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Identification of Cells at Early and Late Stages of Polarization During Odontoblast Differentiation Using pOBCol3.6GFP and pOBCol2.3GFP Transgenic Mice

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Abstract

Transgenic mouse lines in which GFP expression is under the control of tissue-and stage specific promoters have provided powerful experimental tools for identification and isolation of cells at specific stage of differentiation along a lineage. In the present study we used primary cell cultures derived from the dental pulp from pOBCol3.6GFP and pOBCol2.3GFP transgenic mice as a model to develop markers for early stages of odontoblast differentiation from progenitor cells. We analyzed the temporal and spatial expression of 2.3-GFP and 3.6-GFP during *in vitro* mineralization. Using FACS to separate cells based on GFP expression, we obtained relatively homogenous sub-populations of cells and analyzed their dentinogenic potentials and their progression into odontoblasts. Our observations showed that these transgenes were activated before the onset of matrix deposition and in cells at different stages of polarization. The 3.6-GFP transgene was activated in cells in early stages of polarization whereas the 2.3-GFP transgene was activated at a later stage of polarization just before or at the time of formation of secretory odontoblast.

Keywords

Odontoblast differentiation; dental pulp; progenitors; type I collagen; green fluorescent protein

INTRODUCTION

Dentinogenesis is regulated by odontoblasts, highly specialized cells originating from the neural crest derived cells of the dental papilla. The differentiation of odontoblasts from the neural crest cells is a long process involving several intermediate steps that are dependent and regulated by epithelial signals [1–4]. The first epithelial signals from the early oral ectoderm lead to the induction of odontogenic potential in the cranial neural crest cells. The next step in the advancing differentiation within the odontoblast cell lineage is the formation of dental

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papilla during the transition from bud to cap stage of tooth development that is regulated by signals from epithelial bud and primary enamel knot. Next, dental papilla cells in close proximity to the epithelial–mesenchymal interface at the tip of the cusp differentiate into pre-odontoblasts, whereas the rest of dental papilla cells form the dental pulp [1–4].

The differentiation of pre-odontoblasts occurs at the bell stage of tooth development and is regulated by signals from inner enamel epithelium and secondary enamel knots [1–4]. Further differentiation proceeds in a graded fashion from cusp tips towards the inter-cuspal areas and cervical loops and include the withdrawal of pre-odontoblasts from cell cycle and the formation of polarized odontoblasts from pre-odontoblasts in close contact with the epithelial–mesenchymal interface. It has been suggested that pre-odontoblasts located away from the interface, becomes incorporated within the Höhl layer and during tooth injury leading to odontoblast death, differentiate into new odontoblasts for reparative dentinogenesis [5]. The formation of pre-odontoblasts is followed by the formation of polarizing odontoblasts, secretory/functional odontoblasts and finally the formation of mature and terminally differentiated odontoblasts [1–4].

Secretory/functional odontoblasts (also called young odontoblasts) are engaged in the secretion of unmineralized predentin matrix, composed primarily of type I collagen (Col1a1) [4,6]. As these odontoblasts continue their differentiation, they secrete many non-collagenous proteins (NCPs) essential for the mineralization of collagen fibers and crystal growth [7]. The NCPs of dentin include proteins that are also found in bone such as decorin [8], biglycan [8], osteonectin [9,10] and osteocalcin (OC) [10]. Other NCPs in dentin belong to the SIBLING (Small Integrin-Binding LIgand, N-linked Glycoprotein) family that includes osteopontin (OPN), bone sialoprotein (BSP), dentin matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE) [7]. High levels of expression of *DSPP* and *DSP* are the hallmark of odontoblast differentiation and are routinely used to distinguish differentiated odontoblasts from undifferentiated progenitors and osteoblasts [11–13]. *DSPP* expression is first detected in secretory odontoblasts and increases in terminally differentiated odontoblasts. After production of mineralized dentin, odontoblasts recede towards pulp and leave behind cell processes that extend into the mineralized dentin and give dentin matrix its characteristic tubular morphology [2–4]. In mice, the steps between the formation of pre-odontoblasts and mature odontoblasts are completed within 6–10 hours [14,15].

The cellular and molecular mechanisms regulating odontoblast differentiation have been the subject of intense investigation using a variety of *in vitro* and *in vivo* approaches using cells derived from dental pulps [3,11,16]. The differentiation of odontoblasts in these cultures has been characterized by a number of standard morphological and functional methods including the expression of extracellular matrix components. Most often, published studies report end point assays in whole primary dental pulp cultures, which represent a heterogeneous population [3,16] containing cells and nodules at various stages of odontoblast differentiation. The heterogeneity has made it difficult to fully characterize the intermediate steps in the odontoblast lineage and differentiation.

Key to the isolation of cells at intermediate stages during lineage progression is the ability to identify and isolate the cells at specific stages of differentiation. Techniques most often employed include isolation of cells by fluorescence-activated cell sorting (FACS), based on expression of cell surface antigens, and laser capture microscopy based on location of recognizable anatomical markers. In recent years, transgenic animals carrying Green Fluorescence Protein (GFP) coding sequences under the control of tissue-specific or stage-specific promoters and the utilization of FACS-mediated cell isolation and enrichment have provided powerful experimental tools for lineage studies [17–22].

Transgenic mouse lines in which GFP expression is under the control of tissue- and stage specific regulatory elements of genes involved in osteogenesis have provided valuable tools for examining the stepwise progression and differentiation of osteo-progenitors into pre-osteoblasts, osteoblasts and osteocytes [23–25]. Pre-osteoblasts could be identified by the expression pOBCol3.6GFP, osteoblasts by the expression of pOBCol2.3GFP, mature osteoblasts by OC-GFP and osteocytes by the expression of DMP1-GFP transgenes [23–25].

Col1a1 is the most abundant collagen in dentin (approximately 86–90%) [26–28]. The regulatory elements of the Col1a1 gene directing its expression to odontoblasts are similar to those in bone [29–32]. Furthermore, the stepwise progression of progenitor/stem cells into the odontoblast lineage is comparable with the process of osteoblasts differentiation, which starts from stem/progenitor cells differentiation into pre-osteoblasts and finally into terminally differentiated functional osteoblasts. These observations suggest that pOBCol3.6GFP and pOBCol2.3GFP transgenic animals in which 3.6- and 2.3-kb fragments of rat type I collagen promoter drive the expression of GFP, respectively, provide models for examination of the stepwise progression of progenitor/stem cells along the odontoblast lineage.

