Inhibition of the terminal differentiation of odontoblasts and their transdifferentiation into osteoblasts in *Runx2* transgenic mice*

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Summary. *Runx2* is an essential transcription factor for bone and tooth development whose function in odontoblast differentiation remains to be clarified. To pursue this issue, we examined tooth development in *Runx2* transgenic mice under the control of *Colla1* promoter (*Tg(Colla1-Runx2)* mice). Endogenous *Runx2* protein was detected in the nuclei of preodontoblasts, immature odontoblasts, mesenchymal cells in the dental sac, and osteoblasts, while transgene expression was detected in odontoblasts and osteoblasts. Odontoblasts in *Tg(Colla1-Runx2)* mice lost their columnar shape and dentin was deposited around the odontoblasts, which were cuboid or flat in shape. The dentin in *Tg(Colla1-Runx2)* mice was thin and possessed lacunae that contained odontoblasts and bone canaliculi-like structures, while predentin and

dental tubules were absent. We examined the expression of dentin matrix protein genes, *Colla1* and dentin sialophosphoprotein (*DSPP*), by *in situ* hybridization, and dentin matrix proteins, osteocalcin, osteopontin, and dentin matrix protein 1 (DMP1) as well as an intermediate filament, nestin, by immunohistochemistry to characterize odontoblasts in *Tg(Colla1-Runx2)* mice. Results showed *Colla1* expression was down-regulated, *DSPP* expression was lost, and nestin expression was severely decreased in the odontoblasts of *Tg(Colla1-Runx2)* mice. Further, the expressions of osteocalcin, osteopontin, and DMP1 were up-regulated in odontoblasts, although the up-regulation of osteocalcin expression was transient. These findings indicate that *Runx2* inhibits the terminal differentiation of odontoblasts, and that *Runx2* induces transdifferentiation of odontoblasts into osteoblasts forming a bone structure. Thus, *Runx2* expression has to be down-regulated during odontoblast differentiation to acquire full odontoblast differentiation for dentinogenesis.

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Introduction

Dentinogenesis begins when outer cells from the dental papilla, which is derived from neural crest cells, are induced to differentiate into odontoblasts. Odontoblasts start their polarization while elongating and developing synthesis organelles, assume a tall columnar shape with an apical process, and establish a continuous single layer with a clear epithelial appearance

(Arana-Chavez and Massa, 2004). Odontoblasts synthesize and secrete type I collagen and noncollagenous dentin matrix proteins, including dentin sialophosphoprotein (DSPP), which is a large parental protein that subsequently suffers cleavage to originate dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), dentin matrix protein 1 (DMP1), and a small amount of four noncollagenous proteins (osteopontin, bone sialoprotein, osteonectin, and osteocalcin) that are abundantly synthesized and secreted by osteoblasts (Linde and Goldberg, 1993; Butler and Ritchie, 1995). Further, DMP1 is synthesized and secreted by osteocytes as well as odontoblasts (Toyosawa *et al.*, 2001, 2004).

Runx2, a transcription factor that belongs to the Runx family, is essential for osteoblast differentiation (Komori *et al.*, 1997; Otto *et al.*, 1997). The formation of tooth germ in *Runx2*-deficient mice terminates at the cap stage, and the differentiation of ectomesenchymal cells in dental papilla to odontoblasts is blocked, indicating that Runx2 is an essential transcription factor for odontogenesis (D'Souza *et al.*, 1999; Aberg *et al.*, 2004). *Runx2* expression is upregulated in the condensed dental mesenchyme but downregulated in differentiated odontoblasts during cytodifferentiation (Bronckers *et al.*, 2001; Yamashiro *et al.*, 2002; Chen *et al.*, 2005). Since tooth germs in *Runx2*-deficient mice lack differentiated odontoblasts and the mice die soon after birth, the function of Runx2 in the process of odontoblast differentiation remains to be clarified.

