

Connexin43 Deficiency Causes Delayed Ossification, Craniofacial Abnormalities, and Osteoblast Dysfunction

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Abstract. Connexin(Cx)43 is the major gap junction protein present in osteoblasts. We have shown that overexpression of Cx45 in osteoblasts expressing endogenous Cx43 leads to decreased cell–cell communication (Koval, M., S.T. Geist, E.M. Westphale, A.E. Kemendy, R. Civitelli, E.C. Beyer, and T.H. Steinberg. 1995. *J. Cell Biol.* 130:987–995) and transcriptional downregulation of several osteoblastic differentiation markers (Lecanda, F., D.A. Towler, K. Ziambaras, S.-L. Cheng, M. Koval, T.H. Steinberg, and R. Civitelli. 1998. *Mol. Biol. Cell* 9:2249–2258). Here, using the Cx43-null mouse model, we determined whether genetic deficiency of Cx43 affects skeletal development in vivo. Both intramembranous and endochondral ossification of the cranial vault were delayed in the mutant embryos, and cranial bones originating from migratory neural crest cells were also hypoplastic, leaving an open foramen at birth. Cx43-deficient animals also exhibited

retarded ossification of the clavicles, ribs, vertebrae, and limbs, demonstrating that skeletal abnormalities are not restricted to a neural crest defect. However, the axial and appendicular skeleton of Cx43-null animals were essentially normal at birth. Cell to cell diffusion of calcein was poor among Cx43-deficient osteoblasts, whose differentiated phenotypic profile and mineralization potential were greatly impaired, compared with wild-type cells. Therefore, in addition to the reported neural crest cell defect, lack of Cx43 also causes a generalized osteoblast dysfunction, leading to delayed mineralization and skull abnormalities. Cell to cell signaling, mediated by Cx43 gap junctions, was critical for normal osteogenesis, craniofacial development, and osteoblastic function.

Key words: connexin43 • connexin45 • gene knock-out • skeletal development • osteoblast differentiation

Introduction

Skeletogenesis is a highly complex process that leads to the formation of elements of the skull and the axio-appendicular bones (Karsenty, 1998). During development, the shape, number, and proper location of each skeletal element is driven by synchronized genetic programs coordinating the differentiation, function, and interaction of its cellular components (Erlebacher et al., 1995; Karsenty, 1999). Most of the skeletal elements originate from the

mesoderm. Dorsal paraxial and lateral plate mesoderm give rise to the axial and appendicular skeleton, respectively; the cephalic mesoderm gives rise to some cranial bones (interparietal, supraoccipital, basioccipital, and exoccipital). However, the majority of skeletal elements in the head region are derived from migratory neural crest cells, which participate in the formation of the fronto-nasal mass, (frontal, parietal, and squamosal bones), as well as the maxillo-mandibular elements (Couly et al., 1993).

Independent of their ontogenesis, osteogenic cells will ultimately form ossified tissue by two mechanisms: endochondral and intramembranous ossification. During endochondral ossification, precursor cells condense in areas destined to become bone and acquire the general shape of the bone segment, thus providing the template for future skeletal elements (Hall and Miyake, 1992, 1995). This mechanism is prevalent in most of the vertebrate skeleton, leading to the formation of the skeletal axis, limbs, and

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base and caudal part of the skull, or chondrocranium. The rest of the skull, including the cranial vault and the maxillo-mandibular bones, is formed by intramembranous ossification, a process which involves the condensation of mesenchymal precursors and the direct transition to differentiated bone cells, without an intermediate cartilaginous template (Hall and Miyake, 1992). Therefore, the skull represents a unique structure formed by elements derived from both neural crest cells and paraxial mesoderm and undergoes both mechanisms of ossification (De Beer, 1971).

Although the precise mechanisms that lead to the migration, differentiation, and homing of neural crest cells to the specific areas of the skull remains elusive, recent data point to an important role for gap junctional communication in determining a correct cellular specification during development. Connexin(Cx)43,¹ a gap junction protein prevalent during development (Yancey et al., 1992), is abundant in neural crest cells (Lo et al., 1997). Studies in transgenic animals overexpressing Cx43, as well as in Cx43-null mice, have shown that gap junctional communication mediated by Cx43 modulates the migratory rate of cardiac neural crest cells, and that the abnormal migration of these cells is a likely mechanism by which heart malformations develop in mice with either loss or gain of Cx43 function (Huang et al., 1998). Thus, a certain level of Cx43 gap junctions seems to be necessary for controlling neural crest cell migration, perhaps by providing an organized signaling network to guide cells to their final location. Since part of the skull is derived from neural crest cells and Cx43 is the major gap junction protein present in osteoblasts (Schirmacher et al., 1992; Civitelli et al., 1993; Donahue et al., 1995), we asked whether loss of Cx43 would cause skeletal abnormalities during development. Homozygous Cx43-null mice die shortly after birth, as a consequence of severe malformation of the conotruncal heart segment leading to right ventricular outflow obstruction (Reaume et al., 1995; Ya et al., 1998). Although the shape of these animals was reported to be grossly normal at birth (Reaume et al., 1995), a careful analysis of their skeletal development had not been performed.

The idea that Cx43 may be involved in bone development is borne out of our studies in human and murine bone cells, which demonstrated that expression and function of this Cx is directly related to the differentiation state of osteogenic cells (Civitelli et al., 1993; Steinberg et al., 1994). Furthermore, altering the molecular permeability of Cx43 gap junctions, by transfecting cells with Cx45, results in transcriptional downregulation of osteocalcin and bone sialoprotein (Lecanda et al., 1998), two genes that are critical for matrix mineralization and calcification, and decreased permeability to negatively charged dyes (Koval et al., 1995). In contrast, overexpression of Cx43 in communication-deficient cells increases permeability to negatively charged dyes and upregulates the activity of osteocalcin and bone sialoprotein promoters (Lecanda et al., 1998).

We find that mice lacking Cx43 exhibit delayed intramembranous ossification. Delayed ossification is evident in all the skull elements derived from neural crest

cells, resulting in nonclosure of the cranial sutures at birth. We also observed a less pronounced delay of endochondral ossification in the axial skeleton of Cx43-null mice relative to wild-type littermates. Furthermore, osteoblast-enriched cultures from calvaria of Cx43-deficient mice exhibit reduced expression of marker genes and stunted mineralization *in vitro*, associated with poor cell-cell diffusion of negatively charged dyes. These results point to a bone formation defect and strongly suggest that gap junctional communication mediated by Cx43 is critically important in osteogenesis and normal osteoblast function.

Materials and Methods

Animal Model

A heterogeneous 129/C57BL mouse strain, harboring a null mutation of the Cx43 gene, was used (Reaume et al., 1995). Mendelian distribution in the heterozygous animals at birth could be observed only if delivery was performed by Cesarean section. As previously reported, Cx43^{-/-} pups were born with some swelling in the neck and abdominal regions. They rapidly developed muscular contractions and eventually died within one hour of delivery. Genotyping was performed, as previously described (Reaume et al., 1995), from the yolk sac or the tail of embryonic day (E) 15.5–E18.5 embryos or neonates (Hogan et al., 1994). Genomic DNA was extracted in Tris-HCl buffer, pH 8.5, containing 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 µg/ml proteinase K, for 8–16 h at 55°C in an agitator and was precipitated with isopropanol, phenol-chloroform, and ethanol, sequentially. Genomic DNA was resuspended in Tris-EDTA buffer, which was used as a template for PCR analysis. To establish zygosity of the offspring, three primers were used: Cx43-5', 5'-CCC CAC TCT CAC CTA TGT CTC C-3'; Cx43-3', 5'-ACT TTT GCC GCC TAG CTA TCC C-3'; and Neo-5', 5'-GCT TGC CGA ATA TCA TGG TGG A-3' (Reaume et al., 1995). The Cx43-5' and Cx43-3' primers amplify part of exon 2 of the wild-type Cx43 allele, yielding a product of ~500 bp. The combination of Cx43-3' and Neo-5' amplify part of the neo/Cx43 fusion gene, yielding a cDNA fragment of ~1,000 bp. After preheating at 94°C for 15 min, 40 cycles were run, with denaturation at 94°C, and annealing at decreasing temperature from 65°–58°C, and final extension for 10 min at 72°C. PCR products were loaded on a 1.2% agarose gel for electrophoresis. Animals homozygous for the Cx43-null gene and wild-type littermates were identified by the presence of a single band of the respective size, whereas heterozygous animals had both bands, corresponding to the two alleles.

Alizarin Red/Alcian Blue Staining

Alizarin red/alcan blue staining was performed using established methods (McLeod, 1980). In brief, newborn mice were deskinning, eviscerated, and kept in 100% ethanol for 24 h. The carcasses were fixed in acetone for 24 h and then stained for 24 h in a solution containing 0.1% alizarin red, 0.3% alcian blue, acetic acid, and 70% ethanol (1:1:1:17, vol/vol/vol/vol). They were then transferred to a solution of 1% KOH in 20% glycerol until clear and then stored in glycerol.

