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Odontoblast-Targeted Bcl-2 Overexpression Impairs Dentin Formation

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ABSTRACT

Apoptosis has been described extensively in tooth development, which is under tight control of multiple apoptosis regulators, including anti-apoptotic protein Bcl-2. However, it is totally unclear how Bcl-2 is related to odontogenesis, especially dentinogenesis. Using a transgenic mouse Col2.3Bcl-2 in which human Bcl-2 was overexpressed in odontoblasts, the effect of Bcl-2 on dentinogenesis was investigated. Overexpression of Bcl-2 was detected by immunohistochemistry and Western blot. Odontoblast apoptosis was evaluated by TUNEL and Western blot detection of cleaved caspase-3. Odontoblast differentiation was assessed by real-time PCR detection of dentin matrix expression. Dentin mineralization was evaluated by micro-CT in vivo, and alizarin red S staining and calcium content analysis in vitro. Bcl-2 was found to be overexpressed in odontoblasts and prevent their apoptosis. Odontoblast differentiation and mineralization was inhibited by Bcl-2, as evidenced by lower expressions of DMP-1, OC, and DSPP, and decreased odontoblast mineralization in vitro, as well as decreased dentin thickness and mineral density in vivo when compared to the wild-type animals. Inhibition of odontoblast differentiation by Bcl-2 occurs, at least partially, via a suppression of MEK-ERK1/2 signaling pathway. In conclusion, Bcl-2 overexpression prevents odontoblast apoptosis and impairs dentin formation, partially via an inhibition of odontoblast differentiation. This study revealed some novel functions of Bcl-2 in dentinogenesis in addition to its anti-apoptotic effect, which shed some light on the genetic complexity of tooth development. J. Cell. Biochem. 111: 425–432, 2010. © 2010 Wiley-Liss, Inc.

KEY WORDS: ODONTOBLASTS; APOPTOSIS; Bcl-2 OVEREXPRESSION; DENTIN

Tooth development involves complex reciprocal interactions between the oral epithelium and cranial neural-crest-derived mesenchymal cells. A developing tooth bud is composed of enamel organ, dental papilla, and dental follicle, which gives rise to ameloblasts, odontoblasts, and cementoblasts, respectively. Ameloblasts form enamel which is the hard outer shell that covers the crown of the tooth. Dentin, deposited by odontoblasts, is the bone-like substance that is under the enamel in the crown and under the cementum in the root. Cementum covers the root surface and is produced by cementoblasts. The pulp or root canal is the open space inside the tooth which accommodates blood vessel and nerves that provide nutrients and sensation to the tooth [Nanci, 2008].

Apoptosis, a physiological process important for remodeling tissues in multicellular organisms, has been detected during embryonic tooth development [Vaahtokari et al., 1996]. It occurs sequentially in budding epithelium, epithelial enamel knot, and condensed mesenchyme in a developing tooth organ [Matalova et al., 2004]. During enamel formation, approximately 50% of ameloblasts undergo apoptosis, which is associated with decreased height and denseness of remaining cells [Smith and Warshawsky, 1977]. During dentinogenesis, apoptosis is found in odontoblasts,

Abbreviations used: Bcl-2, B-cell lymphoma-2; βGP, β-glycerophosphate; Cbfa-1, core-binding factor alpha-1; Col1a1, type I collagen; DMP-1, dentin matrix protein-1; DPP, dentin phosphoprotein; DSPP, dentin sialophosphoprotein; ECM, extracellular matrix; ERK, the extracellular signal-regulated kinase; ERK-P, phosphorylated extracellular signal-regulated kinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; hBcl-2, human Bcl-2; OC, osteocalcin; RT, room temperature; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; +/+, wild type; tg/+, heterozygous transgenic; tg/tg, homozygous transgenic.

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sub-odontoblastic regions, central pulp fibroblasts, and perivascular endothelial cells [Vermelin et al., 1996; Franquin et al., 1998]. It appears that apoptosis plays an important role in maintaining homeostasis of various cellular compartments of a developing tooth.

Apoptosis is regulated by several groups of proteins, and B-cell lymphoma-2 (Bcl-2) family is a key group among them. It includes both anti- (Bcl-2, Bcl-XL, Bcl-w, Mcl-1 and A1) and pro- (Bax, Bak, Bok, Bad, Bid, Bik, Blk, Hrk, BNIP3 and BimL) apoptotic proteins, which can form heterodimers to modulate each other's functions [Zimmermann et al., 2001]. Bcl-2 is an anti-apoptotic protein that can protect cells from apoptosis elicited by many detrimental stimuli, such as cytokine withdrawal, ultraviolet-irradiation, and cytotoxic drugs [Yang and Korsmeyer, 1996; Strasser et al., 1997]. Bcl-2 has been found in enamel organ [Slootweg and de Weger, 1994], ameloblasts [Kondo et al., 2001], odontoblasts, and subodontoblastic layers [Piattelli et al., 2000]. Bax is an antagonist of Bcl-2, and Bcl-2/Bax ratio determines cell fate. When Bcl-2 outweighs Bax, a cell will survive; otherwise it will die. In mature ameloblasts, increased Bax immunoreactivity and low Bcl-2 expression correlates with massive apoptosis, while in pre-ameloblasts, intense immunohistochemical localization of Bcl-2 and light staining for Bax is consistent with the absence of apoptosis in pre-ameloblasts [Kondo et al., 2001]. Bcl-2 also seems to promote dentinogenesis by preventing trauma-induced odontoblast apoptosis. In mouse molars under artificial cavity preparation, Bcl-2 expression was increased significantly in odontoblasts underlying the cavity, suggesting that elevation of Bcl-2 is an important strategy for odontoblasts to handle injury and maintain the vitality and function of the dentin–pulp complex [Kitamura et al., 2001].