We previously generated *Runx2* transgenic mice under the control of 2.3 kb *Coll1a1* promoter (*Tg(Coll1a1-Runx2)* mice) to examine the function of Runx2 in the late stage of osteoblast differentiation (Liu *et al.*, 2001; Kanatani *et al.*, 2006). In *Tg(Coll1a1-Runx2)* mice, osteoblast differentiation in the late stage was inhibited; the expression of osteopontin, which is highly expressed in immature osteoblasts, was upregulated; the expression of osteocalcin, which is highly expressed in mature osteoblasts, was downregulated; and osteocyte numbers were severely reduced (Liu *et al.*, 2001; Geoffroy *et al.*, 2002; Kanatani *et al.*, 2006). As the 2.3 kb *Coll1a1* promoter specifically drives transgene expression to osteoblasts and odontoblasts (Rossert *et al.*, 1995; Liu *et al.*, 2001) and Runx2 expression is downregulated in mature odontoblasts (Bronckers *et al.*, 2001; Yamashiro *et al.*, 2002; Chen *et al.*, 2005), we examined the effects of the sustained expression of Runx2 in odontoblasts using *Tg(Coll1a1-Runx2)* mice to reveal the function of Runx2 in odontoblast differentiation.

Materials and Methods

Transgenic mice

Tg(Coll1a1-Runx2) mice and *Tg(Coll1a1-lacZ)* mice were generated as previously described (Liu *et al.*, 2001). β -galactosidase activity was detected as described (Ueta *et al.*, 2001). Transgenic lines were maintained in a B6C3H F1 background. Prior to the study, all experiments were reviewed and approved by the Animal Care and Use Committee of Nagasaki University Graduate School of Biomedical Sciences.

Histological analysis

For histological analysis, 0-, 3-, 7-, 14-, 21- and 28-day-old mice were used for examination. After anesthetization with an intraperitoneal injection of sodium pentobarbital, they were perfused with 4% paraformaldehyde in 0.05 M cacodylate buffer (pH7.4) for light microscopic analysis, or with 2% glutaraldehyde-2.5% paraformaldehyde in 0.05M cacodylate buffer (pH7.4) for ultrastructural analysis. Dissected mandibles were further immersed in the same fixatives for 24 h at 4°C and decalcified in 5% EDTA (pH7.4) for 3 days to 4 weeks at 4°C. For light microscopic analysis, tissues were embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin or processed for *in situ* hybridization and immunohistochemical analysis as described below. Sections were treated for silver impregnation staining based on the method by Kurazaki *et al.* (2000) to discern canalicular structures. For ultrastructural analysis, tissues were post-fixed with osmium tetroxide in 0.05M cacodylate buffer (pH7.4) and embedded in Epon-Araldite resin. Ultrathin sections were stained with 2% uranyl acetate and Reynold's lead citrate and examined using a Hitachi H-7100 electron microscope.

In situ hybridization

Digoxigenin-11-UTP-labeled single-stranded RNA probes were prepared using a DIG RNA labeling kit (Roche Biochemica, Mannheim Germany), according to the manufacturer's instructions. A 0.32 kb fragment of mouse *Coll1a1* cDNA (Inada *et al.*, 1999) and a 0.42 kb fragment of mouse *DSPP* cDNA were used to generate antisense and sense probes. Hybridization was carried out as described (Inada *et al.*, 1999). *In situ* hybridization using the sense probes showed no significant signals (data not shown).

Fig. 1. Expression patterns of endogenous Runx2 protein. Runx2 expression patterns in the lower first molars of 3-day-old (**a**, **b**) and 1-week-old (**c**, **d**) wild-type mice were examined by immunohistochemistry. Boxed regions in **a** and **c** are magnified in **b** and **d**, respectively. Runx2 is endogenously localized in the nuclei of preodontoblasts (**a**, short arrows), young odontoblasts (**b**, arrows), dental sac cells, and osteoblasts in tooth germs. It is also found transiently in the nuclei of ameloblasts in the coronal regions at 1 week of age (**d**, arrowheads). Ab: ameloblasts, D: dentin, Dp: dental papilla, Ds: dental sac, E: enamel, Ob: odontoblasts. Bars= 100 μ m (**a**), 20 μ m (**b**), 200 μ m (**c**), 50 μ m (**d**)

