Progression of calvarial bone development requires Foxc1 regulation of Msx2 and Alx4

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Abstract

Calvarial bones form by direct ossification of mesenchyme. This requires condensation of mesenchymal cells which then proliferate and differentiate into osteoblasts. Congenital hydrocephalus (ch) mutant mice lack the forkhead/winged helix transcription factor Foxc1. In ch mutant mice, calvarial bones remain rudimentary at the sites of initial osteogenic condensations. In this study, we have localized the ossification defect in ch mutants to the calvarial mesenchyme, which lacks the expression of transcription factors Msx2 and Alx4. This lack of expression is associated with a reduction in the proliferation of osteoprogenitor cells. We have previously shown that BMP induces Msx2 in calvarial mesenchyme (Development 125, 1241–1251, 1998). Here, we show that BMP also induces Alx4 in this tissue. We also show that BMP-induced expression of Msx2 and Alx4 requires Foxc1. We therefore suggest that Foxc1 regulates BMP-mediated osteoprogenitor proliferation and that this regulation is required for the progression of osteogenesis beyond the initial condensations in calvarial bone development.

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Introduction

Bone formation is an essential part of skull vault (calvarial) development in vertebrates. Calvarial bones, which are composed of two frontal, two parietal, and an interparietal bone, as well as most facial bones, form directly from mesenchyme by intramembranous ossification. Osteogenesis in these bones starts by mesenchymal cell condensation. These cells undergo differentiation into osteoprogenitor cells, which proliferate and ultimately differentiate into osteoblasts that lay down the bone matrix (Hall and Miyake, 1992). The mesenchymal condensations of the frontal and parietal bones form on the lateral aspects of the brain close to the cranial base. The calvarial bones grow as sheets between the brain and epidermis, and extend apically toward the top of the skull (Rice et al., 2000). Osteoblast differentiation occurs at the bone margins, or osteogenic fronts, where osteoblasts invade into and progenitor cells are recruited from the surrounding mesenchyme. Prior to birth, calvarial bones approximate each other with sutures forming between the bone margins. Sutures accommodate brain growth. In mouse, all calvarial sutures, except for the posterior section of the frontal suture, remain patent. In humans, metopic/frontal suture is obliterated by the third year, and others fuse in the third or fourth decade of life. Calvarial bones form in a unique environment to other bones; they form in close contact with dura mater, which is the topmost layer of the meninges. Interactions between the dural cells and calvarial mesenchyme have been shown to be important in the regulation of calvarial bone development (Opperman et al., 1995).

During recent years, the understanding of molecular mechanisms involved in calvarial bone development has increased considerably, and several genes regulating ossification have been identified through analysis of mouse mu-

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Fig. 1. Expression of Foxc1 and Foxc2 in forming calvarial bones, cartilages, meninges, and mandible. (A–O) Analysis of Foxc1 and Foxc2 expression pattern. (A–D) Expression of Foxc1 is intense in the condensing prechondrogenic mesenchyme (*) and the primitive meningial layers (arrowheads). (C) Mesenchymal condensation of frontal bone has only minimal levels of Foxc1 expression (surrounded by dashed line), but calvarial mesenchyme (arrow) between the brain and surface ectoderm is positive. (D) Later, Foxc1 transcripts are localized to osteoblasts and osteoblast-forming mesenchyme and the meninges. (E–H) Foxc2 is not expressed in the calvarial osteogenic mesenchyme (arrows) but it is coexpressed with Foxc1 in the meninges. (I, J) Sagittal suture between the opposing parietal bone margins. (I) Foxc1 is not expressed in the menenchymal condensations of osteogenic fronts, while sutural mesenchyme expresses Foxc1. (J) Fibroblast growth factor receptor 2 IIIc (Fgfr2c) expression was used as a marker for osteogenic condensations in the suture (Rice et al., 2000). (K, L) Expression of Foxc2 in Foxc1−/− animals. Foxc2 expression pattern is unchanged in the developing cartilages in ch mutants. (L) The developing meninges appear to have reduced levels of Foxc2 transcripts in the lateral and dorsal regions at E15. This may be due to thin meningial cell layers in Foxc1−/− mutant embryos. (M) Foxc1 is expressed strongly in the osteoblasts of the mandible and in the perichondrium of Meckel’s cartilage. (N, O) Foxc2 is expressed at lower levels in these tissues compared with Foxc1 both in wild type and Foxc1−/− animals. Scale bars are 200 μm.
tants. Mice deficient for the Runt domain-containing transcription factor Runx2 (previously Cbfal) lack all bones, including those of the skull vault (Otto et al., 1997). Runx2 is necessary for osteoblast differentiation, and in cell culture, it acts as an activator of osteoblast-specific gene expression. The homeobox transcription factor Msx2 has been suggested to be an upstream regulator of Runx2 in osteoprogenitor cells since Runx2 expression is downregulated in Msx2-deficient mice (Satokata et al., 2000). Msx2 deficient mice exhibit a delay in calvarial bone ossification and an overall decrease in bone volume. Msx2 has a pivotal role in mediating the balance between early osteoprogenitor cell proliferation and differentiation in calvarial development (Liu et al., 1999; Satokata et al., 2000). In humans, haploinsufficiency for MSX2 results in parietal foramina, which is a skull ossification defect in parietal bones (Wilkie et al., 2000; Wuyts et al., 2000a), whereas enhanced MSX2 gene function causes premature suture fusion resulting in Boston type craniosynostosis (Jabs et al., 1993; Ma et al., 1996). Parietal foramina can also be caused by mutations in the paired-type homeobox gene ALX4 (Wu et al., 2000; Wuyts et al., 2000b; Mavrogiannis et al., 2001). In mice lacking Alx4, the calvarial bone phenotype resembles that of Msx2 mutants; ossification is delayed and parietal bones do not extend over the superior aspect of the skull in newborns (Qu et al., 1997). In humans, heterozygous loss of TWIST causes craniosynostosis, and in a subset of the affected individuals, it can cause parietal foramina (Thompson et al., 1984).