This possibility was supported by our *in vivo* studies in the developing teeth (molars and incisors) of these transgenic animals [12,33,34] that showed the expression of pOBCol3.6GFP (referred to as 3.6-GFP) and pOBCol2.3GFP (referred to as 2.3-GFP) transgenes at high intensity in secretory and differentiated odontoblasts expressing high levels of *DSPP* [12,34]. Our transplantation studies in which pieces of dental pulp isolated from pOBCol2.3GFP mice were transplanted under the kidney capsule showed the formation of dentin-like and bone-like mineralized tissues by explanted dental pulps [12]. Dentin-like matrices were composed of tubular matrix (characterized by extended expression of 2.3-GFP into the tubular matrices) lined with cells expressing high levels of 2.3-GFP and *DSPP*. On the other hand, bone-like matrices were composed of atubular matrix with cells embedded within the matrix expressing high levels of 2.3-GFP and lacking the expression of *DSPP* [12]. These observations indicate that Col1a1-GFP transgenes (2.3-GFP and 3.6-GFP) provide excellent non-invasive markers for identification of odontoblasts and examination of the progression of odontoblast differentiation from progenitor cells.

In the present study, we have used 3.6-GFP and 2.3-GFP transgenic animals as an experimental model to examine the differentiation of odontoblast from progenitor cells in the dental pulp. We have analyzed the temporal and spatial expression of these transgenes during *in vitro* mineralization. With the ability of FACS to separate cells based on GFP expression, we also obtained relatively homogenous sub-populations of cells and analyzed their dentinogenic potentials and their progression into odontoblasts *in vitro*. Our observations show that these transgenes are activated before the onset of matrix deposition and in cells at different intermediate stages of odontoblast differentiation. The 3.6-GFP transgene was activated in cells in early stages of polarization whereas the 2.3-GFP transgene was activated at a later stage of polarization just before or at the time of formation of secretory odontoblast.

Materials and Methods

Primary dental pulp cultures

The coronal portions of the pulps from first and second molars were isolated from 5–7-day-old hemizygous pOBCol3.6GFP, pOBCol2.3GFP and non-transgenic mice and prepared for primary cultures as described previously [35,36]. Briefly, 520 cells/mm² (5×10^5 cells/well in 35-mm culture plates) were grown first in media containing Dulbecco's modified Eagles' medium (DMEM, Invitrogen), 20% fetal bovine serum (FBS, Hyclone, USA), 40 µg/ml and 40U/ml of Pen-Strep antibiotics. Three days later media was changed to media containing 10% FBS that was switched to mineralization inducing media containing 50 µg/ml ascorbic acid,

and 4 μM β -glycerophosphate in confluent cultures at day 7 [13,35,36]. Media was changed every 2 days for a period of 2 weeks.

Digital imaging and epifluorescence analysis in cell culture

GFP expression in cell culture was visualized using an Olympus IX50 inverted microscope equipped with an IX-FLA inverted reflected light fluorescence (Olympus America, Inc., Melville, NY, USA). A specific excitation wavelength was obtained using filters for GFPtpz (exciter: D500/20; dichroic: 525DCLP; emitter: D550/40) and GFPemd (exciter, D470/40; dichroic, 495LP; emitter, D525/50). Images were captured using a SPOTcamera (Diagnostic Instruments, Inc., Sterling Heights, MI).

Cell cultures were also examined with a Zeiss Axiovert 200 microscope (Carl Zeiss, Thornwood, NY) equipped with the motorized X-Y-Z platform, fluorescent cube, and AxioCam color digital camera controlled by a user-defined computation program using Openlab software (Improvision, Lexington, MA) as described before [37,38]. Fluorescent expression of 3.6-GFP and 2.3-GFP in cultures was examined using a TOPAZ filter (YFP). The Zeiss Axiovert 200 microscope workstation allows the user to reproducibly record images of cultures at the same location and under the same conditions at different time points. Approximately 65% of the 35-mm culture dish can be repeatedly imaged and all the images taken can be concentrated into one image using AxioVision Rel 4.7 software.

Detection and Quantification of mineralization in cultures

Mineralized nodules in live cultures were visualized by Xylenol Orange (XO) staining as described previously [35]. Mineralization in fixed cultures was assayed and quantified using a modified von Kossa silver nitrate and modified Alizarin Red-S (AR-S) staining [36]. Values represent mean \pm SE of at least three independent experiment.

RNA extraction and analyses

Total RNA was prepared using TRI Reagent according to the manufacturer's instructions. For RT-PCR, isolated RNAs were reverse transcribed by Superscript II reverse transcriptase (Life Technologies) with oligo dT primers. Subsequent PCR amplifications were carried out using specific primers as described before [13], including primers for detection of GFP: 5'-GTCCGCCCTGAGCAAAGA and 3'-CTAGTGTACCAGGACGACCT [39]. RT-PCR products were resolved on 1% agarose gels, stained with ethidium bromide, and digitally photographed.

Flow cytometric analysis and Sorting (FACS)

Cultures were prepared for flow cytometric analysis by mild trypsin/EDTA digestion followed by centrifugation at $+4^{\circ}\text{C}$. Cells were then re-suspended in 300–500 μl of staining medium containing 1 $\mu\text{g/ml}$ of propidium iodide (PI) and strained through a 70 μm filter. Flow cytometry analysis was done collecting 20,000–100,000 cells on a BD FACScan/Calibur cytometer (Becton-Dickinson, San Jose, CA, USA) using a 488-nm excitation wavelength generated by a 15 mw argon ion laser. Emission was detected using a 500-nm long pass filter (GFP). The percentage of cells expressing GFP at high and low intensities was determined manually by setting a separation point at 10^2 . Data were processed using Cell Quest software. Values represent mean \pm S.E. determined from at least three independent experiments in which dental pulp cells obtained from non-transgenic mice were used as controls.

The expression of GFP was also examined in combination with various commercially available anti-mouse antibodies in 7 days old pulp cultures including CD45.2-Biotin; Sca-1-PE (D7); and CD90/Thy1.2-FITC (53-2.1) as described previously [13]. Approximately $0.5\text{--}1 \times 10^6$ cells

was incubated with pre-titrated antibodies (1:50-1:800), in the presence of rat Ig (when necessary), washed and resuspended in 300 μ l of staining medium containing 1 μ g/ml of PI (propidium iodide) [39]. Between 20,000 and 100,000 cells was used for analysis.

