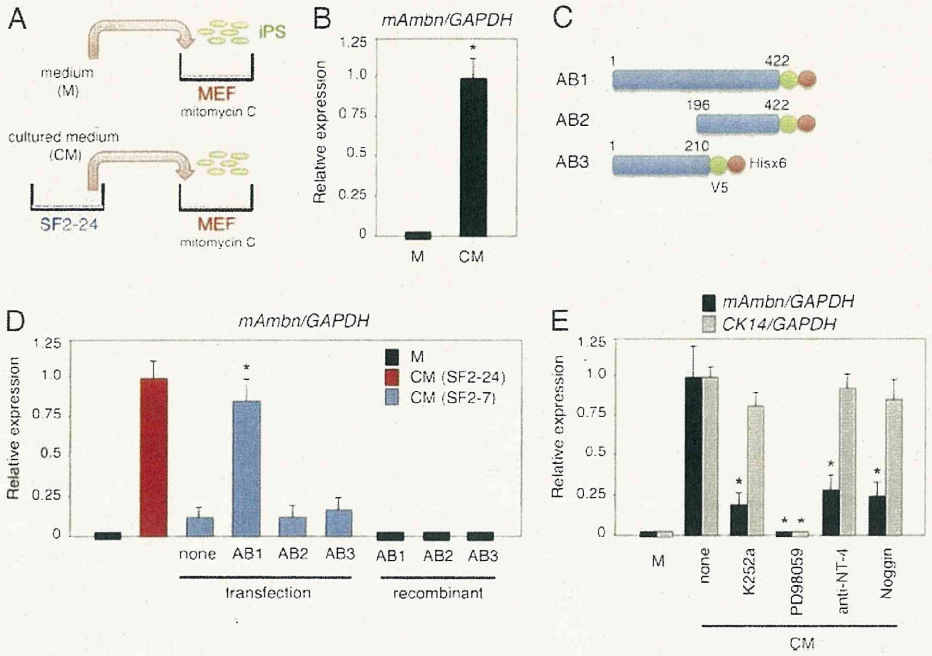

FIGURE 9. **Promotion of ameloblast induction of iPS cells using conditioned SF2-24 cells.** *A*, iPS cells were cultured on mitomycin C-treated MEFs in iPS cell culture medium supplemented with (*CM*) or without (*M*) conditioned medium from SF2-24 cells. *B*, expression of mouse Ambn gene in iPS cells cultured in iPS cell culture medium supplemented with (*CM*) or without (*M*) conditioned medium from SF2-24 cells. *C*, creation of Ambn deletions. All recombinant Ambn proteins have V5 and His tags at the C terminus. *D*, expression of mouse Ambn gene in iPS cells cultured in iPS cell culture medium supplemented with (*CM*) or without (*M*) condition medium from SF2-24 cells, recombinant Ambn-expressing SF2-7 cells or recombinant Ambn proteins. *, $p < 0.05$ (compared with non-transfected SF2-7 cells). *E*, expression of mouse Ambn and CK14 genes in iPS cells cultured in SF2-24 conditioned medium supplemented with K252a, PD98059, anti-NT-4, or Noggin. *, $p < 0.05$ (compared with CM only).

on dental epithelial cell differentiation by iPS cells, we analyzed the expressions of Ambn and CK14 in iPS cells cultured with SF2-24-conditioned medium in the presence of K252a (inhibitor of neurotrophic receptor Trk), PD98059 (MEK inhibitor), anti-NT-4 neutralizing antibody, or Noggin (BMP antagonist). K252a, PD98059, anti-NT-4, and Noggin each inhibited the expression of Ambn in iPS cells. Furthermore, CK14 expression in iPS cells was not inhibited by K252a, anti-NT-4, or Noggin (Fig. 9*E*). These results indicate that NT-4 and BMP signaling are important for differentiation into dental epithelial cells, but not CK14-positive epithelial cells.

DISCUSSION

Tooth development progresses through a number of stages, and the differentiation of dentin matrix-secreting odontoblasts and enamel matrix-producing ameloblasts results in formation of the crown. Ameloblasts and odontoblasts are central cell types involved in tooth development. In developing molars, restricted dental mesenchymal cells interact with the inner dental epithelium through the matrix and differentiate into odontoblasts. In the present study, we established an SP cell line from dental papilla mDP cells using cell sorting with Hoechst staining. SP cells are known to retain multipotency characteristics and can differentiate into various cell types, such as odontoblasts, osteoblasts, adipocytes, and neural cells. Our method for obtaining multipotent SP cells from a single cell line may be useful for development of novel therapeutic strategies that aim at regeneration of oral tissues.

Our co-culture assay of SP cells with dental epithelial cells showed that dental epithelial cells promote SP cell differentiation into DSPP-expressing cells via BMP2 and BMP4, which are secreted from dental epithelial cells (Fig. 5*B*, 5*D*, and 10*A*). Because BMP2 is not highly expressed in dental epithelium, BMP4 may be the dominant signaling regulator during odontoblast differentiation. In the early stages of tooth development, BMP4 is expressed in dental epithelium and induces the transcription factor Msx1 (30). The expression of DSPP is induced via the BMP signaling pathway in cooperation with Runx2, Dlx5, and Msx1 in undifferentiated mesenchymal cells (31). Previously, a bead soak assay of mandibular organ culture showed that BMP4 induced dental mesenchymal cell differentiation (32). Also, a transgenic approach revealed that inhibition of BMP4 by Noggin overexpression, driven by a keratin 14 promoter (K14-Noggin), resulted in the absence of all molars in the mandible. This indicates that BMP4 is essential for tooth bud formation by inducing dental mesenchymal cells (33). As demonstrated, in the present study odontoblastic differentiation of SP cells is completely disturbed by the blocking of BMP signaling. Thus, our finding strongly support the notion that BMP4 signaling is a key factor in induction of dental mesenchymal cells and their differentiation.

Differential synchronization between dental epithelial and mesenchymal cells has been observed during tooth development. Dental epithelial and mesenchymal cells are separated by a basement membrane, which is an essential regulator for epithelial-mesenchymal interaction (34). Both crown and root odontoblasts are induced by interactions with epithelial cells, such as those of the inner dental epithelium, epithelial rest, and epithelial diaphragm (35). Similar to *in vivo* situations, physical

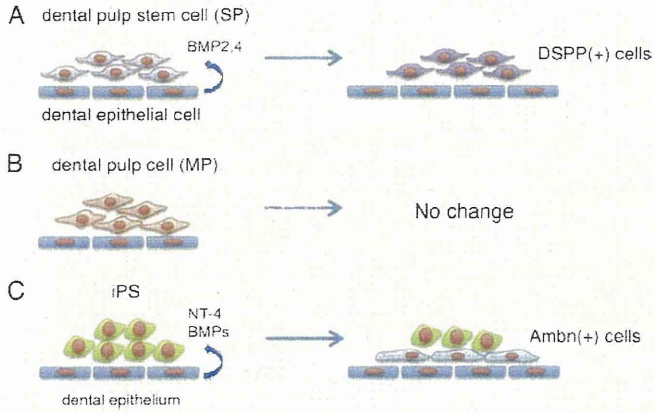

FIGURE 10. **Proposed models of odontogenic induction from dental mesenchymal stem cells and iPS cells by co-culturing with dental epithelial cells.** *A*, dental epithelial cells induce DSPP-expressing odontoblasts from SP cells. *B*, no odontogenic induction was observed in differentiated (*MP*) cells co-cultured with dental epithelial cells. *C*, dental epithelial cells induce Ambn-expressing ameloblasts from iPS cells.

cell attachment of dental epithelial cells was not required for odontogenic induction of SP cells in our experiments, indicating that soluble factors including BMPs are important for odontogenic induction by dental epithelial cells in culture. We also found that MP cells from dental papilla did not differentiate into DSPP-expressing cells, indicating that epithelial-mesenchymal interactions are important for cell fate determination of dental pulp stem cells, but not for differentiated dental pulp cells (Fig. 10, *A* and *B*). It was recently reported that Ambn protein, or a synthetic peptide based on the N-terminal region of the Ambn protein, induced osteoblastic cell differentiation (36). In addition to BMPs, Ambn may also be one of the factors involved in the odontogenic induction process, because the sharing of signaling pathways underlies the mechanism of odontoblastic and osteoblastic induction.