Immunohistochemistry

Immunohistochemical staining was carried out for Runx2, nestin, osteocalcin, osteopontin, and DMP1. A monoclonal mouse anti-Runx2 antibody (Medical Biological Laboratories Co., Ltd., Nagoya), monoclonal mouse anti-nestin antibody (Chemicon International Inc., CA, USA), polyclonal rabbit anti-mouse osteocalcin antibody (Takara Bio Inc., Shiga), polyclonal rabbit anti-mouse osteopontin antibody (Immuno-Biological Laboratories Co., Ltd., Gunma) and polyclonal rabbit anti-DMP1 antibody (Toyosawa *et al.*, 2004) were used as primary antibodies at dilutions of 1:100, 1:100, 1:1000, 1:50 and 1:1000, respectively. As negative controls, normal mouse IgG (Immuno-Biological Laboratories Co., Ltd.) for Runx2 and nestin, and normal rabbit IgG (Immuno-Biological Laboratories Co., Ltd.) for osteocalcin, osteopontin, and DMP1 were used instead of the primary antibody. After blocking endogenous peroxidase activity with 0.3% H₂O₂ in methanol, sections were processed with Histofine Simple Stain MAX-PO(M) (Nichirei) for Runx2 and nestin, and with Histofine Simple Stain MAX-PO(R) (Nichirei) for osteocalcin, osteopontin, and DMP1. Anti-Runx2 antibody binding sites were then

visualized as a black reaction product by applying 0.05% 3,3'-diaminobenzidine (DAB), 0.01% nickel chloride, and 0.01% H₂O₂ in a phosphate buffer for 8 min, while the other antibody binding sites were colored brown by incubation with DAB solution (the solution used above without nickel chloride). Some sections stained with anti-Runx2 were double-labeled with antibodies for nestin, osteocalcin, osteopontin, or DMP1. Finally, sections were counterstained with methyl green.

Results

Expression pattern of Runx2 in wild-type and Tg(Col1a1-Runx2) mice

Runx2 protein was detected in the nuclei of preodontoblasts, immature odontoblasts, mesenchymal cells in the dental sac, and osteoblasts in wild-type mice at 3 days of age (Fig. 1a, b). Runx2 protein was transiently detected in ameloblasts in the coronal regions of wild-type mice at one week of age as previously reported (Fig. 1c, d) (D'Souza *et al.*, 1999; Jiang *et al.*, 1999; Camilleri and McDonald 2006). The expression pattern of the

Fig. 2. Generation of transgenic mice. **a** and **b**: Diagrams of DNA constructs used to generate *Tg(Coll1a1-lacZ)* mice (**a**) and *Tg(Coll1a1-Runx2)* mice (**b**). **c–f**: Histochemical analysis of the odontoblast-specific expression of the transgene, using β -galactosidase staining (**c**, **e**) and Runx2 immunostaining (**d**, **f**), in the lower first molars of 3-day-old *Tg(Coll1a1-lacZ)* (**c**, **e**) and *Tg(Coll1a1-Runx2)* (**d**, **f**) mice. Boxed regions in **c** and **d** are magnified in **e** and **f**, respectively. β -galactosidase and Runx2 are localized specifically in the cytoplasm and nuclei of odontoblasts, respectively, and were found with the onset of dentinogenesis (**e**, **f**, arrows). Ab: ameloblasts, Dp: dental papilla, Ob: odontoblasts. Bars = 200 μ m (**c**, **d**), 50 μ m (**e**, **f**).

transgene in the mandible was examined by β -galactosidase activity using lacZ transgenic mice under the control of the 2.3 kb *Coll1a1* promoter (*Tg(Coll1a1-lacZ)* mice) at 3 days of age (Fig. 2a, c, e). In the tooth germs of the first molars of *Tg(Coll1a1-lacZ)* mice, β -galactosidase activity was localized specifically in odontoblasts and osteoblasts. The β -galactosidase activity was not detected in preodontoblasts, but it appeared with the onset of dentinogenesis (Fig. 2c, e). As the β -galactosidase activity was not detected in periodontal ligament cells or cementoblasts, which produce type I collagen, the 2.3 kb *Coll1a1* promoter region does not contain the binding sites for the transcription factors that are required for the expression in these cells. In *Tg(Coll1a1-Runx2)* mice, Runx2 was strongly detected in the nuclei of odontoblasts and osteoblasts, as seen in *Tg(Coll1a1-lacZ)* mice (Fig. 2b, c–f).

The teeth of *Tg(Coll1a1-Runx2)* mice were fragile, with erupted teeth frequently showing breakage or dental pulp exposure (Fig. 3). The mineral density of incisors in *Tg(Coll1a1-Runx2)* mice at six weeks of age was markedly decreased compared with that of wild-type mice in analysis using peripheral quantitative computed tomography (pQCT) (data not shown).

