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Analysis of developmental potentials of dental pulp *in vitro* using GFP transgenes

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Structured Abstract

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Background – In recent years there has been increasing progress in identifying stem cells from adult tissues and their potential application for tooth replacement/regeneration. Our previous *in vivo* studies show that pOBCol3.6GFP and pOBCol2.3GFP transgenic animals provide a unique model to gain insight into progenitor/stem cells in the dental pulp capable of giving rise to odontoblasts.

Objectives – To characterize the behavior of dental pulp cells derived from pOBCol3.6GFP animals *in vitro*.

Experimental design – Primary cultures were established from the coronal portions of the pulps isolated first molars from 5-day-old pOBCol3.6GFP heterozygous mice and grown for 21 days. In these cultures proliferation, clonogenic capacity, activation of 3.6-GFP and mineralization were examined.

Results – Our observations show that dental pulp cells derived from 3.6-GFP contain a population of proliferative, clonogenic cells with the ability to mineralize. We also show the stage specific activation/upregulation of 3.6-GFP in primary cultures derived from dental pulp. In these cultures, expression of Col1a1-3.6-GFP occurs prior to the appearance of mineralized nodules and is unregulated in mineralized nodules.

Conclusions – Col1a1-GFP transgenes appear to fulfill many of the requirements of a marker gene for cell lineage studies in intact tooth and primary cultures derived from dental pulp.

Key words: Col1a1-GFP; green fluorescent protein; odontogenesis; osteogenesis; progenitor cells; stem cells

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Introduction

Tissue engineering is an interdisciplinary field that attempts to fabricate new tissues and organs for replacements (1–6). In a tissue engineering approach

new tissues are created from cultured cells and biomaterial matrices (synthetic or natural scaffolds) (3, 5, 6). Scaffolds are used to create the three dimensional organization needed for appropriate cell interactions, to serve as vehicles to deliver and retain the cells at a specific site and provide a barrier for the undesired growth of scarring tissues (6–9).

One successful approach in tissue engineering is to foster in stem cells the biological processes that occur during development (1,10,11). Essentials for the stem-cell-based approach are the identification of donor stem cells and a clear understanding of molecular mechanisms regulating the normal development of a given tissue.

Stem cells are present in many of the vertebrate organs and are generally defined as clonogenic cells with the ability of self-renewal for long periods and multi-lineage differentiation (9,12).

In recent years, there has been significant progress towards identification of stem cells for various adult tissues including skin, neural tissue, bone marrow and teeth. In the tooth, post-natal stem cells have been identified in human and mouse dental pulp (7,13–17), dental epithelium of continuously erupting mouse incisors (18–23), and human periodontal ligaments (24). Engineered tooth-like tissues from whole rat and pig dissociated tooth germ (25,26), and from recombined murine tissues (27) have also been reported. These studies, although limited, indicate the viability of a tissue engineering approach for tooth replacement and treatment of periodontal diseases.

We have used pOBCol3.6GFP (referred to as 3.6-GFP) and pOBCol2.3GFP (referred to as 2.3-GFP) transgenic animals to gain insight into stem/progenitor cells in the dental pulp (16,17,28). Our analyses of the pattern of expression of Col1a1-GFP transgenes in the developing teeth of these transgenic mice are detailed in our recent publications (16,17,28). These studies showed that in developing molars, expression of Col1a1-GFP became evident at the bell stage (E18) of tooth development. At this stage low but detectable levels of Col1a1-GFP were expressed in a limited group of polarizing odontoblasts. At the late bell stage of tooth development (E19), high levels of Col1a1-GFP were expressed in functional odontoblasts engaged in the secretion of a thin layer of predentin (16, 17). At the secretary stage of crown formation during postnatal growth (P1–P5), high levels of Col1a1-GFP were expressed in the entire layer of odontoblasts covering the dental pulp. An interesting finding in these *in vivo* studies was the observation that the expression of Col1a1-GFP transgenes extended into the odontoblasts processes (16,17). The patterns of expression of Col1a1-GFP in differentiating odontoblasts were similar to the pattern of expression of type I collagen protein and mRNA and correlated with the pattern of expression of dentin sialophosphoprotein (*DSPP*) and dentin matrix protein-1(*Dmp1*) in functional odontoblasts at E19. During the secretary stages of tooth development, high levels of Col1a1-GFP and *DSPP* were expressed in the entire layer of fully differentiated odontoblasts covering the dental pulp. Hybridization of the adjacent section with a *Dmp1* riboprobe revealed the restricted expression of *Dmp1* in the functional odontoblasts located at the cervical loops and not by fully differentiated odontoblasts. Our studies in the continuously erupting incisor confirmed these observations (16,28).

