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Reduced Expression of Dentin Sialophosphoprotein Is Associated with Dysplastic Dentin in Mice Overexpressing Transforming Growth Factor-β1 in Teeth*

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Transforming growth factor (TGF)-β1 is expressed in developing tooth from the initiation stage through adulthood. Odontoblast-specific expression of TGF-β1 in the tooth continues throughout life; however, the precise biological functions of this growth factor in the odontoblasts are not clearly understood. Herein, we describe the generation of transgenic mice that overexpress active TGF-β1 predominantly in the odontoblasts. Teeth of these mice show a significant reduction in the tooth mineralization, defective dentin formation, and a relatively high branching of dentinal tubules. Dentin extracellular matrix components such as type I and III collagens are increased and deposited abnormally in the dental pulp, similar to the hereditary human tooth disorders such as dentin dysplasia and dentinogenesis imperfecta. Calcium, one of the crucial inorganic components of mineralization, is also apparently increased in the transgenic mouse teeth. Most importantly, the expression of dentin sialophosphoprotein (*dspp*), a candidate gene implicated in dentinogenesis imperfecta II (MIM 125420), is significantly down-regulated in the transgenic teeth. Our results provide *in vivo* evidence suggesting that TGF-β1 mediated expression of *dspp* is crucial for dentin mineralization. These findings also provide for the first time a direct experimental evidence indicating that decreased *dspp* gene expression along with the other cellular changes in odontoblasts may result in human hereditary dental disorders like dentinogenesis imperfecta II (MIM 125420) and dentin dysplasia (MIM 125400 and 125420).

Mammalian development is a complex and highly orchestrated process that involves intricate cross-talk between growth factors and other regulatory molecules. These molecules interact with each other to induce specific molecular and cellular changes leading to organogenesis. Interactions between epithelium and mesenchyme are particularly crucial during the initiation of development of key organs such as teeth, skin, hair, mammary gland, and prostate (1). Tooth development is initiated by epithelial-mesenchymal interactions in the first branchial arch, and several transcription factors and growth factors are known to be expressed by dentin extracellular matrix (DECM)-producing[1] odontoblasts and enamel-producing ameloblasts during tooth development (2–5). Transforming growth factor-β1 (TGF-β1), a prototype of the TGF-β superfamily, is a multi-functional growth factor expressed in a wide variety of developing tissues from the early stages. The regulation of cell proliferation, differentiation, embryonic development, and apoptosis by TGF-β1 is well established (6–8). During mouse tooth development, TGF-β1 is expressed initially in the oral epithelium at embryonic day 13, and later its expression extends into the mesenchymal compartment and then gets restricted to the ectomesenchymal layer (odontoblasts). The odontoblast-restricted expression of TGF-β1 persists throughout life in the mice (9). Odontoblasts produce DECM from embryonic day 16 and subsequently mineralize in an orderly manner. TGF-β1 has been shown earlier to have mitogenic effects in tooth explant cultures (10) and to induce secretion of DECM components. Although it has been suggested that TGF-β1 plays a crucial role in dental tissue repair processes by the induction of reactionary (11) and reparative dentinogenesis (12), the precise *in vivo* functions associated with its continued expression are not clearly understood. Interestingly, subtle changes such as attrition and reduced mineralization of the teeth along with inflammation were observed in TGF-β1 knockout mice (13). However, the maternal transfer of active TGF-β1, multi-focal inflammation, and neonatal lethality in these mice further complicate the clear understanding of the precise role of TGF-β1 in tooth development (14).

To gain more insights into the specific *in vivo* roles of TGF-β1 during tooth development, we targeted the overexpression of active TGF-β1 to odontoblasts, starting from embryonic day 17 using the upstream regulatory sequences of the dentin sialophosphoprotein (*dspp*) gene (15). These animals develop a novel phenotype that resembles hereditary dental disorders such as dentinogenesis imperfecta II (DGI; MIM 125420) and dentin dysplasia (MIM 125400 and 125420). We present here a detailed analysis of this phenotype and molecular mechanisms leading to this phenotype and discuss the role of TGF-β1 in dentinogenesis and the tooth disorders.