Disturbance of odontoblast differentiation in Tg(Coll1a1-Runx2) mice

Morphologies of preodontoblasts and immature odontoblasts were similar between the tooth germs of wild-type and *Tg(Coll1a1-Runx2)* newborn mice (data not shown). At 3 days of age, odontoblasts in the first molar of wild-type mice were columnar. Odontoblasts in *Tg(Coll1a1-Runx2)* mice were also columnar at the cervical loop

where odontoblasts were immature, but they lost this shape in cusp regions where dentinogenesis advanced and dentin was deposited around odontoblasts (Fig. 4a–f). At one week of age, dentin was apparently thin in *Tg(Coll1a1-Runx2)* mice compared with that in wild-type mice, and odontoblasts in *Tg(Coll1a1-Runx2)* mice lost their columnar shape in the whole area and were surrounded by dentin, exhibiting a bone structure (Fig. 4g–l). No formation of predentin and dentinal tubules was observed in *Tg(Coll1a1-Runx2)* mice (Fig. 4k, l).

Although there was little difference in the contour of the first molar between wild-type and *Tg(Coll1a1-Runx2)* mice at 4 weeks of age (Fig. 5a, d), dentin in *Tg(Coll1a1-Runx2)* mice was thinner and did not possess structures peculiar to normal dentin, such as predentin and dentinal tubules (Fig. 5, 6). The dentin possessed lacunae that contained odontoblasts, which were similar to alveolar bone in structure (Fig. 5f). Silver impregnation staining showed that the dentin had bone canaliculi-like structures around their lacunae in *Tg(Coll1a1-Runx2)* mice, but that the alveolar bone had fewer canaliculi than in wild-type mice (Fig. 6). In coronal regions, the bone structures were frequently observed in molars of *Tg(Coll1a1-Runx2)* mice (Fig. 5e). Odontoblasts were polarized and columnar in wild-type mice, while odontoblasts in *Tg(Coll1a1-Runx2)* mice were cuboid or flat (Fig. 5c, f). These findings were clearly visualized by electron microscopy at one week of age (Fig. 7). In *Tg(Coll1a1-Runx2)* mice, structural changes in odontoblasts became apparent where enamel deposition began, and ectopical secretion of collagen fibers by depolarized odontoblasts was seen among odontoblasts (Fig. 7b). At coronal regions where dentinogenesis had advanced, some odontoblasts were embedded in dentin like osteocytes and others were located on the surface of dentin like osteoblasts, with both being flat or cuboid (Fig. 7d). Enamel deposition was partly disturbed in the apical region of *Tg(Coll1a1-Runx2)* mice (Fig. 4l).

Expression of dentin matrix proteins and nestin in *Tg(Coll1a1-Runx2)* mice

To investigate the characteristics of odontoblasts in *Tg(Coll1a1-Runx2)* mice, we examined the expressions of two dentin matrix protein genes, *Coll1a1* and *DSPP*, by *in situ* hybridization, the dentin matrix proteins, osteocalcin, osteopontin, and DMP1, and an intermediate filament, nestin, by immunohistochemistry. *Coll1a1* expression was similarly detected in odontoblasts of wild-type and *Tg(Coll1a1-Runx2)* mice at 3 days of age (Fig. 8a, b, e, f); however, *Coll1a1* expression in odontoblasts was apparently reduced in *Tg(Coll1a1-Runx2)* mice compared

Fig. 3. Appearance of molars in *Tg(Coll1a1-Runx2)* mice. Appearances of the lower left molars of 4-month-old wild-type (wt) and *Tg(Coll1a1-Runx2)* (tg) mice are shown. The teeth of *Tg(Coll1a1-Runx2)* mice are fragile and breakage or dental pulp exposure is seen after eruption (arrows). Bars=1 mm

with wild-type mice at one week of age (Fig. 8i, j, m, n). *DSPP* expression was strongly detected in odontoblasts of wild-type mice at 3 days and one week of age, whereas *DSPP* expression in odontoblasts was severely reduced at 3 days of age compared with that in wild-type mice, and was barely detectable at one-week-old *Tg(Coll1a1-Runx2)* mice (Fig. 8c, d, g, h, k, l, o, p). On the other hand, *DSPP* expression in ameloblasts was similarly observed in wild-type and *Tg(Coll1a1-Runx2)* mice (Fig. 8g, h, o, p).

