Odontoblast Cells Immortalized by Telomerase Produce Mineralized Dentin-like Tissue Both in Vitro and in Vivo*

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Received for publication, December 20, 2001, and in revised form, March 18, 2002
Published, JBC Papers in Press, March 19, 2002, DOI 10.1074/jbc.M112223200

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The formation of dentin provides one well accepted paradigm for studying mineralized tissue formation. For the assembly of dentin, several cellular signaling pathways cooperate to provide neural crest-derived mesenchymal cells with positional information. Further, “cross-talk” between signaling pathways from the mesenchymal derived odontoblast cells and the epithelially derived ameloblasts during development is responsible for the formation of functional odontoblasts. These intercellular signals are tightly regulated, both temporally and spatially. When isolated from the developing tooth germ, odontoblasts quickly lose their potential to maintain the odontoblast-specific phenotype. Therefore, generation of an odontoblast cell line would be a valuable reproducible tool for studying the modulatory effects involved in odontoblast differentiation as well as the molecular events involved in mineralized dentin formation. In this study an immortalized odontoblast cell line, which has the required biochemical machinery to produce mineralized tissue in vitro, has been generated. These cells were implanted into animal models to determine their in vivo effects on dentin formation. After implantation, we observed a multistep, programmed cascade of gene expression in the exogenous odontoblasts as the dentin formed de novo. Some of the genes expressed include the dentin matrix proteins 1, 2, and 3, which are extracellular matrix molecules responsible for the ultimate formation of mineralized dentin. The biological response was also examined by histology and radiography and confirmed for mineral deposition by von Kossa staining. Thus, a transformed odontoblast cell line was created with high proliferative capacity that might ultimately be used for the regeneration and repair of dentin in vivo.

Cells destined to form the mineralized tissues are derived from three distinct lineages. The cranial skeleton develops from neural crest cells. Most of the axial skeleton like skull, ribs, and sternum comes from the sclerotomes. Finally, the appendicular bones are derived from lateral plate mesoderm. Neural crest cells additionally have the plasticity to differentiate into multiple mesenchymal derivatives such as bone, cartilage, dentin, and dental pulp (1). Odontoblasts, which are responsible for the synthesis of dentin (2), arise from neural crest progenitors that have undergone differentiation in a multistep lineage pathway.

At its later stages, odontoblast development involves cells called preodontoblasts, which, after a number of cell divisions, terminally differentiate into postmitotic tall columnar odontoblast cells responsible for the synthesis of a mineralized matrix. This event is highly coordinated and regulated by extracellular matrix molecules, signaling molecules, growth factors, and their receptors. During the differentiation process, precise cell-cell, cell-matrix, and matrix-matrix interactions are responsible for triggering odontoblast differentiation and synthesis of the key components responsible for matrix calcification.

Researchers have implicated several regulatory factors and complex interactions with the epithelially derived ameloblasts in the regulation of odontoblast differentiation (3). Regulatory events that occur during dentinogenesis result in the onset of odontoblast-specific gene expression for dentin matrix protein 2 (DMP2) and dentin sialoprotein (DSP) (4). Various other phosphoproteins synthesized by the odontoblasts, such as dentin matrix protein 1 (DMP1) along with DMP2, may play a structural and/or regulatory role in the calcifying organic matrix by providing sites for apatite nucleation. The steric arrangement of phosphate groups in these phosphoproteins may be optimal for the binding of calcium and presumably for the subsequent heterogeneous formation of apatite crystals.

The differentiation of odontoblasts along with the expression of the dentin sialophosphoprotein (DSPP) or DMP3 and DMP1 are necessary for mineralized dentin formation (5–7). Takagi and Veis (8) first suggested that defects in the genes for dentin extracellular noncollagenous proteins (NCPs) were likely candidates for the dentinogenesis imperfecta Type II, a rare disorder marked by mineralization defects. Takagi et al. (9) further suggested that dentinogenesis imperfecta patients might show reduced levels of acidic phosphoproteins such as dentin phosphophoryn (now known as DMP2). Recently, a nonsense mutation in exon 3 of DSPP gene was reported to be responsible for one case of dentinogenesis imperfecta Type II (10). Only two dentin-specific NCPs, the phosphophoryns and DSP have been identified thus far (4, 6). Although many investigators have demonstrated the abundance of these proteins in the dentin matrix, their precise role is not known.

