



The pro- α 2(XI) collagen gene is expressed in odontoblasts

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ABSTRACT

Since the dentine is analogous to bone, its extracellular matrix shares many similarities to bone tissues. Similar to the bone, type I collagen is the major organic component in dentine. However, little is known about minor fibrillar collagens, which seem to be co-expressed such as type I or II collagen. The present study examined the gene expression of type V and XI collagens, which play important roles in collagen fibril formation and skeletal morphogenesis, using RT-PCR and *in situ* hybridization combined with immunohistochemistry. The transcripts of pro- α 1(XI), pro- α 2(XI), pro- α 1(V) and pro- α 2(V) chains were present, but not pro- α 3(V) and pro- α 1(II) chains, of which an overglycosylated variant is pro- α 3(XI) chain, in mouse incisor tooth, using RT-PCR and *in situ* hybridization. The pro- α 2(XI) protein, which is mainly expressed in cartilage, were observed in the odontoblast using a specific polyclonal antibody. Real-time RT-PCR showed that the transcripts of pro- α 2(XI), pro- α 1(V) and pro- α 2(V) were predominant in crown and that of pro- α 1(XI) in root of the tooth. Finally, the expression of pro- α 2(XI) was confirmed with an odontoblastic cell line transformed with human telomerase reverse transcriptase (hTERT) both *in vitro* and *in vivo*. The data suggest a new subtype of the V/XI collagen molecule containing α 2(XI) chain.

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Introduction

Odontoblasts are responsible for the formation of dentine, the collagen-based mineralized tissue that forms the bulk of teeth. Odontoblasts are derived from ectomesenchymal cells, exhibit a tall columnar shape and establish a continuous single layer with a clear epithelioid appearance [1]. After dentinogenesis, they are aligned along the periphery of the dental pulp, thus playing a role in the maintenance of the tooth integrity owing to their capacity for depositing new layers of dentine throughout life. Since dentine is a tissue analogous to bone, its extracellular matrix shares many similarities with the bone matrix.

Collagen is an important class of extracellular matrix components. The structural integrity and functional properties of different tissues are influenced by the characteristic combinations and amounts of these molecules [2]. Among them, type I, II, III, V and XI collagens are included in the group of fibril-forming collagens, based on their structural and functional features and are divided

Abbreviations: hTERT, human telomerase reverse transcriptase; PBS⁻, phosphate-buffered saline, Mg²⁺ and Ca²⁺ free; DSPP, dentin sialophosphoprotein; DSP, dentin sialoprotein; RT-PCR, reverse transcription-polymerase chain reaction; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay.

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into two subgroups, major (I, II and III) and minor (V and XI) fibrillar collagen based on their contents in tissues [3]. Minor fibrillar collagens type V and XI are incorporated into the fibrils of the much more abundant collagen types I and II, respectively, and act as regulators of the sizes and shapes of the resultant heterotypic fibrils [4–6].

Type I collagen is mainly expressed in odontoblast [7]. However, little is known regarding the expression of minor fibrillar collagens [8]. The expression of minor fibrillar collagen was examined in the tooth. The pro- α 2(XI) collagen chain, which is a component of type XI collagen found in the fibrillar network of cartilage, was observed to be expressed in odontoblasts.

Materials and methods

Animals. The mice and rabbits were purchased from commercial sources (Yoshitomi, Fukuoka, Japan). The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Staging of mouse embryos and preparation of sections. The gestational age was initially determined by the date of formation of the copulation plug and confirmed by crownrump length. For *in situ* hybridization and immunohistochemistry, the head portion of

mouse embryo was fixed overnight in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS⁻), dehydrated and embedded in paraffin and 10 μ m consecutive sections were prepared.

RT-PCR (reverse transcription-polymerase chain reaction) analysis. Total RNA (5 μ g) from a mouse tooth was reverse transcribed using MMLV reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) with random hexamers at 37 °C for 1 h. The reaction sample was then heated at 95 °C for 5 min to stop reaction. After reverse transcription, PCR was performed in a 25 μ l mixture containing 1 μ l of reverse transcription the reaction product, using Go Taq Green Master Mix (Promega, Madison, WI, USA). Twenty-eight cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min and extension at 72 °C for 1 min were performed for *Col1a1*, *Col2a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col11a1*, *Col11a2*, *Dspp* (Dentin sialophosphoprotein) and β -actin genes. The gene-specific primers are listed in Appendix Table 1. PCR products (12 μ l) were resolved electrophoretically on 1.5% agarose gel and the gel was stained with ethidium bromide and photographed under UV light.