For FACS sorting, seven day cultures were prepared by mild trypsin/EDTA digestion followed by centrifugation at 4°C. FACS sorting based on GFP expression was performed on 2.5 \times 10⁶ cells/ml by UCHC FACS facility on a FACS-Vantage BD cell sorter with a 130- μ m nozzle at a speed of 3–5K cells/s. GFP was excited at 488 nm with an argon laser and a 550/30 emission filter was utilized. Upon separation, GFP⁺ and GFP[–] live cells were collected into DMEM with 20% FBS, and plated at 520 cells/mm² (5 \times 10⁵ cells/well in 35-mm culture plates) density. Cultures were grown as described for unsorted population.

Immunocytochemistry and Confocal Microscopy

Dental pulp cells cultured on glass surface for 14 days were fixed and processed for immunocytochemistry as described previously [13]. Briefly, cells were fixed with 2% paraformaldehyde, washed and then incubated with 3% dry milk in PBS to block nonspecific staining. Cells were then incubated with 1:400 dilution of Anti-DSP antibody [LF-153 (kind gift from Dr. Larry Fisher)] for 1 hour at room temperature, and then with 1:800 dilution of Alexa Fluor 568 goat anti-rabbit antibody (Invitrogen, USA) for an additional hour at room temperature. For nuclear detection, samples were incubated with 1:500 dilution of TO-PRO-3 (Invitrogen, USA, 1:500 in PBS) for 30 min. After staining, cells were transferred to a slide with Prolong Gold anti-fade reagent (Invitrogen, A). A Zeiss LSM 510 confocal laser scanning microscope (Thornwood, NY) with a 63 \times , 1.4 numerical aperture oil immersion objective was used to collect images by simultaneous recording in the 5681 and 6471 channels. The expression of DSP was correlated with the expression of 3.6-GFP and 2.3-GFP.

Cell cycle analysis

To analyze the cell cycle and rate of proliferation in Colla1-GFP⁺ and Colla1-GFP[–] populations, three days cultures were washed with PBS, trypsinized and resuspended in a staining media (1 \times HBSS, 2% fetal calf serum, 10mM HEPES, pH7.2) containing 5 μ g/ml of a Hoechst 33342 nuclear dye (Molecular Probes, Invitrogen, USA) and incubated for 90 minutes at 37°C in a dark as described before [40]. Controls included cultures from the same animal but without addition of nuclear dye and cultures from wild type animals. Cells were analyzed in a BD LSRII flow cytometer (Becton-Dickinson). Data was analyzed using ModFitLT software (Verity Software House, Inc., Topshame, ME, USA) and FlowJo software. Values represent mean and \pm SD of percentage of in G0+G1, G2+M and S phases in GFP⁺ and GFP[–] sub-populations in at least three independent experiments.

Statistical analysis

Unpaired, two-tailed t-tests were performed to determine statistically significant differences and p<0.05 was considered statistically significant.

RESULTS

Expression of 3.6-GFP and 2.3-GFP transgenes during mineralization in primary dental pulp cultures

We have previously characterized the sequence of mineralization in dental pulps from unerupted murine molars [13] and showed that when dissociated pulp cells are placed in primary culture, they proliferate rapidly and reach confluence at day 7. The proliferation phase is followed by the formation of distinct multi-layered individual nodules. The first sign of

mineralization is around day 10, with increases thereafter so that by day 21 almost the entire culture dish is covered by a sheet of mineralized tissue [13].

To gain further insight into the stage of activation of 3.6-GFP and 2.3-GFP transgenes during odontoblast differentiation, we examined their expression during the mineralization in primary pulp cultures established from coronal portions of unerupted molars from hemizygous pOBCol3.6GFP and pOBCol2.3GFP transgenic mice (P5-P7). The pattern and intensity of GFP expression was correlated with the onset and subsequent growth of the mineralized nodules at various time points in real time by XO staining in the same cultures.

Epifluorescence analysis of primary pulp cultures from transgenic animals showed expression of the transgenes in scattered cells at day 2 (Figure 1A & B). At day 7, 3.6-GFP was detected in clusters of fibroblastic-like cells (Figure 2A) whereas 2.3-GFP was expressed in isolated cuboidal cells (Figure 2B). After addition of mineralization media, at day 10, 14 and 21, both transgenes were expressed at high intensity in multilayered and mineralized nodules (Figure 1A & B). At day 10, the areas expressing the transgenes at high intensity were larger than the areas of mineralization (XO staining) (Figure 1A & B). At days 14 and 21, the areas of XO staining overlapped more closely with areas expressing transgenes at high intensity. Cells expressing these transgenes at lower intensities were detected between nodules (Figure 1A & B).

These results showed similarities in the temporal and spatial patterns of expression of these transgenes in primary pulp cultures. Both transgenes were expressed at early time points prior to mineralization. Cells undergoing mineralization expressed 3.6-GFP and 2.3-GFP transgenes at high intensity (Figure 1A & B).

Col1a1 transgenes are expressed before the expression of markers of mineralization

Our previous analyses of expression of these transgene in the developing teeth *in vivo* showed that they are expressed at high levels in secretory odontoblasts expressing *DSPP* [12,34]. The expression of these transgenes at early time points (before mineralization) in primary pulp cultures was different from our *in vivo* studies and thus was further examined by RT-PCR analysis of primary pulp cultures from pOBCol2.3GFP and pOBCol3.6GFP transgenic animals.

Expression of both transgenes was detected at day 2 and 7 before the expression of various markers of mineralization including *DSPP* (Figure 2C and data not shown). In these cultures expression of *DSPP*, *OC*, *BSP* and *DMPI* was detected at day 14 and increased at day 21 (Figure 2C).

Expression of Col1a1-GFP in odontoblasts and osteoblasts in primary dental pulp cultures

Our previous characterization of dental pulp cultures from unerupted murine molars showed dentinogenic and osteogenic potentials of these cells [13]. To correlate the expression of transgenes with dentinogenesis, DSP expression was examined in primary dental pulp and primary calvaria osteoblasts cultures from pOBCol3.6GFP and pOBCol2.3GFP transgenic mice. At day 14 expression of both transgenes was detected in mineralized nodules in primary dental pulp and primary calvaria osteoblasts cultures. In pulp cultures, DSP expression was detected in some but not all mineralized nodules expressing transgenes (supplemental Figure 1A–H and data not shown). DSP expression was not detected in mineralized nodules in primary calvaria osteoblasts (supplemental Figure 1I–L and data not shown). These results indicated that in primary pulp cultures these transgenes are expressed by both odontoblasts and osteoblasts.

