M Savontaus
M Rintala-Jämsä
J Morko
O Rönning
M Metsäranta
E Vuorio

Abnormal craniofacial development and expression patterns of extracellular matrix components in transgenic Del1 mice harboring a deletion mutation in the type II collagen gene

Authors' affiliations:
Mikko Savontaus, Jukka Morko, Marjo Metsäranta, Eero Vuorio, Department of Medical Biochemistry and Molecular Biology, University of Turku, Turku, Finland
Marjo Rintala-Jämsä, Olli Rönning, Institute of Dentistry, University of Turku, Turku, Finland
Present addresses: Mikko Savontaus, Mount Sinai School of Medicine, Institute for Gene Therapy and Molecular Medicine, New York, NY 10029, USA; *Marjo Metsäranta,* Hospital for Children and Adolescents, University of Helsinki, FIN-00014 Helsinki, Finland

Correspondence to:
Dr Eero Vuorio
Department of Medical Biochemistry and Molecular Biology
University of Turku
Kiinamyllynkatu 10
FIN-20520 Turku
Finland
Tel.: +358 2 333 7349
Fax: +358 2 333 7229
E-mail: eero.vuorio@utu.fi

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Structured Abstract

Authors – Savontaus M, Rintala-Jämsä M, Morka J, Rönning O, Metsäranta M, Vuorio E

Objective – To analyze the effect of a type II collagen mutation on craniofacial development in transgenic Del1 mice.

Design – Samples from homozygous (+/+) and heterozygous (+/–) transgenic Del1 mice harboring mutations in the type II collagen gene as well as non-transgenic (–/–) littermates were collected at days 12.5, 14.5, 16.5 and 18.5 of gestation. The cartilaginous and bony elements of the craniofacial skeleton were analyzed after staining with alcian blue, alizarin red S and von Kossa. The expression patterns of type II, IX and X collagens and aggrecan were analyzed by immunohistochemistry and *in situ* hybridization.

Results – Several abnormalities were observed in the craniofacial skeleton of transgenic Del1 mice. These include an overall retardation of chondrogenesis and osteogenesis in Del1 +/+ mice, and to a lesser extent also in Del1+/– mice. Characteristic findings in Del1 +/+ mice included a reduced anterioposterior length, a smaller size of the mandible, a palatal cleft and a downward bending snout. We also detected retarded ossification of calvarial bones in Del1 +/+ and +/– mice when compared with Del1 –/– mice. A surprising finding was the presence of both type II and X collagens and their mRNAs in the periosteum of the cranial base.

Conclusion – The present study confirms the important role of type II collagen mutation in craniofacial development and growth. In addition to affecting endochondral ossification, the

type II collagen mutation also disturbs intramembranous ossification in the developing craniofacial skeleton.

Key words: cartilage; chondrodysplasia; collagen; craniofacial development; bone; transgenic mice

Introduction

Disproportionate growth of the craniofacial complex is a common finding in several osteochondrodysplasias (1, 2). As the growth of the craniofacial skeleton proceeds by a combination of endochondral and intramembranous ossification and also involves specific features, such as Meckel's cartilage and secondary cartilages, the role of individual molecular components of cartilage in determining the craniofacial morphology has been difficult to assess. The models employed in previous studies include artificial arrest of sutural growth (3, 4) transplantation of chondro-osseal structures (5) and disruption of cartilage matrix by systemic introduction of papain (6). Additional models include natural mutations affecting murine craniofacial growth, such as *cho* (7), *dmm* (8) and *cmd* (9), later shown to result from mutations in the genes coding for the proα1(XI) collagen chain (10), the proα1(II) collagen chain (11) and aggrecan (12), respectively. The availability of transgenic and knockout mouse models has introduced a new way to address the roles of specific gene-protein systems on skeletal growth. We have previously reported on the consequences of two engineered mutations in the type II collagen gene on the craniofacial development and growth in transgenic mice (13, 14).

Type II collagen is the major collagen in cartilage. It is synthesized as procollagen consisting of three identical proα1(II) collagen chains, coded for by the *Col2a1* gene. In the extracellular space the procollagen molecules undergo proteolytic processing and the resultant rod-like type II collagen molecules associate with each other in a quarter-staggered manner into fibrils which also contain small amounts of type IX and type XI collagen molecules (15). The fundamental role of type II collagen for the structural integrity of cartilage is demonstrated by the different chondrodysplasias and other skeletal and ocular disorders resulting from mutations in the human *COL2A1* or murine *Col2a1* genes (16, 17).

The role of cartilage in endochondral ossification is obvious; it provides the anlage for bone formation. The appearance of ossification centers in specific locations of these cartilages follows a well established temporal and spatial sequence (18). At the base of the skull, specific bipolar growth zones, synchondroses, form between the ossification centers (19). Of the murine calvarial bones, only the occipital bone and part of the temporal bone undergo endochondral ossification. Other calvarial bones undergo intramembranous ossification where cartilage does not play an intermediary role (18). The mandible also ossifies intramembranously. The process is dependent on the presence of Meckel's cartilage, which is not replaced by bone, but serves as a model for bone formation on its external aspect (20). In the facial skeleton, e.g. the maxillary and pre-maxillary bones undergo intramembranous ossification. The anterior portion of the nasal cartilage does not undergo ossification, but remains cartilaginous through life.

