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

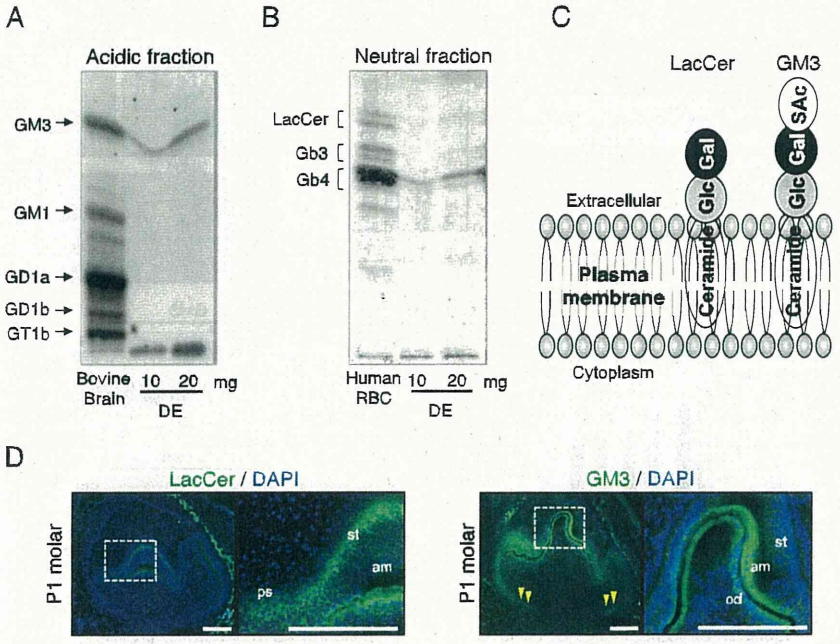

Figure 1. Detection of GSLs in dental epithelial cells, and expressions of GM3, LacCer, and Gb4 in developing mouse molars. TLC findings for **(A)** acidic and **(B)** neutral lipids in 10 and 20 mg of dental epithelial cell pellets. We prepared a standard mixture of neutral glycolipids for TLC, while 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.

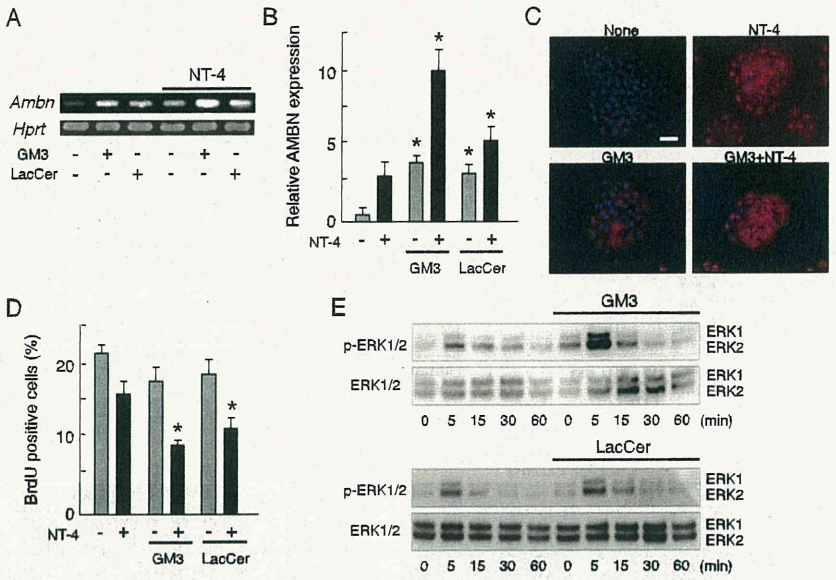

Figure 2. Biological roles of GM3 and LacCer in dental epithelial cells. **(A)** RT-PCR analysis of *Ambn* expression. NT-4 induced *Ambn* expression. Pre-treatment for 1 hr with either GM3 or LacCer in dental epithelial cells induced *Ambn* expression, which was synergistically enhanced by pre-treatments 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