We examined the expression of nestin, a marker of odontoblasts, and Runx2 by double labeling using anti-nestin and anti-Runx2 antibodies. In the tooth germs of wild-type mice, preodontoblasts expressed Runx2 but not nestin, and odontoblasts expressed nestin but almost no Runx2 at 3 days and one week of age (Fig. 9a, c). At these times, nestin was also expressed in cells under the odontoblast layer (Fig. 9a, c). At 4 weeks, nestin was restrictively localized in the cytoplasm of odontoblasts and the odontoblast processes (Fig. 10a, b). In *Tg(Coll1a1-Runx2)* mice, nestin expression in odontoblasts was reduced at 3 days and further reduced at one week of age, while nestin expression in cells under the odontoblast layer was similar to that in wild-type mice (Fig. 9). At 4 weeks of age, nestin was barely detectable in the odontoblasts in the molars of *Tg(Coll1a1-Runx2)* mice (Fig. 10c, d).

We examined the expressions of osteocalcin and Runx2

Fig. 4. Legend on the opposite page.

Fig. 5. Light micrographs of the lower first molars at 4 weeks of age. Sections of lower first molars of 4-week-old wild-type (wt) (a–c) and *Tg(Coll1a1-Runx2)* (tg) (d–f) mice were stained with haematoxylin and eosin. Sections of b, c, e and f are magnifications of boxed areas in a and d. The dentin in *Tg(Coll1a1-Runx2)* mice is thinner than in wild-type mice and shows a bone structure (f), which resembles alveolar bone (c, f, asterisks). The bone structure is also seen in the coronal pulp region (e, long arrow). Odontoblasts in *Tg(Coll1a1-Runx2)* mice lose their columnar structure (f, short arrows). D: dentin, Dp: dental pulp, Pl: periodontal ligament. Bars = 400 μ m (a, d), 100 μ m (b, e), 50 μ m (c, f)

Fig. 4. Light micrographs of tooth germs at 3 days and one week of age. Sections of tooth germs of first molars in wild-type (wt) (a, b, c, g, h, i) and *Tg(Coll1a1-Runx2)* (tg) (d, e, f, j, k, l) mice at 3 days (a–f) and one week (g–l) were stained with haematoxylin and eosin. Sections of b, c, h, and k are magnifications of the cervical loop regions indicated by boxes in a, d, g, and j, respectively. Sections of c, f, i and l are magnifications of the coronal regions indicated by boxes in a, d, g and j, respectively. There is no difference in the contour of tooth germs between wild-type and *Tg(Coll1a1-Runx2)* mice, but the thickness of the dentin is extremely thin in the latter. In the early stage of dentinogenesis, odontoblasts of transgenic mice are morphologically similar to those of wild-type mice (b, e); thereafter, they lose their polarized, columnar shape as dentinogenesis advances (compare f, k and l with c, h and i, respectively). Odontoblasts in *Tg(Coll1a1-Runx2)* mice are surrounded by dentin (f, k, l, arrows). Ab: ameloblasts, D: dentin, Dp: dental papilla, E: enamel. Bars = 400 μ m (a, d, g, j), 50 μ m (b, c, e, f, h, i, k, l)

Fig. 6. Silver impregnation staining. Sections of the lower first molars of 4-week-old wild-type (wt) (a) and *Tg(Coll1a1-Runx2)* (tg) (b) mice were stained with a silver impregnation staining method and the root areas are shown. Bone lacuna structures with canaliculi are seen in the dentin of *Tg(Coll1a1-Runx2)* mice (b, arrows). Asterisks indicate alveolar bone. D: dentin, Dp: dental pulp, PI: periodontal ligament. Bars = 50 μ m

by double labeling using anti-osteocalcin and anti-Runx2 antibodies. In wild-type mice, osteocalcin was expressed weakly in odontoblasts at 3 days but strongly at one week of age, while odontoblasts — which expressed osteocalcin — did not express Runx2, but a significant number of osteoblasts expressed both osteocalcin and Runx2 (Fig. 11a-c, g-i). In 3-day-old *Tg(Coll1a1-Runx2)* mice, osteocalcin expression was increased in odontoblasts as compared with wild-type mice, and Runx2 was strongly detected in odontoblasts (Fig. 11a, b, d, e). At one week of age, however, osteocalcin expression was decreased in most odontoblasts, except for those at the cervical loop in which odontoblasts maintained their columnar shape, although Runx2 was strongly detected in most odontoblasts (Fig. 11j, k). Osteocalcin expression in osteoblasts was lower in *Tg(Coll1a1-Runx2)* mice than in wild-type mice, as previously described (Liu *et al.*, 2001; Kanatani *et al.*, 2006) (Fig. 11c, f, i, l).