Taken together, these studies show that Col1a1-GFP transgenes were expressed initially at low levels in the polarizing odontoblasts that do not express *DSPP* and *Dmp1*. The expression of Col1a1-GFP transgenes was intensified in functional odontoblasts and terminally differentiated odontoblasts that express high levels of *DSPP*. These observations indicated that Col1a1-GFP transgenes provide an excellent non-invasive marker for examining the progression of odontoblast differentiation from progenitor cells.

This possibility was supported by our transplantation studies in which pieces of dental pulp isolated from 2.3-GFP mice were transplanted under the kidney capsule (16,17). In these experiments 2.3-GFP served as an excellent visual marker to ensure the isolation of coronal pulp free of attached odontoblasts. Our studies showed that pieces of dental pulp gave rise to mineralized tissues that were both dentin-like and bone-like. Dentin-like matrices were composed of tubular containing matrix (characterized by extended expression of 2.3-GFP into the tubular-containing matrices) lined with cells expressing high levels of 2.3-GFP and *DSPP* but low to undetectable levels of *Dmp1* and very few if any osteocyte-like cells. On the contrary bone-like matrices were composed of atubular matrices with osteocyte-like cells embedded within the matrix expressing high levels of 2.3-GFP and *Dmp1* but low to undetectable levels of *DSPP*. These powerful applications of GFP allowed us to clearly demonstrate that

dental pulp from postnatal mice (without contaminating odontoblasts) contains competent progenitor cells capable of differentiating into a new generation of odontoblast-like cells secreting tubular containing dentin as well as osteoblast-like cells secreting bone-like matrices.

These studies together showed that Col1a1-GFP transgenes are activated at specific stages of odontoblasts differentiation *in vivo* (16,17,28). The goal of the present study was to examine the stage-specific activation of 3.6-GFP in dental pulps *in vitro*.

Materials and methods

Preparation of dental pulp cultures

The coronal portions of the pulps from first molars from 5-day-old pOBCo13.6GFP heterozygous mice were isolated, minced and digested with enzyme mixture containing 0.05% trypsin (Gibco^{TM}, Invitrogen Corporation, Carlsband, USA) and 0.1% collagenase P (Boeringer Mannheim, Mannheim, Germany) at 37°C for 50 min on rocking platform. Single-cell suspensions were obtained by passing the cells through narrowed Pasteur pipettes, and a 70-μm strainer (Fisher Scientific, Pittsburg, USA). Single-cells of the dental pulp were plated at 5×10^5 cells/well in 35 mm culture plates (Fisher Scientific) in Dulbecco's modified Eagles' medium (Gibco^{TM}, Invitrogen Corporation, Carlsband, USA), containing 10% fetal bovine serum (FBS) (HyClone, Logan, USA), 100 U/ml of penicillin, 100 μg/ml of streptomycin (Gibco^{TM}, Invitrogen Corporation, Carlsband, USA) and incubated at 37°C and 6.5% CO_2. Plating medium was changed after 24 h and after 3 days. The medium was changed to differentiation medium at day 5 containing 50 μg/ml of ascorbic acid and 4 mM of β-glycerophosphate. The medium was and changed every other day for the duration of the experiment (21 days).

Colony forming potential of dental pulp cells

To assess the colony forming efficiency of dental pulp cells dissociated dental pulp cells were plated at different densities (0.5, 1, 3, 5×10^5/well) into 6-well plates (Costar, Cambridge, MA, USA) in alpha-MEM supplemented 10% FBS and 100 U/ml of penicillin, 100 μg/ml streptomycin. Media was changed every other day. At different time points (3 and 5 days) after plating, cells were fixed with acetone/citric acid and stained with 0.05% Crystal Violet (CV) (Sigma Chemical Co., St Louis, MO, USA). Aggregates with ≥30 cells were scored as colonies. Total number of colonies in each culture dish was counted under the inverted microscope using a grid.