MATERIALS AND METHODS

TGF-β1 Transgenic Construct—The transgenic construct consisting of a 6-kilobase *dspp* upstream regulatory sequence (15, 16) and a 1.5-kilobase active porcine TGF-β1 cDNA (17) with a SV40 poly(A)

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[1] The abbreviations used are: DECM, dentin extracellular matrix; DGI, dentinogenesis imperfecta; DSPP, dentin sialophosphoprotein; TGF, transforming growth factor; *d*TGF-β1, *dspp*-TGF-β1 transgenic.

sequence (see Fig. 1*a*) was microinjected into fertilized FVB/N eggs to generate transgenic mice. Mice were genotyped for the presence of the transgene by Southern analysis of the tail DNA using a SV40 poly(A) probe. The *dspp*-TGF-β1 transgenic (*d*TGF-β1) mouse lines varied in the copy number of integrated transgenes (data not shown). Mice were housed in a pathogen-free facility and fed *ad libitum* with dough diet (Bio Serv, Holton Industries Co., Frenchtown, NJ).

Microradiography—Transgenic and wild type mice were euthanized by cervical dislocation, and the heads were dissected out and sliced sagittally into two symmetrical halves. The mineral density of teeth was analyzed by microradiographic technique using x-ray imaging with a standard setting of 120 s × 15KV (model MX20, Faxitron x-ray Corporation, Wheeling, IL). Images were scanned and quantified using a computerized National Institutes of Health image system.

Preparation of Tissue Sections, Histological Analysis, and Immunohistochemistry—Whole jaws from the transgenic and wild type mice were dissected under a stereomicroscope and fixed in 10% buffered formalin overnight. The tissues were decalcified in EDTA-sodium phosphate buffer for 10–15 days, dehydrated, and embedded in paraffin wax, and 5-micron-thick sections were cut and collected onto silanated microscope slides. Immunostainings for TGF-β1, Collagen (Col) I, II, and III were performed using antibodies at 1:400 dilution. Immunohistochemical analysis was performed using a commercial kit according to the manufacturer's suggestions (Vectastain ABC Kit, Vector Laboratories Inc., Burlingame, CA). The sections were counterstained with hematoxylin and eosin and were photographed under light microscopy. Anti TGF-β1 antibody was a gift of Dr. Kathy Flanders (NCI, National Institutes of Health). Collagen I and III antisera were kindly provided by Dr. Larry Fisher (NIDCR, National Institutes of Health).

Northern Blot Analysis—The incisors and molars were dissected out from wild type and transgenic mice, and total RNA was prepared using a RNA STAT-60™ kit according to the manufacturer's recommendations (Tel-Test, Inc., Friendswood, TX). Total RNA (10 μg) was electrophoresed on a 1% formaldehyde gel and transferred onto a nylon membrane. The membrane was hybridized with ^{32}P-labeled *dspp* probe (pSX1.7 Exon IV) (18). Autoradiographs were exposed to Kodak x-ray film (Eastman Kodak Co.) for 24 h at −70 °C.

In Situ Hybridization—Templates for antisense and sense riboprobes for *dspp* gene were generated by digesting pSX1.7 containing exon IV with *Sac*I and *Xba*I, and an *in vitro* transcription assay was carried out to incorporate digoxigenin-11-dUTP with T7 and T3 RNA polymerases according to the manufacturer's recommendations (Roche Molecular Biochemicals). Frozen sections (15 microns) were cut, air dried, and fixed in 4% paraformaldehyde for 10 min at 4 °C. The sections were rinsed with phosphate-buffered saline and treated with 0.2 M HCl, 1 μg/ml proteinase K, 0.25% acetic anhydride in 0.1 M triethanolamine buffer 5 min each with brief rinses in DEPC water in between the treatments. *In situ* hybridization and signal detection were carried out according to Roche Molecular Biochemicals nonradioactive *In Situ* Hybridization Application Manual. Slides were counter stained with hematoxylin and mounted with Crystal Mount (Biomedia, Foster City, CA) for photography.

RESULTS

Generation of dTGF-β1 Transgenic Mice—Five founder mice were generated from the microinjections of the *dspp*/TGF- β1 construct (Fig. 1*a*) into fertilized FVB/N mouse eggs. All of the founders were established as independent lines based on independent integrations of the transgenic construct in the genome. Mouse tail DNA preparations were subjected to restriction enzyme digestion, and the presence of the transgene was determined by Southern analysis using the whole transgene fragment as a probe. Copy number in each line was analyzed by using the endogenous *dspp* gene as an internal control. Transgenic mice were maintained as heterozygotes and mated with either wild type FVB/N or with the transgenic heterozygotes for further analysis. The lines were classified as low, medium, and high expressors, and these lines displayed a general correlation between the level of TGF-β1 expression and severity of the tooth phenotype (data not shown). One of the high expressor lines was further analyzed in detail.

