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Application of spontaneously immortalized odontoblast cells in tooth regeneration

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ABSTRACT

Here, we report on the first attempt to bioengineer tooth using a spontaneously immortalized mesenchymal cell line. To assess the odontogenic potential of this cell line, odontoblast-lineage cells (OLC) were re-associated with competent dental epithelium isolated from E14.5 mice. A novel three-dimensional organ germ culture method was applied to nurture the constructs in vitro. Additionally, recombinants were transplanted under the kidney capsule in host animals for 2 weeks. Transplants developed into tooth tissues in one-third of the cases. OLC-derived GFP-positive cells could be identified in mineralizing tooth germs by immunohistochemistry. OLCs were capable of intercellular and cell-matrix communication, thus they eventually differentiated into functional odontoblasts. In summary, we managed to utilize OLCs for dental mesenchyme substitution in tooth regeneration experiments. Therefore, our spontaneously transformed cell line proved its potential for future complex, tooth developmental and bioengineering studies.

Introduction

Recombination experiments have been widely used in the last decades for tooth engineering purposes. The ultimate objective, a successful tooth regeneration method for biological replacement for lost tooth, has been a long-awaited desire of researchers, as well as, clinicians.

Re-associations form a significant segment of recombinations, where intact dental tissues [1–3] or dissociated cells [4–6] are reconstructed ex vivo. Recombinations can be grouped into homologous [7,8] or heterogonous experiments [9–11]. Recently, along with dental cells [12,13] a variety of non-dental cells has been applied in tooth primordial reconstitutions. The emergence of stem cells (neural stem cells, dental pulp stem cells, bone marrow stromal cells, etc.) in dental tissue engineering [14–16], provides promising expansion of our knowledge about organ developmental mechanism.

Since odontogenesis defined as perpetual interactions [17–19] between the dental epithelium and the ectomesenchymal dental papilla, these components seem to be prerequisite in recombinants. Epithelial–mesenchymal communication eventually triggers cell differentiation and apposition of extracellular matrix. Through recombinant experiments, researchers have gained substantial understanding of the signaling cascade during tooth development; however, to date, the complete description of the matricellular myriad is still out of reach.

A novel, three-dimensional approach [20] provides a unique potential for in vitro, as well as, in vivo recombination studies. A pioneer study [21] reported on successful re-associations between the dental mesenchyme and cultured, clonal, epithelial cell lines. Although, frequent failures of tooth regeneration, indicated certain limitations of this technique. As a consecutive task, in the present study we attempted to investigate the possibility of creating the inverse alternatives of those recombinants. Namely, we aimed tooth regeneration by recombinations of an immortalized, dental mesenchymal cell line with intact dental epithelium. The shortage of human embryonic tissues and the lack of well-established dental stem cell retrieval procedures have led to the introduction of murine immortalized cell lines [22–24]. Employing established cell line sources grants proper consistency, reliability, and reproducibility, which are unequivocally necessary to transform research data into practical organ regeneration strategies. Furthermore, to circumvent the limitations of restorative autologous tissue grafting, future elaborations of tissue grafts developed by progenitor cells are unavoidable [25].

This study utilized a transformed, continuous cell line developed previously in our laboratory [26]. Because OLC cell line fosters a full-ranged odontogenic phenotype in contrast to other existing cell lines [23,27], OLCs represent remarkably useful research materials. The authors believe that, to date, this is the first study to introduce a neural-crest-derived transformed odontoblast-lineage cell line into organogenesis experiments.

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Materials and methods

Animals. ICR mice purchased from CLEA Japan Inc. (Tokyo, Japan) were treated by the guidelines of the Animal Committee at Akita University School of Medicine. The Animal Research Committee previously approved the protocols, and all subsequent animal surgical experiments were adhered to the "Regulation for Animal Experimentation".

OLC cell culture. OLCs were maintained as detailed in our previous report [23]. Brief descriptions of their origin and culturing conditions are described in the followings. Dental papilla cells were isolated from embryonic day 18.5 C57BL/6-TgN (act-EGFP) mouse embryos, and fibroblastic-like outgrowths were cultured as defined by Hanks et al. [23]. Single cell-derived colony cells were routinely transferred over 150 times during the last three years. Those subcultures were periodically monitored and confirmed for odontoblastic features and odontogenic gene expression (e.g., DSPP, DMP-1, MEPE).

