INTRODUCTION

Tooth development begins when regional signals emanating from oral ectoderm induce molecular changes in underlying cranial neural crest-derived ectomesenchyme. The invagination of the dental lamina and its development into a morphologically distinct enamel organ is then influenced by signals from the dental papilla mesenchyme. This reciprocal and inductive exchange of molecular signals between tooth ectoderm and mesenchyme regulates tooth morphogenesis and culminates in the differentiation of odontoblasts, ameloblasts and cementoblasts and the formation of dentin, enamel and cementum matrices respectively. Due to its suitability for in vitro manipulation, the developing tooth organ provides a unique model to study the complex interplay of signaling molecules that confer positional information, determine size and shape and influence the terminal differentiation of matrix-producing cells (reviews: Thesleff et al., 1995; Thesleff and Nieminen, 1996; Maas and Bei, 1997; Thesleff and Sharpe, 1997).

In vivo and in vitro tissue recombination studies have demonstrated the shift of inductive potential from odontogenic epithelium to mesenchyme between mouse embryonic days E11 and E12 (Mina and Kollar, 1987, Lumsden, 1988). Recent investigations have unravelled the molecular basis for the transfer of odontogenic potential between dental epithelium and mesenchyme. Cbfa1 is a critical transcriptional regulator of osteoblast differentiation. Mutations in this gene cause cleidocranial dysplasia (CCD), an autosomal dominant disorder in humans and mice characterized by defective bone formation. CCD also results in dental defects that include supernumerary teeth and delayed eruption of permanent dentition. The dental abnormalities in CCD suggest an important role for this molecule in the formation of dentition. Here we describe results of studies aimed at understanding the functions of Cbfa1 in tooth formation. RT-PCR and in situ hybridization analyses show that Cbfa1 has a unique expression pattern in dental mesenchyme from the bud to early bell stages during active epithelial morphogenesis. Unlike that observed in osteoblast differentiation, Cbfa1 is downregulated in fully differentiated odontoblasts and is surprisingly expressed in ectodermally derived ameloblasts during the maturation phase of enamel formation. The role of Cbfa1 in tooth morphogenesis is further illustrated by the missshapen and severely hypoplastic tooth organs in Cbfa1−/− mice. These tooth organs lacked overt odontoblast and ameloblast differentiation and normal dentin and enamel matrices.}

SUMMARY

Osteoblasts and odontoblasts, cells that are responsible for the formation of bone and dentin matrices respectively, share several molecular characteristics. Recently, Cbfa1 was shown to be a critical transcriptional regulator of osteoblast differentiation. Mutations in this gene cause cleidocranial dysplasia (CCD), an autosomal dominant disorder in humans and mice characterized by defective bone formation. CCD also results in dental defects that include supernumerary teeth and delayed eruption of permanent dentition. The dental abnormalities in CCD suggest an important role for this molecule in the formation of dentition. Here we describe results of studies aimed at understanding the functions of Cbfa1 in tooth formation. RT-PCR and in situ hybridization analyses show that Cbfa1 has a unique expression pattern in dental mesenchyme from the bud to early bell stages during active epithelial morphogenesis. Unlike that observed in osteoblast differentiation, Cbfa1 is downregulated in fully differentiated odontoblasts and is surprisingly expressed in ectodermally derived ameloblasts during the maturation phase of enamel formation. The role of Cbfa1 in tooth morphogenesis is further illustrated by the missshapen and severely hypoplastic tooth organs in Cbfa1−/− mice. These tooth organs lacked overt odontoblast and ameloblast differentiation and normal dentin and enamel matrices. Epithelial-mesenchymal recombinants demonstrate that dental epithelium regulates mesenchymal Cbfa1 expression during the bud and cap stages and that these effects are mimicked by the FGFs but not by the BMPs as shown by our bead implantation assays. We propose that Cbfa1 regulates the expression of molecules in mesenchyme that act reciprocally on dental epithelium to control its growth and differentiation. Taken together, our data indicate a non-redundant role for Cbfa1 in tooth development that may be distinct from that in bone formation. In odontogenesis, Cbfa1 is not involved in the early signaling networks regulating tooth initiation and early morphogenesis but regulates key epithelial-mesenchymal interactions that control advancing morphogenesis and histodifferentiation of the epithelial enamel organ.

Key words: Cbfa1/Osf2, Odontogenesis, Epithelial morphogenesis, Inductive signalling, Knockout mice, Odontoblasts, Ameloblasts

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In vivo and in vitro tissue recombination studies have demonstrated the shift of inductive potential from odontogenic epithelium to mesenchyme between mouse embryonic days E11 and E12 (Mina and Kollar, 1987, Lumsden, 1988). Recent investigations have unravelled the molecular basis for the transfer of odontogenic potential between dental epithelium and mesenchyme.
and mesenchyme. These studies indicate that signaling molecules in the BMP (bone morphogenetic protein), FGF (fibroblast growth factor) Hh (hedgehog) and Wnt families as well as several transcription factors, play a key role in tooth bud formation and progression to the cap stage (Theis and Sharpe, 1997). Despite this progress in understanding the molecular basis for tooth initiation and early morphogenesis, little is known about the molecules involved during advancing tooth morphogenesis and cytodifferentiation.