Osteoblast Isolation

Osteoblast-enriched calvaria cultures were obtained using established methods (Rifas et al., 1994, 1995), with minor modifications. Calvaria were dissected under sterile conditions within 2 h of delivery. After removal of periosteum and endosteum, calvaria from each animal were placed in individual tissue culture wells, which contained α -MEM supplemented with 10% FCS and penicillin-streptomycin, for 24–48 h. After genotyping (see above), two to six calvaria of each genotype group were cut into small pieces (1–2 mm), pooled, and digested in a solution containing 2 mg/ml of collagenase A in serum-free medium at 37°C for 10 min. The medium was discarded and replaced with fresh collagenase-containing medium plus DNase solution (5 µg/ml). After 2 h digestion at 37°C, bone debris was separated by using a 70-µm nylon mesh (Falcon). Cells were precipitated by centrifugation, washed several times to remove excess collagenase and DNase enzymes, resuspended in α -MEM supplemented with 10% FCS, and plated at 2×10^4 cells/cm². After confluence, cells were trypsinized, resuspended, and replated at the same density. All

¹Abbreviations used in this paper: Cx, connexin; E, embryonic day; RT, reverse transcriptase.

the experiments were performed using cells from the first passage. Small fractions of cells were routinely seeded in 35-mm dishes for histochemical determination of alkaline phosphatase activity, using standard methods (Lecanda et al., 1997), to assess for variability among different preparations. A similar method was used to isolate osteoblasts from long bones. In brief, femurs and tibias of neonates were cleaned of soft tissue under a dissecting microscope. The epiphyses were cut and discarded to avoid contamination with cartilaginous tissue. The diaphyses were cut in small pieces and digested with collagenase A, as described above. Cells were counted and seeded in 60-mm dishes until confluent. Results are representative of five independent experiments using five different litters.

Dye Coupling

A technique we previously described (Ziambaras et al., 1998) was used to assess cell to cell diffusion of negatively charged dyes. In brief, cells from each genotype group were mobilized by trypsin digestion and preloaded in suspension with calcein-AM, and then they were added on top of a monolayer of the same cell type. The "parachuted" cells were allowed to settle onto the unlabeled monolayer and returned to the incubator for two hours. The diffusion of calcein from donor to acceptor cells was detected by fluorescence microscopy on an Axiovert inverted microscope (ZEISS) and a calcein filter set. The number of coupled cells per parachuted cell was determined in 15–20 random snapshots in each well (24-well plate), and 4 wells were used per group.

Cell Proliferation

Cell proliferation was estimated by [³H]thymidine incorporation (Cheng et al., 1994). Cells were seeded in 24-well plates at a density of 2×10^5 /well. One day later, cells were washed in serum-free medium and incubated in α -MEM for 24 h. They were then incubated for 24 h in fresh α -MEM containing 2 μ Ci/well of [methyl-³H]thymidine (20 Ci/mmol, 1 mCi/ml) and 10% BSA. The quantity of isotope incorporated in proliferating cells was measured after TCA precipitation and ethanol washing, as previously described (Cheng et al., 1994).

Western Blot Analysis

As previously described (Civitelli et al., 1993; Lecanda et al., 1997), cells were seeded on 100-mm tissue culture plates and cultured in α -MEM with 10% FCS for one week after reaching confluence. They were then solubilized in lysis buffer, containing a cocktail of proteinase inhibitors, and sonicated. The protein content was measured according to the method of Bradford (Bradford, 1976). Proteins were separated using a Novex-Cell two system and precast gels. After blocking with 5% nonfat milk, the membranes were incubated with the appropriate antibody for 1 h, washed at room temperature, and incubated with an anti-rabbit or anti-mouse peroxidase-conjugated antibody. The immune reaction was detected by exposing the membranes to autoradiography film (Hyperfilm; Amersham Pharmacia Biotech) using the ECL detection system (Amersham Pharmacia Biotech), according to the manufacturer's recommendations.

Reverse Transcription (RT) and Polymerase Chain Reaction

Semiquantitative RT-PCR was carried out following the protocol of Estus and co-workers (Estus et al., 1994). mRNA was extracted from confluent 100-mm tissue culture dishes, as previously described (Lecanda et al., 1997). Then, 0.5 μ g of mRNA was reverse transcribed with avian myeloblastosis virus reverse transcriptase in a 20- μ l reaction, using the Reverse Transcription System (Promega). First strand cDNA (0.6 μ l) was used as template for the PCR reaction in the presence of [α -³²P]dCTP, and the reaction products were separated on a 4–20% polyacrylamide gel gradient (Novex). Gels were dried and exposed to radiographic films (Amersham Pharmacia Biotech). Oligonucleotides primers used were (written in the 5' to 3' direction): (a) osteocalcin, OCN1, AAGTCCACACAGCAGCTTG and OCN2, AGCCGAGCTGCCAGAGTTTG (Desbois et al., 1994); (b) glyceraldehyde phosphated dehydrogenase, GAPDH1, ACTTTGTCAAGCTCATTTCC and GAPDH2, TGCAGCGAACTT-TATTGATG (Tong et al., 1994); (c) connexins, Cx43-1, CCCACTC-TCACCTATGTCTCC, Cx43-2, ACTTTTGCCGCTAGCTATCCC (Cx43) (Reaume et al., 1995), Cx45-1, TGCTAGAGGAGATCCCAACCA, and Cx45-2, CCTTCTGTCTGCCTCACCA (Cx45) (sequence data available from EMBL/GenBank/DBJ under accession no.

X63100); (d) osteopontin, OPN1, ACACTTTCACTCCAATCGTCC, OPN2, TGCCCTTCCGTTGTTGTCC (Tong et al., 1994); and (e) type I collagen I COL1A-1, TCTCCACTCTTCTAGTTCCCT and COL1A-2, TTGGGTCATTTCACATGCT (Tong et al., 1994). PCR fragments are 368 bp for OCN, 269 bp for COL1A, 519 bp for Cx43, 306 bp for Cx45, 239 bp for OPN, and 267 bp for GAPDH.

Bone Histology

Bones were fixed overnight in 4% paraformaldehyde and dehydrated before they were embedded in paraffin. 5- μ m sections were stained with hematoxylin/fast green/safranin O (Lillie, 1965).

In Vitro Mineralization

Cells were cultured in six-well culture dishes, in the presence of ascorbic acid (50 μ g/ml) and β -glycerophosphate (10 mM), for 2 or 3 wk. Cells were washed three times with TBS, fixed with 10% neutral formalin solution for 5 min, and rinsed with deionized water for Von Kossa staining. After the addition of 5% silver nitrate solution, the wells were exposed to UV light for 1 h (Lillie, 1965). The plates were rinsed with deionized water, and the residual silver nitrate was neutralized with 5% sodium thiosulphate. Mineralization was quantified by calculating the surface area (in cm²) covered by the black stain in each well (9.6 cm²), using a computerized image analysis package (IPLab; Scanalytics, Inc.).

Results

Defects of Neural Crest-derived Skeletal Elements in Cx43^{-/-} Mice

Since previous studies suggested a role for gap junctional communication in neural crest cell migration (Huang et al., 1998), we first focused our attention on neural crest-derived elements of the skull at different stages of development. As shown in Fig. 1, a and b, ossification centers of neural crest origin forming the cranial vault, including parietal, nasal, frontal, and squamous parts of the temporal bone, were absent in Cx43^{-/-} mice at E16.5. Only small ossification centers in the premaxilla and maxilla, also derived from the neural crest, were noted in the periorbital region in the homozygous mutants compared with the extensive areas of ossification present in the wild-type animals in these regions. No differences were observed in the cartilaginous primordium of the nasal capsule between homozygous mutants and normal littermates. At E18.5, the flat bones of the cranial vault were still hypomineralized, allowing a direct view of the base of the skull (Fig. 1, c and d). In particular, frontal and parietal bones of Cx43-null animals showed more ossified rostral and lateral edges, with the central and caudal portions still nonmineralized. Other bone segments formed by endochondral ossification, including pterygoid, alisphenoid, and basisphenoid, displayed delayed ossification (Fig. 1, e and f). At E18.5, the whole skull was smaller in homozygous mutants than in wild-type littermates, presumably the result of retarded intramembranous ossification.

The maxilla and mandibula are derived from neural crest cells populating the first pharyngeal arch, and they undergo both endochondral (Meckel's cartilage) or intramembranous ossification (vomer, palatine, mandibular, and premaxilla). At E16.5, Meckel's cartilage was the only mandibular element present in the homozygous Cx43^{-/-} mutants, whereas robust ossification within a membranous primordium in the inferior part of the ramus was already present at this stage in normal animals (Fig. 1, a and b).