We have used the congenital hydrocephalus (ch) mouse line to study regulation of calvarial osteogenesis. This is a spontaneous mouse mutant line originally described by Grüneberg (1943). In ch mutant embryos, cartilages and bones are affected throughout the body, but the major skeletal phenotype is the lack of calvarium associated with hydrocephalus. Rudimentary calvarial bones form at the sites of initial mesenchymal condensations but they fail to extend cranially (Grüneberg, 1943, 1953; Green, 1970; Kume et al., 1998; Hong et al., 1999). Other organs and structures affected and studied in ch mutants include the heart (Winnier et al., 1999), eye (Kume et al., 1998; Hong et al., 1999; Kidson et al., 1999), urogenital organs (Green, 1970; Grüneberg and Wickramaratne, 1974; Kume et al., 2000), and leptomeninges (Grüneberg and Wickramaratne, 1974; Kume et al., 1998). Congenital hydrocephalus is caused by a point mutation in the forkhead/winged helix transcription factor Foxc1 (previously Mfh1) (Kume et al., 1998; Hong et al., 1999). The point mutation results in a stop codon at the amino terminus of the DNA-binding winged helix domain, and thus ch mutant animals lack functional Foxc1 protein. Mice with Foxc1 null alleles and ch mutant mice have identical phenotypes (Kume et al., 1998). The expression pattern of Foxc1 has been studied in early embryogenesis (Sasaki and Hogan, 1993) as well as during organogenesis, specifically during development of the eye (Kume et al., 1998; Kidson et al., 1999), heart (Swiderski et al., 1999; Winnier et al., 1999), kidney (Kume et al., 2000), and endochondral skeleton (Kume et al., 1998). Foxc1 is highly homologous to Foxc2, previously Mfh1 (Hiemisch et al., 1998a). Studies of animals with Foxc1 and Foxc2 single null alleles and double and compound heterozygotes suggest that at least partial functional redundancy between the two genes exists during embryogenesis (Winnier et al., 1999; Kume et al., 2000, 2001). Yet, little is known about the function and downstream targets of Foxc1.

In order to examine the function of Foxc1 in calvarial bone development, we have examined the detailed expression pattern of Foxc1 and Foxc2 during calvarial development and studied the pathogenesis of the calvarial phenotype in ch mutant mice. We show that ch mutant embryos lack Msx2 and Alx4 expression in the osteogenic calvarial mesenchyme, as well as Alx4 expression in the meninges. Functional in vitro assays indicate that Foxc1 is required for the induction of Msx2 and Alx4 by BMP in the osteogenic
calvarial mesenchyme. In addition, we show that this mesenchymal cell population exhibits reduced proliferation in ch mutants. Our results demonstrate that Foxc1 plays a critical role in mediating the BMP signal that regulates Mxs2 and Alx4 expression and in the progression of osteogenesis during calvarial bone development.