Real-time RT-PCR. For a quantitative analysis of the expression level of the mRNA, real-time PCR was performed using a Light-Cycler TaqMan Master (Roche, Indianapolis, IN). The thermal cycling conditions included 1 cycle at 95 °C for 10 min, and 45 cycles at 95 °C for 10 s and at 60 °C for 30 s. The relative mRNA expression level of *Dspp*, *Col111*, *Col11a2*, *Col5a1* and *Col5a2* was normalized against that of the β -actin gene from the same RNA preparations using a comparative threshold cycle method. The primer sequences are listed in Appendix Table 1.

In situ hybridization. cDNAs for *Col1a1*, *Col2a1*, *Col5a1*, *Col5a2*, *Col5a3*, *Col11a1*, *Col11a2* and *Dspp* genes were generated by RT-PCR (Appendix Table 1). The amplified fragments were subcloned into the pGEM-T Easy vector (Promega). The inserts were further subcloned into a pBluescript vector for generating riboprobes. All constructs were sequenced on an ABI310 sequencer (Applied Biosystems, Foster, CA, USA). After linearization at the appropriate restriction sites, antisense and sense probes were generated by *in vitro* transcription with T3 or T7 polymerases in the presence of [³⁵S]-dUTP. *In situ* hybridization was performed on 10 μ m sections, as previously described [9].

Production of GST (glutathione S-transferase)-tagged fusion proteins. cDNAs encoding a portion of pro- α 1(XI), pro- α 2(XI) collagen and dentin sialoprotein (Dsp), which is derived from 5' the region of the DSPP gene, were generated by RT-PCR (Appendix Table 1). The amplified fragments were subcloned into the pGEM-T Easy vector (Promega). Following digestion at the appropriate restriction sites, the fragment was subcloned into a pGEX-4T vector (Amersham). The other recombinant proteins, GST-tagged pro- α 1(V) and pro- α 3(V) have all been previously described [9]. The recombinant GST fusion proteins were expressed and prepared according to the manufacturer's instructions.

Production of antibodies. Recombinant GST-pro- α 2(XI) collagen and Dsp protein (0.5 mg) were mixed with Freund's Complete Adjuvant (Difco, Detroit, MI, USA) and subcutaneously injected into female rabbits. The animals were boosted twice with the same amount of protein mixed with Freund's Incomplete Adjuvant (Difco) and the blood was collected 1 week after the second booster. The antibody was purified using GST affinity column chromatography. The specificity of the antibodies was confirmed by ELISA (enzyme-linked immunosorbent assay) as previously described [9].

Immunohistochemistry. Immunohistochemistry was performed as previously described [10].

Cell culture. The root of an incisor tooth was dissected from adult mice and primary cell culture was conducted as described before [11]. Rat ROS 17/2.8 osteosarcoma cells and rat RCS chondrosarcoma cells were also cultured.

Transfection with hTERT gene and isolating a single clone. Eighty percent of the confluent primary cells from a tooth specimen were

transfected with the construct of hTERT (Geron Corporation, Menlo Park, CA, USA) using lipofectamine (Invitrogen, Carlsbad, CA, USA) [12]. Forty-eight hour after transfection, the cells were replated at a low density. They were further expanded into cell lines. Single clones were passed and assayed for the specific marker, DSPP gene. This yielded cell line, MOC-4, which expressed the DSPP gene.

Transplantation of MOC-4 cells into mice. The transformed MOC-4 cells were transplanted into immunodeficient mice [12]. Briefly, the cells were injected subcutaneously into 4-week-old male BALB/cAnNcrj-nu mice (Charles River Japan Inc., Kanagawa, Japan) after incubating the cells (1.5×10^6) in a mixture of 40 mg of hydroxyapatite granules (Apaseram, Pentax, Tokyo, Japan) and fibrin clot (mixture of mouse fibrinogen and thrombin, Sigma, St. Louis, MO, USA). After one month, the transplants were retrieved and prepared for histological analysis. The sections were decalcified using Kalkitox (Wako, Tokyo, Japan).

Statistical analysis. Data were analyzed by *t*-test using Stat Views J-5.0 (Abacus Concepts, Berkeley, CA, USA). A *P* value of <0.05 was considered to be statistically significant.