It has been found that Bcl-2 affects cellular adhesion, proliferation, and differentiation besides its role as an anti-apoptotic effector. Depending on the cell types, Bcl-2 can either promote or inhibit these processes [Linette and Korsmeyer, 1994; Limana et al., 2002; Li et al., 2003; Belanger et al., 2005; Weber and Menko, 2005; Zhang et al., 2007]. For example, Bcl-2 promotes the differentiation of epithelial cells [Lu et al., 1996], lymphocytes [Linette and Korsmeyer, 1994], and neurons [Liang et al., 2003]. However, the terminal differentiation of keratinocytes, monocytes, and lens fiber cells is inhibited by Bcl-2 overexpression, probably due to the disruption of apoptosis-related Bcl-2 and caspase-dependent (ABC) differentiation pathway [Harada et al., 1998; Sordet et al., 2002; Weber and Menko, 2005]. It is unknown yet why Bcl-2 has these opposite effects.

In order to determine how Bcl-2 is related to dentinogenesis, a transgenic mouse Col2.3Bcl-2 was employed in the study. The human Bcl-2 (hBcl-2) gene was driven by 2.3 kb fragment of rat type I collagen promoter and thus targeted to tissues rich in type I collagen including dentin. Dentin is constantly formed throughout a tooth's lifetime, starting with active primary dentinogenesis until closure of root apex, and followed by physiological secondary dentin formation at a continuing but much slower rate [Ten Cate, 1998]. Continuous secondary dentinogenesis makes Col2.3 promoter constantly active, resulting in stable expression of transgene in odontoblasts. The tooth phenotype was evaluated in vivo and in vitro to see how Bcl-2 overexpression affects dentin formation.

MATERIALS AND METHODS

ANIMALS

The Col2.3Bcl-2 transgenic mice and their wild-type littermates were imported from University of Connecticut Health Center. The methods of creating the transgenic mice and a PCR-based genotyping were described previously [Pantschenko et al., 2005]. The results reported herein were from Founder 1, which were confirmed by Founder 2. Founder 2 demonstrates slightly stronger transgene expression in odontoblasts than Founder 1, similarly as what was shown in osteoblasts [Pantschenko et al., 2005]. All animal-related experiments were approved by the Center for Laboratory Animal Medicine and Care at the University of Texas Health Science Center at Houston.

PULP CELL CULTURES

Coronal molar pulps of 5-day-old mice were isolated and digested with 0.05% trypsin and 0.1% collagenase P at 37°C for 50 min. Cells were plated at 10^4 cell/cm^2 in α-MEM containing 10% fetal bovine serum (FBS), 100 U/ml of penicillin, and 100 μg/ml of streptomycin. After confluence (normally on day 7), the cells were induced to differentiate with 10^{-8} M dexamethasone, 8 mM β-glycerophosphate (βGP), and 50 μg/ml ascorbic acid in α-MEM for 1 day, then the medium was switched to α-MEM + 10% FBS + 4 mM βGP + 50 μg/ml ascorbic acid, and was changed every other day thereafter. The day of plating pulp cells was counted as day 1, and on subsequent days 7, 14, and 21, protein or RNA was extracted, or biochemical assay was performed on the cultures as mentioned below.

IMMUNOHISTOCHEMISTRY

Hemi-mandibles of mice were isolated, fixed in 10% neutral buffered formalin, decalcified in 3.4% sodium formate/15% formic acid, and embedded in paraffin. Antigen retrieval was performed using 4 N HCl for 10 min at 37°C. Endogenous peroxidase activity was blocked by incubating 10 min with 3% H_2O_2. Nonspecific proteins were blocked with DAKO protein block (Dako, Carpinteria, CA) for 30 min at room temperature (RT). Sections were incubated at RT for 1 h with 4 μg/ml of monoclonal mouse anti-human Bcl-2 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), monoclonal mouse anti-mouse Bcl-2 (Santa Cruz), monoclonal mouse anti-mouse Bax antibody (Santa Cruz), or mouse immunoglobulin (negative control). Secondary antibody and substrate staining were performed with DAKO LSAB+ kit and liquid DAB+ substrate-chromogen system (Dako).