Materials and methods

Mating and genotyping

Embryos were obtained by mating congenital hydrocephalus (ch) heterozygote mice (Jackson Laboratories, USA). The vaginal plug date was designated as E0. To detect the point mutation in the Foxc1 gene in ch mice (Kume et al., 1998) solid-phase minisequencing was set up to detect the variable nucleotide. A fragment of Foxc1 sequence surrounding the variable nucleotide in Foxc1 gene was amplified from tail DNA samples by PCR. We used GC-rich PCR system kit (Roche) according to the manufacturer’s protocol with the exception of using 10 p mol of 5'-biotin-labeled reverse primer. Forward primer 5'-CTAC-CAGTTCATCATGGACCGC-3' and 5'-biotinylated reverse primer 5'-TGAGGCAGTCCAGTGGCC-3' were used. The PCR cycle was: 95°C for 3 min, 63°C for 1 min, 72°C for 1 min, and 95°C for 1 min. Cycle was repeated 33 times, and the final products were extended at 72°C for 8 min. The PCR products were used as a template for solid-phase minisequencing as described in Suomalainen and Syvanen (1996); detection primer 5'-GGGACATAAG-CAGGGCTGG-3', 3H-labeled dCTP (53.0 Ci/mmol specific activity; Amersham), and 3H-labeled TTP (118 Ci/ mmol specific activity; Amersham) were used. Radiolabeled dCTP was incorporated onto the detection primer bound to the wild type allele of Foxc1, and radiolabeled TTP to the mutated allele. The amount of radiolabel was measured in a scintillation counter (Wallac) using 3H counting program. Counting the ratio of 3H-dCTP and 3H- TTP between duplicate samples indicated the nucleotide present at the variable site of the Foxc1 gene. If the 3H-dCTP/3H-TTP ratio was >10, the genotype was wild type. A ratio between 0.5 and 2 corresponded to a heterozygous, and a ratio <0.1 corresponded to a mutant genotype.

Tissue dissection and culture

E15 calvaria were dissected from heads. Brains and epithelia were removed. After brain removal, the dura mater was still attached to the mesenchyme of the explant. Explants were cultured on filters, supported by grids, in Dulbecco’s minimal essential medium (DMEM). DMEM was supplemented with GlutaMAX-1 (Life Technologies), 10% FCS, 100 µg/ml of ascorbic acid, and 20 IU/ml penicillin-streptomycin. Explants were cultured at 37°C in 5% CO2 for 48 h.

Bead implantation assays

Affi-Gel blue agarose beads (100–200 µm; Bio-Rad) were incubated with 100 ng/µl recombinant human BMP2 (R&D Systems) for 1 h at 37°C. Control beads were soaked with the same concentration of bovine serum albumin under the same conditions. Protein-containing beads were placed on the top of the calvarial explants under a dissecting microscope. Explants were cultured as described above, and fixed in 4% paraformaldehyde overnight at 4°C.

Skeletal staining

E17 calvaria or E15 calvarial explants cultured for 7 days were fixed in 95% ethanol overnight, and stained in alcian blue staining solution (150 µg/ml alcian blue, 80% ethanol, and 20% acetic acid) overnight. Excess alcian blue was removed in 95% ethanol. Tissues were cleared in 2% KOH for 2 h before transfer into the alizarin red staining solution (75 µg/ml of alizarin red in 1% KOH) and incubated 4–15 h. Explants were cleared in 20% ethanol/1% KOH, and transferred into glycerol–ethanol (1:1) solution, and stored at 4°C. The shortest distance between the parietal bone margins was measured as the minimum sagittal suture width. Statistical analysis of the mean values was performed by t test.

BrdU incorporation and TUNEL analysis

Pregnant ch heterozygote females were injected i.p. with 2 ml/100 g body weight of undiluted 5'-bromo-2'-deoxyuridine (BrdU) solution (Zymed). After 2 h, embryos were collected, fixed in Bouin’s fixative, and paraffin-embedded. BrdU incorporation was immunodetected by using BrdU staining kit (Zymed). BrdU-positive cells were counted in the calvarial mesenchyme adjacent to the advancing osteogenic condensations, which were detected morphologically. A minimum of 10 sections were analyzed per mouse. The same size area of the calvarial mesenchyme was marked on each section. Two independent observers performed cell counting. Statistical analysis on average cell counts was performed by t test. Terminal deoxynucleotidyl transferase-mediated dUTP nick- end labeling (TUNEL) assay was performed by using DeadEnd Colorimetric TUNEL System (Promega).

35S in situ hybridization

In situ hybridization on tissue sections was performed as described by Vainio et al. (1993). The preparation of the following RNA probes has been described: Alx4 (Hudson et al., 1998), Bsp (Rice et al., 1999), Fgfr2c (Rice et al., 2000), Foxc1 (Hiemisch et al., 1998b), Mxs1 and Mxs2 (Jowett et al., 1993). Foxc2 probe was a kind gift from Dr. Kirsi Sainio (University of Helsinki). Both bright and dark field images were taken from hybridized sections. Silver grains were
selected from the dark field image, colored red, and superimposed onto the identical bright field image.