Results

Expression of pro α -chains of V/XI collagen

RT-PCR was initially performed using mouse tissue to examine the expression pattern of the α chains constituents of V/XI collagen molecule in the tooth. As shown in Fig. 1A, pro- α 1(XI), pro- α 2(XI), pro- α 1(V) and pro- α 2(V) chains were detectable in an adult mouse incisor tooth, but not pro- α 3(V) and pro- α 1(II). The expression patterns of ROS and RCS cells derived from bone and cartilage, respectively, were different from that of the tooth.

In situ hybridization was performed on mouse neonate, young (Fig. 1B) and embryonic (Fig. 1C) tissue specimens to determine the precise expression of the α chains in the tooth. The DSPP gene was strongly and restrictedly expressed in odontoblasts and preameloblasts. As shown previously [8], pro- α 1(XI) chain was moderately expressed in odontoblasts as well as in bone and cartilage tissues. The pro- α 2(XI) chain was also moderately expressed in odontoblasts. The expression of pro- α 2(XI) was negative in a ROS in an RT-PCR analysis (Fig. 1A), but positive in the surrounding bone tissue using *in situ* hybridization (Fig. 1B). During the developing stage (Fig. 1C), the signal of DSPP gene appeared in odontoblasts at 16.5E of mouse embryo. Very weak signals of the pro- α 2(XI) and pro- α 1(XI) chain were seen in odontoblasts and mesenchymal cells of the interstitial region as well as in bone tissue in the 16.5E embryos (Fig. 1C and Appendix Fig. 1B). The pro- α 1(II) chain was never expressed in odontoblasts in the 16.5E mouse embryo (Appendix Fig. 1B).

Distribution of pro- α 2(XI) collagen chain in tooth

As mentioned above, unexpectedly, we showed transcripts of pro- α 2(XI) collagen gene in odontoblasts for the first time. To detect the protein, we prepared a specific polyclonal antibody against pro- α 2(XI) chain (Appendix Fig. 2A). The specificity of the antibody was confirmed using ELISA (Appendix Fig. 2B). The rabbit antiserum against pro- α 2(XI) polypeptide-GST fusion protein was passed in GST column to eliminate antibody against GST protein. The antibody was not cross-reacted with GST and acidic domains of pro- α 1(V), pro- α 3(V) and pro- α 1(XI) collagen chain. We also generated specific antibody against DSP protein (Appendix Fig. 2C).

Immunohistochemistry was performed using sections of E14.5 embryo and postnatal day 2 mouse (Fig. 2). DSP protein was observed in the dental pulp of the day 2 mouse, but not in E14.5. These findings were consistent with those of *in situ* hybridization.