Histochemical analysis of cell cultures

Histochemical staining for alkaline phosphatase (AP) activity at different time points of cultures was performed using a commercially available kit (86-R Alkaline Phosphatase; Sigma Diagnostics, Inc., St Louis, MO, USA) according to the manufacturer's instructions.

Epifluorescence analysis in cell culture

To visualize Col1a1-GFP expression in cultures, an Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescent light (Olympus America, Inc., Melville, NY, USA) was used. A specific excitation wavelength was obtained using the filters for GFPtpz (exciter, D500/20; dichroic, 525DCLP; emitter, D550/40) and GFPemd (exciter, D470/40; dichroic, 495LP; emitter, D525/50), and photographed by digital camera (SPOT; Diagnostic instruments, Inc., Sterling Heights, MI, USA). Phase contrast images of the same areas of cultures were obtained using the same microscope under phase contrast illumination and converted to grayscale.

Mineral detection in cell cultures

Xylenol orange (XO) was added to living cultures *in vitro* to mark the mineralized nodules. XO produced a red color when visualized under the microscope using a TRITC filter. XO powder was dissolved in distilled water and filtered to make the concentrated 20 mM stock solution and stored at 4°C. XO was added into cultures at a final concentration of 20 mM for 4 h to overnight. XO-containing medium will be replaced by fresh medium prior to photography to avoid a fluorescent background.

Results

Colony forming assay and AP staining

The clonogenic cell population in dental pulp was assayed by CV staining after 3 and 5 days. The numbers of colony-forming units in cultures derived from dental pulp were dependent on initial cell density and time in culture (Fig. 1 and data not shown). As shown in Fig. 1 after 5 days in culture, there were increases in the number of colonies with increasing cell density (Fig. 1). Histochemical staining for AP activity of same culture dishes showed the presence of high percentage of AP positive cells in cultured derived from dental pulp cells (Fig. 1).

The Colony-forming efficiency of cells derived from dental pulp was also compared with bone marrow. After 5 days of cultures at the same densities (5×10^5), the numbers of CV positive colonies formed by dental pulp cells were significantly higher than that in cultures derived from bone marrow stromal cells (Fig. 1). However, the cultures derived from bone marrow contained higher percentage of AP colonies as compared with those in pulp (Fig. 1).

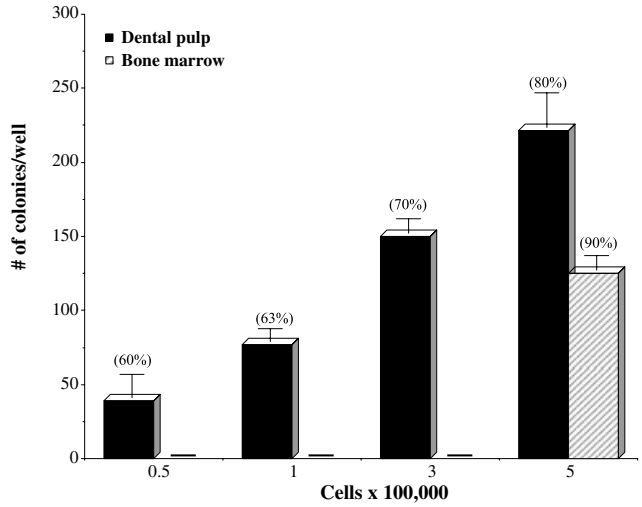

Fig. 1. Histogram showing the total number of colonies formed by dental pulp cells at various densities after 5 days in cultures. Note the increases in total number of colonies with increases in cell density. The number of Alkaline phosphatase (AP)-positive colonies in cultured dental pulp cells at various densities, examined by AP staining of the same cultures are represented in parentheses. Comparison of the colony-forming cells from dental pulp and bone marrow at same plating densities showed differences in the total number of colony assayed by crystal violet staining and AP positive (represented in parentheses) between the two cell populations. The data represent the mean ± standard deviations of 3 of independent experiments.