OLCs were cultured in minimum essential medium with alpha modification (α-MEM) (Sigma, USA), supplemented with 15% (v/v) fetal bovine serum (Sigma), gentamicin (50 μg/ml)–L-glutamine (2 mM) (Sigma) solution, and fibroblast growth factor (FGF)-2 (2 ng/ml) on type I collagen-coated 10 cm culture plates, at 37 °C in a humidified chamber of 5% (v/v) CO_2 in air.

Reconstitution of bioengineered tooth germs. Reconstitutions were conducted according to the recommendations of Nakao et al. [20]. First molar tooth primordia (Fig. 1A) were dissected from the mandibles of 14.5-day-old ICR mouse embryos. Epithelial and mesenchymal portions were dissociated using tungsten needles after enzymatic digestion with 1.2 U/ml dispase II (Roche, Germany) and 70 U/μl DNase I (Takara Bio Inc., Japan) for 20 min (Fig. 1B and C). OLC cultures were harvested by trypsinization, and collected by centrifugation in siliconized microtubes. Cell pellet concentrations were approximately 5×10^8/ml when cells were injected (0.5 μl) into the collagen drops. Cell-matrix type I-A (Nitta gelatin, Japan) drops containing the reconstituted tooth germs (Fig. 2A) were deposited on cell culture inserts (0.4 μm, Beckton Dickinson Labware, USA) then incubated at 37 °C overnight.

Three-dimensional in vitro organ culture. Organ cultures were supplemented with α-MEM medium containing 15% fetal bovine serum and gentamicin–L-glutamine solution, in the presence of 10 mM β-glycerophosphate plus 50 μg/ml ascorbic acid (Sigma, USA). The culture medium was changed in every 3 days. Recombinants were maintained in four experimental groups during in vitro, as well as, in vivo experiments: Group I, positive controls of E14.5 tooth germs; Group II, E14.5 dental epithelium re-associated with E14.5 dental mesenchyme; Group III, negative controls of OLCs or E14.5 dental epithelium, without recombination; and Group IV, E14.5 dental epithelium combined with OLCs.

In vivo transplantation procedures. ICR male mice, aged 8–10 weeks, were used as hosts for transplantations. Subrenal capsule (SRC) assay was performed as previously described [28]. Constructs from each experimental group were aseptically implanted beneath the renal capsule for 14 days.

Histological and immunohistochemical analysis. Implants and organ cultures were fixed in 4% paraformaldehyde, then decalcified in 4.5% EDTA, pH 7.4 (Wako, Japan), at 4 °C for 4–6 days. Specimens were embedded in optimal cutting temperature medium (Tissue-Tek OCT, Sakura-Finetek, USA). Ten micrometers frozen sections were prepared for subsequent histological analysis. Slides were stained with hematoxylin and eosin (HE), Goldner's trichrome [29], and 1% alizarin red S (Sigma).