**Results**

**Foxc1 and Foxc2 expression in the developing calvarium**

We examined the expression pattern of Foxc1 and Foxc2 by in situ hybridization using frontal sections of the developing calvarium (Fig. 1). An early sign of intramembranous bone development is the formation of mesenchymal condensations at E12 (Rice et al., 2000). We focused our studies between E11 and E15, when mineralized bone is formed. Some Foxc1 transcripts were localized in the calvarial mesenchyme already at E11 (Fig. 1A). From E12 onwards, the calvarial mesenchyme stretching across the developing skull had a high level of Foxc1 expression (Fig. 1B–D). In contrast, Foxc1 expression was detected only at a minimal level in the mesenchymal condensations of the frontal bones at E13 (Fig. 1C). At E15, Foxc1 transcripts were localized to mature osteoblasts and the periosteum in the calvarial bones (Fig. 1D). At this stage, parietal bones oppose each other at the top of the calvarium and form a sagittal suture which comprises mesenchymal condensations at osteogenic fronts separated by sutural mesenchyme. We detected only a low level of Foxc1 expression in these condensations, while sutural mesenchyme showed intense Foxc1 expression (Fig. 1I).

In the developing cartilages, the Foxc1 expression was intense in prechondrogenic mesenchymal condensations of the developing cranial base and nasal cartilages at E12 and E13 (Fig. 1B and C). Later, the expression of Foxc1 was limited to the perichondrium (Fig. 1D). In addition, Foxc1 was expressed strongly throughout meningeal development, starting at E12.

Foxc1 and Foxc2 have been shown to have distinct but overlapping expression patterns in many mesodermal tissues (Hiemisch et al., 1998b). Foxc1 and Foxc2 showed coexpression in prechondrogenic mesenchyme in the head and in the developing meninges (Fig. 1B, C, F, and G). Foxc2 transcripts were not detected in calvarial osteogenic mesenchyme (Fig. 1G and H). In ch mutant mice, Foxc2 expression was essentially normal in the calvarial region (Fig. 1K and L). At E15, meninges showed reduced expression of Foxc2 in dorsal and lateral areas. This may be due to thin meningeal cell layers in the ch mutant (Kume et al., 1998). In facial bones, for instance in the mandible, Foxc1 was expressed in osteoblasts and in the perichondrium of Meckel’s cartilage, and Foxc2 transcripts were localized in the same regions but at a reduced level (Fig. 1M–O).

Thus, the expression pattern of Foxc1 is consistent with roles during early and late stages of bone formation. Foxc1 may have an early function in the head mesenchyme destined to become skeletal structures, and a later role may be to regulate osteoblast function. Interestingly, Foxc1 expression was turned off between these early and late stages when mesenchymal cells aggregate and form osteogenic condensations. Foxc1 and Foxc2 were coexpressed in the jaw bones and in the dural cell layer but not in the calvarial mesenchyme.

**The calvarial phenotype of ch mutant mice is not secondary to hydrocephalus and is apparent in ch heterozygotes**

In ch mutant embryos, the hydrocephalus was apparent at E15 with brain tissue protruding anteriorly due to expanded ventricles (Fig. 2A). The calvarial mesenchyme was thin and loosely organized. At this stage, calvarial bone development was progressing toward the apex of the skull in the wild type embryos, while in ch mutants, mature osteoblasts were located only within the area demarcated by the initial osteogenic mesenchymal condensations seen at E13, and did not extend superiorly (Fig. 2B). Bone growth was visualized by in situ hybridization with a probe for Bone sialoprotein, which is expressed in functional osteoblasts, and by whole-mount alizarin red/alcan blue staining of heads and calvarial explants (Fig. 3A–F). Bone was missing from the apical aspect of the skull in ch mutants; only rudimentary calvarial bones formed at the sites of initial osteogenic condensations. These bones were misshapen with the frontal and parietal bones extending posteriorly.

Although heterozygous ch embryos have been reported to exhibit normal calvaria (Green, 1970), abnormalities in their endochondral skeleton have been described (Green, 1970; Hong et al., 1999). Therefore, we analyzed the calvarial phenotype of heterozygous embryos in detail. We measured the minimum width of the sagittal suture between parietal bones in wild type and heterozygous calvarial explants at E17 (Fig. 3G and H). In wild type embryos, the sagittal suture width ranged from 0.03 to 0.47 mm with the mean being 0.23 mm (n = 7; s.d. = 0.14). In heterozygous embryos, the minimum sagittal suture width ranged from 0.17 to 0.60 mm, the mean being 0.39 mm (n = 7; s.d. = 0.14). Thus, the sagittal suture was 41% wider in heterozygotes than in wild type littermates (P < 0.03). Furthermore, all seven heterozygous explants had an irregularly shaped patch of secondary cartilage endocranial to the sagittal suture mesenchyme, while only two out of seven wild type explants (29%) had a similar but smaller secondary cartilage.