WESTERN BLOTTING

On culture days 7, 14, and 21, pulp cells were lysed in RIPA buffer by shaking at 4°C for 15 min, and spun at 10,000*g* for 10 min. The supernatant protein concentration was determined by BCA assay. Proteins (50 μg) was separated by a 10–20% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) system, and transferred to nitrocellulose membrane. The blots were incubated with 1:100 monoclonal mouse anti-human Bcl-2 (Santa Cruz), mouse anti-mouse Bcl-2 (Santa Cruz), mouse anti-mouse Bax (Santa Cruz), rabbit anti-mouse large fragment (19 kDa) of cleaved

caspase-3 (Cell Signaling Technology, Danvers, MA), polyclonal HRP-conjugated goat anti-actin antibodies (Santa Cruz), 1:1,000 monoclonal rabbit anti-mouse ERK1/2 (Cell Signaling Technology), or monoclonal rabbit anti-mouse phosphor-ERK1/2 antibody (Cell Signaling Technology) overnight at 4°C. The blots were washed, followed by incubation with 1:10,000 goat anti-mouse or -rabbit HRP conjugated secondary antibody for 1 h at RT. Protein bands were visualized using an Immun-Star^{TM} HRP substrate kit (BioRad).

TUNEL STAINING

Apoptotic cells on mouse hemi-mandible sections were detected using TACS TBL kit (R&D Systems, Minneapolis, MN). Light microscopy exam revealed apoptotic odontoblasts as having condensed, blue-stained nuclei. Quantification of apoptotic cells was determined in a blinded nonbiased manner and expressed as percentage of total cell counted.

MICRO-CT ANALYSIS

Hemi-mandibles were isolated, fixed in 70% ethanol, and scanned with an Explore Locus SP pre-clinical Specimen Scanner (GE Medical Systems, London, Ontario). The images were reconstructed using a modified Feldkamp method [Feldkamp et al., 1984]. The incisor dentin thickness was the average of labial and lingual dentin thickness at cervical region where alveolar bone first appears. The molar dentin thickness is the average of buccal and lingual dentin thickness at cervical region of middle sagittal planes of mesial root of mandibular first molars. Results from 10 contiguous slices were averaged. Incisor dentin densities were sampled at three sites: in the tip of the crown underneath enamel, on the lingual root surface close to cervical area, and in the root tip close to apex. Molar densities were measured at two sites: in crown underneath the enamel, and on mesial roots close to apex. The measurement of dentin density was calibrated with a hydroxyl apatite phantom.

ALIZARIN RED S STAINING

On days 7, 14, and 21 of cultures, cells were washed with PBS and fixed in 4% paraformaldehyde for 10 min at RT. Cells were washed again and stained with 2% (w/v) alizarin red S for 2 min at RT, and finally washed with water.

CALCIUM CONTENT

On days 7, 14, and 21 of cultures, cells were treated with 5% trichloroacetic acid (TCA) for 30 min twice at RT. The calcium content in the pooled TCA extracts was measured colorimetrically using a calcium kit (Eagle Diagnostics, De Soto, TX) and normalized to cell number for each well.

REAL-TIME PCR

Total RNA was isolated from pulp cultures on days 7, 14, and 21 by Tri Reagent (Molecular Research Center, Cincinnati, OH). Reverse transcriptase treatment of RNA was performed at 42°C for 80 min using 3 μg of RNA with oligo primers (Invitrogen, Carlsbad, CA) and BD Sprint Powerscript (BD Biosciences, San Jose, CA). Real-time PCR was performed in a BioRad iCycler (BioRad, Hercules, CA). The 10 μl PCR reaction consisted of 4 μl cDNA dilution (40 ng cDNA), 1 μl primers (5 μM), and 5 μl 2× iQ SYBR Green Supermix (BioRad). The PCR conditions were as follows: 1 cycle of 15 min at 95°C, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 20 s, 1 cycle of 95°C for 1 min, and finally 1 cycle of 55°C for 1 min. The PCR amplification was performed in triplicate. The transcript expressions of genes were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primers for type I collagen (Col1a1), osteocalcin (OC), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP-1), core-binding factor alpha-1 (Cbfa-1), and GAPDH were 5′-CACCCCAGCCGCAAAGAGT and 5′-CGGGCAGAAAGCACAGCACT, 5′-TGAACAGACTCCGGCG and 5′-GATACCGTAGATGCGTTTG, 5′-AGTTCGATGACGAGTCC and 5′-GTCTTCTCCCGCATGT, 5′-GCGCGGATAAGGATGA and 5′-GTCCC-CGTGGCTACTC, 5′-CTTCATTCGCCTCACAAAC and 5′-GTCACTG-CGCTGAAGA, and 5′-ACCACAGTCCATGCCATCAC and 5′-TCCA-CCACCCTGTTGCTGTA, respectively.

STATISTICAL ANALYSIS

All figures are representative of three independent experiments. Statistical analysis was performed by Student's *t*-test to determine significance between groups ($P \leq 0.05$).

RESULTS

HBcl-2 IS OVEREXPRESSED IN ODONTOBLASTS OF Col2.3Bcl-2 TRANSGENIC MICE AND PREVENTS ODONTOBLAST APOPTOSIS IN VIVO AND IN VITRO

The Col2.3 promoter is activated in cells secreting type I collagen. In teeth, odontoblasts secrete type I collagen which is principal component of extracellular matrix (ECM) in dentin [Butler, 1995], thus presumably the transgene is overexpressed in odontoblasts. To confirm transgene expression, immunohistochemistry was performed on hemi-mandible sections of 1- to 2-month-old mice. Transgenic animals demonstrated positive hBcl-2 staining in odontoblasts of incisors and molars as well as osteoblasts along alveolar bone surfaces, but wild type had no hBcl-2 expression (Fig. 1A). Transgenic mice at E19 and 3-month old also demonstrated hBcl-2 staining (data not shown). In the molar pulp-derived cultures, transgenic odontoblasts demonstrated stable expression of hBcl-2 throughout 3 weeks, whereas wild type was negative (Fig. 1C). The expression of endogenous mouse Bcl-2 and Bax were comparable between the transgenics and wild type (Fig. 1C), suggesting a Bcl-2/Bax ratio favorable for cell survival in transgenic cultures.