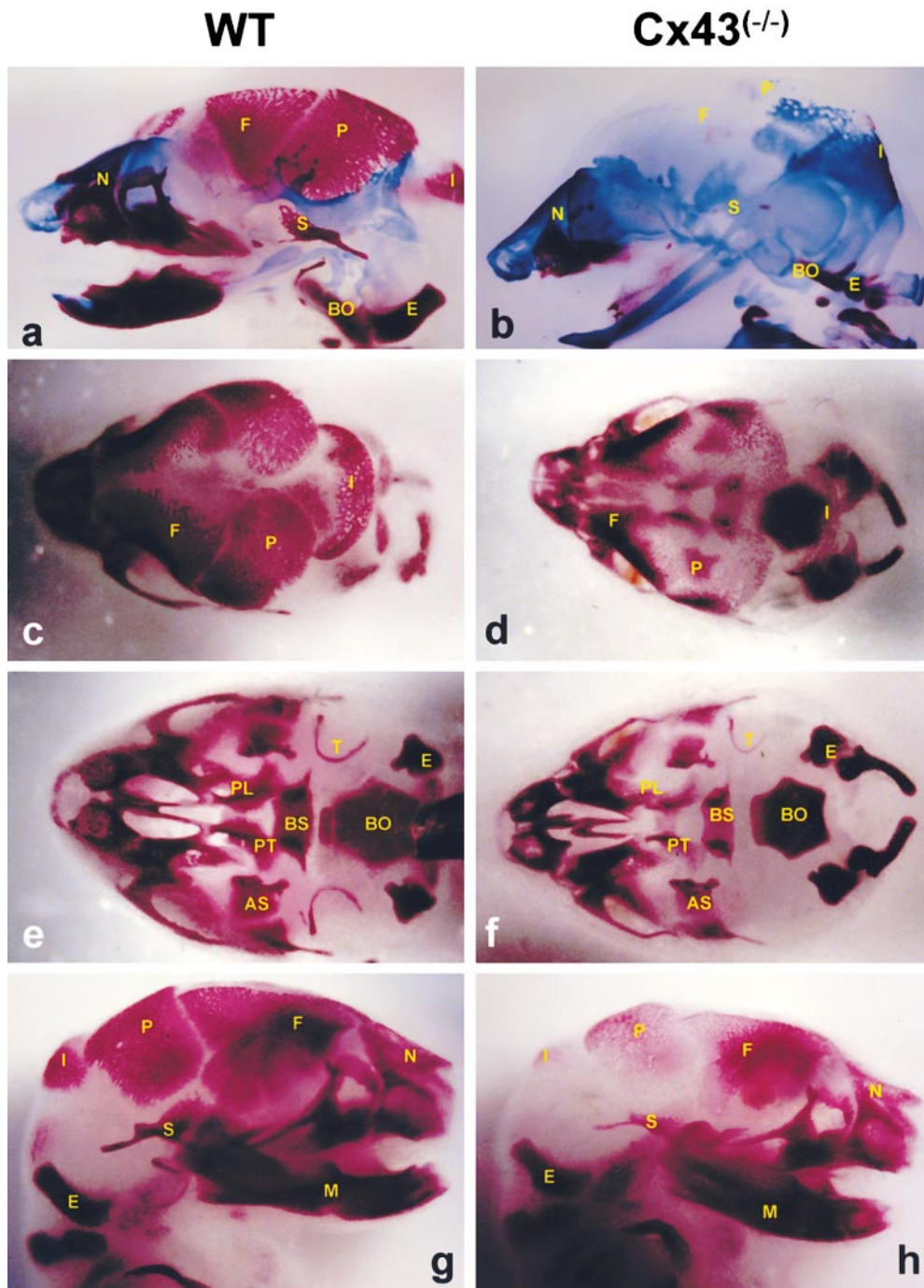


Figure 1. Skull development in *Cx43*^{-/-} embryos. (a and b) Lateral view of the skull of alizarin red/alcian blue-stained E16.5 wild-type (WT) and *Cx43*^{-/-} embryos. (c and d) Dorsal, (e and f) ventral, and (g and h) lateral views of the skull of alizarin red-stained E18.5 (c, e, and g) wild-type and (d, f, and h) *Cx43*^{-/-} embryos. (g and h) Lateral view on the skull at E18.5 shows the incipient mineralization of parietal, nasal, frontal, and squamous parts of the temporal bones in *Cx43*-null mice, though all these elements remain developmentally hypomineralized, especially around their rostral boundaries. F, frontal bone; P, parietal bone; S, squamous part of the temporal bone; I, interparietal bone; E, exoccipital bone; M, mandible; T, tympanic ring; PL, palatine bone; PT, pterygoid bone; BS, basisphenoid bone; AS, alisphenoid bone; and BO, basioccipital bone.

Those differences disappeared at E18.5 when maxillary bones and mandible were almost completely mineralized in both mutant and wild-type mice (Figs. 1, g and h, and 2, a and b). However, the mandible was reduced in size in *Cx43*-null mice at E18.5 (Fig. 2, a and b) and at birth (Fig. 2, c and d). As a consequence, the lower incisors were not as prominent as in normal littermates at birth (Fig. 2, c and d, arrow), suggesting a delay in tooth development and eruption. Nonetheless, the temporo-mandibular joint was apparently normal in the mutant mice.

The lack of proper development of bones forming the roof of the skull, namely the parietal and frontal bones, resulted in an open parietal foramen (Fig. 3, a-d). The much

reduced size of parietal and frontal bones, in conjunction with the hypoplastic interparietal bone, resulted in a smaller calvarium and a more flattened skull (Fig. 3, a and b). This is the most prominent phenotypic feature of *Cx43*^{-/-} mice at birth. Microscopic examination of sagittal sections of the skull of *Cx43*^{-/-} mice at birth revealed thinner, brittle parietal bones with reduced diploic space in the homozygous mutants compared with control littermates (Fig. 4, a and b). In contrast, homozygous mutants displayed more prominent cartilagenous anlage of the occipital bone (Fig. 4, c and d), presumably the consequence of retarded ossification. We conclude that *Cx43* deficiency causes delayed development of all neural crest-derived fa-