Changes in Col1a1-GFP+ population and the intensity of GFP signal in primary dental pulp cultures

Changes in the percentage of 2.3-GFP and 3.6-GFP expressing cells (GFP+) and the signal strength within the GFP+ populations at different time points was examined by flow cytometry analysis (supplemental Figure 2 and Table 1).

Analysis of cultures from pOBCol3.6GFP mice showed that during mineralization there were increases in the percentage of GFP+ cells and about a 3 fold decrease in the GFP- cells (supplemental Figure 2 and Table 1A). At all time points 3.6-GFP was expressed at two levels of intensity (supplemental Figure 2 and Table 1B). Cells that expressed 3.6-GFP at the lower intensity (less or equal to 10^2) constituted 13% of the cells at day 2 and increased about 2.3 fold by day 14 (supplemental Figure 2 and Table 1B). Cells expressing 3.6-GFP at higher intensity (more than 10^2) increased about 1.5 fold between days 2 and 14 (supplemental Figure 2 and Table 1B). These changes were followed by increases in the mean intensity (strength) of GFP signal that were most significant between days 2 (approximately 230) and 7 (approximately 2116). No significant change in the mean intensity was seen in these cultures between days 7 and 14.

FACS analysis of primary cultures derived from pOBCol2.3GFP mice showed that mineralization in these cultures was also associated with increases in the percentage of 2.3-GFP+ cells and 6 fold decreases in the percentage of 2.3-GFP- cells (supplemental Figure 2 and Table 1A). Similar to 3.6-GFP, 2.3-GFP was also expressed at two intensities. Cells expressing 2.3-GFP at lower intensity constituted 29% of the cells at day 2 and decreased by day 14, whereas cells expressing 2.3-GFP at high intensity increased about 3.5 fold between days 2 and 14 (supplemental Figure 2 and Table 1B). There were also continuous increases in the mean intensity of 2.3-GFP between days 2 (450) and day 14 (>7420). At all time points the intensity of the 2.3-GFP exceeded the intensity of 3.6-GFP transgene.

Thus, during mineralization in primary pulp cultures there are increases in cells expressing 3.6-GFP transgene at low and high intensities and increases in the percentage of cells expressing 2.3-GFP at high intensity.

The increases in the percentage of the GFP+ cells in these cultures can be due to activation of transgenes in GFP- cells (did not express these transgenes before) and/or proliferation of existing GFP+ cells present at early time points in cultures. To distinguish between these possibilities, DNA content/cell cycle analysis was performed in three-day-old cultures using flow cytometry and Hoechst 33342 nuclear stain.

Cell cycle analysis in cultures from pOBCol3.6GFP mice showed proliferation in both populations. There were no significant differences in the rates of proliferation (G_2M+S) between 3.6-GFP- (46.7%) and 3.6-GFP+ (42.8%) cells (Table 2). Analysis of cultures from pOBCol2.3GFP mice also showed similar rates of proliferation in 2.3-GFP- (47.5%) and 2.3-GFP+ (42.3%) cells (Table 2). The rates of proliferation (G_2M+S) in 2.3-GFP+ and 2.3-GFP- cells were very similar to 3.6-GFP+ and 3.6-GFP- cells. These results indicated that increases in the number of GFP+ cells in cultures were due to proliferation of GFP+ as well as activation of these transgenes in cells that did not express GFP before.

Stage of activation of 3.6-GFP and 2.3-GFP transgenes in pulp cultures

The presence of a mixture of cells (GFP+ and GFP-) and the high rate of proliferation of GFP+ cells in pulp cultures made it difficult to study the stage of activation of this transgenes during mineralization and dentinogenesis. Therefore, as the next step, we studied the expression of these transgenes in Col1a1-GFP- populations using FACS based on GFP.

Primary pulp cultures were prepared from pOBCol3.6GFP and pOBCol2.3GFP transgenic mice and grown for 7 days. Following growth and expansion, FACS and re-analysis were used for separation of two populations with >97% purity (Figures 3A and 4A). Both populations were plated at same density as unsorted cells (5×10^5 cells/well in 35-mm culture plates). Cells were first grown for 7 days in media supporting their proliferation and then for an additional 7 to 14 days in mineralization inducing media. Controls included cultures from unsorted cells (primary cultures) and cultures from unsorted cells that were re-plated after 7 days (secondary culture).

Cultures from 3.6-GFP⁻ cells remained healthy and proliferated (Figures 3B). In these cultures, expression of 3.6-GFP was not detected during the first 6 days (Figure 3B) and appeared in scattered cells around day 7. Expression of 3.6-GFP was detected in clusters of fibroblastic-like cells at day 10 and in cuboidal cells in mineralized nodules at day 14 and 21. XO staining showed that the onset of mineralization in 3.6-GFP⁻ cultures was at day 14 and later than in primary and secondary unsorted cells (day 10).

The appearance of GFP signal in 2.3-GFP⁻ cultures was similar to 3.6-GFP⁻ cultures. In these cultures, expression of 2.3-GFP appeared at day 7 in isolated cuboidal cells and increased thereafter (Figure 4B). The onset of mineralization in 2.3-GFP⁻ cultures was at day 10 and similar to primary and secondary unsorted cells. Expression of 2.3-GFP was detected in isolated cuboidal cells (Figure 4B).

Comparison of the whole cultures at days 14 and 21 showed that the intensity of the GFP in cultures from 2.3-GFP⁻ and 3.6-GFP⁻ populations was significantly lower than in unsorted cells (supplemental Figure 3). Mineralization in 3.6-GFP⁻ and 2.3-GFP⁻ cultures displayed nodular patterns.

These observations showed similarities in the stage of activation of these transgenes. Both transgenes were activated around days 6/7 prior to mineralization indicating that during odontoblasts differentiation these transgenes are activated prior to the formation of secretory odontoblasts in either pre-odontoblasts or polarizing odontoblasts.

Mineralization and dentinogenic potentials of Col1a1-GFP+ populations

To gain further insight into stage of activation of these transgenes, mineralization and dentinogenic potentials of cultures from different populations were examined and compared. Controls in these experiments also included primary and secondary pulp cultures.

Cultures from 3.6-GFP⁺ cells remained healthy and proliferated (Figures 3C & 4C). In these cultures GFP expression was maintained throughout the entire culture period. The first sign of mineralization in these cultures was at day 10 with increases thereafter (Figures 3C & 4C). At days 14 and 21, extensive mineralization was detected as a sheet over the entire culture dishes (Figure 5C & E and data not shown). The extent of mineralization in 3.6-GFP⁺ cultures after 21 days was lower than primary cultures but similar to the secondary cultures (Figure 5G). The extent of mineralization in secondary cultures was about 66% lower than in primary cultures showing the effects of replating on the extent of mineralization (Figure 5G).