The failure of calvarial bone growth to progress in ch mutant animals has been suggested to be secondary to the hydrocephalus and caused by increased intracranial pressure (Grüneberg, 1943; Kume et al., 1998). To test this, we followed the development of E15 calvarial explants in organ cultures for 7 days and then stained them for bone and cartilage (Fig. 3I–N). In these conditions, the possible mechanical effects of intracranial pressure are eliminated. Calvarial bones increased in size in wild type
Fig. 3. Calvarial bone development is inhibited in Foxc1+/- animals and the inhibition is not secondary to hydrocephalus. (A–H) Skeletal staining of E17 heads and calvarial explants. Mineralized bone is stained with alizarin red and cartilages with alcian blue. (A–C) Lateral view of heads. The difference between wild type and Foxc1+/− calvarial development is not apparent, but the Foxc1−/− animal lacks skull top. (D–F) Calvarial explants (two view) show that wild type and Foxc1+/− embryos have two frontal and two parietal bones, and an interparietal bone. In the Foxc1−/− embryo, their growth has been arrested. Note the mesenchymal tissue stretching across the skull top in the Foxc1−/− calvarial explant (*). (G, H) High magnification images of the wild type and Foxc1+/− explants show that sagittal suture is wider in Foxc1+/−. (H) Secondary cartilage (arrow) has formed in Foxc1+/− embryo endocranial to the suture. (I–N) In vitro culture of E15 calvarial explants, top view. Seven day (t7) cultures show that elimination of the intracranial pressure did not rescue the growth of the calvarial bones in Foxc1+/− and Foxc1−/− explants in comparison with t7 wild type cultures and day zero (t0) cultures. Images shown are representative samples. f, frontal bone; ip, intraparietal bone; p, parietal bone. (Note that t0 and t7 explants are different since no skeletal staining can be done during culture period.) Scale bars are 1 mm.
explants (n = 3), and the sagittal suture became narrower. In heterozygous explants (n = 12), the sagittal suture stayed wide in all explants. We did not observe any bone growth in ch mutant explants (n = 4), and the calvarial mesenchyme between rudimentary calvarial bones regressed in ch mutant explants. Hence, we did not observe any rescue of bone growth in heterozygous or ch mutant explants when cultured in vitro.

These data suggest that the arrested calvarial development in ch mutants is not secondary to mechanical influences of the hydrocephalus, and that Foxc1 directly regulates the growth of calvarial bones. Lack of one Foxc1 allele is sufficient to cause a delay in the ossification of parietal bone plates.

Msx2 and Alx4 expression are downregulated in calvarial mesenchyme of Foxc1 mutant mice

We searched for potential downstream targets of Foxc1 by in situ hybridization analysis of ch mutant calvarial tissue (Fig. 4). Runx2, a marker for preosteoblasts, is expressed in osteogenic condensations at E13, and also in the preosteoblasts between the brain and epidermis at E15. The expression pattern of Runx2 appeared normal in ch mutant...
animals at E13, but Runx2 transcripts were limited to regions surrounding the rudimentary calvarial bones at E15, and no transcripts were seen more superiorly in the calvarial mesenchyme (Fig. 4A–F).

Msx2 null mice have defective skull ossification and a persistent bone-free area between the parietal bones (Satokata et al., 2000). Our previous work has shown that Msx2 is expressed in sagittal suture mesenchyme and at the osteogenic fronts of parietal bones that are active sites of osteoblast differentiation (Kim et al., 1998). In wild type embryos, Msx2 was expressed throughout the calvarial osteogenic mesenchyme at E13 (Fig. 4G and H). In bone, Msx2 was expressed in the periosteum (Fig. 4K). In ch mutant embryos, Msx2 expression was limited to the calvarial mesenchymal condensations and a region extending a short distance into the osteogenic mesenchyme beyond the condensation sites. No expression was detected more superiorly in the osteogenic mesenchyme (Fig. 4I and J). At E15, Msx2 expression was further limited to the periosteum of the rudimentary bones (Fig. 4L). Other craniofacial structures exhibited a normal Msx2 expression pattern in ch mutant tissue.

Msx1, a gene related to Msx2, is expressed in a pattern similar to Msx2 during calvarial development (MacKenzie et al., 1991), and mice lacking Msx1 exhibit multiple craniofacial abnormalities (Satokata and Maas, 1994). In ch mutant animals, the Msx1 expression pattern was unchanged (Fig. 4M–R).

The Alx4 null mice have a localized bone defect in the skull, and their calvarial phenotype is reminiscent of the Msx2 mutant phenotype. Alx4 has been reported to be expressed in osteoblastic precursor cells of most bones and in the developing meninges (Hudson et al., 1998). We detected Alx4 expression in condensations of calvarial bones, and in the osteogenic mesenchyme in a pattern similar to that of Msx2 (Fig. 4S, T, and W). In addition, Alx4 was expressed in the developing meninges. In ch mutant embryos, Alx4 expression was detected in the condensations of calvarial bones, but it was greatly reduced in the calvarial mesenchyme similarly to Msx2 (Fig. 4U, V, and X). The meningeal expression of Alx4 was also limited to inferior region in ch mutants. Other craniofacial structures exhibited a normal expression pattern of Alx4.