Localization of 3.6-GFP in the primary cultures derived from coronal portion of the pulp

Cells derived from dental pulp proliferated and reached confluence at around day 7 with many clusters of AP positive cells (Fig. 1). The formation of distinct multi-layered individual nodules was seen at day 7 (not shown). At this time point, the nodules were separated by fibroblast-like cells (not shown). The first sign of mineralization occurred around day 10. Over the next few days in culture, there were significant increases in the size and number of mineralized nodules (Fig. 2).

Epifluorescence analysis of cultured cells showed weak but detectable levels of 3.6-GFP expressions in scattered cells in day 3 (Fig. 2A and C). The level of 3.6-GFP expression increased throughout the culture

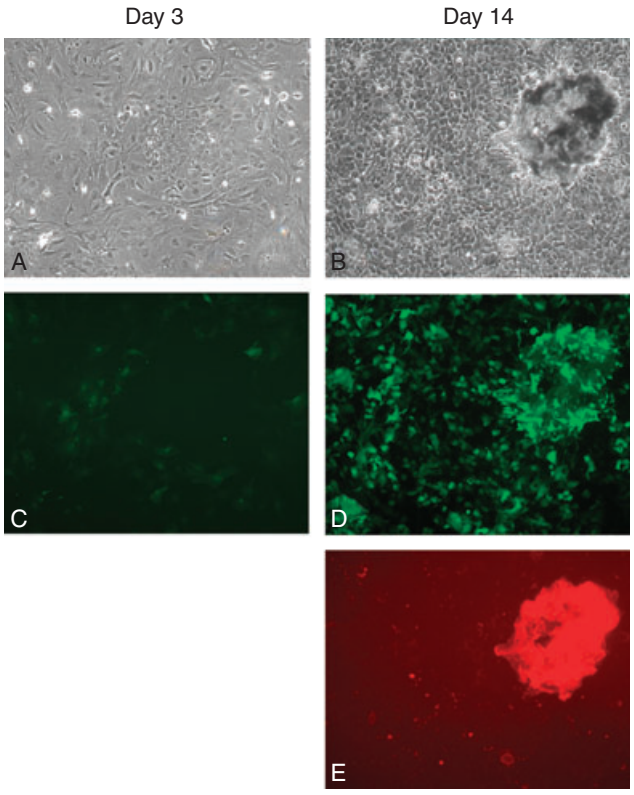

Fig. 2. Expression of green fluorescent protein (GFP) in differentiating primary dental pulp cells derived from Col1a1-3.6-GFP transgenic animals. Primary dental pulp cells were grown for 14 days in differentiation medium containing 50 μg/ml of ascorbic acid and 4 mM of β-glycerphosphate. Each panel represent the phase contrast (A and B) and epifluorescent (C, D, E) images of the same positions in 3 (A, C) and 14 (B, D, E) days old cultures. 3.6-GFP is expressed in few cells in 3-day-old cultures derived from dental pulp (C). There are high levels of 3.6-GFP expression in 14-day-old cultures. In 14-day cultures 3.6-GFP expression is detected in mineralized nodules (D, E) assayed by xylenol orange-staining (E). Note the expression 3.6-GFP expression in cells surrounding the nodules.

period. At day 7, elevated levels of 3.6-GFP were expressed in clusters of cells that were destined to become a nodule (not shown). The 3.6-GFP signal was intensified in well-demarcated mature nodules at days 10, 14, and 21 (Fig. 2 and data not shown). Low levels of 3.6-GFP were also expressed in the periphery of nodules and between individual nodules (Fig. 2).

The *in vitro* mineralization in these cultured were examined by XO staining that is a red fluorescence dye that stains newly deposited mineral without affecting the growth of the culture. Our observations showed a close correction between the areas of the cultures stained with XO and 3.6-GFP expressions in mineralized nodules.