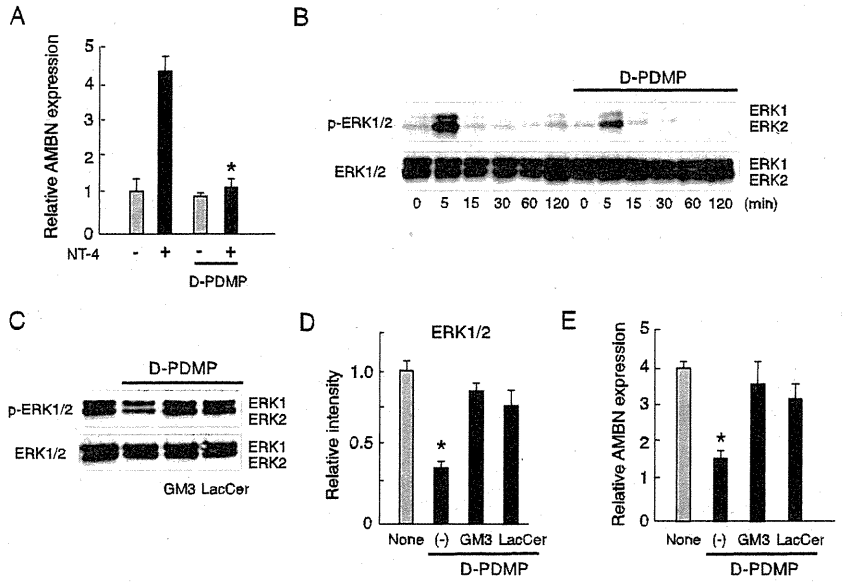

Figure 3. D-PDMP blocks NT-4-mediated action in dental epithelial cells, and exogenous administration of GM3 and LacCer restores NT-4-mediated ERK signaling attenuated by depletion of GSLs. **(A)** Real-time PCR analysis of NT-4-mediated *Ambn* expression with or without D-PDMP pre-treatment. D-PDMP 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, $\sim 0.5–1 \times 10^6$ 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

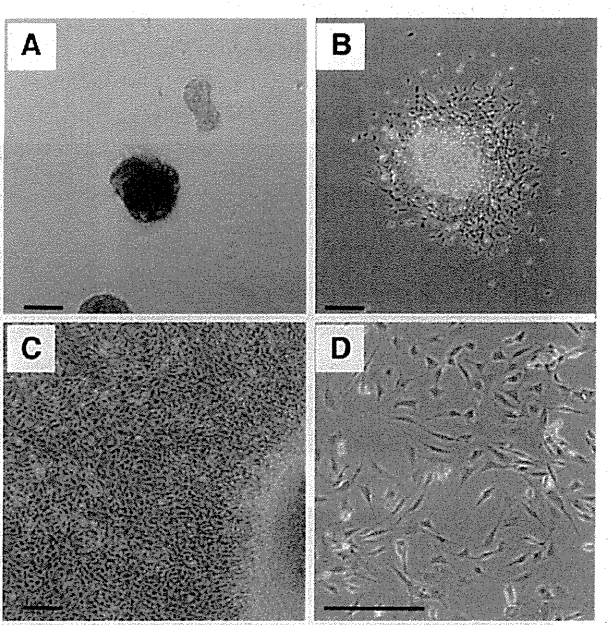

FIG. 1. Derivation of NCLC from mouse iPS cells. **(A)** iPS cells were differentiated in suspension to form neural spheres. **(B)** The spheres spontaneously attached and formed rosette-like structures. **(C)** The cells migrated away from the rosette. **(D)** The migratory cells formed a uniform population.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