To see if the transgene was functional, a TUNEL assay was performed on the tooth sections of 4- to 6-week-old animals (Fig. 1B). Wild type had higher apoptotic rate than the transgenics in incisors ($12 \pm 2.5\%$ vs. $5 \pm 0.37\%$ as a percent of total cells counted, respectively, $P = 0.043$) and in molars ($14 \pm 2.8\%$ vs. $6 \pm 1.3\%$, as a percent of total cells counted, respectively, $P = 0.037$). To confirm in vivo observation, Western blot for large fragment (19 kDa) of cleaved caspase-3 was run for proteins extracted from pulp cultures. Caspase-3 is an apoptotic executioner, and its cleavage indicates activation of apoptotic cascade [Nicholson and Thornberry, 1997; Ashkenazi and Dixit, 1998]. Wild-type cultures demonstrated stronger cleaved caspase-3 expression than the transgenics on

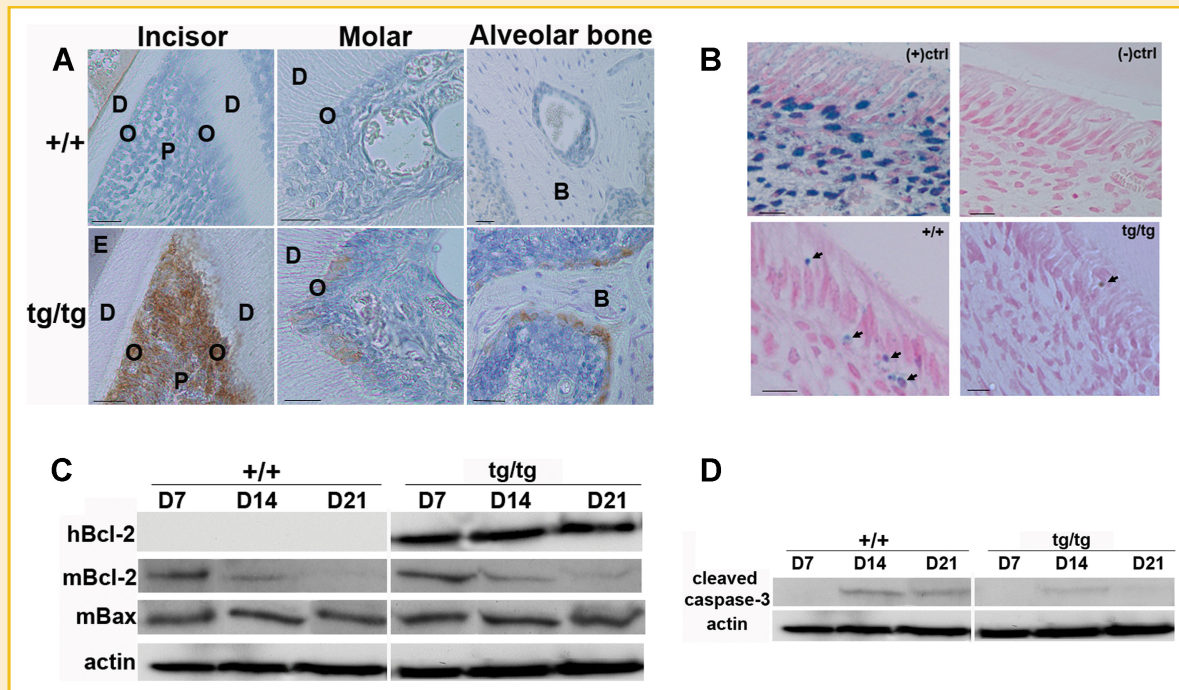

Fig. 1. Human Bcl-2 (hBcl-2) is overexpressed in the odontoblasts of Col2.3Bcl-2 mice and prevents their apoptosis in vivo and in vitro. A: Immunohistochemistry demonstration of hBcl-2 overexpression in the odontoblasts of incisors and molars of 40-day-old Col2.3Bcl-2 mice. The brown color indicates positive staining for hBcl-2. Stronger hBcl-2 staining is noticed in the odontoblasts of the incisors than that of the molars, probably due to the continuous eruption of the incisors and hyperactivity of the Col2.3 promoter. Osteoblasts along alveolar bone surfaces also show positive hBcl-2 staining in the transgenic animals. Wild-type animals demonstrate negative staining for odontoblasts and osteoblasts. +/+, wild type; tg/tg, homozygous transgenic; B, bone; D, dentin; E, enamel; O, odontoblasts; P, pulp. Scale bar = 100 μm. B: TUNEL assay shows a decreased odontoblast apoptosis in the tooth sections of 4- to 6-week-old Col2.3Bcl-2 mice compared with wild type. Black arrows point to apoptotic cells with condensed, blue-stained nuclei. Nuclease treatment or exclusion of TdT is used as (+) and (−) control, respectively. Scale bar = 100 μm. C: Western blot demonstration of hBcl-2 overexpression in the pulp cell cultures derived from Col2.3Bcl-2 transgenic mice. The expression of hBcl-2 is stable in the transgenic cultures over 3-week period. No hBcl-2 is detected in the wild-type cultures. The expression of endogenous mBcl-2 and mBax is not affected by the transgene. Both wild type and transgenic odontoblasts demonstrate decreased mBcl-2 expression with time. Actin is the loading control. +/+, wild type; tg/tg, homozygous transgenic; D, day. D: Western blot demonstrates a stronger cleaved caspase-3 expression in the wild-type cultures compared with transgenics on days 14 and 21, with the difference at day 14 being moderate and at day 21 being more significant. This result indicates a prevention of odontoblast apoptosis by Bcl-2, especially at later culture period. Actin is the loading control. +/+, wild type; tg/tg, homozygous transgenic; D, day.