The condensation of odontogenic mesenchyme at E12 is coincident with the aggregation of cells in surrounding preosteogenic mesenchyme. As development progresses, osteogenesis and odontogenesis remain intimately associated. The terminal differentiation of osteoblasts and odontoblasts from mesenchymal condensates is marked by the expression of type I collagen, a structural protein of bone and dentin. Despite these similarities there are several differences that distinguish dentin from bone (Linde and Goldberg, 1993). Whether odontogenesis and osteogenesis share common signaling pathways and how divergence, if any, is achieved between odontoblasts and osteoblasts remain central issues in skeletal biology.

Recent discoveries have revealed that a transcription factor called Cbfa1 (core binding factor A1) also referred to as Osf2 (osteoblast specific factor 2), is a critical regulator of osteoblast differentiation (Ducy and Karsenty, 1995). Cbfa1 is the mouse homologue of the Drosophila pair-rule gene runt (Ogawa et al., 1993) and its expression in mouse is largely restricted to areas of mesenchymal condensation that form the future skeleton (Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997; Ducy and Karsenty, 1995). Osteoinductive growth factors like the BMPs, regulate Cbfa1 expression in osteoblasts. The transcriptional molecule is also able to regulate the expression of multiple extracellular matrix genes in osteoblasts and its overexpression can induce osteoblast-specific gene expression in fibroblasts and myoblasts (Ducy et al., 1997). Mice homozygous for a targeted deletion of Cbfa1, show no osteoblast differentiation and hence completely lack bone (Komori et al., 1997; Otto et al., 1997). Thus, Cbfa1 is a transcriptional activator of osteoblast differentiation that is needed for in vivo bone formation.

Genetic studies have recently identified the lack of expression of one allele of Cbfa1 as the basis for the cleidocranial dysplasia syndrome (CCD) in mice and humans (Otto et al., 1997; Mundlos et al., 1997; Lee et al., 1997). CCD is an autosomal-dominant inherited disorder characterized by delayed ossification, patent fontanelles and Wormian bones. In addition, the dentition is affected with multiple supernumerary teeth arising from the permanent dentition that fail to erupt (Jones et al., 1997). The critical importance of Cbfa1 in osteogenesis and the dramatic changes affecting dentition in CCD patients suggest an important yet poorly understood role for this molecule in tooth development. Furthermore, it is unclear whether the defects in CCD dentition are linked to disturbances in osteogenesis or whether Cbfa1 directly influences key events during tooth development.

To gain a better understanding of Cbfa1’s function we analyzed its temporal and spatial expression from early to late phases of odontogenesis and performed a histologic analysis of tooth development in mice lacking Cbfa1. We further explored the regulation of its expression in mandibular and tooth mesenchyme by using tissue recombination and bead implantation assays. Our data indicate that Cbfa1 expression in dental mesenchyme is intimately associated with epithelial-mesenchymal interactions during tooth development and is affected by epithelial signals that involve the FGFs. Taken together, our results suggest that Cbfa1 regulates the expression of mesenchymal factors that influence the morphogenesis and histodifferentiation of the epithelial enamel organ.

MATERIALS AND METHODS

RT-PCR analysis

Mandibular and maxillary molar segments/molars, long bone, calvaria and liver were dissected from E15 and E18 embryos as well as from newborn and adult (postnatal day (P) 21) mice. Tissues were snap-frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using RNA STAT-60 (Tel-Test Inc., Texas) and first strand cDNA synthesized using oligo(dT) primers and reverse transcriptase (Perkin Elmer Cetus, Norwalk, CT, USA). cDNAs were amplified using Cbfa1-specific primers and PCR conditions as described by Komori et al. (1997). PCR products were fractionated on a 2% agarose gel and transferred to nitrocellulose membrane, Hybond N+ (Amersham Co., Illinois).

Tissues for in situ hybridization

Heads from B6D2F2 and (CBAT6T6 × NMRI) F1 hybrid mouse embryos staged from E10 to E18 (vaginal plug appearance = E0) as well as from newborn (NB) and 2-, 4-, 7-day old (P2, P4 and P7) mice, were fixed in 4% paraformaldehyde or 10% neutral buffered formalin (NBF) for up to 48 hours at 4°C. Postnatal tissues were demineralized in 12.5% buffered-EDTA with 2.5% paraformaldehyde for 2 weeks and then processed for paraffin wax embedding. Serial sections prepared in either the sagittal or coronal/frontal planes were mounted on silane-coated slides.

Probes and in situ hybridization

The following cDNAs were used to generate [α-35S]UTP sense and antisense riboprobes using either T3, T7 or SP6 RNA polymerases: 336 bp 5’ EcoRI-XbaI of murine Cbfa1 (Ducy et al., 1997) and a 600 bp 5’ PvuII-HindIII fragment of murine Cbfa1 (kindly provided by Dr Komori, Osaka University Medical School, Osaka, Japan); 321-bp 3’ EcoRI-HindIII mouse prot(1) collagen (Metsäranta et al., 1991) and a 500 bp 5’ NcoI-PvuII murine DSP (K). Genotyping was performed as previously described (Otto et al., 1997). Tissues were fixed in 10% NBF overnight at 4°C and then processed for paraffin wax embedding. Serial sections prepared in the frontal plane were stained with hematoxylin and eosin. Comparisons were made with Cbfa1+/− and Cbfa1+/+ littermates.