The behaviour of 2.3-GFP⁺ cells in culture was similar to 3.6-GFP⁺ cells (Figures 3C & 4C). The intensity of the GFP in cultures from 2.3-GFP⁺ and 3.6-GFP⁺ populations exceeded those in unsorted and Col1a1- GFP⁺ cultures (Supplemental Figure 3).

The extent of mineralization in 2.3-GFP⁺ cultures was similar to 3.6-GFP⁺ cultures (Figure 5G). However, the relative levels of *DSPP* in 2.3-GFP⁺ cultures were significantly higher than in 3.6-GFP⁺ cultures (Figure 5H) suggesting that 2.3-GFP⁺ population contains either

increased number of odonto-progenitors (pre-odontoblasts and/or polarizing odontoblasts) and/or odonto-progenitors at more advanced stage of differentiation.

Mineralization and dentinogenic potentials of Col1a1-GFP⁻ populations

Analyses of 3.6-GFP⁻ and 2.3-GFP⁻ cultures after 21 days showed distinct differences in the extent of mineralization and relative levels of *DSPP* expression (Figure 5). The extent of mineralization and relative levels of *DSPP* expression in 3.6-GFP⁻ cultures were significantly lower than in 2.3-GFP⁻ cultures (Figures 5G & 5H). Interestingly, the extent of mineralization and relative levels of *DSPP* in 2.3-GFP⁻ cultures was similar to 3.6-GFP⁺ cultures (Figures 5G & 5H).

The differences in the dentinogenic potentials of various subpopulations can be due to differences in the number of odonto-progenitors. To test this possibility, the distribution of CD90 and Sca-1, markers shown to be expressed by progenitors in dental pulp [13] in different populations were examined and compared. As shown in Supplemental Figure 4, Sca1⁺/CD45⁻ and CD90⁺/CD45⁻ cells were present in all four sub-populations. The percentage of Sca1⁺/CD45⁻ cells was highest in 2.3-GFP⁻ populations. (Supplemental Figure 4). Our interpretation of these results is that the differences in the dentinogenic potentials of different populations are not related to the number of odonto-progenitors and are related to differences in the stage of differentiation of odonto-progenitors in different populations.

DISCUSSION

The goal of our studies was to use pOBCol3.6GFP and pOBCol2.3GFP transgenic mice to distinguish and identify populations of cells at early stages of odontoblast differentiation. In our previous studies we characterized the expression of 3.6-GFP and 2.3-GFP transgenes in the developing teeth *in vivo* and showed similarities in the temporal and spatial expression of these transgenes during odontoblast differentiation [12,33,34].

In the present study we examined the expression of these transgenes during the mineralization and odontoblast differentiation in primary cultures derived from the coronal portions of dental pulp from these transgenic animals. Our studies showed the expression of these transgenes in scattered cells at day 7 before initiation of mineralization and expression of *DSPP*. These observations appear to be different from our previous *in vivo* studies that showed the expression of both transgene at high intensity in functional and in fully differentiated odontoblasts expressing high levels of *DSPP* [12,33,34]. However, in our *in vivo* studies, we reported the expression of low but detectable levels of both transgenes in differentiating odontoblasts at the tip of the mesio-lingual cusp of the first mandibular molar at the late bell stage (E18) of tooth development [12,33,34]. These observations together with previous tissue distribution studies showing that Col1a1 is first expressed in polarizing odontoblasts and its expression is maintained in secretory and terminally differentiated odontoblasts [26–28] indicated that during odontoblasts differentiation 2.3-GFP and 3.6-GFP are activated in polarizing odontoblasts.

The activation of these transgenes in polarizing odontoblasts is further supported by our studies in Col1a1-GFP⁺ populations. Our results showed that 2.3-GFP⁺ and 3.6-GFP⁺ populations contain highly proliferative cells giving rise to sheets of mineralized tissue with very little if any Col1a1-GFP⁻ cells indicating their enrichment in progenitors committed to mineralization. Our further studies showed the formation of odontoblasts expressing *DSPP* in both populations. However, the levels of *DSPP* in 2.3-GFP⁺ cultures were significantly higher than in 3.6-GFP⁺ cultures. This difference most likely is related to increased number of polarizing odontoblasts in 2.3-GFP⁺ population as compared to 3.6-GFP⁺ population.

Furthermore, our results showed similarity in the levels of *DSPP* in 2.3-GFP⁻ and 3.6-GFP⁺ cultures. This together with the lack of significant differences in the distributions of CD90⁺ and Sca1⁺ cells in these populations suggested that similarities in the levels of *DSPP* were related to similarities in the stage of differentiation of odonto-progenitors in these populations. These observations suggest differences in the stage of activation of the transgenes during odontoblast differentiation in that the 3.6-GFP transgene is activated at an earlier developmental stage (i.e; early stage of polarization) than the 2.3-GFP transgene (i.e; later stage of polarization just before transition to secretory odontoblasts) (see Figure 6). The differences in the stage of activation of these transgenes during odontoblast differentiation is also supported by the differences in the morphology of the 3.6-GFP⁺ and 2.3-GFP⁺ cells in early time points and before the mineralization in pulp cultures. These differences were not appreciated in the developing teeth *in vivo* because of the close proximity of cells in the early and late stage of polarization. Further experiments are in progress to examine the expression of these two transgene in dual GFP reporter mice in which different color GFPs (topaz and cyan) are driven by 3.6 and 2.3 kb of the *Col1a1* promoter, respectively.

The patterns of expression of these transgenes in developing teeth and in dental pulp cultures share both similarities and differences with previous observations in the developing bones and calvaria that indicated that *Col1a1*-GFP transgenes can identify distinct sub-populations of cells during osteoblast differentiation [24,38,41,42]. Histological analyses of calvaria and long bones showed that 3.6-GFP was expressed in the fibroblastic layer of the periosteum whereas 2.3-GFP was expressed in the osteoblastic layer of periosteum [41,42]. Furthermore, *in vitro* studies revealed distinct differences in the time of activation of 3.6-GFP and 2.3-GFP transgenes during osteogenesis [38,41]. In primary cultures derived from neonatal calvaria and bone marrow stromal cells, expression of 3.6-GFP was detected early prior to the appearance of mineralized nodules and was correlated with the expression of markers of pre-osteoblasts (i.e. mRNAs for alkaline phosphatase and *Col1a1*). On the other hand, expression of 2.3-GFP was not detected at early stages of differentiation and was detected at the time of mineralization. The expression of 2.3-GFP was correlated with the expression of markers of mineralization (i.e. mRNAs for bone sialoprotein and osteocalcin) [38,41]. These observations together led to the conclusion that during osteogenesis activation of these transgenes is associated with different stages of osteoblast differentiation [38,41]. 3.6-GFP is activated in pre-osteoblasts whereas 2.3-GFP is activated in osteoblasts [38,41].