Ameloblasts secrete enamel-specific extracellular matrices including Ambn, which are lost upon tooth eruption. This makes it impossible to repair or replace damaged enamel in an erupted tooth. Therefore, identifying alternative sources of these cells becomes important. Bone marrow-derived cells can give rise to different types of epithelial cells. In mixed cultures with c-Kit+-enriched bone marrow cells, embryonic dental epithelial cells, and dental mesenchyme, bone marrow cells might be reprogrammed to give rise to ameloblast-like cells (37). Our strategy to create ameloblasts from mouse iPS cells may have direct application in tooth regeneration. We succeeded in establishing a co-culture system using cells derived from two different species, mouse iPS cells and rat derived enamel matrix secreting ameloblasts. This is the first demonstration of differentiation of iPS cells into ameloblasts through interactions with dental epithelium (Fig. 10*C*). However, a set of stem cell markers was continuously expressed in iPS cells after 7 days of co-culturing (Fig. 7*C*), indicating that a portion of the iPS cells had differentiated into enamel-secreting ameloblasts and some still retained stem cell potential. Thus, the efficacy of iPS cell differentiation into ameloblasts by enamel-secreting ameloblasts feeder cells must to be improved prior to for clinical application.

A number of factors are thought to give iPS cells the capacity for direct or indirect differentiation into ameloblasts. Possible direct effectors include gap junctions, intercellular binding molecules, adhesion factors, and extracellular matrices secreted by dental epithelium. Growth factors might also be involved, because conditioned medium from SF2-24 cells induced Ambn expression in iPS cells. Ambn is also a candidate factor for dental cell differentiation of iPS cells, as SF2 cells expressing low levels of Ambn did not induce differentiation of iPS cells. Furthermore, overexpression of full-length Ambn in cells expressing low levels of Ambn induced iPS cells into ameloblast-like differentiation (Fig. 9*D*). Ambn has diverse functions in various cellular physiologies, such as cell growth, differentiation, cell polarization, and attachment, though the detailed mechanisms of Ambn signaling require additional investigation. Ambn-null mice display severe enamel hypoplasia due to impaired dental epithelial cell proliferation, polarization, and differentiation into ameloblasts, as well as loss of cell attachment activity with immature enamel matrix (2). These results suggest that Ambn, especially full-length, is necessary for both *in vivo* and *in vitro* ameloblast differentiation.

There were differences in cell lineage determination of the dental pulp stem cells and iPS cells when co-cultured with dental epithelial cells. RT-PCR analysis showed that co-culturing induced SP cells to form odontoblastic cells, whereas iPS cells were induced to form ameloblastic cells. In addition, the expression of Brachyury, a mesodermal marker, in iPS cells was down-regulated by co-culturing with SF2-24 cells (Fig. 7*C*). Conversely, expressions of the epithelial markers p63 and CK14, as well as the dental epithelial marker epiprofin/Sp6 were up-regulated (Fig. 7*C*, supplemental Fig. S5) (28). These results suggest that the cell lineage of the iPS cells in our co-culturing system was effectively guided into an epithelial cell lineage. It has been reported that the default cell lineage of ES cells is the ectodermal cells, except when cultured in the presence of BMP antagonists (38, 39). Because BMPs promote ectodermal differentiation of ES cells, the expression of BMP observed in SF2 cells (Fig. 5*D*) may also contribute to dental epithelial cell differentiation of iPS cells. A previous our reported that NT-4 induced Ambn expression in dental epithelium, while NT-4 knock-out mice showed delayed expression of enamel matrices in the early stage of ameloblast differentiation (29). In the present study, the presence of the anti-NT-4 neutralizing antibody or Noggin in conditioned medium from SF2-24 cells inhibited Ambn expression, but not that of CK14 (Fig. 9*E*). On the other hand, SP cells strongly expressed the endogenous Sox2 protein, one of the reprogramming factors involved in generation of iPS cells (data not shown). Recently, iPS cells were generated from human dental pulp cells with a high level of efficiency in comparison to dermal fibroblasts, possibly due to a high expression level of Sox2 in dental pulp stem cells. However, additional reprogramming factors are required for creation of iPS cells from dental pulp cells. Thus, SP cells themselves did not have the same degree of multipotency as seen with ES and iPS cells. SP cells are considered to be mesenchymal stem cells that originate from dental pulp cells, which are derived from cranial neural crest cells. Neural crest cells can differentiate into several different cell lineages, such as neuron, glia, melanocyte,

osteoblast, chondrocyte, and odontoblast cells (40, 41). We believe that SP cells are not able to gain multipotency beyond the potential of neural crest cells. Thus, SP cells preserve some degree of multipotency that is different in an undifferentiated state as compared with ES and iPS cells. In co-cultures with SF2-24 cells, SP cells did not differentiate into ameloblasts, whereas iPS cells did (Fig. 10). Comparative analysis between SP and iPS cells is essential to clarify the mechanisms involved in directional cell fate determination.

In this study, we sought to clarify the role of dental epithelium and stem cell interactions by culturing rat dental epithelium with mouse iPS cells and SP cells. Rodent incisors grow throughout the lifespan of the animal by maintaining stem cells in the cervical loop, located at the end of incisor. A dental epithelial cell niche also exists in the cervical loop of the incisor. Analysis of gene knock-out mice for epiprofin/Sp6, an essential transcription factor for dental epithelial cell differentiation and enamel formation, has revealed that supernumerary teeth are formed by interactions between dental mesenchyme and undifferentiated dental epithelium (4, 42). In addition, those studies showed continuous signals from dental epithelial cells of mutant mice induced the continued differentiation of dental mesenchymal cells into odontoblasts (4, 42). Together these findings suggest that dental epithelial cells can induce dental mesenchymal cells to differentiate into odontoblasts. Therefore, rat dental epithelial cells may provide an *in vitro* niche environment for surrounding mouse iPS cells and SP cells. Elucidation of the mechanism of cell fate determination by dental epithelial cells may facilitate development of novel therapeutic approaches for regenerative dentistry.

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RESEARCH REPORTS
Biological

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Glycosphingolipids Regulate Ameloblastin Expression in Dental Epithelial Cells

ABSTRACT

Neurotrophin 4 (NT-4) and its receptors regulate the differentiation of ameloblasts in tooth development. Gangliosides, sialic acids that contain glycosphingolipids (GSLs), are involved in a variety of membrane-associated cell physiological functions such as ligand-receptor signal transmission. However, the expression patterns and functions of GSLs during tooth development remain unclear. In this study, we identified strong expressions of GM3 and LacCer in dental epithelium, which give rise to differentiation into enamel-secreting ameloblasts. Exogenous GM3 and LacCer in dental epithelial cells induced the expression of *ameloblastin* (*Ambn*), while it was also interesting that GM3 synergistically exerted enhancement of NT-4-mediated *Ambn* expression. In addition, consistently exogenous GM3 and LacCer in dental epithelial cells induced distinct activation of extracellular signal-regulated kinase 1/2 (ERK1/2), an event upstream of the expression of *Ambn*. Furthermore, depletion of GSLs from dental epithelial cells by D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-PDMP) inhibited *Ambn* expression as well as phosphorylation of ERK1/2. In contrast, exogenous addition of GM3 or LacCer rescued the phosphorylation of ERK1/2 repressed by pre-treatment with D-PDMP. Taken together, these results suggest that GM3 and LacCer are essential for NT-4-mediated *Ambn* expression, and contribute to dental epithelial cell differentiation into ameloblasts.