Deciphering the regulatory mechanisms involved in the terminal differentiation and synthesis of odontoblast-specific matrix molecules requires a homogenous population of cells. However, primary odontoblast cells cannot be maintained in cultures for a long time due to their finite life span (3, 11). Several methodologies have been employed by investigators to

* This work was supported by the Department of Orthodontics at University of Illinois at Chicago (to C. E.) and NIDCR, National Institutes of Health Grant DE11657 (to A. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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§ The abbreviations used are: DMP, dentin matrix protein; DSP, dentin sialoprotein; DSPP, dentin sialophosphoprotein; NCP, noncollagenous proteins; hTERT, human telomerase reverse transcript.
develop cell lines from various mineralized tissues, the most common being SV-40 large "T" antigen gene (12–15); others have achieved proliferative potential by immortalizing primary cells with various oncogenes (16, 17). It has been demonstrated that cultured primary cells can circumvent senescence and continue to proliferate when transformed by a number of agents. Here we report the successful immortalization of rat odontoblasts using a gene for telomerase, a multicomponent enzyme that regulates chromosomal length upon cell division. As telomerase is composed of a template RNA plus an essential catalytic subunit of human telomerase reverse transcript (hTERT) (18), functional telomerase activity can be reconstituted by providing the catalytic subunit absent from somatic cells through ectopic expression of hTERT (19). In this study, odontoblast cells have been immortalized by reconstitution of telomerase activity. The result was immortal cell cultures that are highly proliferative and can grow without senescence. These immortalized cells demonstrate the presence of fully differentiated odontoblast-specific markers, namely DMP1, DMP2, and DMP3, and also have the potential to produce a mineralized dentin matrix both in vitro and in vivo. The availability of this cell line will provide a unique opportunity to determine the mechanisms responsible for differentiation of the neural crest-derived cells into odontoblast-like cells. These cells could ultimately be manipulated for tissue engineering of damaged dentin and to identify novel genes that are odontoblast-specific.

MATERIALS AND METHODS

Isolation of Primary Tooth Germs and Cell Cultures—First and second molar tooth germs were dissected aseptically from 3–5-day-old Sprague-Dawley rats, and primary cell culture was conducted (20). Briefly, the tissues were cut into small particles (about 0.1 cm³) and incubated with 10 ml of 0.25% trypsin and 1 mM EDTA (Invitrogen) at 37 °C for 30 min. The released cells were passed through a 100-μm cell strainer (BD Labware) and pelleted by centrifugation (3600 rpm for 10 min). The pellet was resuspended and maintained in the culture medium Dulbecco’s modified Eagle’s medium/ F-12 (Invitrogen) supplemented with 15% (v/v) fetal bovine serum (Cellgro), 100 IU/ml penicillin/streptomycin, 5 μg/ml fungizone, and 50 μg/ml ascorbic acid. The medium was changed every 3 or 4 days.

Transduction of Primary Cell Culture—80% confluent primary tooth germ cells were transected with pCI-Neo-hTERT (kind gift from Weinberg Laboratories, Whitehead Institute for Biomedical Research, MIT) and pMX1-SV40T-Neo-195 (kind gift from Dr. S. R. Winn, Division of Plastic and Reconstructive Surgery, Oregon Health Sciences University, Portland, OR) by using FuGENE (Roche Molecular Biochemical, Basel, Switzerland) and Polyfect (Qiagen), as described by the manufacturer. Forty-eight hours after transfection, the cells were replated at a low density and were then selected with 0.7 mg/ml G418 sulfate (Sigma).