Novel Tooth Phenotype of dTGF-β1 Mice—All the transgenic mouse lines displayed tooth-specific phenotypes with varying degrees of severity. The *d*TGF-β1 mice were born with no apparent defects and grew normally on the dough diet. However, from the age of 2 weeks, the *d*TGF-β1 mice displayed progressive discoloration of teeth (Fig. 1, *b* and *c*). Initially, both the mandibular and maxillary incisors of the *d*TGF-β1 mice appeared opaque, turned chalky white, and fractured, leaving behind stumps. The high resolution radiographic images of the incisors and molars of the *d*TGF-β1 mice exhibited remarkably reduced mineralization (Fig. 1, *d* and *e*). The quantitation of *d*TGF-β1 teeth by x-ray image analysis indicated a reduction of opacity by about 90% in the incisors (wild type, 145.5 ± 15.3 au (arbitrary units); *d*TGF-β1, 14.8 ± 4, au; $n = 6$, $p < 0.001$) and 62% in molars (wild type, 229.8 ± 16.2 au; *d*TGF-β1, 87.3 ± 15.7, $n = 6$; $p < 0.001$).

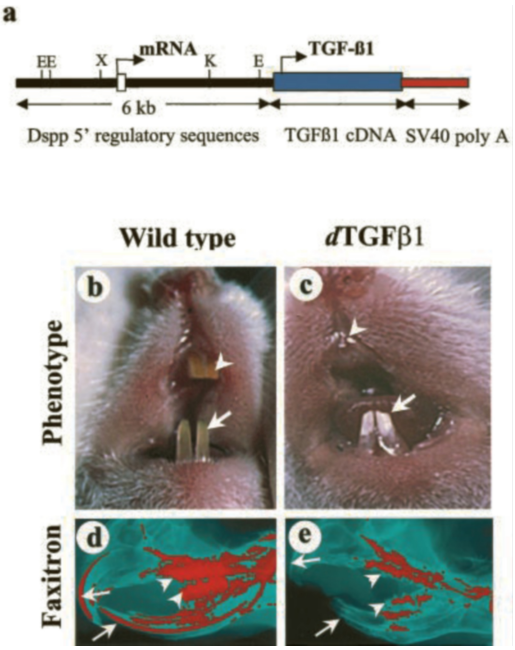

FIG. 1. **Strategy for generation of *d*TGF-β1 mice and gross tooth abnormalities in these mice.** *a*, schematic representation of the TGF-β1 transgenic construct. The transgenic construct was constructed by fusing a 6-kilobase dentin sialophosphoprotein (*dspp*) upstream regulatory sequence with active porcine TGF-β1 cDNA in which Cys^{223} and Cys^{225} codons are replaced with serine codons. *b*, normal maxillary and mandibular incisors in wild type mice. *c*, loss of maxillary incisors (*arrowhead*) and discolored and fractured mandibular incisors (*arrows*) in *d*TGF-β1 mice. *d*, normal opacity in the craniofacial region of 15-day-old wild type mice as measured by radiographic analysis. *e*, significantly reduced opacity in the incisors (*arrows*) and molars (*arrowheads*) of *d*TGF-β1 mice.

In *d*TGF-β1 animals, the teeth displayed irregular dentin formation with a significant number of cellular inclusions (Fig. 2, *b*, *c*, *e*, and *f*). Compared with the wild type (Fig. 2, *a* and *d*), the transgenic mice displayed a highly disorganized odontoblast layer and irregular dentinal tubules all along the dentinal layer (Fig. 2, *b* and *c*). The dentinal tubules were short in length and sparsely distributed (Fig. 2, *c* and *f*). Electron microscopic analysis of wild type mouse teeth showed normal dentin architecture with dentinal tubules coursing from the dentin-enamel junction in a parallel organization toward the dental pulp (Fig. 3, *a* and *c*), whereas *d*TGF-β1 mouse incisors showed a thin layer of relatively normal mantle dentin and markedly abnormal dentin with reduced numbers of dentinal tubules (Fig. 3, *b* and *d*). The coronal area of the transgenic tooth pulp was obliterated with a disorganized dentin, similar to the structural abnormalities observed in the incisors.

