Molecular Regulation of Craniofacial Bone and Palate Development

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Academic Dissertation

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ORIGINAL PUBLICATIONS I-III

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are also presented.

I Progression of calvarial bone development requires Foxc1 regulation of *Msx2* and *Alx4*. (2003) **Ritva Rice**, David P.C. Rice, Bjorn R. Olsen, and Irma Thesleff. *Developmental Biology*. 262:75-87.

II *Fgfr* mRNA Isoforms in Craniofacial Bone Development. (2003) David P.C. Rice, **Ritva Rice**, and Irma Thesleff. *Bone* 33:14-27.

III Disruption of *Fgf10/Fgfr2b* coordinated epithelial-mesenchymal interactions causes cleft palate. **Ritva Rice**, Bradley Spencer-Dene, Elaine C. Connor, Amel Gritli-Linde, Andrew P. McMahon, Clive Dickson, Irma Thesleff, and David P.C. Rice. Submitted to J. Clin. Inv.

ABBREVIATIONS

Ach	Achondroplasia
AER	Apical ectodermal ridge
Alx	Vertebrate homologue of *Drosophila* Aristaless gene
Alp	Alkaline phosphatase
Apaf	Apoptotic protease-activating factor
Bax	Pro-apoptotic gene
Bcl	Anti-apoptotic gene
Bey	Bulgy-eye mouse mutant
Bmp	Bone morphogenetic protein
Bmpr	Bone morphogenetic protein receptor
BSA	Bovine serum albumin
Bsp	Bone sialoprotein
BrdU	5'-bromo-2'-deoxyuridine
ch	congenital hydrocephalus
CNC	Cranial neural crest
Col I	Type I collagen
Col II	Type II collagen
Cre	Cre recombinase
dCTP	deoxycytidine-5'- triphosphate
Dlx	Vertebrate homologue of *Drosophila* distal less gene
DNA	Deoxyribonucleic acid
E	Embryonic day
Egf	Epidermal growth factor
FCS	Fetal calf serum
Fgf	Fibroblast growth factor
Fgfr	Fibroblast growth factor receptor
Fox	Forkhead box
Id	Inhibitors of differentiation
Ihh	Indian hedgehog
Hh	Hedgehog
Hox	Homeodomain box
Ht-PA	Human tissue plasminogen activator
MEE	Medial edge epithelium
MMP-2	Matrix metalloproteinase-2
Msx	Vertebrate homologue of *Drosophila* muscle segment (*Msh*) gene
MT-MMP	Membrane type matrix metalloproteinase
OB1	mouse osteoblastic cell line
Oc	Osteocalcin
Op	Osteopontin
P	Postnatal day
PCR	Polymerase chain reaction
PF	Posterior frontal
PFA	Paraformaldehyde
PKC	Protein kinase C
Ptc	Patched
PTHrP	Parathyroid hormone related peptide
RA	Retinoic acid
Runx	Runt-domain containing gene
SADDAN	Severe achondroplasia with developmental delay and acanthosis nigricans
Shh	Sonic hedgehog
Smo	Smoothened
Stat	Signal transducer and activator of transcription
Tbx	T-box gene
Tgfβ	Transforming growth factor beta
Tgfbr2	Transforming growth factor beta receptor type II
TTP	Thymidine-5'-triphosphate
TUNEL	Terminal deoxynucleotidyl transferase mediated nick end labelling
UTP	Uridine-5'-triphosphate
Wnt	Vertebrate homologue of *Drosophila* Wingless gene

Genes are stated in italics, and proteins in roman. Mouse genes/proteins are in lower case letters. Human genes/proteins are in upper case letters unless otherwise stated.

ABSTRACT

The development of cranial bones and palate are complex processes where the development of multiple elements needs to be coordinated spatiotemporally to produce a working unit such as calvarium, cranial base, or palate. I have shown here that a common theme in the early development of craniofacial structures is balancing the level of proliferation and differentiation of progenitor cell populations by growth factor signalling so that large enough population of progenitor cells is formed before differentiation, or that proliferation is maintained for the outgrowth of the element.

Here I show that a reduced proliferation of osteoprogenitor cells in mice lacking a forkhead/winged helix transcription factor Foxc1 prevents the outgrowth of calvarial bones. I further demonstrate that Foxc1 is required for bone morphogenetic protein (Bmp) regulation of *Msx2* and *Alx4* to maintain this proliferation.

Fibroblast growth factor (Fgf) signalling also plays an important role in regulating the proliferation of progenitor cell populations during craniofacial development as shown here by the expression patterns of different Fgf receptor isoforms in the proliferative osteoprogenitor cells in calvarial sutures, and in the proliferative chondrocytes in the cranial base synchondroses and mandibular condyles. Furthermore, I show that reiterative Fgf signalling between palatal shelf epithelium and mesenchyme is essential for maintaining proliferation and cell survival in these tissues to ensure outgrowth and morphogenesis of the palatal shelves.

INTRODUCTION

The hard tissues in the head, the bones, cartilages, and teeth, play many essential roles for our survival. Calvarial and cranial base bones protect the brain and sense organs from external shocks. Three small bones in the inner ear are needed for us to hear. Jaws allow us to talk, and together with teeth they enable us to chew. Palate works as a barrier between mouth and nose, thus ensuring that we can eat and breathe at the same time.

The craniofacial skeleton differs from the axial and appendicular skeleton in many aspects. The craniofacial skeleton is derived from both cranial neural crest and paraxial mesoderm while the axial skeleton is of paraxial mesoderm origin. During development cranial bones use both endochondral and intramembranous ossification, but the only intramembranous bones in the axial skeleton are the medial parts of the clavicles (Huang et al., 1997). Also, secondary cartilages are not found in the axial skeleton. Sutures and synchondrosis are only part of the craniofacial skeleton. They function as fibrous joints between adjacent skeletal elements; sutures between intramembranous flat bones, and synchondrosis between cranial base endochondral bones. Calvarial and cranial base bones are unique in being in contact with the meninges covering the brain.

On a molecular level, an important difference between craniofacial and axial and appendicular skeleton is the Hox code. It is needed for the anterior-posterior patterning and positional identity of the axial and appendicular skeleton. But Hox genes are primarily not expressed in craniofacial regions forming skeletal elements (Whiting, 1997). Interestingly, Creuzet et al. (2002) showed that it is essential for the development of the facial skeleton to stay Hox negative. A reverse is true too: downregulation of Hox genes in a subset of trunk neural crest cells allows these cells to produce chondrocytes that they normally do not generate (Abzhanov et al., 2003). Hox genes contributing to the craniofacial skeleton are those expressed in the occipital somites and the 2nd branchial arch. Therefore, Hox code contributes to the posterior cranial base and lower face, respectively.

Despite its differences, craniofacial skeletal development requires the same set of basic developmental signals as other developing tissues. Studies in tooth, mandible, palate, and calvarium models have shed light onto the importance of epithelial-mesenchymal tissue interactions that guide the morphogenesis and growth of many tissues. The bone morphogenetic (Bmp), fibroblast growth factor (Fgf), Wingless (Wnt), and hedgehog (Hh) signalling pathways interact in the formation of a tooth (reviewed in Jernvall and Thesleff, 2000). Similarly, Bmp, Fgf, and Hh pathways have been shown to interact in the calvarial suture development to regulate ossification (Kim et al., 1998; Iseki et al., 1999; Rice et al., 2000). It seems that it is the spatiotemporal activation of a specific set of transcription factors by growth factor signalling molecules that determine the differentiation and growth of tissues.

REVIEW OF THE LITERATURE

The origins of craniofacial structures

The skeletal elements of the skull are derived from mesoderm and cranial neural crest (CNC). CNC cells originate from the neural epithelium in the neural folds. These cells undergo epithelial-to-mesenchymal transition, and migrate to their final destinations in the neck and craniofacial regions (Trainor and Nieto, 2003). In avian, quail-chick chimaeras have allowed detailed studies of the fate of CNC cells (Noden, 1983; Couly et al., 1993; Huang et al., 2000) These studies have demonstrated that the facial skeleton and anterior cranial base are entirely of cranial neural crest origin, and that the posterior cranial base skeleton is derived from paraxial and somitic mesoderm (Huang et al., 2000).

The origin of calvarium

The origin of calvarial tissue layers has remained debatable. In the developing chick, Noden (1986) showed that intramembranous skull bones, their periosteum and sutures are derived from paraxial mesoderm, and that the underlying dura mater is derived from CNC cells. On the other hand, Couly et al. (1992, 1993) showed that all tissues in the calvarium are from early migratory populations of cranial neural crest cells. Thus, we can safely say that at least dura mater is derived from the neural crest in the calvarium of the avian. In mouse, CNC cell destinations have been studied by histological analysis of early embryos and DiI-labeling experiments (Nichols 1981; Serbedzija et al., 1992; Osumi-Yamashita et al., 1994). The techniques used in these studies have allowed a detailed study of the migratory pathways of neural crest cells. Similarly to chick, mouse cranial neural crest cells populate the first, second, and third branchial arches, and the frontonasal mass. Therefore, CNC cells contribute to the facial structures also in the mouse. Recently, the creation of a transgenic mouse *Wnt1*-Cre/R26R, which carries a permanent neural crest cell lineage marker, allowed the analysis of the origin of mesenchymal tissues in the mouse cranium (Jiang et al., 2002). The authors showed that the alisphenoid, squamosal, and frontal bones of the calvarium, the metopic suture mesenchyme between the frontal bones, and the meninges underlying them are of cranial neural crest origin. They also showed that the sagittal suture mesenchyme between the parietal bones and the dura mater underneath these bones are of neural crest origin. The parietal bones themselves and the meninges covering the mid- and hindbrain are of mesodermal origin. The interparietal bone was shown to be of dual origin: the medial part of the bone is derived from the neural crest while the lateral parts are mesodermal. Thus, it seems that in mouse, calvarial tissue layers caudal to frontal bones arise from mesoderm with the exception of the meninges underneath the parietal bones. The disadvantage of using *Wnt1*-Cre/R26R mice is that *Wnt1* expression is not specific to neural crest cells but also to parts of the brain (Jiang et al., 2002). Pietri et al. (2003) have established a transgenic line, the human tissue plasminogen activator-Cre mouse (Ht-PA) that targets specifically neural crest cells that have undergone epithelial-mesenchymal transdifferentiation and that are beginning to migrate. The calvarium of Ht-PA mice was analyzed only prior to calvarial bone formation. Thus, at the moment we do not know whether results from the Ht-PA mice would be comparable with the results from *Wnt1*-Cre/R26R mice for the origin of calvarial tissue layers.

Importance of tissue origin

Does the tissue origin matter? It becomes important when deficiencies in neural crest cell formation, migration or proliferation could occur. For instance when the transcription factor *Dlx2*, which is expressed in the migratory and postmigratory neural crest cells, is "knocked out", the mutant embryos exhibit first and second arch defects (Qiu et al., 1995).

The proposal that the origin of the embryonic mesenchyme is relatively unimportant is supported by the finding that as neural crest cells reach their destinations they have become indistinguishable from cells of mesodermal origin (Serbedzija et al., 1992). Also, Schneider (1999) demonstrated that when midbrain level neural crest cells were transplanted into somitomeres 4, 5, and 6, the transplanted neural crest cells participated in the formation of the lateral wall of the brain case. The formed skeletal elements were morphologically indistinguishable from those of control embryos. Also, Trainor and Krumlauf (2000, 2001) have shown that mouse cranial neural crest cells have plasticity, and that they can be patterned by their environment. Ito et al. (2003) studied the role of transforming growth factor beta receptor type II (Tgfbr2) in cranial neural crest cell population under *Wnt1* promoter. Their results demonstrated that aberrant Tgfβ signalling results in abnormal development of the dura mater, and that this leads to calvarial defects irrespective of tissue origins. Thus, it seems that the susceptibility of the mesenchyme to appropriate inductive signals from surrounding tissues and the presence of the right inductive signals can be more important than the tissue origin of craniofacial skeletal elements.