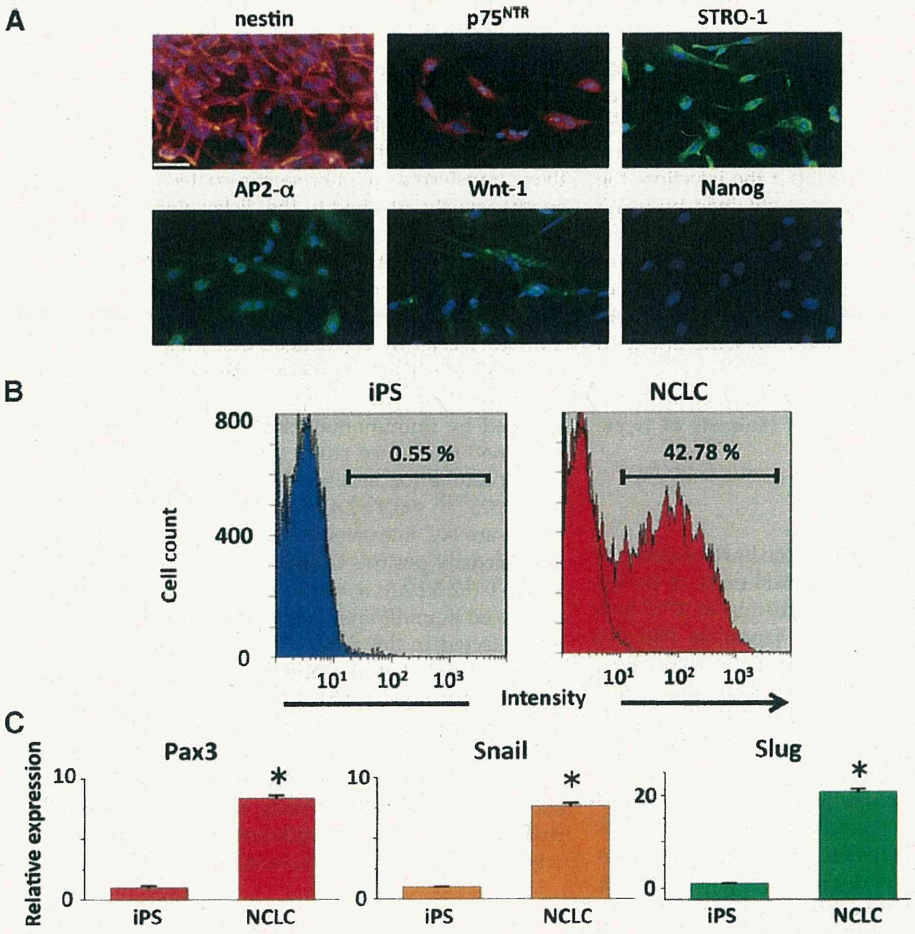

FIG. 2. Characterization of NCLC induced from iPS cells. **(A)** NCLC were immunostained with the indicated antibodies. Nanog-GFP was not detected in NCLC. Nuclei are shown in *blue*. Scale bar = 50 μm. **(B)** Representative flow cytometric analysis for HNK-1-positive cells among undifferentiated iPS 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

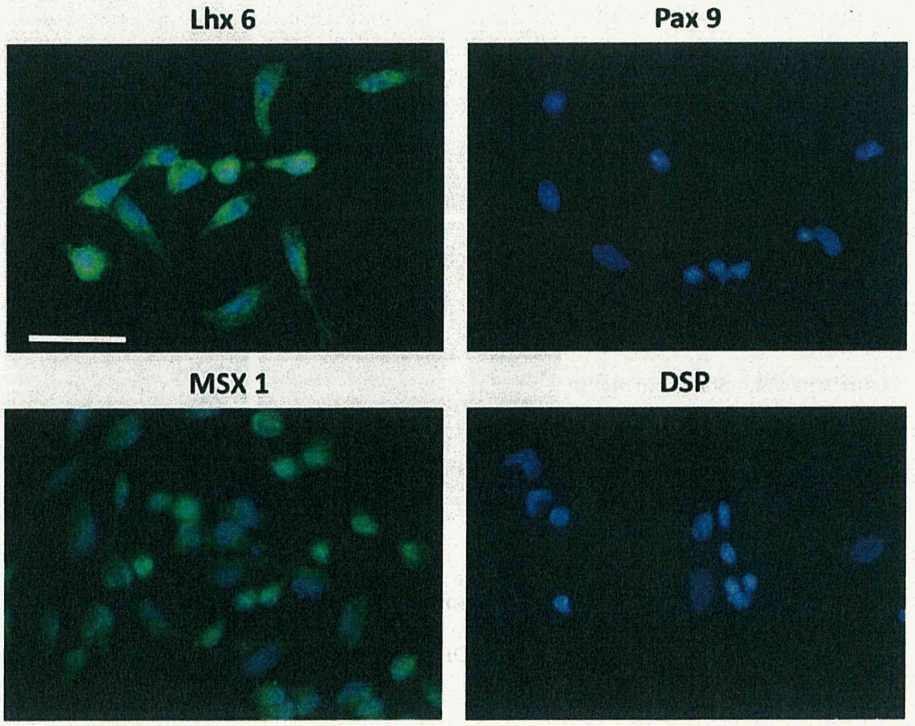

FIG. 3. Expression of DMC and odontoblast marker proteins in NCLC. NCLC were immunostained with the indicated antibodies. Nuclei were stained with Hoechst 33342 (*blue*). Scale bar = 50 μm. DMC, dental mesenchymal cells. Color images available online at www.liebertonline.com/scd