KEY WORDS: cell differentiation, enamel, developmental biology, tooth development, cell signaling, gene expression.

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INTRODUCTION

Glycosphingolipids (GSLs) are ubiquitously expressed in all eukaryotic cells and are mainly localized in the outer leaflet plasma membrane (Hakomori, 1990). They are integral for the dynamics of many cell membrane events, including cellular interactions, signaling, and trafficking. Recently, GSLs were demonstrated to be clustered with sphingomyelin and cholesterol to form a microdomain platform of caveolae and lipid rafts, which have been termed 'detergent-resistant microdomains' and have been found to modulate cellular signal transduction, cell migration, and cell adhesion (Anderson, 1998; Hakomori *et al.*, 1998; Simons and Toomre, 2000). Since GSLs are dominantly expressed in the brain and significantly change their levels of expression during development of the central nervous system, their functions for determination of neural cell fate and differentiation have been well-investigated (Inokuchi, 2009). GSLs, including nerve growth factor (NGF) and NT-4, are critical for neurotrophin family signaling. Neurotrophins bind the p75 receptor and 3 trk tyrosine kinase receptors, TrkA, TrkB, and TrkC (Barbacid, 1994; Chao, 1994; Friedman and Greene, 1999). Elucidation of the molecular interactions of gangliosides and neurotrophins would improve understanding of nerve development, and provide opportunities to enhance recovery after nerve injury and degenerative brain disorders (Mitsiadis *et al.*, 1993; Hirata *et al.*, 2001; Ribeiro-Resende *et al.*, 2007).

In the field of dentistry, the GD3 synthase gene has been identified in dental epithelial cells and found to be responsible for cell proliferation (Yamada *et al.*, 2005). Neurotrophins and their receptors are expressed in developing teeth (Mitsiadis *et al.*, 1993). It was recently reported that molars in NT-4-deficient mice developed a thinner enamel layer, while NT-4 inhibited the expression of ameloblastin through TrkB (Yoshizaki *et al.*, 2008). Ameloblastin, an early marker of ameloblasts, plays multiple roles in dental epithelial cellular physiologies and differentiation, and *Ambn* knockout mice show severe enamel hypoplasia (Fukumoto *et al.*, 2004), indicating that *Ambn* is essential for ameloblast differentiation and enamel formation.

In the nervous system, regulation of the neurotrophin family has been well-investigated. However, the expression patterns and roles of GSLs in hard tissue development are poorly understood. In the present study, we investigated the roles of GSLs in hard tissue, and found GM3 and lactosylceramide (LacCer) abundantly present in dental epithelial cells, where they displayed synergistic activities to induce NT-4-mediated *Ambn* expression in dental epithelial cells. Our results suggest that GM3 and LacCer enhance NT-4 signaling and are involved in *Ambn* expression.

MATERIALS & METHODS

Cell Culture and Conditions

SF2 cells, a rat-derived dental epithelial cell line, were maintained as previously described (Yoshizaki *et al.*, 2008). As pre-treatment, 1.0 μM of GM3 (Sigma, St. Louis, MO, USA), LacCer (Sigma), or Gb4 (Sigma) was added 1 hr before stimulation with NT-4 (R&D Systems, Minneapolis, MN, USA). Cellular GSLs were depleted after 24 hrs of treatment with 20 μM D-PDMP (Sigma), an inhibitor of glucosylceramide synthase.

Sphingolipid and Glycosphingolipid Extraction, and Thin-layer Chromatography (TLC)

Sphingolipids and glycosphingolipids were extracted as previously described (Usuki *et al.*, 1996; Fukumoto *et al.*, 1999). Each of the natural and acidic lipid fractions was dissolved in chloroform/methanol, after which fractions of dental epithelial cells were applied to high-performance TLC plates (Merck, Darmstadt, Germany). For standards, a bovine brain ganglioside mixture (Wako, Tokyo, Japan) and neutral glycolipids from human erythrocytes were used.

Preparation of Tissue Sections and Immunohistochemistry

For immunohistochemistry, we prepared frozen sections from mouse heads obtained on post-natal day 1 (P1). Sections were incubated with the primary antibodies anti-GM3 (M2590) and anti-LacCer (Nakayama *et al.*, 2008), which were visualized with an Alexa 488 conjugated anti-rabbit IgG antibody (Invitrogen, Carlsbad, CA, USA). Nuclear staining was performed with DAPI dye (Invitrogen).

RNA Isolation and Real-time RT-PCR Analysis

Total RNA from SF2 was purified with TRIzol reagent (Invitrogen). After cDNA synthesis, real-time PCR analysis was performed with a StepOne™ with SYBR green PCR reagent (Applied Biosystems, Foster City, CA, USA). We repeated 3 independent experiments, and statistical differences were analyzed by Student's *t* test.

Cell Proliferation Assay

A BrdU incorporation assay was performed as previously described, with a 5-bromo-2'-deoxyuridine labeling and detection kit (Roche, Tokyo, Japan) (Nakamura *et al.*, 2004). BrdU-incorporated SF2 was detected with anti-BrdU mouse antibody and visualized by use of an FITC-conjugated anti-mouse IgG antibody (Roche).

Western Blotting

Cells were pre-treated with or without 100 ng/mL of NT-4 for 0-120 min at 37°C. Western blotting was performed with anti-p44/p42 (ERK1/2) or anti-phospho p44/p42 (p-ERK1/2) (Tyr 202/Tyr204) antibodies (CST). Data quantification analysis of Western blotting results was conducted with a MultiGauge image analyzing system (Fujifilm-GE Healthcare, Tokyo, Japan). We repeated 3 independent experiments, and statistical differences were analyzed with Student's *t* test.

RESULTS

Detection of GSLs in Dental Epithelial Cells

To determine total GSLs synthesized in dental epithelial cells, we performed TLC analysis using acidic and neutral lipid fractions from dental epithelial cell pellets. With the acidic fractions, a clear band was detected that was the same size as GM3, while no other obvious bands were detected. The band intensities of these reactions were increased in a volume-dependent manner (Fig. 1A). In contrast, a major band the size of Gb4 and a weak band corresponding to LacCer were detected in the neutral fractions, as well as additional minor bands with weak densities (Fig. 1B). Ganglioside biosynthesis begins with ceramide formation, which takes place in the endoplasmic reticulum followed by synthesis of glucosylceramide (GlcCer). LacCer is synthesized by the GalT-1 enzyme from GlcCer and GM3 is synthesized by α2,3-sialyltransferase from LacCer (Fig. 1C). To characterize which types of cells in the tooth germ express LacCer and/or GM3, we performed immunohistochemical analysis of developing molars using anti-LacCer (T7A7) and anti-GM3 (M2590) antibodies. LacCer expression was detected in epithelial-derived ameloblasts, though it was restricted to the side of the stratum intermedium (Fig. 1D). GM3 was more widely and strongly detected in the developing tooth germs as compared with LacCer, since its expression was detected in ameloblasts and the stratum intermedium of epithelial tissue, as well as in odontoblasts. A clear difference in expression pattern between LacCer and GM3 was observed in the early stage of ameloblasts, with the latter detected in both presecretory ameloblasts located at the cervical loop and secretory ameloblasts, while LacCer was preferentially detected in secretory ameloblasts (Fig. 1D).