Morphogenesis of craniofacial structures

The development of the craniofacial structures is a complex process of patterning and growth of different units arising from cranial neural crest and paraxial mesoderm. The skull can be divided into the **neurocranium**, which consists of the calvarium and the cranial base, and the **viscerocranium**, which consists of the face, palate, jaws, and teeth (Meikle, 2002; Wilkie and Morriss-Kay, 2001). During early embryonic development the viscerocranium arises from five different processes. These processes grow, meet and fuse. The **frontonasal process** contributes to the formation of the nose, upper incisors, and primary palate. **Two maxillary processes** become the secondary palate, the rest of the upper teeth, and maxilla. **Two mandibular processes** develop into the mandible and lower teeth. The development of the neurocranium occurs as formation of localized chondrification/ossification centres in head mesenchyme due to local and systemic inductive signals.

Development of the secondary palate

The mammalian **secondary palate** forms as bilateral shelves from maxillary processes (Fig. 1). In mouse, the mesenchymal cells within the maxillary processes proliferate and form the palatal shelf primordia, which start to grow downwards lateral to the tongue at E12. Two days later the palatal shelves have begun to elevate to a horizontal position above the tongue. By E14.5 the shelves have met at midline and begin to fuse to form a single continuous palate (Ferguson, 1988). The **palate epithelium** can be divided into three regions: the nasal, oral, and medial edge epithelium (MEE). The fusion of palatal shelves involves adhesion, migration, apoptosis, and transdifferentiation of MEE cells into mesenchymal cells (Cuervo et al., 2002; Ferguson, 1988; Griffith and Hay, 1992; Martinez-Alvarez et al., 2000; Shuler et al., 1991, 1992).

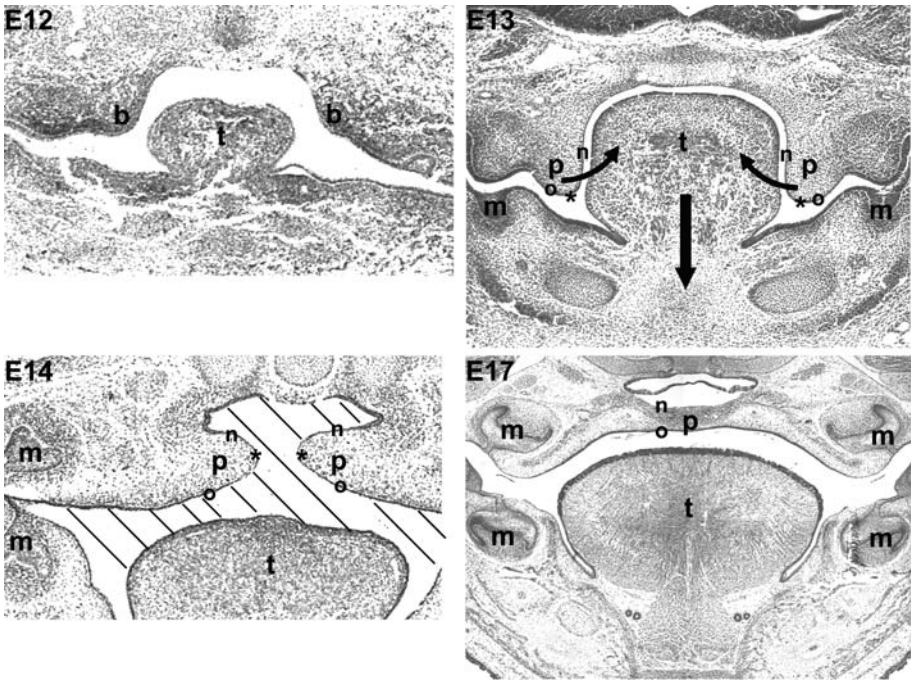

Figure 1. Palate development in mouse. Palatal shelves begin to develop as a bud (b) from maxillary processes at E12. They grow downwards next to the tongue (t), and by E13 palatal shelves (p) are visible. The epithelium surrounding the palatal shelf can be divided into oral (o), medial edge (*), and nasal (n) epithelium. At E13 the developing tongue depresses, and palatal shelves start to elevate. By E14 the palatal shelves have elevated. The hatched area indicates the continuous oral and nasal cavities before palatal shelves have met and fused. E17 shows a fused, continuous palate with separated oral and nasal cavities. m, molar tooth.

Molecular pathogenesis of cleft palate

A defect at any of the developmental stages can cause a cleft palate (Fig. 1b-c, k-l in Publication III). Cleft palate may also arise as a secondary defect. For instance, *Hoxa2* null allele mice exhibit a cleft palate due to abnormal attachment of the hyoglossus muscle to the greater horn of the hyoid; this does not allow depression of lateral edges of the tongue (Gendron-Maguire et al., 1993; Rijli et al., 1993; Barrow and Capecchi, 1999). Interestingly, deletion of *Hoxa1* alleles in *Hoxa2* null mutants restores the hyoid and tongue musculature connections, and rescues the cleft palate (Barrow and Capecchi, 1999). These studies demonstrate the importance of tongue posture in the elevation of palatal shelves. Furthermore, ectopic expression of trunk level *Hoxa7*, *Hoxb6*, or *Hoxb7*, or of posterior hindbrain level *Hoxd4*, in the anterior hindbrain level leads to a cleft palate. It is thought that the occipital and/or basisphenoid bone abnormalities in these transgenic mice interfere with the elevation of the palatal shelves (reviewed in Whiting, 1997).

Mutations in many genes have been shown to cause clefting of the secondary palate in mice (Table I). Most of these mouse mutants have multiple other craniofacial abnormalities, and the pathogenesis of the cleft palate has not been well studied. The only mouse mutant that exhibits a partial or complete cleft palate that is not associated with other craniofacial abnormalities is *transforming growth factor β3* (*TGFβ3*) null allele mice (Kaartinen et al., 1995; Proetzel et al., 1995). Kaartinen et al. (1997) later showed that *TGFβ3* null mutants lack the initial adhesive interactions between palatal shelves, and

that the basement membrane of MEE cells is not degraded. Taya et al. (1999) showed that the initial adhesive interactions are abnormal due to lack of proteoglycan-rich filopodial structures on the surface of the MEE. Thus, transdifferentiation of the MEE cells to mesenchyme, which requires the adhesion of opposing MEE (Griffith and Hay, 1992) and the proteolytic degradation of basement membrane in midline epithelial cells (Kaartinen et al., 1997; Blavier et al., 2001), is deficient in these mice. Finally, Martinez-Alvarez et al. (2000) have shown evidence that TGFβ3 also plays a role in inducing the apoptosis of MEE cells during palatal shelf fusion. Furthermore, Cecconi et al. (1998) showed that *Apaf1* mutant mice, which lack apoptosis due to disrupted caspase signalling, exhibit a cleft palate where palatal shelves develop and elevate normally, but fail to fuse.

Table I. Mouse mutants with cleft palate.

Mouse mutation	Defects	Molecular mechanism	References
Transcription factor			
Alx3-/-;Alx4-/-	Increased apoptosis in frontonasal mass, abnormal lateral position of nasal processes at E10.5		Beverdam et al., 2001
Alx4+/-;Cart1-/-, Alx4-/-;Cart1+/-, and Alx4-/-;Cart1-/-	Midline fusion defect		Qu et al., 1999
δΕΦ1−/−	Cleft palate		Takagi et al., 1998
dHAND-/-	Disruption of branchial arch mesenchyme		Yanagisawa et al., 2003
Dlx5-/-	Reduced proliferation	Loss of Goosegoid in mandible and frontonasal mass	Acampora et al., 1999; Depew et al., 1999
Foxc2 (Mfh1)-/-	Cleft palate	Disrupted Shh signalling?	Iida et al., 1997
Gli2-/-	Palatal shelves do not elevate		Mo et al., 1997
Hic1-/-	Small palatal shelves		Carter et al., 2000
Hoxa2-/-	Palatal shelves do not elevate due to tongue musculature defects		Gendron-Maguire et al., 1993; Rijli et al., 1993
Lhx8-/-	Small palatal shelves		Zhao et al., 1999
Msx1-/-	Impairment of proliferation in palatal mesenchyme and epithelial-mesenchymal interactions	Lack of Bmp4 expression in mesenchyme leads to lack of Shh in epithelium which leads to lack of Bmp2 in mesenchyme	Zhang et al., 2002
Mhox-/-	Cleft palate		Martin et al., 1995
p63-/-	Epithelial-mesenchymal interactions affected		Mills et al., 1999, Yang et al., 1999
Pax9-/-	Cleft palate	Required for expression of Bmp4, Msx1, and Lef1	Peters et al., 1998
Pitx1-/-	Palatal shelves do not elevate		Lanctot et al., 1999, Szeto et al., 1999
Pitx2-/-	Small palatal shelves	Lack of Fgf8 expression, misexpression of Bmp4, Msx1, and Msx2	Lu et al. 1999
Prx1-/-;Prx2-/-	Small palate due to reduced proliferation	Control of Shh expression in epithelium	ten Berge et al., 1998; Lu et al., 1999; ten Berge et al., 2001
Rae28-/-	Cleft palate	Misexpression of some Hox genes	Takihara et al., 1997

Tbx1-/-	Cleft palate		Jerome and Papaioannou, 2001
Titf2-/-	Cleft palate		De Felice et al., 1998
Growth factors and receptors			
α5–ιντεγριν–/–	Small palatal shelves		Bader et al., 1998
Activinb-A-/-	Cleft palate		Matzuk et al., 1995a,b
ALK4-/-	Cleft palate		Matzuk et al., 1995a
Efgr-/-	MEE persists	Metalloproteinase expression	Miettinen et al., 1999
Ephb2-/-;Ephb3-/-	Small palatal shelves		Orioli et al., 1996
ET-1-/-	Epithelial-mesenchymal interactions affected	Lack of dHAND expression	Kurihara et al., 1994, Yanagisawa et al., 1998
Fgf10-/-	Epithelial-mesenchymal interactions affected, reduced proliferation	Lack of Shh expression	Publication III
Fgf18-/-	Failure of palatal shelf elevation?		Liu et al., 2001
Fgfr1 hypomorph	Cleft palate due to tongue blocking palatal shelf elevation		Trokovic et al., 2003
Fgfr2b-/-	Epithelial-mesenchymal interactions affected, reduced proliferation	Lack of Shh expression	De Moerlooze et al., 2000; Publication III
Follistatin-/-	Small palatal shelves		Matzuk et al., 1995c
Jagged 2-/-	Palatal shelves do not elevate and tongue fuses with them	Disrupted Notch signalling	Jiang et al., 1998a
PDGFRa-/- (Patch mutant)	Decreased neural crest migration, abnormal tissue remodelling	Decrease in MMP-2 and MT-MMP	Morrison-Graham et al., 1992, Robbins et al., 1999
γPAP–/–	Cleft palate		Lohnes et al., 1993
Ryk-/-	Highly positioned tongue obstructs elevation of one of the palatal shelves	Interacts with Ephb2 and b3 receptors	Halford et al., 2000
Shh-/-	Decreased cell survival and proliferation, failure of midline	Lack of Tbx1 expression in branchial arches	Chiang et al., 1996
TGFb2-/-	Palatal shelves do not elevate		Sanford et al., 1997
TGFb3-/-	Lack of filopodia structures on the surface of MEE prevents adhesion		Kaartinen et al., 1995, Proetzel et al., 1995, Taya et al., 1999
Growth factor antagonist			
Chordin-/-	Defects in axial mesendoderm and pharyngeal arches 2-6	Misexpression of Fgf8 and Tbx1 in pharyngial region	Bachiller et al., 2003
Extracellular matrix molecule			
(a1) II collagen (Dmm) mutant	Cleft palate	Thick collagen fibrils	Pace et al., 1997
Perlecan (Hspg2)-/-	Cleft palate	Disorganized collagen fibrils	Arikawa-Hirasawa et al., 1999
Intracellular molecule			
3b-hydroxysterol D7-reductase-/-	Small palatal shelves (cleft palate)	Shh signaling?	Wassif et al., 2001
Apaf1-/-	No palatal shelf fusion due to lack of apoptosis	Blockage in caspase signalling	Cecconi et al., 1998, Honarpour et al., 2000, Yoshida et al., 1998

CASK mutant	Sex linked cleft palate		Wilson et al., 1993; Laverty and Wilson, 1998
IKK1-/-	Cleft palate	NFkB activation diminished	Li et al., 1999
p57kip2-/-	Cleft palate	Altered cell proliferation and differentiation	Zhang et al., 1997; Caspary et al., 1999
Tattered (D8-D7 sterol isomerase) mutant	Abnormal palatine process and anterior palatine foramen fails to join		Derry et al., 1999
Neurotransmitter			
GABA67-/-	Palatal shelves do not fuse		Asada et al., 1997; Condie et al., 1997; Homanics et al., 1997