Histologic examination of dentition in Osf2/Cbfa1−/− mice

Heads from Cbfa1−/− mice at E16.5 and neonatal stages were obtained from intercrosses of heterozygous breeding pairs in our laboratory (G. K.). Genotyping was performed as previously described (Otto et al., 1997). Tissues were fixed in 10% NBF overnight at 4°C and then processed for paraffin wax embedding. Serial sections prepared in the frontal plane were stained with hematoxylin and eosin. Comparisons were made with Cbfa1+/− and Cbfa1+/+ littermates.

Mandibular arch and tooth epithelial-mesenchymal recombinants

Mandibular processes were dissected from Ell (CBA × NMRI) F1 embryos. They were cultured as whole explants or the epithelium was separated from mesenchyme and then recombined as described by Vainio et al. (1993). When E14 molar mesenchyme was separated...
from epithelium it consisted of the papilla and follicle mesenchyme. For E14 experiments, the papilla was carefully dissected free from the follicle and surrounding mesenchyme. At earlier stages, it was not possible to cleanly dissect the dental papilla from follicle mesenchyme.

Beads were implanted on mesenchyme that was cultured on Nuclepore filters (pore size 0.1 μm; Costar, Pleasanton, CA) using conditions described previously (Vainio et al., 1993). Tissues were treated with 100% methanol for 1-2 minutes and fixed in 4% paraformaldehyde overnight for whole-mount in situ hybridization. Some whole mounts were embedded in gelatin/albumin and sectioned at 200 μm with a vibratome.

**Recombinant proteins and bead implantation assays**

Heparin acrylic beads (Sigma H-5263) were used with FGF-4 and FGF-8 peptide (R&D Systems, Abingdon, Oxon, UK) and Affi-gel blue (BioRad) beads with recombinant BMP-2, BMP-4 and BMP-7 proteins (kind gift from J. Wozney, Genetics Institute, MA, USA). Beads were counted (100 beads/tube), washed and pelleted by centrifugation and then incubated 45 minutes at 37°C with FGF-4 or FGF-8 (25 ng/μl), BMP-2, BMP-4 and BMP-7 (100 ng/μl) or bovine serum albumin (BSA, 1 μg/μl). Beads were placed on explants using a capillary pipette. Whole-mount in situ hybridization was done as described by Henrique et al. (1995) and modified by Jernvall et al. (1998) using probe concentrations of 0.5 μg/ml. The tissues were treated with proteinase K at 10 μg/ml at 37°C for 7 to 20 minutes.

**RESULTS**

**Cbfa1 is temporally and spatially regulated during craniofacial development**

RT-PCR analyses showed Cbfa1 transcripts of the expected size in E15 mineralizing tissues, expression being most dominant in calvaria (Fig. 1A). Relatively high levels of Cbfa1 expression were evident in first molars, long bone and calvaria at E18 and at the neonatal stage. At 21 dpn (days of postnatal development), lower Cbfa1 expression was observed in first molar and long bone when compared to the second molar and calvaria. Southern blots of select RT-PCR products (Ducy et al., 1997) confirmed the presence of the gene in all mineralizing organs, studied. No Cbfa1 signal was detected in an E15 liver sample (data not shown).

In situ hybridization analysis revealed no Cbfa1 expression in any craniofacial tissues at E11 (data not shown). Cbfa1 expression became evident at E12 in areas of future osteogenesis (Fig. 2A,B). In contrast, non-mineralizing tissues including Meckel’s cartilage, muscle, epithelium and nerve were negative. Higher magnification views revealed Cbfa1 in the mesenchyme near the invaginating dental epithelium that was contiguous with that in preosteogenic mesenchyme. Analysis of serial sections revealed fewer Cbfa1 transcripts in mesenchyme nearest the tooth epithelial-mesenchymal interface (Fig. 2B).

At E13, the Cbfa1 expression had enlarged to include the preosteogenic mesenchyme surrounding the nasal capsule (Fig. 2C). Intense hybridization was observed in mesenchyme contacting the dental epithelium at the tip of the tooth bud (Fig. 2D). At the cap stage (E14), high levels of Cbfa1 expression were evident in dental papilla and follicle as well as in osteogenic zones clearly demarcating these regions from surrounding tissues (Fig. 2E). The mesenchyme between the tooth germ and the oral epithelium was consistently negative for Cbfa1 expression. By E16, this region showed positive hybridization with the Cbfa1 riboprobe (Fig. 2G). At this early bell stage, Cbfa1 expression was markedly downregulated in cuspal regions of the dental papilla mesenchyme (Fig. 2G). Higher levels of Cbfa1 expression appeared in a distal section of the apical area of the dental papilla between the cervical loop epithelia (Fig. 2H). At the late bell stage (E18), Cbfa1 expression was downregulated throughout the dental papilla. In contrast, intense hybridization remained in the dental follicle and in osteoblasts within alveolar bone (Fig. 2I).