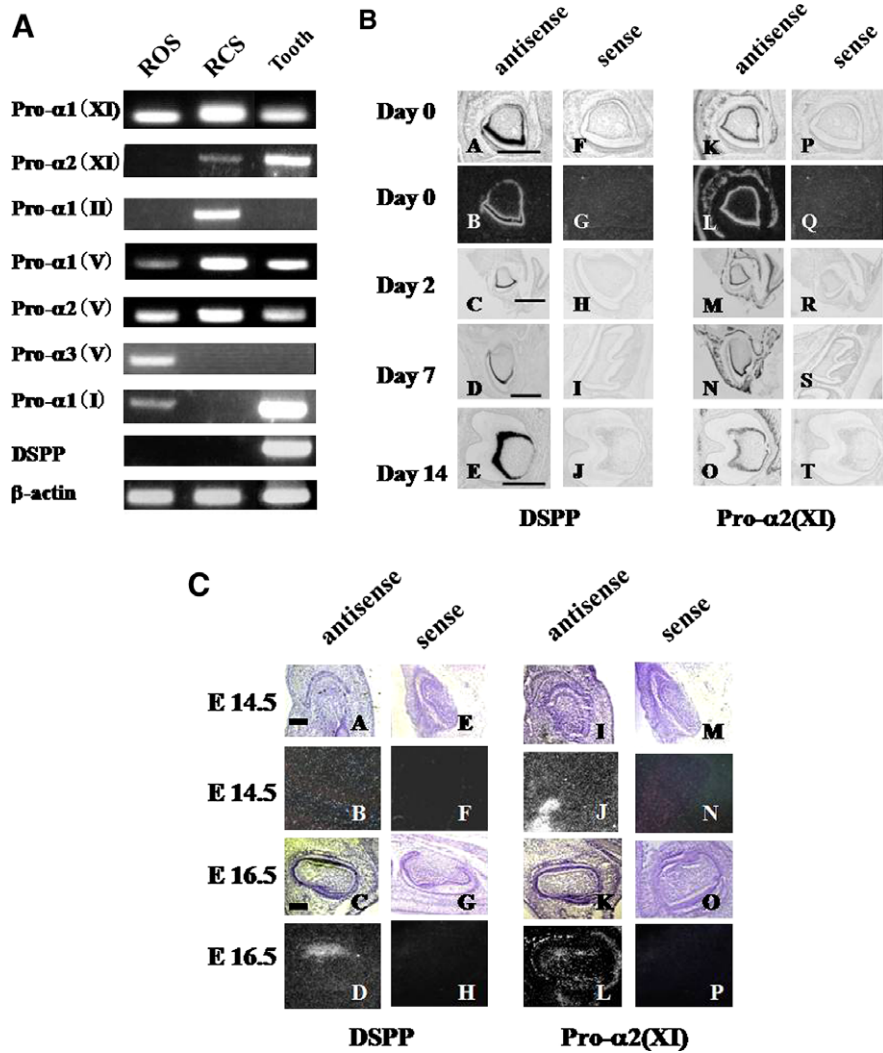


Fig. 1. (A) RT-PCR analysis of pro- α 1(XI), pro- α 2(XI), pro- α 1(II), pro- α 1(V), pro- α 2(V), pro- α 3(V) and pro- α 1(I) collagen chains and DSPP gene expression in ROS cells, RCS cells and teeth. RNA from teeth at day 21 or older mice was used. RT-PCR was performed using specific primers. β -Actin was used as an internal control. (B) *In situ* hybridization of the tissues at a 0, 2, 7 and 14 day mouse. The sections were hybridized with radioactively labeled DSPP and pro- α 2(XI) collagens antisense (A–E, K–O) and sense (F–J, P–T) riboprobes. Photomicrographs are shown with brightfield (days 0, 2, 7 and 14) or darkfield (day 0: panel B, G, L and Q). There is no signal in control panels (F–J, P–T). Scale bar: 100 μ m. (C) *In situ* hybridization of the tissues from E14.5 and E16.5 mouse embryos. The sections were hybridized with radioactively labeled DSPP and pro- α 2(XI) antisense (A–D, I–L) and sense (E–H, M–P) riboprobes. Photomicrographs are shown with brightfield (panels A, C, E, G, I, K, M and O) or darkfield (panels B, D, F, H, J, L, N and P). There is no signal in control panels (E–H, M–P). Scale bar: 100 μ m.

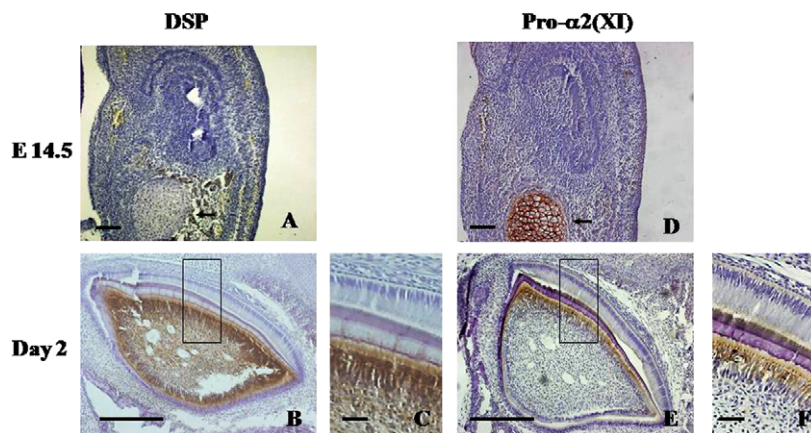


Fig. 2. Immunohistochemical localization of DSP protein and pro- α 2(XI) collagen chain in E14.5 mouse embryos and day 2 mice. Photomicrographs were shown for sections of an E14.5 mouse embryo (A and D) and a day 2 mouse (B, C, E and F). The portions of square in B and D were magnified in C and F, respectively. Paraffin sections were stained with anti-DSP (A, B and C) and anti-pro- α 2(XI) antibody (D, E and F). The arrows in A and D show the cartilage tissue. Note that anti-pro- α 2(XI) antibody stained the cartilage tissue in the E14.5 mouse embryo (D). Scale bar in A and D: 50 μ m, B and E: 100 μ m, C and F: 10 μ m.