Induction of *Ambn* Expression through ERK1/2 Signaling in Dental Epithelial Cells by Either GM3 or LacCer, and Their Synergistic Enhancement Mediated by NT-4

To investigate the biological functions of GM3 and LacCer expressions in dental epithelium, we examined *Ambn* expression by adding either GM3 or LacCer in an exogenous manner to culture media. RT-PCR analysis revealed strong induction of

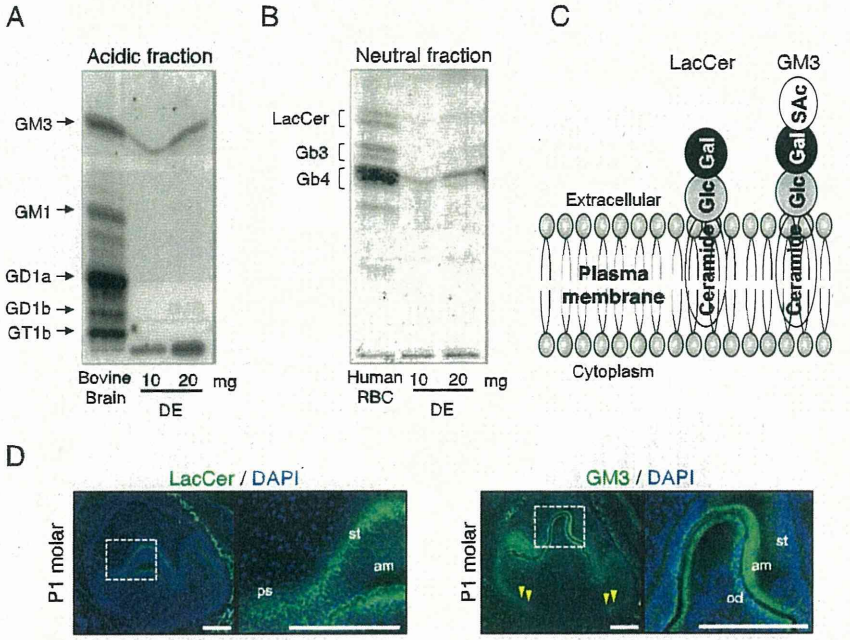

Figure 1. Detection of GSLs in dental epithelial cells, and expressions of GM3, LacCer, and Gb4 in developing mouse molars. TLC findings for **(A)** acidic and **(B)** neutral lipids in 10 and 20 mg of dental epithelial cell pellets. We prepared a standard mixture of neutral glycolipids for TLC, while a crude ganglioside mixture was prepared from bovine brain tissue. **(C)** Structures of LacCer and GM3. The LacCer synthesis enzyme attaches a galactose by the GalT-1 enzyme, and GM3 is synthesized by α-2,3-sialyltransferase from LacCer. **(D)** Localization of LacCer and GM3 in the lower first molar of post-natal day 1 mice was analyzed with anti-LacCer (left panel, green) and anti-GM3 (right panel, green) antibodies. Nuclear staining was performed with DAPI dye (blue). am, ameloblast; st, stratum intermedium; od, odontoblasts. Arrowheads indicate cervical loop of molar. Bar = 100 μm.

Ambn by both GM3 and LacCer, while weak expression was observed in their absence (Fig. 2A). Previously, we showed that the expression of *Ambn* in dental epithelial cells was promoted by NT-4 (Yoshizaki *et al.*, 2008). Interestingly, NT-4-mediated *Ambn* expression was synergistically enhanced by GM3 and promoted by LacCer in an additive manner. Also, real-time PCR analysis showed that pre-treatment with NT-4 resulted in a 6-fold increase and that with GM3 an 8-fold increase in *Ambn* expression, while that expression was significantly increased by 22-fold following pre-treatment with both NT-4 and GM3 (Fig. 2B). Similar results were obtained in immunocytochemical analysis with the anti-Ambn antibody (Fig. 2C). In addition, pre-treatment with LacCer also induced *Ambn* expression by nearly 7-fold, and NT-4 additively increased *Ambn* expression (Fig. 2B). In contrast, Gb4 did not have an effect on *Ambn* expression in SF2 cells (data not shown). These results suggest that either GM3 or LacCer promotes dental epithelial cell differentiation into ameloblasts.

A BrdU incorporation assay revealed that dental epithelial cell proliferation was down-regulated by pre-treatment with either GM3 or LacCer, and cell proliferation activities were significantly disturbed in the presence of NT-4 (Fig. 2D), indicating that these GSLs along with NT-4 accelerate dental epithelial differentiation.

In dental epithelial cells, NT-4 binds their receptor, TrkB, and up-regulates *Ambn* expression by activating the ERK1/2 pathway (Yoshizaki *et al.*, 2008). To analyze the further synergistic effects of GM3 and LacCer on activation of ERK1/2 by NT-4, we performed Western blotting using an anti-phospho ERK1/2 (p44/p42) antibody with and without pre-treatment with GM3, LacCer, or Gb4. ERK1/2 phosphorylation by NT-4 was detected at 5 min after stimulation and became weak at 60 min. Pre-treatment with GM3 at 1 hr before NT-4 stimulation significantly promoted ERK1/2 phosphorylation from 5 to 15 min after stimulation (Fig. 2E). Importantly, ERK1/2 phosphorylation was observed immediately following pre-treatment with GM3, which indicated a constitutive activation of ERK1/2 signaling without NT-4. Since dental epithelial cells endogenously express NT-4, an autocrine loop of NT-4 activated ERK1/2 signaling (data not shown). Similar results were obtained with LacCer (Fig. 2E). Gb4 did not have an effect on NT-4-mediated ERK signaling in SF2 cells (Appendix Fig. 1). These results suggest that pre-treatment with either GM3 or LacCer promotes NT-4-mediated ERK1/2 activation, and consequently contributes to the promotion of ameloblast differentiation.

Essential Roles of Gangliosides in NT-4-mediated Dental Epithelial Differentiation

We found that GM3 and LacCer are functional GSL fractions in NT-4-mediated ERK activation that participate in biosynthesis of dental epithelial cells in developing teeth. To investigate the roles of these GSLs in differentiation of dental epithelial cells, we used an inhibitor of glucosylceramide synthase, D-PDMP. We carefully determined the optimal concentration of D-PDMP at minimum toxicity in SF2 cultures. As expected, following depletion of GSLs on the cell surface of dental epithelium by D-PDMP, NT-4-mediated *Ambn* expression was completely abolished (Fig. 3A) and NT-4-mediated phosphorylation of ERK1/2 was significantly reduced by D-PDMP (Fig. 3B). These results suggest that the presence of gangliosides is essential for induction of dental epithelial cell differentiation into ameloblasts by NT-4.

Following D-PDMP treatment, ERK1/2 phosphorylation was remarkably reduced (Figs. 3C, 3D). Surprisingly, exogenous GM3 completely rescued down-regulated ERK phosphorylation caused by D-DPMP in dental epithelial cells (Figs. 3C, 3D), which was also observed with LacCer at slightly lower levels as compared with GM3 treatment (Fig. 3D). To evaluate the biological activities involved in the rescue of ERK phosphorylation, we examined the expression of *Ambn*. D-PDMP treatment of dental epithelial cells led to a 60% reduction in NT-4-mediated *Ambn* expression. As expected, addition of GM3 and LacCer restored *Ambn* expression down-regulated by NT-4, due to the lack of GSLs present in dental epithelial cells (Fig. 3E). These results suggest that the presence of GM3 and LacCer is essential for NT-4 signaling during dental epithelial cell differentiation.