Osteopontin protein was detected in the alveolar bone and cementum but not in dentin in wild-type mice, while it was strongly detected in the alveolar bone, cementum, and dentin in *Tg(Coll1a1-Runx2)* mice (Fig. 12a b, e, f, i, j, m, n). DMP1 protein was detected strongly in the alveolar bone, especially around canaliculi, and in the cementum, but weakly in dentin in wild-type mice, while the DMP1 protein was detected weakly in the alveolar bone but

strongly in the dentin and cementum in *Tg(Coll1a1-Runx2)* mice (Fig. 12c, d, g, h, k, l, o, p). The deposition of osteopontin and DMP1 was prominent in the bone structures in the coronal regions of *Tg(Coll1a1-Runx2)* mice (Fig. 12f, h, n, p).

Discussion

Odontoblasts in *Tg(Coll1a1-Runx2)* mice lost their polarized, columnar features, and the dentin did not show its typical structures — such as dentinal tubules or predentin — but did show the appearance of bone, in which bone lacuna-like structures containing cellular inclusion were present. The gene expression of DSPP, which is known to be a tooth-specific protein (D'Souza *et al.*, 1997; Begue-Kirn *et al.*, 1998), was down-regulated in odontoblasts of *Tg(Coll1a1-Runx2)* mice. Further, an immunohistochemical analysis of nestin, which is also an odontoblast marker protein and is not expressed in osteoblasts (Terling *et al.*, 1995; About *et al.*, 2000; Struys *et al.*, 2005), showed distinct down-regulation in odontoblasts in *Tg(Coll1a1-Runx2)* mice. In contrast to the reduction of these odontoblast-specific proteins, osteopontin and DMP1, which are noncollagenous

Fig. 7. Electron micrographs. Section of tooth germs of lower first molars in wild-type (wt) (**a, c**) and *Tg(Coll1a1-Runx2)* (tg) (**b, d**) mice at 1 week of age were examined by transmission electron microscopy, and odontoblasts/dentin/enamel layer interfaces at early (**a, b**) and late (**c, d**) stages of dentinogenesis are shown. Odontoblasts in wild-type mice exhibit a polarized, columnar shape (**a, c**), whereas those in *Tg(Coll1a1-Runx2)* mice begin to lose their configuration and dentin is deposited in the odontoblast layer (**b**, asterisk) when enamel deposition starts (**a, b**, arrowheads). In the late stage of dentinogenesis, odontoblasts are enclosed by dentin (**d**, short arrows) or located on the surface of dentin (**d**, long arrows), being cuboid or flat. Ab: ameloblasts, D: dentin, E: enamel, Ob: odontoblasts, Pd: predentin. Bars = 5 μ m

Fig. 8. *In situ* hybridization using *Col1a1* and *DSPP* probes. Expression of *Col1a1* (a, b, e, f, i, j, m, n) and *DSPP* (c, d, g, h, k, l, o, p) mRNAs in the tooth germs of first molars in wild-type (wt) (a, c, e, g, i, k, m, o) and *Tg(Coll1a1-Runx2)* (tg) (b, d, f, h, j, l, n, p) mice at 3 days (a–h) and one week (i–p) of age were examined by *in situ* hybridization. Boxed regions in a, b, c, d, i, j, k and l are magnified in e, f, g, h, m, n, o, and p, respectively. *Col1a1* signals are strongly observed in odontoblasts of both wild-type and *Tg(Coll1a1-Runx2)* mice at 3 days of age (a, b, e, f, arrows), but are apparently reduced in *Tg(Coll1a1-Runx2)* mice at one week (i, j, m, n, arrows). *DSPP* signals are detected in odontoblasts in wild-type mice at 3 days and one week (c, g, k, o, arrows), but are severely reduced at 3 days (d, h, arrows) and barely detectable at one week (l, p, arrows) in *Tg(Coll1a1-Runx2)* mice. *DSPP* signals are similarly observed in ameloblasts of wild-type and *Tg(Coll1a1-Runx2)* mice (g, h, o, p, arrowheads). Ab: ameloblasts, Dp: dental papilla. Bars = 200 μ m (a–d, i–l), 50 μ m (e–h, m–p).