Isolating a Single Clone—A standard sterile disc cloning (BelArt Products) method selectively removed well isolated colonies. They were replated at low densities to obtain secondary colonies, which were then expanded into cell lines. Two single clones (T4-4 and T3-2) from hTERT transfection and one (A4-4) from SV40 large T antigen transfection were identified by this method, and they exhibited characteristic odontoblast morphology. These cells were expanded, passaged, and assayed for different phenotypic markers.

Reverse Transcription-PCR—Total RNA was extracted from A4-4, T3-2, and T4-4 cell lines and rat tooth germs using Trizol reagent (Invitrogen) and treated with DNaseI (RNase-free, RQ1, Promega). Three micrograms of total RNA was reverse-transcribed for 90 min at 42 °C with Superscript II (Invitrogen). PCR Supermix (Invitrogen) was used in all of the PCR reactions. Primers for the PCR reaction for DMP1, DMP2, DMP3, alkaline phosphatase, collagen type I, ameloblastin, CBFA1, cytomegalovirus, and glyceraldehyde-3-phosphate dehydrogenase were designed from available sequences at the National Center for Biotechnology Information gene data bank. The PCR products were verified by sequencing. For DMP2 Northern blot the primers were designed from the untranslated region.

Analysis of Gene Expression by Northern Blot—Northern blots were performed as described by Sambrook et al. (21). mRNA was extracted from cultured cells at the 22nd passage using a FastTrack Kit (Invitrogen). Five-microgram samples of mRNA were resolved on 0.8% agarose gels containing formaldehyde. The RNA was transferred to a Hybond nylon membrane (Amersham Biosciences). The membrane was prehybridized with the use of HyperHb (Research Genetics, Huntsville, AL) and probed with randomly labeled (Decaprime kit; Ambion) appropriate probes. The probes used were for DMP1, DMP2, DMP3, and glyceraldehyde-3-phosphate dehydrogenase.

Immunohistochemistry—Transfected cell clones at passage 23 were seeded on glass chamber slides (BD Labware) and maintained in standard medium supplemented with 0.2 mg/ml G418 for 7 days. Cells were fixed with 10% formalin in neutral buffer (Sigma) and incubated overnight with monoclonal antibodies against DMP1 (22), DMP2 (gift from Dr. A. Veis), and DMP3 (gift from Dr. W. Butler) and hTERT polyclonal antibody (sc-7214, Santa Cruz Biotechnology). Thereafter, cells were incubated with fluorescein isothiocyanate-conjugated appropriate secondary antibody (Sigma) for another 2 h. Microphotographs were taken using a confocal microscope.

Detection of Telomerase Incorporation—The pCI-Neo-hTERT expression vector carries a strong constitutive cytomegalovirus promoter and the neomycin gene to confer resistance to G418 sulfate for selection of stable transfectants. The stable transfectants were confirmed by reverse transcription-PCR with primers specific for cytomegalovirus promoter and immunostaining with hTERT antibody.

In Vitro Induction of Mineralized Nodule Formation—The mineralization microenvironment was created by treating T4-4 cells at passage 24 (80–90% confluent) with 100 μg/ml ascorbic acid and 10 mM β-glycerophosphate along with 10 nm dexamethasone for 45 days. mRNA was extracted from the T4-4 cell line at 0, 15, 30, and 45 days for Northern blot analysis to check the expression of DMP’s during the mineralization process.

Fon Kossa Staining—The T4-4 cells undergoing mineralization (40 days in culture) were fixed with 10% formalin in neutral buffer (Sigma) for 15 min. The slides were washed with distilled water and then treated with 1% AgNO₃ for 1 h, washed again with distilled water, and treated with 2.5% sodium thiosulfate for 5 min. The specimens were counterstained and then examined under a light microscope.

X-ray Powder Diffraction—The samples were dried at room temperature and mounted on slides. X-ray diffraction patterns of different mineralized samples was carried out with a Siemens D5000 diffractometer with nickel-filtered CuKa radiation (I = 1.54 Å), at a scanning rate of 1°/min.