Previous systematic studies on matrix production during abnormal craniofacial development in transgenic mice harboring mutations in matrix genes are not available. This study was initiated to obtain such information. Our hypothesis was that genetically disturbed production of the major structural component of cartilage, type II collagen, interferes with the development and growth of the craniofacial complex, and the resultant growth abnormalities reflect the relative importance of chondrogenesis and endochondral ossification for the respective parts of the craniofacial complex. The consequences of the mutation were analyzed by histology for the schedule of chondrogenesis and ossification. Furthermore, *in situ* hybridization and immunohistochemistry were used to characterize the production of specific matrix components during embryonic growth of the craniofacial complex.

Materials and methods

Experimental animals

This study was performed on the offspring of one transgenic founder mouse Del1 carrying six copies of a

mouse type II collagen transgene with a 150-bp deletion containing the 45-bp exon 7 and intron 7 (21). Mice heterozygous for the transgene locus were mated and the embryos were removed by Caesarean section at days 12.5, 14.5, 16.5 and 18.5 of gestation (marked E14.5, E16.5 and E18.5, respectively). This experimental set-up produced embryos homozygous (+/+) and heterozygous (+/−) for the transgene locus as well as non-transgenic (−/−) littermates, which served as controls. DNA was extracted from the tails and/or placentae of the animals for genotyping by polymerase chain reaction (PCR) and Southern hybridization. For visualization of cartilaginous and bony elements of the craniofacial skeleton, 12 complete litters were stained with alcian blue and alizarin red S (22). The rest of the litters were used for histology, immunohistochemistry and *in situ* hybridization. The samples used in this study were selected from a series of 41 litters used for an investigation on the effects of the Del1 mutation on endochondral ossification and growth of long bones as reported earlier (23).

Histology and *in situ* hybridization

For histologic examination and *in situ* hybridization, the heads of mouse embryos were fixed overnight in 4% formaline in phosphate buffered saline (PBS) at room temperature, dehydrated in a graded ethanol series and embedded in paraffin for sectioning. The sections were stained with hematoxylin–eosin, alcian blue or von Kossa. For *in situ* hybridization, the sections were pretreated with proteinase K (3.5 μg/ml for 30 min at 37°C) and acetylated as described earlier (23). The hybridizations were performed at 52°C for 18 h. Hybridization was followed by washes and autoradiography for 2.5–34 days, and staining of the sections with hematoxylin.

Preparation of probes

The mRNAs for proα1(II), α1(IX), and α1(X) collagens and aggrecan were analyzed using cDNA clones pMCol2a1-1, pMCol9a1-1 (24), pMCol10a1-1 (25) and pMAgg-1 (26), respectively. Antisense and sense riboprobes were synthesized using Trans-Probe-T™ kit (Pharmacia LKB, Uppsala, Sweden) and ^{35}S-(thio)UTP (>1000 Ci/mmol; Amersham International, Amersham, UK), and an extended incubation time of 1.5 h for transcription. After DNase I digestion the reactions were heated at 65°C for 15 min, cooled on ice, and fractioned on a Sephadex G-50 column (Pharmacia).

Immunohistochemistry

The distribution of type II and X collagens was studied using monoclonal antibodies against chick type II collagen (27) and against human recombinant type X collagen (28). Deparaffinized tissue sections were rehydrated and digested for 1 h with hyaluronidase (2 mg/ml) in PBS (pH 5). After washing, the anti-type X or II collagen primary antibodies (1:20 and 1:100 dilutions, respectively) were applied overnight at 4°C, and detected using a biotin-labeled secondary antibody and horseradish peroxidase-conjugated streptavidin (Links and Label™ kit; BioGenex, San Ramon, CA, USA) as described earlier (29).

Results

Time schedule of craniofacial ossification

Development of the craniofacial skeleton was first investigated by alcian blue/alizarin red staining of Del1 +/+, +/− and −/− embryos at E14.5, E16.5 and E18.5. Of the calvarial bones, formation of the supraoccipital bone and the petrous part of the temporal bone was seen to proceed via endochondral ossification, while the rest of the temporal bone, and the parietal and frontal bones ossified via the intramembranous route (Fig. 1). An overall retardation of ossification was seen in Del1 +/+ mice, and to a lesser degree also in Del1 +/− mice (Fig. 1). This delay was evident already at the onset of ossification at E14.5 in bones undergoing mineralization by both the endochondral (e.g. vertebral column and base of skull) and intramembranous route (e.g. mandible). Retarded ossification of calvarial bones of Del1 +/+ mice undergoing intramembranous ossification was consistently seen at E16.5. Histologic analysis of sagittal sections of the heads confirmed these observations, as shown for the cranial base in Fig. 2.