The dentinogenic and osteogenic potentials of dental pulp cells *in vitro* [12,13,43] and the expression of 2.3-GFP and 3.6-GFP in both osteoblasts and odontoblasts in dental pulp cultures makes the distinction between activation of these transgenes in odontoblasts vs. osteoblasts difficult. However, it is important to note that our studies showed that in pulp cultures both 3.6-GFP and 2.3-GFP are expressed prior to mineralization. The early expression of 3.6-GFP in pulp cultures is similar to the expression of this transgene in primary cultures derived from neonatal calvaria and bone marrow stromal cells suggesting that the expression of 3.6-GFP at day 7 in pulp cultures reflects the activity of this transgene in pre-osteoblasts and polarizing odontoblasts. On the other hand, the early expression of 2.3-GFP in pulp cultures is different from the expression of this transgene in primary cultures derived from neonatal calvaria and bone marrow stromal cells. This suggest that the expression of 2.3-GFP at day 7 in pulp cultures reflects the activation of this transgene in polarizing odontoblasts and not in the cells in the osteogenic lineage.

In summary, our studies showed that 2.3-GFP and 3.6-GFP transgenes mark cells first at polarizing stage of odontoblast differentiation and later in secretory and terminally differentiated odontoblasts. Furthermore, the differences in the extent of mineralization and levels of *DSPP* expression in sorted population also suggests that these transgene mark cells at different stages of polarization. The transition from pre-odontoblasts to polarizing

odontoblasts and secretory odontoblasts involves many important changes, including changes in cell shape, reorganization of the cytoplasmic organelles, polarization and changes in cell-cell interactions. Recent studies showed expression of several transcription factors including *Klf4* [44], *Dlx3* [45], *Wnt10a* and *Runx2* [46] in polarizing odontoblasts. Abnormalities in dentinogenesis in *Odf1* mutant mice that mimic the X-linked orofacial digital type I syndrome also have reveal the roles of cilium components in polarizing odontoblasts [47]. Identification of a marker for polarizing odontoblasts and two different *Col1a1* promoter fragments with stage-specific expression in cells in odontoblast lineage would allow characterization of their gene expression profiles and facilitate many experimental approaches for studying odontoblasts *in vivo* and *in vitro*.

Previous studies showed that during odontoblast differentiation, expression of *Col1a1* is followed by osteocalcin and osteopontin, which are expressed in late polarizing odontoblasts and secretory odontoblasts [27,28,48]. *DSPP* and *DMP1* expression are first detected in secretory odontoblasts and increased in terminally differentiated odontoblasts [27,28,48]. Based on these reports, further experiments are in progress to examine the expression of OC-GFP, *DMP1*-GFP and *DSPP*-GFP during odontoblast differentiation *in vivo* and *in vitro* to identify and isolate cells at additional intermediate stages during odontoblast differentiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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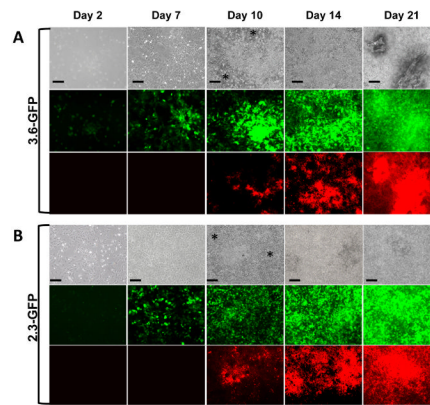


Figure 1. Expression of 3.6-GFP and 2.3-GFP transgenes in primary dental pulp cultures during *in vitro* mineralization and dentinogenesis

Primary dental pulp cultures obtained from pOBCol3.6GFP (A) and pOBCol2.3GFP (B) transgenic animals grown for 21 days in culture. Images of the same areas in cultures at different time points analyzed under phase contrast (upper rows in A and B), epifluorescent light using filters for GFP_{tpz} and GFP_{emd} for detection of GFP (middle rows in A and B) and epifluorescent light using TRITC Red filter for detection of XO staining (lower rows in A and B). Note the presence of GFP⁺ cells early in the culture (day 2), in cell clusters at day 7 and in differentiating and differentiated nodules between days 10–21. Note that areas of the cultures expressing GFP at day 10 extend beyond the areas of XO staining. There is a closer correlation of GFP expression and XO staining at days 14 and 21. Note the expression of low levels of 3.6-GFP and 2.3-GFP in the inter-nodular areas (indicated by asterisc). Scale bars=100μm.

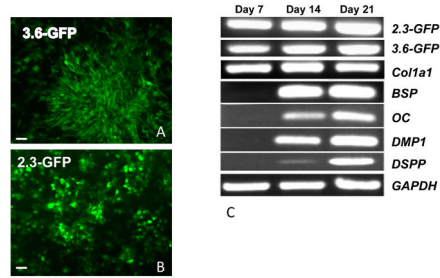


Figure 2. Expression of early and late markers of mineralization in primary pulp cultures and their correlation with 3.6- and 2.3-GFP expression

A and B are epifluorescent images of cultures at day 7 showing the expression of 3.6-GFP in fibroblastic cells and 2.3-GFP in cuboidal cells. Scale bars=100 μ m.

(C) RT-PCR analysis of RNA extracted from primary dental pulp pulps at different time points from pOBCol3.6GFP and pOBCol2.3GFP mice. *GFP* and *Col1a1* expression were detected at day 7 and increases at days 14 and 21. Markers of mineralization (*BSP*, *OC* and *DMP1*) and dentinogenesis (*DSPP*) were detected at day 14 and increased at day 21.