Assay for in Vivo Mineralization—About 5 × 10⁶ of T4-4 cells at passage 25 were mixed with 0.5 × 0.5 cm² of Collagraft (Zimmer, Warsaw, IN), incubated at 37 °C for 0.5 h, and then transferred subcutaneously on the dorsal surface and intramuscularly (quadriceps muscles) in 15-week-old Sprague-Dawley rats. The procedures were performed in accordance with specifications of an approved small animal protocol (UCIC No. 99–040). Radiographs were closely examined for signs of mineralized tissue formation every 10 days. At 12 weeks post-transplantation, the transplants were removed, fixed with 10% formalin, decalcified with buffered 10% EDTA (pH 7.4) for a short duration, and then embedded in paraffin. Sections (5 μm) were deparaffinized, and stained for histological and immunohistological evaluation. Collagraft with RPC-C2A, a dental pulp cell line (23), was used as a control.

RESULTS

Transformation of Primary Odontoblast Cells—The telomerase-containing plasmid was transfected into 3-day-old rat molar tooth germ cells. Cells were selected for neomycin by G418 in the medium. Several individual single cell clones were isolated, and integration of the telomerase gene was detected by PCR and immunostaining. Two of these transfected clones, T4-4 and T3-2, are currently being maintained in culture for over 60 passages. Fig 1A and B, depicts the primary cells from the rat tooth germ after 1 h and 2 days in culture, respectively. The immortalized cells during G418 selection are shown in Fig 1C. Fully differentiated odontoblasts are characterized by a polarized morphology with the nucleus situated at the basal third of the cell body and a long odontoblastic process (gift from Dr. A. Veis), as depicted in Fig 1D. These are single telomerase-expressing cells which has the characteristic odontoblastic phenotype. Fig 1E and F, shows transformed single cells having the same characteristic odontoblast-like morphology. As the morphology of the transformed cells resembled the native odontoblasts, telemorase appears to have conferred upon primary cells the

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ability to express phenotypic characteristics of differentiated odontoblasts.

**Transformed Cells Have Odontoblast-like Phenotype**—To confirm that the identified cell clones were functioning as differentiated odontoblasts we used reverse transcription-PCR to measure gene expression of various NCPs. Fig. 2A shows that the transformed cell line synthesized various dentin NCPs like dentin matrix protein 1, 2, and 3. The transformed cells also expressed other extracellular matrix proteins such as collagen type I, alkaline phosphatase, and CBFA1 (Fig. 2A) as soon as the cells were confluent (6 days in culture). Northern blot analysis further confirmed the expression of odontoblast-specific non-collagen genes such as DMP1, DMP2, and DMP3, indicating odontoblastic differentiation (Fig. 2B). Immunocytochemical studies further confirmed that the cell line (T4-4) expressed the major NCPs in dentin, namely DMP1, DMP2, DMP3, and hTERT (Fig. 3). Thus, the transformed cells appear to function as differentiated odontoblasts on the molecular level.

**Expression of DMPs during Mineralization Activity in Vitro**—To determine whether the T4-4 cells could make a mineralized dentin matrix, the cells were cultured in the presence of differentiation medium containing ascorbic acid, β-glycerophosphate, and dexamethasone. Results from the Northern blot data demonstrated that these transformed cells are able to induce the expression of dentin matrix proteins 1, 2, and 3 in the extracellular matrix. Between 0 and 15 days in culture the expression of DMP1 increased but then dropped after 15 days. In situ data showed a similar time course of gene expression (data not shown). Between 0 and 45 days in culture, transformed cells linearly increased their expression of DMP2 and -3 (Fig. 4). Thus, the pattern of expression of the dentin matrix proteins by transformed odontoblasts mimicked the expression pattern by native differentiated odontoblasts.
A2 staining method used for the detection of phosphates (Fig. 5, A1). This was confirmed histologically by the von Kossa staining method for the detection of phosphates (Fig. 5, A2). Transformed cells in culture for 40 days produced calcified nodules, as depicted by black-stained particles in Fig. 5, A2. We further characterized the deposited mineral by x-ray diffraction. The analysis identified the nodules’ composition as crystalline hydroxyapatite, due to the presence of a sharp peak at 2θ = 31.8 (Fig. 5B). Thus the calcification of the extracellular matrix under in vitro mineralization conditions demonstrates that the entire process is a dynamic process resulting from the synthesis of specific matrix molecules and formation of mineralized nodules. Also the formation of mineralized nodules provides a model for dentin-like tissue formation in vitro. Thus the transformed cells behaved just like differentiated cells in vitro.