days 14 and 21 (Fig. 1D), with the difference at day 14 being moderate and at day 21 being more significant. These data indicates a prevention of odontoblast apoptosis by Bcl-2 in vitro.

Bcl-2 OVEREXPRESSION IMPAIRS DENTIN FORMATION

Micro-CT showed that transgenic animals had increased sizes of pulp chamber and root canal, decreased dentin thickness, and increased porosity of alveolar bone (Fig. 2A). Both tg/+ and tg/tg animals had significantly thinner dentin than wild type, and so does tg/tg compared with tg/+ (Fig. 2B), suggesting a dose-dependent effect of Bcl-2.

For dentin density, transgenic animals demonstrated decreased density in molars compared with wild type, indicating an impairment of dentin formation by Bcl-2 (Fig. 2C,D). In incisors, both transgenics and wild type showed increased dentin densities at the apical–incisal direction, as reported previously [Savage et al., 2006], but no difference was noticed between the two genotypes (Fig. 2E,F).

Bcl-2 OVEREXPRESSION INHIBITS ODONTOBLAST DIFFERENTIATION AND MINERALIZATION

Real-time PCR was employed to evaluate odontoblasts differentiation in the cultures. Both wild type and transgenics demonstrated increased expressions of matrix proteins with time, indicating a progressive maturation of odontoblasts under the culture conditions (Fig. 3A). Transgenics showed significantly lower expressions of DMP-1, OC, and DSPP, especially at later culture period, but the expression of early differentiation marker Col1a1 was not affected. To further explore the possible mechanism for the inhibition, the expression of Cbfa-1 was detected. Cbfa-1 is involved in the regulation of ECM expression during dentinogenesis [Aberg et al., 2004]. Similarly as matrix proteins, Cbfa-1 showed increased expression with time, but no difference was noticed between wild type and transgenics (Fig. 3A). The MEK-ERK1/2 signaling pathway has been shown to play a role in regulation of mesenchymal cell differentiation [Bobick and Kulyk, 2004; Yagi et al., 2005]. As shown in Figure 3B, ERK 1/2 pathway was activated in the odontoblast cultures, and tg/tg pulp cells demonstrated significantly decreased