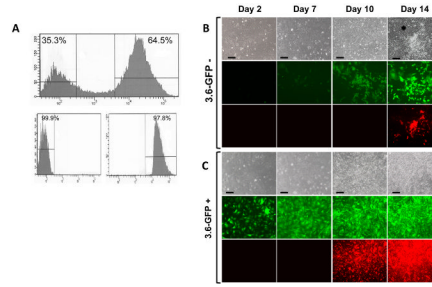


Figure 3. Comparison of behavior of 3.6-GFP+ and 3.6-GFP- populations

Primary pulp cultures from pOBCol3.6GFP were grown for 7 days and processed for FACS sorting. (A) Histogram showing that FACS sorting resulted in clear separation of 3.6-GFP+ (approximately 64%) and 3.6-GFP- (approximately 35%) populations. Histogram of the FACS re-analysis shows that the purity of isolated cell populations was higher from 97%.

B and C represent images of the same areas in live cultures at different time points analyzed under phase contrast (upper rows), epifluorescent light using filters for GFPtpz (middle rows in B and C) and epifluorescent light using TRITC Red filter for detection of XO staining (lower rows in B and C). Note that in 3.6-GFP+ cultures, all cells continuously expressed this transgene. XO staining was detected at day 10 and increased at day 14. In 3.6-GFP- cultures, GFP expression was not detected at day 2. At day 7, 3.6-GFP was expressed in some cells at low intensity. 3.6-GFP was expressed in the cell clusters at day 10 and increased at day 14. XO staining was detected at day 14. Cells in the inter-nodular area are indicated by asterisk. Scale bars=100 μ m.

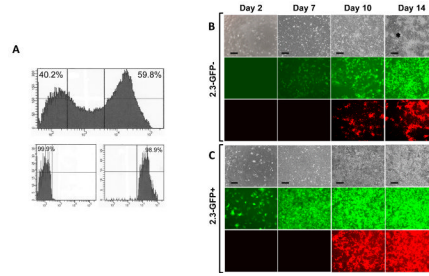


Figure 4. Comparison of behavior of 2.3-GFP+ and 2.3-GFP- sub-populations

Primary pulp cultures derived from pOBCo12.3GFP mice were grown for 7 days and processed for FACS. (A) Histogram showing that FACS sorting resulted in clear separation of 2.3-GFP+ (approximately 60%) and 2.3-GFP- (approximately 40%) sub-populations. Histogram of FACS re-analysis on isolated cell sub-populations showed that the purity of both was higher than 98%.

B and C represent images of the same areas in live cultures at different time points analyzed under phase contrast (upper rows), epifluorescent light using filters for GFPemd (middle rows in B and C) and epifluorescent light using TRITC Red filter for detection of XO staining (lower rows in B and C). Note that in cultures from 2.3-GFP+, all cells continuously expressed this transgene. XO staining was detected at day 10 and increased at day 14.

In 2.3-GFP- cultures, expression of 2.3-GFP was not detected at day 2. At day 7, 2.3-GFP was expressed in some cells. Expression of 2.3-GFP was intensified at day 10 and 14. XO staining is detected at day 10 and 14. Cells in the inter-nodular area are indicated by asterisk. Scale bars=100µm.

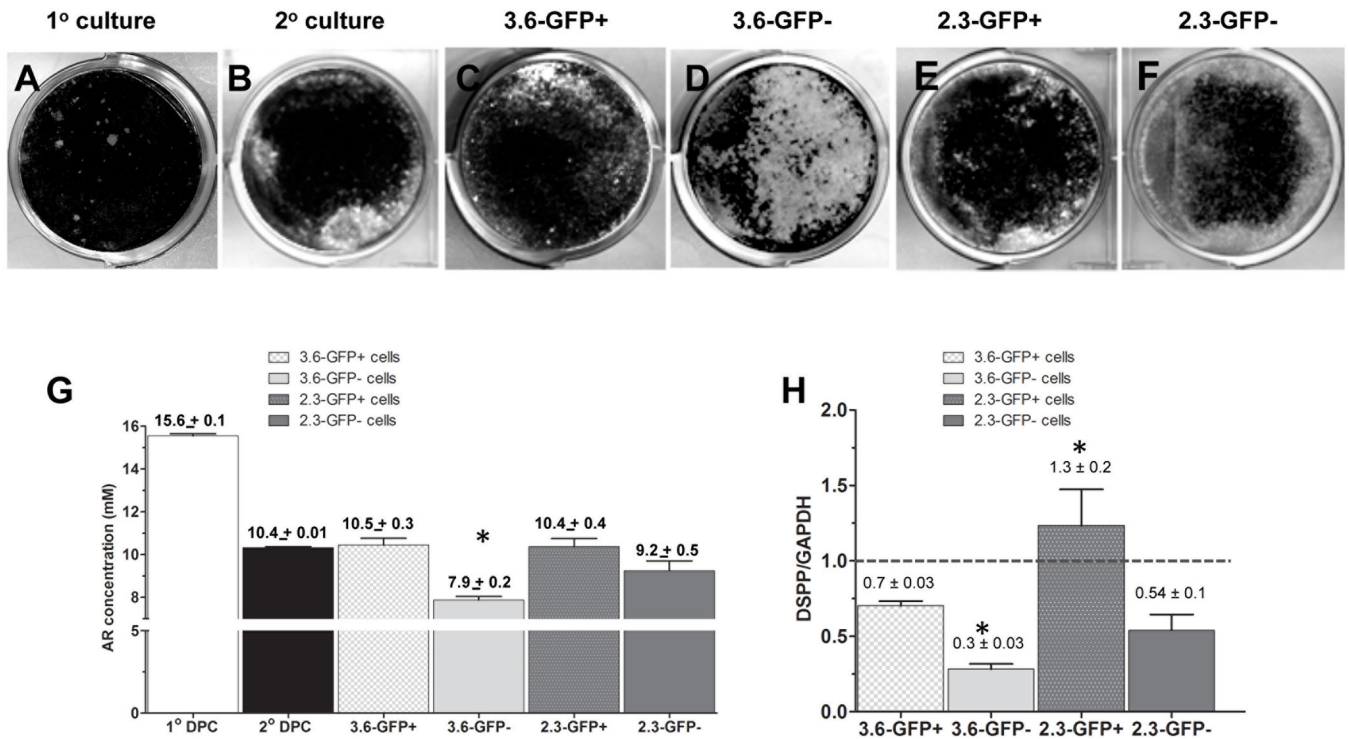


Figure 5. Differentiation potentials of cultures from Colla1-GFP+ and Colla1-GFP- populations
A–F represent images of von Kossa staining of mineralized tissue in cultures established from unsorted cells without re-plating (primary cultures) (A), cultures from unsorted cells that were re-plated after 7 days (secondary culture) (B), 3.6-GFP+ (C), 3.6-GFP- (D), 2.3-GFP+ (E) and 2.3-GFP- (F) cells after 21 days.