basis of these observations, we termed these derived cells mouse iPS cell-derived NCLC. Further, we examined the expression of DMC markers Lhx6, Msx1, and Pax9, and the odontoblast marker DSP, [13,14,23,24], in the NCLC by immunofluorescence. Although Lhx6, Msx1, and Pax9 are expressed in cells other than dental mesenchyme, the combined expression of these genes is specific to DMC [25]. Lhx6 and Msx1 were detected in the NCLC, whereas Pax 9 and DSP were not (Fig. 3).

Further, to evaluate their tumorigenicity we subcutaneously injected NCLC into immunodeficient mice. After 4 weeks, these transplanted NCLC did not form visible teratomas (Fig. 4A), as expected from the absence of Nanog expression; whereas parental undifferentiated iPS formed teratomas including those containing various types of tissues such as bone, cartilage, epidermis, muscle, adipose tissue, and gut-like epithelium (Fig. 4A, B). To further confirm that the NCLC did not form tumors, we performed in vivo imaging to assess tumor angiogenesis. The mice injected with undifferentiated iPS cells showed significant fluorescence of the blood pool-imaging agent at the teratoma site, whereas the NCLC-injected ones showed no imaging signals, indicating the absence of a tumor.

Together, these results suggest that we could obtain non-tumorigenic NCLC efficiently from mouse iPS cells by using this protocol.

