BONE MORPHOGENETIC PROTEIN SIGNALING MOLECULES IN FACIAL MORPHOGENESIS AND DYSMORPHOGENESIS

by

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ABSTRACT

Facial morphogenesis is a dynamic multi-step process involving the formation of the neural crest cell derived facial prominences, their coordinated outgrowth, fusion and differentiation to generate the final facial form. Defects in each of these processes are associated with facial dysmorphogenesis. The identification of genes that are involved in cleft lip is the first aim of this thesis. PCR based differential display was used to compare gene expression profiles in cleft and non-cleft chicken embryos. The second manner to identify candidate genes involved in orofacial clefting was by examining expression patterns of previously cloned genes at the specific time and place where lip fusion occurs. This approach identified Bone morphogenetic proteins (BMPs) as potential genes that are involved with the outgrowth and fusion of facial prominences. Little was known about where BMP receptors and BMP antagonists are expressed in the face or the function of endogenous BMPs in the face. The expression of BMP ligands (*Bmp-2*, *Bmp-4* and *Bmp-7*) was correlated with the expression of BMP receptors (*BmpR-IA*, *BmpR-IB* and *BmpR-II*) and *noggin*, a BMP antagonist, in order to predict where endogenous BMP signaling occurs in the face. *Bmp-2*, *Bmp-4* and *Bmp-7* are expressed in the zone of fusion between the frontonasal mass and maxillary prominences. *Noggin* expression is restricted to the frontonasal mass epithelium and is downregulated at the corners of the frontonasal mass just prior to fusion. Later in development, *Bmps* are expressed in the perichondrium of cartilage whereas *noggin* is expressed within differentiating cartilage. The *BmpR-IA* and *BmpR-II* are expressed ubiquitously whereas *BmpR-IB* is expressed in a subset of tissues. This expression data was used to design studies that examined BMP function during primary palate closure and facial cartilage morphogenesis.

Noggin protein was applied to regions with high BMP expression in the zone of fusion and subsequently induced clefts. These experiments show for the first time that endogenous BMPs regulate two aspects of lip closure; outgrowth of facial prominences and thinning of the frontonasal epithelium prior to fusion. We also examined the well-established retinoic acid model for cleft lip to see whether BMPs were mediating this defect. Ectopic retinoic acid increases *Bmp-2* and *Bmp-4* expression prior to the induction of cleft lip in chickens. Mimicking this result by applying BMP-2 to the face also induced clefts. These results are related to non-syndromic human orofacial clefting and demonstrate the utility of studying cleft lip in non-mammalian model systems.

Finally, experiments were carried out that would directly test the function of the BMP receptors in craniofacial development. Whereas expression of dominant-negative (dn) and constitutively active (ca) BMP type I receptors seldom affected early facial morphology, there were significant effects on chondrogenesis. These mutant forms of the type IA and IB receptors regulated the size and shape of cartilage elements. The dn form of the BMPRIB inhibited chondrogenesis, whereas ca-BMPRIA and ca-BMPRIB increased cartilage formation. The embryos injected with active viruses also lacked feather germs over much of the head and about 50% of specimens did not form an egg tooth. It appears that type IA and type IB receptors play similar roles in regulating cartilage and feather formation in the skull. Overall, these findings identify BMP signalling molecules as critical regulators of facial morphogenesis and dysmorphogenesis.

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LIST OF ABBREVIATIONS

BMP	Bone morphogenetic protein
BMPR	BMP receptor
°C	degrees centigrade
ca	constitutive active
CDMP	cartilage-derived morphogenetic protein
cDNA	complimentary DNA
CL/P	cleft lip with or without cleft palate
CO_2	carbon dioxide
CRBP	cellular RBP
D	aspartic acid
dNTP	deoxynucleotide triphosphate
DEPC	diethyl pyrocarbonate
DD	differential display
DMSO	dimethyl sulfoxide
dn	dominant negative
DNA	deoxyribonucleic acid
DPP	decapentaplegic
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
FGFR2	FGF receptor 2
FHF	Fibroblast growth factor homologous factor

GDF	growth and differentiation factors
GITC	guanidium isothiocyanate
GS	glycine-serine
h/hr	hour
Hox	homeobox
L/ml/µl	liter/milliliter/microliter
mRNA	messenger RNA
Msx	Muscle-specific homeobox
Na	sodium
N-CAM	neural-cell adhesion molecule
OP	osteogenic protein
ptc	patched
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PNA	peanut agglutinin
Q	glutamine
R	rhombomere
RA	retinoic acid
RALDH	retinal dehydrogenase
RAP	RNA arbitrarily primed
RAR	retinoic acid receptor
RARE	RA response elements

RBP	retinol binding protein
RBPr	RBP receptor
RDH	retinol dehydrogenase
RT	reverse transcription
RNA	ribonucleic acid
Rpm	revolutions per minute
RXR	retinoid X receptors
Sax	Saxophone
SHH	sonic hedgehog
^{3}H-TdR	labeled tissue Noden, 1975
TGF-β	transforming growth factor-beta
Tkv	Thick veins
TEM	transmission electron microscopy
TUNEL	terminal deoxynucleotide transferase (TdT) mediated dUTP-digoxygenin nick end labelling
VAD	vitamin A deficiency
%	percentage

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For giving me the patients to persevere I thank God, who is, incidentally, the only teacher who is always right. He revealed to us over 1400 years ago what we learn with amazement today:

> *"We created humans from a quintessence of mud. Thereafter we cause him to remain as a drop of sperm in a place of rest (ie. the womb). Thereafter we fashioned the sperm into something that clings (to the endometrium), which We fashioned into a chewed lump (somite stage). The chewed lump is fashioned into bones, which are then covered with flesh. We develop out of it another creation. Blessed is God, the best of artisans. After that, at length ye will die. Again ye, on the Day of Judgement, will be raised up (and be held accountable for your actions)."*
>
> *Al-Quran 23:12-16*

CHAPTER I

REVIEW OF THE LITERATURE

Normal face development

In vertebrates, the face is formed during several sequential phases of development. The first phase involves the formation of cranial neural crest cells in the dorsal neural tube. Second, the neural crest cells migrate ventrally, populating the presumptive facial prominences. These prominences are buds of mesenchyme encased in epithelium that surround the primitive oral cavity. Third, the neural crest-derived mesenchyme undergoes a period of rapid proliferation and the facial prominences quickly enlarge in size. Fourth, the facial prominences fuse together at the base of the nasal pit, which establishes the basic plan of the face. In the fifth phase, the facial mesenchyme differentiates to form muscle, cartilage and bone (Sperber, 2001).

Origin of Tissues

The origins of mesenchyme in the face are unique compared to other areas of the body. The majority of trunk mesenchyme is derived from the middle germ layer or mesoderm. In the face there is an additional contribution from cranial neural crest cells.

Neural crest formation and migration

In early brain development, three bulges form at the cephalic end of the neural tube: forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain

(rhombencephalon). The rhombencephalon is divided into eight periodic swellings called rhombomeres (r1-8; Lumsden et al., 1991). As the neural tube closes along the dorsal midline, the cells occupying the crest of the neural folds, known as neural crest cells, migrate to different locations and have a wide variety of fates. Cranial neural crest cells migrate ventrally beneath the head ectoderm, through the paraxial mesoderm (somitomeres) and into the face between the 6 and 9 somite stages (24-30 h incubation; Tosney, 1982; Noden, 1975). By grafting ^{3}H-TdR labeled tissue (Johnson, 1966; Noden, 1975) and, more recently, using DiI labeling (Lumsden et al., 1991) the pathways of migration of cranial neural crest into the developing facial prominences have been elucidated. Neural crest cells from regions anterior to rhombomere 3 contribute to the face. The frontonasal mass lies between the nasal placodes and is populated by neural crest cells from the diencephalon (Le Lièvre, 1978; Couly et al., 1993). The lateral nasal prominences are lateral to the nasal placodes and are populated by diencephalic and mesencephalic crest cells (Le Lièvre, 1978). The maxillary prominences are inferior to the lateral nasal prominences and are populated by posterior diencephalic and mesencephalic crest (Le Lièvre, 1978). The mandibular prominences form the inferior border of the stomodeum and have a dual contribution. The distal portion of the mandible is populated by mesencephalic (Couly et al., 1996; Noden, 1978; Le Lièvre, 1974) and the proximal portion is derived from r1 and r2 neural crest cells (Couly et al., 1996; Köntges and Lumsden, 1996).

Somitomeric mesoderm

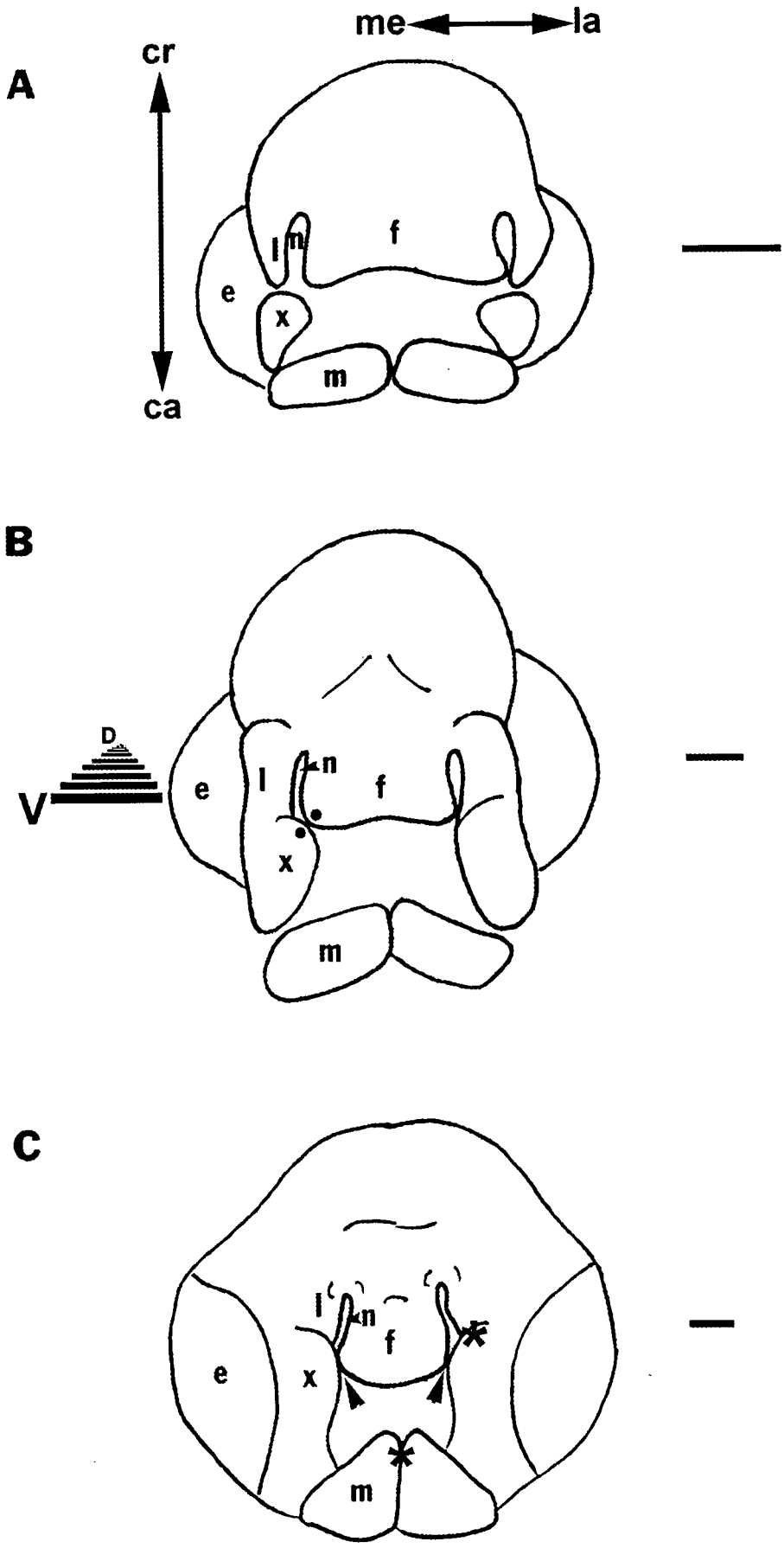
The paraxial mesoderm becomes segmented into whorls of cells called somitomeres, which form along the length of the embryo during gastrulation (Meier, 1979). In the trunk, the somitomeres become compacted and bound together by epithelium to form individual somites (Keynes and Stern, 1988). Cranial somitomeres do not become somites, but rather remain adjacent to the neural tube or disperse to the central regions of the branchial arches (Hacker and Guthrie, 1998; Trainor and Tam, 1995). In chickens, the first pair of somitomeres form next to the prosencephalon while the remaining somitomeres extend caudally and are adjacent to more posterior brain regions (Meier, 1982; Noden, 1983a).

Outgrowth

The difference in embryonic origin may be important in the behaviour of the facial mesenchyme as it grows and differentiates. Following neural-crest cell migration to the face, the facial prominences quickly enlarge (stage 20-28; Hamburger and Hamilton, 1951; equivalent to 3.5 to 5.5 days post-fertilization). The prominences proliferate at different levels and proliferation within the facial mesenchyme of each individual prominence is not uniform (McGonnell et al., 1998; Peterka and Jelinek, 1983; Patterson and Minkoff, 1985; Minkoff and Kuntz, 1977; 1978). These regional differences in proliferation as well as the direction of expansion of the facial mesenchyme (McGonnell et al., 1998) contribute to the changes in shape of the embryonic face as development proceeds (Figure 1.1). Growth of the facial prominences is dependent on epithelial-

Figure 1.1 Schematic diagram of the embryonic chicken faces during outgrowth and differentiation of the facial prominences. Sketches are of the frontal view at stages 20 (A), 24 (B) and 28 (C). Depicted in A is the superior (or cranial)-inferior (or caudal) and medial-lateral axis. Depicted in B is the dorsal-ventral axis, and positions (●) of protein soaked bead placement in the corner of the frontonasal mass and cranial maxillary prominence for studies described in Chapter 4. Arrowheads in C identify regions where fusion occurs between the frontonasal mass and maxillary prominences and * indicates areas where merging occurs. Take note of the changes in shape of the facial prominences as development proceeds. This is a modified version of Fig. 1 in Francis-West et al., 1994.

Key: ca, caudal; cr, cranial; d, dorsal; e, eye; f, frontonasal mass; l, lateral nasal prominences; la, lateral; m, mandibular prominences; me, medial; n, nasal slit; v, ventral; x, maxillary prominences. Scale bars = 500 μm.


A
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mesenchymal interactions, as the epithelium regulates outgrowth (Wedden, 1987; Richman and Tickle, 1989; Hu and Helms, 1999).

Fusion and merging of facial prominences

Two different processes consolidate the facial prominences that form the upper lip, merging and fusion. Merging prominences have a groove or furrow between them, which is filled in by the rapid proliferation of mesenchymal cells. Fusion, however, occurs between regions that are not connected, and therefore requires removal of the double layer of epithelium that forms when the prominences come into contact. Following removal of the epithelial seam there is ingrowth of mesenchyme to form a mesenchymal bridge.

In the face, a combination of fusion and merging takes place. Merging occurs between the paired mandibular prominences, and between the maxillary and lateral nasal prominences. In chickens, fusion takes place between the premaxilla and the distal end of the palatal shelves. The remainder of the secondary palate remains open in birds, unlike in mammals. Fusion also takes place between the lateral corners of the frontonasal mass and the maxillary prominences. The lateral edges of the frontonasal mass (the globular processes) in birds are equivalent to the medial nasal prominences in mammals. The frontonasal mass and maxillary prominences are already joined in proximal regions of the head but merge distally as cell proliferation and outgrowth proceeds. Fusion only occurs at the distal tips of the maxillary and lateral frontonasal regions. The frontonasal mass, lateral nasal and maxillary prominences must all come together in order to close

the primary palate and establish a smooth upper lip. Failure of the frontonasal mass and the maxillary prominences to fuse results in cleft lip.

Complete fusion involves removal of the apposed epithelia and the basement membrane components before mixing of mesenchymal cell populations can proceed (Iamaroon and Diewert, 1996). In the chicken embryo the distal tips of the facial prominences have not yet fused at stage 28. At stage 29, however, fusion is complete and mesenchymal breakthrough has taken place. Three theories have been proposed to explain the disappearance of the double layer of epithelium: 1) epithelial cells migrate to adjacent areas (Carette and Ferguson, 1992); 2) epithelial cells transform to mesenchymal cells (Fitchett and Hay, 1989; Martinez-Alvarez et al., 2000; Sun et al., 2000); and 3) epithelial cells undergo apoptosis (Clarke, 1990; for review see Shuler, 1995). Epithelial apoptosis can be detected in the anterior maxillary prominences, the groove between the lateral nasal and maxillary prominences and the corners of the frontonasal mass in stage 24 and 28 chicken embryos (McGonnell et al., 1998; Shen et al., 1997). Recently, using TUNEL assay and transmission electron microscopy (TEM), Sun et al. (2000) showed that apoptosis in the primary palate is restricted only to the outer epithelial layer, the periderm, and functions to thin the epithelium during fusion. The basal epithelial layer makes up the epithelial seam and is removed soon after by epithelial-mesenchymal transformation. Therefore, in the chicken primary palate, the epithelium between the fusing prominences is removed by a combination of processes. Their TUNEL procedure may not be very sensitive, however, since others have reported that apoptosis is not only in the epithelium but also in the mesenchyme near regions where fusion occurs

(McGonnell et al., 1998; Shen et al., 1997). The molecular regulators of endogenous programmed cell death in the fusing facial prominences have not been identified.

Evidence of bidirectional cell mixing was reported in proximal face regions between the anterior maxillary prominences and the distal frontonasal mass while unidirectional migration of the cells occurred from the frontonasal mass into the lateral nasal process (McGonnell et al., 1998). These studies were performed between stages 20 and 28, prior to fusion of the distal tips of the prominences. In addition, their labeling was done near the base of the prominences, in regions that undergo merging as opposed to fusion. Little is known about how the mesenchymal bridge forms (see Sun et al., 2000). What is known is that there needs to be a sufficient volume of mesenchyme or the resulting bridge may break down (shown in A/WySn mouse embryos; Wang and Diewert, 1992; Diewert and Wang, 1992).

Skeletal derivatives of the facial prominences

The frontonasal mass forms most of the upper beak and gives rise to the interorbital septum, the nasal septum, the prenasal cartilage and the premaxilla. The lateral nasal processes form the alae of the nose and give rise to the nasal conchae, nasal and lachrimal bones. Externally, the maxillary prominences form the sides of the upper beak and internally, the palatal shelves. The maxillae form exclusively membranous bones, including the pterygoid, jugal, quadratojugal, palatine and maxillary bones. The paired mandibular prominences give rise to the lower beak. The skeletal derivatives of the mandibular prominences include, Meckel's cartilage, the retroarticular process, the

mentomandibular bone, the dentary bone, the supraangular bone and the angular bone. Each facial prominence therefore contributes to a distinct region of the face (Figure 1.2). The prominences retain their distinct derivatives even when transplanted to ectopic sites (Wedden et al., 1987; Richman et al., 1997; Richman and Tickle, 1989, 1992). By stage 20, pattern is completely specified in the facial mesenchyme.

Differentiation

Chick/quail chimeras have been extensively used to decipher the extent to which the cranial neural crest and mesodermal populations contribute to the tissues of the face. Derivatives of cranial neural crest cells include cartilage and bone, which are unique to the cranial region, in addition to derivatives also made by trunk neural crest cells such as pigment cells (melanocytes), sensory ganglia, parasympathetic ganglia and endocrine cells (Couly et al., 1996; Köntges and Lumsden, 1996; Couly et al., 1993; Noden 1983b; reviewed in Le Douarin et al., 1994; Hall and Hörstadius, 1988).