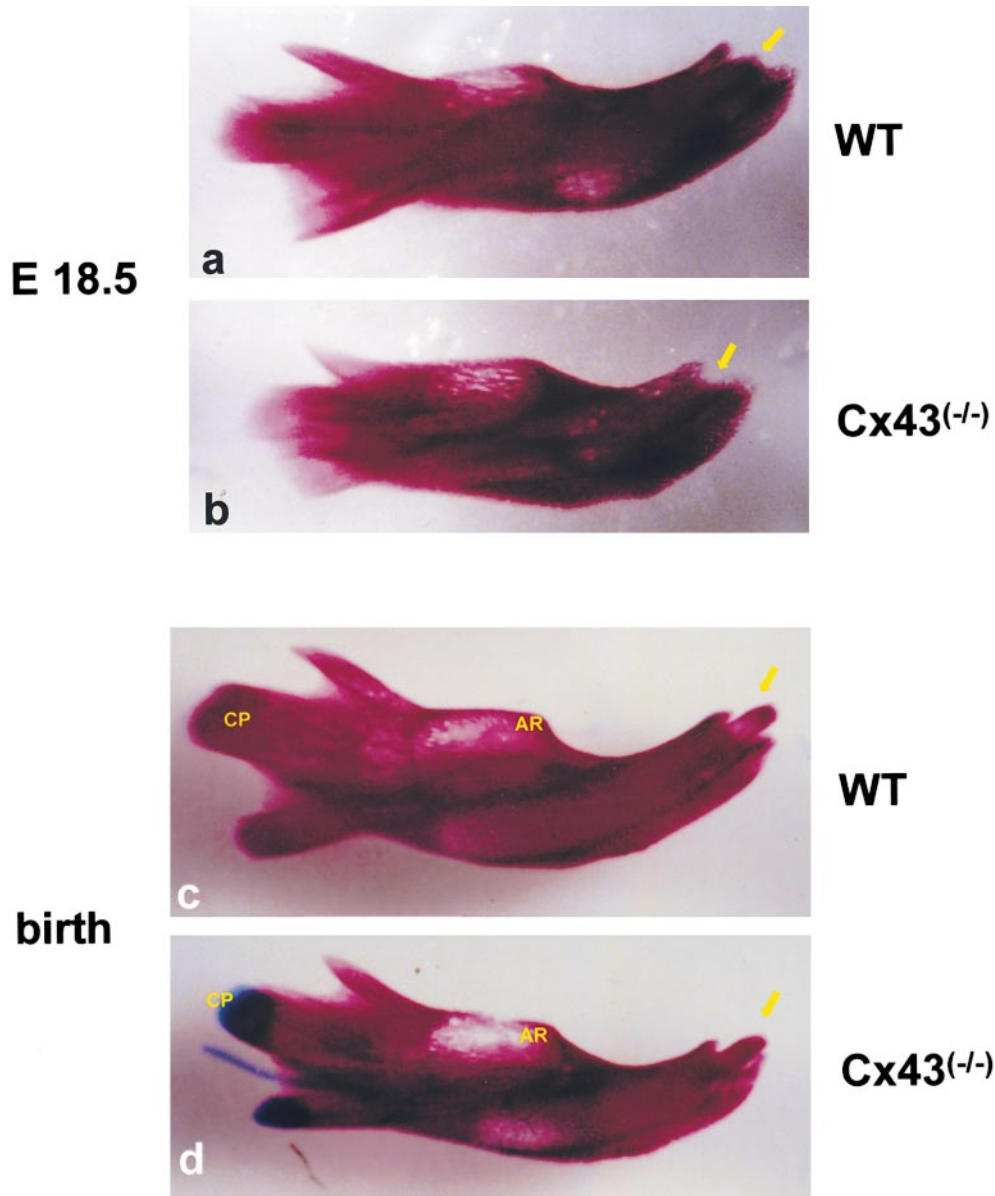


Figure 2. Mandible development in Cx43^{-/-} mice. Alizarin red/alcian blue staining of the mandibles at (a and b) E18.5 and at (c and d) birth of wild-type (WT) and Cx43^{-/-} littermates. In newborn mutants, the mandible was smaller, with a more rounded alveolar ridge and a flatter arch. This malformation contributes to the slightly smaller and more pointed snout in mutant animals. In some Cx43-null newborn mice, cartilaginous primordia of the temporal bone and the condylar region of the mandible were still present at (d) birth. Note, (b, arrow) the lack of the incisor tooth at E18.5 in the Cx43^{-/-} embryo and (d) the less prominent tooth at birth. Also, note the less pronounced alveolar ridge (AR) in the mutant animals and the cartilaginous coronoid process (CP).

cial (nasal, maxillary, vomer, palatine, and mandibular) and cranial vault elements (frontal, parietal, and squamosal).

Delayed Ossification of Mesoderm-derived Skeleton in Cx43^{-/-} Mice

Although neural crest-derived elements contribute largely to the formation of the skull, rostral segments in the occipital region originate from the cephalic mesoderm. Among these, the basioccipital and exoccipital bones appeared normally mineralized at E18.5 (Fig. 1, e and f), but a slight delay was observed at E16.5 (Fig. 1, a and b). In contrast, ossification was retarded in the two centers that form the supraoccipital bone (Fig. 1, g and h), and, at birth, these two centers were still distinguishable in the homozygous mutants, whereas they were fused in wild-type animals (not shown). Interestingly, the interparietal bone, which develops within a cartilaginous primordium derived from the cephalic paraxial mesoderm, was significantly reduced

in size and hypomineralized (Fig. 3, a–d), thus contributing to the reduced size of the skull.

The axial skeleton also revealed retarded ossification in these mutants. At E15.5, only the cartilaginous vertebrae and ribs were present in mutant animals; normally, the pedicles and laminae of the vertebrae and the dorsal half of the ribs are ossified at this stage (Fig. 5, a and b). At E16.5, areas of endochondral ossification were visible in the pedicles and laminae of the thoracic vertebrae and in the dorsal aspect of the ribs of Cx43-null littermates (Fig. 5 c). The ribs of homozygous mutants appeared jagged-shaped and slightly thinner, and the thoracic cage was more brittle than in normal mice. A comparison of the axial skeleton of mutant and wild-type animals, between E15.5 and E18.5, revealed a delay of endochondral ossification of ~1–2 d (Fig. 5, a–e). Thus, the developmental abnormalities in Cx43-deficient animals are not restricted to skeletal elements of neural crest origin. They generally involve many mesoderm-derived skeletal segments. Whereas

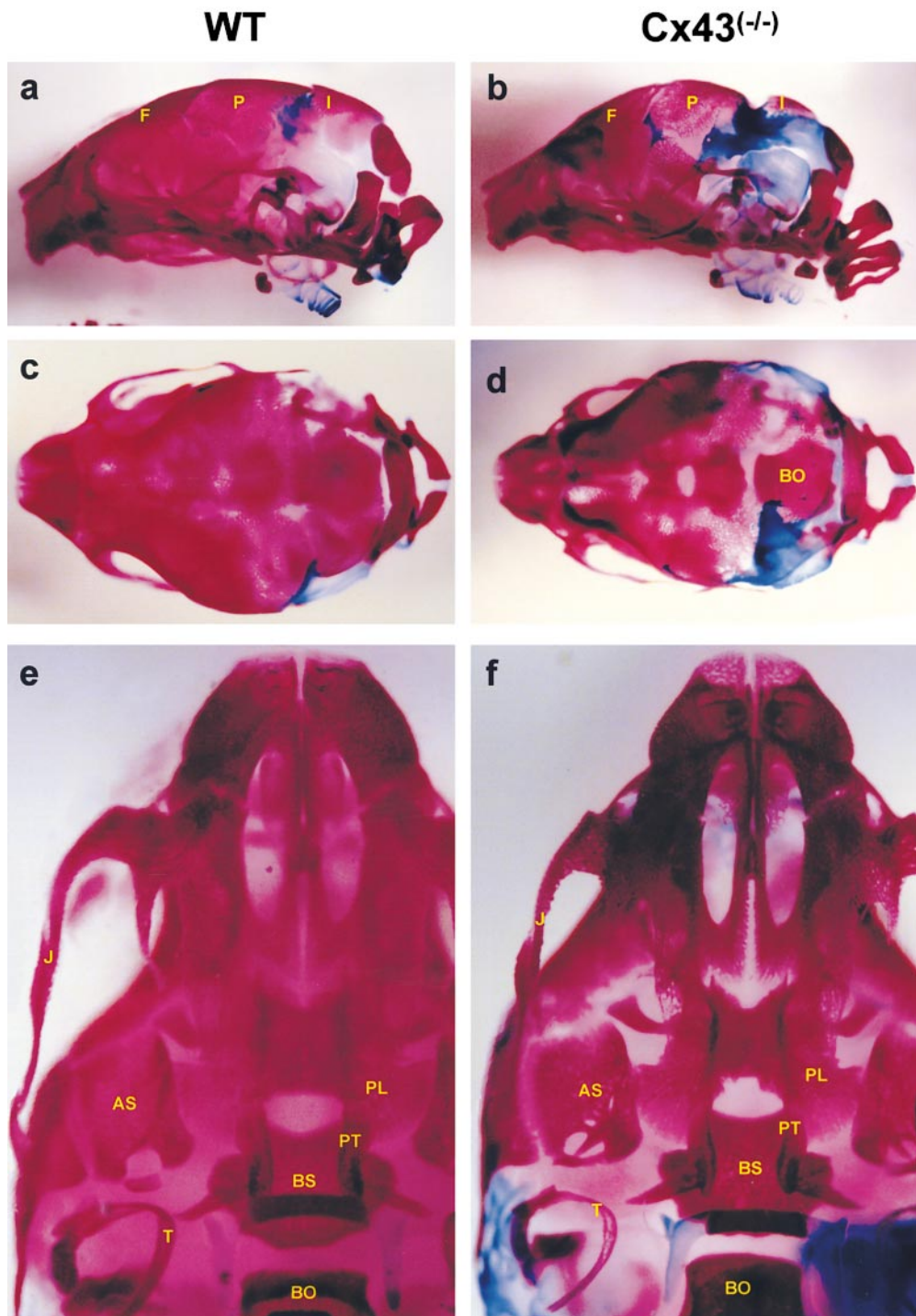


Figure 3. Skull development in $Cx43^{-/-}$ at birth. (a and b) Lateral and (c and d) dorsal views of the cranial vault at birth of (a and c) wild-type and (b and d) $Cx43^{-/-}$ littermates at birth. Note the prominent cartilaginous primordia of the occipital bone in the homozygous mutants, whereas the parietal, interparietal, and supraoccipital bones are thinner and less developed, allowing a direct view of the cranial base. A ventral view of the cranial base of (e) wild-type and (f) $Cx43$ -null littermates at birth. The horizontal laminae of the palatine bones are slightly smaller, and the alisphenoid was misshapen, leaving a larger unmineralized area delimited by the palatine, alisphenoid, and temporal bones. The horizontal portion of the palatine bones and the palatine processes of the maxillary bone, collectively referred to as palatal shelves, were present. The tympanic ring was reduced in size and thinner. The jugal bone of the zygomatic arch was shorter, resulting in a misshapen zygomatic arch. Overall, the nasal and maxillary bones were slightly smaller, resulting in a more pointed and smaller snout in the $Cx43$ -null animals. J, jugal part of the zygomatic bone; F, frontal bone; P, parietal bone; S, squamous part of the temporal bone; I, interparietal bone; E, exoccipital bone; M, mandible; T, tympanic ring; PL, palatine bone; PT, pterygoid bone; BS, basisphenoid bone; AS, alisphenoid bone; and BO, basioccipital bone.

ossification of the calvarium was still retarded and incomplete at E18.5 (Fig. 5, d and e) and at birth (see above), the axial skeleton appeared normally mineralized in newborn $Cx43^{-/-}$ animals at these stages. Accordingly, ossification of the ribs and vertebral laminae were essentially complete at E18.5, though the ribs remained deformed, with a jagged appearance in the homozygous mutants compared with control littermates (Fig. 5, d and e). At birth, there were no differences in number, size, or spacing of the vertebrae and ribs (data not shown), except for the rib deformities just described.