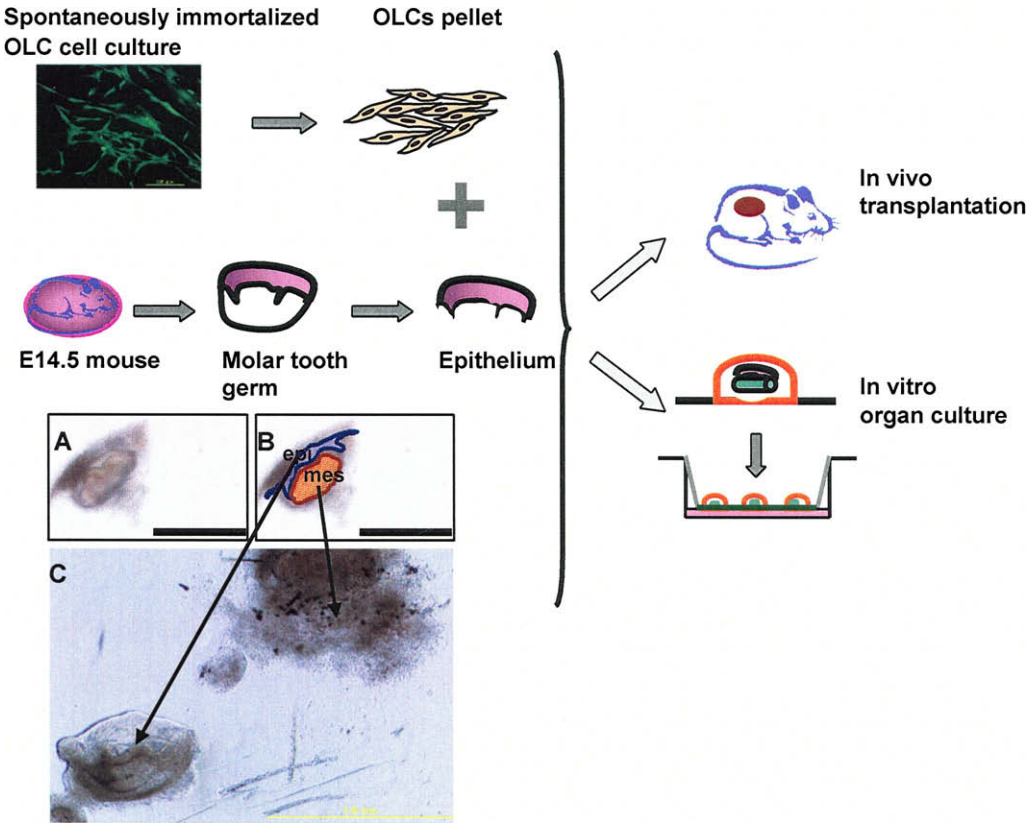

Fig. 1. Schematic illustration of recombinant tooth germ generation. The epithelial portion of E14.5 dental primordium associated with OLCs, then transplanted or cultured in collagen drops. (A) E14.5 cap-stage tooth germ. (B) Dental epithelium (blue) and dental mesenchyme (yellow) before separation, epi, epithelium; mes, mesenchyme. Scale bar, 1 mm. (C) Epithelial and mesenchymal components separated by enzyme treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