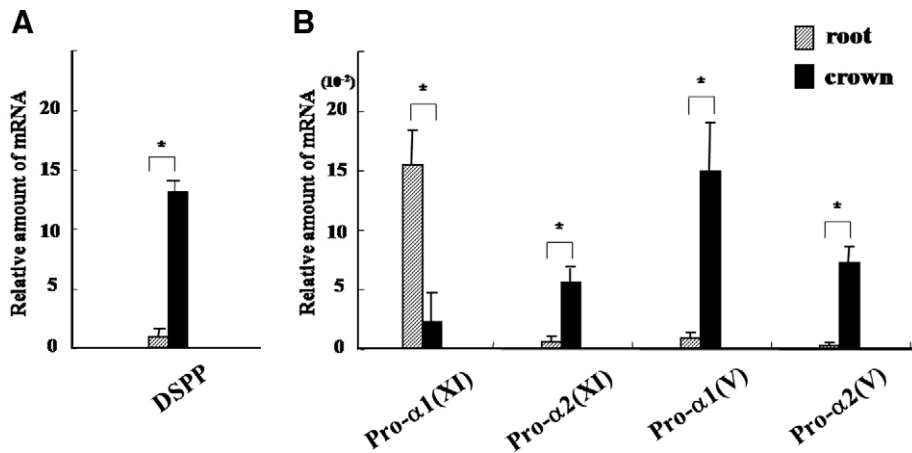


Fig. 3. The relative amount of mRNA of pro-α1(XI), pro-α2(XI), pro-α1(V) and pro-α2(V) collagen chains and DSPP in crown and root of tooth using real-time RT-PCR. (A) The values of the relative ratio of mRNA of DSPP against that of β-actin are the mean ± SD for three independent wells. **p* < 0.05 (B) The values of the relative ratio of mRNA of pro-α1(XI), pro-α2(XI), pro-α1(V) and pro-α2(V) collagen chains against that of β-actin are the mean ± SD for three independent wells. **p* < 0.05 Note that the values of pro-α1(XI), pro-α2(XI), pro-α1(V) and pro-α2(V) collagen chains are less than 10² times than that of DSPP.

The pro-α2(XI) chain was observed in odontoblasts. At E14.5 it was seen in cartilage tissue (an arrow in Fig. 2D), but never in the odontoblasts.

Relative amount of mRNA in tooth

Real-time RT-PCR was used to examine the relative amount of mRNA of each gene in tooth. The tissues specimens from a tooth of 5-week-old mouse were divided into two portions, namely the root and crown. The mean value of the relative amount of transcripts of pro-α1(XI), pro-α2(XI), pro-α1(V), pro-α2(V) and DSPP genes against that of β-actin were 16.30 × 10⁻², 0.53 × 10⁻², 0.98 × 10⁻², 0.16 × 10⁻² and 0.98 in the root, 1.58 × 10⁻², 6.33 × 10⁻², 14.70 × 10⁻², 6.67 × 10⁻² and 13.30 in the crown (Fig. 3), respectively. The amount of transcripts of the pro-α2(XI), pro-α1(V) and pro-α2(V) in the crown were 11.9, 15.0 and 41.7 times greater than in the root.

In contrast, that of pro-α1(XI) in the crown was 0.1 times lower than in the root.

Expression of pro-α2(XI) chain in the immortalized odontoblast

There is a quantitative and qualitative limitation to using primary culture cells from tooth. To avoid those problems, a cell line was established that retains the nature of the odontoblasts using hTERT. The established cell line, MOC-4, was morphologically similar to the non-transfected original cells (Appendix Fig. 4) and expressed the DSPP gene as well as the hTERT gene (Fig. 4A). The transcript of the pro-α2(XI) chain was also expressed in MOC-4 cells. To examine *in vivo*, MOC-4 cells were transplanted subcutaneously into immunodeficient mice. Alkaline phosphatase activity was detectable in the growing tumor cells (Fig. 4B-b). The tumor cells expressed the pro-α2(XI) collagen chain as well as the DSP protein (Fig. 4B-c and B-d).