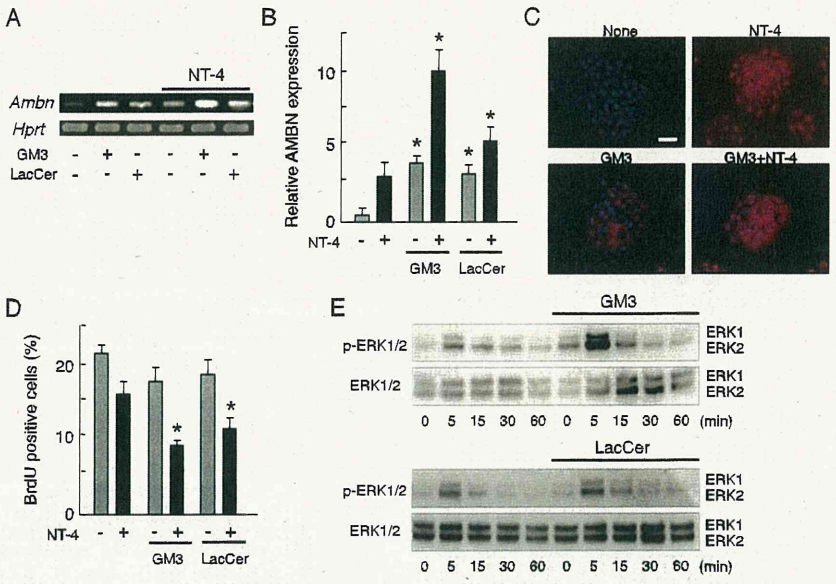

Figure 2. Biological roles of GM3 and LacCer in dental epithelial cells. **(A)** RT-PCR analysis of *Ambn* expression. NT-4 induced *Ambn* expression. Pre-treatment for 1 hr with either GM3 or LacCer in dental epithelial cells induced *Ambn* expression, which was synergistically enhanced by pre-treatments with GM3 or LacCer in the presence of NT-4. **(B)** Real-time PCR analysis of *Ambn* expression. NT-4-mediated *Ambn* expression was significantly enhanced by the presence of GM3 or LacCer. *$p < 0.05$. **(C)** Immunocytochemistry with rabbit anti-*Ambn* antibody. SF2 cells were pre-treated with or without GM3, and cultured in the presence or absence of NT-4 for 48 hrs. An anti-rabbit *Ambn* antibody (sc-50534, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used with an Alexa 594-conjugated anti-rabbit IgG secondary antibody (Invitrogen). Nuclear staining was performed with DAPI. Bar = 100 μm. **(D)** Cell proliferation was examined by a BrdU incorporation assay. NT-4 inhibited dental epithelial cell proliferation, while single pre-treatment with either GM3 or LacCer repressed cell growth. GM3 or LacCer pre-treatment performed 1 hr prior to NT-4 stimulation induced a significant reduction in cell proliferation activity. *$p < 0.05$. **(E)** The time-courses of phosphorylation (p-ERK1/2) and total ERK1/2 (ERK1/2) protein after stimulation with NT-4 were analyzed by Western blotting with or without pre-treatment with GM3 or LacCer. p-ERK1/2 was induced at 5 min after NT-4 stimulation. A marked synergistic induction of ERK1/2 phosphorylation was observed following pre-treatment with GM3 or LacCer at 5 min after NT-4 stimulation in dental epithelial cells.

DISCUSSION

In the present study, we found that GM3 and LacCer are expressed in dental epithelial cells and tooth germs, where they enhance NT-4-induced ameloblastin expression, in part, through ERK activation. Among the members of the ST2Gal subfamily, GM3 and GD3 synthases were shown to contain unique sequences (Kolter *et al.*, 2002). Those findings indicated that GM3 and GD3 synthases are highly specific for their lipid substrates, while other sialyltransferases can induce sialylation of a large number of lipid structures. Our previous study demonstrated that GD3 synthase is highly expressed only in the early stage of dental epithelial cell development, but not in ameloblasts (Yamada *et al.*, 2005; Fukumoto *et al.*, 2007). Furthermore, exogenous GM3 inhibits dental epithelial cell proliferation, whereas overexpression of GD3 synthase induces mitogenic activity and blocks differentiation of dental epithelial cells (Yamada *et al.*, 2005). Therefore, in dental epithelial cells, the expressions of GM3 and GD3 syntheses are synchronized with the promotion of differentiation and proliferation, respectively. In immunohistological analyses, GM3 consistently showed dominant expression in the late stage of tooth development, suggesting an accumulation of GM3 due to depletion of GD3 synthase in differentiating ameloblasts, which arrests cell proliferation and leads to cell differentiation.

Sphingolipids have a natural predisposition for lateral segregation within the membrane and formation of membrane microdomains such as lipid rafts, which play important roles in various cellular physiologies. Previous studies have demonstrated diverse roles of GSLs in signal transduction by controlling the dimerization of certain receptors on lipid raft structures. Thus, the formation of lipid rafts directly affects cellular functions. Exogenous administration of GM3 or LacCer may induce an excess number of lipid rafts, and alter membrane dynamics. Indeed, in the present study, cells with exogenous GM3 formed a large number of GM3 cluster microdomain platforms on plasma membranes (Appendix Fig. 2B). Elucidation of the

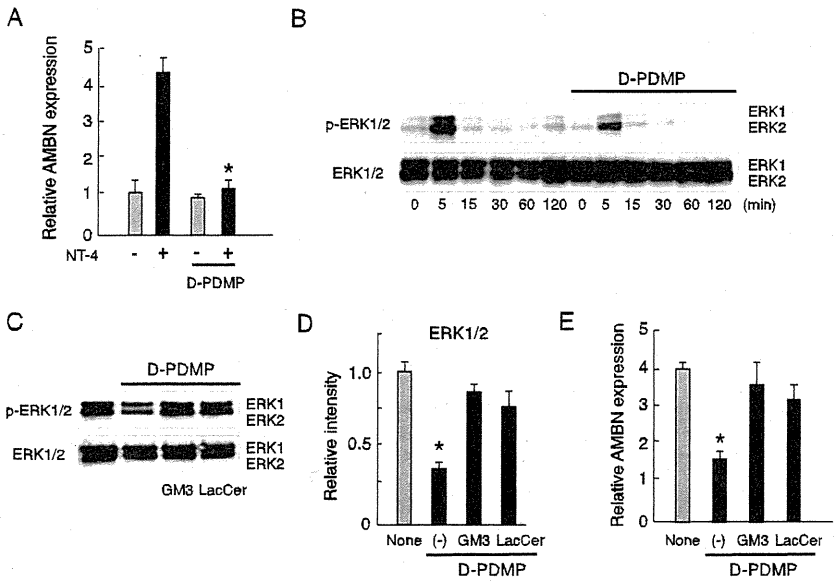

Figure 3. D-PDMP blocks NT-4-mediated action in dental epithelial cells, and exogenous administration of GM3 and LacCer restores NT-4-mediated ERK signaling attenuated by depletion of GSLs. **(A)** Real-time PCR analysis of NT-4-mediated *Ambn* expression with or without D-PDMP pre-treatment. D-PDMP completely canceled *Ambn* expression stimulated by NT-4. *$p < 0.05$. **(B)** The time-courses of phosphorylation (p-ERK1/2) and total ERK1/2 (ERK1/2) protein after stimulation with NT-4 were analyzed by Western blotting with or without D-PDMP pre-treatment. **(C)** ERK1/2 phosphorylation (p-ERK1/2) of dental epithelial cells stimulated with NT-4 for 5 min was decreased by D-PDMP pre-treatment, while exogenous administration of GM3 and LacCer rescued ERK1/2 phosphorylation. *$p < 0.05$. **(D)** Quantification of Western blotting images. **(E)** Real-time RT-PCR analysis of *Ambn* expression. *Ambn* expression in dental epithelial cells was attenuated by D-PDMP pre-treatment, then recovered by addition of either GM3 or LacCer. *$p < 0.05$.

relevance of enhancement of dental epithelial cell differentiation by GM3/LacCer and lipid raft structures would be interesting.

D-PDMP leads to extensive depletion of endogenous GSLs as well as gangliosides biosynthesized from GlcCer. We found that *Ambn* expression in dental epithelium induced by NT-4 was remarkably suppressed by pre-treatment with D-PDMP, while the expressions of TrkB and p75NGFR remained intact (data not shown). Down-regulation of NT-4-mediated *Ambn* expression might be caused by depletion of GSLs on the surfaces of dental epithelial cells. In fact, exogenous GM3 or LacCer may completely restore NT-4 signaling in D-PDMP-treated cells, indicating that those GSLs are essential for dental epithelium differentiation induced by NT-4.