Fig. 9. Immunohistochemical analysis of nestin at 3 days and one week of age. Expressions of nestin and Runx2 proteins in the tooth germs of first molars in wild-type (wt) (**a**, **c**) and *Tg(Coll1-Runx2)* (tg) (**b**, **d**) mice at 3 days (**a**, **b**) and one week (**c**, **d**) were examined by immunohistochemistry. Sections were double-labeled with anti-nestin and anti-Runx2 antibodies. The localization of nestin is stained brown and that of Runx2 is stained black. Nestin immunostaining is observed in odontoblasts (Ob) and cells under the odontoblast layers (asteriks) in wild-type mice (**a**, **c**), while staining in odontoblasts (Ob) but not that in cells under the odontoblast layers (asteriks) is markedly reduced in *Tg(Coll1-Runx2)* mice (**b**, **d**). Arrows indicate preodontoblasts, which express endogenous Runx2. Ab: ameloblasts, Dp: dental papilla. Bars = 50 μ m

proteins present in both bone and teeth but with higher expression levels in the former (D'Souza *et al.*, 1997; Toyosawa *et al.*, 2001; Aguiar and Arana-Chavez, 2007), were increased in the dentin of *Tg(Coll1-Runx2)* mice. These findings indicate that odontoblasts in *Tg(Coll1-Runx2)* mice lost the phenotypes of odontoblasts and acquired those of osteoblasts.

The mRNA of type I collagen, a major organic component of bone and dentin, was similarly expressed in immature odontoblasts of both wild-type and *Tg(Coll1-Runx2)* mice; however, it decreased after the transdifferentiation from odontoblasts to osteoblasts in *Tg(Coll1-Runx2)* mice. The expression of osteocalcin,

another protein found in bone and dentin, was upregulated in immature odontoblasts, but it was also downregulated after the transdifferentiation in *Tg(Coll1-Runx2)* mice. Similarly, the expression of type I collagen and osteocalcin in individual osteoblasts was reduced in *Tg(Coll1-Runx2)* mice because of the inhibition of osteoblast maturation (Liu *et al.*, 2001; Kanatani *et al.*, 2006). After the transdifferentiation from odontoblasts to osteoblasts, therefore, maturational inhibition also seems to have occurred in the osteoblasts. In addition, Runx2 triggers osteocalcin expression in osteoprogenitor cells, although Runx2 is not essential for the steady-state expression of osteocalcin in mature osteoblasts

Fig. 10. Immunohistochemical analysis of nestin at 4 weeks of age. Expressions of nestin proteins in lower first molars of 4-week-old wild-type (**a, b**) and *Tg(Coll1-Runx2)* (**c, d**) mice were examined by immunohistochemistry. Boxed regions in **a** and **c** are magnified in **b** and **d**, respectively. Nestin is exclusively localized in the cytoplasm and process of odontoblasts in wild-type mice (**a, b**), while it is absent in *Tg(Coll1-Runx2)* mice (**c, d**, arrows). D: dentin, Dp: dental pulp, Ob: odontoblasts, Pl: periodontal ligament. Bars = 400 μ m (**a, c**), 50 μ m (**b, d**).

(Maruyama *et al.*, 2007). At an early stage of the transdifferentiation from odontoblasts to osteoblasts, therefore, osteocalcin may have been upregulated transiently. The transdifferentiation also partly affected amelogenesis, indicating that a failure of terminal differentiation of odontoblasts could affect amelogenesis. This implies that the interaction between mesenchymal cells and epithelial cells still plays an important role at their terminal differentiation stages.

DSPP is a vital non-collagenous protein for tooth development. The teeth of *Dspp*-deficient mice show

defective and reduced dentin mineralization and pulp exposure similar to those of human dentinogenesis imperfecta III (Sreenath *et al.*, 2003). In newly differentiated and functional odontoblasts, it has been shown that Runx2 expression is down-regulated, whereas *DSPP* is up-regulated (Gaikwad *et al.*, 2001; Chen *et al.*, 2005). *Runx2* overexpression in odontoblast cell lines, such as MDPC-23 cells and MO6-G3 cells, resulted in the selective down-regulation of *DSPP* (Gaikwad *et al.*, 2001; Chen *et al.*, 2005). Our findings, combined with previous findings, suggest that the down-regulation of Runx2 in odontoblasts