Thus, ch mutant animals exhibit a location-specific lack of Msx2 and Alx4 expression in the calvarial mesenchyme, and in addition Alx4 was reduced in the meninges. It would therefore appear that the failure of calvarial bone growth in ch mutants is caused by a lack of expression of early differentiation factors, such as Msx2 and Alx4, in the calvarial mesenchyme and the dura mater.

Foxc1 is required in the calvarial mesenchyme for the induction of Msx2 and Alx4 by BMP

Bone morphogenetic proteins (BMPs) are signaling molecules that were discovered by their ability to induce ectopic bone formation (Urist, 1997). Since Msx2 has been associated with BMP signaling (Kim et al., 1998), we analyzed the expression patterns of Bmp2, −4, and −7 in frontal head sections at E15. Although the expression of these Bmps has been reported in these tissues at different stages, the precise expression patterns in the calvarial mesenchyme and the surrounding tissues have not been described. Bmp2, −4, and −7 are expressed in the calvarial mesenchyme, and Bmp4 and −7 also in the meninges (Fig. 5A). Hence they are all potential candidates for regulating calvarial bone development. In ch mutant tissues, expression patterns of these Bmps were not altered (Fig. 5A), indicating that Foxc1 is not involved in the regulation of their expression. We next asked whether BMPs act upstream of Foxc1, and whether exogenous BMP regulates the expression of Foxc1 in calvarial mesenchyme. We placed BMP2-containing agarose beads onto the calvarial mesenchyme of E15 wild type explants, and cultured the explants for 48 h. We were unable to show induction of Foxc1 around the BMP2-releasing beads (data not shown). Foxc2 was not expressed in the osteogenic head mesenchyme (Fig. 1), and no induction of Foxc2 was observed in the mesenchyme surrounding BMP2 beads (data not shown): Bovine serum albumin (BSA)-soaked beads were used as negative controls. BSA-soaked beads showed no induction of Foxc1 or Foxc2 (data not shown).

Kume et al. (1998) have shown that Foxc1-deficient cells from sternal primordium have a reduced response to added TGFβ or BMP2 in chondrogenic micromass cultures. Hence, we next analyzed whether Foxc1 would affect the response of calvarial mesenchyme to BMP signals. We have shown previously that exogenous BMP2 and BMP4 both induce Msx2 and Msx1 expression in calvarial mesenchyme in vitro (Kim et al., 1998; Rice, 1999). We placed BMP2-containing beads on the calvarial mesenchyme of wild type, ch heterozygous, and ch null explants, and analyzed Msx2 expression after 48 h by in situ hybridization. Intense expression of Msx2 was seen around the beads in the wild type (Fig. 5B) and heterozygous (data not shown) explants (n = 20). The induction was weaker in the mesenchyme near the parietal bone margin. In contrast, BMP2 did not induce Msx2 expression in ch mutant calvarial explants (n = 5) (Fig. 5B). Beads soaked in BMP7 had a similar effect as BMP2 and induced the expression of Msx2 in wild type calvarial explants but not in ch mutant explants (data not shown). Exogenous BMP2 (Fig. 5B) and BMP7 (data not shown) induced the expression of Alx4 in E15 wild type calvarial explants but not in ch mutant explants. This indicates that Alx4, like Msx2, is regulated by BMP and that this regulation requires Foxc1. BSA-soaked control beads showed no induction of Msx2 or Alx4 (data not shown).

The calvarial mesenchyme is thin and disorganized in ch mutant animals (Kume et al., 1998), and it is possible that the lack of Msx2 and Alx4 induction by BMP is not specific but rather a general inability to respond to BMP signals. To exclude this possibility, as well as to examine whether ch mutant cells are generally unresponsive to BMP signals, we analyzed whether BMP2 could induce the expression of
Msxl which is also regulated by BMP in calvarial mesenchyme (Kim et al., 1998). Msxl was induced in ch mutant explants by BMP2-containing beads in a similar manner to wild type explants (Fig. 5B), indicating that the mesenchyme is indeed competent to respond to BMP and expresses BMP receptors and other molecules needed for the transduction of the BMP signal.

Taken together, our results suggest that Foxc1 is required for the induction of Msx2 and Alx4 by BMP in the developing calvarium. Also, we show that BMP induces Alx4 in calvarial mesenchyme.

Cell proliferation

We have shown previously that BMP4-containing beads inducing Msxl and Msx2 expression in calvarial sutures also significantly increase the number of mesenchymal cells (Kim et al., 1998). Furthermore, overexpression of Msx2 increases cell proliferation in the osteogenic fronts and promotes growth of the parietal bones (Liu et al. 1999), and conversely, lack of Msx2 decreases the number of BrdU-positive cells at the osteogenic fronts during late embryonic and postnatal calvarial development (Satokata et al. 2000). To test whether the early proliferative pool of osteogenic cells was affected in ch mutant mice, we assayed for BrdU incorporation in the developing frontal bones. We found a 30% decrease in the number of BrdU-positive cells in the calvarial mesenchyme of ch mutants in comparison to heterozygous littermates (no wild type embryos in the litters collected) at E13 (Fig. 6A and B). At E15, the ch mutants had 50% less BrdU-positive cells when compared with heterozygous and wild type embryos (Fig. 6A and B). Due to the small litter sizes, the wild type and heterozygous embryos were combined for this analysis. The reduced BrdU incorporation correlates with the lack of Msx2 and Alx4 expression in the ch mutant calvarial mesenchyme (Fig. 4I and S).