Increased Levels of Dentin Extracellular Matrix Proteins in dTGF-β1 Mice—To confirm whether the observed phenotype was due to the increased TGF-β1 levels in teeth of the trans-

genic mice, we performed immunohistochemical analysis on the cross-sections of the incisors from 1-day-old mice using anti-TGF-β1 antibodies (Fig. 4, *a* and *e*). High levels of TGF-β1 were detected in the dentin matrix, around the odontoblasts and also in the dental pulp (Fig. 4*e*). Transgenic TGF-β1 was also detected transiently in the ameloblasts similar to the endogenous *dspp* (data not shown). DECM components are among the most prominent molecules that are regulated by TGF-β1. Increased and abnormal accumulation of DECM was detected in the teeth of transgenic mice by Masson's trichrome staining (Fig. 4, *b* and *f*). Further, we examined the expression of collagens I and III in the dentin and the dental pulp by immunohistochemistry. Increased levels of collagens I and III were observed in the tooth pulp of *d*TGF-β1 mice (Fig. 4, *g* and *h*). However, in the dentin, the staining of collagen I appeared to be either unchanged or slightly reduced, whereas in the dental pulp the expression was increased (Fig. 4, *c* and *g*). The expression of collagen was not uniformly distributed in the dental pulp. Interestingly, the collagen III level was increased in both dentin and dental pulp (Fig. 4, *d* and *h*). It has been reported that the collagen III levels are elevated in osteogenesis imperfecta dentin and also in DGI dentin, suggesting the incomplete differentiation or maturation of the odontoblasts (19).

The phenotypic characterization and histological analysis along with the radiographic profile of *d*TGF-β1 teeth suggested a defect in the mineralization. Therefore, we examined the calcium levels in the undemineralized ground sections from the wild type and transgenic mice teeth by von Kossa's staining (Fig. 5*A*). Uniform distribution of calcium was detected in the mineralized dentin and also in enamel of wild type mice (Fig. 5*A*, *panel a*). Interestingly, in *d*TGF-β1 mouse teeth, the overall expression of calcium appeared to be elevated and unevenly distributed (Fig. 5*A*, *panel b*).

Regulation of Dentin Sialophosphoprotein Gene Expression by dTGF-β1 in the Teeth—Because the *d*TGF-β1 tooth phenotype resembles dentin dysplasia and DGI, we examined the

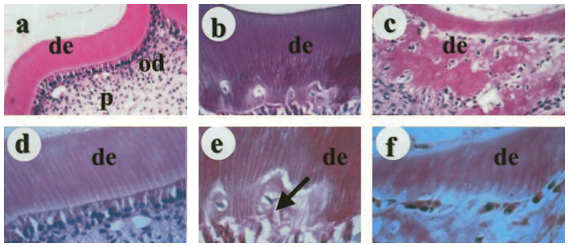

FIG. 2. **Histological analysis of *d*TGF-β1 mouse teeth.** *a*, representative sagittal section of the molar of 15-day-old wild type mouse stained with hematoxylin and eosin. *b*, molar of 15-day-old transgenic mice with a low TGF-β1 expression. *c*, molar of 15-day-old transgenic mice with higher TGF-β1 expression. *d–f* are the high magnifications of *a–c*, respectively. Normal organization of odontoblast layer and orientation of dentinal tubules are seen in the wild type molars. Irregular odontoblast layer and dentinal tubules with cellular inclusions (*arrow*) are present in *d*TGF-β1 molars. *de*, dentin; *od*, odontoblasts; *p*, pulp.