In the early stages of ossification (E14.5), a delay was also observed in the limbs of $Cx43^{-/-}$ embryos (Fig. 6 a). The limbs of the mutant animals were also smaller compared with wild-type littermates. Interestingly, at this stage, the clavicle was not ossified in the $Cx43^{-/-}$ mice, however, in normal mice, both the cartilaginous and membranous sections of the clavicle were already mineralized, for the most part (Fig. 6 a, arrows). However, these differences became less prominent with time, so that at birth the clavicle of $Cx43^{-/-}$ mice was completely ossified (Fig. 6 b) and the limbs were morphologically normal (Fig. 6, c and

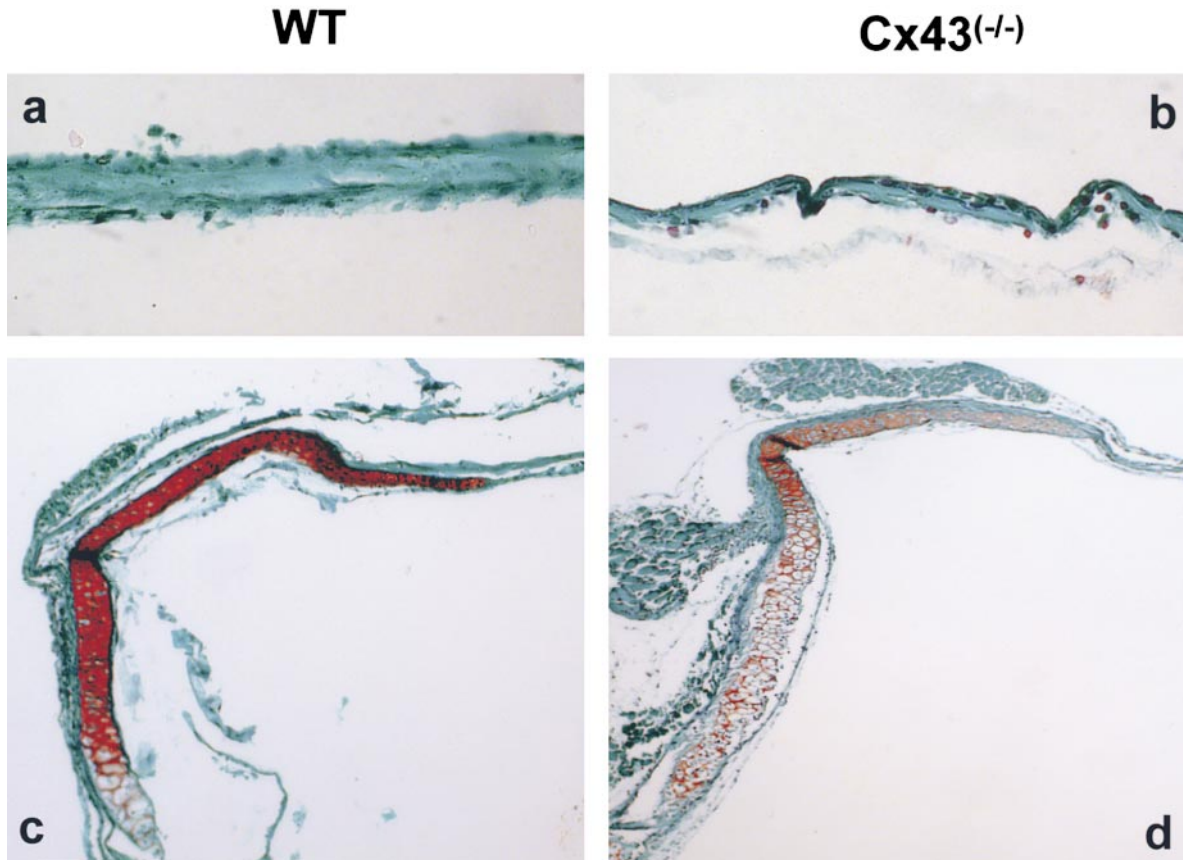


Figure 4. Sagittal sections of the (a and b) parietal and (c and d) occipital bones of the (a and c) wild-type and (b and d) $Cx43^{-/-}$ mice stained with safranin/fast green. Note that the parietal bone is thinner and more brittle in the $Cx43$ homozygous mutants. (d) The cartilaginous anlage of the occipital bone is more prominent in the $Cx43^{-/-}$ animals.

d). Histological sections of long bones of homozygous mutant animals did not reveal detectable differences compared with wild-type bones (Fig. 6 e). The morphology and size of the growth plate was normal, as was mineralization of the primary spongiosa (Fig. 6 e).

Gap Junctional Communication Is Impaired in $Cx43$ -null Osteoblasts

The skeletal phenotype described above is suggestive of a partial-osteoblast defect. Although absence of $Cx43$ severely reduces gap junctional communication in cells derived from mutant animals (Reaume et al., 1995), osteoblasts express $Cx45$ and $Cx46$, as well as $Cx43$ (Koval et al., 1997). Therefore, we asked whether expression and/or localization of these Cxs might be upregulated in osteoblastic cells lacking $Cx43$ as a partial compensatory mechanism. Calvaria cells were isolated from $Cx43^{-/-}$, heterozygous $Cx43^{+/-}$, and wild-type newborn animals, using standard procedures, to obtain osteoblast-enriched cultures (see Materials and Methods). As expected, the $Cx43$ protein was absent in confluent $Cx43^{-/-}$ calvaria cells, and its abundance was lower in heterozygous $Cx43^{+/-}$ cells, compared with wild-type calvaria cells (Fig. 7 a), suggesting a gene-dosage effect, as reported in other tissues (Guerrero et al., 1997). Interestingly, the abundance of $Cx45$ protein was increased in homozygous mutants compared with wild-type and heterozygous cells (Fig. 7 a). On

the other hand, no difference in abundance or distribution of $Cx46$ was seen in $Cx43$ -null cells (not shown). The distribution of $Cx46$ in $Cx43$ -null cells had a similar appearance to normal cells, where $Cx46$ is localized to a late Golgi/TGN compartment (Koval et al., 1997), arguing against a compensatory role of this Cx.

Since gap junctions formed by $Cx45$ display a decreased molecular permeability to negatively charged dyes, compared with those formed by $Cx43$ (Steinberg et al., 1994; Veenstra et al., 1994), $Cx43^{-/-}$ calvaria cells would be expected to allow less intercellular diffusion of small negatively charged molecules than wild-type cells, as demonstrated for embryonic fibroblasts (Reaume et al., 1995). Thus, osteoblast cells isolated from calvaria of wild-type, heterozygous, or homozygous mutants were preloaded with calcein (donor cells) and then added on top of a monolayer culture of unlabeled cells of the same genotypic group (acceptor cells). As anticipated, calcein diffusion was minimal among homozygous mutant cells, whereas abundant cell to cell transmission of the dye was evident in wild-type cultures (Fig. 7 b). Heterozygous $Cx43^{+/-}$ cells were also coupled, though to a lesser degree than homozygous mutants, whereas cells lacking $Cx43$ diffused the dye very poorly (0.8 ± 0.5 coupled cells per cell). Furthermore, no dye diffusion was seen when using the osteogenic sarcoma ROS 17/2.8 cells as donors (data not shown). Since these cells are very well coupled and express