We performed TUNEL assay to test whether lack of calvarial bone development was due to an increase in apoptosis in calvarial mesenchyme. No difference was detected between wild type and ch mutant littermates in the number of apoptotic cells (data not shown).

Thus, these data suggest that Foxc1 regulates cell proliferation in the osteoprogenitor cell population in the calvarial mesenchyme. We suggest that this regulation is mediated by Msx2 and also by Alx4.

Discussion

Foxc1 is expressed at early and late stages of bone development

We have demonstrated that Foxc1 expression is turned on early in both chondrogenic and osteogenic cell populations during craniofacial development. Interestingly, Foxc1 was expressed only at minimal levels in the osteogenic condensations at E13 although the chondrogenic mesenchymal condensations expressed it intensely. Osteogenic condensations at suture bone margins did not express Foxc1 either. Later in development, Foxc1 was expressed in mature osteoblasts. The expression patterns are consistent with a role for Foxc1 in the early specification of skeletal cell lineages in the developing embryo. In mature osteoblasts, Foxc1 may regulate osteoblast-specific extracellular matrix production.

Studies of Foxc1 and Foxc2 double heterozygous and compound heterozygous embryos have suggested that Foxc1 cooperates with Foxc2 to activate target genes during eye, heart, and kidney development, and during somitogenesis (Winnier et al., 1999; Smith et al., 2000; Kume et al., 2001). These organs show extensively overlapping expression of Foxc1 and Foxc2. Here, we show that the expression patterns of Foxc1 and Foxc2 overlap in the cartilaginous skeleton in the head, in the mandible, meninges, and periosteum, but not in the calvarial mesenchyme, indicating that there is no interaction between Foxc1 and Foxc2 at this site, and therefore functional redundancy is unlikely. This may partly explain why the phenotype is more severe in calvarial bones than in other bones in ch mutants.

Calvarial phenotype in ch animals

The ch mutant embryo displays a variety of skeletal abnormalities. Both endochondrally developing bones, such as vertebrae and the sternum, and intramembranously forming bones are affected. Grüneberg (1943) suggested that the primary defect was a cartilage anomaly and that the failure of calvarial bone development was a mechanical consequence of hydrocephalus in the mutants. However, later, Grüneberg and Wickaramatne (1974) concluded that defects in the subarachnoid space, which is part of the meninges, caused the lack of bone development. These suggestions were based on skeletal and histological analyses of ch mutant mice. Kume et al. (1998) analyzed the Foxc1 null mutant mice and proposed that Foxc1 is required for chondrogenesis and for meningeal development. The authors favored Grüneberg’s original hypothesis that the absence of intact skull bones is secondary to the hydrocephalus. This was based on the finding that E11 dorsal head mesenchyme micromass cultures derived from ch mutant embryos gave rise to both cartilage and bone.

We examined the calvarial phenotype by skeletal staining, and show that a calvarial phenotype (a widened sagittal suture at E17) is also seen in ch heterozygotes. We also show that mechanical factors caused by intracranial pressure are not likely to cause the lack of calvarial bone growth in ch mutant animals. Calvarial bone development was not rescued when explants of ch heterozygous or mutant animals were grown in organ culture where possible mechanical effects of hydrocephalus are eliminated. These results, together with the finding that Foxc1 is expressed in osteo...
progenitor cells and mature osteoblasts, support the view that the defect in calvarial bones in ch mutant mice is caused by the lack of Foxc1 in calvarial mesenchyme.

Osteoblast lineage marker analysis by in situ hybridization indicated that the calvarial mesenchyme is defective in ch mutant animals. The expression of Msx2 and Alx4, two transcription factors associated with calvarial bone development and expressed in osteoprogenitor cells, was greatly reduced in calvarial mesenchyme. In the calvarial bones of ch mutant mice, osteoblast differentiation takes place only within the initial mesenchymal condensations next to the cranial base cartilages. In ch mutants, the Msx2 and Alx4 expression was limited to these condensations and to a narrow region of surrounding calvarial mesenchyme.