Neural crest cells contribute to ventral head skeletal structures, including all cartilage and bone in the face, while the mesoderm differentiates to form bones of the chordal skull and cranial vault (Noden, 1983a, Noden, 1988; Table 1.1). The avian head is unique because it forms bone via both endochondral and intramembranous formation and has cartilage that does not ossify (Fig. 1.2; Table 1.1). Derivatives of the somitomeric mesoderm also include the muscles (myoblasts) and angioblasts, the posterior cranial base, otic capsule and calvaria (Noden, 1983a, Noden, 1988; Couly et al., 1995; Hacker and Guthie; 1998).

Figure 1.2 Schematic diagram of the skeletal elements in the chicken head. Cartilagenous elements are in light grey while membrane bones are in dark grey. Picture is from Francis-West et al., 1998.

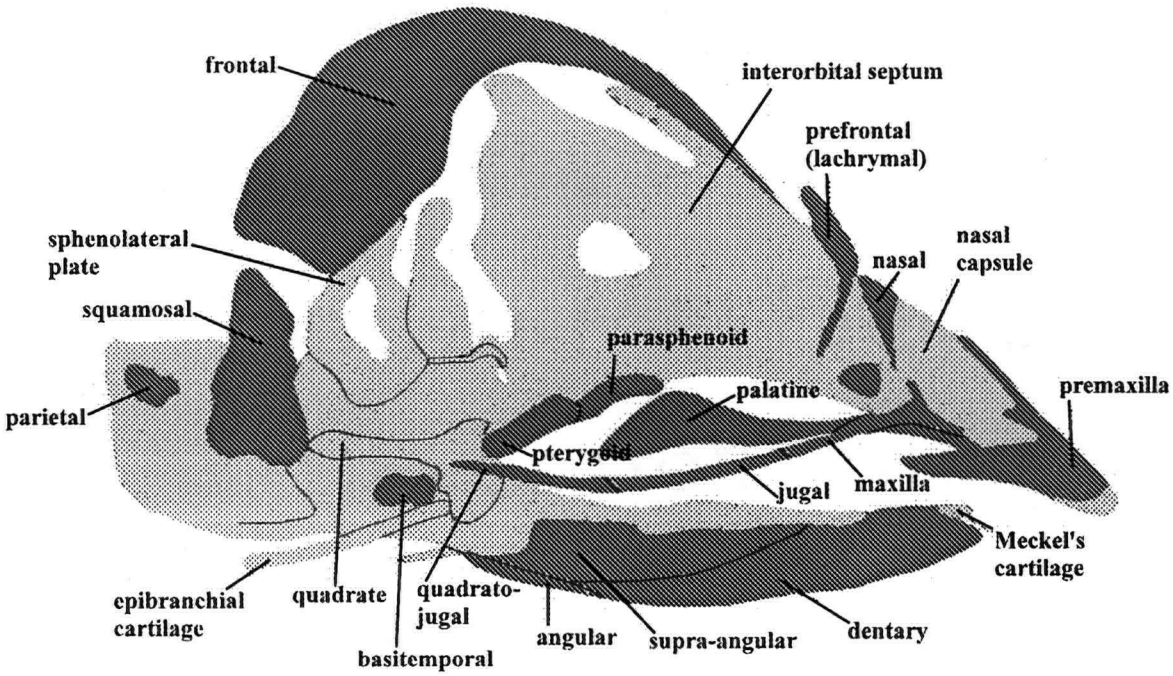

frontal
interorbital septum
prefrontal
(lachrymal)
sphenolateral
plate
nasal
nasal
capsule
squamosal
parasphenoid
palatine
premaxilla
parietal
pterygoid
jugal
maxilla
Meckel's
cartilage
epibranchial
cartilage
quadrate
quadrato-
jugal
basitemporal
angular
supra-angular
dentary

Table 1.1 Origin of the chicken head skeleton (from Couly et al., 1993; Hall, personal communication)

A. Neural crest derived skeletal components

Membrane bones	*Cartilaginous bones*	*Cartilage*
Nasal	Basipresphenoid	Scleral cartilage
Maxillary	Proximal Meckel's	Meckel's cartilage
Palatine	Quadrate	Interorbital septum
Quadratojugal	Hyoid	Nasal conchae
Jugal	Pars cochlearis of otic capsule	Nasal septum
Pterygoid	Columella	Prenasal cartilage
Scleral ossicles		
Retroarticular process		
Mentomandibular bone		
Dentary bone		
Supraangulare bone		
Angulare bone		
Frontal		
Vomer		
Premaxillary bone		
Lachrimal bones		

B. Paraxial mesodermal derived skeletal components

Membrane bones	*Cartilaginous bones*	*Cartilage*
Postorbital	Temporal	
Supraoccipital	Basi postsphenoid	
Squamosal	Pars canalicularis	
Parietal	Cochlearis of otic capsule	
	Occipital	

Skeletal growth via intramembranous ossification involves bone formed directly from mesenchymal cells (reviewed in Fang and Hall, 1997). This process is initiated when mesenchymal cells condense into compact regions. Some of these cells develop into capillaries while most differentiate into osteoblasts, bone precursor cells which deposit an osteoid matrix that is able to bind calcium and become calcified (Hall, 1997). The calcified matrix grows and osteoblasts that get trapped within the bone matrix become osteocytes (bone cells). Eventually, compact mesenchymal cells form a membrane (periosteum) that surrounds the bone. The inner surface of periostial cells that line the bone can also become osteoblasts and deposit additional matrix, resulting in many layers of bone. Thus, the periosteum functions to regulate the rate of bone formation (Hall, 1997).

Cartilage formation begins with the condensation of mesenchymal cells (Hall and Miyake, 1992; 1995; 2000), which aggregate together into compact nodules, enlarge in size and produce several characteristic condensation specific markers such as peanut agglutinin (PNA; Stringa and Tuan, 1996), N-cadherin (Chimal-Monroy and Diaz de Leon, 1999), neural-cell adhesion molecule (N-CAM; Tavella et al., 1994), SOX9 (Ng et al., 1997; Zhao et al., 1997), type II collagen mRNA (Kosher et al., 1986) and FGF receptor 2 mRNA (FGFR2 or Cek3; Matovinovic and Richman, 1997; Wilke et al., 1997; Noji et al., 1993; Patstone et al., 1993). Precartilagenous condensations represent a rough outline of the future skeletal elements (Grüneberg, 1963). These prechondrocytes differentiate into chondrocytes and switch from the production of mesenchymal matrix (type I and type III collagens) to cartilagenous matrix (types II, IX and XI collagen).

Cartilage may remain as such, in which case it does not undergo hypertrophy or invasion by osteoblasts. In ossifying cartilage, the chondrocytes proliferate and continue to elaborate cartilage-specific extracellular matrix. The chondrocytes then stop dividing and increase in volume dramatically, becoming hypertrophic chondrocytes. Collagen X and fibronectin are added to the matrix, which enables the cartilagenous template to become mineralized by calcium carbonate. Soon after, the hypertrophic chondrocytes die by apoptosis. The vacated space eventually becomes bone marrow and allows for invasion of blood vessels, which bring in bone depositing osteoblasts. In endochondral ossification, all the cartilage is removed and replaced by bone and bone marrow (for review see Karsenty, 1998).

Abnormal facial development

Orofacial clefts in humans

Cleft lip with or without cleft palate (CL/P) occurs approximately 1 in 1300 live births and thus is one of the most commonly occurring birth defects in humans (Tolarova, and Cervenka, 1998). Clefts may be classified as syndromic or non-syndromic. Syndromes associated with clefts include, Van der Woude syndrome (1q32; Murray et al., 1990), Treacher Collins syndrome (5q; Dixon et al., 1992) and Waardenburg syndrome (PAX3; Tassabehji et al., 1992). Deletions or duplications in all chromosomes have also been associated with clefts, suggesting that many genes are involved in facial development and that cleft lip and palate can represent a common end-point in the disruption of processes involved in facial morphogenesis (Brewer et al., 1998; 1999). We are interested in the

non-syndromic forms of clefting, which are much more prevalent (~70%; Jones, 1988). Individuals affected by non-syndromic clefting have no other physical or developmental anomalies, no recognized maternal teratogen exposure or identifiable environmental characteristics (Murray, 1995).

The etiology of non-syndromic orofacial clefts is complex and is thought to include multiple environmental and genetic factors. Environmental factors that correlate with cleft lip and palate include insufficient vitamin intake and teratogens such as cigarette smoking, alcohol use, and prescription drugs (Wyszynski and Beaty, 1996; Romitti et al., 1999). Genetic analysis of cleft lip in a congenic mouse strain indicates that nonsyndromic CL/P is caused by two loci with epistatic interactions (Juriloff, 1995). Similar conclusions are reached from genetic research on humans, which include segregation analysis (Marazita et al., 1986; Mitchell, 1997) and twin studies (Mitchell and Risch, 1992). A few candidate genes associated with cleft lip or cleft palate have been identified (for review see Schutte and Murray, 1999) using genetic linkage and association studies in humans (TGFβ3 and MSX1, Lidral et al., 1998; TGFα, Ardinger, 1989; Mitchell, 1997; RARα, Chenevix-Trench et al., 1992) and transgenic mouse models (TGFβ3, Proetzel et al., 1995; Kaartinen et al., 1995; MSX1, Satokata and Maas, 1994). In the future, it might be possible to identify and reverse such defects early in development and thereby avoid costly surgical care and the social difficulties encountered by affected individuals.

The first step in preventing such birth abnormalities is understanding the processes involved in normal facial morphogenesis and the genes that regulate them. Errors at any step in facial morphogenesis can potentially result in clefting. Adding further complexity to these processes is that attaining a critical size of the prominences, removal of the epithelia, fusion and sufficient merging of the mesenchyme all need to occur within a narrow time window for normal development to occur. Craniofacial developmental biology therefore has direct relevance to the clinical treatment of cleft lip and other facial syndromes. Unfortunately, the majority of orofacial clefts in mutant mouse embryos involve the secondary palate or are midline clefts between the medial nasal prominences, and therefore are not particularly informative for understanding the etiology of cleft lip (see Francis-West et al., 1998). Therefore, a reproducible cleft lip that resembles the human defect would be an extremely valuable model system.

Phenocopying cleft lip in chickens

Chicken model system

The five phases of face development are very similar in all vertebrates and are highly conserved through evolution. Similarities in the origin of embryonic tissues, general morphology (Hall and Horstadius, 1988), patterns of gene expression (Francis-West et al., 1998), and mechanisms of development of the face have allowed other vertebrates, notably rodents and chickens, to be used as valuable model systems to understand normal human craniofacial development and related disorders. The chicken model is particularly advantageous because of the ease with which the embryo can be directly manipulated in

ovo at all critical phases of face development. Several methods, including manipulations and/or exposure to teratogens have been used to generate unilateral or bilateral cleft lip in chickens (reviewed in Young et al., 2000). Similarly, exposure to teratogens in rodents prevents fusion and result in clefts of the lip, midface and palate (reviewed in Diewert and Wang, 1992). Stage and position-specific manipulations can act as 'conditional knockouts' or 'knockdowns'. In general these are much easier to perform in chickens than in mice. Traditional knockout studies potentially disrupt several processes in facial morphogenesis or facial defects may even be secondary to abnormal brain development (Opitz and Gilbert, 1982; Couly and Le Douarin, 1985; 1987). The chicken embryo allows us to target specific processes in primary palate formation in a spatial and temporal specific manner. By studying the molecular and cellular basis by which teratogens exert their effects, we can gain a better understanding of both normal and abnormal development. Below are described several experimental approaches used to study cleft lip in chicken embryos.

Hydrocortisone treatment

An example of a teratogen that causes cleft lip in chickens is hydrocortisone (Peterka and Jelinek, 1983). A single (3μl) intraamniotic injection of hydrocortisone (0.1μg/μl) on day 4 (stage 21-24) resulted in a full bilateral cleft. Clefts were formed due to hypoplasia of the frontonasal mass prior to fusion. The reduced size of the frontonasal mass prevents contact with the maxillary prominence and fusion fails to occur. The suppressed growth must be temporary and/or restricted to the lateral regions of the frontonasal mass since, in every case, a full-length upper beak and egg tooth form.

Excision of epithelium

Tissue (epithelial-mesenchymal) recombination studies have shown that the facial epithelium is required for outgrowth of the facial prominences (Wedden, 1987; Richman and Tickle, 1989). When the frontonasal mass or maxillary prominence was stripped of *Shh* expressing ectoderm in ovo, at stage 25-26, outgrowth of the corresponding prominence was interrupted and clefts resulted (Hu and Helms, 1999). Extirpation at earlier stages, or of adjacent *Shh*-negative frontonasal ectoderm, had no effect on subsequent facial morphology. These data show that insufficient signaling from the epithelium will prevent fusion and at later stages not all-epithelial regions are equal in supporting facial outgrowth.

SHH antibody

By targeting genes specifically expressed in the ectodermal region that supports outgrowth of the frontonasal mass, we are able to understand the genetic requirements for outgrowth and fusion. From the extirpation studies above, Hu and Helms (1999) predicted that SHH might be supporting outgrowth. To test this they implanted SHH antibody-soaked beads into the frontonasal mesenchyme. Embryos treated in this manner mimicked the epithelial stripping defect; cleft lip occurred due to reduced outgrowth of the frontonasal mass.

Retinoic acid

The discovery that retinoid excess causes dysmorphogenesis implicates retinoids as key patterning factors. Excess retinoic acid (RA, all-*trans,* 13-*cis*, or 9-*cis*) is a potent teratogen that causes an assortment of birth defects in many animals, including vertebral and craniofacial malformations (Cohlan, 1953; Kalter, 1960; Hassell et al., 1977; Yip et al., 1980; Jelinek and Kistler, 1981; Wiley et al., 1983; Fernhoff and Lammer, 1984). In humans, defects associated with excess retinoic acid include micrognathia, microtia and cleft palate but not cleft lip (Lammer et al., 1985). Early studies in chickens examining the role of excess retinoic acid on facial clefting were relatively non-specific. A retinoic acid solution was added systemically, leading to global effects on development (Hassell et al., 1977; Jelinek and Kistler, 1981). While some specificity is introduced by varying the timing of administration, it is difficult to identify primary versus secondary effects. Defects were much more consistent and easier to interpret once a carrier, formate-ion exchange beads, was used to locally apply retinoic acid (Eichele et al., 1984). At high concentrations, retinoic acid-bead implantations into the limb resulted in limb truncations and reproducible, full bilateral cleft lip (Tamarin, 1984; Wedden and Tickle, 1986). In chickens, retinoic acid inhibits outgrowth of the frontonasal mass (Wedden, 1987), maxillary (Helms et al., 1997) and lateral nasal prominences (Richman, unpublished data). The mechanisms by which outgrowth is restricted in response to RA treatment include changes in gene expression (Shen et al., 1997; Helms et al., 1997; Brown et al., 1997; Rowe et al., 1991), reduced proliferation (McGonnell et al., 1998) and the induction of apoptosis (Shen et al., 1997). By placing beads directly into the face (Richman and Leon Delgado, 1995; Shen et al., 1997) instead of under the apical

ectodermal ridge (Wedden and Tickle, 1986; Tamarin, 1984; Helms et al., 1997; Brown et al., 1997) a unilateral or bilateral cleft lip can be created in a dose-dependent manner (Richman and Delgado, 1995). To study phenotypes that more closely resemble human cleft lip, low doses of retinoic acid in the nasal pit can be used, which does not affect outgrowth of the nose. The disadvantage of the lower dose is that it is difficult to control the quantity of RA that is applied and as a result the magnitude of the cleft varies considerably (Richman and Delgado, 1995). Therefore, in order to generate consistant clefts we used higher dosages for our experiments.

Retinoic acid physiology

Retinoic acid (RA), retinol and retinaldehyde, collectively known as retinoids, are biological derivatives of vitamin A and serve important roles in embryogenesis. The major form of vitamin A is retinol, which is stored in the liver or bound to retinol binding protein (RBP) in the circulation. Retinol uptake is mediated by a cell surface receptor for RBP (RBPr; Båvik et al., 1997; Ward et al., 1997). Accumulation of retinol within embryonic cells occurs in cells that express a high-affinity intracellular RBP, cellular CRBP I (Ong et al., 1994; Gustafson et al., 1993). Target cells oxidize retinol to retinaldehyde and subsequently to RA, which are enzymatic reactions carried out by retinol dehydrogenase (RDH) and retinal dehydrogenase (RALDH) respectively (Bhat et al., 1995; Penzes et al., 1997). There are two isoforms of retinoic acid, all-*trans*-RA and 9-*cis*-RA. The physiological effects of RA, the active form of vitamin A, are transduced by binding to its intracellular receptors.

RA Receptors

The discovery of retinoic acid receptors (RARs) unraveled the mechanism by which RA induces its biological effects (Giguere et al., 1987; Petkovitch et al., 1987). RARs are ligand-inducible transcriptional regulators classified structurally and functionally as members of the steroid/thyroid hormone receptor superfamily of transcription factors. A second family of retinoic acid receptors, retinoid X receptors (RXRs), was isolated soon after (Mangelsdorf et al., 1990). Numerous isoforms of the receptors are known today, which are derived by differential promoter usage and alternative splicing. The RARs (RARα, RARβ and RARγ) can be activated by all-*trans*-RA or 9-*cis*-RA but RXRs (RXRα, RXRβ, RXRγ) are activated only by 9-*cis*-RA. The RXRs are unique in that they function as heterodimeric partners for not only RARs but also a number of other nuclear receptors. RARs therefore bind cooperatively with RXRs in the promoter region of target genes that contain RA response elements (RARE). RAR homodimers bind weakly to RAREs or not at all (Gudas et al., 1994), whereas RXR homodimers can bind to certain promoter sequences containing RARE and stimulate transcriptional activation (Lee et al, 1993; Wilson et al., 1992). RA has been shown to regulate Hox (homeobox) gene expression, genes that specify anterior-posterior polarity in vertebrates, through receptor binding to RAREs (Hoxa1; Langston and Gudas, 1992; Hoxb1, Marshall et al., 1996; Hoxd4, Popperl and Featherstone, 1993).

RAR and RXR are expressed in a wide range of organs, with much overlap in receptor expression during development, suggesting a role for RA in the morphogenesis of such tissues. RARα is almost ubiquitously expressed in all tissues (Ruberte et al., 1990; 1991)

however more specific localization is observed for RARβ (head and face mesenchyme, nasal epithelium, eyes, ears, limb mesenchyme, kidney; Dollé et al., 1989; 1990; Ruberte et al., 1991; Smith and Eichele, 1991; Rowe et al., 1991) and RARγ (in cartilage, sclerotome and keritinizing epithelia; Ruberte et al., 1990). RXR expression patterns show less tissue specificity than RARs (Dollé et al., 1994). In the chick face, *RARβ* is expressed in all the facial prominences (Rowe et al., 1991; Rowe et al., 1992; Smith et al., 1991). The localized function of RA is not only dependent on the presence of the RA receptors, but also on the availability of retinoids, ie. RA. RALDH-2 transcription in the chicken face is localized to the nasal placodes and the developing branchial arches of stage 17 embryos (later stages were not reported; Berggren et al., 1999). The localization of RALDH-2 to the face provides further evidence to support a role for endogenous RA in face morphogenesis.

Biological functions of endogenous retinoic acid

Retinoids play essential roles in vertebrate development. Evidence to support this comes from a spectrum of congenital malformations exhibited by mouse fetuses from females with inadequate dietary intake of vitamin A. The features of vitamin A deficiency (VAD) syndrome include defects in heart and aortic arches, urogenital tract, respiratory tract, diaphragm, eye and craniofacial morphogenesis (cleft face, palate and lip; Morriss-Kay and Sokolova, 1996). Vitamin A deprivation in quails causes development to arrest at about stage 20/21, prior to face formation. Embryos exhibit defects in limb (Stratford et al., 1999), hindbrain (Maden et al., 1996), heart (Heine et al., 1985) and somite formation (Maden et al., 2000).