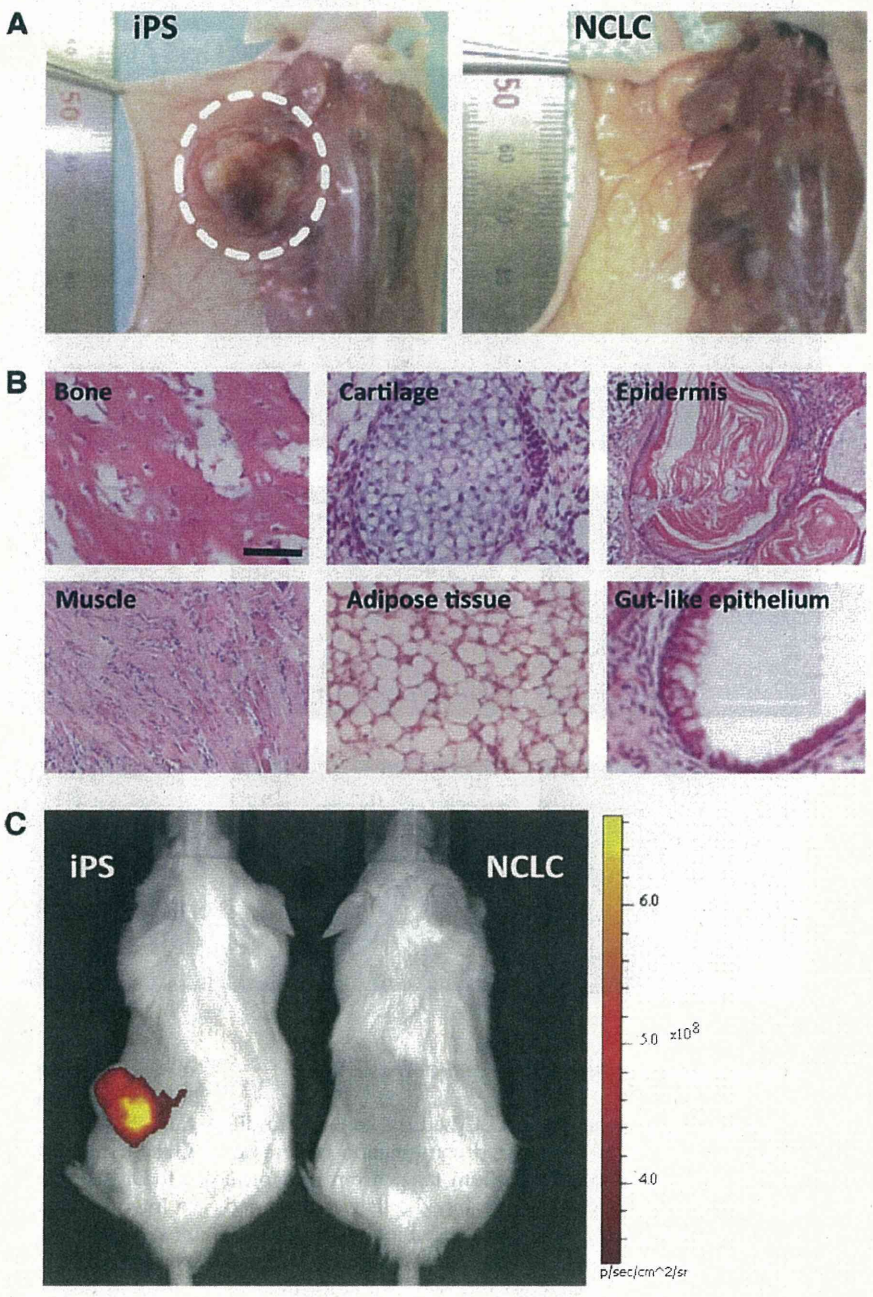

FIG. 4. Teratoma formation of NCLC. **(A)** Tumor monitoring 4 weeks after the injection of NCLC (*right*) into nude mice compared with that after injection of undifferentiated iPS cells (*left*). The *white-dotted circle* indicates the teratoma. **(B)** H-E staining of the teratoma derived from undifferentiated iPS cells. Scale bar = 50 μm. **(C)** Four weeks after mice had been subcutaneously injected with NCLC or undifferentiated iPS cells, Angiosence 750 was injected via a tail vein. A representative mouse injected with undifferentiated iPS cells (*left*) shows significant fluorescence at the teratoma site, whereas the NCLC-injected mouse (*right*) shows no imaging signals. H-E, hematoxylin and eosin. Color images available online at www.liebertonline.com/scd