Growth retardation in transgenic mice

In addition to the delayed ossification schedule, both the alcian blue/alizarin red staining (Fig. 1) and histologic examination (Fig. 2) revealed reduced longitudinal growth of different craniofacial cartilages and

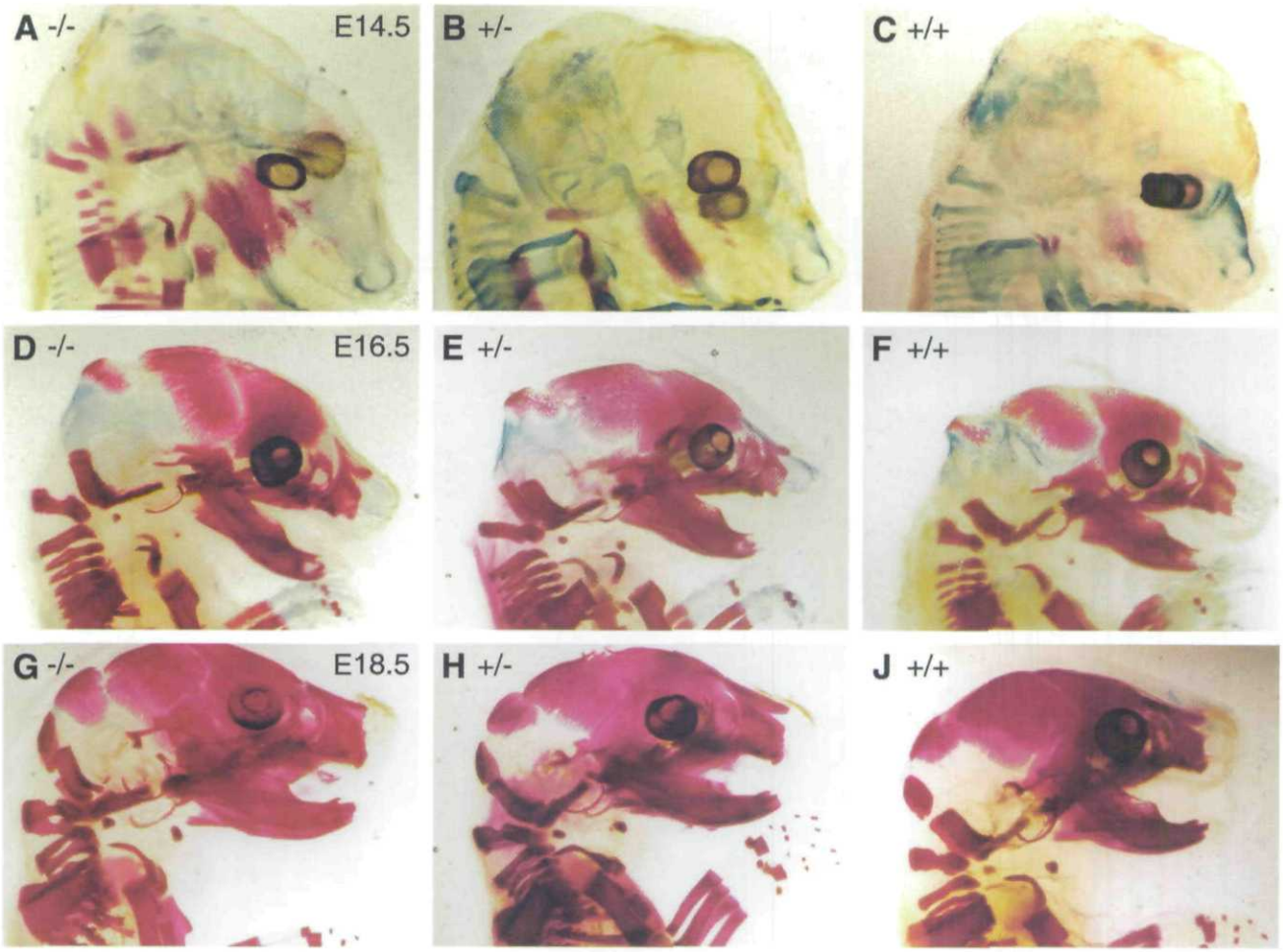

Fig. 1. Craniofacial development of Del1 −/− (A, D and G), +/− (B, E and H) and +/+ mice (C, F and J) at 14.5 (A–C), 16.5 (D–F) and 18.5 days (G–J) of embryonic development. The genotypes (Del1 −/−, +/− and +/+) are shown in each panel, and the embryonic stage (E14.5, E16.5 and E18.5) for each horizontal line in the panel to the left. Alizarin red/alcian blue staining.

bones, and suggested an abnormal shape of the entire head. These differences were seen through the observation period from E14.5 to E18.5, and were most obvious between Del1 +/+ and Del1 −/− mice, Del1 +/− mice representing an intermediate. In association with the reduced anterioposterior length of the skull and Meckel's cartilage, all Del1 +/+ mice exhibited a cleft palate (Fig. 2C,F,J), whereas none of the Del1 +/− mice presented with such a defect.

A typical feature in Del1 +/+ mice was a downward bending snout, which was often in contact with the thorax. A morphological difference was also observed at the junction between the cranial base and the vertebral column: in Del1 +/+ mice the cranial base was located at the same level with the atlas, while in the Del1 +/− and −/− mice the occipital part of the cranial base was located above the atlas (Fig. 2).

In many instances the cartilaginous synchondroses in Del1 +/+ mice appeared shorter and thicker than in non-transgenic control mice, making the entire base of the skull appear shorter and thicker. No gross abnormalities were seen in the cartilages of Del1 +/− or +/+ mice in the larynx, pharynx and nose.