Fig. 11. Immunohistochemical analysis of osteocalcin. Expressions of osteocalcin and Runx2 proteins in the tooth germs of first molars in wild-type (wt) (a–c, g–i) and *Tg(Coll1a1-Runx2)* (tg) (d–f, j–l) mice at 3 days (a–f) and one week (g–l) of age were examined by immunohistochemistry. Sections were single-labeled with an osteocalcin antibody (a, d, g, j) or double-labeled with osteocalcin and Runx2 antibodies (b, c, e, f, h, i, k, l). The localization of osteocalcin is stained brown and that of Runx2 is stained black. b, e, h and k show cusp regions in sections adjacent to a, d, g and j, respectively (short arrows). c, f, i and l show alveolar bone in sections adjacent to a, d, g and j, respectively (arrowheads). Differentiated odontoblasts in wild-type mice do not express Runx2 but express osteocalcin weakly at 3 days and strongly at one week of age (a, b, g, h), while a significant number of osteoblasts express both osteocalcin and Runx2 (a, c, g, i). Osteocalcin expression is enhanced in odontoblasts, which are positive for Runx2, in *Tg(Coll1a1-Runx2)* mice at 3 days of age (d, e), and is decreased in most odontoblasts, except young odontoblasts in the cervical loop region (j, long arrows) at one week (j, k). Osteocalcin expression in osteoblasts is reduced in *Tg(Coll1a1-Runx2)* mice (d, f, j, l). Ab: ameloblasts, B: bone, D: dentin, Ob: odontoblasts. Bars = 200 μ m (a, d, g, j), 20 μ m (b, c, e, f, h, i, k, l)

Fig. 12. Immunohistochemical analysis of osteopontin and DMP1. Expressions of osteopontin (a, b, e, f, i, j, m, n) and DMP1 (c, d, g, h, k, l, o, p) proteins in the tooth germs of first molars in wild-type (wt) (a, c, e, g, i, k, m, o) and *Tg(Coll1a1-Runx2)* (tg) (b, d, f, h, j, l, n, p) mice at one week (a–h) and 2 weeks (i–p) of age were examined by immunohistochemistry. Boxed regions in a–d and i–l are magnified in e–h and m–p, respectively. Both osteopontin and DMP1 are localized in the alveolar bone and cementum but not in the dentin in wild-type mice (a, c, e, g, i, m), although a weak immunoreactivity for DMP1 antibody is detected in the coronal dentin at 2 weeks of age (k, o, arrowhead). Both are strongly detected in the bone structure in the coronal regions of *Tg(Coll1a1-Runx2)* mice (b, d, f, h, j, l, n, p, arrows), but immunoreactivity for DMP1 is decreased in the alveolar bone (compare d and l with c and k, respectively, asterisks). E: enamel, D: dentin, Dp: dental papilla (e–h) or dental pulp (m–p). Bars = 400 μ m (a–d and i–l), 50 μ m (e–h and m–p)

is required for DSPP expression and dentinogenesis.

Histological characteristics of the bone structure formed during dentinogenesis in *Tg(Coll1a1-Runx2)* mice were similar to those of reparative dentin, which has an atubular matrix with entrapped cells and exhibits a bone-like appearance. Although the mechanisms of

the reparative dentine formation remain unclear, it has been shown that reparative dentin is formed by newly differentiated odontoblast-like cells that derive from pulp cells and share some structural characteristics with bone (Smith *et al.*, 1995; Tziafas, 1995; Aran-Chavez and Massa, 2004; Aguiar and Aran-Chavez, 2007).

Furthermore, it has been reported that osteopontin, DMP1, and osteocalcin are up-regulated in the formation of reparative dentin or in newly formed bone-like tissues in the pulp cavity after tooth replantation (Braut *et al.*, 2003; Almushayt *et al.*, 2006; Aguiar and Aran-Chavez, 2007; Mei *et al.*, 2007; Zhao *et al.*, 2007), and that the cells forming the bone-like tissues are Runx2-positive and nestin-negative (Ogawa *et al.*, 2006; Zhao *et al.*, 2007). Combined with previous findings, therefore, our observations imply that Runx2 might be involved in the formation of bone-like tissues in reparative dentin.

In conclusion, the continuous expression of *Runx2* in odontoblasts disturbed odontoblast differentiation at a late stage and resulted in the formation of a bone structure. Runx2 down-regulated odontoblast-specific proteins, nestin and DSPP, but induced osteopontin and DMP1, indicating that Runx2 induced the transdifferentiation of odontoblasts into osteoblasts. Thus, our findings indicate that Runx2 expression has to be down-regulated during odontoblast differentiation to maintain the phenotype of odontoblasts and acquire full odontoblast differentiation for dentinogenesis.

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