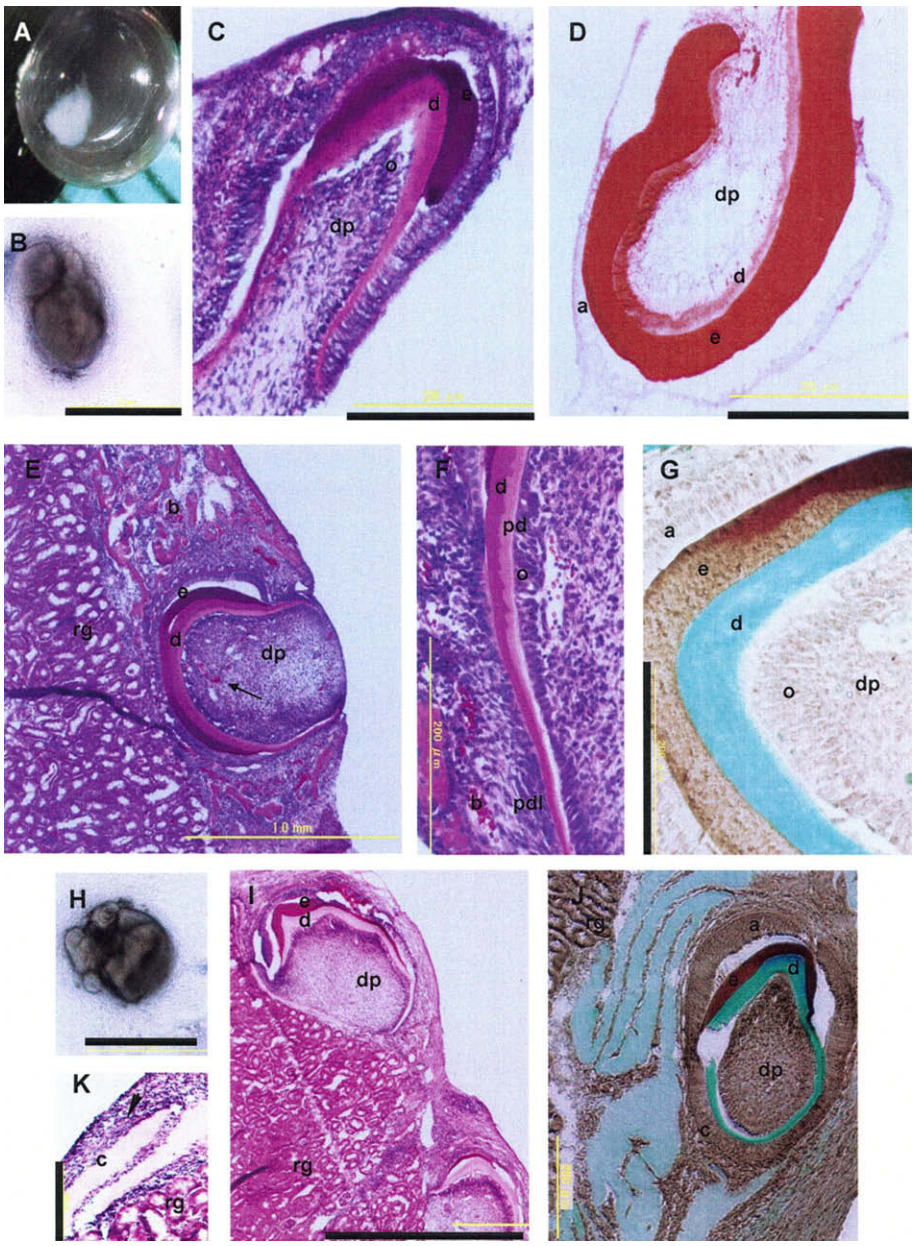

Fig. 2. Group I, II, and III controls in three-dimensional in vitro organ cultures and in vivo subrenal capsule assay. (A) E14.5 molar primordium in collagen matrix. Scale represents 1 mm. (B) Occlusal view of E14.5 molars after 14 days in vitro growing. Scale bar, 1 mm. (C) Histological section of an in vitro developed tooth germ, HE staining. Scale bar, 200 μm. (D) Biomineralization of in vitro cultured tooth, visualized by Alizarin red staining, scale bar, 200 μm. (E) Whole tooth regeneration after 2 weeks of transplantation into kidney. The arrow shows capillaries in the dental papilla. (F) Higher magnification shows regenerated periodontal ligaments and bone surrounding the root. (G) Histological aspects (Goldner's staining) of columnar ameloblasts and elongated, polarized odontoblasts in the thickening enamel–dentin complex. Scale bar, 200 μm. (H) Group II tooth germ after 14 days in collagen drop in vitro. Scale bar, 1 mm. (I) Renal implants of E14.5 epithelium and E14.5 mesenchyme recombination. Scale bar, 1 mm. HE, and (J) Goldner's trichrome staining. Scale bar, 200 μm. (K) Group III implant (OLCs marked by arrowhead) in subrenal transplant, HE staining, Scale bar, 200 μm. (a, ameloblast; b, bone; c, collagen; d, dentin; dp, dental papilla; e, enamel; epi, epithelium; mes, mesenchyme; o, odontoblast; pd, predentin; pdl, periodontal ligament; rg, renal glomeroli.)

For immunohistochemical analysis, samples were incubated at room temperature with primary polyclonal antibody against GFP (MBL, Japan), diluted 1:500. Then, samples were incubated with fluorescently labeled secondary antibody Alexa Fluor 546 (Molecular Probes, USA) anti-rabbit antibody, diluted 1:200. Labeled sections were observed using an Olympus IX70 fluorescence light microscope.

Results

Group I and Group II explant-derived regenerated tooth germs

The outcomes of different types of reconstitutions are summarized in Table 1. Tooth germ development resembling that of a natural tooth showed extracellular matrix calcification in the predentin and in enamel layers (Fig. 2C and D), which proved sufficient nutrition into the collagen drops from the culture media. In vivo, Group I implants revealed complete reconstitution of tooth primordium with attached periodontal ligaments, and surrounding bone formation (Fig. 2F). We also detected adequate blood supply by in situ vascularization into the dental papilla (Fig. 2E).

In Group II implants, we found 100% occurrence of regenerated tooth germs. E14.5 epithelium combined with E14.5 mesenchyme (Fig. 2H–J) represented a strategically important control, since in Group IV implants the dental papillae were substituted with OLCs. In Group III recombinants, enamel organs and OLCs (Fig. 2K) were

Table 1
Distribution of different types of implants used in recombination experiments.