Matrix production and deposition during craniofacial development

To better understand the mechanisms leading to abnormal craniofacial development of Del1 mice, the mRNA levels for major cartilage components were studied by *in situ* hybridization. At day E14.5, type II collagen mRNA was localized throughout the cranial base in the Del1 +/+ and +/− embryos. In Del1 −/− embryos the occipital part of the base did not contain type II collagen mRNA as this part was already

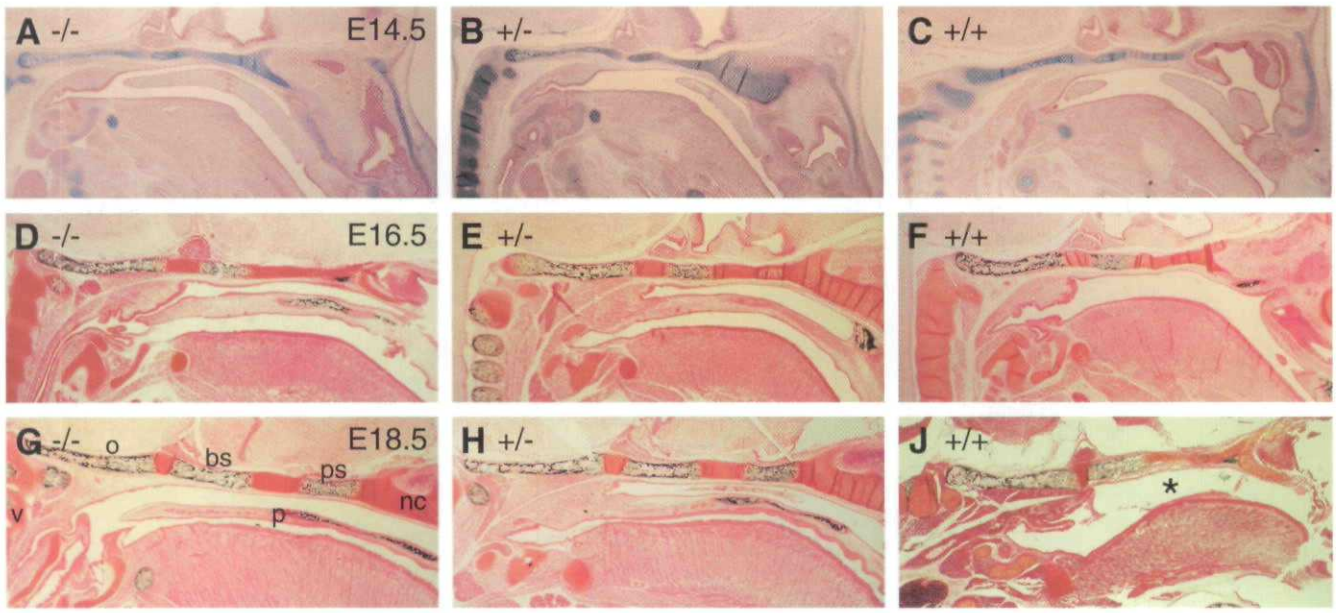

Fig. 2. Development and growth of the cranial base in Del1 −/− (A, D and G), +/− (B, E and H) and +/+ mice (C, F and J) at 14.5 (A–C), 16.5 (D–F) and 18.5 days (G–J) of embryonic development. The genotypes (Del1 −/−, +/− and +/+) are shown in each panel, and the embryonic stage (E14.5, E16.5 and E18.5) for each horizontal line in the panel to the left. Key anatomical structures are marked in panel G: bs, basisphenoidal bone; nc, nasal cartilage; o, occipital bone; p, palate; ps, presphenoidal bone; v, vertebral column. The asterisk in panel J denotes the palatal cleft. Histologic sections were stained with alcian blue (A–C) or von Kossa (D–J).

undergoing endochondral ossification (Fig. 3D–F). This finding provides additional proof for the retarded development of Del1 +/+ and +/− embryos. This is further demonstrated by the comparable expression patterns of mRNAs for two other cartilage matrix components, type IX collagen (Fig. 3G–J) and aggrecan (Fig. 3K–M). The presence of type X collagen mRNA was restricted to hypertrophic chondrocytes. In Del1 −/− embryos type X collagen mRNA was expressed widely through the occipital bone and in the basisphenoidal bone, whereas in Del1 +/+ and +/− embryos this mRNA was localized only in the anterior part of the occipital bone (Fig. 3N–P).

At day E16.5, the ossification of the cranial base had progressed as demonstrated by the more restricted pattern of type II collagen mRNA expression (Fig. 4D–F). The mRNA could no longer be detected in the basioccipital or basisphenoidal bone in Del1 −/−. The reduced length of these structures is an example of the retarded skeletal development in Del1 +/+ and +/− embryos. Type IX collagen (Fig. 4G–J) and aggrecan mRNAs (Fig. 4K–M) exhibited similar cartilage-specific expression patterns than type II collagen mRNA, except for enhanced perichondrial expression of the α1(IX) collagen mRNA in the occipital and sphenoidal bones of Del1 +/+ and +/− embryos. In addition to the presence of type X collagen mRNA in hypertrophic chondrocytes of the synchondroses, the mRNA was also seen in the periosteal region of occipital and sphenoidal bones where no hypertrophic cells could be identified.