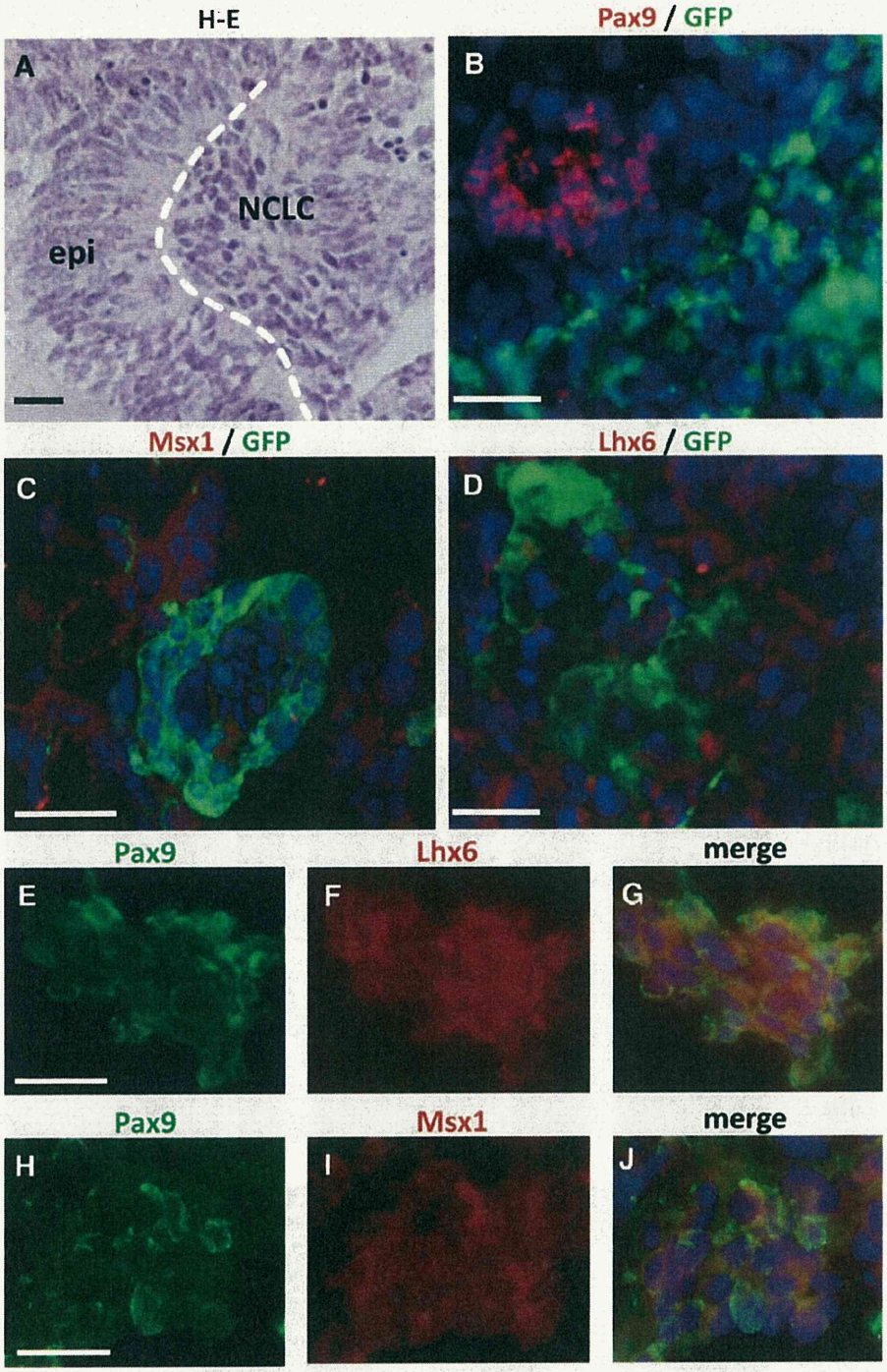

FIG. 5. Combination cultures of NCLC and dental epithelium. **(A)** The image shows an H-E stained 2-week culture. The *white-dotted line* indicates the border between NCLC and dental epithelium (epi). **(B–D)** The cultures were double-stained with the indicated antibodies. GFP-expressing dental epithelium did not express DMC markers. **(E–J)** Pax9 was co-localized with Lhx6 **(E–G)** or Msx1 **(H–J)**. Nuclei are shown in *blue*. Scale bar = 20 μm. Color images available online at www.liebertonline.com/scd

Odontogenic response of iPS cell-derived NCLC

To determine whether the NCLC had the capacity to differentiate into DMC, we prepared combination cultures of NCLC and mouse dental epithelium taken from the apical ends of the lower incisors. The combined cells were cultured in vitro and analyzed for the expression of molecular markers of tooth development. After 2 weeks in culture, NCLC aggregation adjacent to the dental epithelium was observed (Fig. 5A). Pax9, Msx1, and Lhx6 were expressed in the NCLC but not in the GFP-expressing dental epithelium (Fig. 5B–D), and Pax9 was co-expressed with Msx1 and Lhx6 (Fig. 5E–J), suggesting the differentiation of NCLC into DMC.

Interestingly, some NCLC also expressed the odontoblast marker DSP (Fig. 6A). In contrast, no marker expression was observed in combination cultures of undifferentiated iPS cells and dental epithelium (data not shown). Additionally,