**Cbfa1 expression is downregulated in differentiated odontoblasts and upregulated in maturational ameloblasts**

Fig. 3 depicts the expression profile of Cbfa1 mRNA in early postnatal life. At the newborn stage (NB; Fig. 3A) and at 4 dpn (Fig. 3B) Cbfa1 expression was intense in all osteoblasts and in the dental follicle that will give rise to cementoblasts and periodontal ligament (Fig. 3B). Newly differentiated odontoblasts and dental pulp cells near the cervical loop showed weaker levels of Cbfa1 expression while mature odontoblasts were negative. Epithelium remained negative including the stellate reticulum and secretory ameloblasts (Fig. 3B). At 7 dpn, high levels of Cbfa1 expression remained in the dental follicle (Fig. 3D). Unlike secretory ameloblasts that appeared negative, maturational ameloblasts appeared strongly positive for Cbfa1 as seen in Fig. 3C (box E), Fig. 3E and in a section through an incisor at 2 dpn (Fig. 3F).

Cbfa1 expression was downregulated in differentiated
odontoblasts prior to the expression of dentin matrix genes. As indicated in Fig. 3G, dentin sialophosphoprotein (Dsp) was expressed transiently in preameloblasts and in newly differentiated odontoblasts but not in osteoblasts at the newborn stage. At 2 dpn, \( \alpha \)d(l) collagen was expressed in differentiated odontoblasts and osteoblasts (Fig. 3H). Thus, the developmental profiles of \( \text{Cbfa1} \) expression in odontoblasts and osteoblasts appear different suggesting that the gene may be differentially regulated in these cells.

\( \text{Cbfa1} \) is required for normal tooth development

Fig. 4 illustrates our phenotypic analysis of tooth development in E16.5 and newborn \( \text{Cbfa1}^-/- \) mice. At E16.5, first molars in a \( \text{Cbfa1}^{+/+} \) littermate appeared normal and showed cuspal morphogenesis (mandibular molar; Fig. 4A,B). E16.5 \( \text{Cbfa1}^-/- \) molars were significantly smaller with poor differentiation of the enamel organ that had only advanced to the cap stage. As previously reported, the absence of \( \text{Cbfa1} \) resulted in a complete lack of osteoblast differentiation and bone formation (Fig. 4C,D). Similar differences existed between \( \text{Cbfa1}^{+/+} \) (Fig. 4E) and \( \text{Cbfa1}^-/- \) incisors, the latter appearing markedly hypoplastic and developmentally delayed (Fig. 4F). Interestingly, tissue separation at the epithelial-mesenchymal interface was seen in all \( \text{Cbfa1}^-/- \) sections suggesting that the integrity of this region was compromised in the absence of \( \text{Cbfa1} \) (Fig. 4C,D,F).

At birth, \( \text{Cbfa1}^{+/+} \) first molars were at the late bell stage (Fig. 4G). In contrast, \( \text{Cbfa1}^-/- \) molar organs had only progressed up to the cap/early bell stage. The defective enamel organ showed poorly delineated cuspal outlines. Although the dental papilla had formed, no overt differentiation of odontoblasts was observed (Fig. 4H). In a neonate wild-type incisor, well differentiated ameloblasts and odontoblasts and an organized layer of predentin and dentin were visible. In contrast, \( \text{Cbfa1}^-/- \) incisor organs were hypoplastic with poorly differentiated cells and a highly disorganized layer of an osteodentin-like matrix (Fig. 4I).

These results indicate that the absence of \( \text{Cbfa1} \) affected both molar and incisor development suggesting a non-redundant role for \( \text{Cbfa1} \) in tooth organogenesis. It is likely that differences in the severity of the molar and incisor phenotype are due to the earlier developmental schedule of incisors.

The onset and maintenance of \( \text{Cbfa1} \) expression in mandibular osteogenic mesenchyme at E11 is not dependent on epithelial signals

There is evidence that bone formation in the embryonic facial processes is dependent on signals from the epithelium (Hall, 1978). In order to analyze whether the onset of \( \text{Cbfa1} \) expression in mesenchyme requires the presence of epithelium, we cultured E11 mandibular arches as explants in the presence and absence of epithelium. \( \text{Cbfa1} \) expression was not seen in...
the mandibular arches at this stage in vivo. When mandibles were cultured as whole explants for 24 hours, intense Cbfa1 expression was seen in the lateral aspects of the arch (Fig. 5A). The expression domain was significantly extended after two days, and the molar and incisor regions appeared less intense (Fig. 5B). When E11 mandibular mesenchyme was cultured without epithelium for 24 hours, Cbfa1 expression was apparent in both halves of the mandible (Fig. 5C). This indicates that the mesenchyme had been committed to Cbfa1 expression prior to E11 and that epithelium was not needed for the induction or maintenance of Cbfa1 expression in mesenchyme.

The dental epithelium is capable of stimulating Cbfa1 expression in dental mesenchyme during the bud stage

To specifically address whether odontogenic epithelium could stimulate the induction of Cbfa1 expression in dental mesenchyme, we separated the presumptive dental epithelium from mesenchyme at E11, prior to the appearance of Cbfa1. Tissues were then cultured in recombination for 24 hours. In E11 presumptive molar mesenchyme cultured without epithelium, a patch of Cbfa1 expression likely representing osteogenic mesenchyme was visible (Fig. 5D). When recombined with E11 epithelium, a translucent zone appeared in the mesenchyme contacting the epithelium indicating that interactions between the tissues had occurred (Fig. 5E). However, whole-mount hybridization showed an absence of Cbfa1 in this region of mesenchyme in all 21 explants studied (Fig. 5F). Similar results were observed in twelve recombinant cultures of E12 dental epithelium and mesenchyme (data not shown). In E13 recombinants, a clear induction of Cbfa1 was evident throughout the translucent mesenchyme in two out of ten explants (Fig. 5G,H). In five explants, expression was seen in part of the translucent zone (Fig. 5I) while three explants showed no expression (data not shown). Patches of Cbfa1 expression were seen in E11, E12 (not shown) and E13 mesenchyme when cultured alone as well as in the recombinants, in areas not correlating with the location of the epithelium. These presumably represent osteogenic mesenchyme.