Genetic studies of RA endogenous functions

Mouse knockouts of various RARs and RXRs have been used to evaluate the function of RA and, more precisely, the RARs and RXRs in vivo. Mice lacking one RA receptor are normal and fertile (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Mendelsohn et al., 1995). Either the phenotype is too subtle and is not manifested in the detection procedures or some of the functions of the mutated receptor are taken over by another. Disruption of all isoforms of the RAR receptors and double receptor knockout mice are more informative. RAR compound mutants exhibit all malformations typical of fetal VAD, including in limb, axial and craniofacial morphogenesis (in embryos with disruption of all isoforms of RARα; Lufkin et al., 1993, or RARγ; Lohnes et al., 1993). In the face, RARα/γ double knockout mice exhibit median clefts of the primary palate (Lohnes et al., 1994). This defect was duplicated in embryos exposed to a RAR pan-antagonist, confirming that endogenous RA is required for facial morphogenesis (Kochhar et al., 1998).

The above studies identify a developmental requirement for retinoids and suggest that a mechanism to maintain a critical balance of retinoid activity must be present during normal face morphogenesis. Severe vitamin A deficiency is uncommon in humans, but some investigators feel that mild deficiencies combined with genetic defects may be an underestimated cause of human abnormalities (Morriss-Kay and Ward, 1999). This may be especially true for those with a genetic disposition to cleft lip.

Genes downstream of RA teratogenicity

The mechanism by which RA induces craniofacial defects at a molecular level is starting to be understood. Retinoic acid decreases the expression of *shh* (Helms et al., 1997) and fibroblast growth factor homologous factor-4 (*FHF-4;* Muñoz-Sanjuan et al., 2001). Expression of *fgf8* (Helms et al., 1997), *fgf receptors* (*Fgfr2* and *Fgfr3*; Richman and Delgado, 1995) and *Bmp-2* (Munoz-Sanjuan et al., 2001) are unaffected. However, in my opinion *Bmp-2* appears to be upregulated in Figure 3L of Munoz-Sanjuan et al. (2001). Retinoic acid also alters the expression of numerous transcription factors. For example, *RARβ* is ectopically expressed (Rowe et al., 1991) while activating protein-2 (*AP-2*; Shen et al., 1997), *Msx-1* and *Msx-2* (Brown et al., 1997) are all downregulated following RA application. Several genes shown to be downregulated by RA treatment have later been shown to be associated with cleft lip in chickens (Shh, Hu and Helms, 1999), cleft lip and/or palate in humans (MSX1; van den BooGaard et al, 2000), cleft palate (Msx-1, Ichiro and Maas, 1994) or cleft lip and palate in mice (Msx-1 and Msx-2, Maas, personal communication). Thus, understanding the molecular mechanism by which RA induces cleft lip has proven to be an excellent model system to identify genes involved in facial morphogenesis/dysmorphogenesis. This approach also provides another method to identify candidate genes potentially involved in orofacial clefting.

Screening strategies to isolate novel, differentially expressed genes

Control of gene expression lies at the heart of embryology, cell biology and neoplasia. The examination of expression changes in embryos treated with teratogens or other manipulations is an excellent way to identify genes that may mediate normal and

abnormal morphogenesis. Genes whose expression is altered in the position of the future defect are studied. The advantage is that genes selected in this manner are likely to be causally related to the defect. The disadvantage is that these techniques examine genes that have already been identified and characterized, thus missing many other potentially important genes. A number of techniques are available for the detection and cloning of novel transcripts that are differentially expressed.

Subtractive hybridization

Subtractive hybridization enriches those sequences of RNA that are expressed at different levels between two cell populations (Sargent, 1987). Complementary DNA (cDNA) prepared from the tissue of interest (for example, the frontonasal mass) is hybridized to an excess of (complementary) poly (A)+ RNA from a second, similar tissue type, for use as a subtractive driver. The unique cDNAs that do not anneal to the driver poly A+ RNA are isolated (for example, by column chromatography). The enriched cDNAs are then used to generate subtractive cDNA libraries for screening, or to make radiolabelled probes to screen libraries differentially. Subtractive hybridization is a well-established technique that has the potential to clone previously unidentified transcripts. Limitations in this technique include that it is labor intensive, requires use of unstable poly A+ RNA, is prone to picking up abundant sequences, and either up-regulated or down-regulated genes can be identified, but not both. The advantage of this procedure is that, even if it doesn't identify significant genes, the tissue-specific cDNA library can be used for other experiments.

Differential display

Differential display (Liang and Pardee, 1992) compares the mRNA content of different tissue or cell culture extracts. Following reverse transcription (using a primer anchored in the poly-A region) and polymerase chain reaction (PCR; with degenerate primers) a population of radioactive cDNAs are generated that are representative of the mRNA species present in each extract. Different primer pairs are used in independent reactions in order to amplify a distinct subset of mRNA transcripts. The PCR is performed with a radiolabelled nucleotide so that the products of the reactions can be resolved by electrophoresis and then visualized using autoradiographic film. The genes corresponding to differential band intensities can then be purified, reamplified and either cloned or used as probes to screen cDNA libraries. Differential display uses small tissue amounts, total RNA and allows the simultaneous analysis of multiple tissues. This technique, however, can also be labor intensive (especially when many primer combinations are used for adequate representation of mRNAs), often generate false positive clones, and true positive clones are sometimes identified as rRNA or house-keeping genes. This strategy also preferentially amplifies 3' non-coding parts of the message, which restricts direct database sequence comparisons. At the end of the procedure, a library-screening step is required to clone the full length coding sequence.

RNA arbitrarily primed (RAP-) PCR

RAP-PCR is another RNA fingerprinting strategy similar to differential display (Welsh et al., 1992). RAP-PCR, however, uses an arbitrary primer in the reverse transcription reaction instead of a primer anchored in the poly A region. The PCR reaction is then

performed with this same primer along with many possible second arbitrary primers. The advantages and limitations of using the differential display technique also apply to RAP-PCR. RAP-PCR, however, eliminates the problem of preferentially amplifying the 3'-untranslated region of transcripts.

Microarrays

Microarray technology allows the simultaneous monitoring of gene expression for tens of thousands of genes and has been described as a recent breakthrough in experimental molecular biology (see Duggan et al., 1999). A microarray is a glass slide, onto which single-stranded DNA molecules are attached at fixed locations. There may be tens of thousands of spots on an array, each representing a single expressed sequence from a particular tissue. Microarrays exploit the preferential binding of complementary single-stranded nucleic acid sequences and thereby allow the comparison of mRNA abundance from two different samples. Briefly, isolated RNA from a sample population and control or reference populations are labeled with a red and a green dye, respectively. Both RNA extracts are washed over the array, allowing complementary gene sequences to hybridize with sequences on the spots. Excitation from laser emissions causes the dye labels to flouresence, allowing the estimation of the relative expression levels of the hybridized genes in the sample compared to the reference population. For example, if the mRNA complementary to a spot on the microarray is most abundant in the sample the spot will be red but if the mRNA is greater in control populations it will be green. If RNA from both populations bind equally well the spot will be yellow and if there is no binding it will be black. By obtaining the 'gene expression profile' from nearly all-possible

conditions (that is, different developmental stages, assorted tissues or dysplasia) and combining all of this information in a database, the dynamic expression of each gene in the genome can be characterized.

There are several advantages of using microarrays over other differential hybridization techniques: 1) in a single run, microarrays allows the analysis of up to 40,000 genes (depending on the size of the array); 2) each of the cDNAs has already been cloned so the identity of the differentially-expressed gene is clear ie. housekeeping genes can be eliminated from consideration; 3) there are built in positive controls with genes that are known to be differentially expressed; 4) the cDNA on the slide has been 'normalized' so that only rare, interesting sequences are represented. The disadvantages are that a species and preferably stage specific array is preferred because the stringency for hybridization is very high. Thus, for our work we need a chicken face microarray, which we would have to make ourselves.

Candidate genes involved in facial development

The expression patterns of many genes have been described in the face, several of which may have roles in patterning, epithelial-mesenchymal interactions, proliferation and differentiation (Francis-West et al., 1998). Examples of potential signaling molecules that are localized to specific regions and tissues within the face include FGFs (fibroblast growth factors; Wall and Hogan, 1995; Barlow and Francis-West, 1997; Richman et al., 1997; Munoz-Sanjuan et al., 2001; Bachler and Neubüser, 2001), BMP (bone morphogenetic protein; Francis West et al., 1994; Wall and Hogan, 1995; Wang et al.,

1999; Shigetani et al., 2000) SHH (Sonic hedgehog) families (Wall and Hogan, 1995; Helms et al., 1997; Ahlgren and Bronner-Fraser, 1999), Wnt5a (Wingless) family (Dealy et al., 1993; Yamaguchi et al., 1999; Ladher et al., 2000). All of the aforementioned genes are secreted proteins that signal through cell surface receptors. In addition, there are many transcription factors (Msx-1, Msx-2, AP-2, Slug, Tbx-2, Tbx-3, Dlx-1,2,3,5, Barx-1, RARα, RARβ, RARγ, Gli-2,3, Gsc) expressed in the face (Francis-West et al., 1998; Davideau et al., 1999; Barlow et al., 1999; Shen et al., 1997), some of which lead to facial defects when they are knocked out (for review see Francis West et al., 1998). Subsets of these genes have been shown to work within the same signalling pathway (eg. BMPs and Msx genes) in the face (Barlow and Francis-West, 1997; Wang et al., 1999). In choosing genes to study in detail in this thesis, we selected a growth factor family, BMPs, which have known expression in the primary palate (Francis-West et al., 1996) and for whom reagents exist to study function in facial morphogenesis.

TGF-β superfamily

Over the last 20 years, a large family of secreted signaling molecules, the transforming growth factor-β family, has been isolated that regulates a plethora of biological activities during normal growth and development. An invariant feature of these growth factors is the presence of seven cysteine residues, six of which are closely grouped to make a rigid structure called a cysteine knot (Daopin et al., 1992; Schlunegger and Grutter, 1992) within the mature peptide region. Based on sequence comparisons between their bioactive domains, members of this family are divided into subfamilies (Massagué, 1998). A major subdivision is the BMP family.

Bone morphogenetic proteins

Pioneering work by Urist and his colleagues demonstrated that demineralized bone or dentin extracts could induce ectopic bone and cartilage when implanted either subcutaneously or intramuscularly in rats (Urist 1965: Urist et al., 1973, 1979). The search for factors responsible for this effect resulted in the cloning of the first BMPs more than 20 years later (Wozney et al., 1988). To date, more than 30 members of this family have been identified in organisms ranging from sea urchin to mammals, and are termed BMPs, osteogenic proteins (OPs), cartilage-derived morphogenetic proteins (CDMPs), growth and differentiation factors (GDFs) or inhibin. These subclassification are segregated according to structural similarities (for review see Ducy and Karsenty, 2000).

Biologically active BMPs are glycosylated dimeric molecules (Griffith et al., 1996). Whether BMPs normally exist solely as homodimers is not known but BMP-4/7 recombinant heterodimers have greater bioactivity than homodimers (Hazama et al., 1995; Suzuki et al., 1997). The homologs of many mammalian BMPs have been identified in invertebrates such as *Drosophila* (decapentaplegic, dpp) and *Caenorhabditis elegans* (Daf-7; Ren et al., 1996), and in vertebrates such as zebrafish and *Xenopus* (Vg1; Weeks and Melton, 1987). BMP-2 and BMP-4 are so similar to dpp, sharing 84-88% sequence homology, that they can substitute for one another in biological assays. BMP-4 can rescue embryonic defects in dpp mutant flies (Padgett et al., 1993) and recombinant dpp can induce endochondral bone formation in mammals (Sampath et al., 1993).

The name, bone morphogenetic protein, is misleading because there is tremendous genetic and experimental evidence that these molecules regulate numerous biological processes. Expression and functional studies have identified BMP to be involved in patterning, morphogenesis and differentiation of embryonic organs and tissues where reciprocal interactions between epithelial and mesenchymal cells are important (for review see Hogan, 1996; Kingsley, 1996; Wozney, 1998).

BMP Receptors and BMP signaling molecules

The biological effects of BMPs are mediated by transmembrane serine/ threonine kinases cell surface receptors, classified structurally and functionally as type I and type II. The receptor types are approximately 55 kDa (~500 amino acids) and 70 kDa (~570 amino acids) glycoproteins, respectively. Two type I receptors have been identified, BMPR-IA (ALK-3, BRK-1) and BMPR-IB (ALK-6, BRK-2; for review, see Massague, 1998). Heterotetramers of type IA, type IB and type II receptors form when ligand binds (Yamashita et al., 1994). BMPRs are evolutionarily conserved and homologs of the type I and type II receptors have been isolated in non-vertebrates like *Drosophila* (Thick veins (Tkv), Saxophone (Sax), and Punt; Nellen et al., 1994; Brummel et al., 1994; Xie et al., 1994) and *C. elegans* (Daf-1 and Daf-4; Georgi et al., 1990; Estevez et al., 1993).

The extracellular and intracellular domains of the BMP receptors are approximately 150 and 475 amino acids, respectively. Immediately preceding the type I receptor kinase domain is a highly conserved 30 amino acid region call the glycine/serine-rich (GS)

domain because of its characteristic SGSGSG sequence (Wrana et al., 1994). The GS domain regulates the receptors' kinase activity. Mutation of a glutamine (Q) residue to aspartic acid (D) in the GS domain activates the kinase domain and constitutive signaling activity in the cell (Zou et al., 1997). Downstream of the receptors are receptor-activated Smads (1,5,8) that can activate gene transcription (Heldin et al., 1997; Massagué, 1998; Hoodless and Wrana et al., 1998).

BMPs in embryonic development

Studies involving the localization of BMP signaling molecules, ectopic gain-of-function and loss-of-function studies demonstrate a broad range of biological functions for BMPs in addition to post-fetal cartilage and bone induction. These functions include cell proliferation, differentiation, apoptosis, morphogenesis, and organogenesis.

Early in development, BMPs regulate dorsal-ventral axis formation. In *Drosophila*, dpp acts as a dorsalizing factor (Ferguson and Anderson, 1992; Wharton et al., 1993) while in *Xenopus*, BMP-4 is a ventralizing factor during mesoderm formation (Jones et al., 1996). BMPs therefore act as morphogens, by providing positional information necessary for pattern formation (Kay and Smith, 1989; Wolpert, 1989). In addition, BMPs are implicated in the patterning of other organs such as the somites, teeth, feathers, limbs, forebrain and head (Drossopoulou et al., 2000; Golden et al., 1999; Tonegawa and Takahashi, 1998; McMahon et al., 1998; Capdevila and Johnson, 1998; Noramly and Morgan, 1998; Marcelle et al., 1997; Vainio et al., 1993).

The role of endogenous BMPs can be studied with knockout mice or with the application of antagonists. Knockout mice have not been particularly informative for facial morphogenesis since they either die very young or have no facial phenotype (BMP-2, Zhang and Bradley, 1996; BMP-4, Winnier et al., 1995; BMP-7, Dudley et al., 1995; Luo et al., 1995; BMPR-IA, Mishina et al., 1995; BMPR-IB, Baur et al., 2000; Li et al., 2000; BMPRII, Beppu et al., 2000). The list of BMP antagonists is growing and includes Noggin, Follistatin, Chordin, Gremlin/Drm, Caronte/Cerberus and Dan (Smith, 1999). The antagonists bind to BMPs and prevent them from interacting with and activating their receptors (Zimmerman et al., 1996; Piccolo et al., 1996, 1999; Hsu et al., 1998; Yokouchi et al., 1999; Esteban et al., 1999). Developmental processes regulated by BMP antagonists include, joint formation (Noggin, Brunet et al., 1998), patterning the neural tube and somites (Noggin, McMahon et al, 1998), hair-follicle induction (Noggin, Botchkarev et al., 1999), limb patterning and outgrowth (Gremlin, Zuniga et al., 1999; Capdevila et al., 1999; Merino et al., 1999), left-right asymmetry (Caronte, Esteban et al., 1999; Yokouchi et al., 1999; Cerberus, Zhu et al., 1999), overall growth, secondary palate formation, tooth and skeletal development (follistatin; Matzuk et al., 1995), ear development (Chordin; Bachiller et al., 2000) and primitive streak formation (Chordin; Streit et al., 1998). In *Xenopus*, dorsalizing factors from the dorsal lip of the blastopore, Spemann's organizer, were identified as BMP antagonists (Noggin, Chordin and Follistatin) and later the level of BMP activity was shown to be a key regulator of dorsal-ventral axis formation (De Robertis and Sasai, 1996). BMP activity, balanced by its antagonists, also governs whether ectoderm will form epidermis (in the presence of BMP activity), or neural tissue (in its absence; Lamb et al., 1993; Hemmati-Brivanlou et al.,

1994; Sasai et al., 1994, 1995; Wilson and Hemmati-Brivanlou, 1995). Only *noggin* and *gremlin/drm* have been localized to the chicken face. *Noggin* is expressed in frontonasal mass epithelium (see Chapter 2). At stages 22-26, *gremlin* is expressed in the maxilla, mandible, second and third branchial arches but not the prominences giving rise to the upper beak (Bardot et al., 2001). In the mouse face, chordin is ubiquitously expressed (Scott et al., 2000). Follistatin is expressed in the mesenchyme surrounding the nasal epithelium and in the tongue (Feijen et al., 1994). *Noggin* is expressed in the face of older mouse embryos, after cartilage differentiation (Brunet et al., 1998). *Cerberus-like* does not appear to be expressed in the mouse face (Stanley et al., 2000), although DAN is (Pearce et al., 1999).

The complexity of the BMP signaling system indicates that their activity is very closely regulated. The various defects following mutations in BMP signaling molecules attest to critical functional roles for BMPs in a wide range of organs and phases of development. Their intricate functioning makes them good candidates for the complex control of facial morphogenesis.

AIMS OF THE PRESENT STUDY

In the present study we explore the functions of BMPs during chicken facial morphogenesis.

The general aims of this project were:

1. To identify candidate genes involved in cleft lip;
2. To test the function of the candidate genes in normal and abnormal facial development;
3. To identify genes that are important in craniofacial skeletal development.

The specific aims were:

1. Use differential display to identify genes associated with RA-induced cleft lip in chickens;
2. To localize the expression of *BMP ligands*, *BMP receptors* and *antagonists* during the different phases of craniofacial morphogenesis;
3. To study the function of BMP signaling in primary palate morphogenesis and dysmorphogenesis;
4. To determine the roles of BMP receptor signaling in craniofacial skeletogenesis.

CHAPTER II

Isolation of genes associated with retinoic acid-induced cleft lip in chickens

Introduction

Cleft lip with or without cleft palate (CL/P) is a commonly occurring birth defect that occurs when the facial prominences surrounding the nasal pit fail to fuse together. Fusion takes place primarily between the frontonasal mass (or medial nasal prominences in mice and humans) and the maxillary prominences. Morphological defects early in face development, such as reduced size (or outgrowth) of the facial prominences, have been associated with orofacial clefts (Trasler, 1968; Diewert and Wang, 1992). The reduced size prevents contact between the prominences and fusion is unable to take place. Identifying the molecular regulators of facial outgrowth would help understand normal morphogenesis and disorders resulting from alterations in this developmental process.