The surprising observation of cartilage collagen mRNAs in the periosteal regions of the cranial base prompted immunohistochemical analyses of serial sections of E16.5 embryos for type II and X collagens (Fig. 5). In addition to staining all cartilages, the antibodies to type II collagen also recognized the matrix surrounding hypertrophic chondrocytes, parts of the septae of primary spongious bone, and periosteum (Fig. 5C,D). The antibodies to type X collagen also stained the matrix around hypertrophic chondrocytes, parts of the cancellous bone septae, and periosteum (Fig. 5E,F).

At day E18.5, ossification of the base of the skull had progressed in all genotypes but the retardation in the ossification schedule persisted in Del1 +/+ and +/− mice (Fig. 2G–J). This observation was supported by the expression patterns of type I and II collagen mRNAs, determined by *in situ* hybridization which resembled those observed at day E16.5 (data not

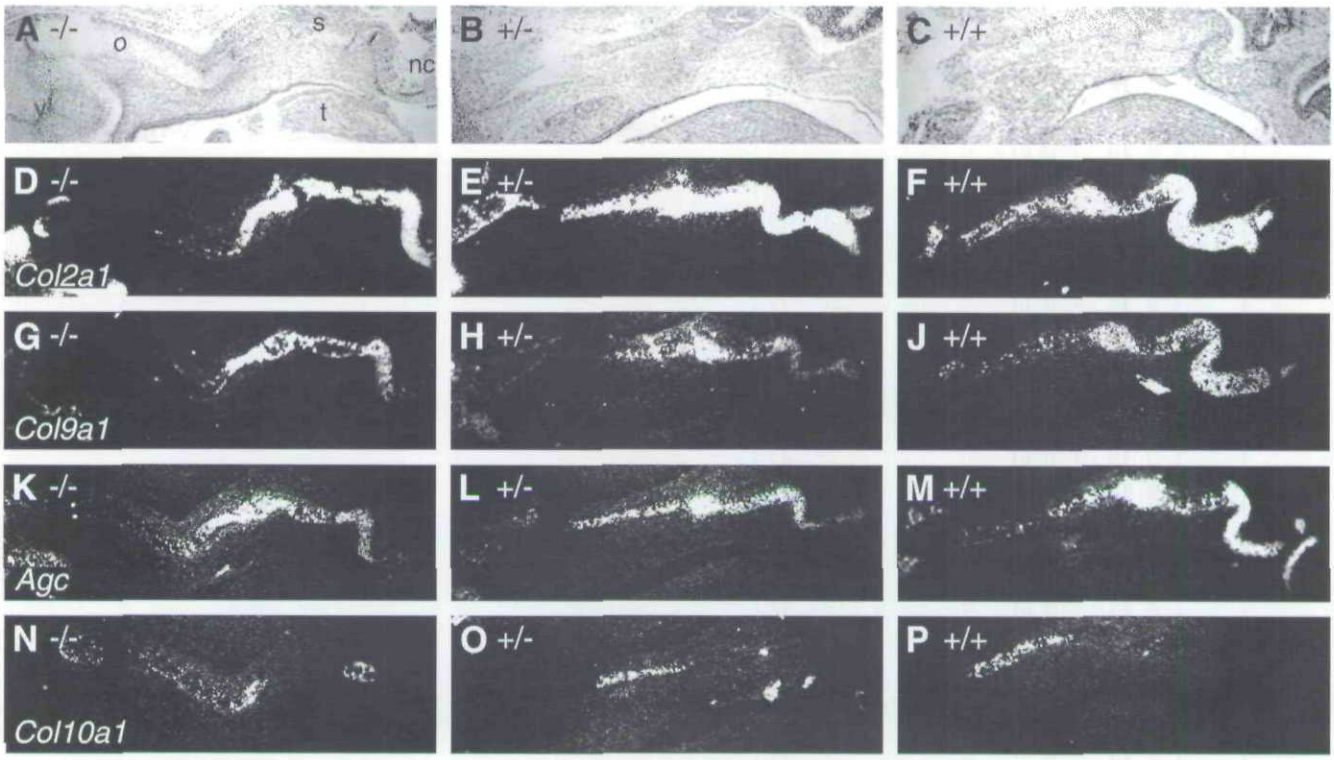

Fig. 3. In situ hybridization of sagittal sections of E14.5 Del1 −/− (A, D, G, K and N), Del1 +/− (B, E, H, L and O) and Del1 +/+ embryos (C, F, J, M and P) illustrating the localization of mRNAs for proα1(II) collagen (D–F), α1(IX) collagen (G–J), aggrecan (K–M) and α1(X) collagen (N–P). Panels A–C show light microscopic views of hematoxylin/eosin-stained serial sections. The other panels are dark field views of the autoradiographic grains. The genotypes (Del1 −/−, +/− and +/+) are shown in each panel, and the probes used for each horizontal line in the panel to the left: Col2a1, type II collagen; Col9a1, type IX collagen; Col10a1, type X collagen; Agc, aggrecan. Key anatomical structures are marked in panel A: o, occipital bone; s, basi/presphenoidal bone; t, tongue; v, vertebral column; nc, nasal cortilage.

shown). The proportion of hypertrophic cells was clearly increased, and the endochondral formation of trabecular bone retarded in Del1 +/+ embryos when compared with Del1 −/− littermates (Fig. 2).