Several studies of ganglioside synthases in insects and mice have used gene-targeting approaches and revealed functional redundancy in multiple cellular events and organogenesis (Haltiwanger and Lowe, 2004; Hennet, 2002; Yoshikawa *et al.*, 2009), which explains why GM3 synthase (St3gal5) mutations do not cause abnormalities in humans, while LacCer-deficient mice are embryonic-lethal. Although functional redundancy exists among GSLs, the expression pattern of the sugar chain shows a cell-type-specific distribution, since we found expressions of GM3 and LacCer in molars. In addition, GM3, Gb4, and LacCer are major GSL populations, and no other GSLs were detected in tooth germs (Figs. 1A, 1B). Our results also clearly suggest distinct roles of GM3 and LacCer in NT-4-mediated *Ambn* expression. Alteration of the pattern of sugar chains could modify cellular actions in specific cell types and at an adequate developmental stage of dental epithelial cells. For analysis of the specific function of LacCer in enamel formation, conditional knockout of LacCer with epithelial cells would be interesting. Furthermore, elucidation of the functions of GM3 and LacCer in tooth development might aid in the development of a novel therapeutic approach for diseases with enamel abnormalities, such as amelogenesis imperfecta.

In the present study, we clarified the expressions and localizations of GM3 and LacCer in developing teeth. GM3 and LacCer were found localized in dental epithelium, and shown to contribute to ameloblast differentiation and enamel matrix production. Empirical findings show that consumption of milk or breast-feeding during tooth development promotes mineralization of enamel by supplementation of minerals such as fluoride and calcium. Interestingly, a high concentration of GM3 has been found in both cow and human milk (Iwamori *et al.*, 2008). Analysis of our data provides an additional bridge between empirical and experimental findings in regard to enamel formation. In conclusion, our results revealed important roles for GM3 and LacCer in developing teeth, and may be useful for *in vivo* differentiation of ameloblasts.

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Differentiation of Induced Pluripotent Stem Cells Into Dental Mesenchymal Cells

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Similar to embryonic stem cells, induced pluripotent stem (iPS) cells can differentiate into various cell types upon appropriate induction, and thus, may be valuable cell sources for regenerative medicine. However, iPS cells have not been reported to differentiate into odontogenic cells for tooth regeneration. Here we demonstrated that neural crest-like cells (NCLC) derived from mouse iPS cells have the potential to differentiate into odontogenic mesenchymal cells. We developed an efficient culture protocol to induce the differentiation of mouse iPS cells into NCLC. We confirmed that the cells exhibited neural crest (NC) cell markers as evidenced by immunocytochemistry, flow cytometry, and real-time reverse transcription-polymerase chain reaction. Further, in recombination cultures of NCLC and mouse dental epithelium, NCLC exhibited a gene expression pattern involving dental mesenchymal cells. Some NCLC also expressed dentin sialoprotein. Conditioned medium of mouse dental epithelium cultures further enhanced the differentiation of NCLC into odontoblasts. These results suggest that iPS cells are useful cell sources for tooth regeneration and tooth development studies.

Introduction

ONCE TEETH ARE DAMAGED by bacterial infection, traumatic injury, or tooth wear, their repair is difficult without using artificial materials. Therefore, particular interest in the therapeutic application of stem cells has emerged from the possibility that they can differentiate into odontogenic cells. To date, there are several reports on the odontoblastic transformation of stem cells isolated from adult and neonatal tissues [1–3]. However, the clinical application of these cells is limited, since they exist in small numbers and slowly grow in culture.

Induced pluripotent stem (iPS) cells are generated from somatic cells by the simultaneous introduction of several factors; and they differentiate into the 3 embryonic germ layers with an extensive proliferative capacity [4–6]. This technique allows us to generate pluripotent stem cells without the use of embryos, to overcome rejection problems after implantation of non-autologous cells, and to avoid ethical issues associated with the use of embryonic stem (ES) cells. Recently, many researchers have reported that iPS cells can differentiate into different cell types, such as neurons, cardiac myocytes, and renal lineage cells, under appropriate conditions [7–9]. Therefore, iPS cells have emerged as potential cell sources for regenerative medicine. However, their potential to differentiate into odontogenic cells has not yet been investigated.

Tooth development involves a series of reciprocal epithelial–mesenchymal interactions between the mandibular epithelium and neural crest (NC)-derived ectomesenchyme [10,11]. The NC-derived ectomesenchymal cells contribute to the dental mesenchymal cells (DMC) that differentiate into dentin-secreted odontoblasts [12]. Targeted mutations in transcriptional factor genes of NC cells results in craniofacial and tooth development abnormalities [13,14]. Therefore, NC cells are critical for tooth organogenesis. Recently, several reports have proposed effective induction protocols to promote the differentiation of ES cells into NC cells [15,16]. The induced NC cells generate neurons, Schwann cells, and mesenchymal cells as derivatives. Further, the mesenchymal cells differentiate into adipocytes, chondrocytes, osteoblasts, and smooth muscle cells. From these findings, we speculate that iPS cell-derived NC cells can be suitable cell sources for tooth regeneration.

In this study, we established a culture protocol to induce the differentiation of mouse iPS cells into NC-like cells (NCLC), and demonstrated that iPS cell-derived NCLC can differentiate into DMC and odontoblasts upon stimulation by dental epithelial cells.

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Materials and Methods

Antibodies

Anti-nestin mouse monoclonal antibodies were purchased from Chemicon (Billerica, MA). Anti-$p75^{NTR}$ rabbit polyclonal antibodies, anti-Wnt-1 goat polyclonal antibodies, anti-Pax9 rat monoclonal antibodies, and anti-dentin sialoprotein (DSP) goat polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-STRO-1 mouse monoclonal antibodies were obtained from R&D Systems (Minneapolis, MN); and anti-AP2-α mouse monoclonal antibodies, from Cosmo Bio (Tokyo, Japan). Anti-HNK-1 mouse monoclonal antibodies came from Sigma-Aldrich (Tokyo, Japan). Anti-green fluorescent protein (GFP) monoclonal antibodies were a product of Cell Signaling Technology (Danvers, MA). Anti-Lhx6 rabbit polyclonal antibodies were purchased from Abgent (San Diego, CA); and anti-Msx1 rabbit polyclonal antibodies, from Lifespan Biosciences (Seattle, WA). Hoechst 33342, Alexa Fluor 488, and Alexa Fluor 546 secondary antibodies were procured from Invitrogen (Carlsbad, CA).

Cell culture and NC induction

Cells of the mouse iPS cell line iPS-MEF-Ng-20D-17 (RIKEN BRC, Tsukuba, Japan) [4] were cultured on mitotically inactivated mouse embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 15% fetal bovine serum, 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma-Aldrich), 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen), and 1,000 U/mL mouse leukemia inhibitory factor (Chemicon). The cultures were maintained at 37°C in a 5% CO_2 humidified incubator, and the medium was changed every other day.

NCLC were derived from mouse iPS cells by a previously described method [17] with modifications. In brief, iPS cells were dissociated with 0.25% trypsin/EDTA and transferred to nonadherent culture dishes (HydroCell, CellSeed, Tokyo, Japan) with neural induction medium for suspension culture to form neuroectodermal spheres. The neural induction medium consisted of a 1:1 ratio of DMEM/F12 (Invitrogen) and neurobasal medium (Invitrogen) supplemented with 0.5× N_2 (Invitrogen), 0.5× B27 (Invitrogen), 5 μg/mL insulin (Sigma-Aldrich), 20 ng/mL basic fibroblast growth factor (bFGF) (R&D Systems), 20 ng/mL epidermal growth factor (R&D Systems), 50 U/mL penicillin, and 50 μg/mL streptomycin. After 4 days in suspension culture, the spheres were transferred to fibronectin-coated culture dishes (BD Biosciences, San Jose, CA). After 8–10 days, the spheres or rosettes of NCLC were manually removed, and the resultant NCLC were dissociated with 0.25% trypsin/EDTA, and plated on fibronectin-coated culture dishes. The isolation of HNK-1-positive cells was carried out with a magnetically-activated cell sorting separator (Miltenyi Biotec, Bergisch-Gladbach, Germany) using magnetic microbeads (Miltenyi Biotec) coupled to anti-HNK-1 mouse monoclonal antibody.