Palatal shelf development requires epithelial-mesenchymal interactions

The development of palatal shelves has also been shown to require epithelial-mesenchymal interactions which suggest that also paracrine mode of regulation plays a role in secondary palate formation. Tyler and Pratt (1980) performed E12 mouse palatal cultures where they separated epithelium from underlying mesenchyme, and cultured the separated tissues in culture medium with or without added epidermal growth factor (EGF), which was known to affect epithelial cell differentiation and DNA synthesis. The results showed that oral and nasal regions of palatal epithelium undergo limited differentiation when cultured alone and without EGF. Isolated epithelial cultures with EGF showed some epithelial cell differentiation. Recombination of epithelium and mesenchyme in culture showed normal histodifferentiation of the epithelium without EGF. When recombinant tissues were cultured in the presence of EGF, oral and nasal epithelia became continuous with each other and the midline epithelium was lost. Most importantly, only the epithelial-mesenchymal cultures with added EGF showed DNA synthesis in the epithelium. This experiment was the first to indicate that epithelial-mesenchymal interactions are needed for palatal growth; the epithelium requires mesenchyme to respond to the mitogenic effect of EGF (Tyler and Pratt, 1980). A few years later, Ferguson and Honig (1984) published a study that further supported an important role for epithelial-mesenchymal interactions and for their right timing during the development of the secondary palate. The authors performed cultures with mouse, chick and alligator palates and mandibles. Intraspecies recombination cultures of mandibular epithelium with palatal mesenchyme, and vice versa, showed that in alligator and chick the mesenchyme directs the epithelial cell fate: the palatal epithelium adopted mandibular phenotype when it was cultured with mandibular mesenchyme, and vice versa. However, in mouse the results differed from alligator and chick, and showed that both mandibular and palatal epithelial cell fate was determined by E12: when differentiated E12/E13 mandibular epithelium was cultured with the same age palatal mesenchyme, the mandibular epithelium maintained its original phenotype. Similarly, cultures of E12/E13 palatal epithelium recombined with mandibular mesenchyme kept their palatal phenotype. Intra- and interspecies palatal epithelium – palatal mesenchyme recombination cultures, where mesenchyme and epithelium were placed either parallel or perpendicular to their medial edges, demonstrated that the orientation of palatal mesenchyme directs the epithelial differentiation into nasal, oral, and MEE regions. Such epithelial-mesenchymal interactions are common to many developing organ, and for instance regulate the morphogenesis and cell differentiation of developing teeth: the early dental epithelium directs the tooth morphogenesis but during budding stage this role is shifted to dental mesenchyme (reviewed in Thesleff and Sharpe, 1997).

Growth factor signalling controls palate development

Some of the molecular signalling pathways involved in the epithelial-mesenchymal interactions have been unravelled recently. The importance of TGFβ signalling during palatogenesis was further emphasized in a study by Ito et al. (2003). The authors generated a cranial neural crest cell specific null allele of *TGFβ receptor type II* (*TGFbr2*) that can bind all Tgfβ ligands and also some Bmps, *Tgfbr2*$^{fl/fl}$*;Wnt1-Cre*. These animals exhibited cleft palate and calvarial abnormalities. This receptor is expressed both in the MEE cells and in the mesenchyme adjacent to MEE during secondary palate development. The cleft palate in the conditional *Tgfbr2* mutants was shown to be due to about 10% decrease in cell proliferation in the CNC derived mesenchymal cell population in the palatal shelves from E14.5 onwards. Interestingly, in these mutants the palatal fusion was not disturbed; when palatal shelves were placed in organ culture they fused. Thus, in these mutants the epithelial function of Tgfβ3 may not be fully blocked, and there may be a cell autonomous requirement for Tgfβ signalling in the CNC derived palatal mesenchyme (Ito et al., 2003). Decreased cell proliferation in the *Tgfbr2* mutants was correlated to reduced *cyclin D1* expression in the mesenchyme of the palatal shelves in the mutant. The authors discuss that the altered expression of *cyclin D1* is not likely to be the sole cause of the cleft palate phenotype since *cyclin D1* null allele animals do not exhibit cleft palate (Fantl et la., 1995).

The *Tgfbr2* mutants showed 2.5 times elevated levels of *Msx1* expression in the palatal mesenchyme. This finding seems contradictory to reports that *Msx1* null-allele animals exhibit a cleft palate (Satokata and Maas, 1994). Cleft palate in *Msx1-/-* animals has been studied by Zhang et al. (2002). They demonstrated that the mesenchymal proliferation in the anterior palatal shelves is achieved by epithelial-mesenchymal interactions that combine Bone morphogenetic (Bmp) and Sonic hedgehog (Shh) signalling pathways, and that disruption of this signalling network leads to a cleft palate. In these mice, the primordial palatal shelves formed and elevated normally but failed to contact each other and did not fuse (Zhang et al., 2002). When *Msx1*-deficient palatal shelves were placed in contact with each other in organ culture, the shelves fused indicating that this was not the cause of the cleft palate. The cleft palate phenotype was rescued by crossing the *Msx1*-deficient mice with transgenic mice that overexpress human *BMP4* under the control of *Msx1* promoter. Furthermore, the authors showed that palatal mesenchyme expresses *Bmp4*, and that ectopic BMP4 protein induces expression of *Shh* in palatal epithelium. Ectopic Shh protein turns on the expression of *Bmp2* in the mesenchyme, thus indicating that Shh signals from the epithelium back to the mesenchyme. Bmp2 then acts as a mitogen to stimulate proliferation in the mesenchyme. A similar signalling network has been established previously to tooth development (Chen et al., 1996; Bei et al., 2000; Dassule et al., 2000; Gritli-Linde et al., 2002). It is important to keep in mind that both Bmp2 and Bmp4 use the same receptors (BmprI and II in homo- and heterodimers) for their signalling. Interestingly, even though *Msx1*, *Bmp2*, *Bmp4*, and *Shh* were found to be expressed only in the anterior region of the palatal shelves (anterior to first molar tooth) at E12.5 and E13.5, posterior regions were also affected (Zhang et al., 2002). Work from the Thesleff laboratory has shown that *Shh* is expressed along the entire anteroposterior axis of palatal epithelium at E13 (Keränen et al., 1999; Fig. 5d,g in Publication III), and that mesenchymal Fgf10 regulates epithelial expression of *Shh* through the epithelial receptor Fgfr2b (Fig. 5c in Publication III). It is possible that the expression of *Shh* is under complex regulation where Bmp signalling primarily regulates the anterior expression of *Shh*, and Fgf signalling along the whole anterior-posterior

length of the MEE. In the developing tooth it has been shown that Fgfs and Bmp4 induce both Msx1-independent and –dependent signalling pathways (Bei and Maas, 1998).

Development of cranial base

In the mammalian skull, the **chondrocranium** is made up of the cranial base together with the otic and nasal capsules (Fig. 2). The main function of the chondrocranium is to protect the brain, cranial nerves and sense organs. In addition, the growth of the cranial base is important to the growth of the middle third of the face (Meikle, 2002).

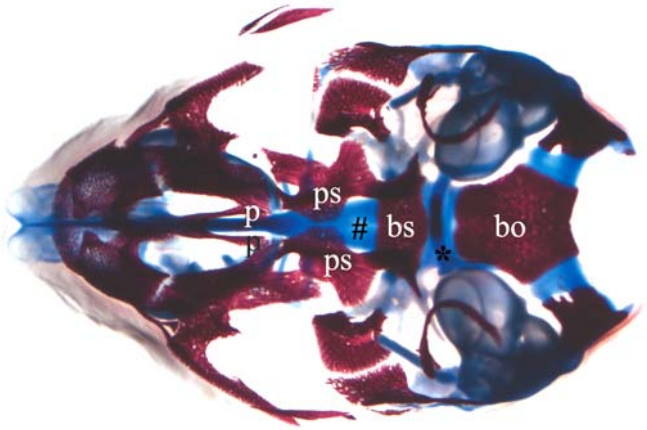

Figure 2. Skeletal staining of cranial base in E17 mouse, caudal view. Mineralized bones are stained with alizarin red, and cartilages with alcian blue. Calvarium and mandibles have been removed. p, palatal shelves, ps, presphenoid, bs, basisphenoid, bo, basioccipital bone, #, intersphenoidal synchondrosis, *, spheno-occipital synchondrosis. The hyoid bone goes over the spheno-occipital synchondrosis; the centre of the hyoid bone is mineralized.

Endochondral ossification in cranial base

Cranial base bones form through endochondral ossification. Their development starts as cartilaginous condensations within the head mesenchyme. Condensation is a pivotal stage in skeletogenesis, and it is the main resource from which skeletal elements are built. Condensations have predictable sizes which affect whether skeletogenesis will be initiated, and what will be the final size and shape of the elements. In addition, genes specific to differentiation are upregulated at this stage (Hall and Miyake, 2000). There are three main condensations in the developing cranial base: (1) **prechordal or trabecular condensation** giving rise to the sphenoid bones, (2) **parachordal condensation** giving rise to the occipital bones, and (3) **otic and nasal capsule condensation**. These condensations differentiate into chondrocytes that will proliferate, and the prechordal and parachordal condensates will join and fuse to form a continuous plate of cartilage extending from the front of the skull to the **foramen magnum** to support the developing brain (Meikle, 2002). As chondrocytes mature they cease cell proliferation and become hypertrophic. The hypertrophic chondrocytes lay down calcified cartilaginous matrix. The mid-hypertrophic zone becomes vascularized. Blood vessels bring osteoclasts and osteoblasts into the cartilage. Osteoclasts will remove the cartilaginous matrix, and osteoblasts will replace it by bone (Mundlos and Olsen, 1997; Ornitz and Marie, 2002).

The joints between adjacent mineralized cranial base bones will stay cartilaginous, and are called **synchondroses** (Fig. 2). A synchondrosis has a resting zone of chondrocytes in the middle, which is surrounded by proliferative zones on both sides (Fig. 2 in Publication II). The proliferative zones are followed by hypertrophic zones, and finally erosive zones are found at both ends of the synchondrosis closest to the bone ends where hypertrophic chondrocytes are replaced by bone. Thus, synchondrosis can be regarded as two growth plates placed back to back, and consequently, growth at the synchondrosis is bidirectional (Meikle, 2002). Roberts and Blackwood (1984) performed a proliferation study and demonstrated that there is a caudorostral growth gradient in the cranial base cartilages; the highest growth rate being in the basioccipital and the lowest in the rostral edge of the presphenoid.

The spheno-occipital synchondrosis is of special importance in humans since it remains open until the teenage years, unlike the other synchondroses which fuse early in life, and thus influences skull growth for a long period of time (Ingervall and Thilander, 1972). For instance, maxillary hypoplasia in achondroplasia patients is caused by short cranial base, which is due to impaired growth at the spheno-occipital synchondrosis (Cohen et al., 1985).

Molecular control of cranial base bone development

The molecular mechanisms that regulate the development of cranial base are poorly studied. The occipital region normally expresses *Hox* genes from the paralogous groups 1 and 2 suggesting that they are at least partly patterned by *Hox* genes (Whiting, 1997). Evidence towards the susceptibility of occipital and sphenoid bones to patterning by *Hox* genes has been given by transgenic mice misexpressing these genes. Ectopic expression of trunk level *Hoxb7* (previously *Hox-2.3*) under chick *β-actin* promoter caused malformations in occipital bones and in basisphenoid (McLain et al., 1992). Ectopic expression of *Hoxd4* (previously Hox-4.2) more rostrally than its normal mesodermal anterior boundary resulted in a homeotic transformation of the occipital bones towards a more posterior phenotype of neural arches, and in a partial or complete agenesis of the basisphenoid bone (Lufkin et al, 1992). Similarly, ectopic expression of *Hoxb6* (previously *Hox-2.2*) in the head region led to missing or malformed occipital bones, basisphenoid, or squamosal (Kaur et al., 1992).