Discussion

Several consistent findings were made on the development and growth of the skulls of transgenic Del1 +/+ and +/− mice when compared with their non-transgenic control littermates. An overall retardation of chondrogenesis and osteogenesis was seen in Del1 +/− mice, and to a greater extent in Del1+/+ mice. Analogous findings have been made on endochondral ossification of long bones and vertebral column in these mice (21, 23, 30). In addition to delayed appearance of ossification centers, the growth of the entire skeleton was delayed in Del1 +/+ mice, and to a smaller extent also in Del1 +/− mice. This was particularly well demonstrated by the retarded longitudinal growth of the skull (Figs 1 and 2), which is likely to contribute to the other abnormalities seen in the heads of Del1 +/+ and +/− mice. Although we have successfully performed cephalometric measurements on mice at the age of 3, 6 and 9 months (14), the difficulty of identifying the anatomical structures used for cephalometry in embryonic skulls, the tendency of embryonic skulls to deform during sample preparation and the dependency of the measurements on the plane of sectioning, made such measurements too unreliable for statistical analyses.

The size of the mandible also appeared reduced in Del1 +/+ embryos. This was obvious already in day E14.5 Del1 +/+ mice as a smaller size of Meckel's cartilage and associated retardation of intramembranous ossification of the mandible (Fig. 1). Both the formation of Meckel's cartilage and the ossification process have previously been linked with innervation of the area (20, 31, 32). As the prechondrogenic form of type II collagen, type IIA, has been identified in early neuroepithelium (33–35), it is possible that the retarded

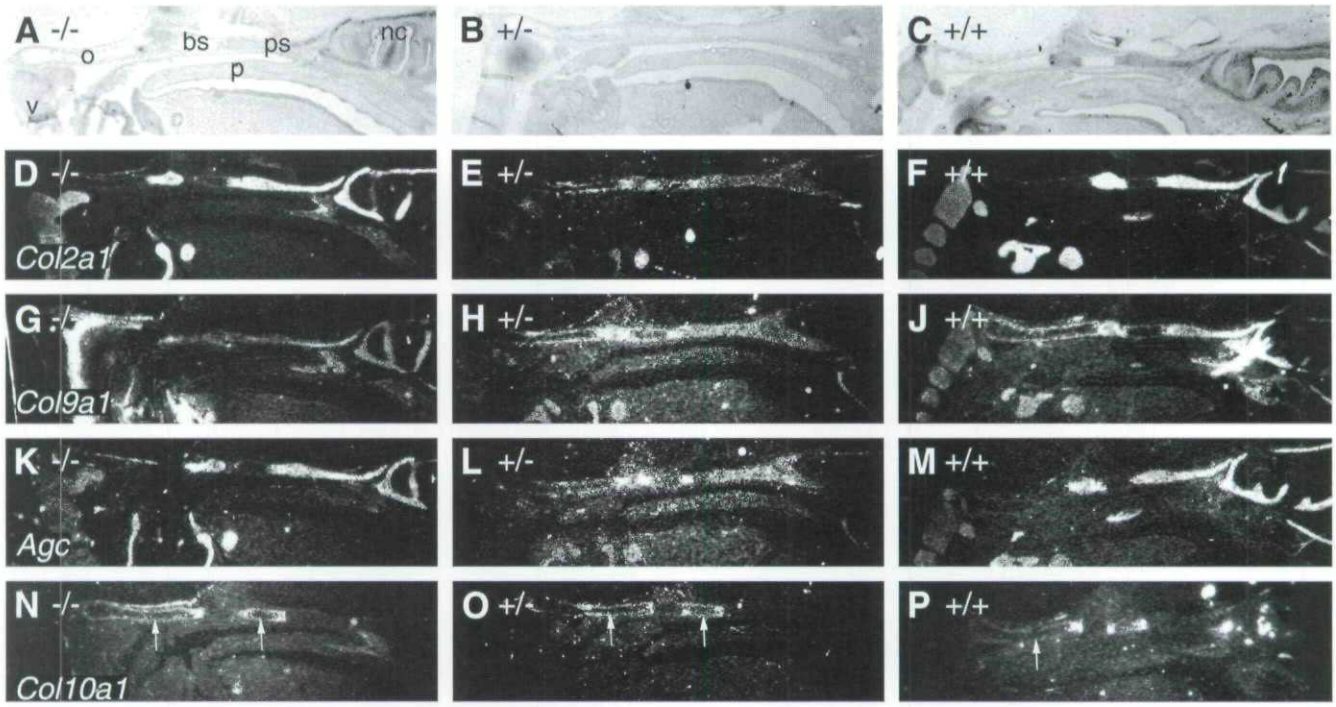

Fig. 4. In situ hybridization of sagittal sections of E16.5 Del1 −/− (A, D, G, K and N), Del1 +/− (B, E, H, L and O) and Del1+/+ embryos (C, F, J, M and P) illustrating the localization of mRNAs for proα1(II) collagen (D–F), α1(IX) collagen (G–J), aggrecan (K–M) and α1(X) collagen (N–P). Panels A–C show light microscopic views of hematoxylin/eosin-stained serial sections. The other panels are dark field views of the autoradiographic grains. The genotypes (Del1 −/−, +/− and +/+) are shown in each panel, and the probes used for each horizontal line in the panel to the left: Col2a1, type II collagen; Col9a1, type IX collagen; Col10a1, type X collagen; Agc, aggrecan. Key anatomical structures are marked in panel A: bs, basisphenoidal bone; nc, casal cartilage; o, occipital bone; p, palate; ps, presphenoidal bone; v, vertebral column. The arrows in panels N, O and P highlight periosteal expression of type X collagen mRNA.

expression of type IIA collagen in Del1 +/+ contributes to delayed chondrogenesis and intramembranous ossification, although this could not be resolved in the present study.