To address this issue we used the RA-cleft lip-chicken model system, which has numerous advantages. The chicken embryo was used because it resembles mammalian embryonic face and is easily manipulated at nearly all stages of face development. When the chicken embryo is exposed to excess RA a full bilateral cleft lip is generated in a consistent and reproducible manner (Tamarin et al., 1984, Richman and Delgado, 1995). The RA-induced clefts are the result of reduced outgrowth of the prominences giving rise to the upper beak (frontonasal mass (Wedden, 1997) and the paired-lateral nasal (McGonnell et al., 1998) and maxillary prominences (Helms et al., 1997; McGonnell et

al., 1998). The frontonasal mass forms most of the upper beak and is most affected by RA (Tamarin et al., 1984). In spite of being in close proximity the paired-mandibular prominences outgrow normally and the lower beak is unaffected even at very high RA concentrations. Careful analysis, shortly after RA treatment, shows that outgrowth is reduced due to elevated levels of apoptosis, reduced proliferation and changes in the directional expansion of the face (McGonnell et al., 1998). These changes also correlate with changes in expression of several transcription factors and secreted signaling molecules (see thesis introduction for details). This method however only allows analysis of genes that have already been isolated. Here we combined the RA-cleft lip-chicken model system with differential display-polymerase chain reaction (DD-PCR) in order to identify new and novel genes downstream of RA that are potentially involved in outgrowth of the facial prominences.

Materials and Methods

Treatment of embryos with retinoic acid. Fertile, white leghorn chicken embryos are incubated at 38^{O}C until they reach stage 20. AG1X-2 beads (Biorad, formate form) of 100μm diameter are soaked in 5mg/ml retinoic acid (dissolved in dimethyl sulfoxide, DMSO) for 20 minutes. Beads are rinsed 3 times in culture medium for a total of 20 minutes and implanted into the right nasal pit of the host embryo (Eichele et al., 1984; Richman and Leon Delgado, 1995). Treated embryos are returned to the incubator for 24 hours. In order to generate irreversible full bilateral clefts the embryos required a minimum exposure of 16 hours (Wedden and Tickle, 1986; Richman and Leon Delgado, 1995).

Dissection of tissue. Once embryos reach stage 24 (24 hours post-bead implantation), they are removed from the egg and the frontonasal process is dissected in chilled phosphate buffered saline (PBS) solution using iridectomy scissors and fine forceps. Batches of explanted frontonasal tissue are rapidly frozen in liquid nitrogen and stored at –70^{0}C.

RNA extraction. Two separate batches of frozen RA treated frontonasal prominences and two batches of non-treated frontonasal promiences were homogenized in guanidium isothiocyanate (GITC) and then RNA was extracted in acid phenol (Chomzynski and Saachi, 1987). Briefly, tissue was solubilized in denaturing solution (4M GITC, 25 mM NaCitrate pH 7.0, 0.5% N-Lauryl Sarcosine, 0.1M β-mercaptoethanol) with a hand-held glass homogenizer. To this homogenized mix was added, 1 vol water saturated phenol, 0.2 vol chloroform:isoamyl alcohol (49:1), 0.1 vol 2M NaAcetate pH 4. The combined

mix was vortexed, set on ice for a minimum of 15 minutes and then centrifuged in a microfuge at 10,000 rpm for 20 minutes (4^{O}C). The upper aqueous layer was removed precipitated with 1 vol isopropanol (mix, let sit for 1 hour at –20^{O}C, spin at 10,000 at 4^{O}C to pellet). The pellet was then resuspended in denaturing solution, precipitated and pelleted as described before. At this time the pellet was washed with 75% ethanol to remove salts and resuspend in DEPC-H_20. Any contaminating genomic DNA was removed from all samples by treating the total RNA for 30 minutes at 37^{O}C with 10 U RNase-free DNase I followed by phenol-chloroform extraction and ethanol precipitation. In parallel, total RNA was extracted from normal stage 24 frontonasal prominences.

Northern blot analysis. Total RNA (10μg) was resolved by electrophoresis on a 1.2% formaldehyde gel and transferred to a nitroplus 2000 membrane (Micon Separations Inc.). Blots were prehybridized with 50% formamide, 5x SSC, 10xDenhardt's solution, 0.1 mg/ml denatured salmon sperm DNA, 0.2% SDS, 0.05 M sodium phosphate buffer pH 6.8, and 0.001M EDTA for 2 hours at 42^{O}C. Hybridization was performed for 16 hours at 42^{O}C in the above prehybridization mix plus 10% dextran sulphate with 2-5x10^{7} cpm of ^{32}P-labelled probes prepared according to methods described in the T7-random priming kit (Pharmacia). Filters were washed twice at 65^{O}C with 2xSSC containing 0.5% SDS for 30 minutes, followed by 0.2xSSC and 0.5% SDS for 40 minutes before exposure to X-ray film at -70^{O}C.

Reverse transcription-polymerase chain reaction (RT-PCR) differential display (DD). Differential display was performed essentially as described by Liang et al., 1993. Primers anchored to the poly A-region are used in the reverse transcription reactions. Three RT reactions were performed using the total DNA-free RNA (0.5 μg RNA/reaction) in 1x

transcription buffer; 10 mmol/L dithiothreitol; 20 μmol/L each deoxynucleotide triphosphate (dNTP; dGTP, dATP, dCTP, dTTP); and 1 μmol/L of either H-T11G, H-T11C, H-T11A oligonucleotide primer (where H is a *Hin*dIII restriction site). Samples were heated to 65^{O}C for 5 minutes and cooled to 37^{O}C for 10 minutes before addition of 100U Moloney Murine Leukemia Virus RTase. After incubation at 37^{O}C for one hour, the reactions were heated to 95^{O}C for 5 minutes before storage at –20^{O}C. A proportion of the reverse transcribed products was amplified by PCR using the original cDNA primer plus one of the five arbitrary primers. PCR was performed on a Perkin-Elmer Thermal Cycler (94^{O}C for 30 seconds, 52^{O}C for 2 minutes, 72 minutes for 30 seconds for 40 cycles, followed by a 5-minutes extension at 72^{O}C). The 20μl PCR reaction mix contained 2 μl RT cDNA template, 50 mmol/L KCL, 10 mmol/L Tris-HCL (pH 8.3), 2.5 mmol/L MgCl2, 0.5 μmol/L H-T11N primer, 0.2 μmol/L arbitrary primer, 2 μmol/L each dNTP, 10 deoxynucleotide triphosphate (dNTP; dGTP, dATP, dCTP, dTTPCi 35S-ATP (Dupont NEN, Albany, NY), and 1 U *Taq* DNA plymerase. Arbitrary primers used were as described in the GeneHunter Kit (E-JR-A, gcgaattctatccatg; E-JR-B, gcgaattctgattgcc; E-JR-C, gcgaattctgctctca; E-JR-D, gcgaattcttgcttga; E-JR-E, gcgaattctagtatgg; where E is an *Eco*RI restriction site). PCR products from both samples were electrophoresed together to enable identification of bands with differing intensities. PCR amplification products (4μl) were denatured by heating for 3 minutes to 80^{O}C in sequencing gel stop buffer and electrophoresed at 55 W constant power on a 6% denaturing polyacrylimide gel. Gels were dried onto Whatman 3MM filter paper (VWR, Seattle, WA) and exposed for 1 to 4 days to Kodak XAR-5 film (CMX, Seattle, WA) with fluorescent ink orientation markers. These markers were used to accurately position the radiograph over

the gel during excision of the band. Differential expression was verified by multiple DD-PCR reactions, using different RNA preparations and numerous gel electrophoesis. All DD-PCR reactions were repeated at least twice using each reverse transcription reaction product. Two reverse transcription reaction products were generated from two separate RNA preparations. Each DD-PCR reaction was run on a sequencing gel twice.

Amplification and cloning of differential display gel bands. Bands that appeared to consistently have different band intensities from control samples were excised from the dried sequencing gels with a razor blade. Therefore, only bands clearly differential on at least 4 gels were only selected. The gel piece was soaked for 15 minutes in 100 μl H_2O, then boiled for 15 minutes to elute the DNA. The eluate was transferred to a fresh tube. The DNA was precipitated by addition of 3mmol/L sodium acetate (pH 5.2) to a concentration of 0.3 mol/L and 2.2 volumes of 100% ethanol, followed by 30 minutes of incubation at $-80^{O}C$, and centrifugation at 13000 rpm for 15 minutes in a microfuge. The DNA pellet was washed in 70% ethanol and resuspended in water. Reamplification by PCR was performed in 40 μl using the appropriate primers and conditions as described above except for dNTP concentrations of 20 μmol/L and no isotope. In parallel a fraction of the eluate was amplified in the presence of ^{35}S-ATP and electrophoresed, on a sequence gel, next to the original DD-PCR product to ensure that the re-amplified product was the exact size of the band that was cut out from the gel. Four microliters of the above reaction was reamplified a second time under the same conditions for 40 cycles. The products of both amplifications were digested with HindIII and EcoRI, than separated on a 2% agarose gel. A well was made below the bands of interest by excising a strip of agarose and the DNA bands were electrophoresed into the well containing 5x

TAE buffer. The DNA/TAE buffer was removed using a pasteur pipette and precipitated as above with sodium acetate. The resuspended DNA was used directly for cloning into the EcoRI/HindIII site of a pBS+ vector (Stratagene, La Jolla, CA). For each excised band, a minimum of 5 white (recombinant) colonies was picked. Recombinant plasmid DNA was purified using the miniprep procedure described by Sambrook et al., (1989) and tested for inserts of appropriate size by excising the insert with *Eco*RI and *Hin*dIII and viewing on a 2% agarose gel. DNA sequencing was performed by the Nucleic Acid Protein Service (UBC).

Database searches. The combined EMBL and GenBank databases were searched with BLAST.

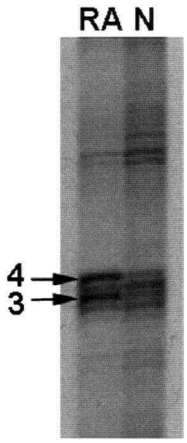
Results

Tissue collection and RNA purification. A stage 24-frontonasal prominence yields approximately 0.16 μg of total RNA. In order to attain sufficient quantities of total RNA for northern analysis and differential display, approximately 500 RA treated and 400 control frontonasal prominences were collected. RNA extraction procedures yielded a sum of 200 μg and 135 μg of total RNA, respectively. Two separate preparations for each sample set were made and kept separately. The quality of the RNA samples was determined for each sample preparation by assessing the integrity of 18s and 28s bands when total RNA (20 μg) was electrophoresed on a MOPS-formaldehyde gel. In addition, northern hybridization using a cyclophilin or type I collagen probe indicated 'tight' bands. The absence of tailing indicated that the RNA was of high quality and was not partially degraded.

Display of differentially expressed sequences. Thirteen bands were identified as being differentially expressed (Fig. 2.1). Six bands were upregulated and seven downregulated following RA treatment. DNA eluted from the bands of interest was amplified by PCR using the original primers. Selected clones were taken from the top of the gel and determined to be 200-250 basepairs in length. Two of the 13 differential expressed products were subcloned into pBS+ vector and sequenced. The sequencing information obtained was used to compare with gene sequences in Genbank and EMBL databases, but unfortunately no significant homology to any reported sequence was found.

Isolated clones are ubiquitously expressed in the face. Radiolabelled probes were made from the two sequenced clones and used in sectioned in situ hybridization experiments. In

Figure 2.1 Autoradiography of patterns of gene expression follow DD-PCR. Left and right lanes represent RA-treated and normal frontonasal tissues, respectively. DD-PCR was performed using the H-T11A anchored primer and E-JR-A arbitrary primer. Note that samples were not loaded evenly; there are greater amounts of normal PCR products. Despite the uneven loading, band numbers 3 and 4 were still upregulated in RA frontonasal mass tissue.


RA N
4
3

situs were performed on stage 20, 24 and 28 chicken face sectioned in the frontal and sagittal planes. Both genes were ubiquitously expressed.

Discussion

Our study shows that the differential display technique can be potentially used to identify genes that are differentially expressed between RA treated and normal frontonasal prominences. We were able to detected 13 potential genes whose transcription was altered in response to RA application. We had proposed to confirm differential expression of the remaining 11 clones using northern and in situ hybridization techniques. If the clones were differentially expressed in the RA treated face we would then screen a cDNA library to pull out a longer clone. This library-based clone would have no PCR errors in the sequence and would include coding sequence that can be used to identify the gene. Genes that are not differentially expressed in in situ hybridization screening but found to be expressed in the face may still be of interest as they may be novel genes.

There are however numerous limitations to differential display. This technique preferentially amplifies abundant transcripts. The differential expression of isolated clones is often found to contain false positives and are not actually expressed at different levels in the two cell populations when examined using an independent technique (like northern hybridization; Donohue et al., 1994; Aiello et al., 1994). The procedure requires a lot of subcloning (11 of our clones still needed to be subcloned), which is a difficult procedure. Clones are often ribosomal RNA or housekeeping genes and therefore uninformative. Clones are predominantly, if not exclusively, localized to the 3'-untranslated portion of a gene as a result of the oligo-dT-based cDNA primers. This is particularly a disadvantage when studying the chicken model system because the

untranslated flanking regions of chicken genes are very seldom reported in the DNA database. The aforementioned limitations in differential display lead us to reconsider whether it would in our best interest to continue with this project.

Despite all the problems mentioned, classical differential display still offers several advantages over screening methods, like subtractive-hybridization: 1) it requires only a relatively small amount of DNA; 2) it is a relatively simple technique and does not require special reagents or materials; 3) uniquely expressed RNAs enriched or underexpressed in two or more different tissues or cell types can be identified simultaneously, in contrast to subtractive hybridization, which selects for those overexpressed in a single tissue; and 4) it eliminates the danger of removal of novel genes sharing stretches of sequence with abundant members of gene families. A particularly advantageous modification to the classic differential technique uses degenerate oligos specific for conserved protein motif-encoding sequences instead of arbitrary primers (Stone and Warton, 1994). A similar modification was considered early in the project where the total RNA would be reverse transcribed, using a poly-T primer, and the resulting cDNA would be used for differential amplification by PCR using 2 arbitrary primers. This modification would have avoided the selective amplification of the 3'-end of a gene. This method, however, still preferentially amplifies abundant genes.

Examination of differential expression in the year 2001.

Differential display continues to be used but contains modifications to avoid the difficulties accompanying this procedure. For example, a combination of differential display and long-distance PCR protocols have been used to amplify and comparatively

display highly reproducible cDNA fragments ranging in size from 150 bp to 2 kb (Jurecic et al., 1998). This combined method assures that the coding region of a gene is amplified. The recent advent of monitoring gene expression using microarrays is in many ways an extension of differential display. cDNA microarray technology provides a format to simultaneously compare the relative differences in expression levels of thousands of genes between samples, and in a single hybridization assay. Numerous improvements have been made to this procedure (microarray preparation, some human and mouse arrays are now commercially available, probe labeling, hybridization, software for image analysis and data analysis; Duggan et al. 1999; Cheung et al., 1999; Bassett et al., 1999; Loftus et al., 1999; Tamayo et al., 1999; Zweiger, 1999). Besides technological improvements, cDNA microarrays have been combined with other screening methods to facilitate the analysis of gene expression. For example, microarrays were used for expression analysis of cDNA clones from breast cancer cell lines that were generated by a modified subtractive hybridization procedure (Yang et al., 1999). By incorporating a preliminary subtraction step (called library normalization), this depletes sequences common to the target and driver samples. The application of this technology to development has been hampered by the lack of appropriate microarrays. Some have been reported for mouse embryos (Tanaka et al., 2000). For our studies, we would require a chicken embryo cDNA microarray. One was used to identify genes downstream of Lyn, a member of the Src family of cytoplasmic signal transducers (Korade-Mirnics et al., 2000). However, pre-made microarrays are generally of utilitarian design and do not sufficiently accommodate organ specific research. The most important feature of a microarray is that it be species specific, then stage specific and

finally organ specific. Ideally we would require a chicken embryo, stage 20-28 face microarray. Microarrays have only just begun to be applied but they have already generated a wealth of information.

Contributions to this chapter

The scientific ideas for the preceding chapter were primarily the supervisor's. I contributed in selecting the procedure for isolating differentially expressed genes. The data presented in this chapter were almost completely collected by me. Dr. Todd Wilke performed the in situ experiment.

CHAPTER III

Bone Morphogenetic Proteins, BMP Receptors and Noggin: Expression during Craniofacial Morphogenesis

Summary

Bone Morphogenetic Proteins (BMPs) are secreted growth factors that may play a role in craniofacial morphogenesis. Here we correlate the expression of BMP ligands (*Bmp-2*, *Bmp-4* and *Bmp-7*) with the expression of BMP receptors (*BmpR-IA*, *BmpR-IB* and *BmpR-II*) and *noggin*, a BMP antagonist, in order to predict where endogenous BMP signaling occurs in the face. *In situ* hybridization studies on stage 10-32 chicken embryos showed that *Bmps* are expressed in facial epithelium and mesenchyme during outgrowth and patterning of the facial prominences. Later, *Bmps* are expressed peripheral to cartilage condensations and in the perichondrium, tongue and egg tooth. *BmpR-IA* and *BmpR-II* are ubiquitously expressed up to stage 30 when they are specifically excluded from differentiating cartilage. *BmpR-IB* transcripts are present in early head mesenchyme, and later in prechondrogenic condensations, the perichondrium and olfactory epithelium, suggesting a more specialized function for this receptor. *Bmp-7* is also expressed in the olfactory epithelium, overlapping the expression of *BmpR-IB*. *Noggin* is expressed - where it may act to suppress BMP activity - in the early head mesenchyme, frontonasal mass epithelium and differentiating cartilage. We conclude that the specificity of BMP signaling is more dependent on the localized expression of ligands and antagonists than it is on localized receptor expression.

Introduction

Bone Morphogenetic Proteins (BMPs) are secreted signaling molecules that are structurally related to the transforming growth factor beta (TGFβ) super family (Wozney et al., 1988). BMPs regulate an array of diverse embryonic processes, in part through reciprocal epithelial-mesenchymal interactions (limb, Niswander and Martin, 1993; Pizette and Niswander, 1999; teeth, Vainio et al., 1993; Jernvall et al., 1998; Wang et al., 1999; lungs, Weaver et al., 2000; face, Barlow et al., 1999; Wang et al., 1999). Epithelial-mesenchymal interactions are necessary for outgrowth of the facial prominences (Wedden, 1987; Richman and Tickle, 1989; 1992; Hu and Helms, 1999) and for induction of bone (Tyler and Hall, 1977). *Bmp-2*, *Bmp-4* and *Bmp-7* have been localized to the epithelium and mesenchyme of the developing facial prominences (Francis-West et al., 1994; Wall and Hogan, 1995; Wang et al., 1999; Shigetani et al., 2000). Only a few of the molecules involved in BMP signaling have been mapped and the data emphasizes expression during outgrowth of the facial prominences. Identifying the regions of the face that are responsive to BMP ligands (express BMP receptors) and modulate BMP activity (express BMP antagonists) would aid in our understanding of regional endogenous BMP signaling in the head. The comparative expression data would further explain biological differences following exogenous BMP application.

There are several key stages in avian head development; 1) neural crest cell production, specification and migration, 2) formation of branchial arches and the nasal placodes, 3) formation of the maxillary and mandibular divisions of the first branchial arch, 4) enlargement of the facial prominences, 5) fusion and merging of the upper facial

prominences to form the primary palate and 6) differentiation of skeletal tissues. Cranial neural crest cells arise and migrate from the dorsal margin of the neural tube ventrally through the paraxial mesoderm (somitomeres) into the face between the 6 and 9 somite stages (Tosney, 1982; Noden, 1975). *Bmp-4* is expressed at high levels in r3 and r5 (Watanabe and Le Douarin, 1996) and may be locally restricting production of neural crest cells (Graham et al., 1994; but see also Fairlie et al. 1999). Derivatives of cranial neural crest cells include components of the sensory nervous system (there is also a placodal contribution to the ganglia and sensory neurons), melanocytes, cartilage and bone (Le Douarin, 1982; Hall and Horstadius, 1988). The somitomeric mesoderm gives rise to bones of the chordal skull and skeletal muscles (Couly et al, 1992, 1993; Noden, 1983; Hacker and Guthrie, 1998). The mesenchyme adjacent to the developing brain is unique compared to other areas of the body, since it is both neural crest- and mesodermally-derived. Hence it would be interesting to know where other *Bmps* are expressed during stages when cranial neural crest cells are migrating.