**Transformed Cells Exhibit Mineralization Activity in Vivo**—To confirm that the in vitro data accurately recapitulate mineralization events in vivo, we implanted the T4-4 cells both subcutaneously and intramuscularly in Sprague-Dawley rats. Collagraft, a three-dimensional scaffold, was used to immobilize the T4-4 cells. The cell-impregnated scaffold had the potential to proliferate and synthesize a mineralized matrix when implanted in rats. After subcutaneous implantation in Sprague-Dawley rats the immobilized cells showed the potential to maintain their differentiated state, proliferate, and synthesize a mineralized matrix within 6 weeks. After 12 weeks of implantation, the cellular scaffolds were harvested and subjected to histological and immunohistochemical analysis. Morphological examination showed no evidence of inflammation or rejection. Transformed, explanted cells appeared well organized surrounded by a mineralized matrix that was laid down in a polarized manner (Fig. 7A). Protein expression using polyclonal antibodies directed against DMP1 (Fig. 7D) and the DSP portion of DMP3 (Fig. 7E) shows that the transformed cells not only expressed the proteins intracellularly but also in the mineralized matrix. An antibody directed against telomerase confirmed the identity of the matrix-producing cells, as the implanted transformed cells (data not shown). Terminal differentiation of native odontoblasts is accompanied by the synthesis of NCPs such as DMP1, DMP2, and DMP3, which have been hypothesized to play a functional role in mineralization.

Radiographic evaluation further demonstrated that the transformed cells formed mineralized tissue. The intensity of the matrix synthesized by the implanted cells increased with time compared with the control group (Fig. 6). von Kossa (Fig. 7B) and Masson’s trichrome blue (Fig. 7C) stain confirmed the presence of calcium phosphate and collagen in the mineralized matrix. As a control, we implanted RPC-C2A dental pulp cells, while these cells also secreted matrix proteins the level of secretion was much lower and there were no obvious mineralized tissues after von Kossa staining. Further immunostaining for DMP1 and DMP3 was negative in the transplanted scaffold and cells (Fig. 7, F and G). In summary, the in vivo results demonstrated that T4-4 transformed cells could differentiate into odontoblast-like cells and exhibited the gene products and mineralization events characteristic of terminally differentiated, native odontoblasts.
respectively. The representative immunostaining for DMP1 and the DSP portion of DMP3, stained for collagen fiber, higher magnification of trichrome (A), Masson's trichrome (B), Masson's trichrome-stained for collagen fiber, higher magnification of A (C). D and E represent immunostaining for DMP1 and the DSP portion of DMP3, respectively. The box in A indicates the region shown in B to E. F and G represent immunostaining for DMP1 and the DSP portion of DMP3 in control cells.

**DISCUSSION**

Odontogenic differentiation in culture has not been well characterized due to two major limitations: the limited life span of primary cells and the paucity of differentiation markers. Odontoblasts are terminally differentiated cells, thus they cannot be further induced for differentiation. Recent advancements in identification and cloning of dentin matrix proteins by other laboratories and ours have helped tremendously in the area of identifying markers for terminally differentiated odontoblasts (5, 6). The understanding of signaling mechanisms involved in dentin formation remains in its infancy even though substantial progress has been made in identifying the fundamental pathway required for tooth patterning. We anticipate that the development of an odontoblast cell line will not only be useful for identifying novel targets and signaling pathways for dentin formation but will also help in the field of gene therapy for regeneration of dentin.