Immunofluorescence

Immunofluorescence was performed according to our previously described method [18]. In brief, the cultured cells were fixed with 4% paraformaldehyde, rendered permeable in 0.1% Triton X-100 (Sigma-Aldrich), and blocked with 5% serum. The cells were then incubated with primary antibodies for 1 h at room temperature. Alexa Fluor 488- and Alexa Fluor 546-conjugated secondary antibodies were used for detection of the target. Secondary antibodies were added to frozen sections that had been incubated with primary antibodies overnight at room temperature. Fluorescent images were obtained by using a fluorescence microscope (IX71; Olympus, Tokyo, Japan). Images were analyzed with software supplied with the confocal microscope or standard image analysis software (Metamorph; Universal Imaging Corporation, Downingtown, PA). All imaging data were obtained from experiments replicated at least 4 times.

Flow cytometry

The cells were detached with 0.25% trypsin/EDTA. For cellular staining, ~$0.5–1 \times 10^6$ cells were incubated with the primary antibody on ice for 30 min. Phycoerythrin-conjugated secondary antibody was then added. Cells were analyzed with a flow cytometer (EPICS XL ADC; Beckman Coulter, Tokyo, Japan) and the supplied software. Experiments were performed in triplicate.

Real-time reverse transcription-polymerase chain reaction

Real-time reverse transcription (RT)-polymerase chain reaction (PCR) was performed according to our previously described method [18]. In brief, total RNA was isolated by using an RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. Reverse transcription of total RNA was performed by use of a PrimeScript RT reagent kit (Takara Bio, Siga, Japan). cDNA amplification was performed by real-time RT-PCR using SYBR® Premix Ex Taq™ (Takara Bio) with a Thermal Cycler Dice (Takara Bio) according to the manufacturer's protocol. Primer sequences for each cDNA were as follows: for *GAPDH*, 5′-GTC TCC TCT GACTTC AAC A-3′ (forward) and 5′-CAG GAA ATG AGC TTG ACA AA-3′ (reverse); for *Pax3*, 5′-AAC AAG CTG GAG CCA ATC AAC TG-3′ (forward) and 5′-CTG AGG TCT GTG GAC GGT GCT A-3′ (reverse); for *Snail*, 5′-TCT GAA GAT GCA CAT CCG AAG C-3′ (forward) and 5′-TTG CAGTGG GAG CAG GAG AAT-3′ (reverse); for *Slug*, 5′-GGC TGC TTCAAG GAC ACA TTA GAA C-3′ (forward) and 5′-GGT CTG CAG ATGTGC CCT CA-3′ (reverse); for *Foxd3*, 5′-CGG CGC CTG TGT TCT CAG TA-3′ (forward) and 5′-CTG CGC AGA GTG AAC CTT CAA A-3′ (reverse); for *DSPP*, 5′-GAG CAA CAC GGA TGG ATG ATT TC-3′ (forward) and 5′-CAC TCT TGT CAC GCA CAG CCT TA-3′ (reverse); and for *DMP1*, 5′-GAT GTC AGA GCA CAC ATG GTG AGA-3′ (forward) and 5′-CAG GTT GGT GAA CCA GAG CAT C-3′ (reverse). Target gene expression levels were normalized to those of GAPDH. Further, relative gene expression levels were calculated relative to levels in control cultures by using the comparative Ct ($2^{-\Delta\Delta Ct}$) method. Experiments were performed in triplicate.

Teratoma formation

Teratoma formation was assessed according to our previously described method [19]. In brief, iPS cells (1×10^6 cells) were mixed with 500 μL of Cellmatrix type I-A (Nitta Gelatin, Osaka, Japan). The mixture was subcutaneously injected

into the lower flank of age-matched male KSN/Slc nude mice (Japan SLC, Shizuoka, Japan) or SCID-CB17 mice (CLEA Japan, Tokyo, Japan). After 4 weeks, Angiosense 750 (Olympus) was injected via a tail vein to highlight perfused vessels within the teratoma. One day after the injection, the fluorescence intensities and images were obtained by using an IVIS imaging system (Xenogen, Alameda, CA). The teratomas were then surgically extracted, fixed in 4% paraformaldehyde, decalcified, dehydrated through an ethanol series, embedded in paraffin, and serially sectioned at a 6-μm thickness. Finally the sections were placed on slides and then stained with hematoxylin and eosin.

All experiments were performed in accordance with the Protocols for the Humane Treatment of Animals of Iwate Medical University.

Tissue recombination

Dental epithelium was dissected from the lower incisors of transgenic mice expressing GFP. The apical ends of the incisors were removed and incubated for 10 min at 37°C in a solution of 1% Dispase (Roche, Tokyo, Japan) in PBS (−) containing 20 U/mL DNase I (Invitrogen). After incubation, the epithelium was mechanically separated by using fine forceps and needles. The absence of mesenchymal cell contamination was confirmed by visual inspection under a stereomicroscope.

After NCLC had been detached by using 0.25% trypsin/EDTA, they were centrifuged. The cell pellet (0.3–0.5 μL) was seeded in a drop of 30 μL of polymerized Cellmatrix type I-A on transplant filters (0.1-μm pore size; Millipore, Billerica, MA). A piece of dental epithelium was then placed over the gel, and the recombinant explants were cultured by the Trowell technique [20]. After 2 weeks in culture, the explants were sequentially fixed with 4% paraformaldehyde, immersed in a series of graded sucrose solutions, embedded in Tissue-Tek OCT (Sakura-Finetek USA, Torrance, CA), and frozen.

Conditioned medium of dental epithelial cell cultures

For preparation of medium conditioned by dental epithelial cells, we used the dental epithelial cell line HAT-7 [21]. The cells were maintained in DMEM/F12 medium containing 10% fetal bovine serum, 50 U/mL penicillin, and 50 μg/mL streptomycin. The culture medium was replaced every 2 days until the cells had reached 80%–90% confluence. The supernatant was filtered and stored at −80°C before use.

Statistics

All data were reported as the means ± SD. Statistical significance was assessed by using the 2-tailed Student's *t*-test for 2 groups or analysis of variance (Tukey's test) for >2 groups. $P < 0.05$ was considered as statistically significant.

Results

Derivation of NCLC from mouse iPS cells

Since DMC are generated from cranial NC cells [22], we hypothesized that the induction of iPS cells to differentiate into NCLC could be an effective method to obtain DMC. Previous studies have shown that human ES cell (hESC)-derived neural rosettes can differentiate into NC cells [16,17]. Here we modified the differentiation protocol for mouse iPS cells. Mouse iPS cells were cultured in suspension for 4 days to form neural spheres (Fig. 1A). The neural spheres were then transferred to fibronectin-coated dishes, and they spontaneously attached to the dishes within 1 day (Fig. 1B). The spheres formed rosette-like structures and gave rise to migratory cells with a stellate morphology (Fig. 1B, C). The rosettes were then mechanically removed, and the remaining migratory cells formed a uniform cell population. These migratory cells were passaged and maintained on fibronectin-coated dishes for subsequent experiments (Fig. 1D). Expression of NC marker protein in the derived cells was assessed by immunofluorescence and flow cytometry. The immunofluorescence study showed that >90% of the cells were positive for nestin (91.9 ± 4.1, $n = 4$), AP2-α (90.2 ± 3.4, $n = 4$) $p75^{NTR}$ (90.9 ± 2.4, $n = 4$), and Wnt-1 (94.5 ± 4.8, $n = 3$), which are NC lineage-specific markers (Fig. 2A). They were also partially positive for the mesenchymal stem cell marker STRO-1 (62.5 ± 2.8, $n = 4$). In contrast, Nanog-GFP, which is expressed in undifferentiated iPS cells (data not shown), was not detected in the derived cells (Fig. 2A). Flow cytometry demonstrated that the number of HNK-1 (marker of migrating NC cells)- positive cells among the derived cells was increased compared with that among the undifferentiated iPS cells (Fig. 2B). Real-time RT-PCR further confirmed that mRNA expression of NC-specific transcriptional factors, such as Pax3, Snail, and Slug, was markedly higher in derived cells than in undifferentiated iPS cells (Fig. 2C); whereas Foxd3 was not detected (data not shown). On the