By stage 14, the branchial arches and the mesenchyme ventral to the forebrain and midbrain are filled with proliferating neural crest cells, some regions of which express *Bmp-7* (Wall and Hogan, 1995). The appearance of the nasal placodes at stage 15 demarcates the position of the frontonasal mass and the lateral nasal prominences, which form medial and lateral to the nasal placodes, respectively. Expression of *Bmp-2* and *Bmp-4*, but not *Bmp-7*, has been described in the developing nasal pit (Francis-West et al., 1994). The ectoderm of the first branchial arch expresses *Bmp-4* as soon as it is formed (Shigetani et al., 2000). At stage 18, the maxillary and mandibular prominences

become distinct divisions of the first branchial arch (Yee and Abbot, 1978; Tamarin et al., 1984) and all the facial prominences are established. *Bmp-2* and *Bmp-4* are both expressed in the presumptive maxillary region and in the maxillary prominence (Francis-West et al., 1994).

Between stage 20 and 28, the facial prominences enlarge and take shape through cell proliferation and directional cell expansion (McGonnell et al., 1998; Peterka and Jelinek; 1983; Minkoff and Kuntz, 1977; 1978). Overlying epithelium is necessary for proper facial outgrowth while the mesenchyme contains the information for skeletal patterning (Wedden, 1987; Richman and Tickle, 1989; Hu and Helms 1999). Frontonasal mass epithelium also has unique polarizing capability when transplanted under the apical ectodermal ridge of the limb bud (Helms et al., 1997). This organizing activity is correlated with *sonic hedgehog* (*Shh*) expression in the frontonasal mass epithelium, a molecule that is also expressed in several other regions with polarizing activity (Sanz-Ezquerro and Tickle, 2000; Riddle et al., 1993). *Bmp-2* and *Bmp-4* are expressed in all the facial prominences during outgrowth (Francis-West et al., 1994), while the expression of *Bmp-7* has not been examined in all the facial prominences during this entire period (Wang et al., 1999; Wall and Hogan, 1995).

The frontonasal mass, lateral nasal prominences, and maxillary prominences merge and fuse to close the primary palate and establish the upper beak. The formation of the primary palate is a critical phase in facial morphogenesis. The most important step is the formation of the mesenchymal bridge between the corners of the frontonasal mass and

maxillary prominences. Fusion requires sufficient enlargement of the facial prominences, removal of the epithelial seam between the prominences and migration of the underlying mesenchyme to form the mesenchymal bridge (McGonnell et al., 1998; Wang et al., 1995). While the molecular basis for the formation of the bridge is not well understood, it is possible that *Bmps* are involved. In support of this hypothesis is the expression of *Bmp-2* and *Bmp-4* in the contact region between the frontonasal mass and maxillary prominences prior to fusion (Francis-West et al., 1994). Moreover TGF-β3, a related family member, regulates fusion of the secondary palate by similar mechanisms to those used in the primary palate (Taya et al., 1999; Kaartinen et al., 1995; Proetzel et al., 1995).

In the avian embryo, fusion and merging of facial prominences coincide with the onset of cell differentiation. The mesenchyme of the frontonasal mass gives rise to the prenasal cartilage and the premaxillary bone, while the epithelium forms the egg tooth. The lateral nasal prominences form the nasal chonchae. Derivatives of the maxillary prominences include the pterygoid, quadratojugal and palatine bones. The mandibular prominences form Meckel's cartilage and the surrounding membrane bones of the lower beak. BMPs stimulate the formation of prechondrogenic condensations from mesenchyme and later regulate the differentiation of chondroprogenitors to chondrocytes (Pizette and Niswander, 2000). Unlike the limb skeleton, which is mesoderm-derived, the facial skeleton is neural crest cell-derived. It is not known whether BMPs are expressed during facial skeletogenesis in similar patterns to those observed during limb differentiation.

Functional evidence to support a role for BMPs in patterning the chick face has been reported (Barlow and Francis-West, 1997; Ekanayake and Hall, 1997; Barlow et al., 1999; Shigetani et al., 2000). For example, application of BMP-2 and BMP-4 soaked beads to the maxillary prominence or to the medial mandibular prominence leads to changes in gene expression and the formation of a supernumerary palatine bone or branching of Meckel's cartilage (Barlow and Francis-West, 1997). Application of BMP-7 beads to isolated mandibular mesenchyme interferes with normal elongation of the cartilage (Wang et al., 1999); therefore BMP-7 is not able to replace the facial epithelium. At present we did not know whether facial mesenchyme expresses Bmp receptors (*BMPRs)* and which receptors are mediating the aforementioned responses to exogenous BMPs.

BMPs signal via transmembrane serine/ threonine kinases cell surface receptors, which are classified due to structural and functional differences, as type I and type II (BRK-3) receptors. Two type I receptors have been identified, BMPR-IA (ALK-3, BRK-1) and BMPR-IB (ALK-6, BRK-2; for review, see Massague, 1998). The type I receptors act as signal transducers by phosphorylating cytoplasmic targets, including members of the Smad family, on serine/threonine residues (Heldin et al., 1997; Massagué, 1998; Hoodless and Wrana, 1998). BMPR-II enhances ligand binding. BMPs bind weakly to each receptor type when expressed alone, but high affinity binding is observed when both receptor types are present (Koenig et al, 1994; ten Dijke et al, 1994; Liu et al 1995; Nohno et al., 1995). In the presence of BMPR-II, BMP-4 and BMP-7 bind BMPR-IB more efficiently than BMPR-IA (Nohno et al., 1995; Rosenzweig et al., 1995). In the

limb, *BmpRIA* and *BmpRII* are ubiquitously expressed throughout early morphogenesis, while *BmpRIB* is more specifically localized in prechondrogenic aggregates, immature chondrocytes and the perichondrium (Kawakami et al., 1996; Zou et al., 1997; Merino et al., 1998).

The actions of endogenous BMPs are likely controlled by naturally occurring antagonists including noggin (Capdevila and Johnson, 1998; Merino et al., 1998), chordin (Streit et al., 1998), gremlin (Merino et al., 1999; Capdevila et al., 1999), cerberus (Zhu et al., 1999) and caronte (Yokouchi et al., 1999; Esteban et al., 1999). These antagonists bind to BMPs and prevent them from interacting with their receptors, thus blocking BMP signaling (Zimmerman et al., 1996; Piccolo et al., 1996; Yokouchi et al., 1999; Esteban et al., 1999). Noggin can induce secondary axis formation in *Xenopus* (Smith and Harland, 1992; Smith et al., 1993); inhibit neural crest cell delamination (Kanzler et al., 2000; Sela-Donenfeld and Kalcheim, 1999), inhibit joint formation (Brunet et al., 1998), inhibit programmed cell death (Pizette and Niswander, 1999; Capdevila and Johnson, 1998; Merino et al., 1998; McMahon et al., 1998), inhibit chondrogenesis (Capdevila and Johnson, 1998) and alter patterning of the neural tube and somites (McMahon et al., 1998). Biochemical studies indicate that noggin binds BMP-2 and BMP-4 strongly, and BMP-7 with lower affinity (Zimmerman et al., 1996). The *Noggin* knockout mouse exhibits craniofacial abnormalities thus suggesting there is *noggin* expression in the normal developing head. To date, there have been no detailed studies mapping expression of noggin in mouse or chicken head development.

In this chapter we correlate the expression of *BMPs* with the expression of *BMPRs* and *noggin* in order to predict the sites where endogenous BMP signaling occurs. Our expression data shows that *noggin* is expressed in specific locations in the facial epithelium and early head mesenchyme. We also demonstrate that facial mesenchyme and epithelium express *BMPRs*, suggesting mechanisms by which cells respond to exogenous and endogenous BMP protein during craniofacial development.

Materials and Methods

Chicken embryos

Fertilized white leghorn chicken eggs were obtained from Coastline Chicks, Abbotsford, B.C. Eggs were incubated at 37.5°C until they reached the desired stage of development. Embryos were staged according to Hamburger and Hamilton (1951).

In situ hybridization probes

The chicken cDNAs were kindly provided by the following individuals: *BmpR-IA*, *BmpR-IB* (L. Niswander; Zou and Niswander, 1996; Zou et al., 1997), *BmpR-II* (T. Nohno; Kawakami et al, 1996), *Bmp-2* and *Bmp-4* (P. Brickell; Francis-West et al., 1994), *Bmp-7* (B. Houston; Houston et al., 1994), *noggin* (R. Johnson; Capdevila and Johnson, 1998) and *shh* (C. Tabin, Riddle et al., 1993). Plasmids were linearized with appropriate restriction enzymes in order to transcribe sense or antisense riboprobes labelled with [^{35}S]-UTP (Rowe et al., 1991).

Autoradiographic in situs

Specimens used for *in situ* hybridization were fixed in 4% paraformaldehyde (PFA) and processed into wax. RNA *in situ* hybridization on wax sections was done according to Rowe et al., (1991). Serial sections were divided between several slides so that expression patterns for different genes could be compared within the same embryo. Slides were left to develop for 1 to 3 weeks. Photographs were taken on a Zeiss compound microscope under darkfield illumination with Kodak Gold 100 print film or with a Minolta RD-175 digital camera. Near-adjacent sections were stained with

Toluidine Blue and photographed with brightfield illumination. Plates were composed using Adobe Photoshop.

Results

Radioactive *in situ* hybridization was performed on a total of 17 stage 10, 24 stage 15, 16 stage 20, 18 stage 24, 16 stage 28, 5 stage 30 and 3 stage 32 embryos over 7 experiments. Adjacent sections were hybridized to *Bmp-2*, *Bmp-4*, *Bmp-7*, *BmpR-1B*, *BmpR-1A*, *BmpR-II*, and *noggin* in order to compare patterns of expression within the same embryo. We refer to the cranial-caudal or superior-inferior axis as being from the top of the head to the tail, the medio-lateral axis as being from the center of the embryo towards the eyes, dorso-ventral axis as being from the back to the front of the embryo and the proximo-distal axis as being from the jaw-joint region to the tip of the beak (Fig. 1.1). Expression was compared to background levels observed in non-expressing tissue on the same slide.

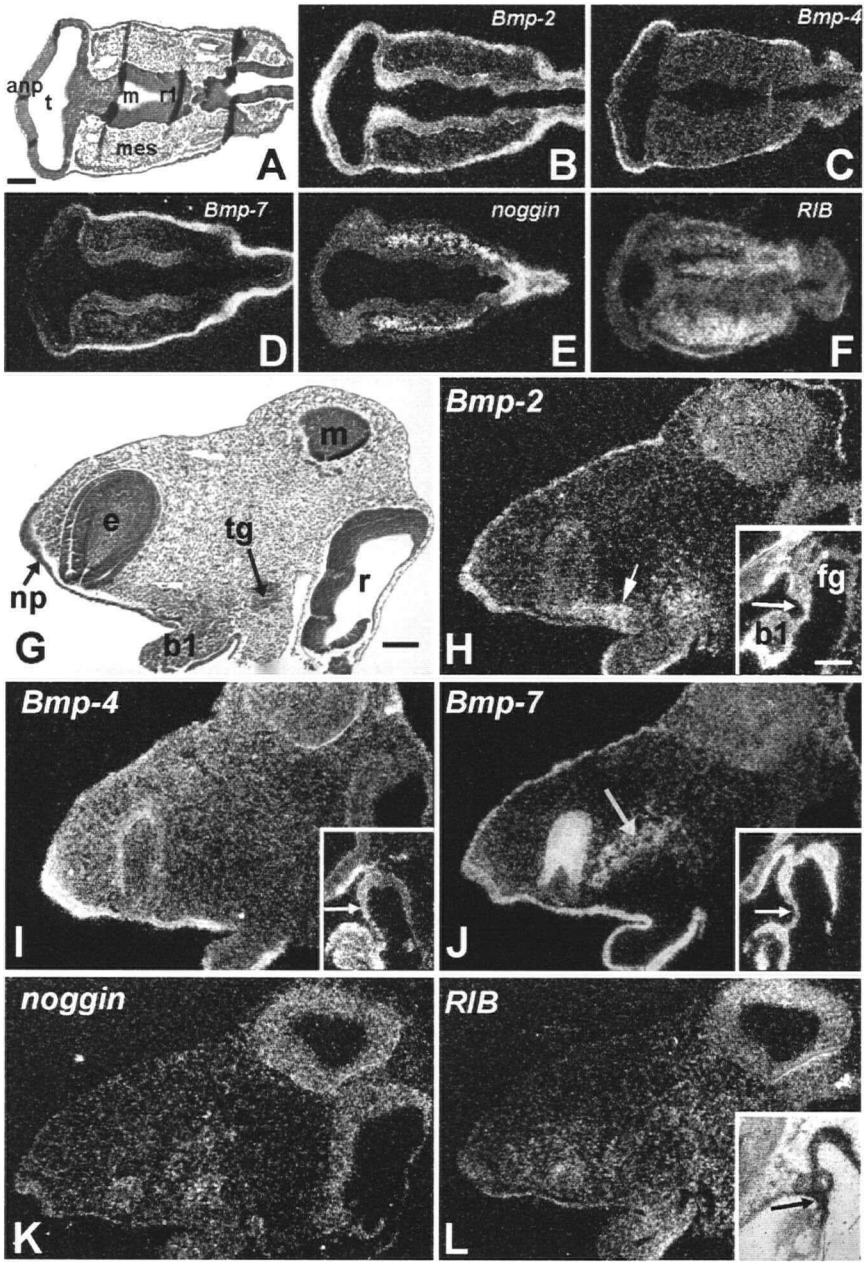
Expression during neural crest cell migration

At stage 10, *Bmp-2* and *Bmp-4* transcripts are not detected in early head mesenchyme (Fig. 3.1B,C). In contrast, *Bmp-7* transcripts are found in the mesenchyme rostral to the mesencephalon-rhombencephalon isthmus, with expression fading caudal to r1 (Fig. 3.1D). *Noggin* and *BmpR-IB* transcripts are expressed abundantly at all axial levels of the craniofacial mesenchyme (Fig. 3.1E,F) and in the notochord (data not shown). *BmpR-IA* and *BmpR-II* are ubiquitously expressed in mesenchyme and epithelium (data not shown).

Epithelial expression is detected for the three ligands. *Bmp-2* and *Bmp-4* transcripts are detected throughout the head epithelium from the anterior neuropore and extends well into the hindbrain (Fig. 3.1B,C). *Bmp-7* expression is not observed in the anterior neuropore but is seen in the presumptive lens ectoderm covering the optic stalks and in

Figure 3.1 Expression in stage 10 and 15 chicken embryos. A-F are frontal sections through the head of the embryo. A to E are adjacent sections from the same embryo while F is from a different embryo. G-L are parasagittal sections from a single stage 15 embryo. A,G are Toluidine Blue stained sections that were not hybridized. H'-J' and L' (insets) are sagittal sections through the buccopharyngeal membrane, positive signal is seen as white silver grains in darkfield and black silver grains in bright field. J: arrow indicates expression in the mesenchyme proximal to the eye. K: *Noggin* did not appear to be expressed in stage 15 embryos. L: *BmpR-IB* continues to be expressed in the head mesenchyme, and is also in the epithelium in the roof of the stomodeum and caudal first branchial arch.—H'-J', L': *Bmp-2*, *Bmp-4*, *Bmp-7* and *BmpR-IB* are all expressed in the buccopharyngeal membrane (arrows in H', I', J', L').

KEY: anp - anterior neuropore; b1 - first branchial arch; e - eye; f - forebrain; fg - foregut; m - midbrain; mes - mesenchyme; np - nasal pit; r1 - rhombomere 1; t - telencephalon; tg - trigeminal nerve. Scale bars for stage 10 embryos = 50 μm; stage 15 embryos = 100 μm.


anp
t
m
r1
mes
A
Bmp-2
B
Bmp-4
C
Bmp-7
D
noggin
E
RIB
F
m
e
tg
r
np
b1
G
Bmp-2
fg
b1
H
Bmp-4
I
Bmp-7
J
noggin
K
RIB
L

the remaining ectoderm extending caudally to the hindbrain (Fig. 3.1D). *BmpR-IB* transcripts are present caudal to the optic stalks and extend past the hindbrain (Fig. 3.1F). *BmpR-IB* transcripts in the surface epithelium overlap the expression of all three BMPs examined. *Noggin* transcripts are not detected in the head epithelium (Fig. 3.1E).

Localized expression of BMP signaling molecules in the neuroepithelium is also observed at stage 10. *Bmp-2* is abundantly expressed in the anterior neuropore, the optic stalks and also in the future diencephalon (Fig. 3.1B). *Bmp-7* is found at low levels in the neuroepithelium of the mesencephalon and anterior rhombencephalon (Fig. 3.1D). *BmpR-IB* transcripts are only detected in the anterior neuropore (data not shown). *Noggin* message is localized to the optic stalk regions (Fig. 3.1E).

Onset of face morphogenesis

At stage 15, *Bmp-2* and *Bmp-7* are expressed in complementary patterns in the head mesenchyme. *Bmp-2* transcripts are located proximal to the first branchial arch, within the first branchial arch, and adjacent to the ectoderm of the presumptive maxillary region (Fig. 3.1H). *Bmp-4* is expressed at background levels in the head mesenchyme (Fig. 3.1I). *Bmp-7* transcripts are more rostral than those of *Bmp-2* and are located just proximal to the eye (Fig. 3.1J). This is the location of the future ophthalmic branch of the trigeminal nerve. *Noggin* is transcribed at low levels in the head mesenchyme (Fig. 3.1K). *BmpR-IB* message is abundant in mesenchyme ventral to the brain where the cranial base will form (Fig. 3.1L). *BmpR-IB* transcripts are spread over a larger area of the head and overlap with *Bmp-2*, *Bmp-4* and *Bmp-7* transcripts. *Bmp-2* transcripts also

extend into the first branchial arch and overlap with *BmpR-IB* mRNA (compare Fig. 3.1H and L). *BmpR-IA* and *BmpR-II* are expressed in both epithelium and mesenchyme but are not concentrated in any particular locations (data not shown).

Bmp-4 expression is predominantly in the surface epithelium with the highest levels in the first branchial arch, presumptive maxillary region, roof of the stomodeum, and in the epithelium covering the forebrain (Fig. 3.1I; note that *Bmp-4* transcripts are also in the epithelium around the forebrain at stage 10). *Bmp-2, Bmp-4* and *Bmp-7* are expressed in the epithelium of the first branchial arch and the presumptive maxillary region (Fig. 3.1H-J). *Bmp-4* is also expressed in the presumptive palatal epithelium in mouse (E9.5; LaMantia 2000), similar to the roof of the stomodeum in stage 15 chicken embryos.

BmpR-IB and *Bmp* transcripts overlap in several epithelial regions at stage 15. *BmpR-IB* is highly expressed in the caudal surface of the first branchial arch and overlaps all three *Bmps* examined (Fig. 3.1L). *BmpR-IB* transcripts are expressed in the roof of the stomodeum in the same location as *Bmp-4* and *Bmp-7* (data not shown). The nascent nasal placode expresses *Bmp-2*, *Bmp-4* and *Bmp-7*, but neither *BmpR1B* nor *noggin* (Fig. 3.1H-J,L). The buccopharyngeal membrane separates the stomodeum from the foregut and is only a transitory structure. Shortly after stage 15, the membrane breaks down, allowing communication between the gut and the oral cavity. All three *Bmps* are expressed in the buccopharyngeal membrane, as is *BmpR-1B* (Fig. 3.1H'-J', L').