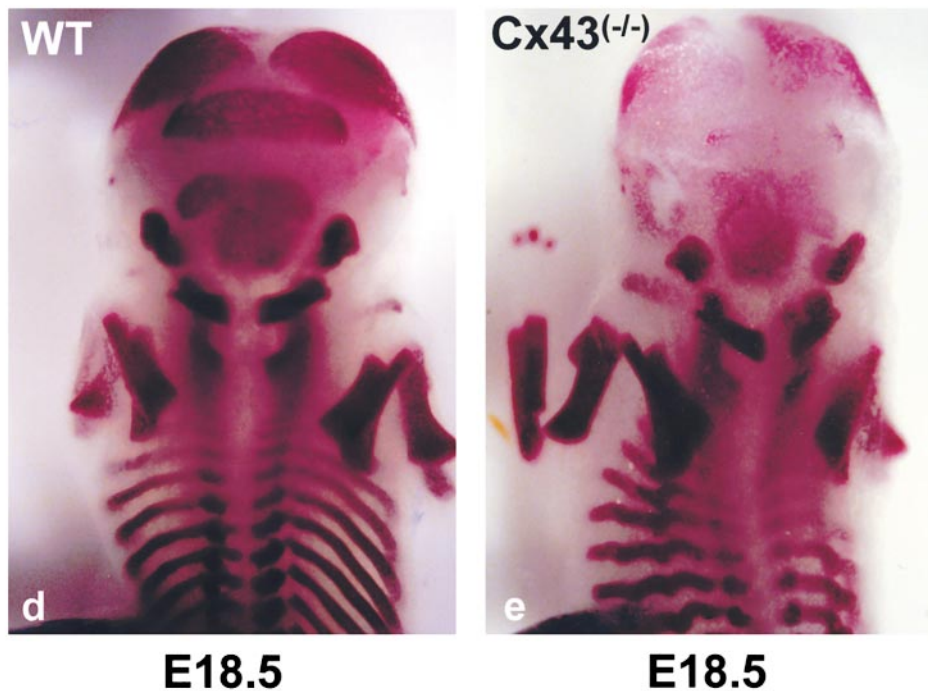
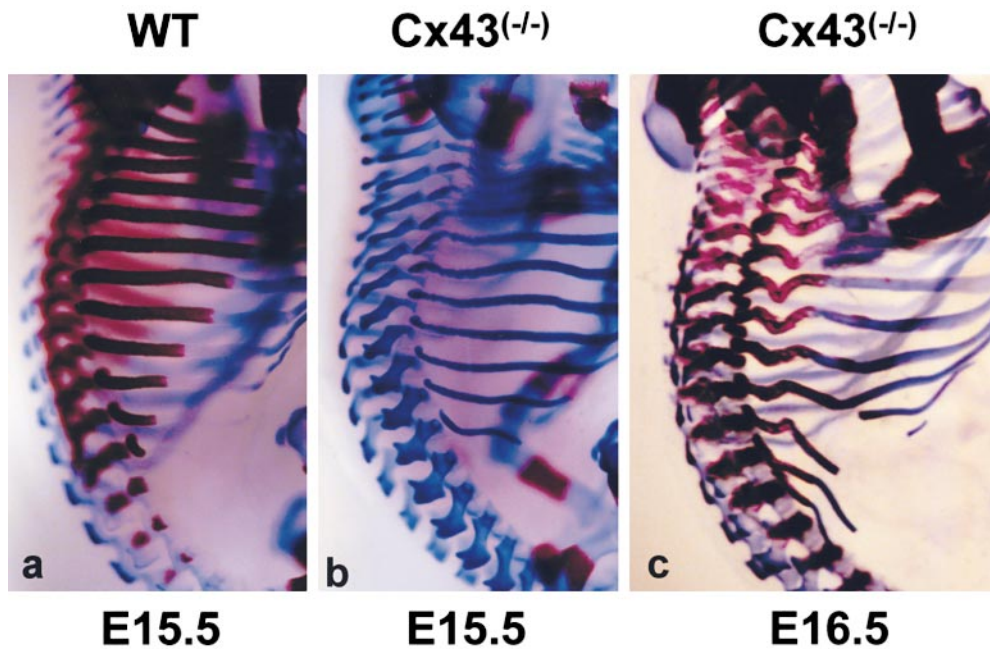


Figure 5. Axial development in $Cx43^{-/-}$ embryos. Lateral view of the thoracic vertebrae stained with alizarin red/alcian blue of (a and b) E15.5 and (c) E16.5 (a and d) wild-type embryos and (b, c, and e) $Cx43^{-/-}$ homozygous mutants. (b and c) Note the delayed ossification of the vertebrae and ribs and the thinner and deformed ribs in the mutant animals. Posterior view of the head and thorax of alizarin red-stained (d) wild-type and (e) $Cx43^{-/-}$ embryos at E18.5 showing a similar ossification of the vertebrae, limbs, and exoccipital bones, but delayed ossification of the parietal, interparietal, and supraoccipital bones. Note that the two ossification centers of the supraoccipital bone have not fused in the $Cx43^{-/-}$.

high levels of Cx43, but no Cx45, the results prove that the decreased cell-cell diffusion of negatively charged dyes among Cx43-null calvaria osteoblasts is the consequence of a lack of Cx43.

Cx43-null Osteoblasts Are Dysfunctional

As previously noted, the cardiac malformations in Cx43-deficient animals seem to originate from a neural crest abnormality (Huang et al., 1998), whereas in the skeleton, a more generalized defect of osteoblast differentiation and/or function seems to underline the delayed ossification of

the skull and the axio-appendicular bones. Thus, we studied the ability of Cx43-null cells to proliferate and differentiate in culture. Consistent with the original report in embryonic fibroblasts (Reaume et al., 1995), cultured calvaria osteoblasts from newborn $Cx43^{-/-}$ animals exhibited a cell proliferation rate, assessed by thymidine incorporation after 24 h, similar to that of wild-type cells ($2,180 \pm 183$ versus $1,961 \pm 360$ cpm/well, respectively, $n = 4$). In contrast, alkaline phosphatase activity, a marker of osteoblastic differentiation, was significantly decreased in Cx43-null cells, compared with wild-type cells, after 5 d once confluence was reached (274.3 ± 75.4 versus $732.2 \pm$

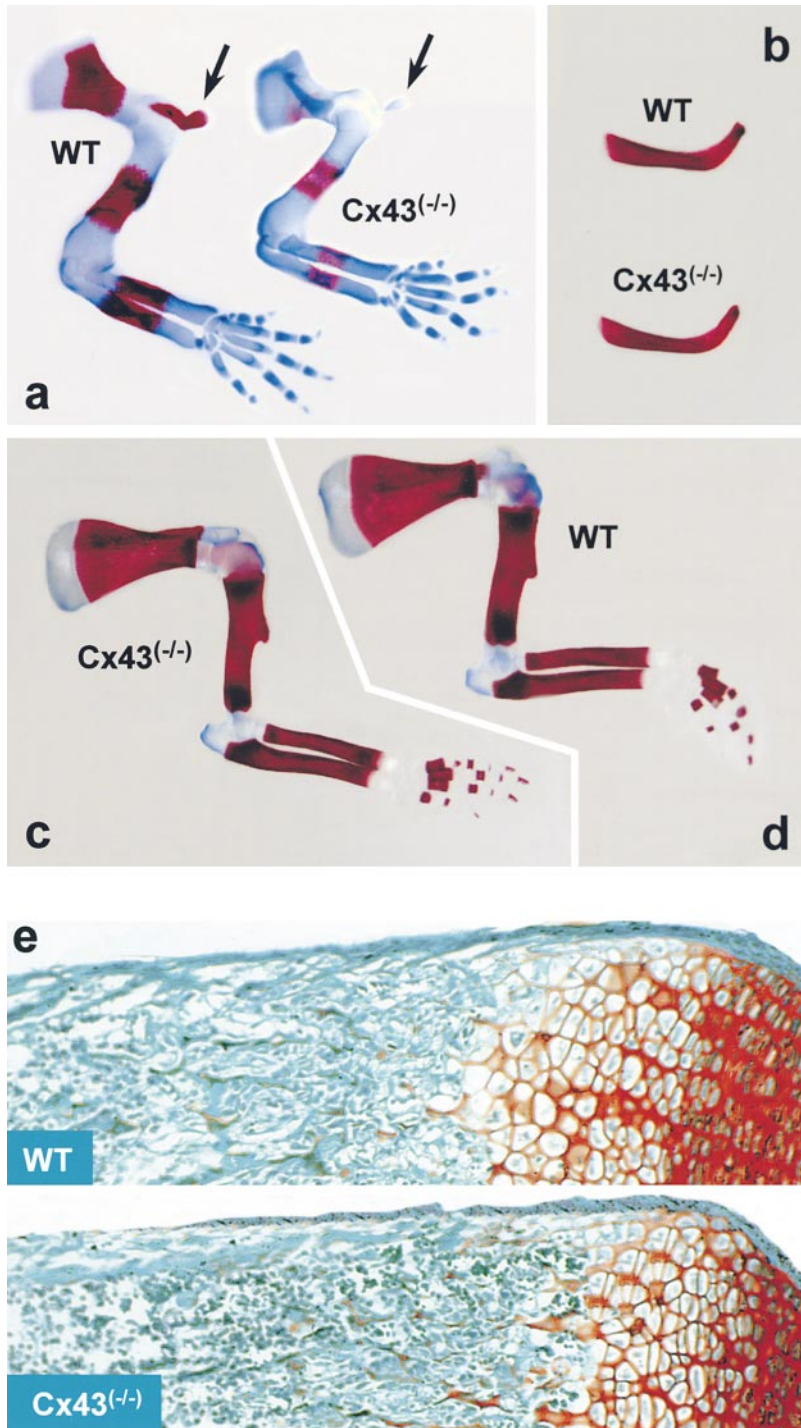


Figure 6. Development of the appendicular skeleton in $Cx43^{-/-}$ embryos. Front limbs at (a) E14.5, (b) clavicle at birth, and (c and d) front limbs at birth were stained with alizarin red/alcian blue. Note, the delayed mineralization of the scapula and the long limb bones (humerus, radius, and cubitus) in the mutant embryos at (a) E14.5 compared with (c and d) normal birth. Also, note the lack of both intramembranous and endochondral ossification of the clavicle, present only as a small cartilaginous template in the (a, arrows) $Cx43^{-/-}$ animals at E14.5. However, the clavicles appeared normal at (b) birth. (e) Longitudinal sections of safranin O/fast green-stained femurs of wild-type and $Cx43^{-/-}$ neonates. The size and morphology of the growth plate is apparently normal, as is the primary spongiosa in the homozygous mutant bone.