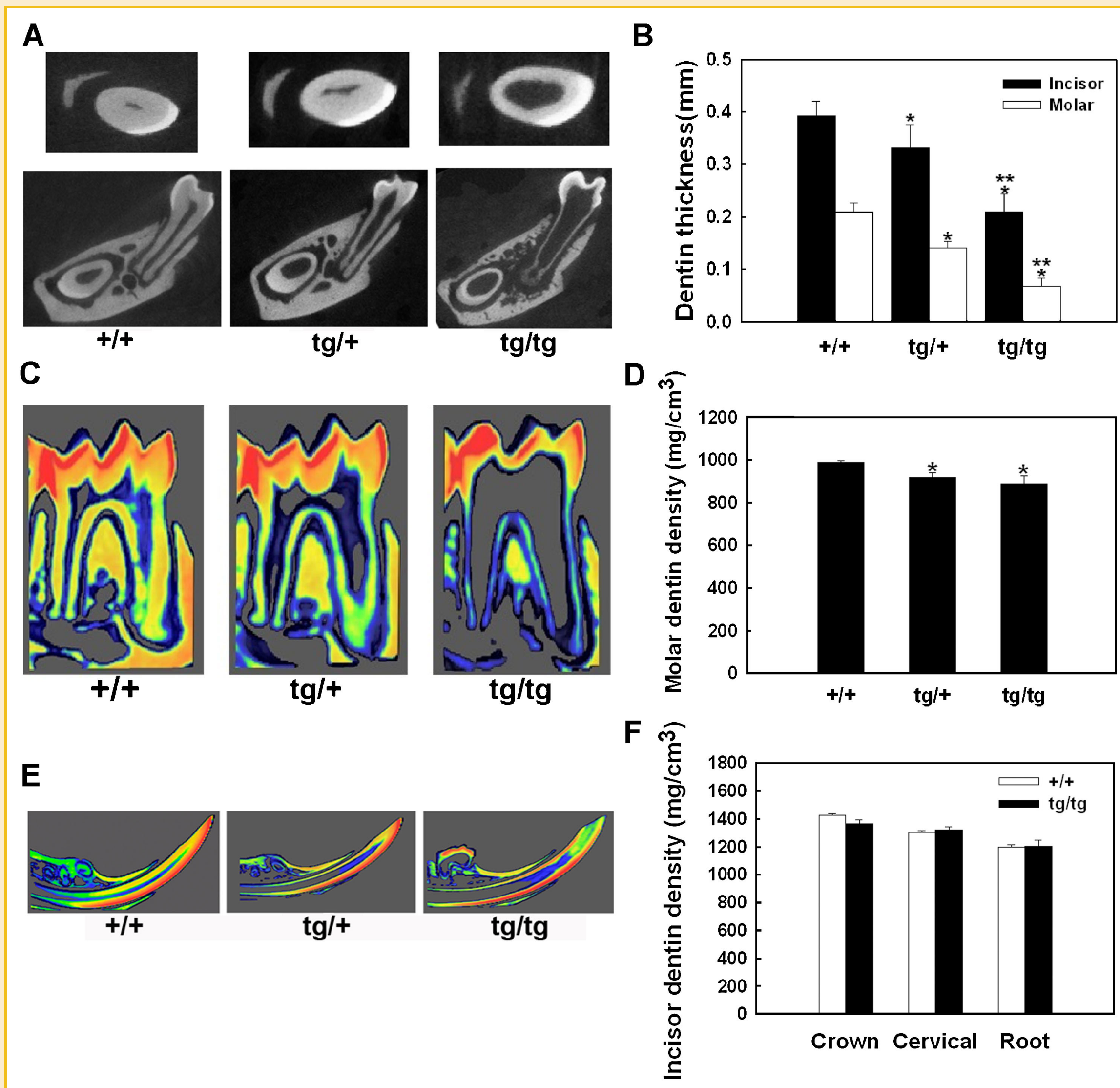

Fig. 2. Odontoblast-targeted Bcl-2 overexpression results in decreased dentin thickness and mineral density in Col2.3Bcl-2 transgenic mice. A: The top panel shows the cross-sections of incisors where alveolar ridges just appear. The lower panel shows the middle sagittal sections of mesial roots of mandibular first molars. Notice the decreased dentin thickness, increased pulp chamber size and porosity of alveolar bone with the increased doses of Bcl-2. B: Both heterozygous and homozygous Col2.3Bcl-2 animals have significantly decreased dentin thickness in their incisors and molars compared with those of wild type, and homozygous have significantly thinner dentin than heterozygous. C: Color-rendered images show the differences in dentin density in the molars. Red represents the highest density, yellow = intermediate and blue = lowest. D: Both heterozygous and homozygous Col2.3Bcl-2 animals have significantly decreased dentin density in their molars compared with those of the wild type. E: Color-rendered images show the dentin density in the incisors. F: Both wild type and transgenic animals show progressive increment of dentin density in the incisors from the apical areas to the incisal surfaces, but no difference is found in the corresponding regions between the two genotypes. +/+, wild type; tg/+, heterozygous; tg/tg, homozygous; $^{*}P < 0.05$ transgenic compared with wild type; $^{**}P < 0.05$ homozygous compared with heterozygous. Data are reported as means ± standard error of the mean (n = 10 for each genotype).

phosphor-ERK 1/2 expressions compared to the wild type on days 7 and 14, suggesting the inhibition of odontoblast differentiation by Bcl-2 occurs, at least partially, via a suppression of ERK 1/2 pathway.

For mineralization, wild type and transgenic cultures progressively deposited more mineral with time as assayed by alizarin red S staining and calcium content analysis (Fig. 4A,B), but transgenics produced much less mineralization than wild type, indicating an inhibition of odontoblast mineralization by Bcl-2.

DISCUSSION

In this study, it has been found that certain fraction of odontoblasts undergo apoptosis without exogenous apoptogenic stimuli, as shown by 12–14% of TUNEL positive odontoblasts in vivo in the wild-type animals and activation of caspase-3 in wild-type mice-derived pulp cultures. It is speculated that odontoblasts need to maintain certain level of turnover to get rid of aged or damaged cells as well as to stimulate the progenitors to differentiate into