Expression in stage 20 facial prominences

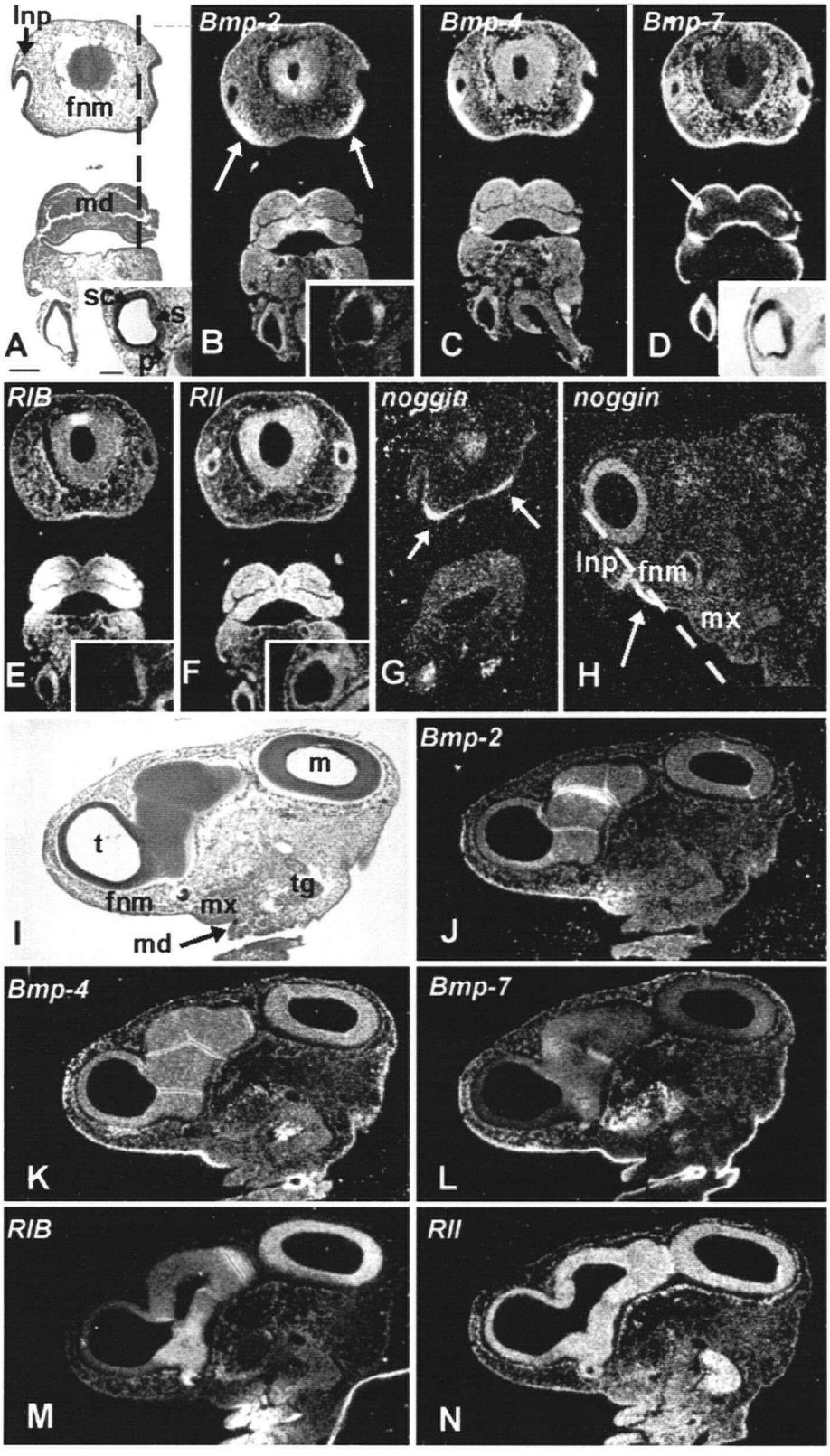
Frontonasal mass and lateral nasal prominence expression

In the stage 20 frontonasal mass, the three *Bmps* are localized to the mesenchyme at the lateral edges of the frontonasal mass. *Bmp-4* and *Bmp-7* are also expressed in the center of the frontonasal mass, while *Bmp-2* is not expressed (Fig. 3.2B-D and J-L; see also stage 20, *Bmp-2* and *Bmp-4* expression pattern in Francis-West et al., 1994). *BmpR-IB*, *BmpR-IA*, and *BmpR-II* are all expressed in the lateral frontonasal mass mesenchyme and overlap the expression of the three ligands (Fig. 3.2E,F and data not shown). *Noggin* is not present in the frontonasal mass mesenchyme (Fig. 3.2G,H). The lateral nasal prominence mesenchyme does not express *Bmp-2* or *Bmp-4* (Fig. 3.2B,C and data not shown) but does express *Bmp-7*, *BmpR-1B* and *BmpR-II* (Fig. 3.2D-F).

The frontonasal mass and lateral nasal prominences express all three *Bmps*, *BmpR-II* and *BmpR-IA* throughout the epithelium (Fig. 3.2B-D, F, J-K, N and data not shown). In contrast, transcripts for *BmpR-IB* are most abundant in the medial frontonasal mass epithelium and relatively lower in the lateral nasal prominence epithelium (Fig. 3.2E). *Noggin* is expressed in the epithelium covering the caudal edge of the frontonasal and extends into the nasal slits (Fig. 3.2G,H). The invaginating nasal pit epithelium does not express *Bmp-2*, *Bmp-4* or *noggin* transcripts except at the extreme distal edges (Fig. 3.2G and data not shown). *Bmp-7* and *BmpRII* are expressed throughout the nasal pit epithelium (Fig.3. 2D and F).

Figure 3.2 Expression in stage 20 embryos. A and I are Toluidine Blue stained sections that were not hybridized. A-F are frontal sections of a single embryo while G is from a different embryo. Arrows in B and G highlight expression in the lateral frontonasal mass regions. Arrow in D shows expression in the mandibular branch of the trigeminal nerve. The angle at which embryos were cut in the frontal plane does not include the maxillary prominences. Expression patterns in the maxillary prominence are displayed in parasagittal sections (H-N). Sections I-N are from the same embryo. H: Note restricted *noggin* expression to the narrow band in the frontonasal epithelium (arrow). Insets A', B', D', E' and F' are frontal sections of the otic vesicle within the same embryo, photographed in dark field (except D which is in bright field). Planes of section are described by the lines in A and H.

KEY: fnm - frontonasal mass; lnp - lateral nasal prominence; m - midbrain; md - mandibular prominences; mn - mandibular branch of trigeminal nerve; mx - maxillary prominence; p - posterior crista ampullaris; s - macula sacculi; sc - superior crista ampullaris; t - telencephalon; tg - trigeminal nerve. Scale bars for frontal and parasagittal sections = 100 μm; insets = 50 μm.


lnp
fnm
md
sc
s
p
A
Bmp-2
B
Bmp-4
C
Bmp-7
D
RIB
E
RII
F
noggin
G
noggin
lnp
fnm
mx
H
m
t
fnm
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tg
md
I
Bmp-2
J
Bmp-4
K
Bmp-7
L
RIB
M
RII
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Maxillary prominence expression

The only BMP detected in the maxillary mesenchyme at stage 20 is *Bmp-2*. *Bmp-2* is also expressed in the distal cranial mesenchyme (Fig. 3.2J and Francis-West et al., 1994). *BmpR-IB* is localized to the caudal edge of the proximal maxillary prominence and thus is complementary to *Bmp-2* (Fig. 3.2M). *BmpR-IA* and *BmpR-II* demonstrate local concentrations of intense signal in the maxillary mesenchyme (Fig. 3.2N; *BmpR-IA* not shown).

In the epithelium, *BmpR-IB* transcripts are detected in the caudal edge of the maxilla, and thus are complementary to *Bmp-2* and *Bmp-4* (compare Fig. 3.2M with 3.2J,K). *Noggin* is not detected in the maxillary epithelium (Fig. 3.2H). *BmpR-IA* and *BmpR-II* are expressed throughout the maxillary epithelium (Fig. 3.2N and data not shown).

Mandibular prominence expression

At stage 20, *Bmp-2* and *Bmp-4* are expressed at low levels throughout the mandibular mesenchyme except adjacent to the midline, where *Bmp-2* transcripts are much more abundant than in lateral regions (Fig. 3.2B,C). The mandibular branch of the trigeminal nerve expresses *Bmp-7* transcripts (Fig. 3.2D). *BmpR-IB* is most abundant in the lateral mesenchyme of the mandibular prominences, complementary to *Bmp-2* transcripts (compare Fig. 3.2B with E). *BmpR-IA* (not shown) and *BmpR-II* are both very abundant in the mandible (and maxilla) compared to the frontonasal mass at stage 20 (Fig. 3.2F,N). *Noggin* message is not detected above background levels in the mandibular prominence (Fig. 3.2G).

All three *Bmps* are also localized to the mandibular epithelium at stage 20. *Bmp-2* and *Bmp-4* (Fig. 3.2B,C) transcripts are more medial, while *Bmp-7, BmpR-IA*, *BmpR-IB* and *BmpR-II* are very abundant throughout the mandibular epithelium (Fig. 3.2D,F and data not shown). *Noggin* transcripts are not detected in the mandibular epithelium (Fig. 3.2G).

BMP expression during morphogenesis of the otic vesicle

A description of where *Bmp-4*, *Bmp-5*, and *Bmp-7* are expressed during sensory organ differentiation in the chicken inner ear can be found in other published work (Wu and Oh, 1996; Oh et al., 1996; Gerlach et al., 2000). Here, we compare *Bmp-4* and *Bmp-7* localization to *Bmp-2* and the BMP receptors at stage 20, when three foci of *Bmp* expression are observed. The anterior, posterior and medial part of the otocyst corresponded to regions that give rise to the superior and posterior, crista ampullaris, and macula sacculi, respectively. *Bmp-2* is also transcribed in all three regions (Fig. 3.2B'), similar to other BMPs examined. We confirm that *Bmp-7* expression is less restricted than the other *Bmps* (Fig. 3.2D'). *BmpR-IB* transcripts are localized to the medial otocyst region; expression begins just inferior to the anterior focus and extends to the posterior focus (Fig. 3.2E'). As in other tissues, *BmpR-IA* and *BmpR-II* are ubiquitously expressed (Fig. 3.2F' and data not shown). *Noggin* transcripts are not detected in the stage 20 otocyst (also reported by Chang et al., 1999).

Expression in the stage 24 face

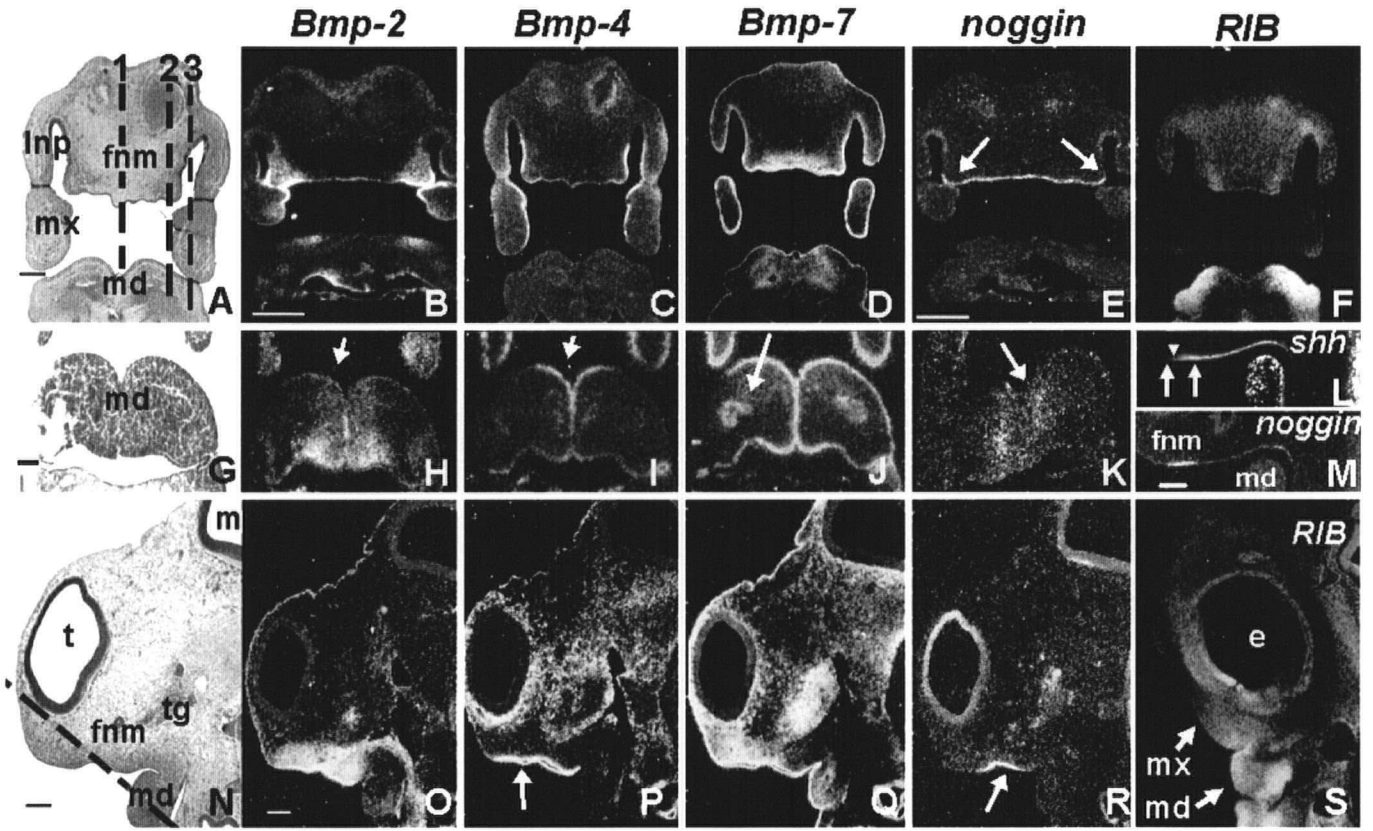
The expression patterns of the examined genes at stage 24 are for the most part similar to those observed at stage 20. Here, we will highlight the differences at stage 24. In the frontonasal mass, *Bmp-7* expression is in the mesenchyme along the inferior edge of the frontonasal mass and becomes more localized, rather than being spread all across as it was at stage 20 (Fig. 3.3D). *Bmp-2* is also more localized in the corners of the frontonasal mass (Fig. 3.3B). *BmpR-IB* expression is more prominent in the mesenchyme of the frontonasal mass and in the region surrounding the cranial end of the nasal slit (Fig. 3.3F). *BmpR-IB* is no longer expressed in the medial frontonasal mass epithelium. *Noggin* is expressed in the caudal epithelium of the frontonasal mass just as at stage 20, and overlaps the most distal extent of *shh* expression (compare Fig. 3.3L and M).

In the lateral nasal prominences, *Bmp-4* transcripts are mostly localized to the mesenchyme in the furrow between the lateral nasal and maxillary prominences (Fig. 3.3C). *Bmp-4* is also in the lateral epithelium but at lower levels than in the medial epithelium (Fig. 3.3C). The expression patterns of *Bmp-2*, *Bmp-7*, *BmpR-IB*, and *Noggin* in the lateral nasal epithelium are unchanged from stage 20. *Bmp-7* is expressed in the lateral regions of the lateral nasal prominences and overlaps *BmpR-IB* (compare Fig. 3.3D with F).

Bmp-4 and *Bmp-7* transcripts are first detected in the maxillary mesenchyme at stage 24. *Bmp-2* is expressed in the medial and rostral maxillary mesenchyme (Fig. 3.3B), while

Figure 3.3 Expression in stage 24 embryos. Sections in frontal (A to K) and parasagittal (L to S) planes. A,G,N – Toluidine Blue stained, non-hybridized sections. Adjacent sections within single embryos are as follows (A, C, D, F = embryo1), (B,E = embryo 2), (G - J = embryo 3), (L-R = embryo 4) and (S = embryo 5). E: Note *noggin* expression in the epithelium around the lateral corners of the frontonsal mass (arrows). G - K are sections through the mandibular prominence. H, K: Overlapping *Bmp-2* and *noggin* transcripts in the mandibular mesenchyme (arrows). J: *Bmp-7* expression in the mandibular branch of the trigeminal nerve (arrow). L, M: *Noggin* transcripts in the frontonasal mass overlap and extend slightly distal (arrows in L) to the start of *shh* expression (arrowhead in L). P: *Bmp-4* is highly expressed in the frontonasal mass epithelium (arrow) and overlaps with *noggin* transcripts (arrow in R). Planes of section are described by the lines in A and N. Sagittal sections in L,M were cut along line 1 in A. Sagittal sections in N,O,P,Q,R were cut along line 2 in A. Sagittal sections in S were cut along line 3 in A.

KEY: e, eye; fnm, frontonasal mass; lnp, lateral nasal prominence; m, midbrain; md, mandibular prominences; mx, maxillary prominence; t, telencephalon; tg, trigeminal nerve. Scale bars for frontal sections (A-F) = 200 μm; frontal sections of the mandible (G-K) = 100 μm; sagittal sections (N-S) = 200 μm and (L, M) = 200 μm.


Bmp-2
Bmp-4
Bmp-7
noggin
RIB
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2
3
lnp
fnm
mx
md
A
B
C
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E
F
md
G
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I
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K
shh
L
fnm
noggin
md
M
m
t
tg
fnm
md
N
O
P
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R
RIB
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Bmp-4 and *Bmp-7* transcripts are present in the lateral and rostral maxillary mesenchyme (Fig. 3.3C,D). *BmpR-IB* message is expressed in the mesenchyme of the caudal half of the maxilla (Fig. 3.3F,S). *Bmp-2* and *Bmp-4* continue to be expressed in the maxillary epithelium, but transcripts are more prominent in the medial surface compared to the lateral sides (Fig. 3.3B,C Francis-West et al., 1994). *Noggin* message is not present in maxillary epithelium or mesenchyme (Fig. 3.3E).