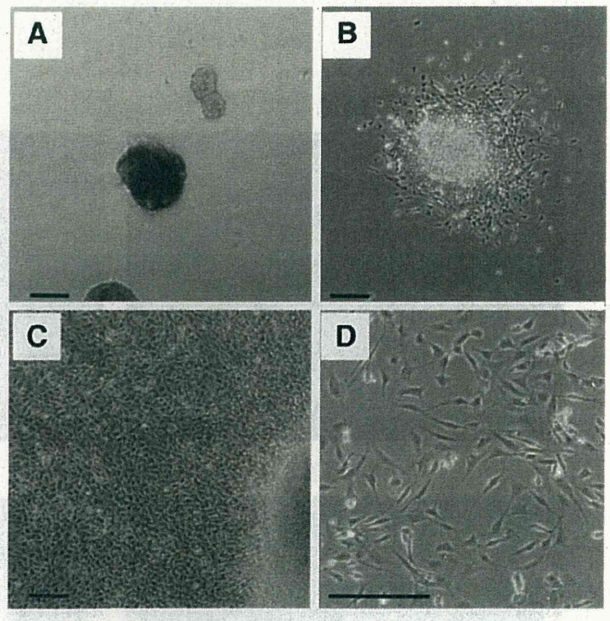

FIG. 1. Derivation of NCLC from mouse iPS cells. **(A)** iPS cells were differentiated in suspension to form neural spheres. **(B)** The spheres spontaneously attached and formed rosette-like structures. **(C)** The cells migrated away from the rosette. **(D)** The migratory cells formed a uniform population. Scale bar = 200 μm. iPS, induced pluripotent stem; NCLC, neural crest-like cells.

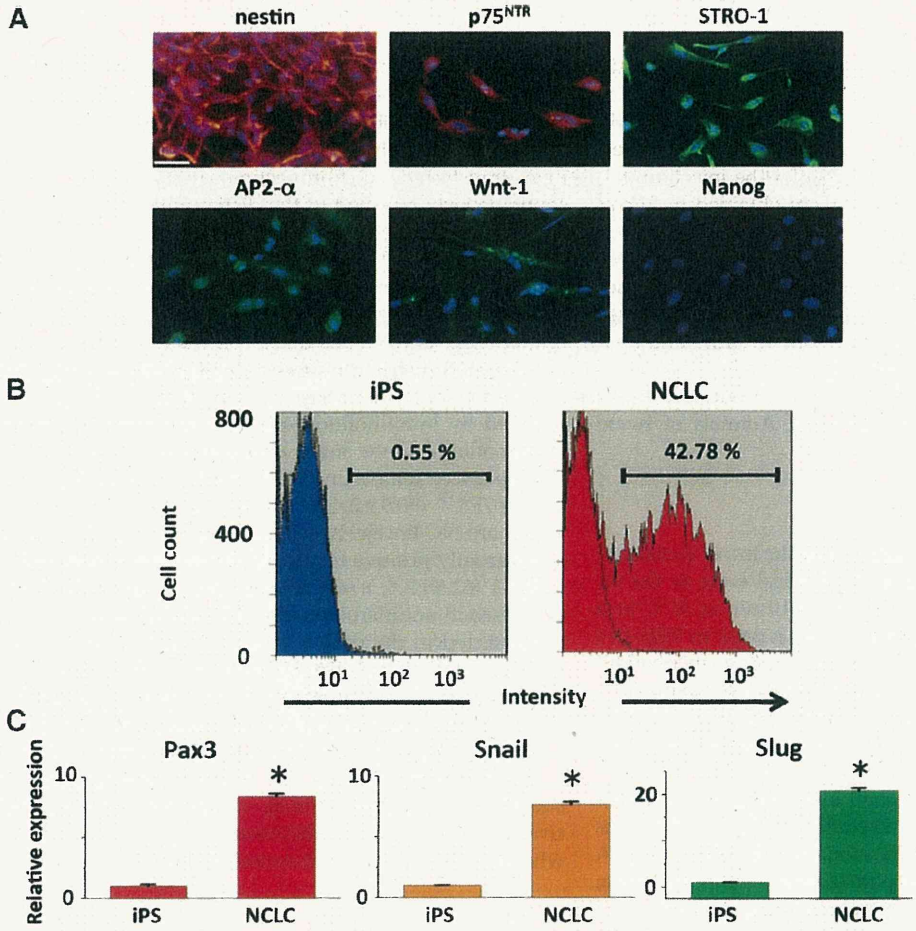

FIG. 2. Characterization of NCLC induced from iPS cells. **(A)** NCLC were immunostained with the indicated antibodies. Nanog-GFP was not detected in NCLC. Nuclei are shown in *blue*. Scale bar = 50 μm. **(B)** Representative flow cytometric analysis for HNK-1-positive cells among undifferentiated iPS and NCLC. **(C)** Pax3, Snail, and Slug mRNA expression in undifferentiated iPS and NCLC. GFP, green fluorescent protein. $^{*}P < .05$, significantly different from iPS. Color images available online at www.liebertonline.com/scd

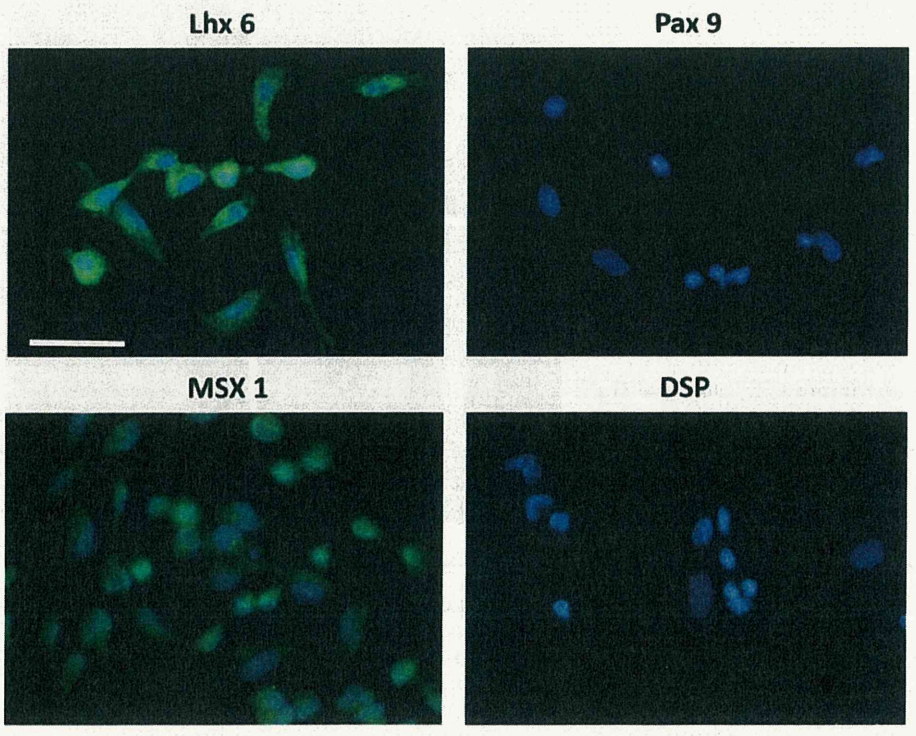

FIG. 3. Expression of DMC and odontoblast marker proteins in NCLC. NCLC were immunostained with the indicated antibodies. Nuclei were stained with Hoechst 33342 (*blue*). Scale bar = 50 μm. DMC, dental mesenchymal cells. Color images available online at www.liebertonline.com/scd