127.4 nmol/min/mg protein, respectively, $n = 6$). Accordingly, we observed a significantly lower abundance of collagen type I and osteocalcin mRNA in homozygous mutant cells compared with wild-type and heterozygous cells, whereas osteopontin mRNA was slightly higher in $Cx43$ -null osteoblasts, compared with wild-type and heterozygous cells (Fig. 8 a). Interestingly, type I collagen and osteocalcin mRNA abundance was also reduced, albeit modestly, in heterozygous $Cx43^{+/-}$ cells compared with wild-type cells, with no appreciable differences in osteopontin mRNA. To determine whether the observed osteoblast dysfunction is generalized or restricted to the cal-

varia, we isolated osteoblasts from long bones of newborn mice (see Materials and Methods). Similar to the calvaria cells, steady-state type I collagen and osteocalcin mRNAs were both reduced in confluent, postproliferative osteoblast cultures derived from the femur and tibia. In contrast, osteopontin mRNA was less abundant in $Cx43$ -null cells from the long bone, compared with wild-type cells, a result at variance with the calvaria cells (Fig. 8 a). This may reflect the relative insensitivity of osteopontin gene expression to changes in gap junctional communication, compared with osteocalcin and type I collagen (Lecanda et al., 1998). Nonetheless, analysis of protein expression

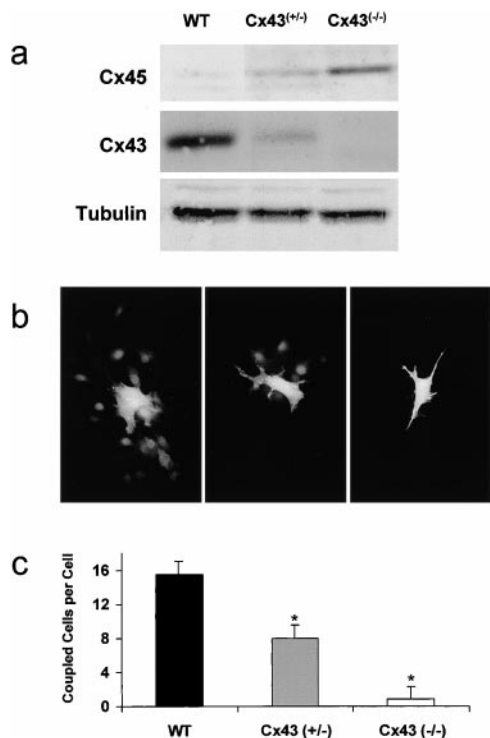


Figure 7. Cx expression and cell coupling in calvaria osteoblasts derived from wild-type (WT), heterozygous Cx43^{+/-}, and homozygous Cx43^{-/-} mice at birth. (a) Immunoblot of whole cell lysate using antibodies against Cx45 and Cx43. Tubulin was used to control for protein loading in each lane. (b) Diffusion of calcein among calvaria osteoblasts of the different genotype groups. Dye coupling was assessed two hours after “parachuting” calcein-loaded donor cells onto an unlabeled monolayer and by counting neighboring cells that have taken the fluorescent dye in 20 microscopic fields in each cell preparation. Cells of the same genotypic group were used as donor cells after calcein loading. (c) Quantitation of dye coupling in each genotype group. The number of cells taking calcein from a single preloaded donor cell was counted as a measure of dye coupling. Data represent the average ± SD of eight random microscopic fields in 13-mm coverslips (ten coverslips per isolate, pooled data from four cell isolates per genotype group). **P* < 0.05, Mann-Whitney U test.

in whole cell lysates of calvaria cells corroborated the mRNA data, confirming a decrease in the level of type I collagen and slight increase in the level of osteopontin present in these cells (Fig. 8 b).

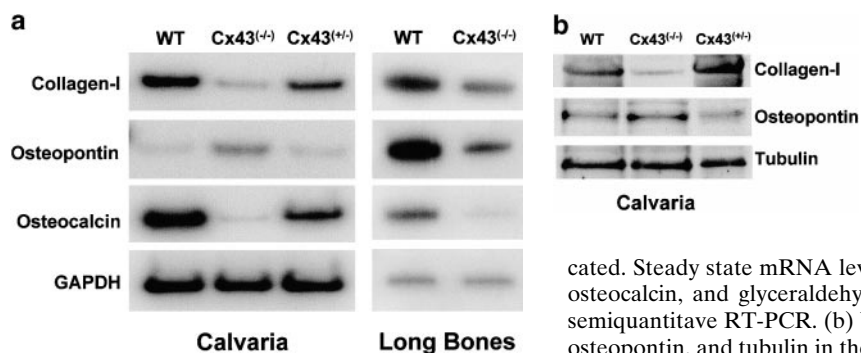


Figure 8. Production of bone matrix proteins by osteoblasts derived from either calvaria or long bones (femur and tibia) of newborn animals. (a) Total RNA was isolated from cultures of wild-type, heterozygous Cx43^{+/-}, and homozygous Cx43^{-/-} cells, after they reached confluency, as indicated. Steady state mRNA levels of type I collagen (Collagen-I), osteopontin, osteocalcin, and glyceraldehyde phosphate dehydrogenase were assessed by semiquantitative RT-PCR. (b) Western analysis of type I collagen (Collagen-I), osteopontin, and tubulin in the three genotype groups using calvaria cells.

Finally, we tested the mineralization potential of Cx43^{-/-} calvaria osteoblasts grown in mineralizing medium, containing ascorbic acid and β-glycerolphosphate, for three weeks. Consistent with the previous observation on osteoblast gene expression, the number of mineralized nodules after two weeks in culture was substantially lower in Cx43-null cells than in wild-type calvaria cells (65 ± 17 versus 278 ± 73 nodules per well, respectively, *P* < 0.001), and the mineralized area was reduced >80% after 3 wk of culture (Fig. 9). Mineralization was moderately lower in heterozygous Cx43^{+/-} calvaria osteoblast cultures relative to wild-type cells, which is in line with the lower level of type I collagen and osteocalcin mRNA noted above. Microscopic examination did not reveal any nonmineralized nodules in the cultures of Cx43-null osteoblasts.

Discussion

Here, we demonstrate that the genetic deletion of Cx43 results in delayed skeletal ossification and craniofacial abnormalities. Both intramembranous and endochondral ossification are retarded, irrespective of the embryonic tissue of origin, and are related to impaired osteoblast maturation and function. Therefore, in addition to the reported neural crest cell defect, the lack of Cx43 also causes a generalized osteoblast dysfunction, leading to delayed mineralization and skull abnormalities.

Previous studies have shown that the migratory potential of neural crest cells emerging from neural tube explants is impaired in Cx43-null mice, whereas their proliferation rate is normal (Huang et al., 1998). The authors linked the major heart malformation of Cx43-null animals to the reduced migration of neural crest cells to sites of conotruncal development. Migration defects were observed in both preotic hindbrain neural crest cells, from which the cranial vault originates, as well as in postotic crest cells (Huang et al., 1998). Of note, defects in the enteric ganglia and thymus, which originate from postotic crest cells, have been recently reported in Cx43^{-/-} mice, in addition to the heart malformations (Huang et al., 1998). Therefore, a defect in preotic neural cell migration to sites that give rise to the cranial vault (parietal, frontal, and squamous bones) would contribute to the hypoplastic skull of Cx43-null mice. In fact, the reduced capacity to form mineralized nodules in Cx43-deficient calvaria cells, despite a normal proliferation rate, supports the idea that parietal and frontal bones of Cx43^{-/-} animals contain a reduced number of osteoprogenitor cells, which is consistent