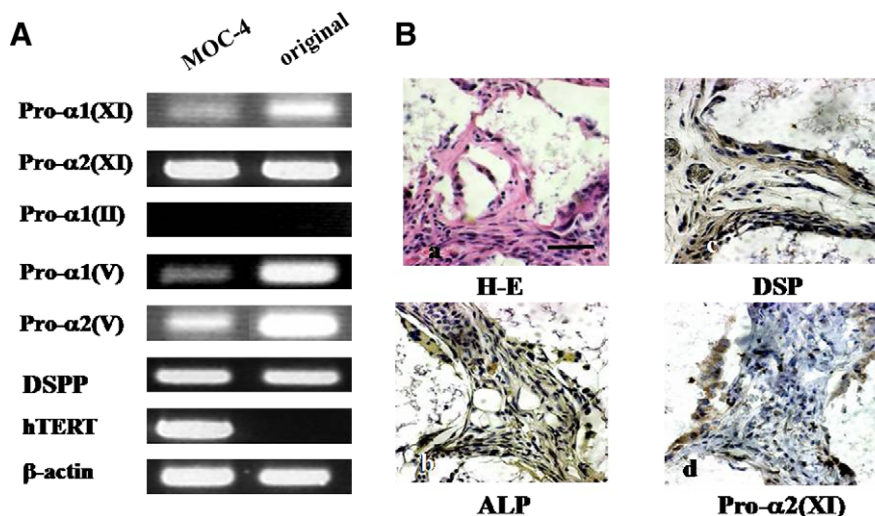


Fig. 4. The gene expression in the transformed odontoblast. (A) Expression of pro-α1(XI), pro-α2(XI), pro-α1(II), pro-α1(V) and pro-α2(V) collagen chains, hTERT and DSPP genes in MOC-4 and original cell. β-Actin was used as an internal control. (B) Immunohistochemical localization of DSP and pro-α2(XI) collagen chain in a tumor that was transplanted MOC-4 cell. The photomicrographs show hematoxylin and eosin (H-E) staining (a), staining for alkaline phosphatase activity (b) and immunohistochemical staining with anti-DSP protein (c) and anti-pro-α2(XI) collagen (d) antibodies. Scale bar: 10 μm.

Discussion

The present study examined the expression of type V/XI collagen in the tooth. Unexpectedly, a moderate expression of the pro- $\alpha 2(XI)$ chain was seen in the tooth using RT-PCR and *in situ* hybridization combined with immunohistochemistry methods. The data were confirmed using an established cell line that possesses osteoblastic characteristics in regard to morphology and gene expression. Type XI collagen, which is composed of $\alpha 1(XI)$, $\alpha 2(XI)$ and $\alpha 3(XI)$ chain, is expressed in cartilage. However, the $\alpha 1(XI)$ collagen chain is widely expressed in non-cartilage tissues [8]. In comparison to the $\alpha 1(XI)$ chain, the expression of the $\alpha 2(XI)$ and $\alpha 3(XI)$ chains is rather restricted to cartilage.

Type XI collagen could nucleate and promote the assembly of type II collagen into thin fibrils in cartilage [13]. On the other hand, the molecule containing $\alpha 1(V)$ chain can form banded fibrils with type I collagen [14]. Previous biochemical studies have documented the presence of heterotypic collagen molecules consisting of types V and XI collagen chains. The [$\alpha 1(XI)$] $\alpha 2(V)$ molecule is present in a human rhabdomyosarcoma cell line (A204) and the bovine vitreous [15,16]. Niyibizi and Eyre have previously suggested that $\alpha 1(V)$ chain is involved in forming heterotypic V/XI molecules in bovine bone [17]. In the chondrodysplasia mouse (*cho*), which is a neonatal lethal mouse caused by absence of $\alpha 1(XI)$ collagen chain, Fernandes et al. hypothesized the substitution of $\alpha 1(V)$ for $\alpha 1(XI)$ collagen chain and expected $\alpha 1(V)\alpha 2(XI)$ in the mouse [18]. The present study has showed the $\alpha 1(XI)$ and $\alpha 2(XI)$ chains to be expressed, but never $\alpha 1(II)$ chain in dentin. Therefore, type XI collagen, which contain $\alpha 3(XI)$ chain which is overglycosylated variant of $\alpha 1(II)$ chain, is never present. Instead, a homotrimer of $\alpha 2(XI)$ chain or heterotypic V/XI molecule containing $\alpha 2(XI)$ chain may therefore be present in dentin. A real-time PCR analysis showed the amount of pro- $\alpha 2(XI)$, pro- $\alpha 1(V)$ and pro- $\alpha 2(V)$ mRNA to be similar in the root and crown. This may therefore suggest the occurrence of the molecule of $\alpha 2(XI)\alpha 1(V)\alpha 2(V)$.

The current data suggest a new subtype of V/XI collagen molecule containing $\alpha 2(XI)$ chain and the complexity of the molecular assembly in dentin. The different combination of type V/XI collagen chains could confer different physiological properties to the fibril network in the matrix.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.001.

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