At E14, the cap stage, Cbfa1 expression was intense throughout the dental papilla and surrounding follicle and osteogenic mesenchyme (see Fig. 2E and F). When the entire dental mesenchyme (papilla and surrounding mesenchyme) was cultured in isolation, Cbfa1 expression was downregulated in most of the mesenchyme but distinct patches of expression remained (Fig. 5J). When dental papillae were cleanly dissected from the mesenchyme and then cultured in isolation, Cbfa1 was absent in six of the seven explants (Fig. 5K). These results correlate with results of the expression analysis that show Cbfa1 expression in follicle and osteogenic mesenchyme but not within the dental papilla (see Fig. 2G-I). The culture of dental epithelium in contact with dental papilla mesenchyme stimulated Cbfa1 expression in single and double recombinants where two pieces of epithelia were sandwiched between two mesenchymes (Fig. 5L,M respectively). Vibratome sections showed that expression had been induced in the mesenchyme immediately underlying the epithelium (Fig. 5N). None of the control explants showed positive hybridization with Cbfa1 sense probes (Fig. 5O).

Interestingly, in mandibular arch and tooth recombinant cultures, we observed that the presence of epithelium favored the size of the mesenchyme that appeared larger than when cultured without epithelium (Fig. 5). This suggests that the dental epithelium had a positive stimulatory effect on mesenchymal survival.

Fig. 3. Cbfa1 expression in tooth organs during postnatal development.

(A) A mandible of a newborn mouse showing high levels of Cbfa1 mRNA within bone and dental follicle. Although dental papilla is negative, Cbfa1 expression is pronounced in the apical region of the tooth organ. (B) During matrix formation at 4 dpn, Cbfa1 remains highly expressed in dental follicle and surrounding bone. Secretory odontoblasts and ameloblasts do not express Cbfa1 mRNA. (C) At 7 dpn, Cbfa1 transcripts persist in dental follicle and surrounding bone (boxed areas in C shown in higher magnification in D and E). Interestingly, hybridization signal is visible in a restricted population of ameloblasts (am, long arrow in E) in the maturation stage of amelogenesis. Ameloblasts lateral to this zone (short arrow in E) that have presumably not entered the maturative stage appear negative. (F) In a 2 dpn incisor Cbfa1 is seen in a comparable zone of mature ameloblasts. The downregulation of Cbfa1 in odontoblasts (see A) coincides with the expression of Dsp, an odontoblast-specific marker. (G) Transient expression of Dsp is seen in a group of pre-ameloblasts (am). (H) In contrast to Cbfa1, type I collagen is expressed in all odontoblasts and osteoblasts at 2 dpn. df, dental follicle; inc, incisor; mb, mandibular bone; am, secretory ameloblasts; od, odontoblasts; e, enamel; d, dentin. Scale bar: (A,H) 500 µm; (B,C,G) 250 µm; (D,E,F) 100 µm.
Cbfa1 expression in dental mesenchyme is stimulated by FGFs but not by BMPs

At E11, the dental epithelium expresses Fgf-8 as well as Bmp-2 and Bmp-7 in vivo and these signaling molecules regulate a number of genes in dental mesenchyme (review: Thesleff and Sharpe, 1997). Since the E11 epithelium did not stimulate Cbfa1 expression in recombinant cultures, it was unlikely that these signal proteins would have stimulatory effects. Indeed, no stimulation of Cbfa1 expression in E11 mesenchyme was seen with beads releasing BMP-2 and BMP-7 in the twelve (BMP-2) and six (BMP-7) explants analyzed (Fig. 6A and B respectively). BMP-4 beads also did not stimulate Cbfa1 expression in six explants (data not shown). The activities of BMP-2 and BMP-7 were confirmed by the earlier well documented induction of Msx1 expression in E11 mesenchyme (Fig. 6C,D; Vainio et al., 1993).

A total of twelve (BMP-2), six (BMP-4) and six (BMP-7) E14 mesenchyme explants were analyzed. At this developmental stage (E14) in vivo, all three Bmps are expressed in the dental epithelium (Vaahtokari et al., 1996). Interestingly, BMP-2, BMP-4 and BMP-7 releasing beads consistently failed to stimulate Cbfa1 expression in dental mesenchyme (Fig. 6E-G) indicating that they are not required for the maintenance of Cbfa1 expression. Controls with BSA-soaked beads were completely negative (Fig. 6H).

Beads soaked with FGF-8 failed to stimulate Cbfa1 expression in any of the seven E11 mesenchymes analyzed (Fig. 7A). FGF-4 beads also failed to elicit a clear zone of Cbfa1 expression in any of the 24 cultures of E12 mesenchyme studied (Fig. 7C). In most cultures, Cbfa1-positive patches were seen in explants with FGF-4, FGF-8 as well as control BSA beads, but the analysis of a large number of explants indicated that these patches of Cbfa1 expression near the beads were not induced by FGF. Rather they represent Cbfa1-expressing cells in areas of contaminating osteogenic mesenchyme (Fig. 7A-D).