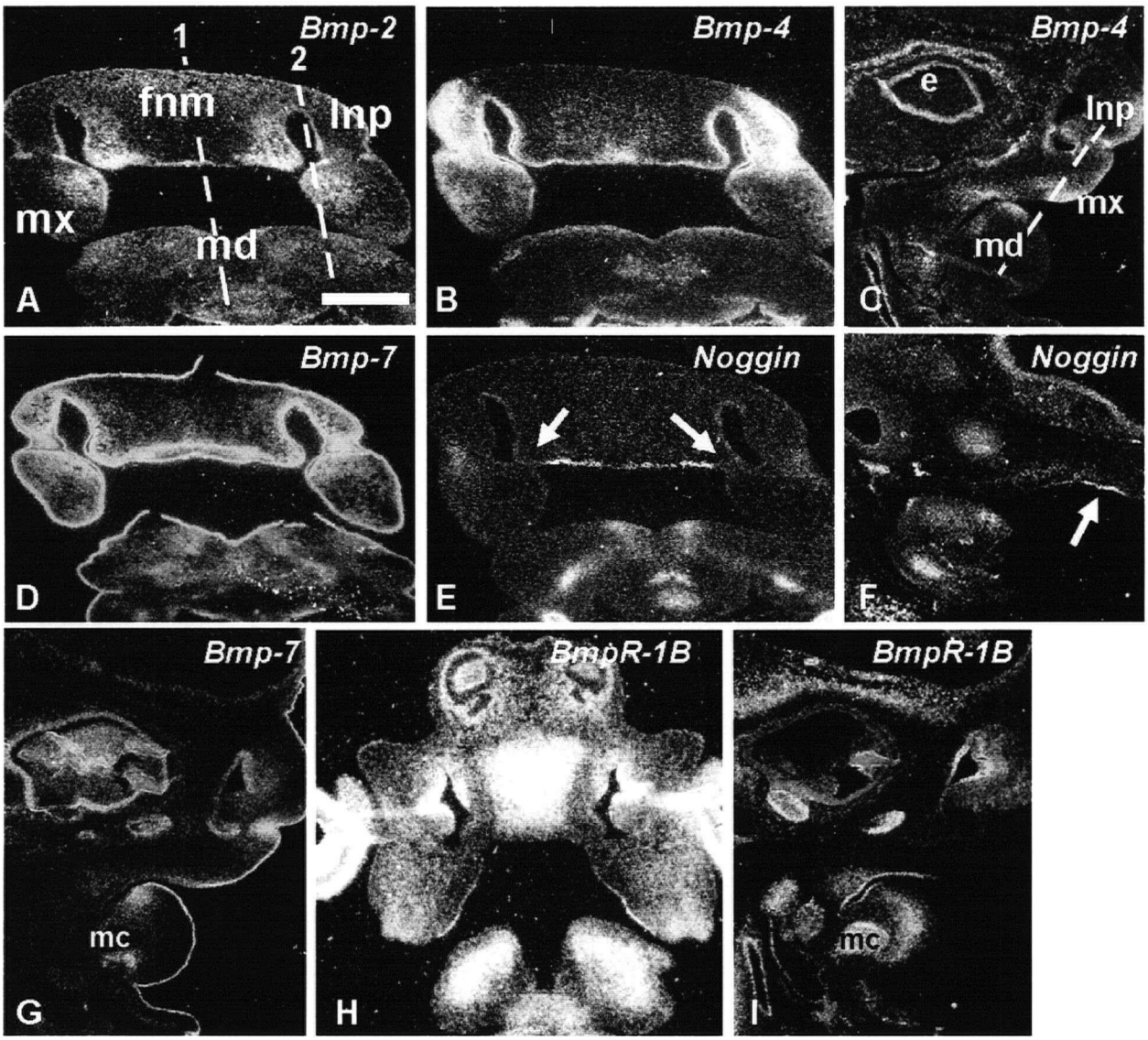
In the mandibular prominences at stage 24, the expression of *Bmp-2*, *Bmp-7*, and *noggin* is restricted to the medial mandibular mesenchyme while *BmpR-IB* becomes restricted to the superior-lateral mesenchyme (Fig. 3.3F, H-K). *Bmp-7* is expressed in the maxillary and mandibular branches of the trigeminal nerve (Fig. 3.3J, Q). *Noggin* is also expressed at low levels in the trigeminal nerve and in the otic vesicle (Fig. 3.3R).

Expression in stage 28 embryos

At stage 28, *Bmp-2, Bmp-4*, and *Bmp-7* are expressed in the epithelium and mesenchyme of the extreme corners of the frontonasal mass (now called the globular processes; Romanoff, 1960), and at the medial edge of the maxillary prominence where fusion takes place. The mesenchymal expression of *Bmp-2* in the corners of the frontonasal mass is much higher than that of *Bmp-4* (Fig. 3.4A,B). *Bmp-4* expression is primarily epithelial in this zone of contact (Fig. 3.4B). *Bmp-7* is also expressed at high levels in the frontonasal mass epithelium and subjacent mesenchyme (Fig. 3.4D). All three *Bmps* are also transcribed in the deep furrow between the merging lateral nasal and maxillary mesenchyme.

Figure 3.4 Expressions in stage 28 embryos. A, B, D and E are adjacent frontal sections within the same embryo while H is from a different embryo. Arrows in E highlight the absence of *noggin* transcripts in the corner epithelium of the frontonasal mass. C, G and I are parasagittal sections within the same embryo. F is a mid-sagittal section from a different embryo. F: Shows localized *noggin* expression in a narrow band of frontonasal mass epithelium (arrow).

KEY: e - eye; fnm - frontonasal mass; lnp - lateral nasal prominence; mc - Meckel's cartilage; md - mandibular prominences; mx - maxillary prominence. Scale bar = 500 μm.


Bmp-2
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Bmp-4
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C
Bmp-7
D
Noggin
E
Noggin
F
Bmp-7
mc
G
BmpR-1B
H
BmpR-1B
mc
I

Bmp-2, *Bmp-4*, and *Bmp-7* are expressed in overlapping domains in the medial mesenchyme and epithelium of the maxillary process (Fig. 3.4A,B, D). *Bmp-4* is located in a caudal domain within the maxillary prominence (Fig. 3.4C and Francis-West et al., 1994).

Noggin transcripts are localized to the caudal frontonasal mass epithelium but no longer extend around the globular processes (N=3/3; compare Fig. 3.3E with Fig. 3.4E). Mid-sagittal sections show that *noggin*-expressing epithelium separates the caudal edge of the frontonasal mass from the roof of the stomodeum (Fig. 3.4F). *BmpR-IB* is also present in the lateral mesenchyme of the globular processes (Fig. 3.4H). *BmpR-IA* and *BmpR-II* are ubiquitously expressed at stage 28 in the epithelium and mesenchyme (data not shown).

Cartilagenous condensations

Prechondrogenic condensations are beginning to form in the center of the frontonasal mass, the location of the future prenasal cartilage and nasal septum. *BmpR-IB* transcripts are very abundant in the center of the frontonasal mass and define the chondrogenic region (Fig. 3.4H). *Bmp-2* is expressed in mesenchymal cells lateral to the condensation (Fig. 3.4A and data not shown). *Bmp-4* expression is found caudal to the condensation (Fig. 3.4B). *Bmp-7* is expressed in the mesenchyme adjacent to the condensation on the distal, caudal and lateral sides (Fig. 3.4D and data not shown). *Noggin* transcripts are not present in the center of the frontonasal mesenchyme at stage 28 (data not shown).

Condensing mesenchymal cells in the medial edge of the lateral nasal prominences and surrounding the cranial nasal pit form the nasal conchae. The most

abundant transcripts found in the lateral nasal prominences are *Bmp-4*, *Bmp-7*, and *BmpR-IB*. *Bmp-4* and *Bmp-7* transcripts are localized to the lateral and caudal regions of the lateral nasal prominences and overlap *BmpR-IB* transcripts (Fig. 3.4B-D and G-I). In shallow frontal sections, *BmpR-IB* message is found in most of the lateral nasal mesenchyme corresponding to the superior choncha (data not shown). In deeper sections, the region of expression becomes restricted to the mesenchyme next to the nasal slits (Fig. 3.4H,I). *Bmp-7* transcripts overlap with *BmpR-IB* (compare Fig. 3.4D,G with H, I). *Noggin* transcripts are localized to the mesenchyme in the lateral nasal- maxillary furrow (Fig. 3.4E).

In the mandible, Meckel's cartilage condensation is further advanced than the frontonasal mass cartilages (Matovinovic and Richman, 1997). Meckel's cartilage expresses high levels of *BmpR-IB* (Fig. 3.4H,I) and *noggin* (Fig. 3.4E,F) transcripts. In contrast, the three BMPs are expressed in complementary patterns to that of *noggin*. *Bmp-4* is expressed in a narrow region at the superior edge of the mandibular prominence (Fig. 3.4B,C and Francis-West et al., 1994). *Bmp-2* is expressed in the mesenchyme superior and inferior to Meckel's cartilage. *Bmp-7* is expressed adjacent to the condensations of Meckel's cartilage (Fig. 3.4G), in the presumptive perichondrium.

Expression in stage 30 embryos

Expression in maxillary mesenchyme, sites of intramembranous ossification

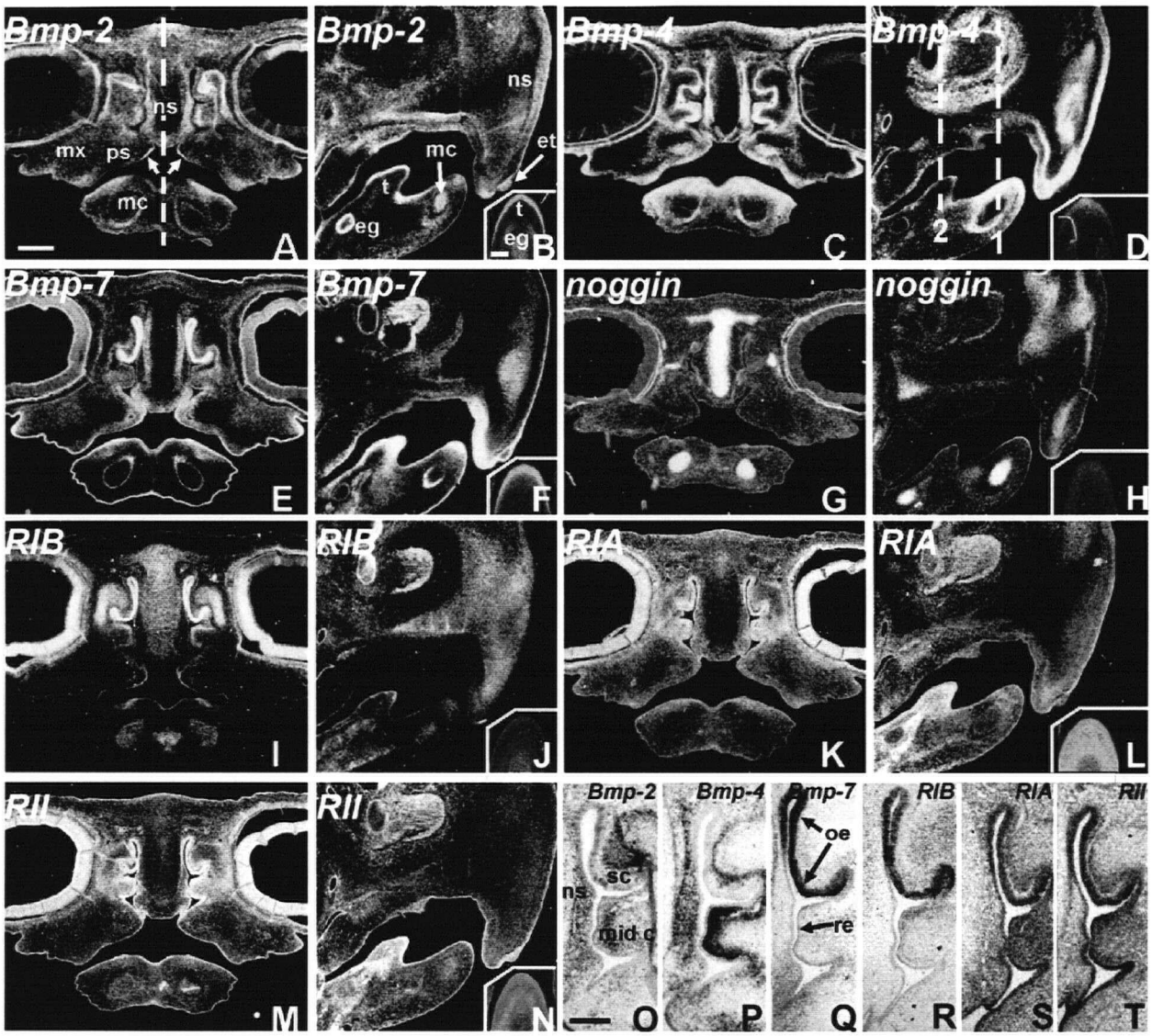
The maxillary prominences make membranous bone that condenses directly from mesenchyme, starting at stage 31 (day 7; Murray, 1963; Romanoff, 1960) without a cartilagenous template. The fate of discrete regions of the maxillary prominences is not

known, but the interesting patterns of gene expression suggest that some degree of molecular prepatterning exist as early as stage 28. These regional concentrations of gene expression are more noticeable at stage 30. Areas of transcript overlap in the maxillary prominence include lateral mesenchyme (*Bmp-2, -4, -7*, and *noggin*; Fig. 3.5E,G), medial and cranial mesenchyme (*Bmp-2, -4, –7*, and *BmpR-IB*; Fig. 3.5A,C, E) and caudal mesenchyme (*Bmp-2, -4, -7 and BmpR-1A*; Fig. 3.5A,C, K). The central mesenchyme expresses *Bmp*-2, *Bmp-4*, and *Bmp-7*. *Noggin* transcripts were also detected in the caudal mesenchyme in deeper frontal sections (data not shown).

Expression in cartilage and perichondrium

By stage 30, cartilage differentiation is underway in the chondrocranium. *BmpR-IB* is expressed within cartilage elements and the developing perichondrium of the nasal septum, nasal conchae, and Meckel's cartilage (Fig. 3.5I,J). *Noggin* message is very abundant within the nasal septum, nasal conchae, and Meckel's cartilage, but nested within the larger *BmpR-1B* expressing region (Fig. 3.5G). In the nasal conchae, *Noggin* signal is prevalent in the more distal frontal sections and extends cranially and merges with *noggin* expression in the nasal septum (data not shown). *Bmp-2, Bmp-4*, and *Bmp-7* are expressed in the perichondrium, bordering *Noggin* and in all the facial cartilages but overlapping the outer edges of the *BmpR-IB* expression domain (compare Fig. 3.5G-J with A-F). *Bmp-2* but not *Bmp-4* or *Bmp-7*, is present above background levels within the cartilage (Fig. 3.5A-F). *BmpR-IA* and *BmpR-II* are expressed at low levels in the cartilage compared to the surrounding mesenchyme (Fig. 3.5K-N; with the exception of *BmpR-II* in Meckel's cartilage; Fig. 3.5M,N).

Figure 3.5 Expressions in stage 30 embryos. Serial sections within embryo 1 were made in the frontal (A,C,E,G,I,K,M) and embryo 2 in the mid-sagittal plane (B,D,F,H,J,L,N). The medial edge epithelium of the palate (white arrows in A) expresses *Bmp2*, *Bmp-7* and *BmpR-IB*. Insets (B',D',F',H',J',L',N') are frontal sections of the tongue. O-T are brightfield, higher power views of darkfield frontal sections showing expression in the left nasal epithelium and nasal chonchae. Note the very abundant and specific expression of *Bmp-7* (Q) and *BmpR-IB* (R) in the olfactory epithelium. Planes of section are described by the lines in A and D. Frontal sections in A,C,E,G,I,K,M were cut along line 2 in D. Frontal sections in A',C',E',G',I',K',M' were cut along line 1 in D. KEY: e - eye; eg - entoglossum; mid c - middle concha; ns - nasal septum; oe - olfactory epithelium; mc - Meckel's cartilage; mx - maxilla; ns - nasal septum; ps - palatal shelf; re - respiratory epithelium; sc - superior concha; t - tongue. Scale bars for frontal and sagittal sections = 500 μm; nasal conchae sections = 250 μm; tongue insets = 100 μm.


Bmp-2
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Expression in nasal epithelium

The nasal epithelium gives rise to two distinct, sensory and non-sensory, epithelial domains. The sensory olfactory epithelium arises in the cranial-proximal regions of the nasal passage, while the respiratory epithelium forms more caudal-distally. *BmpR-IA* and *BmpR-II* are ubiquitously expressed throughout the nasal epithelium (Fig. 3.5K',M'). The olfactory epithelium expresses high levels of *Bmp-7* and *BmpR-IB* (Fig. 3.5E',I'). *Bmp-7* transcripts precisely overlap those of *BmpR-IB* in the superior and middle nasal chonchae epithelium (compare Fig. 3.5E with I). *Bmp-2*, *Bmp-4*, and *Noggin* are not expressed in the nasal epithelium (Fig. 3.5A,C, G).

Maxillary and mandibular epithelium

The medial edge of the maxillary prominences does not fuse in birds (unlike in mammals), hence the epithelium persists throughout life. Molecules that are restricted to the medial edge epithelium along the palatal shelves at stage 30 include *Bmp-2* and *BmpR-IB* (Fig. 3.5A,I). All other cDNAs used in the present study were expressed throughout the maxillary epithelium, with the exception of *noggin. Noggin* message is not detected in the maxillary epithelium (Fig. 3.5G).

The mandibular ectoderm expresses BMPs, their receptors, and *noggin*. The cranial or oral side of the mandibular ectoderm expresses *Bmp-2, Bmp-4, Bmp-7, noggin* and *BmpR-IB* (Fig. 3.5A-I). Within the oral ectoderm there is slightly higher expression of *Bmp-2*, *noggin* and *BmpR-IB* in the medial compared to the lateral epithelium. *Bmp-7*, *BmpR-IA* and *BmpR-II* are expressed throughout the intraoral and extraoral mandibular epithelium (Fig. 3.5E,F, K-N).

Expression in the tongue

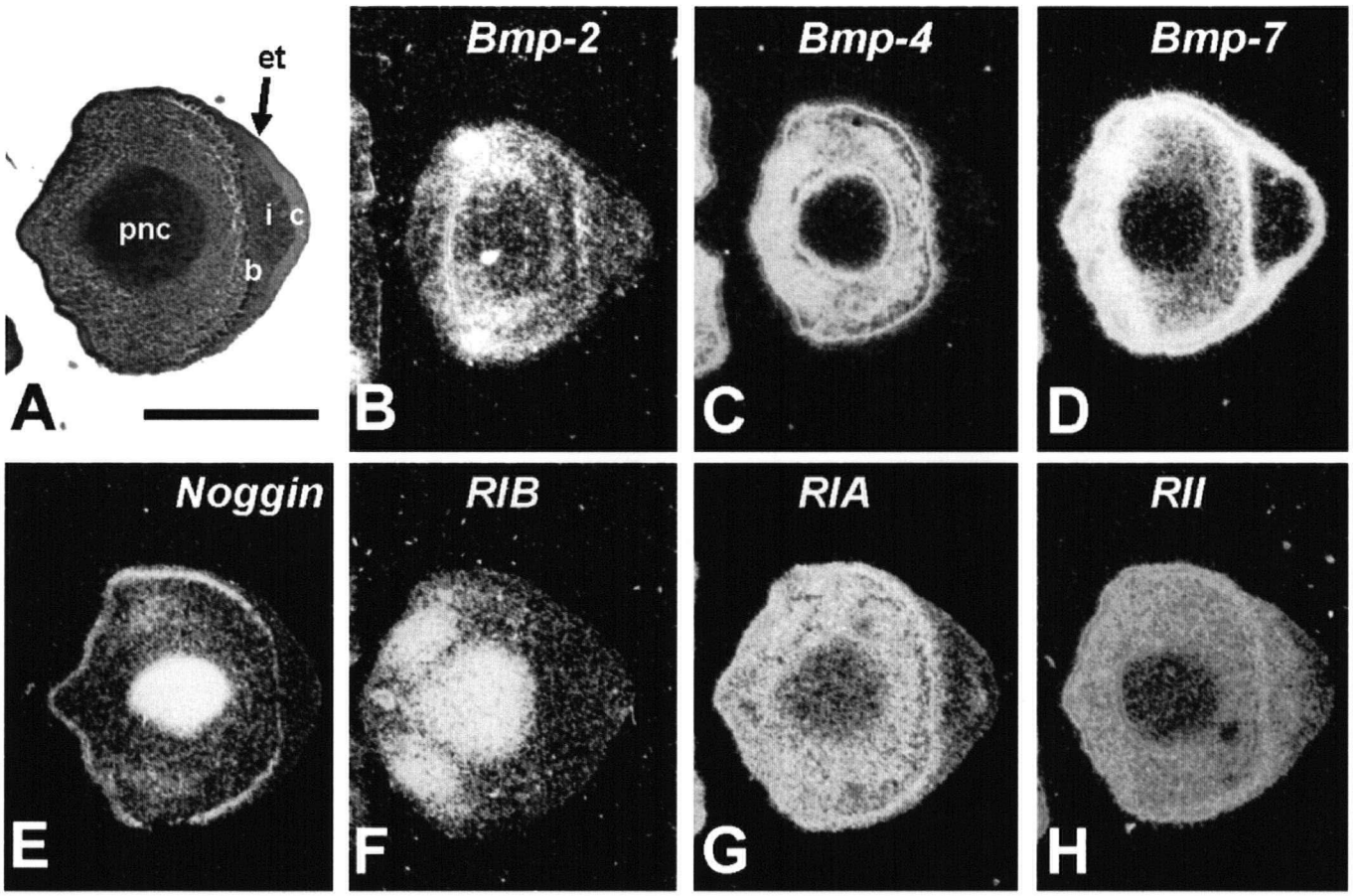
Our *in situ* hybridization studies suggest that BMPs may also be involved in formation of the specialized tongue epithelium. At stage 30, the BMP ligands display distinct expression patterns within the tongue. *Bmp-2* transcripts are found in the epithelium, predominantly in the medial regions, while *Bmp-4* is in the mesenchyme underlying the epithelium (Fig. 3.5B,D and 3.5B',D'). *Bmp-7* is expressed throughout the epithelium and underlying mesenchyme Fig. 3.5F and F'). *BmpR-IA* and *BmpR-II* are expressed in the entire developing tongue mesenchyme and epithelium (Fig. 3.5L,N and 3.5L',N'). *Noggin* message is not detected in the tongue epithelium but is expressed at low levels in the underlying mesenchyme (Fig. 3.5H and H'). *BmpR-IB* is only expressed in the mesenchyme and overlaps entoglossum condensations (data not shown).