In humans, missense, gain-of-function mutations in *fibroblast growth factor receptor 3* (*Fgfr3*) result in chondrodysplasias many of which also affect the cranial base (Ornitz and Marie, 2002). A mouse model for human achondroplasia, which carries G369C mutation in *Fgfr3*, showed premature fusion of synchondroses (Chen et al., 1999). Similarly, transgenic mice carrying a heterozygous mutation S365C in *Fgfr3*, which is equivalent to a mutation causing thanatophoric dysplasia type I in humans, had prematurely fused and ossified synchondroses causing a short cranial base (Chen et al., 2001). Mouse models harbouring K644E and K644M mutation reflecting the human thanatophoric dysplasia type II and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), respectively, have been generated but only axial skeletons were studied (Iwata et al., 2000; Iwata et al., 2001). In both mouse models, the heterozygous mutants appear to have rounded heads (figure 2D in Iwata et al., 2000; figure 3D in Iwata et al., 2001), which is suggestive of cranial base malformations. All these mutant mice showed fewer proliferative chondrocytes in postnatal growth plates in limbs. This was caused by increased levels of Stat (signal transducer and activator of transcription) proteins. Stats activate cell cycle inhibitors, and thus prevent proliferation. Also, *Indian hedgehog* (*Ihh*) and *parathyroid hormone related peptide* (*PTHrP*) expression domains and intensities were reduced in growth plates indicating reduced

numbers of prehypertrophic chondrocytes, and thus impaired chondrocyte differentiation. Interestingly, Iwata et al. (2000) showed that K644E mutation in *Fgfr3* increases proliferation of growth plate chondrocytes during embryonic chondrogenesis at E15. This coincided with a transient upregulation of *patched* (*Ptc*) expression, which is a receptor for Ihh. By E18 the proliferation rate and expression of *Ptc* were similar to wild type littermates. The expression pattern of *Fgfr3*, which is expressed in the proliferating chondrocytes, and *Ihh*, which is expressed in the neighbouring prehypertrophic cells, further supports the interaction between these two signalling pathways during embryonic development (Ornitz and Marie, 2002). *Fgfr3* and *Ihh* are expressed in a similar pattern in spheno-occipital synchondrosis; *Fgfr3c* in prehypertrophic and proliferating cells, and *Ihh* in prehypertrophic and hypertrophic cell populations (Publication II). Thus, it is likely that Fgfr3 regulates the expression of *Ihh* also in the cranial base cartilages to keep chondrocytes immature and allow further growth. In long bones, Fgfr3 signalling through Stats is independent of Ihh (Chen et al., 2001). Whether Stats are involved in the growth of the synchondroses remains to be seen.

Mice deficient in *Fgfr2IIIc* isoform showed reduced size of the bones of the cranial base, and premature fusion of the occipital and spheno-occipital synchondroses (wrongly termed ‘sutures’ in the publication) (Eswarakumar et al., 2002). Interestingly, these null allele mutants showed decreased expression of *Ihh*, *PTHrP*, and *Runx2* similarly to gain-of-function mutations in *Fgfr3c*. Thus, Fgfr2c appears to have an opposite role to Fgfr3c, and to be a positive regulator of ossification (Eswarakumar et al., 2002).

Development of calvarium

Calvarial bones, which are composed mainly of two frontal, two parietal and an interparietal bone, form directly from mesenchyme by intramembranous ossification (Fig. 3). Intramembranous ossification starts by mesenchymal cell condensation. Condensed mesenchymal cells undergo differentiation into osteoprogenitor cells which proliferate, and ultimately differentiate into osteoblasts that lay down bone matrix (Hall and Miyake, 2000). Just as for chondrogenic condensations, osteogenic condensations affect the final size and shape of the bone, and are the sites where differentiation pathway commences. The mesenchymal condensations for calvarial bones form close to the cranial base and the brains. The calvarial bones grow as sheets between the brain and epidermis, and extend apically towards 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 osteogenic fronts. **Sutures** are fibrous joints that accommodate brain growth. In mouse all calvarial sutures, except 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 (Meikle, 2002).

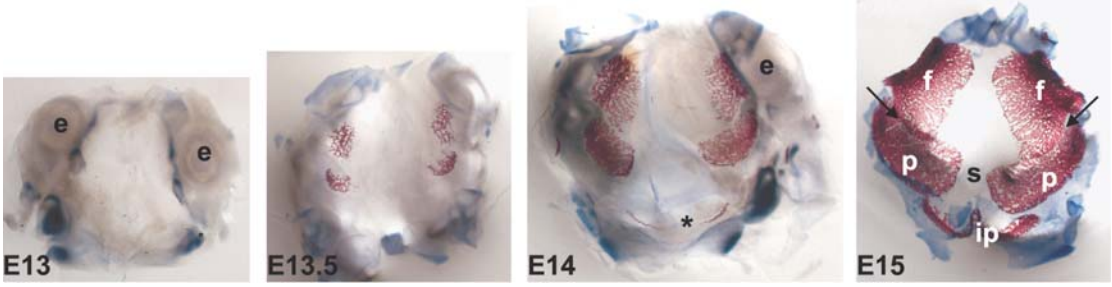

Figure 3. Calvarial bone development in mouse. Skeletal stain by alizarin red (mineralized bones) and alcian blue (cartilages) in calvarial explants. Calvarial bones start developing from osteogenic condensations at E12-E13. Mineralized frontal (f) and parietal (p) bones are visible by E13.5. By E14 the interparietal (ip) bone is visible as two separate ossification centres. These lateral ossification centres grow medially to form the interparietal bone. By E15 the sagittal suture (s) between the opposing parietal bone plates has formed, as have the coronal sutures (arrows) between the frontal and parietal bone plates. e, eye. Images are the same magnification.

Tissue interactions during calvarial suture formation

Calvarial bones grow in close contact with **dura mater** which is the outermost layer of the meninges covering the brain. Interactions between the dural cells and calvarial mesenchyme have been shown to be important in the regulation of osteogenesis within a suture, and thus in the suture fate (i.e. patent or ossified). Opperman et al. (1993) performed in vitro organ cultures of E19 and postnatal day 1 (P1) rat calvaria with or without the underlying dura mater. This study answered some fundamental questions about calvarial growth and suture formation. The authors demonstrated that calvarial bone growth was not influenced by biomechanical forces originating from the cranial base as bone growth was not affected in culture where there were no mechanical tensions; they showed that tissue interactions between the calvarial mesenchyme and the dura mater were required to maintain coronal sutures patent as these sutures were obliterated by bone in cultures without dura mater, and that foetal dura mater was not required for initial suture formation. Opperman et al. (1995) continued their studies by showing that in E19/P1 rat calvarial cultures dura mater maintains patent sutures by soluble factors since separation of mesenchyme and dura mater by a polycarbonate filter kept the coronal sutures patent. Further evidence for the requirement of dura mater in the suture fate was published on posterior frontal (PF) suture on postnatal rats (Roth et al., 1996). These authors performed operations on P8 rats to separate dura mater from the PF suture by a silicone sheet, and studied the effect at the start (P15), during (P20), and after (P30) fusion. When dura mater was separated from the PF suture by a silicone sheet, the PF suture ossification was delayed. Bradley et al. (1997) and Levine et al. (1998) studied whether the dura mater underneath the suture specifies the suture patency. They showed that 180º rotation and translocation of murine sagittal suture onto the PF dura mater caused the normally patent sagittal suture to fuse and ossify. The PF suture, which now overlaid sagittal dura mater, stayed patent. The sagittal suture, which overlaid the PF dura mater, fused. Thus, these authors demonstrated that there are regional differences in the dura mater in adult animals. Opperman et al. (1998) showed that the function of the dura mater in the suture fusion involves induction of cell proliferation prior to fusion which is followed by increased collagen synthesis in fusing sutures. At the same time, work from the Thesleff laboratory demonstrated that a similar mechanism regulates the suture fate already during embryogenesis (Kim et al., 1998). This study showed that E16 mouse sagittal suture without dura mater fused within 72 hours in culture while control explants still had patent sagittal sutures. Thus, there is ample evidence from many laboratories that paracrine cell signalling mechanisms between the dura mater and overlying suture mesenchyme modulate suture ossification throughout the development.

Regulation of calvarial suture fate by growth factor signalling

The first publication that identified some of the soluble factors signalling from the dura mater to the suture was by Opperman et al. (1997) where the authors localized

Tgfβ isoforms 1-3 to frontal and coronal sutures in postnatal rats by immunohistochemical analysis, and importantly showed that Tgfβ3 levels increased in the patent coronal sutures when PF suture was fusing. To better understand the role of different Tgfβ isoforms in the suture fate, Opperman et al. (1999) performed in vitro calvarial cultures in the presence or absence of neutralizing antibodies against the isoforms. Control coronal sutures did not fuse when the function of Tgfβ1 or β2 was blocked, but fused when the function of Tgfβ3 was blocked. Coronal sutures cultured in the absence of the dura mater were rescued from fusion by blocking the function of Tgfβ2 but not Tgfβ1 or β3. These studies demonstrated that Tgfβ isoforms play differential roles in the regulation of suture fate so that isoform β2 plays a role in the suture fusion while β3 is needed to keep sutures patent. Further support for these findings came from a recent study where injection of Tgfβ3 into the PF suture prior to fusion delayed the fusion (Opperman et al., 2002). This study also demonstrated that in Tgfβ3-treated PF sutures the levels of Tgfβ2 and transforming growth factor beta receptor type I (TβR-I) were downregulated. Tgfβ isoforms were shown to control the number of cells; Tgfβ2 increased cell proliferation while Tgfβ3 reduced it (Opperman et al., 2000). Also, this study showed that patent suture has higher numbers of apoptotic cells than fusing one. This finding is in accordance with Rice et al. (1999) where apoptotic cells were found in calvarial bones, intervening sutures and fontanelles from E16 onwards. Thus, cell confluency plays a role in the regulation of suture fate. Most et al. (1998) and Mehrara et al. (1999) showed that increased levels of Tgfβ1 and Fgf2 proteins were produced in the dura mater underlying the PF suture just prior and during the fusion while patent sagittal suture produced only low levels of these growth factors during the same time period. Greenwald et al. (2001) targeted an adenoviral construct of secreted form of Fgf2 to PF or coronal dura mater in utero to demonstrate that Fgf2 was responsible for the increased proliferation, extracellular matrix molecule synthesis, and for the synthesis of Tgfβ1. They also showed that a similar targeting of a truncated form of Fgfr1, which blocks Fgf signalling, caused the infected PF sutures to stay patent. Thus, this elegant study showed that Fgf signalling regulates postnatal suture fate. The role of Fgf signalling in the regulation of suture patency had previously been shown in embryonic mouse tissue (Kim et al., 1998). This study demonstrated that recombinant human FGF4 soaked beads, which were placed on osteogenic fronts of parietal bones of E16 calvarial explants, accelerated sagittal suture fusion by increasing the amount of proliferative cells. Interestingly, FGF4-containing beads placed in the mid-sutural mesenchyme did not alter suture patency. Kim et al. (1998) further demonstrated that Bmp signalling does not regulate embryonic suture patency since BMP4-containing beads placed either on osteogenic fronts or in the mid-sutural mesenchyme of sagittal suture did not alter the patency of the sagittal suture but increased sutural tissue thickness.

Some of the target molecules and functions of Fgf and Bmp signalling that control the osteogenesis in the developing suture have been discovered. In the sagittal suture, Fgf signalling has been shown to induce the expression of transcription factors *Msx1* (but not *Msx2*) and *Twist*, and the extracellular matrix protein *bone sialoprotein* (*Bsp*) in the suture mesenchyme, and Bmp signalling has been shown to upregulate the expression of transcription factors *Msx1*, *Msx2*, and *Inhibitors of differentiation* (*Id*) (Kim et al., 1998; Rice et al., 2000). Twist was shown to be both an upstream regulator and a downstream target of Fgf signalling; not only did exogenous FGF2 induce the expression of *Twist*, but lack of Twist also caused Fgfr2 protein to be misexpressed in the sagittal suture (Rice et al., 2000). Work from the Morriss-Kay laboratory has shown that FGF2 beads upregulate the expression of *osteopontin* (*Op*) and *Fgfr1* at high concentrations and *Fgfr2* at low

concentrations in coronal sutures (Iseki et al., 1997; Iseki et al., 1999). The authors concluded that a concentration gradient of Fgf ligand modulates the differential expression of *Fgfr1* and *-2*, and that these receptors have differential roles so that signalling through Fgfr2 is responsible for the proliferation of osteogenic cells, and signalling through Fgfr1 regulates osteogenic differentiation. Kim et al. (1998) showed that FGF2-containing beads induced cell proliferation in the sagittal suture.