Loss-of-function mutations in Msx2 or Alx4 cause similar calvarial phenotypes, i.e., defective calvarial bone development resulting in parietal foramina both in humans and mice due to deficient bone formation and the lack of closure of calvarial sutures. The calvarial phenotype in ch mutant embryos is much more severe than in either Msx2 or Alx4 null mice as the growth of calvarial bone is arrested after the initial condensation stage. We suggest that Msx2 and Alx4 have similar functions in calvarial mesenchyme and that the combined lack of their function produces the dramatic cal-

Fig. 5. Foxc1 is required for BMP induced expression of Msx2 and Alx4 in the developing calvarium. (A) Expression patterns of Bmp2, -4, and -7. All three Bmps are expressed in the head mesenchyme, and Bmp4 and -7 also in the meningeal cell layers. No difference was detected between wild type and Foxc1−/− embryos. Scale bars are 200 μm. (B) Transverse sections of calvarial explants cultured for 48 h with BMP2-containing agarose beads placed onto the mesenchyme. In wild type explants, BMP2 has induced Msx2 and Alx4 expression in the mesenchyme around the bead. In Foxc1−/− tissue, BMP2 has not induced Msx2 and Alx4 expression. Endogenous Msx2 expression is seen in the peristeum of parietal bones both in wild type and Foxc1−/− explants. BMP2 beads have induced Msx1 expression both in wild type and Foxc1−/− explants. Arrows indicate beads that have induced expression. Arrowheads indicate the parietal bone margins. Beads were colored blue to aid visualization. Scale bars are 1 mm.
varial phenotype in \( ch \) mutants. Interestingly, \( Alx4 \) expression, which unlike \( Msx2 \) was coexpressed with \( Foxc1 \) also in the meninges, was absent from the meninges in \( ch \) mutant embryos. This suggests that \( Foxc1 \) regulates \( Alx4 \) expression in both calvarial mesenchyme and meninges. The role of the dura mater in the regulation of sutural ossification is well established (Opperman et al., 1995; Kim et al., 1998; Levine et al., 1998). Hence, \( Alx4 \) may have a dual function in calvarial osteogenesis. It may control production of a signal in the dura mater, regulating bone formation in the neighboring calvarial mesenchyme and control the response of the calvarial mesenchyme. Since calvarial bones are the only bones that interact with dura mater during development, the lack of meningeal signal may partly explain why the calvarial bones are affected more severely than other bones in \( ch \) mutants.

\( Foxc1 \) mediates BMP induction of \( Msx2 \) and \( Alx4 \)

We show evidence that \( Foxc1 \) participates in BMP-induced expression of \( Msx2 \) and \( Alx4 \) in the calvarial mesenchyme. \( Msx2 \) maintains early osteoprogenitor cell proliferation in sutures (Liu et al., 1999; Satokata et al., 2000), and it regulates cell proliferation and differentiation in skin (Jiang et al., 1999). We show that lack of \( Foxc1 \) is associated with a reduced amount of BrdU-positive cells in the osteoprogenitor cell populated areas in calvarial mesenchyme. It would appear that \( Foxc1 \) is necessary for \( Msx2 \) and \( Alx4 \) expression only in proliferative calvarial mesenchyme. This we base on findings that, for instance, mesenchymal condensations and periosteum express \( Msx2 \) and \( Alx4 \) in \( ch \) mutants. Both the condensations and periosteum have no changes in their BrdU-positive cell counts. The site-specific lack of proliferation in the osteoprogenitor cell areas in calvarial mesenchyme may be responsible for the lack of further calvarial bone development beyond the rudimentary bones in \( ch \) mutants. Thus, our results indicate that the initial calvarial bone formation is under different controls than the subsequent progression of osteogenesis. We suggest that \( Foxc1 \) plays an important part in the progression of calvarial bone development by regulating \( Msx2 \)- and \( Alx4 \)-driven osteoblastic proliferation. Premature differentiation would lead to a depletion of osteoprogenitor cells and prevent further bone development. Other Fox family members have been associated with cellular proliferation. Foxe3 promotes proliferation of epithelial cells in the developing lens (Blixt et al., 2000), Foxg1 regulates proliferation in the telencephalic neuroepithelial cells (Xuan et al., 1995), and Foxn1 in the epithelial cells of the hair follicle (Brissette et al., 1996; Prowse et al., 1999).

We show that \( Foxc1 \) participates in the Bmp regulation of \( Msx2 \) and \( Alx4 \). The source of the Bmp signal may be in the mesenchyme or in the dura mater. Most of the affected organs and structures in \( ch \) mutants form in a mesenchymal environment and need inductive interactions with the surrounding tissues during morphogenesis. Many of these in-
teractions involve Bmp signaling. Hence, it is possible that mediating Bmp signaling may be a general function of Foxc1 during development.

We have shown that Foxc1, Msx2, and Alx4 are coexpressed in calvarial mesenchyme, and that Foxc1 is required for the induction of Msx2 and Alx4 expression by BMP in this tissue (Fig. 6C). Since Foxc1 and Alx4 are also coexpressed in the meninges, which are affected in Foxc1 mutants, it is possible that Foxc1 regulates calvarial bone development not only by autocrine signaling but also in a paracrine manner by influencing the production of a dural signal involved in bone formation.

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