Interestingly, in E13 dental mesenchyme, i.e. the stage when dental epithelium was first shown to stimulate Cbfa1 expression, a clear zone of Cbfa1 induction was observed around the FGF-4 beads in 17 out of 22 explants (Fig. 7E). The control BSA beads had no observable effects (Fig. 7F). At E14, FGF-4 had a clear stimulatory effect on Cbfa1 expression in all ten explants analyzed (Fig. 7G,H). In explants which included surrounding mesenchymal tissue, patches of Cbfa1 expression were observed in the periphery (Fig. 7G). FGF-8 had a similar stimulatory effect as FGF-4 in 5 out of the 6 explants studied (data not shown).

Hence, the results from the bead assays are consistent with those from epithelial-mesenchymal tissue recombinations and indicate that epithelial signals do not induce Cbfa1 expression in the dental mesenchyme prior to E13. Therefore, FGFs are needed for the stimulation of expression in the dental papilla.
DISCUSSION

Our results demonstrate that \textit{Cbfa1} plays an important role in tooth development, in particular, epithelial morphogenesis. Furthermore, \textit{Cbfa1} may indirectly influence tooth cytodifferentiation and function through its control of tooth morphogenesis. Our tissue recombination and bead treatment experiments further support these findings.

Fig. 5. Effects of epithelium on the initiation of \textit{Cbfa1} expression in E11 whole mandibular arch explants and in E11-E14 tooth explants. Gene expression in each explant was monitored by whole-mount in situ hybridization. \textit{Cbfa1} was not detected at the onset of culture (not shown). (A,B) E11 intact mandibular arch cultured for 24 and 48 hours respectively show expression of \textit{Cbfa1} in the osteogenic areas. After 48 hours (B) expression had extended fully into the osteogenic area that has assumed the shape of the mandibular arch. Tooth bearing areas show fainter hybridization (arrows). (C) E11 mandibular arch mesenchyme cultured without epithelium for 24 hours shows \textit{Cbfa1} expression, although the arch is reduced in size as compared to A and B. D-O show the effects of dental epithelium on the induction of \textit{Cbfa1} expression in tooth mesenchyme. Epithelial and mesenchymal tissues from first molar organs were separated and then recombined in culture for 24 hours. \textit{Cbfa1} expression was analyzed by whole mount in situ hybridization. (D) In E11 molar mesenchyme a patch of \textit{Cbfa1} expression was seen which presumably represents osteogenic mesenchyme that was included in the dissection. (E) E11 epithelial-mesenchymal recombinant shows a translucent zone of epithelial induction in mesenchyme (arrow). (F) Whole-mount in situ hybridization shows an absence of \textit{Cbfa1} expression within the translucent zone (arrow). (G) In an E13 epithelial-mesenchymal recombinant, a well defined translucent zone of induction (arrow) is seen nearest the epithelial interface. (H) \textit{Cbfa1} expression (arrow) is clearly localized to the translucent zone in the explant in G. (I) A similar explant as in H showing induction of \textit{Cbfa1} expression (arrow) only in a restricted location within the translucent zone. (J) \textit{Cbfa1} expression in the periphery of cultured E14 mesenchyme (representing follicle and osteogenic mesenchyme) but not in dental papilla mesenchyme. (K) E14 papilla mesenchyme without any follicle shows an absence of \textit{Cbfa1} expression. (L) E14 recombinant culture showing stimulation of \textit{Cbfa1} expression in mesenchyme adjacent to epithelium. (M) E14, \textit{Cbfa1} expression in two dental papilla mesenchymes recombined with two epithelia. (N) A vibratome section (along the plane indicated in M) shows \textit{Cbfa1} expression in mesenchyme surrounding epithelia. (O) E14 papilla mesenchyme treated with a \textit{Cbfa1} sense riboprobe showing the absence of hybridization. m, mesenchyme alone; e+m, recombined epithelium and mesenchyme. +1 = 24 hours; +2 = 48 hours. Scale bar: 450 µm.

from E13 onwards when epithelial morphogenesis advances from the bud to the early bell stage.
implantation assays show that *Cbfa1* expression in dental mesenchyme is stimulated by epithelial signals which include FGF family members but not the BMPs.

**Cbfa1 is a mesenchymally expressed gene regulating epithelial morphogenesis**

The pattern of expression of *Cbfa1* in dental mesenchyme correlates with key developmental events. As tissue recombinations have demonstrated, tooth development is initiated by odontogenic epithelium and the inductive potential is transferred from the epithelium to mesenchyme by E12 (Mina and Kollar, 1987; Lumsden, 1988; review: Thesleff and Nieminen, 1996). The onset of *Cbfa1* expression in dental papilla mesenchyme follows its acquisition of odontogenic potential. Furthermore, the gene is downregulated in the dental papilla at the early bell stage, E16, when epithelial morphogenesis is advanced and cuspal development is underway. Thus, from E13 to E16, *Cbfa1* is apparently needed for progress of tooth development from the bud to the bell stage during which time there is rapid growth and folding of the enamel organ epithelium.