According to Diewert (36, 37), linear growth of the mandible is needed to change the position of the tongue during the closure of the secondary palate. As reported earlier (21) and also shown here, all Del1 +/+ mice were found to have a palatal cleft with the tongue remaining between the palatal shelves. If there was any growth retardation in the body of the mandible in Del1 +/− mice, its extent was insignificant, because the closure of the palate was normal in all these mice. A disturbance in the growth of the mandibular condyle has been shown to have no effect on the closure of the palatal shelves, because the ectomesenchymal blastema of the condylar cartilage only appears at a time when the fusion of the shelves is already complete (38, 39).

Another typical feature in Del1 +/+ mice was a downward bending snout, which was often in contact with the thorax. It has been observed that the amount of extension of the cranial base cannot contribute to formation of the cleft palate (40). On the contrary, it has been suggested that the flexure of the head and neck changes the position of the tongue, and thus contributes to the formation of the palatal cleft. In Del1 mice, a flexure of the vertebral column (30) and a downward bending snout, together with a short cranial base and a small mandible, were sufficient to cause a palatal cleft although the inferior angulation of the cranial base and the vertebral column were increased.

The junction between the cranial base and the vertebral column has been described to be very sensitive for developmental abnormalities, including structural disharmony and fusion between the foramen magnum and the upper vertebrae (41). In Del1 +/+ mice, the upper vertebrae were pushed into the foramen magnum, and the cranial base was located at the same level with the atlas, while in the Del1 +/− and −/− mice, the occipital part of the cranial base was located above the atlas. This morphological abnormality most likely explains the abnormal angulation of the cranial base and the vertebral column.