(G) Histogram showing the amounts of extracted Alizarin Red staining in different cultures after 21 days. Note the decrease in the mineralization in secondary pulp cultures as compared to primary pulp cultures. The amounts of extracted Alizarin Red staining were similar between secondary unsorted cells, 3.6-GFP+, 2.3-GFP+ and 2.3-GFP- populations. There was mineralization in 3.6-GFP- cultures. Values represent the concentration of the extracted Alizarin Red calculated from the mean absorbance ± S.E. for at least three independent experiments with multiple samples in each experiment (* $p < 0.05$).

(H) Histogram showing the relative levels of *DSPP* after 21 days in various cultures. Note the increased levels of *DSPP* in cultures from 2.3-GFP+ as compared to other cultures. Also note that the relative levels of *DSPP* were similar in 2.3-GFP- and 3.6-GFP+ cultures. The relative levels of *DSPP* in 3.6-GFP cultures were lower than all other cultures. The dash line represents the *DSPP/GAPDH* in secondary cultures that was arbitrary set to 1. Values represent the mean ± S.E. of *DSPP/GAPDH* for at least three independent experiments (* $p < 0.05$).

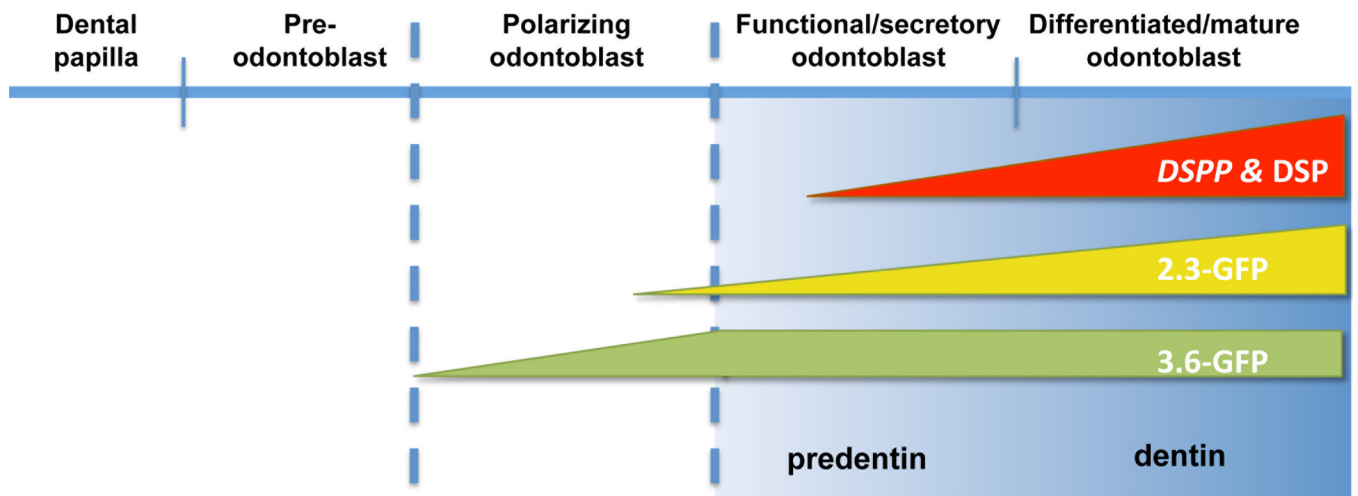


Figure 6. Schematic representation of proposed stages of activation of 3.6-GFP and 2.3-GFP transgenes during odontoblast differentiation. *DSPP* was used as a marker of early and later stages of mineralization.

Table 1

Expression of GFP in primary pulp cultures obtained from pOBCol3.6GFP and pOBCol2.3GFP animals. FACS analysis of dental pulp cultures at different time points (days 2, 7, 10 and 14). Values represent means \pm S.E. from at least three individual experiments. A) Percentage of GFP+ and GFP- populations. B) Distributions of cells expressing of GFP at high and low intensity within the GFP+ populations. Arrows in Figure 2 demonstrate the separation border between cells expressing high and low levels of GFP.

A			
	Days in culture	% of GFP+ cells	% of GFP- cells
3.6-GFP	2	44.67 \pm 2.7	55.3 \pm 2.7
	7	57.3 \pm 1.5	42.7 \pm 1.5
	10	61.3 \pm 1.8	38.7 \pm 1.8
	14	79.3 \pm 1.5	20.7 \pm 1.5
2.3-GFP	2	49.6 \pm 1.8	50.4 \pm 1.8
	7	72.9 \pm 11.8	27.1 \pm 11.8
	10	83.6 \pm 10.5	16.4 \pm 10.5
	14	92 \pm 3.0	8.0 \pm 3.0

B			
		GFP+ cells	
	Days in culture	High intensity	Low intensity
3.6-GFP	2	31.3 \pm 4.3	13.3 \pm 3.5
	7	32.3 \pm 2.2	25 \pm 2.6
	10	39.7 \pm 0.9	21.3 \pm 4.5
	14	48.3 \pm 2.0	30.3 \pm 1.5
2.3-GFP	2	20.5 \pm 3.7	29.1 \pm 2.4
	7	45.1 \pm 9.7	27.7 \pm 4.4
	10	65.0 \pm 16.9	18.6 \pm 6.5
	14	70.1 \pm 12.8	21.9 \pm 10.4

Table 2
Analysis of cell proliferation using Hoechst 33342 dye

Three day old primary pulp cultures derived from pOBCol3.6GFP and pOBCol2.3GFP animals were trypsinized and incubated with Hoechst 33342 for 90 minutes. Between 10,000–20,000 cells were FACS analyzed and results were obtained using ModFit Software. Results represent mean \pm S.E. from at least three experiments.

	Phases of the cell cycle	GFP–	GFP+
Col3.6GFP	G ₀ G ₁	53.6 \pm 1.8	56.8 \pm 2.4
	S	27.8 \pm 1.8	21.8 \pm 1.5
	G ₂ M	18.6 \pm 1.2	21.0 \pm 1.2
Col2.3GFP	G ₀ G ₁	52.6 \pm 1.8	57.8 \pm 2.4
	S	6.9 \pm 1.6	18.1 \pm 1.9
	G ₂ M	40.6 \pm 0.4	24.2 \pm 0.7