Discussion

Because adult teeth lack the ability to reconstruct and/or regenerate missing tissues, tooth loss resulting from multiple factors including congenital abnormalities, disease and trauma, is currently being treated by a variety of traditional clinical therapies such as use of prostheses and implants. However, these devices are incapable of replacing all the functions of the original tooth, often fail overtime, and are incapable of undergoing remodeling. As is true in many other organs, one of the major problems in reconstruction and/or regeneration of tooth structures is the lack of clearly identified progenitor/stem cells from adult tissues that are able to replace or regenerate the effete mature cells and structures.

In recent years, promoter-green fluorescent protein (GFP) reporter transgenic mice has become one of the most widely-used, non-invasive protein markers for studying lineage determination and progression *in vivo* and *in vitro* (29,30). The availability of transgenic animals carrying GFP coding sequences under the control of tissue-specific or stage-specific promoters have provided an excellent animal model for these studies.

We have used transgenic mouse lines in which GFP expression is under the control of 3.6- and 2.3-kb fragments of the rat Col1a1 promoter to gain insight into stem/progenitor cells in the dental pulp (16,17,28). Previous studies have shown that the 3.6-kb promoter directs strong expression of GFP mRNA and protein in bone and other non-osseous type I collagen-expressing tissues, while the 2.3-kb promoter directs expression of GFP mRNA and protein predominantly in bone and tendon (31,32). *In vivo* analyses of developing long bones in 2.3-GFP and 3.6-GFP mice and *in vitro* analyses of expression of the transgenes in primary osteoblast cultures indicates that Col1a1-GFP transgenes can identify different subpopulations of cells during osteoblasts differentiation (31,32). In the long bones, Col3.6GFP is expressed in both pre-osteoblasts and differentiated osteoblasts, whereas Col2.3GFP expression is limited to differentiated osteoblasts and osteocytes (31,32).

The stage-specific activation of GFP by these two Col1a1 promoter fragments has also been confirmed by *in vitro* studies (31,32). In primary cultures derived from neonatal calvaria and bone marrow stromal cells, low level 3.6-GFP expression occurs prior to the appearance of mineralized nodules. At later stages of differentiation, intense 3.6-GFP expression is observed in the mineralized areas of the nodules. On the contrary, expression of 2.3-GFP is seen at the onset of mineralization and is correlated with the appearance of differentiated osteocytes expressing bone sialoprotein and OC (32).

Col1a1 is also one of the earliest markers of odontoblast differentiation. The terminal differentiation of odontoblasts is accompanied by a dramatic increase in type I collagen synthesis (33). The regulatory elements of the Col1a1 gene responsible for type I collagen expression in teeth are similar to those directing expression to bone. Our previous studies and studies reported in this paper indicate that pOBCol3.6GFP and pOBCol2.3GFP transgenic animals provide powerful models for direct examination of the underlying mechanisms and the signaling pathways involved in odontoblast differentiation.

Our studies on patterns of expression of 3.6-GFP and 2.3-GFP in developing teeth of transgenic animals have shown that these transgenes are expressed at low levels in polarizing odontoblasts. Transgene expression is upregulated in functional and terminally differentiated odontoblasts *in vivo*. In the present study we show that dental pulp cells derived from 3.6-GFP contain a population of proliferative, clonogenic cells with the ability to mineralize. The behavior of dental pulp cells derived from this transgenic animal is similar to the previously characterized behavior of dental pulp isolated from un-erupted third molars (13,14), exfoliating primary

teeth (34) and periodontal ligament (24) in human. We also show the stage specific activation/upregulation of 3.6-GFP in primary cultures derived from dental pulp. In these cultures, expression of Col1a1-3.6-GFP occurs prior to the appearance of mineralized nodules and is unregulated in mineralized nodules. Thus, Col1a1-GFP transgenes appear to fulfill many of the requirements of a marker gene for cell lineage studies in intact tooth and primary cultures derived from dental pulp.

The stage-specific activation of Col1a1-GFP transgenes can be used as a tool 1) to mark cells at early stages of differentiation and separate them from the heterogeneous background by FACS sorting for further lineage analysis (differentiation potential and expression profiling); and 2) to characterize specific progenitor cell populations for their ability to give rise to odontoblasts secreting a dentin-like tubular containing matrix.

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