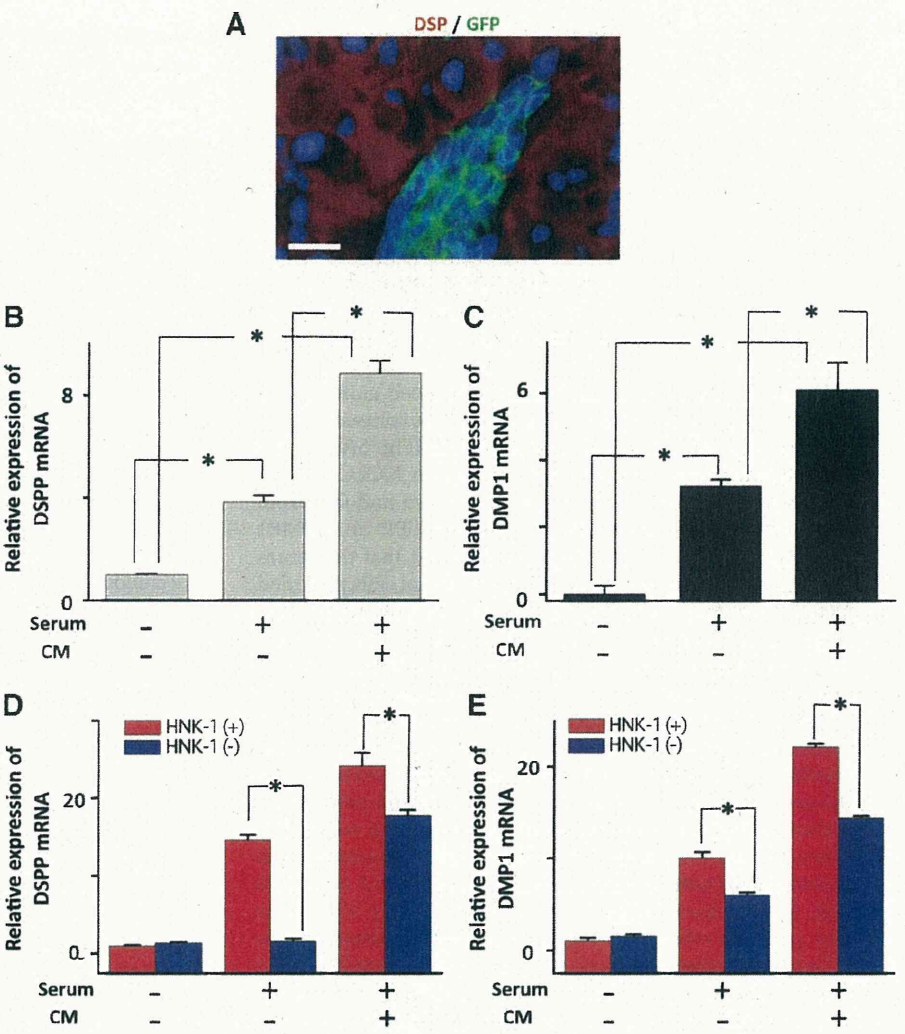

FIG. 6. Odontogenic response of NCLC to dental epithelium. **(A)** Cultures of NCLC combined with GFP-expressing dental epithelium were double-stained with DSP and GFP antibodies. Dental epithelium did not express DSP. Nuclei are shown in *blue*. Scale bar = 10 μm. **(B** and **C)** DSPP and DMP1 mRNA expression of NCLC after 2 weeks of culture with serum or conditioned medium (CM) from cultures of dental epithelial cells. **(D** and **E)** DSPP and DMP1 mRNA expression of HNK-1 positive (+) and HNK-1 negative (−) cells isolated from an NCLC population and cultured for 2 weeks with serum or CM from dental epithelial cell cultures. *Asterisks* indicate significant differences ($^*P < 0.05$) between values indicated by the brackets. DSP, dentin sialoprotein; DSPP, dentin sialophosphoprotein; DMP1, dentin morphological protein 1. Color images available online at www.liebertonline.com/scd

when NCLC were cultured in the presence of serum, dentin sialophosphoprotein (DSPP) and dentin morphological protein 1 (DMP1) mRNA expression increased; and this increase was further enhanced with the conditioned medium (CM) of dental epithelial cell cultures (Fig. 6B, C). Moreover, the HNK-1-positive cells isolated from the NCLC population presented higher expression of DSPP and DMP1 than the HNK-1-negative cells when cultured with serum or with CM from dental epithelial cell cultures (Fig. 6D, E). Taken together, these results indicate that iPS cell-derived NCLC, especially the HNK-1 positive cells, had the potential to differentiate into odontoblast progenitor cells upon stimulation with dental epithelium.