The expression of *Cbfa1* in the dental mesenchyme is unique when compared to other genes like *Msx1*, *Bmp-4* and *Pax9* (see the www database at http://honeybee.helsinki.fi/toothexp, 1999). These regulatory molecules are induced by the early dental epithelium (E10-11) and are associated with the acquisition of odontogenic potential in the mesenchyme. Furthermore, they are expressed within the translucent zone underlying the epithelium in tissue recombination cultures. Here we showed that *Cbfa1* in dental mesenchyme was not induced by E11 odontogenic epithelium and that it was not expressed in the translucent zone.

A different function for *Cbfa1* is also indicated by the unique tooth phenotype in *Cbfa1*-deficient mice. *Cbfa1-/-* molars developed beyond the bud stage unlike those of mice with targeted inactivations of the *Msx1*, *Pax9*, *Lef1* and *activin-βA* genes which arrested at the bud stage (Satokata and Maas, 1994; van Genderen et al., 1994; Matzuk et al., 1995; Peters et al., 1998). *Dlx1/2* compound mutants arrest at the lamina phase in maxillary molar development while in *Gli 2/3* and *Msx1/2* all teeth are arrested at the lamina stage (Qiu et al., 1997; Hardcastle et al., 1998; Bei and Maas, 1998 respectively). *Cbfa1-/-* molars were severely hypoplastic and misshapen but underwent epithelial morphogenesis to reach an aberrant bell stage at birth. Newborn mutant incisors that develop earlier showed abnormal odontoblasts, dentin formation and lacked enamel. Thus, it is likely that *Cbfa1-/-* molar organs would have progressed beyond the bell stage if pups had survived past birth. In extrapolating findings from our expression analysis to our interpretation of the *Cbfa1-/-* tooth phenotype, it is reasonable to speculate that the defect (delay) in molar odontogenesis was initiated earlier in development, most likely at E13.

It is possible that *Cbfa1* affects advancing tooth morphogenesis by acting upstream of diffusable signaling molecules within the mesenchyme, such as the BMPs, that in turn regulate gene expression within the enamel organ (Jernvall et al., 1998). Furthermore, *Cbfa1* may independently affect the expression of mesenchymal genes involved in the formation or degradation of basement membrane or other extracellular matrix components. The lack of a stable epithelial mesenchymal interface has been shown to affect proper tooth morphogenesis (Ruch et al., 1983) and could account for the abnormal molar enamel organs seen in *Cbfa1-/-* mice.

Our expression analysis also revealed that *Cbfa1* is initially restricted to the buccal aspect of the maxillary and mandibular arches (see Figs. 2A,C). This may be significant, since in biphyodont mammalian dentition, succedaneous tooth organs develop as a lingual extension of the dental lamina. Our expression data together with the finding of supernumerary teeth in CCD patients (Jones, 1997) may reflect *Cbfa1’s* potential role as a negative regulator of dental lamina outgrowth.

**Role of *Cbfa1* in the differentiation of mineralized tooth matrix-producing cells**

Because *Cbfa1* is critical for osteoblast differentiation, we explored its expression during odontoblast differentiation. The marked downregulation in differentiated odontoblasts contrasted with the high levels of *Cbfa1* expression in osteoblasts throughout craniofacial ossification. Our phenotypic analyses in newborn *Cbfa1* mutants revealed the presence of poorly differentiated odontoblasts and a highly

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**Fig. 7.** The FGFs stimulate *Cbfa1* expression in dental papilla mesenchyme during advancing epithelial morphogenesis. (A) FGF-8 does not stimulate *Cbfa1* expression in E11 dental mesenchyme. (B) E11, control BSA bead; (C) E12, absence of *Cbfa1* induction around FGF-4 bead. (D) E12, control BSA bead. (E) E13, clear stimulation of *Cbfa1* expression around FGF-4 bead. (F) E13, control BSA bead. (G) E14, strong induction of *Cbfa1* around FGF-4 bead in dental mesenchyme. Note expression in peripheral cells from surrounding mesenchyme. (H) E14, dental papilla mesenchyme showing stimulation of *Cbfa1* expression around FGF-4 bead. In most explants treated with either FGF or BSA, patches of hybridization were visible that correspond to endogenous *Cbfa1* expression in osteogenic mesenchyme. Scale bar: 450 μm.
disorganized dentin matrix (in incisors alone). How Cbfa1 at the molecular level affects odontoblast differentiation has yet to be elucidated. One possibility is that inductive signals are turned on in dental epithelium by a Cbfa1-dependent pathway and that these molecules then act back upon the mesenchyme to stimulate odontoblast differentiation. Alternatively, Cbfa1 may regulate the expression of other inductive molecules in the mesenchyme that directly influence odontoblast differentiation.

The finding of Cbfa1 transcripts in maturative phase ameloblasts is the first demonstration of the presence of this transcription factor in ectodermal cells involved in mineralization. It is possible that Cbfa1 affects enamel maturation and/or the fate of ameloblasts which is believed to be one of programmed cell death. Our analysis of neonate mutant incisors showed that ameloblasts whose differentiation is dependent on the presence of preodontin failed to develop. This implies that the osteodentin-like matrix seen in Cbfa1 mutant incisors was defective. That the absence of Cbfa1 compromised the differentiation of odontoblasts and ameloblasts was not surprising since in tooth development cytodifferentiation is dependent on morphogenesis.