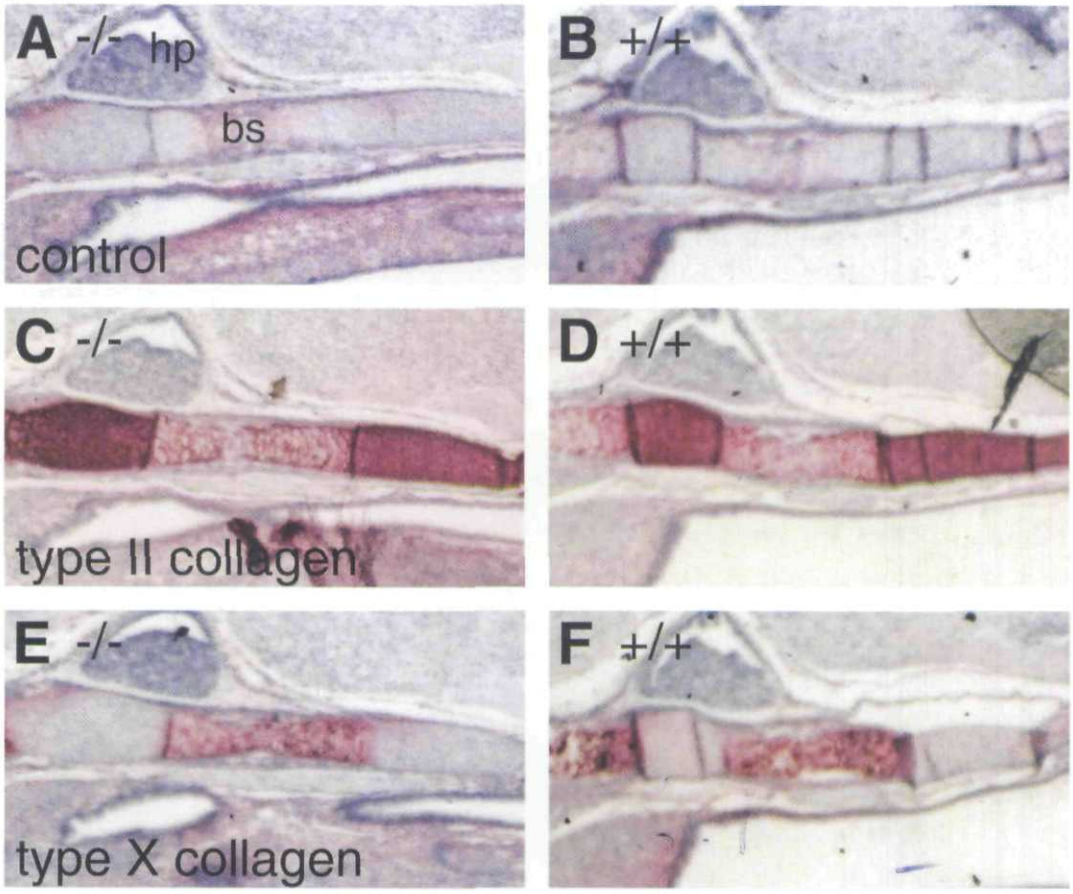

Fig. 5. Immunohistochemistry of sagittal sections of E16.5 Del1 +/+ (A, C and E) and Del1 −/− embryos (B, D and F) for type II collagen (C and D) and type X collagen (E and F). Control stainings were performed without the primary antibody (A and B). The genotypes (Del1 −/−, +/− and +/+) are shown in each panel, and the immunostaining (control; type II collagen; type X collagen) in the left panels of each horizontal line. Key anatomical structures are marked in panel A: bs, basisphenoidal bone; hp, hypophysis. The secondary antibody was recognized using the peroxidase method and counterstaining was performed with hematoxylin.

Comparison of the craniofacial growth data with other skeletal growth (21, 23, 30) in Del1 +/+ mice reveals a relationship between the extent of growth retardation in long bones and the base of the skull. This is contrary to the study of Kjaer (42) on humans which demonstrated that neither the linear size nor the age of the fetus correlate with the developmental stage of the cranial base.

As in humans, the appearance of ossification centers has been used to time embryonic development (18). For example, the ossification center of the anterior arch of atlas has been considered to be a typical mark of stage E16 mouse embryos (18). In the present study, this ossification center was observed in the E16.5 Del1 −/− and +/− mice, but not in Del1 +/+ mice. In E18.5 Del1 +/+ mice, the ossification center of the dens basis was just appearing, while in the −/− and +/− mice the whole atlas-axis area was already well developed. According to Theiler (18) the ossification center of the dens basis appears during the 17th embryonic day. Comparative studies on skeletal development in the mouse are, however, hampered by variations in the ossification schedule in different reports and mouse lines [cf. the present study and (40)]. The present study clearly demonstrates that the retardation of chondrogenesis, chondrocyte hypertrophy and osteogenesis results from retarded expression of genes coding for the major structural components of the respective matrices. The fact that the temporospatial expression patterns of mRNAs for type II and IX collagens and aggrecan were maintained, suggests that the entire chondrogenic differentiation program is delayed in Del1 mice. Analogously, the delayed appearance of type X collagen and its mRNA in synchondroses, and that of type I collagen mRNA in trabecular bone, confirmed that the endochondral

ossification process was similarly delayed in Del1 mice.

Somewhat surprisingly we also detected retarded ossification of calvarial bones in E16.5 Del1 +/+ and +/− mice when compared with Del1 −/− mice. This finding suggests that type II collagen has some kind of an inductive role also in intramembranous ossification. According to Müller and O'Rahilly (43) the first ossification centers of the calvarial bones appear at the upper part of the supraoccipital region. This area is in close contact with the chondroblastic area of the supraoccipital bone. The presence of secondary cartilage at various sites of facial and calvarial skeleton during suture development (44) suggests that secondary cartilage may also affect intramembranous ossification. Alternatively, retarded expression of type IIA collagen in neuroepithelium could contribute to retarded ossification of calvarial bones.

Another surprising finding was the presence of both type II and X collagens and their mRNAs in the periosteum of the cranial base. Type II collagen mRNA has also been observed in the periosteal region (45) and the ossification groove of Ranvier of long bones (46). Periosteal ossification has earlier been reported to play an important role in the ossification of the basioccipital bone (47). Youssef (48) has also suggested that the surface of the bones of the cranial base undergo intensive intramembranous ossification before the appearance of endochondral ossification. However, the production of type X collagen, as seen in the developing murine cranial base, has been associated with endochondral, not intramembranous, ossification. Type X collagen expression has also been observed in the zone of periosteal ossification (49) and in non-chondrogenic cells of grafted chick periosteum (50). The reactivity of periosteum in healing bone defects also involves activation of type X collagen production (29).

The observation of mRNAs for cartilage-specific type II, IX, X and XI collagens along the periphery of the basioccipital bone is in agreement with the concept of the primarily interstitial growth pattern of this bone until days 18–19 of mouse embryonal development (40). In the present study, the highest mRNA levels were seen in chondrocytes located in the synchondroses emphasizing the gradually increasing role of these structures in the growth of the skull. In synchondroses, cell proliferation, matrix production and cell hypertrophy, which all seem to be affected in Del1 +/+ mice, have been shown to contribute to growth of the cranial base (51). In Del1 +/− mice these defects seemed to be less severe and made nearly normal embryonic development and growth of the synchondroses possible. We have previously reported on premature fusion of synchondroses in adult Del1 +/− mice (14).

In summary, several abnormalities were observed in the craniofacial skeleton of transgenic Del1 +/+ mice harboring mutant type II collagen transgenes. This is not surprising, as endochondral ossification plays an important role in craniofacial development and growth. In addition to disturbing the structure of hyaline cartilage, the production of mutant type II collagen chains seems to affect also intramembranous ossification. Further studies are needed to determine whether this effect is mediated by retarded production of type IIA collagen by prechondrogenic mesenchyme and neuroepithelial structures, or aberrant production of mutant type II collagen during intramembranous ossification.

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