Discussion

In this study, we succeeded in inducing the differentiation of mouse iPS cells into NCLC in vitro, and demonstrated for the first time that NCLC could further differentiate into odontogenic mesenchymal cells, including odontoblasts progenitor cells. We modified a culture protocol for the differentiation of hESCs [17] into NC cells, and showed that it was suitable for use with mouse iPS cells, though the species (murine and primate) and cell types (iPS and ES) were different. With regard to species, the signaling pathways that regulate primate ES cell differentiation are similar to those operating in murine ES cells [25]. Therefore, this confirms the success of transferring differentiation strategies from the primate to the murine system. In fact, the method for inducing differentiation of murine ES cells into neural cells functions with primate ES cells as well [26,27]. Concerning cell types, iPS cells are virtually equivalent to ES cells in terms of pluripotency, ES cell marker expression, and teratoma formation in vivo [4–6]. Previous reports demonstrated that iPS cells differentiate into various cell types, including NC cells, under the same culture conditions as used for ES cells [7–9]. However, some reports documented that the differentiation capability of iPS cells is lower than that of ES cells [8,9]. Hence, further improvements are needed for enhancing iPS cell differentiation capacity.

Our iPS cell-derived NCLC expressed NC markers such as nestin, $p75^{NTR}$, AP2-α, and Wnt-1 (Fig. 2). However, interestingly, Foxd3 was not expressed. In Mundell's report, Foxd3 is downregulated in cranial NC mesenchyme, and Foxd3-null cranial NC cells show accelerated differentiation into mesenchymal cells [28]. Using our differentiation

protocol, we found that NCLC expressed the mesenchymal stem cell markers STRO-1, Lhx6, and Msx1, which are expressed in dental mesenchyme. Hence, NCLC may have preferentially differentiated into mesenchymal cells. In addition to Lhx6 and Msx1 expression, NCLC expressed Pax9 (Fig. 5), when cultured with dental epithelium. This result is consistent with Ohazama's report showing that ES cells, neural stem cells, and adult bone marrow-derived cells express Lhx6, Msx1, and Pax9 when cultured with embryonic oral epithelium [29]. Thus, similar to ES and other stem cells, our iPS cell-derived NCLC showed the capacity to differentiate into dental mesenchyme upon stimulation with dental epithelium.

We further showed that NCLC in combined culture with dental epithelium differentiated into DSP-expressing cells, indicating differentiation into odontoblasts (Fig. 6A). In this experiment, we detected DSP expression in NCLC around the dental epithelium. In addition, the serum and CM from dental epithelial cell cultures enhanced DSPP and DMP1 expression (Fig. 6B–E). These results suggest that the serum and soluble components from dental epithelial cells provided NCLC with an environment suitable for odontoblast differentiation. Since NCLC derived from hESCs differentiate into several mesenchymal lineages in the presence of serum [15], our iPS cells-derived NCLC may have been induced to differentiate into DMC lineages in the presence of serum and further to differentiate into odontoblasts in the presence of soluble components of the dental epithelium. Medium conditioned by tooth germs or ameloblasts has the potential to induce stem cells to differentiate into odontogenic cells [1,30]. Thus, CM, including ours, may contain important signal molecules for tooth development, such as Notch-1 and FGFs [20]. Further investigation is needed to identify the factors that promote effective odontoblast induction. Since the detailed mechanism of the spatio-temporal regulation of odontoblast differentiation is still unclear, our culture protocol will be most useful for studying odontoblast differentiation. In addition to odontoblasts, dental pulp cells and dental follicle cells are also believed to arise from DMC during tooth germ development. Although not addressed here, it would be interesting to know whether NCLC have the capability to differentiate into those 2 cell types.

Recent advances in tissue engineering techniques indicate that the bioengineering approach may be successful for the regeneration of dental tissue. The main concept of tooth regeneration is to mimic the process of natural tooth development, either in vitro or in vivo. By taking advantage of reciprocal epithelial–mesenchymal interactions, numerous studies have shown that dental epithelial and mesenchymal cells in fetal tooth germs can form bioengineered teeth [31–33]. However, regarding clinical applications, the use of fetal tissue and/or cells gives rise to the same ethical issues as those encountered with ES cells. Therefore, iPS cell-derived DMC generated in this study have the potential to overcome the problems related to cell sources for stem cell-based tooth regeneration and treatment of tooth-related diseases.

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Author Disclosure Statement

No competing financial interests exist.

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