The expression of Cbfa1 by the dental follicle through late odontogenesis suggests that the gene may be involved with cementum formation. Our tissue recombination experiments suggest that the regulation of Cbfa1 expression in dental follicle may be different from that in dental papilla. It is possible therefore, that Cbfa1’s function in cementogenesis is similar to its role in osteogenesis and may differ from its potential role in dentin formation. This is an important observation because it indicates that the mechanisms regulating the differentiation and function of tooth mineralized tissue cells (odontoblasts and ameloblasts) may differ from that of osteoblasts.

**Cbfa1 expression in dental mesenchyme is stimulated by epithelial-mesenchymal interactions and by the FGFs**

Our tissue recombination studies with whole E11 mandibular arches showed that the initiation and maintenance of Cbfa1 expression in osteogenic mesenchyme did not require epithelium at this stage. The cultures of isolated mesenchyme from the tooth region in E11 to E13 embryos in which patches of Cbfa1 expression were frequently seen also supported the conclusion that the maintenance of Cbfa1 in the osteogenic mesenchyme does not depend on epithelial signals. It is possible that epithelial signals regulate the initiation of Cbfa1 expression and subsequent osteogenesis at an earlier stage, as has been suggested previously in cranial bones (Hall, 1978). We have in fact observed that in E10 mandibular explants, epithelium is required for the onset of Cbfa1 expression in osteogenic mesenchyme (unpublished observations). However, as the epithelium significantly enhanced the growth of the mandibular mesenchyme in culture, these positive effects of the epithelium on Cbfa1 expression and bone formation may relate to non-specific trophic effects permitting the advancing differentiation of predetermined osteogenic mesenchyme. Hence, our studies do not allow definite conclusions to be made concerning the requirement of epithelium for the initiation of Cbfa1 expression and bone development for the mandibular arch.

Earlier studies indicate that E11 dental epithelium stimulates the expression of numerous developmental regulatory genes in the mesenchyme (Thesleff and Sharpe, 1997) and that these stimulatory effects can be mimicked by signaling molecules including FGF-8 and BMP-2, BMP-4 and BMP-7. Our tissue recombinations demonstrate that dental epithelium of E11 and E12 embryos did not induce Cbfa1 in the underlying mesenchyme. From our bead implantation assays we conclude that neither BMPs nor FGFs stimulated Cbfa1 expression in E11 and E12 mesenchyme, while the BMPs did induce Msx1 expression (Vainio et al., 1993). These experiments as well as the unique expression pattern of Cbfa1 indicate that it is not involved in the transfer of inductive potential from epithelium to mesenchyme. Rather we suggest that Cbfa1 is an important regulatory molecule in the subsequent interactions between the dental epithelium and mesenchyme which regulate epithelial morphogenesis and thus the shape of the tooth.

Our data indicate that after the initiation of odontogenesis, epithelium had a clear stimulatory effect on Cbfa1 expression in dental mesenchyme (see Fig. 5). This is consistent with our expression analyses that showed Cbfa1 expression in E13 dental mesenchyme under the epithelial bud and at the cap stage (E14), when expression was intense throughout the dental papilla. Our bead assays showed that the epithelial induction of Cbfa1 expression in E13 and E14 dental mesenchyme was mimicked by FGFs whereas the BMPs had no inductive effects. Interestingly, at E13, FGF beads induced Cbfa1 expression in most explants whereas the epithelium had a clear stimulatory effect in only two cultures and a weak effect in five out of ten explants. This is consistent with the fact that the expression of Fgf-4 and Fgf-9 appear at E13 at the tip of the epithelial tooth bud in vivo, coincident with the start of Cbfa1 expression in the subjacent underlying dental mesenchyme. These Fgfs are expressed through the cap stage in the epithelial enamel knot, a structure expressing several other signaling molecules and believed to act as a signaling center regulating tooth crown formation (Jernvall et al., 1998), and in E14 mesenchyme the epithelium as well as FGFs stimulated Cbfa1 expression in all explants. The enamel knot curiously disappears at a time coincident with the down regulation of Cbfa1 in dental papilla. Based on our experiments we conclude that dental epithelium and FGFs stimulate the expression of Cbfa1 in E13 and E14 dental papilla mesenchyme. We cannot conclusively distinguish from these studies whether this stimulation reflects the induction of de novo expression of Cbfa1 or whether it represents the maintenance of existing Cbfa1 expression.

In conclusion, these studies suggest that Cbfa1 plays a unique and essential role in tooth development. Cbfa1’s expression in dental mesenchyme is controlled by signals emanating from the dental epithelium. In turn, Cbfa1 regulates the expression of mesenchymal molecules that act reciprocally on epithelium to control the histogenesis and morpho-differentiation of the enamel organ. Thus, Cbfa1 may indirectly influence odontoblast and ameloblast differentiation by regulating genes that control the terminal differentiation of these cell types. In light of these data we interpret the abnormalities in tooth formation seen in Cbfa1-/- mice as a demonstration of its distinct role in tooth development that is not indirectly linked to the absence of osteogenesis. The molecular mechanisms underlying Cbfa1’s role/s in tooth development may thus differ
from its function in bone formation and offers new insights into the process of cell differentiation in mineralized tissues.

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REFERENCES