Many *Fgf* ligands are expressed broadly by suture mesenchyme and the underlying dura mater both in embryonic and postnatal tissues (Iseki et al., 1997; Kim et al., 1998; Rice et al., 2000; Hajihosseini and Heath, 2002) while *Bmp2* and *Bmp4* show restricted expression pattern to osteogenic fronts and to dura mater (Kim et al., 1998). *Fgf receptors* show a specific spatiotemporal expression pattern in the developing sutures. *Fgfr1*, *2*, and *3* are expressed in coronal sutures (Iseki et al., 1999). *Fgfr1c*, *Fgfr2b* and *-2c*, and *Fgfr3c* are expressed by the osteogenic fronts and to a lesser degree in the suture mesenchyme in sagittal (Kim et al., 1998; Rice et al., 2000) and in lambdoidal sutures (Fig. 6 in Publication II). Kim et al. (1998) and Rice et al. (2000) further showed that the same localization of *Fgfr* transcripts continues on postnatal tissues. These expression patterns together with the results from the bead assays support the role of Fgf signalling in the regulation of the proliferative osteogenic cell population.

The molecules downstream of Fgf and Bmp signalling also show restricted expression patterns during suture development. *Msx1* and *Msx2* are expressed in a very similar pattern in suture mesenchyme and in osteogenic fronts from E15 onwards (Kim et al., 1998). After birth the expression of *Msx* genes is downregulated so that by P5 the expression of *Msx2* has disappeared while *Msx1* is still expressed in a very low level. *Bsp* is expressed throughout the calvarial bones during embryogenesis, and in postnatal tissues the expression of *Bsp* is stronger in osteogenic fronts (Rice et al., 2000). *Twist* is expressed by suture mesenchyme and osteogenic fronts, and *Id* expression is localized to suture mesenchyme. Both *Twist* and *Id* expression decreased with increasing maturity of osteogenic cells (Rice et al., 2000). Thus, the functional assays together with the expression pattern analysis indicate that Bmp signalling regulates mostly proliferative (*Msx1+*, *Msx2+*, *Id+*) osteoblast cell populations in the developing sutures while Fgf signalling can regulate both proliferative osteoblastic cells (*Msx1+*, *Twist+*) and mature (*Op+*, *Bsp+*) osteoblast cell populations.

These two growth factor signalling pathways can either be linked, or they can use parallel pathways. Both pathways can regulate the expression of *Msx1*. Also, Id can link the pathways as its expression is regulated by Bmps, and it can suppress the expression of Twist, which is involved in Fgf signalling (Pesce and Benezra, 1993; Rice et al., 2000). More evidence to support the idea that Fgf and Bmp signalling pathways are linked during osteogenesis was given by Warren et al. (2003) who showed that Bmp antagonist *noggin* was expressed in suture mesenchyme and dura mater in patent coronal and sagittal sutures but not in fusing PF suture. The authors showed that in the PF suture *noggin* expression was suppressed by Fgf2 prior to suture fusion. The expression of *noggin* was regulated in a suture-specific manner since Fgf2 was present only in the dura mater of the fusing PF suture (Warren et al., 2003). It seems that Fgf2 regulates the activity of Bmp signalling through noggin so that when the time is right Fgf signalling will suppress noggin, and allow more Bmp activity, which will then commence osteoblastic differentiation in fusing sutures. A complementary in vitro study on primary murine bone cell cultures showed that Fgf2 and noggin both inhibit differentiation of osteoblastic cells in a stage specific manner (Kalajzic et al., 2003). This study demonstrated that treatment of osteoblastic cell cultures with Fgf2 protein prevented the cells to express early markers of osteoblasts such as *type I* and *III collagen* while treatment with noggin protein blocked

the expression of later markers of *Bsp* and *osteocalcin* (*Oc*). In other words, Fgf2 kept the osteoblastic cells at progenitor stage, while noggin inhibited further differentiation into mature osteoblasts.

Molecular control of osteogenesis during calvarial development

The studies discussed above relied on the wild type animals. The understanding of molecular mechanisms involved in calvarial bone development has also increased considerably through analysis of mouse mutants that show ossification abnormalities in the skull (Table II). Mice deficient for the Runt-domain containing transcription factor *Runx2* (previously *Cbfa1*) lacked all bones including those of the skull vault (Otto et al., 1997). Runx2 was shown to be necessary for osteoblast differentiation, and to act as an activator of osteoblast-specific gene expression. The transcription factor Msx2 has been suggested to be an upstream regulator of Runx2 in osteoprogenitor cells since *Runx2* expression is down-regulated in *Msx2*-deficient mice (Satokata et al., 2000). *Msx2*-deficient mice exhibited a delay in calvarial bone ossification and an overall decrease in bone volume. Overexpression of *Msx2* on the other hand caused overgrowth of parietal bones, and increased the amount of proliferative cells in the osteogenic fronts (Liu et al., 1999). Calvarial abnormalities are also found in *Msx1* null allele mice which exhibit overlapping parietal bones, and enlarged anterior fontanel (Satokata and Maas, 1994), and calvarial phenotype of *Msx2* mutant is enhanced by genetic combination with *Msx1* loss of function (Satokata et al., 2000). Thus, Msx proteins have a pivotal role in mediating the balance between early osteoprogenitor cell proliferation and differentiation in calvarial development. In humans, haploinsufficiency of *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). Mice lacking *Alx4* exhibit a calvarial bone phenotype similar to 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 Saethre-Chotzen craniosynostosis, and in a subset of the affected individuals it can cause parietal foramina (Thompson et al., 1984). In cultured human mutant calvarial osteoblasts from Saethre-Chotzen patients, the reduced Twist dosage increased cell growth, collagen synthesis and matrix production, inhibited expression of *Runx2* and *Oc*, and induced apoptosis (Yousfi et al., 2001, 2002a, 2002b). Mice heterozygous to *Twist* have been proposed as a model of the human disease as they exhibit fusions of coronal sutures (Carver et al., 2002). A recent study from the Maxson laboratory demonstrated that Msx2 and Twist function in parallel to control the proliferation and differentiation of early osteoblast cell populations in frontal bone primordia, and that the growth and patterning of the frontal bone requires cooperative contributions from both Bmp and Fgf pathways (Ishii et al., 2003). Mice lacking a forkhead/winged helix transcription factor *Foxc1* develop only rudimentary calvarial bones and also show other skeletal abnormalities (Grüneberg, 1943; Kume et al., 1998; Hong et al., 1999). Heterozygous *Foxc1* mutants have delayed ossification of skull bones (Fig. 3h in Publication I). Thus, it seems that a proper dosage of many transcription factors is essential for regulating osteogenesis during calvarial development.

Gain-of-function mutations of *Fgfr*s lead to chondrodysplasia due to impaired chondrocyte proliferation and differentiation. At sutures, which are osteogenic, gain-of-function mutations in *Fgfr*s lead to premature fusion of some or all sutures (Ornitz and Marie, 2002). Of human craniosynostosis syndromes caused by mutations in *FGFR*s only

Crouzon syndrome has no associated limb abnormalities (Carlton et al., 1998). Mice carrying a Pro250Arg mutation in *Fgfr1*, which is the most commonly found mutation in Pfeiffer syndrome (acrocephalosyndactyly) patients, have synostosed frontal, sagittal, and coronal sutures (Zhou et al., 2000). These animals exhibited accelerated bone formation in calvarial sutures which was manifested by increased expression of *Runx2*, *Op*, *Oc*, and *Bsp* in calvarial sutures, and by both increased proliferation and differentiation of osteoblastic cells. Interestingly, no differences were noted in growth plates of mutant and wild type animals; neither were cranial base synchondroses affected making the Pro250Arg mutation in *Fgfr1* to be osteoblast cell lineage specific. To support the role of Fgfr1 in the control of calvarial suture ossification, adenoviral expression of dominant-negative form of *Fgfr1* was shown to inhibit calvarial suture fusion (Greenwald et al., 2001). When *Fgfr2c* hemizygote mice were produced the phenotype of the mutant animals resembled that of Apert/Pfeiffer patients (Hajihosseini et al., 2001). This would seem contradictory since these patients have gain-of-function mutations in *FGFR2b* or in *FGFR2c*. The authors demonstrated that the heterozygotic deletion of exon 9, which is specific to the *Fgfr2c* isoform, caused a splicing switch that resulted in ectopic *Fgfr2b* expression. The authors also showed that Fgf10, which is one of the ligands that can activate Fgfr2b, is present in coronal sutures, and ectopic signalling is possible. This misexpression of *Fgfr2b* thus caused the excessive ossification in coronal sutures. Revest et al. (2001) showed that mice deficient for *Fgfr2b* (and unaffected expression of *Fgfr2c*) exhibited a delay in intramembranous ossification and a reduction in skull bone mass. Mice lacking both alleles of *Fgfr2c* without splice switching also demonstrated delayed ossification of skull bone plates that was caused by decreased cell proliferation at coronal suture from E16.5 onwards (Eswarakumar et al., 2002). Thus, both isoforms of Fgfr2 appear to function as positive regulators of ossification during calvarial development. Mansukhani et al. (2000) demonstrated that when OB1 cell line, which is a proliferative mouse osteoblastic cell line capable of differentiating into nodule-forming mature osteoblasts, was retrovirally transfected to overexpress *Fgfr2* carrying activating Apert (Ser252Trp) or Crouzon (Cys342Tyr) mutations, the cells exhibited increased proliferation, and suppressed differentiation into nodule-forming mature osteoblasts.

Of Fgf ligands, Fgf2, 3, 4, and 18 have been shown to be involved in regulating calvarial suture ossification. Transgenic mice overexpressing *Fgf2* show increased apoptosis in the osteogenic fronts of the posterior frontal suture (Mansukhani et al., 2000). Fgf2-treated posterior frontal sutures cultured in vitro show induced cell proliferation and enhanced suture fusion (Moursi et al., 2002). In chicks, blocking endogenous Fgf2 activity in calvarial bones caused inhibition of proliferation and osteoblastic differentiation in a dose-dependent manner (Moore et al., 2002). Heterozygous *Bulgy-eye* (*Bey*) mice, which were produced by retroviral insertional mutagenesis, led to the finding that overexpression of both *Fgf3* and *Fgf4* in calvarial sutures caused them to fuse prematurely (Carlton et al., 1998). Ectopic adenoviral expression of *Fgf2* in coronal sutures also caused premature fusion (Greenwald et al., 2001). Two publications demonstrated at the same time that Fgf18 is required for both osteogenesis and chondrogenesis. Ohbayashi et al. (2002) and Liu et al. (2002) showed that *Fgf18*-/- mice exhibited delayed calvarial ossification and suture closure due to decreased proliferation of osteogenic cells, and delayed terminal differentiation of osteoblasts. In the skull vault, the expression of *Runx2* and *Op* were decreased in the mutant animals. Fgf18 appears to control the maturation of osteoblasts from *Runx2*-positive, *Op*-negative, and *Oc*-negative cells to *Runx2*-, *Op*-, and *Oc*-positive cells (Ohbayashi et al., 2002). In chondrogenesis, the role of Fgf18 appeared to be the opposite of osteogenesis. In *Fgf18* null allele mice the growth plates were enlarged, and more cells were undergoing proliferation and

differentiation (Ohbayashi et al., 2002; Liu et al., 2002). The authors concluded that Fgf18 is a positive regulator of osteogenesis but a negative regulator of chondrogenesis. Both groups suggested that the differential role in chondro- and osteogenesis may be based on a differential receptor usage; Fgf18 could use Fgfr3c in chondrocytes and Fgfr2c in osteoblasts (Ohbayashi et al., 2002; Liu et al., 2002). In cell culture it has been shown that Fgf18 may compensate for the action of Fgf2 in proliferation, differentiation and matrix synthesis in bone and cartilage (Shimoaka et al., 2002). This study also demonstrated that Fgf10 had no effect on osteoblasts or chondrocytes in cell culture. Interestingly, expression pattern analysis has shown that Fgf10 is expressed in coronal sutures (Hajihosseini et al., 2001).