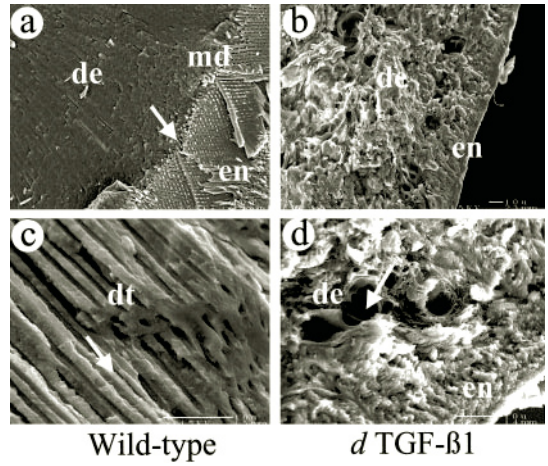

FIG. 3. **Ultrastructural analysis of *d*TGF-β1 mouse teeth.** Ground sections of incisors from wild type (*a* and *c*) and *d*TGF-β1 (*b* and *d*) mice. The *arrow* indicates dentin-enamel junction. Note the highly abnormal and irregular dentin in *b*. *c* and *d*, higher magnifications of dentin in *a* and *b*, respectively. The *arrow* indicates regular dentinal tubules in *c* and highly disorganized tubules with voids in *d*. Note the abnormal deposition of DECM with void spaces (*arrow*) in *d*. *de*, dentin; *dt*, dentinal tubules; *en*, enamel; *md*, mantle dentin.

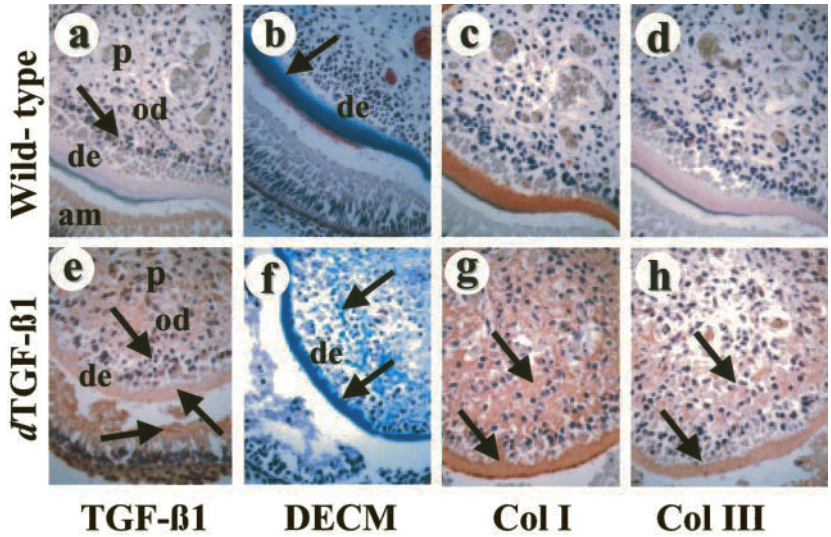

FIG. 4. **Immunohistochemical analysis of TGF-β1 and collagenous components of DECM in *d*TGF-β1 mice.** Incisors from 1-day-old wild type (*a*) and transgenic (*e*) mice were cross-sectioned and immunostained with anti-TGF-β1 antibodies. *a*, the *arrow* indicates TGF-β1 staining in the odontoblasts. *e*, the *arrows* indicate intense staining of TGF-β1 in the dental pulp of *d*TGF-β1 mice. *b* and *f*, Masson's trichrome staining for DECM components. *b*, *blue* staining of DECM (*arrow*) is noticeable in dentin of the wild type mouse incisors. *f*, massive deposition of DECM is seen as *blue stain* (*arrows*) in the dental pulp and dentin region of *d*TGF-β1 mice. Immunostaining of collagen I (*c* and *g*) and collagen III (*d* and *h*) in DECM. Collagen I staining in the control animals (*c*) follows a similar pattern as DECM staining seen in *b*. Incisors of the *d*TGF-β1 mice show excessive collagen I staining (*arrows*) in the dental pulp and dentin region (*g*). Collagen III staining in the control group is meager (*d*), whereas in the *d*TGF-β1 group, there is increased staining (*arrows*, *h*). *am*, ameloblasts; *de*, dentin; *od*, odontoblasts; *p*, pulp.