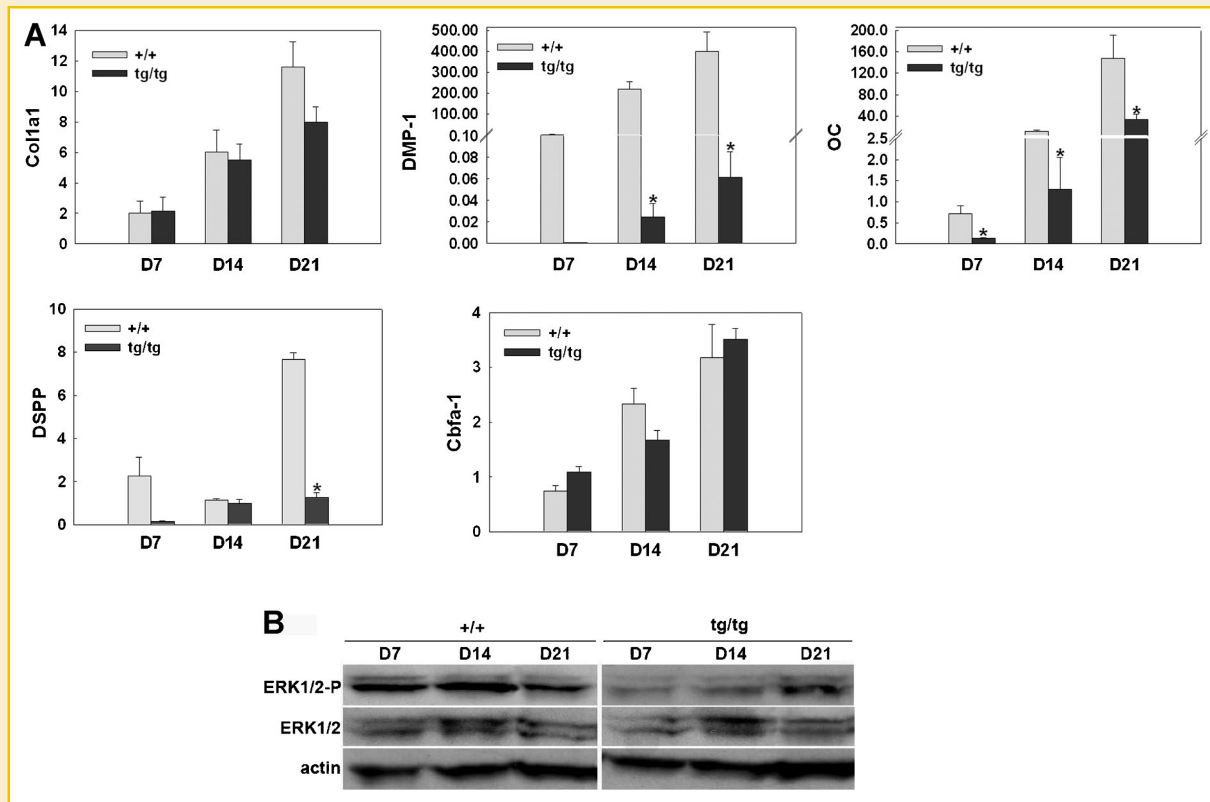

Fig. 3. Bcl-2 overexpression inhibits odontoblast differentiation via a suppression of ERK 1/2 signaling pathway. A: Real-time PCR shows that both wild type and transgenic odontoblasts demonstrate increased expressions of the extracellular matrixes with time, but transgenic odontoblasts show lower levels of expressions, especially DMP-1, OC, and DSPP. There is no difference in Col1a1 and Cbfa-1 expression between the two. +/+, wild type; tg/tg, homozygous transgenic; D, day; Col1a1, type I collagen; OC, osteocalcin; DSPP, dentin sialophosphoprotein; DMP-1, dentin matrix protein-1; Cbfa-1, core-bonding factor alpha-1; $^{*}P < 0.05$ transgenic compared with wild type. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is the internal control. The error bars represent the standard error of the mean. B: Western blot shows that transgenic odontoblasts expressed much lower phosphor-ERK 1/2 expressions than wild type, especially on days 7 and 14, demonstrating that suppression of MEK-ERK 1/2 pathway contributes to the inhibition of odontoblast differentiation by Bcl-2. ERK, the extracellular signal regulated kinase; ERK-P, phosphorylated extracellular signal regulated kinase. Actin is the loading control.

fully functional odontoblasts to maintain a productive odontoblast pool.

Since odontoblast apoptosis is prevented in the Col2.3Bcl-2 transgenic mice, it is speculated that these animals will demonstrate an expansion of the odontoblast compartment unless they undergo a cell-cycle arrest or premature senescence. However, H&E staining of E18 and postnatal day 7 tooth sections do not reveal any significant difference in odontoblast densities between the transgenic and wild-type animals (data not shown). In addition, neither proliferation nor senescence rate of odontoblasts seems to be affected by Bcl-2, as Ki-67, and β-galactosidase staining for proliferative and senescent odontoblasts, respectively, showed similar results in day 7 pulp cell cultures derived from wild type and transgenic animals (data not shown). The seemingly unaffected odontoblast density may result from a proportional alteration of pulp chamber volume (transgenics have larger pulp chambers than the wild type) or other unidentified mechanisms. A thorough analysis of odontoblast behaviors at different time points is expected to help us better understand the phenomena.

Bcl-2 has been shown to facilitates differentiation of osteoblasts via an up-regulation of Cbfa-1, osterix, and Wnt/β-catenin in Col2.3Bcl-2 animals [Pantschenko et al., 2005; Zhang et al., 2007], whereas the results reported herein show that the differentiation of odontoblasts is inhibited by Bcl-2 in a Cbfa-1 independent manner. This is consistent with other reports in the literatures on the opposite effects of Bcl-2 on the differentiation of many other cell types [Lu et al., 1996; Harada et al., 1998; Liang et al., 2003; Weber and Menko, 2005]. Bcl-2 appears to affect the osteoblast lineage at an earlier stage than odontoblasts, since early differentiation markers such as alkaline phosphatase and Col1a1 are elevated in osteoblasts [Zhang et al., 2007], whereas only relatively later markers, such as DMP-1, OC, and DSPP, are inhibited in odontoblasts. Histological study of developing teeth at prenatal stage did not reveal any gross abnormality of tooth structures in the transgenic animals (data not shown), which correlates with relatively late activation of the transgene in dentition. The Col2.3 promoter will not activate until the periphery dental papilla cells adjacent to enamel organ are induced to differentiate into mature odontoblasts and secrete type I collagen, which occurs very late in embryonic development (at approximately embryonic stage E18.5) [Oka et al., 2007]. It is speculated that absence of effect on type I collagen expression and on prenatal dentin phenotype is partially related to the timing when