Roles of Bmp and Fgf signalling in human calvarial osteoblast cells have been shown to be similar to murine models. Human studies have focused on materials obtained from craniosynostosis patients, and to human neonate calvarial osteoblastic cell cultures. Calvarial cells cultured from Apert patients have demonstrated that these mutant cells show increased levels of alkaline phosphatase (Alp) and type I collagen expression (Col I), and increased matrix formation which indicate increased osteogenesis in mutant cells (Lomri et al., 1998). These cells also show reduced levels of Fgfr2 protein but unaltered levels of mRNA (Lemonnier et al., 2000). Coronal sutures of Apert patients also show increased levels of Col I, Op and Oc proteins, and reduced levels of Fgfr2 (Lemonnier et al., 2000). The function of Fgf signalling in human calvarial osteoblasts is similar to murine models: Fgf signalling has a dual role so that it promotes proliferation of preosteoblasts and prevents their maturation while in mature osteoblast cells it promotes differentiation and matrix mineralization (Debiais et al., 1998). The increased osteogenesis by activating Fgfr mutations, such as found in Apert syndrome, has been linked to increased levels of *N-Cadherin* and cell-cell adhesion (Debiais et al., 2001; Lemonnier et al., 2001b). Lemonnier et al. (2001a) have also shown that mature osteoblasts and osteocytes in Apert coronal sutures show increased levels of apoptosis through activation of protein kinase C (PKC)-pathway leading to activation of caspase-8. Also Bmp signalling has been shown to regulate osteoblast differentiation and apoptosis in human calvarial osteoblasts: it induces transient *N-Cadherin* expression which is required for the expression of osteoblast specific genes such as *Runx2* and *Oc* (Haÿ et al., 2000), and it actives apoptosis via Smad-independent, PKC-dependent pathway similarly to Fgf signalling (Haÿ et al., 2003). These authors showed further evidence that Bmp-induced apoptosis is mediated through bone morphogenetic factor receptor type Ib (BmprIb) in osteoblasts, and that this occurs independently of Bmp-induced differentiation of osteoblasts.

Table II. Mouse mutants with calvarial phenotype.

Mouse mutant	Calvarial phenotype	Defect	Molecular mechanism	References
Transcription factor				
Alx3-/-;Alx4-/-	Reduced frontal and parietal bones	Increased apoptosis in frontonasal mass,		Beverdam et al., 2001
Alx4-/-	Calvarial foramen	Delayed ossification of parietal bones		Qu et al., 1997
Alx4-/-;Cart1-/-	Absence of skull bones			Qu et al., 1999
Cart1-/-	Absence of skull bones			Zhao et al., 1996

Foxc1-/- and Congenital hydrocephalus (Foxc1) mutant	Rudimentary calvarial bones	Reduced proliferation of osteoblastic cells	Misexpression of Msx2 and Alx4 in calvarial mesenchyme	Gruneberg, 1943; Kume et al., 1998; Publication I
Extra toes (Gli3) mutant	Open skull vault			Johnson 1967; Mo et al., 1997
Gli2-/-	Open skull vault	Delayed ossification		Mo et al., 1997
Hic1-/-	Absent skull bones			Carter et al., 2000
Msx1-/-	Enlarged anterior fontanelle	Delayed ossification, reduced proliferation		Satokata and Maas, 1994
Msx1-/-;Msx2-/-	Loss of membrane bones	Delayed ossification, reduced proliferation		Satokata et al., 2000
Msx2-/-	Calvarial foramen	Delayed ossification, reduced proliferation		Satokata et al., 2000
Msx2-/-;Twist+/-	Calvarial foramen	Delaye ossification, reduced proliferation		Ishii et al., 2003
Runx2-/-	Absence of bones	Osteoprogenitor cell development arrested		Komori et al., 1997
Ski-/-	Absent skull bones	Increased apopotosis in craniofacial mesenchyme		Berk et al., 1997
Twist+/-	Calvarial foramen	Delayed ossification, reduced proliferation		Ishii et al., 2003
Growth factors and receptors				
Bulgy-eye (Bey) mutant	Synostosis of several cranial sutures		Overexpression of Fgf3 and Fgf4	Carlton et al., 1998
Fgf18-/-	Delayed calvarial ossificiation	Transient reduction of osteogenic proliferation at E14.5, and delay of terminal differentiation of osteoblasts		Ohbayashi et al., 2002
Fgfr1 Pro250Arg mutant	Synostosis of several cranial sutures	Increased proliferation and differentiation of osteoblasts	Increased expression of Cbfa1	Zhou et al., 2000
FgfR2-IIIc hemizygote	Coronal synostosis		Splicing switch to ectopic expression of FgfR2-IIIb	Hajihosseini et al., 2001
Shh-/-	Absent of hypoplastic craniofacial bones	Midline failure, decreased cell survival and proliferation		Chiang et al., 1996
TGFb2-/-	Small and dysmorphic calvarial bones			Sanford et al., 1997
Gap junction molecule				
Connexin 43-/-	Hypoplastic cranial bones	Delayed ossification		Lecanda et al., 2000
Extracellular matrix molecule				
Perlecan (Hspg2)-/-	Dilated sutures at midline	Reduced ossification	Disorganized collagen fibrils and glycosamino-glycans	Arikawa-Hirasawa et al., 1999

AIMS

The main aim was to study how growth factor signalling regulates the development of craniofacial structures, in particular the formation of calvarial bones and secondary palate.

I What are the molecular mechanisms that control the progression of calvarial bone growth from the initial sites of bone formation? Specifically, does Foxc1 regulate osteogenic cell proliferation in the calvarial mesenchyme? What growth factor signalling pathway does Foxc1 regulate in calvarial mesenchyme, and what are the downstream targets?

II What are the molecular mechanisms that control the growth of cranial base synchondroses during embryonic development? Specifically, are *fibroblast growth factor receptor* (*Fgfr*) isoforms expressed in synchondroses? In which cell populations are the *Fgfr* isoforms expressed, and what do the expression patterns tell about their possible functions in synchondroses?

III What are the molecular mechanisms that control the formation of secondary palate? Specifically, does reiterative Fgf10/Fgfr2b signalling play a role in the development and outgrowth of a palatal shelf bud? Is Fgf10/Fgfr2b signalling needed for the proliferation of cells in the early palatal shelf? What are the epithelial downstream target(s) of Fgf10/Fgfr2b signalling, and do they signal back to the mesenchyme?

MATERIALS AND METHODS

Mating and genotyping

Mouse embryos were obtained by mating NMRI mice (Jackson Laboratories, USA); *Fgfr2b* null allele embryos were obtained from mating heterozygous *Fgfr2b* mice (De Moerlooze et al., 2000); *Foxc1* mutant embryos were obtained from mating heterozygous *congenital hydrocephalus* (*ch*) mice (Jackson Laboratories, USA). The vaginal plug date was designated as E0. Mice carrying a *Fgf10* null allele (Min et al., 1998) were kindly provided by Dr. Clive Dickson's laboratory, Cancer Research, UK. *K-14Cre;Shh$^{c/n}$* (Dassule et al., 2000) and *K-14Cre;Smo$^{c/n}$* (Gritli-Linde et al., 2002) were from Dr. Amel Gritli-Linde, Göteborg University, Sweden.

Fgfr2b null allele mice were genotyped by PCR as described previously (De Moerlooze et al., 2000). In *ch* mice, a nonsense point mutation in the amino terminus of the DNA binding domain of *Foxc1* gene creates an early stop codon, and a non-functional Foxc1 protein (Kume et al., 1998; Hong et al., 1999).

To detect the C→T substitution in the *Foxc1* gene in *ch* mice solid-phase minisequencing was set up. 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 pmol of 5'-biotin-labeled reverse primer. Forward primer 5'-CTACCAGTTCATCATGGACCGC-3' and 5'-biotinylated reverse primer 5'-7CGAGCGTCCAGTAGCTGCCC-3' were used (7=methylation). PCR cycle: 95°C for 3 minutes, 63°C for 1 minute, 72°C for 1 minute, and 95°C 1 minute. Cycle was repeated 33 times, and the final products were extended at 72°C for 8 minutes. The PCR products were used as a template for solid-phase minisequencing as described in Suomalainen and Syvänen (1996); detection primer 5'-GGGACAATAAGCAGGGCTGG-3', ^{3}H-labelled dCTP (53.0 Ci/mmol specific activity, Amersham), and ^{3}H-labelled TTP (118 Ci/mmol specific activity, Amersham) were used. Radiolabelled dCTP was incorporated onto the detection primer bound to the wild type allele of *Foxc1*, and radiolabelled TTP to the mutated allele. The amount of radiolabel was measured in a scintillation counter (Wallac) using ^{3}H counting program. Counting the ratio of ^{3}H-dCTP and ^{3}H-TTP between duplicate samples indicated the nucleotide present at the variable site of the *Foxc1* gene. If the ^{3}H-dCTP/^{3}H-TTP ratio was >10, the genotype was wild type. A ratio between 0.5-2 corresponded to a heterozygous, and a ratio <0.1 corresponded to a mutant genotype.

Tissue dissection and culture

Calvarial explants: 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 Nuclepore polycarbonate filters, supported by grids, in Dulbecco's minimal essential medium (DMEM). DMEM was supplemented with GlutaMAX-1 (Life Technologies), 10% fetal calf serum (FCS), 100 μg/ml of ascorbic acid, and 20 IU/ml penicillin-streptomycin. Explants were cultured at 37°C in 5% CO_2 for 48 hours.

Palatal explants: E12.5 and E13.5 palatal shelf primordia were dissected from maxillary tissue and cultured adjacent to each other in DMEM supplemented as described. Palatal explants were cultured 24 - 48 hours.

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 hour at 37°C. Heparin coated acrylic

beads (Sigma) were used for recombinant human FGF2 or FGF10 (50 ng/μl, R&D Systems). 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 (PFA) 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 hours before transfer into the alizarin red staining solution (75 μg/ml of alizarin red in 1% KOH) and incubated 4-15 hours. 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

Calvaria: Pregnant *ch* heterozygote females were injected intraperitoneally (i.p.) with 2 ml/100 g body weight of undiluted 5-bromo-2'-deoxyuridine (BrdU) solution (Zymed) for E13 and E15 embryos. After 2 hours, embryos were collected, fixed in 4% PFA, and paraffin embedded. BrdU incorporation was immunodetected 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 per mouse were analyzed. 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 labelling (TUNEL) assay was performed using DeadEnd Colorimetric TUNEL System (Promega).

Palates: Mice were injected i.p. with 1ml/100 g body weight BrdU and sacrificed after 2 hours to collect E12 and E13 embryos. In palatal shelf explants in culture, the cell proliferation was assessed also by BrdU incorporation. Explants from E13 embryos were cultured 20 hours, and then BrdU pulsed for 4 hours (1:200 BrdU in culture medium). Embryos and palate explants were then treated as above for immunodetection of BrdU-positive cells, which were counted by 2 individuals.

^{35}S in situ hybridization

In situ hybridization on tissue sections was performed as described by Vainio et al. (1993). Briefly, paraffin sections were cleared from paraffin by xylene and rehydrated in ethanol series. Tissue sections were then permeabilized with proteinase K treatment, and hybridized overnight at 52 ºC with ^{35}S-UTP labelled riboprobes. After hybridization unspecifically bound and excess probe was removed by washes and RNase A treatment, dehydrated in ethanol series, and coated with autoradiography emulsion. Slides were exposed for 14 days, developed, fixed, counterstained with haematoxylin, dehydrated, and mounted with xylene-based DePex (BDH). The preparation of the following RNA probes has been described: *Alx4* (Hudson et al., 1998), *Bsp* (Rice et al., 1999), *Fgf3*, *Fgf7*, *Fgf10* (Kettunen et al., 1998; Kettunen et al., 2000), *Fgfr1b*, *Fgfr1c*, *Fgfr2b*, *Fgfr2c* (Rice et al., 2000), *Foxc1* (Hiemisch et al., 1998b), *Foxc2* (a kind gift from Dr. Kirsi Sainio), *Msx1* and *Msx2* (Jowett et al., 1993), *osteopontin* (a kind gift from Dr. Marian Young), *Shh* (Vaahtokari et al., 1996). Both bright and dark field images were taken from hybridized

sections. Silver grains were selected from the dark field image, coloured red, and superimposed onto the identical bright field image.

RESULTS AND DISCUSSION

Molecular biology of Foxc1 during calvarial development (Publication I)

The calvarial bones develop through intramembranous ossification so that first an osteogenic condensation must form followed by bone growth from the condensate towards the apex of the skull. To study what regulates and maintains this outgrowth and development of calvarial bones from the condensates, we studied *congenital hydrocephalus* (*ch*) mutants that develop only rudimentary calvarial bones, which do not grow beyond the sites of condensations. (Publication I).