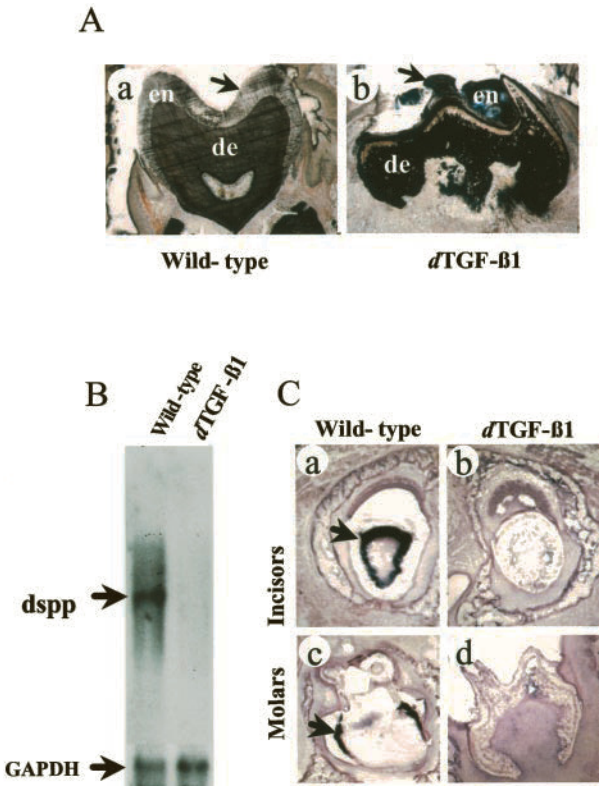

FIG. 5. **Tooth calcification studies on *d*TGF-β1 mice.** *A*, undemineralized sections from 1-month-old wild type (*panel a*) and *d*TGF-β1 (*panel b*) molars stained by von Kossa's method. *en*, enamel; *de*, dentin. *B*, analysis of expression of *dspp*; Northern blot analysis of total RNA from teeth of wild type and *d*TGF-β1 mice. The *dspp* mRNA level was significantly reduced in teeth of *d*TGF-β1 mice as compared with controls, whereas the GAPDH level remained unaffected. *dspp* and GAPDH probes were as described earlier (18). *C*, expression of *dspp* mRNA by *in situ* hybridization. Frozen cross-sections of the tooth from 1-week-old animals were processed and probed with a digoxigenin-labeled *dspp* ribo-probe for *in situ* hybridization. Distinct dark staining pattern for the presence of *dspp* transcripts is observed in the incisors (*panel a*) and molars (*panel c*) of the wild type animal. Significant reduction in *dspp* transcripts is observed in the transgenic incisors (*panel b*) and molars (*panel d*).

expression of the *dspp* gene that has been implicated in the etiology of the DGI II subtype (28). Northern analysis of tooth RNA using *dspp* exon-IV DNA as a probe revealed a significant reduction in the levels of *dspp* transcripts in *d*TGF-β1 mice (Fig. 5*B*). Furthermore, we also examined the odontoblast specific expression of the *dspp* gene by *in situ* hybridization using the same *dspp* riboprobe. The expression of *dspp* mRNA was detected only in the odontoblasts of both incisors and molar teeth of the wild type mice (Fig. 5*C*, *panels a* and *c*). The odontoblast specific expression of *dspp* gene (Fig. 5*C*, *panels b* and *d*) was significantly reduced in the transgenic mouse teeth, confirming the reduction seen in the Northern analysis.

DISCUSSION

To analyze *in vivo* functions of the multi-functional growth factor TGF-β1 in tooth development, we generated transgenic mice overexpressing active TGF-β1 from embryonic day 17 in the teeth. We achieved the tissue-specific expression by driving the transgene with mouse *dspp* gene regulatory sequences, which were well characterized for the presence of tooth-specific expression in both *in vitro* and *in vivo* model systems (16).

The *d*TGF-β1 teeth displayed a gradual discoloration of teeth, finally resulting in an opalescent appearance. The teeth were worn progressively or fractured, leaving short stumps. These changes were associated with significantly decreased mineralization of teeth and abnormal dentin formation. Defective mineralization has been identified in human autosomal tooth disorders such as DGI (MIM 125490 and 125500), and dentin dysplasia (MIM 125400 and 125420). These disorders are generally characterized by discoloration and fractures of teeth associated with poor mineralization of DECM. Also, mutations in the *Col1A1* and *Col1A2* genes encoding collagen I that result in increased deposition and altered assembly of collagen fibers, major components of DECM, have been described for DGI-I associated with osteogenesis imperfecta (MIM 166240) (21–23).