Transplant type	Total number (in vitro)	Reconstituted tooth germ (in vitro)	Other ectopic tissues (in vitro)
Group I (E14.5 tooth germ)	10 (5)	10 (5)	– (–)
Group II (E14.5 epithelium + E14.5 mesenchyme)	10 (5)	10 (4)	– (1)
Group III (E14.5 epithelium/OLCs)	6 (5)	– (–)	6 (5)
Group IV (E14.5 epithelium + OLCs)	60 (10)	19 (4)	41 (6)

cultured without their counterpart of the epithelial–mesenchymal interface, which did not yield tooth development.

Bioengineered tooth structure derived from OLC cells

Three days after setting the constructs in vitro, the injected columnar volume of OLCs was still presented (Fig. 3A). Apparently, after 2 weeks, the OLC-mass shrank around the epithelium (Fig. 3B). Sections from Group IV in vitro and in vivo samples are illustrated in Fig. 3C–H. Using the Goldner's trichrome staining, the collagen-containing blue-colored dentin layers are clearly distinguished from the decalcified red-brownish enamel layers (Fig. 3D and G).

GFP-positive cells, detected in transplants by immunohistochemical investigation, evidenced the presence of OLCs in the bioengineered tooth germs (Fig. 3F and H). However, functional odontoblasts showed only a faint signal. Control slides of C57BL/6-TgN (act-EGFP) post-partum day-1 tooth germs also showed decreased GFP intensity of differentiated odontoblasts (data not shown).