The *ch* mutants lack functional winged helix/forkhead transcription factor Foxc1 due to a nonsense point mutation that causes an early stop codon at the amino terminus of the DNA binding domain (Kume et al., 1998; Hong et al., 1999). The *ch* mutant animals produce a truncated Foxc1 peptide consisting only of the amino terminal winged helix domain. It lacks the central DNA binding domain and carboxyl terminal winged helix domain. The lack of the DNA binding domain prevents the truncated Foxc1 peptide to bind to any target DNA. The *ch* mutants have an identical phenotype to *Foxc1* knock out mice (Kume et al., 1998). The major skeletal phenotype is the rudimentary calvarial bones but other skeletal elements are also affected including the ribs, sternum, and cranial base bones (Grüneberg, 1943; Grüneberg, 1953; Green, 1970; Kume et al., 1998; Hong et al., 1999).

Few years ago, Jiang et al. (2002) demonstrated that mouse calvarium is derived from two different origins so that the frontal bones and the dura mater underlying it are neural crest-origin, the parietal bones are of mesodermal origin while the dura mater underlying them is derived from the neural crest, and the interparietal bone and the underlying dura mater are mesodermal. In *ch* mutant animals the calvarial bones were affected similarly despite their differential mesenchymal origin or the origin of the underlying dura mater (Fig. 3C, F in Publication I). The initial osteogenic condensations form in *ch* mutants although Grüneberg (1943) showed that their size and shape is altered. The condensations for the frontal bones appeared broader and shorter in *ch* mutants (Fig. 2B in Publication I) but this may partly be due to a differential plane and angle of sectioning in comparison to wild type sections. We showed that in *ch* mutant calvaria the osteogenesis within condensations started at the right spatiotemporal window as indicated by the correct timing and location of expression of *Msx2*, *Alx4*, *Runx2* and *Bsp* at E13 (Fig. 2B, 4A-L, 4S-X in Publication I). But the osteogenesis at the growing edges of the condensations were suppressed as indicated by the reduced expression of *Msx2* and *Alx4* (Fig 4G-J, 4M-P in Publication I).

The massive hydrocephalus has been thought to prevent the growth of the calvarial bones in *ch* mutants due to increased intracranial pressure (Grüneberg, 1943; Kume et al., 1998). In vitro culture free of pressure should rescue the calvarial phenotype, at least partly, if the increased intracranial pressure is responsible for the lack of bone growth. When calvarial explants from *ch* mutants were cultured for seven days, their calvarial bones did not further their growth (Fig. 3 in Publication I). Further support for the intrinsic and primary role for Foxc1 in the calvarial phenotype came from detailed analysis of *ch* heterozygous embryos; they had an enlarged sagittal suture indicating delayed ossification (Fig. 3H in Publication I). These results together with the expression pattern of *Foxc1* in calvarial mesenchyme and in osteoblasts (Fig. 1A-D in Publication I) and the reduced expression of osteoprogenitor cell markers *Msx2* and *Alx4* (Fig. 4 in Publication I) support a dosage-sensitive role for Foxc1 in calvarial bone development and growth.

Msx2 regulates proliferation at the osteogenic fronts of postnatal sutures (Satokata et al., 2000), and as *Msx2* and *Alx4* transgenic animals show calvarial bone defects similar to *ch* mutant animals,. we hypothesized that Foxc1 maintains proliferation of osteoprogenitor cells in the early calvarial bones by positively regulating the expression of *Msx2* and *Alx4* in the osteogenic calvarial mesenchyme. To support this hypothesis, we showed that the proliferation of osteoprogenitor cells in the forming calvarial bones was significantly reduced in *ch* mutant animals (Fig. 6A and B in Publication I), and that Foxc1 was required for Bmp-induction of *Msx2* and *Alx4* in calvarial mesenchyme (Fig. 5B in Publication I). What is of special interest and highlights the importance of Msx2 and Alx4 in regulating the proliferation of osteoprogenitor cell population is that the calvarial mesenchyme in *ch* mutants did not lack osteogenic potential. For instance *Msx1* expression was normal in *ch* mutants, and it was BMP-inducible in the *ch* mutant calvarial mesenchyme unlike *Msx2* and *Alx4* (Fig. 4M-R, 5B in Publication I). Also, the expression of *type I collagen* was normal in *ch* mutant calvarial mesenchyme, as was *Twist* expression that is regulated by Fgf signalling (data not shown, unpublished results). Importantly, ectopic FGF2 induced expression of *osteopontin*, which is expressed by osteoblasts, in *ch* mutant calvarial mesenchyme (Fig. 4, unpublished results).

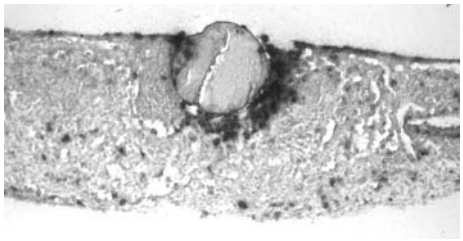

Figure 4. Induction of *osteopontin* by rhFGF2 does not require Foxc1. E15 calvarial explant of *ch* mutant shows induction of *osteopontin* by rhFGF2 bead. This induction demonstrates that the calvarial mesenchyme has osteogenic potential in *ch* mutants.

The combination of lack of *Msx2* and *Alx4* expression and cell proliferation in the calvarial mesenchyme in *ch* mutant animals suggests that Foxc1 plays an important role in maintaining osteoprogenitor cells proliferative and thus preventing too early differentiation and depletion of osteoprogenitor cells. In support of this, the calvarial mesenchyme in the *ch* mutant animals responded to osteoblast-inducing signals. Similar functions have been proposed to other Fox family members: Foxc2 has been shown to control the proliferation of sclerotome-derived cells (Winnier et al., 1997), and Foxe3 has been shown to be essential for maintaining lens epithelial cell proliferation and preventing their premature differentiation (Blixt et al., 2000).

An intriguing aspect of *Foxc1* expression pattern is that although the transcripts localized to osteoprogenitor cells and in mature osteoblasts, they were not found in osteogenic condensations. One possibility is that osteogenic condensations need to stop proliferation to allow differentiation to occur. Another possibility is that Foxc1 participates in the regulation of adhesive properties or composition of extracellular matrix of osteogenic cells. This would not be mutually exclusive with regulation of cell proliferation levels. Interestingly, Foxc1 appears to play differential roles in chondrogenesis as it is expressed in chondrogenic mesenchymal cells and condensations but not in mature chondrocytes.

During calvarial bone formation the Bmp signal may arise from both the calvarial mesenchyme itself or it may come from the underlying dura mater as at least *Bmp2*, *4*, and *7* are expressed in both locations (Fig. 5A in Publication I). Some inductive signals originating from dura mater may play a role in directing the growth of calvarial bones since their growth in *ch* mutants appears to have stopped just prior to contact with the underlying dura mater (Fig. 3C, F in Publication I). Dura mater and subarachnoid layer of the meninges have been reported to be abnormal in *ch* mutants (Green, 1970; Kume et al., 1998) yet their meningial layers express *Bmp*s (Fig. 5A in Publication I). Regulating Bmp signalling may be a general function of Foxc1 as sternal micromass cultures from *ch* mutants failed to differentiate even with added BMP2 (Kume et al., 1998).

Foxc1 and a closely related gene *Foxc2* have distinct yet extensively overlapping expression patterns in neural crest and paraxial mesoderm and in their derivatives (Hiemisch et al., 1998; Publication I). Null allele *Foxc2* animals show a phenotype that is both similar to and different from the phenotype of *Foxc1* mutant animals: the main difference is that the appendicular skeleton and the sternum are intact in *Foxc2* mutants while they are affected in *Foxc1* mutants; *Foxc2* mutants exhibit a complete cleft of the secondary palate while *Foxc1* mutants have not been reported to have any defects in their palatal development; and importantly, no calvarial abnormalities have been reported in *Foxc2* animals (Iida et al., 1997; Winnier et al., 1997; Kume et al., 1998; Hong et al., 1999). Studies on compound heterozygous *Foxc1*+/-;*Foxc2*+/- embryos during heart, kidney, eye, and somite development has shown that Foxc1 and Foxc2 have similar, dose-dependent functions, and that they can compensate for each other (Kume et al., 2000, 2001; Smith et al., 2000). In double heterozygous *Foxc1*+/-;*Foxc2*+/- animals no gross malformations have been detected in the developing skeleton indicating that these genes do not interact during skeletogenesis (Winnier et al., 1999). The compound homozygous *Foxc1*-/-;*Foxc2*-/- animals have a very small first branchial arch, and they lack second branchial arch and segmented paraxial mesoderm (Kume et al., 2001). Severe defects in the first and second branchial arches indicate that facial skeleton would be defective, and that both Foxc1 and Foxc2 function during facial development. Skeletal phenotype in compound homozygous animals can not be studied as these embryos die at E9.0 - 9.5 due to heart failure (Kume et al., 2001). Although the expression patterns of *Foxc1* and *Foxc2* are extensively overlapping during embryonic development, we have shown that *Foxc2* was not expressed in the calvarial mesenchyme and thus can not compensate for the lack of *Foxc1* in that tissue (Fig. 1 in Publication I). *Foxc2* was expressed in the dura mater together with *Foxc1* (Fig. 1 in Publication I).

Publication I showed that Foxc1 regulates Bmp signalling but Foxc1 itself does not appear to be a positive downstream target of Bmp signalling as BMP beads in the calvarial mesenchyme at E15 did not induce expression of *Foxc1* (data not shown). *Foxc* genes have been shown to be under Shh regulation in the developing head mesenchyme at E9 and to direct expression of *Tbx1* in the developing facial structures and the aortic arch (Yamagishi et al., 2003). Mahlapuu and her colleagues (Mahlapuu et al., 2001) demonstrated that *Shh* null allele animals lack expression of *Foxf1* in the developing lung, and that Shh-secreting cell pellets on lung mesenchyme activated the expression of *Foxf1*. Furthermore, Mahlapuu et al. (2001) showed that lung epithelial Bmp signalling negatively regulates the mesenchymal expression of *Foxf1*, and that Fgf signalling inhibits the expression of *Foxf1* by inhibiting Shh signalling (FGF7) and by activating Bmp signalling (FGF10). Both of these Fgf ligands use the epithelial receptor Fgfr2b (Mahlapuu et al., 2001). In the mouse calvarium, the major Fgf receptor isoform has been shown to be Fgfr2c during suture formation (Kim et al., 1998; Rice et al., 2000; Publication II). Fgf signalling can activate Bmp signalling during suture fusion by

suppressing the expression of Bmp antagonist *noggin* (Warren et al., 2003). Hedgehog signalling has also been shown to play a role during suture fusion (Kim et al., 1998). Presently, I am investigating possible roles for Foxc1 as a link between Fgf, Hh, and Bmp signalling during calvarial development.

Isoform-specific Fgfr expression patterns in craniofacial skeleton during development (Publication II)

To understand where Fgf signalling pathway may function during development, it is essential to study the tissue localization of the receptors in detail. Four different Fgf receptor genes (Fgfr1-4) have been identified of which receptors 1-3 show alternative splicing that creates different receptor isoforms. Especially, the alternative splicing at the coding region of the extracellular ligand-binding immunoglobulin-like domain III changes the expression domain and the ligand specificity of the receptor isoform so that the same gene yields two different receptors: the epithelial IIIb and mesenchymal IIIc isoforms (Kannan and Givol, 2000; Ornitz and Marie, 2002). Recognizing differential or overlapping expression patterns of *Fgfr* isoforms is important when aiming to understand human pathological conditions caused by gain-of-function mutations in different *FGFR* isoforms. These mutations can cause ligand-independent dimerization of the receptor and thus activate signalling; they can extend the duration of the signalling, alter ligand specificity, or cause isoform switching (Ornitz and Marie, 2002.).

In the craniofacial region, the *Fgfr* isoform expression patterns have been carefully studied during molar (Kettunen et al., 1998) and sagittal suture (Kim et al., 1998; Rice et al., 2000) development in the Thesleff laboratory. Coronal sutures have been shown to express *Fgfr*s (Iseki et al., 1997) but this study did not distinguish between b and c isoforms. In Publication II we showed that also coronal and lambdoidal sutures express *Fgfr* isoforms in the same pattern as sagittal suture: the proliferative preosteoblast cell population in osteogenic fronts of the calvarial bones express mainly *Fgfr2c*, with weaker intensity *Fgfr1c*, and very weakly *Fgfr2b* (Fig. 6 in Publication II). Based on the *Fgfr* isoform expression patterns, it appears that all established calvarial sutures utilise Fgf signalling similarly through *Fgfr2c* and *Fgfr1c*.