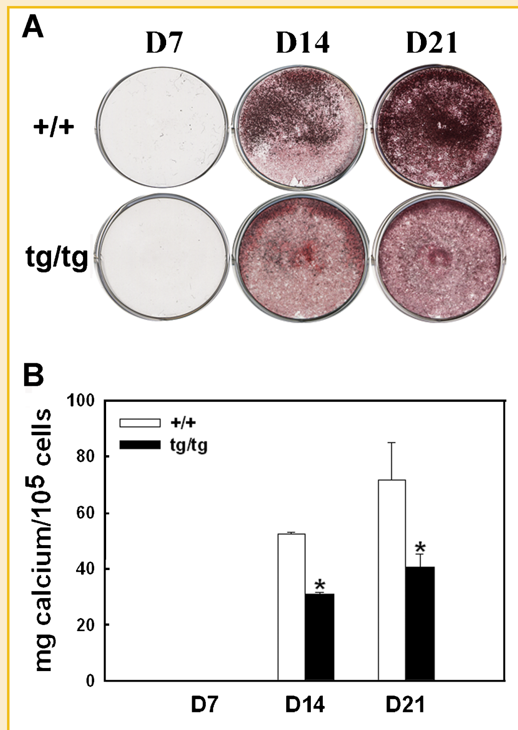

Fig. 4. Col2.3Bcl-2 transgenic pulp cells produced significantly less mineral than wild type in vitro. A: Alizarin red S stains mineralization in the dishes dark red. Both wild type and transgenic odontoblasts progressively produce more mineral with time, but wild type produced more than transgenics on days 14 and 21. B: Calcium content analysis shows significantly lower calcium concentrations in the transgenic cultures than those of the wild type on days 14 and 21. +/+, wild type; tg/tg, homozygous transgenic; D, day; $^{*}P < 0.05$ transgenic compared with wild type. The experiments were repeated independently three times. Error bars represent the standard error of the mean.

the promoter is turned on. Bcl-2 has been reported to positively regulate chondrocyte differentiation by suppressing ERK 1/2 signaling pathway [Yagi et al., 2005]. Our result shows a suppression of ERK 1/2 pathway by Bcl-2 in odontoblasts as well, but interestingly ERK signaling appears to positively regulate odontoblast maturation. There are other transcription factors or signaling molecules which may also be critical for odontoblast differentiation, such as Msx-1, Msx-2, c-Jun, Jun-B, or TGF-β [Begue-Kirn et al., 1992; Begue-Kirn et al., 1994; Kitamura and Terashita, 1997]. Exploring the interactions between Bcl-2 and these factors may shed more light on how Bcl-2 inhibits later stage of odontoblast differentiation.

It has been shown that Bcl-2 dose-dependently inhibits dentin formation, with increasing Bcl-2 resulting in decreased dentin thickness and increased pulp chamber and root canal size. Manipulation of Bcl-2 appears to be a new way of controlling dentin thickness and tooth configuration, which has potential clinical applications in tooth bioengineering.

Transgenic animals demonstrated thinner and less dense dentin in vivo and less mineralization in cultures. Inhibition of odontoblast differentiation by Bcl-2 definitely contributes to impaired dentinogenesis seen in transgenic animals. The noncollagenous matrix proteins, such as dentin phosphoprotein (DPP), have been found to play an initiation and regulatory role in the formation of dentin hydroxyapatite [Boskey, 1991; Butler, 1995]. Defective matrix deposition may contribute to dentin dysplasia observed in transgenic animals. In addition, Bcl-2 seems to regulate apoptosis via an influence on intracellular calcium repartition and homeostasis [Baffy et al., 1993; Magnelli et al., 1994; Rong et al., 2008]. Regulation of calcium trafficking may be another important mechanism for Bcl-2 to regulate biomineralization, as mineralization is inhibited in both bone and teeth although the effect of Bcl-2 on osteoblast/odontoblast differentiation has been identified as opposite.

It is interesting to note that the incisor dentin density is not affected by the transgene. Mouse incisors are continuously erupting, and the dynamic nature of odontoblasts may contribute to different phenotypes observed in incisors versus molars of Col2.3Bcl-2 mice. This observation correlates with the findings in several other transgenic mouse models demonstrating unique features of incisor dentin, and phenotypic differences between their incisors and molars [Opsahl et al., 2005; Savage et al., 2006].

In conclusion, this study demonstrates the prevention of dentinogenesis by odontoblast-targeted Bcl-2 overexpression, partially via an inhibition of odontoblast differentiation. It provides new insight in dental developmental biology, and points to an alternative strategy of controlling the quantity of artificial dentin in tooth bioengineering via a manipulation of Bcl-2.

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