Although *dspp* regulatory sequences are odontoblast-specific, an intense staining for TGF-β1 was observed in dental pulp, indicating apparent secretion of this growth factor from odontoblasts into the pulp. In agreement with earlier reports on the inductive effects of TGF-β1 on differentiation of pulpal cells into odontoblasts (24, 25) and also on vasculogenesis (26), we observed an apparent increase in pulpal cell mass and also differentiation into odontoblast-like cells in *d*TGF-β1 teeth. In these transgenic mice, the dentinal tubules through which the DECM and other components are secreted to form dentin were disorganized and reduced in length as a result of improper polarization and alignment of odontoblasts (data not shown). This improper organization may impair the orderly secretion and deposition of collagenous and other molecules into the mineralization front. Increased vasculature in the pulp and around the odontoblasts was also observed in the mice (data not shown). However, unlike the wild type mice, the pulpal cells of *d*TGF-β1 mice displayed patchy and ocular staining for collagen I and III, indicating the differentiation of dental pulp into odontoblast-like cells as a result of secreted TGF-β1 protein. Differentiation of dental pulp into collagen producing odontoblast-like cells has been suggested in *in vitro* and *ex vivo* culture systems by addition of exogenous growth factors, either alone or in combination, to understand the development of odontoblasts (10). Increased levels of collagen I and III and their abnormal accumulation in the pulp seen in *dspp*/TGF-β1 transgenic mice are reminiscent of similar changes observed in dentinogenesis imperfecta and dentin dysplasia (27, 28).

Interestingly, calcium, a major component of hydroxyapatite crystals in the teeth, was apparently elevated in the transgenic mice. The distribution in the dentin appeared to be patchy and reduced or absent in certain regions. In addition to the collagen trimers and calcium ions, inorganic phosphates are essential for proper mineralization of teeth. The growth and proper organization of hydroxyapatite crystal formation resulting from tri- or hexa-calcium phosphate provides the strength to the dentin. Hence, we examined the expression of DSPP, a highly phosphorylated tooth-specific phosphoprotein, in the teeth of these mice. Additionally, the *dspp* gene has been mapped to the DGI locus and is implicated as a potential candidate gene for DGI-II (20). The *dspp* gene product also has been demonstrated to function as a nucleator in hydroxyapatite crystal formation in dentin (29). Our studies demonstrate for the first time a significant reduction in the levels of *dspp* mRNA in the presence of high levels of TGF-β1. *In situ* hybridization studies have confirmed the reduced expression of *dspp* mRNA in the odontoblasts of *d*TGF-β1 mouse teeth. *dspp* expression was undetectable in the odontoblast-like cells in the pulp canal of transgenic mice. This observation clearly indicates, despite the high levels of calcium and collagen I in the pulp canal in *d*TGF-β1 mouse teeth, that no mineralization in the dental pulp is detected. In addition to the dentin defects, the transgenic mice also displayed a defect in enamel mineralization. Because the main components essential for mineralization in the enamel are calcium and phosphates, the defect in

the enamel mineralization may not be associated with the reduced expression of the *dspp* gene. Moreover, *dspp* expression has been shown to be transient during early stages of amelogenesis. Therefore, the transgenic TGF-β1 is expressed transiently, under the control of *dspp* gene regulatory sequences in ameloblasts during early dentinogenesis, and hence, we speculate the involvement of regulatory molecules other than *dspp,* in the enamel mineralization. Most importantly, our studies clearly provide direct experimental evidence, suggesting that the reduction in the *dssp* gene expression is associated with the tooth phenotype similar to human hereditary conditions such as DGI. However, the direct correlation between either a mutation or decreased expression of the *dspp* gene in DGI disorders needs to be investigated. Interestingly, our preliminary studies on the double transgenic mice (*d*TGF-β1X *dspp*-LacZ generated by crossing *d*TGF-β1 mice with *dspp*-LacZ mice (16)) indicate down-regulation of the LacZ expression suggesting the increased levels of TGF-β1 negatively regulate the *dspp* promoter activity.[2] However, in *d*TGF-β1 mice there is a clear overexpression of TGF-β1 in teeth presumably because of multiple copies of the transgene encoding active TGF-β1. The increased level of TGF-β1 in teeth is likely to accelerate its signaling cascade resulting in decreased *dspp* gene expression, which may result in DGI and dentin dysplasia. A detailed examination of the downstream TGF-β1 signaling pathway will be important in identifying the molecular events underlying these dental disorders.

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