Craniosynostosis patients exhibit multiple skeletal malformations including abnormalities in the limbs, cranial base and face (Wilkie and Morriss-Kay, 2001). Thus, we also detailed *Fgfr* expression patterns in cranial base synchondroses, mandibular condyle, body and alveolus. Synchondroses are growth sites where endochondral ossification regulates the growth of the cranial base bones. *Fgfr1c* was expressed only by osteoblast-forming cells in the erosive zones of the synchondroses while *Fgfr2b* and *Fgfr2c* had overlapping expression patterns in the periosteum and perichondrium suggesting paracrine mode of function for chondrocytes (Fig. 3A-D in Publication II). *Fgfr2b* and *Fgfr2c* were also expressed very weakly by the resting and proliferative chondrocytes (Fig. 3A-D in Publication II). *Fgfr3b* and *c* isoforms were expressed by prehypertrophic and hypertrophic chondrocytes so that the *Fgfr3b* transcript domain was more restricted than that of *Fgfr3c* (Fig. 3E-F in Publication II). This expression pattern suggests that *Fgfr3* isoforms play a cell-autonomous role in the function of chondrocytes. Similar expression domains were seen in mandibular condyle, which is a secondary cartilage that functions as a joint connecting the mandible to the temporal bone: *Fgfr2* isoforms in the perichondrium and *Fgfr3* isoforms in the (pre)hypertrophic chondrocytes (Fig. 4 in Publication II). It is of interest to note that the expression pattern of *Fgfr*s in synchondroses and mandibular condyles is similar to that in growth plates in long bones (reviewed in Ornitz and Marie, 2002).

Wang et al. (2001) demonstrated in *ColII-Fgfr3*^{ach} transgenic mice that suppression of mitogenic activity by Fgf signalling is a unique property of growth plate chondrocytes. As chondrocytes in synchondroses and growth plates express *Fgfr*s in a similar pattern we suggest that some of the cranial base malformations, such as short cranial base or premature fusion of synchondrosis, in craniosynostosis patients arise by similar mechanisms as their limb phenotypes.

Fgfr expression pattern was shown to be the same in all calvarial sutures despite their tissue origins, and the same was true for cranial base synchondroses (Figs 3 and 6 in Publication II). For instance the basioccipital synchondrosis is between the somitomere-derived occipital bone and the paraxial mesoderm-derived basisphenoid bone, and intersphenoidal synchondrosis is between the paraxial mesoderm-derived basisphenoid bone and the cranial neural crest-derived presphenoid bone (Couly et al., 1993).

The expression patterns studied in Publication II help to explain various craniofacial malformations found in patients with *FGFR* mutations. Overlapping expression of b and c isoforms by the same cell population in the developing calvarial sutures and cranial base synchondrosis allows usage of multiple ligands, especially if a mutation has caused aberrant isoform switching. This has been shown to be the case in *Fgfr2c* hemizygous mice in which partial inactivation of an exon specific to the *Fgfr2c* isoform caused ectopic activation of the *Fgfr2b* isoform (Hajihosseini et al., 2001).

Interestingly, Pro250Arg mutation in Fgfr1, which causes craniosynostosis, does not cause any significant differences in long bones or synchondroses (Zhou et al., 2000). The mutation may be specific to osteoblast lineage or intramembranous ossification. This is supported by our *Fgfr* expression pattern analysis in various craniofacial structures which indicated that *Fgfr1* was only expressed by osteoblast-forming cell populations and not by chondrocytes.

Fgf signalling is essential for palatal shelf outgrowth (Publication III)

Fgf signalling has been shown to direct the distal outgrowth of facial prominences in chick (Richman et al., 1997). The vertebrate face develops through frontonasal, two lateral nasal, two maxillary, and two mandibular prominences that are mesenchymal buds surrounded by ectoderm. Palatal shelves again are outgrowths of the two maxillary prominences (Ferguson, 1998).

We used *Fgf10* and *Fgfr2b* null allele animals, which both exhibit cleft palate (Publication III and De Moerlooze et al., 2000, respectively), as model systems to study the role of Fgf signalling in palatal development. Both mutant animals exhibited a wide cleft of the secondary palate so that anterior and posterior palate were equally affected at birth (Fig. 1A-C in Publication III). We first identified abnormalities in the development of secondary palatal shelves in these mutants at E13 when the morphology of the palatal shelves was short and square, and the epithelium was thin and non-stratified (Fig. 1D-I in Publication III). Both in the *Fgf10* and *Fgfr2b* null allele animals the stubby palatal shelves elevated to a horizontal position correctly but were too short to meet with each other at the midline. To study whether the mutant palatal shelves were able to fuse, we placed palatal shelves from *Fgfr2b* null allele litter next to each other in a correct anterior-posterior orientation in organ culture. All genotypes fused in culture and showed a continuous mesenchyme, which indicated that fusion had been complete (data not shown). This ruled out the possibility of defective palatal shelf fusion being responsible for the cleft palate.

We analyzed the expression patterns of *Fgfr2b* and its ligands *Fgf10*, *Fgf7*, and *Fgf3* during palatal development. Fgf22 is also a ligand for Fgfr2b but it is not expressed before E16.5 in mouse embryos (Beyer et al., 2003). This is after palatal shelves have

fused, and thus its expression pattern was not analyzed. *Fgfr2b* was expressed in the epithelium while ligands were expressed in the mesenchyme (Fig. 2 in Publication III and data not shown). *Fgf3* was not expressed in the developing palate, but *Fgf7* and *Fgf10* showed a differential mesenchymal expression pattern so that *Fgf10* expression was found mainly in the mesenchyme adjacent to the oral epithelium, and the expression of *Fgf7* was restricted to the mesenchyme adjacent the nasal epithelium until E14 when it was also expressed in the mesenchyme adjacent to the oral epithelium. As nasal and oral epithelium in palatal shelves differ from each other it is possible that Fgf7 and Fgf10 may play a role in setting this difference. Fgf10 transcripts were also found in the mesenchyme in the 'bend' region on the nasal side of the developing palate. Thus, overlapping expression domains for *Fgf7* and *Fgf10* were the 'bend' region and the medial edge epithelial region (Fig. 6 in Publication III). These expression patterns support a specific function for Fgf signalling in the palatal development.

Previously *Fgf10* and *Fgfr2b* null allele animals have been studied in detail during limb and lung development (Min et al., 1998; Sekine et al., 1999; De Moerlooze et al., 2000; Revest et al., 2001). These studies demonstrated that Fgf10/Fgfr2b signalling pathway regulates cell proliferation, apoptosis and survival, and that it is required for outgrowth of a bud through epithelial-mesenchymal interactions. We studied these mutants for possible changes in the level of proliferation in the palate (Fig. 3 and 4 in Publication III). It is of interest to note that in wild type animals at E12 and E13, the posterior MEE region had more proliferative cells than the anterior MEE region. The palatal mesenchyme reflected the same results (Fig. 3 in Publication III). Similarly, cranial base bones have been shown to have a posterior to anterior growth gradient (Roberts and Blackwood, 1984). In both mutants the epithelial cell proliferation was reduced significantly while cell proliferation was reduced only slightly in the mesenchyme. As Fgf10 signals from the mesenchyme to its receptor in the epithelium, it made sense that the level of proliferation was affected in the epithelium. The reduction in the level of mesenchymal cell proliferation suggested that there may be a signal coming back to mesenchyme from the epithelium to maintain its survival and proliferation. To confirm that Fgf10 plays a proliferative role in the developing palate, we placed FGF10-containing beads on the epithelium of palatal explants cultured in isolation. Ectopic FGF10 induced proliferation in the epithelium and also to a lesser extent in the underlying mesenchyme (Fig. 5 in Publication III).

We also performed TUNEL analysis on the mutants to test whether cell survival was altered when Fgf10/Fgfr2b signalling was blocked. In wild type animals prior to E14 there were no apoptotic cells in the oral epithelium while mutants showed isolated areas of TUNEL-positive, apoptotic cells in the patches of thickened epithelium already at E13 (Fig. 4 in Publication III). This suggested that Fgf10/Fgfr2b signalling pathway maintains outgrowth of the palatal shelves by acting both as a proliferative and a survival factor.

Fgf10/Fgfr2b signalling has been shown to regulate the expression of *Shh* in limb buds (Sekine et al., 1999; Revest et al., 2001). Similarly, we showed that *Shh* is a downstream target of Fgf10/Fgfr2b signalling in the epithelium of the developing palate as its expression was downregulated in the palatal epithelium in both *Fgf10* and *Fgfr2b* null allele animals (Fig. 5D-I in Publication III), and FGF10-containing beads induced expression of *Shh* in the palatal epithelium (Fig. 5B-C in Publication III). To study the role of Shh in the early palatal development, and especially to see whether it has a reiterative effect back to the palatal mesenchyme, we placed human recombinant SHH-containing beads on palatal explants in culture. SHH-containing beads induced proliferation in the palatal mesenchyme (Fig. 5, Publication III). To further support the role of Shh regulating mesenchymal proliferation, we demonstrated that *patched (Ptc)*, the

receptor of Shh, was not expressed in the palatal epithelium but in the mesenchyme. Interestingly, also FGF10-containing beads were sufficient to induce *Ptc* expression in the mesenchyme (Fig. 5, Publication III). These results further support our hypothesis that Shh is a downstream target of Fgf10/Fgfr2b signalling in the early palate, and that this signalling network is responsible for the growth and survival of the epithelium and mesenchyme in the palatal shelves.

The mesenchymal Bmp4/Msx1 signalling has been shown to induce *Shh* expression in the palatal epithelium (Zhang et al., 2002). Thus, it appears that *Shh* is under complex regulation by both Bmp and Fgf signalling pathways during palatal shelf growth. Further support for Shh signalling regulating mesenchymal proliferation and survival and/or differentiation in the developing palate comes from transgenic animals lacking *Shh* specifically in the epithelia (*K-14Cre;Shh*$^{c/n}$, Dassule et al., 2000). These conditional *Shh* null allele animals exhibited a complete cleft palate similar to *Fgf10*-/- or *Fgfr2b*-/- animals (Fig. 5I-J in Publication III). The role of Shh as a mesenchymal regulator was emphasized in transgenic animals where a Shh downstream signalling target *smoothened (Smo)* was removed from epithelia (*K-14Cre;Smo*$^{c/n}$, Gritli-Linde et al., 2002). These animals had normal palatal development (data not shown).

Taken together, we have shown that palatal epithelium functions to direct growth and survival of the adjacent mesenchyme. This signalling requires a network that combines Fgf10/Fgfr2b and Shh signalling.

CONCLUSIONS

The publications composing this thesis were aimed at looking early stages of craniofacial bone and palate development and how their growth is regulated. I have shown that the level of proliferation in the progenitor cell populations is an important factor in the growth and patterning of an organ as either calvarial bones or palatal shelves will not develop if level of proliferation is altered. Proliferation is regulated by growth factor signalling so that an accurate balance of proliferation and differentiation is achieved in the appropriate progenitor cell populations.

I have demonstrated a novel role for transcription factor Foxc1 in controlling Bmp regulation of transcription factors Msx2 and Alx4 in calvarial mesenchyme, and how this regulation is needed for proliferation of osteoprogenitor cells in early calvarial bones. There are still many unanswered questions. What regulates Foxc1? Does Foxc1 regulate Bmp signalling through contact with Smads? What is the role of Foxc1 in the developing meninges and does this relate to calvarial bone formation?

I have also shown that the outgrowth and morphogenesis of the palatal shelves are regulated and maintained through reiterative Fgf and Shh signalling networks in palatal buds. This work raises the developing palate into the category of organs where morphogenesis starts as a bud, and the growth is patterned through network of growth factor signals that dart back and forth between the epithelium and mesenchyme. It will be exciting to see in the future whether the differential expression of *Fgf7* and *Fgf10* determines the nasal and oral epithelium in the developing palatal shelves, and why they are coexpressed in the medial edge epithelium and the ‘bend’ region. Could there be some signalling centre function in these locations?

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