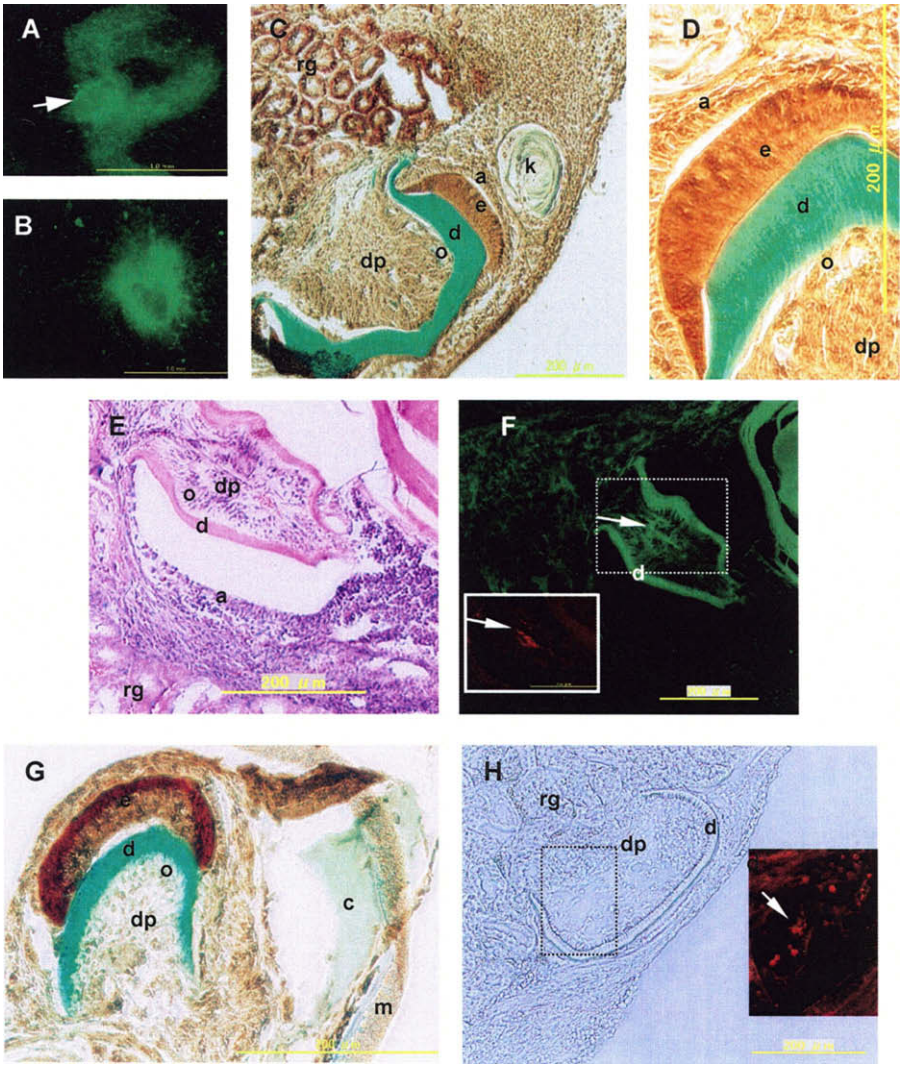

Fig. 3. Bioengineered tooth germs by OLCs associated with E14.5 dental epithelium (Group IV recombinations). (A) OLCs in three-dimensional organ culture after 3 days of cultivation (the arrow indicates the enamel organ). Scale bar, 1 mm, fluorescent image. (B) The same construct after 14 days of in vitro organ culture. Scale bar, 1 mm. (C) Kidney capsule-grafted Group IV reconstituted molar tooth germ by Goldner's staining. Magnification (D) shows the characteristic tubular structure of dentin with odontoblasts. Adjacent to dentin, the thickening enamel produced by ameloblasts is shown. (E) HE staining of Group IV tooth-like primordium in vivo, and it's (F) fluorescence photograph. Dentin, functional odontoblasts, and dental papilla cells show intensive fluorescence. The dotted box image recorded from an anti-GFP-treated section. (G) In vitro grown Group IV recombination (Goldner's staining). (H) Light microscopic image of Group IV recombination. The enlarged view of the dotted box signifies anti-GFP-positive dental papilla cells. (a, ameloblast; c, collagen; d, dentin; dp, dental papilla; e, enamel; epi, epithelium; k, keratin; mes, mesenchyme; o, odontoblast; pd, predentin; rg, renal glomeroli.)

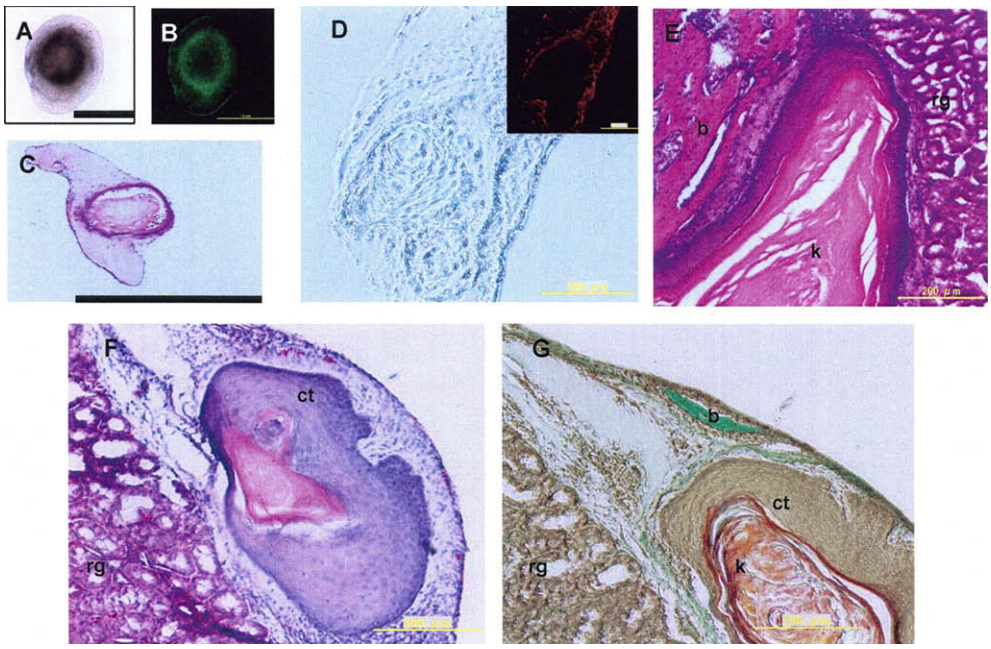

Fig. 4. Group IV recombinant implants with ectopic tissue formation. (A) Light microscopic and (B) fluorescence images of an in vitro explant. Scale bars, 1 mm. (C) HE staining of the same explant. (D) In vitro collagen drop without the sign of tooth development. Anti-GFP section shows GFP-positive OLCs around the central mass. Scale bar, 200 μm. (E) Group IV in vivo recombination grown for 2 weeks in kidney. The implant developed keratin layers and bone, HE staining. (F) Numerous hematoxylin-stained chondroblast nuclei are shown. (G) Atypical tissue formation visualized by Goldner's trichrome technique. (b, bone; ct, cartilagenous tissue; k, keratin; rg, renal glomeroli.)