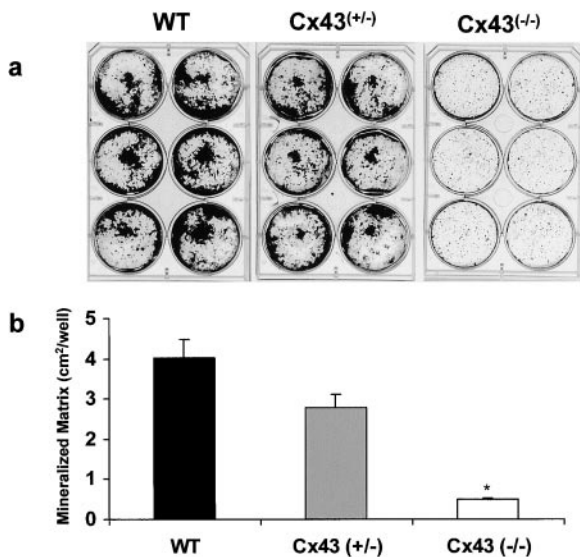


Figure 9. In vitro mineral deposition by calvaria osteoblasts. Cells isolated from calvaria of newborn wild-type (WT), heterozygous *Cx43*^{+/-}, and homozygous *Cx43*^{-/-} mice were grown in mineralizing medium, containing β -glycerophosphate and ascorbic acid, for three weeks. (a) Cultures were stained with Von Kossa. (b) The extent of mineral deposited in the extracellular matrix was quantitated as the surface area (in cm²) covered by black stain in each well (9.6 cm²) using a computerized image analyzer. The illustration and bar chart are representative of identical experiments performed in four independent cell isolates per genotype group.

with a reduced number of embryonic neural crest precursors at ossification sites. Nonetheless, phenotypic characterization of these cells (which has low but detectable type I collagen and osteocalcin, but abundant osteopontin expression relative to wild-type cells) also indicates that *Cx43*-null calvaria cells do contain committed osteogenic precursors whose differentiation potential into matrix secreting and mineral depositing cells is blunted. In addition, the reduced level of type I collagen and osteocalcin expression by osteoblastic cells isolated from long bones of *Cx43*^{-/-} mice, which originate from the mesoderm, demonstrates that the osteoblast defect is not restricted to neural crest-derived cells, and that it affects the whole osteogenic compartment.

Additional findings from the skeletal phenotype of *Cx43*^{-/-} mice support a generalized osteoblast defect. First, delayed ossification is observed not only in the cranial vault, but also in craniofacial segments of cephalic mesoderm origin, i.e., supraoccipital and interparietal bones, as well as in the axial skeleton. Second, though the clavicles are normally mineralized at birth, at earlier stages of bone development (E14.5), both the intramembranous and endochondral parts of the clavicle are not mineralized in *Cx43*^{-/-} mice. Since the clavicle is derived from the mesoderm, the results demonstrate that defective intramembranous bone formation is not restricted to neural crest-derived elements, or to a certain mode of ossification, but instead reflect a generalized functional impairment of *Cx43*-deficient osteoblasts. In the cranial vault, this osteoblast dysfunction is combined with a neural crest cell de-

fect (Huang et al., 1998), leading to the delayed ossification (with open sutures) and skull hypoplasia that characterize the *Cx43*^{-/-} phenotype at birth. In contrast, the delayed ossification of the axial skeleton and limbs is no longer evident at birth, suggesting that the abnormalities in nonneural crest-derived skeleton may be accounted for by the osteoblastic defect only.

It could be argued that the different progression of ossification between the skull and the axial skeleton reflects different requirements for cell-cell communication at different skeletal sites, or that compensatory mechanisms are present in the axial and appendicular skeleton. In this case, the loss of *Cx43* function may alter spatially defined inductive signals that direct craniofacial morphogenesis. Although this possibility remains speculative, recent reports in an avian model indicate that *Cx43* “knockdown,” using antisense strategies, results in craniofacial defects, which mimic our findings (Becker et al., 1999). Interestingly, *msx1*, a homeobox transcription factor critical for craniofacial development, was found to be downregulated in the malformed craniofacial areas where the *Cx43* antisense oligonucleotide is expressed (Becker et al., 1999). More to the point, hypomineralized calvarium with open foramen, mandibular defects, less pronounced alveolar ridge, and short mandibles are all features reminiscent of loss of function mutations of *msx1* (Chen et al., 1996). Although teeth are present in *Cx43*-null animals, whereas they are absent in *msx1*-null mutants (Chen et al., 1996), incisor teeth were less prominent at birth in *Cx43*^{-/-} mice. In contrast, mutations of *msx2*, a transcriptional repressor, that occur in Boston-type craniosynostosis (Jabs et al., 1993) causes premature suture closing (Liu et al., 1995). Abnormal suture closing is also seen in genetic defects of other transcription factors involved in skeletal morphogenesis. For example, loss of function mutations of *lmx1b* give rise to a cranial vault phenotype similar to that seen in *Cx43*-null mice with open sutures (Chen et al., 1998). Based on our previous report demonstrating that gap junctional communication regulates osteoblast gene transcription (Lecanda et al., 1998), one could speculate that signals permeating gap junction channels modulate the spatio-temporal expression of transcription factors required for differentiation of osteogenic precursors. Interestingly, *msx2* is not only an important regulator of osteocalcin gene transcription (Towler et al., 1994), but also participates in transcriptional complexes that bind the proximal region of the osteocalcin promoter, a region we demonstrated to be sensitive to gap junctional communication (Lecanda et al., 1998). The accumulated data lead to the hypothesis that the expression or function of certain transcription factors critical for osteoblast development are sensitive to the intercellular communication environment provided by gap junction channels. Therefore, abnormal modulation of gene transcription is a potential mechanism by which lack of *Cx43* results in skeletal abnormalities.

Our experiments also show that *Cx45* is upregulated in *Cx43*-null osteoblastic cells from calvaria. We have previously shown that *Cx45* gap junctions display a different molecular permeability to negatively charged dyes compared with *Cx43* gap junctions (Steinberg et al., 1994), and that overexpression of *Cx45* in cells expressing endogenous *Cx43* causes decreased gap junctional permeability

and transcriptional downregulation of genes critical for the full expression of the osteoblastic phenotype (Koval et al., 1995; Lecanda et al., 1998). Although skeletal ossification does proceed in Cx43^{-/-} mice, it is significantly delayed in most skeletal areas and is not complete at birth in the skull, where permanent defects are present. Consequently, increased levels of Cx45 in homozygous mutants cannot completely compensate for the lack of Cx43, though we cannot exclude that the two Cxs may serve some common functions in bone. Recent studies on avian development seem to support this premise. Antisense oligonucleotides for Cx43 or Cx45 altered bone formation in explants of chick mandible mesenchyme when they were used in combination, but each antisense oligonucleotide used individually was not a very effective inhibitor (Minkoff et al., 1999). Thus, Cx43 and Cx45 may compensate for each other in early steps of development and tissue differentiation, but they also serve specific functions as cells become more differentiated.

Likewise, we cannot exclude that gap junction-independent compensatory mechanisms, such as upregulation of soluble factors, may override Cx43 deficiency in specific areas of the skeleton. Our results also support the hypothesis that it is the lack of Cx43, rather than the overexpression of Cx45, that causes the osteoblast phenotypic defects observed in previous studies, in that both dominant (Cx45 overexpression) and recessive (Cx43-null mutation) loss of Cx43 result in decreased expression of osteoblast differentiation genes, i.e., osteocalcin, type I collagen, and reduced mineralization potential. Therefore, the type of gap junctional communication provided by Cx43 is very important for normal function of a differentiated osteoblast and for bone formation.

Unfortunately, the lethality of the homozygous loss of Cx43 precludes analysis of the skeleton and bone remodeling beyond birth. Considering the delayed ossification in vivo and the stunted matrix mineralization by Cx43-deficient osteoblasts in vitro, one would anticipate that the consequences of Cx43 gene deletion will be evident postnatally, presumably as defective bone remodeling. Selective deletion of Cx43 in bone cells will be necessary to establish the role of this gap junction protein in an adult remodeling skeleton. A single allele deletion of Cx43 produces slow ventricular conduction in the heart (Guerrero et al., 1997). Although we did find a reduced abundance of Cx43 in heterozygous calvaria cells, this gene dosage effect did not translate into a detectable phenotype, at least until birth. However, it did result in a slight impairment of osteoblast differentiation and function in vitro. Adult Cx43^{+/-} mice are viable and appear indistinguishable from wild-type littermates. However, we cannot exclude that a phenotype, perhaps a reduced accumulation of bone mass, may develop at skeletal maturity in these animals as a consequence of the reduced Cx43 expression and attendant blunted osteoblast function. This hypothesis is currently being investigated.

In summary, the absence of Cx43 results in delayed intramembranous and endochondral ossification, which are results of a generalized osteoblast dysfunction. In the skull, the combined osteoblast abnormality and the known migratory defect of neural crest cells result in craniofacial defects and nonclosure of the cranial foramen. Therefore,

gap junction communication mediated by Cx43 is functionally involved in skeletal development and in normal function of bone forming cells.

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