Various tissue-specific cell lines have been immortalized using telomerase (24–26). In our studies, we transformed cells from the developing tooth germ of rats, the primary repository for odontogenic cells, with a human gene encoding the reverse transcriptase subunit telomerase. Incorporation of the gene in rat odontoblasts resulted in a considerable extension of their life span and permitted the cells to proliferate without limitation. Morphological features of the transformed odontoblast-like cells were similar to in vivo odontoblasts: both show long cytoplasmic processes attached to one end of the cell body and a polarized distribution of the nucleus, the rough endoplasmic reticulum, and the Golgi stack at the other (3). We also observed that the transformed cells had the necessary biochemical program for differentiation and were capable of producing a mineralizing extracellular matrix both in vitro and in vivo.

In particular, we looked at a transcription factor known to be essential for tissue-specific gene expression. Researchers have identified CBFA1 as an essential transcription factor for osteogenesis and chondrogenesis (27, 28) and showed that the gene product plays an essential role in osteoblast differentiation. Mutations of CBFA1 gene result in abnormal skeletal genesis and various dental disorders. The expression of CBFA1 in our immortalized cells corroborates well with a recent report that demonstrates the high level of expression of this transcription factor in differentiated odontoblasts (29). Having a cell line such as the telomerase-transformed rat odontoblasts will be a valuable asset for pinpointing additional transcription factors that control differentiation of the ectomesenchymal neural crest cells into odontoblasts.

The immortalized cells had the potential to synthesize principal extracellular matrix components that are necessary for the assembly of dentin matrix, namely type I collagen, which forms the scaffold for mineral deposition, and NCPs, which are responsible for nucleating and regulating the hydroxyapatite crystal size. The dentin matrix proteins have been proposed to function as regulatory molecules during the ordered deposition and organization of hydroxyapatite. Specifically DMP2 and DMP3 or DSPP defines the phenotypic characteristics of dentin. *In situ* hybridization studies during development have demonstrated that the extracellular matrix genes DMP1, DMP2, and DMP3 are synthesized and secreted by odontoblasts at the mineralization front only after they enter the secretory stage. Therefore, identification of these terminally differentiated odontoblast-specific markers would establish the presence of a true odontoblastic cell line. As T4-4 and T3-2 odontoblast-like cell lines have all the characteristics of an odontoblastic phenotype, they will likely provide an excellent reproducible in vitro system for studying odontoblast-specific gene expression.

The biological potential for these cells to form a mineralized tissue was demonstrated by the *in vivo* implantation studies. The implanted cells were completely polarized and functional. We observed a distinct calcified tissue phenotype coinciding with the expression of the dentin matrix proteins within the microenvironment of the implanted site. We confirmed the presence of phosphate in the mineralized tissue with von Kossa staining. Phosphate is a major component of mineralized tissue matrix that functions to sequester increased calcium ions and helps cells maintain homeostasis (30). Recently Gronthos et al. (31) showed that dental pulp stem cells, when transplanted into immunocompromised mice, generate a dentin-like mineralized structure. These results support our evidence that telomerase-transformed cells function as bona fide differentiated odontoblasts.

The main advantage of developing an odontoblast cell line is that these cells maybe used to study the signaling mechanisms involved in terminal differentiation of odontoblasts, isolation...
and characterization of odontoblast-specific genes, and tissue engineering of these cells for the formation of reparative dentin. The molecular mechanisms during reparative dentin formation have not been systematically investigated. The transformed cells reported here could be efficiently propagated in culture with sufficient proliferative capacity to produce enough dentin.

In conclusion, the present study demonstrates that the T4-4 immortalized cells have unique odontogenic potential along with the capability to form mineralized matrix both in vitro and in vivo. The ultrastructural morphology of matrix mineralization and expression of odontoblast-specific differentiation markers are some of the salient features of the T4-4 odontoblast cell line. This cell line will be a valuable tool for identifying and characterizing the different signaling molecules and transcription factors responsible for the complete differentiation of the odontoblasts as the process of odontoblast differentiation in vitro recapitulates the in vivo situation. An important functional application would be the manipulation of these cells for tissue engineering of mineralized tissues especially in the reparative formation and regeneration of dentin.

Acknowledgments—We thank Verna J. Brown for histological staining, Isac D. Johnson for x-ray photography, and Mei Ling Chen for assisting in confocal laser-scanning microscopy.

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