In two-thirds of Group IV recombinants, however, we observed irregular tissue formation. A globular compound of OLCs, expressing GFP-positive areas, was the most common finding in vitro (Fig. 4A–C). Samples from in vivo experiments showed keratin layers (Fig. 4E), surrounded by cartilaginous-like regions with round-shaped chondroblast-like cells (Fig. 4F and G).

Discussion

In vitro experiments in transparent collagen drops offer a unique model of organ cultures; hence, surveillance is feasible and allows direct, consistent monitoring. Collagen also represents a sufficient biodegradable scaffold for transplantation procedures with easy handling and excellent permeability. We would like to emphasize that the soft-type matrix supports cell motility, which allows directional cell positioning during cell arrangement.

Our 100% efficiency in Group I and Group II in vivo recombinations confirmed the results published previously [21] with a slight difference, that we not detected massive, ectopic bone formation. Group III investigations proved that solely epithelial or mesenchymal parts of the developing tooth germ could not be used to regenerate tooth primordial. Therefore, we concluded that OLCs were involved in the recombinations, rather than traces of odontogenic mesenchyme remained on the enamel organs.

Among Group IV kidney transplants, 32% of the samples developed into tooth structures. We suppose that these changes were induced by functional odontoblast cells, which in this study emerged from OLCs. The onset of dentin apposition signifies the terminal differentiation of odontoblasts derived from preodontoblasts [30,31]. These cells induced by preameloblasts, which differentiates into functional ameloblasts because of sequential and reciprocal cell–cell interaction from the dental papilla mesenchyme [32,33]. We isolated teeth primordia from embryonic day 14.5, which refers the cap-stage in tooth development. Physiologically, during the cap-stage preodontoblasts are not presented yet, whereas their progenitors rooted in mesenchymal dental papilla cells. Hence, we concluded that OLCs were capable of overtaking the task of the ectomesenchyme, and subsequent cellular interactions provoked the cascades of cytological and functional changes, that eventually led to hard tissue formation. In an attempt to improve the results of the earlier findings of a related study [20], we utilized only one dental epithelium per implant to avoid multiple induction of primordia and consequent multiple tooth growth. Since cuspal patterning seems to be modified by the number of dissociated mesenchymal cells [34], it indicates a possible path of future experiment on OLCs to design tooth and cusp formation.

The absence of growing dental primordium in the failed transplants indicates disturbed tooth germ development. Other researches reported similar disturbance in primordial development [1,21], yet they suggested unsuccessful reciprocal interactions following implantation as the cause. The importance of direct cell–cell contact between the epithelial and mesenchymal components has been brought into focus previously [8]. We suspect that this fine interface thus the apposition of the basement membrane rebuilt insufficiently. OLC cell condensation around the central, cornified mass indicates insufficient communication between OLCs and dental epithelium. Additionally, OLCs are E18.5 dental pulp derived cells, identified as most likely precursors of odontoblast-lineage cells. Therefore, their potential might differ from those normal, natural E14.5 dental mesenchyme cells. Since the ability of cell reprogramming plays a key role in dental histo-morphogenesis [4], further investigations with dental mesenchyme from other developmental stages will have to be performed.

This study brought into practice a spontaneously immortalized established dental mesenchymal cell line in recombinations. The experiments assure the applicability of OLCs in bioengineered organ germ method, since OLC cells induced dental development under in vitro, as well as, in vivo conditions. Additional efforts are required to reduce failures of tooth regeneration, realize proper dental morphology, tooth number, and tooth type. Besides, the approach offers possible strategies for the modification of matricellular interactions by bio-active molecules in guided organ regeneration.

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