Egg tooth formation at stage 32

The egg tooth is an epithelial specialization that forms at the tip of the upper beak at stage 30. The egg tooth is keratinized at stage 32. The chick uses the egg tooth during hatching to break through the shell. The outer cornified layer of the egg tooth expresses *Bmp-7, BmpRIA*, and *BmpR-II* (Fig. 3.6D,G, H). The intermediate layer expresses *Bmp-2, BmpR-IA*, and *BmpR-II* (Fig. 3.6B,G, H). The basal cell layer expresses *Bmp*-2, *Bmp-4*, *Bmp*-7, *Noggin*, *BmpRIA*, and *BmpR-II* (Fig. 3.6B-E, G, H).

Figure 3.6 Expression in the egg tooth of stage 32 embryos. Serial sections are in the coronal plane. A is a non-hybridized, Toluidine Blue stained section photographed in brightfield and B-H are darkfield photos.

KEY: b - basal cell layer; c - cornified layer; et - egg tooth; i - intermediate layer; pnc - prenasal cartilage. Scale bar = 500 μm.


et
pnc
i
c
b
A
Bmp-2
B
Bmp-4
C
Bmp-7
D
Noggin
E
RIB
F
RIA
G
RII
H

Discussion

Our data indicate that *BmpR-II* and *BmpR-IA* are ubiquitously expressed during craniofacial development. In contrast, *BmpR-IB* expression is much more specific and therefore suggests more specific functions. We identify novel regions where *noggin* is expressed in the early head mesenchyme and caudal frontonasal mass epithelium, not previously reported in chicken or mouse embryos. The expression patterns suggest various locations where interactions between ligands and receptors and between ligands and *noggin* are taking place in the developing head.

Noggin is expressed in head mesenchyme and possibly migrating neural crest cells

We observe abundant *noggin* transcripts in stage 10 head mesenchyme, a finding not reported previously (Capdevila and Johnson, 1998; Tonegawa and Takahashi, 1998). At earlier stages of head development *noggin* is only expressed in the notochord (6 somites; Tonegawa and Takahishi, 1998). We observe a striking down-regulation of *noggin* in the mesenchyme between stage 10 and 15. Thus, there is a critical phase during neural crest production where high levels of noggin may be required. Several investigators have described noggin's role in the production of neural crest cells (Kanzler et al., 2000; Selleck et al., 1998). An increase in *noggin* expression in neural crest cells destined for the second branchial arch leads to hypomorphic skeletal elements, due to inhibition of neural crest formation and/or migration (Kanzler et al., 2000). Overexpression of *noggin* next to the dorsal neural tube inhibits delamination of trunk neural crest cells (Kanzler et al., 2000; Sela-Donenfeld and Kalcheim, 1999). Interestingly, mice lacking *noggin* have increased deposition of cartilage and bone in the jaws, but show no obvious changes in

cranial neural crest cell migration or production (R. Harland, personal communication). In addition, *Bmp2* knockout mouse embryos lack detectable migratory cranial neural crest cells and branchial arches (Kanzler et al., 2000; Zhang and Bradley, 1996), reinforcing the necessity for BMP signaling in neural crest cell production.

Noggin and *Bmp-4* transcripts are concentrated in the dorsal neural tube (Liem et al., 1995, 1997; Sela-Donenfeld and Kalcheim, 1999; Sela-Donenfeld and Kalcheim, 2000). The BMP most likely to interact with Noggin in the head mesenchyme is BMP-7. Neither *Bmp-2* nor *Bmp-4* transcripts are detected in the paraxial mesoderm at stage 10, concurring with data from other groups (Farlie et al., 1999; Watanabe and Le Douarin, 1996; Liem et al., 1995). *Bmp-7* is however, expressed throughout the mesenchyme cranial to the mid-hindbrain and might also be expressed in the migrating neural crest cells. The effects of BMP-7 on migrating NCC may be attenuated by the presence of Noggin. BMP-7 can stimulate delamination of trunk neural crest but at higher concentrations than BMP-4 (Sela-Donenfeld and Kalcheim, 1999). The effects of BMP-7 on cranial neural crest cell production have not been examined.

A role for BMPs in nasal pit invagination and differentiation of olfactory epithelium

Bmp-2, *Bmp-4*, and *Bmp-7* are all expressed in the nasal placode at stage 15. When the presumptive placodal epithelium from mice is explanted and grown in cell culture it remains as a single cell layer and fails to thicken (LaMantia et al., 2000). Formation of the nasal placodes requires signaling from the mesenchyme (LaMantia et al., 2000). The pattern of *Bmp* expression suggests a role in the formation or maintenance of the nasal

placode. At stage 16 when invagination begins, *Bmp-2* and *Bmp-4* are expressed at the edges of the nasal pit epithelium, but not in the center (Francis-West et al., 1994; Wu and Oh, 1996). At later stages, the same pattern continues with *Bmp-2* and *Bmp-4* being expressed at the outermost edges of the nasal pit. It is possible that reduced BMP expression in the depths of the nasal pit is associated with invagination. In contrast to *Bmp-2* and *Bmp-4*, *Bmp-7* is expressed throughout the nasal pit epithelium at stage 20 and may be involved in the determination of the undifferentiated (proximal) nasal epithelium to become olfactory epithelium.

At stage 30, *Bmp-7* transcripts (not *Bmp-2* and *Bmp-4*) are exclusively found in the olfactory epithelium and not in the respiratory epithelium. Other markers for olfactory epithelium such as NCAM, keratin sulfate and certain lectins are initially expressed throughout the placode and then become restricted to olfactory epithelium (Croucher and Tickle, 1989). *BmpR-IB* is also expressed specifically in the olfactory epithelium, however, unlike *Bmp-7*, *BmpR-IB* is not expressed in the placode. *BmpR-IB* is similarly expressed in the mouse olfactory epithelium (Zhang et al., 1998; Dewulf et al., 1995). In the chicken embryo, it is possible that BMP-7 signals are transduced via BMPR-IB to differentiate the olfactory epithelium from the respiratory epithelium. There are species differences however, and in the mouse embryo *Bmp-4* is expressed in olfactory epithelium (Shou et al., 2000). BMP activity is also required for neurogenesis from the olfactory epithelium, since exogenous Noggin protein completely inhibits the formation of neural colonies in olfactory epithelium-stromal cell co-cultures (Shou et al., 2000).

In contrast, the respiratory epithelium does not express any of the BMPs examined here, but the sub adjacent mesenchyme expresses *Bmp-2* and *Bmp-4* at high levels. The absence of BMPs in the respiratory epithelium may specify or maintain the respiratory epithelium. Further support for this idea comes from our observation that *chordin* is exclusively expressed in respiratory epithelium at stage 30 (data not shown). It seems likely that complete absence of BMP activity is a necessary prerequisite for respiratory epithelium formation.

Potential functions of BMPs in the facial prominences

Depending on their concentration and time in development, exogenous BMPs can induce growth (Barlow and Francis-West, 1997), apoptosis (Zhao and Rivkees, 2000; Barlow and Francis-West, 1997; Ekanayake and Hall, 1997; Ganan et al., 1996; Graham et al., 1994), differentiation (Wu et al., 1997; Denker et al., 1999; Buckland 1998; Merino et al., 1998; Macias et al., 1997; Zou et al., 1997; Duprez et al., 1996a; Roark and Greer, 1994), alter patterning (Dahn and Fallon, 2000; Duprez et al., 1996b) or even suppress growth (Shigetani et al., 2000; Barlow and Francis-West, 1997; Niswander and Martin, 1993). It is likely that endogenous BMPs can also have a variety of effects during morphogenesis of the facial prominences.

An example of data that supports a patterning role for BMPs in the face is the unique expression of *Bmp-2* in the mesenchyme of the presumptive maxillary region (our data and Francis West et al., 1994). While there is no fate map of the stage 15 face, we speculate that BMP-2 is involved in specifying the maxillary division of the first

branchial arch. At stage 20, *Bmp-2* expression persists in the maxillary mesenchyme while all three *Bmps* are expressed in the maxillary epithelium. The specific expression patterns of the BMPs in the maxillary prominence at stage 24, 28, and 30 suggest that bone patterning may depend on BMPs. The first and second branchial arches fail to form in *Bmp2-/-* knockout mouse (Kanzler et al., 2000) therefore we are unable to draw conclusions about the role of BMP-2 in later maxillary prominence patterning. However, excess BMP applied to the maxillary prominence can cause an increase in proliferation and a duplication of the palatine bone (Barlow and Francis-West, 1997). It remains to be seen whether bones can also be respecified through localized inhibition of BMPs.

Bmp-2, *Bmp-4*, *Bmp-7*, and *BmpR-IB* are all expressed in the stomatodeal plate just prior to its breakdown. The expression of BMPs, known regulators of apoptosis (Pizette and Niswander, 1999; Macias et al., 1997; Zou and Niswander, 1994; Ganan et al., 1996; Graham et al., 1994), suggests that apoptosis may be the mechanism for removal of the buccopharyngeal membrane. *Bmp* expression at stage 24 and 28 also correlates with several areas of apoptosis in the face. Higher levels of programmed cell death are present at the corners of the frontonasal mass in both the epithelium and mesenchyme (McGonnell et al., 1998; Shen et al., 1997; Wedden, 1991). There are high levels of *Bmp-2* transcripts in this region in both tissue layers. There is also programmed cell death in the midline mandibular epithelium (Shen et al., 1997) overlapping *Bmp-4* expression. The groove separating the lateral nasal prominence and maxillary prominence has increased apoptosis (McGonnell et al., 1998; Shen et al., 1997) as well as *Bmp-4* and *Bmp-7* expression. Furthermore, the caudal edge of the mandible expresses

Bmp-2 and has increased apoptosis (Shen et al., 1997; Mina et al., 1995). Apoptosis appears to be regulated by different members of the BMP family in different regions of the face.

It has been suggested that BMPs are able to stimulate cell proliferation in the face (Barlow and Francis-West, 1997). Regions with high proliferation include the medial maxilla (McGonnell et al., 1998), lateral edges of the frontonasal mass (Peterka and Jelinek, 1983) and the medial-caudal edge of the mandible (McGonnell et al., 1998) - all of which specifically overlap regions of high *Bmp-2* expression. The expression data argues in favour of a role for BMP-2 in stimulating regional growth in the developing face.

Noggin expression in caudal frontonasal mass epithelium is correlated with epithelial survival

Noggin may be involved in survival of the frontonasal mass epithelium. In the limb, BMPs induce apical ectodermal ridge (AER) breakdown/regression, while Noggin promotes AER survival/expansion (Pizette and Niswander, 1999). The frontonasal mass epithelium may be similar to the limb AER. Tissue recombination studies have previously shown that the limb and facial epithelium can substitute for one another in epithelial-mesenchymal recombinations (Richman and Tickle, 1992). *Noggin* expression is specifically lost in areas where the epithelium does not survive (corners of the frontonasal mass) and where there is increased programmed cell death (Shen et al., 1997).

Presumably, the absence of Noggin allows for increased activity of BMP-2, BMP-4, and BMP-7, facilitates the breakdown of epithelium in the mesenchymal bridge region.

Shh is expressed in the frontonasal mass ectoderm and extends proximally into the intraoral ectoderm. *Noggin* transcripts in the frontonasal mass ectoderm overlap with *Shh* and extend just slightly distally. All three BMPs overlap the proximal ectoderm region of *Shh* expression. *Noggin* expression, and the potential suppression of BMP activity, may determine how far distally the expression of *Shh* extends. Exogenous Noggin can alter the response of neural tube cells to Shh (Liem et al., 2000). Therefore it is possible that *noggin* is defining a unique region of frontonasal mass extraoral ectoderm and distinguishing it from intraoral ectoderm.

Mesenchymal bridge formation in the primary palate

Just prior to fusion of the frontonasal mass and maxillary prominences we detect an increase in *Bmp-2*, *Bmp-4*, and *Bmp-7* expression in the area of contact. *Noggin* expression is present in the epithelium prior to fusion but shortly thereafter, is locally down regulated in the lateral corners of the frontonasal mass. Removal of the epithelial seam between the prominences and sufficient merging of the underlying mesenchyme are both necessary to establish the mesenchymal bridge. Mesenchymal cells from the frontonasal mass have been shown to migrate to the maxillary prominence (McGonnell et al., 1998), although the precise time at which this exchange of cells begins has not been determined. Our data suggest that the BMPs are especially active at stage 28, when mesenchymal bridge formation is occurring.

Noggin-BMP interactions in chondrogenesis

Mesenchymal condensation is the earliest sign of the initiation of chondrogenesis. The size of the condensation is pivotal to the size of the resulting skeletal element. If condensations are smaller than a critical threshold, chondrogenesis may not begin. Similarly, an abnormally large condensation is associated with an overly large skeletal element (Hall and Miyake, 1992; Gruneberg, 1963). Condensation formation requires BMP signaling. Overexpression of noggin reduces cartilage size by suppressing condensation formation (Capdevila and Johnson, 1998; Pizette and Niswander, 2000).

During cartilage maturation we found high expression of *noggin* in the stage 30 and 32 facial cartilages, coinciding with the establishment of the final size. A similar correlation with high *noggin* expression and differentiating cartilage was observed in the chicken limb (Pathi et al., 1999). In homozygous -/- *noggin* mice, the cartilage elements and bones are enlarged. In the head, the mandible and cranial base also appear to be enlarged in the *noggin* nullizygous embryos (Brunet et al., 1998). Since elevated BMP activity suppresses later stages of cartilage differentiation (Zou et al., 1997; Duprez et al., 1996a), expression of *noggin* in facial cartilage may allow differentiation to proceed.

Receptor-ligand interactions in facial development

As BMPR-II cooperates with type I receptors in order to bind BMPs it is therefore not surprising to find ubiquitous expression of *BmpR-II*. The ubiquitous expression of *BmpR-IA*, also reported in mice (Dewulf et al., 1995), indicates that all tissues are

potentially responsive to BMPs and that regions of endogenous BMP activity are not governed by *BmpR-IA* localization. Regions expressing all three receptors may exhibit cooperative signaling from both type-I receptors. Dual signaling from both BMPR-IA and BMPR-IB would likely have unique cellular responses compared to signaling with only BMPR-IA. At present it is not known whether specific combinations of receptor complexes can bind to distinct Smads in vivo (Nakayama et al., 2000).

There may be distinct roles for BMPR-IA and for BMPR-IB in embryo development. For example, decreased BMPR-IA activity inhibits programmed cell death (Yokouchi et al., 1996) while increased BMPR-IA signalling inhibits cartilage maturation (Zou et al., 1997). The clearest role for BMPR-IB is in mediating the progression from cartilage condensation to differentiating chondroblasts (Baur et al., 2000; Yi et al., 2000). There is quite strong evidence that BMPR-IB is not required for programmed cell death. Evidence from the BMPR-IB knockout mouse shows that programmed cell death can occur in the absence of this receptor (Baur et al., 2000). Moreover, BMPR-IB is not expressed in the interdigital region at the time when programmed cell death is abundant (Baur et al., 2000; Zou et al., 1997).

There are numerous specific regions that express *BmpR-IB*, including the paraxial mesenchyme and early branchial arch mesenchyme. These data suggest that this receptor has other functions in addition to its role in chondrogenesis. At stage 10, *Bmp-7* is expressed at higher levels than *Bmp-2* and *Bmp-4*, and is the most likely ligand for BMPR-1B. In stage 15 embryos, *BmpR-IB* transcripts overlap *Bmp-2* in the maxillary

region and *Bmp-7* in mesenchyme proximal to the eye. At stage 20, *BmpR-1B* is expressed in the medial otocyst epithelium of the developing ear. *Bmp-2*, *Bmp-4*, and *Bmp*-7 transcripts overlap with *BmpR-IB* in this region. The lateral otocyst also expresses all three *Bmps* overlapping *BmpR-IA* transcripts. The localization of the BMP ligands (Wu and Oh, 1996; Oh et al., 1996; Gerlach et al., 2000) and receptors may determine the fate of the individual regions of the otocyst. During primary palate formation, *BmpR-IB* is not expressed in the contact region between the fusing prominences and does not appear to be involved in fusion and merging of the upper beak facial prominences. Further studies interfering with individual receptor function would clarify the role of each of the type I receptors during development.

The individual role of the type I BMP receptors in regulating cell growth is not well understood. Outgrowth of the mandible occurs from proliferation in the medial and distal tips, outgrowth of the maxillary prominences is primarily medial and cranial, whereas expansion of the frontonasal mass occurs primarily at the lateral edges. All three of these regions express low levels of *BmpR-IB*, suggesting that BMPR-IA and BMPR-II complexes are mediating the involvement of BMPs in outgrowth.

Later, during cartilage differentiation, the expression patterns of the three *Bmps* and the expression patterns of *BmpR-IB* are largely mutually exclusive. The one exception is that the three BMPs and BMPRIB overlap in the perichondrium (also seen in the limb, Zou et al., 1997). Retrovirus-mediated expression of a constitutively active BMPR-1B construct leads to increased size of cartilage elements (Zou et al., 1997), consistent with a change

in the function of the perichondrium. *Noggin* is not expressed in the perichondrium and therefore interactions between BMPs and BMPR1B are permitted in this region.

Our expression data suggest new functions for the BMP receptors at early stages of development prior to cell differentiation, an intriguing role for Noggin in the formation of early head mesenchyme and during the fusion of the primary palate, and furthermore a role for BMP-7 and BMPR-IB in olfactory epithelial differentiation. Future experiments will address these interesting findings.

Contributions to this chapter

The preceding chapter represents a manuscript that has been submitted to the journal Developmental Dynamics. The scientific ideas of both my supervisor and myself comprise this manuscript. Examining the expression of *Bmp-2*, *Bmp-4*, *Bmp-7*, *BmpR-IA* and *BmpR-IB* during facial outgrowth (stages 20, 24, 28) was Dr. Richman's idea. Including *BmpR-II*, *noggin*, *chordin* and *tolloid* genes in this study and also examining stages where neural crest cell migration (stage 10), initiation of facial prominences (stage 15), differentiation (stage 30, 32) occurs was my idea. The majority of the work and analysis presented in this chapter was done by me. The writing of this manuscript and assembly of accompanying figures was also a joint effort between Dr. Richman and me. I received technical assistance from Sandra Babich, Steve Ritchie and Les Grad who purified plasmids and linearized DNA used to make riboprobes.

CHAPTER IV

Bone morphogenetic proteins (BMPs) regulate primary palate closure

Summary

The basis for cleft lip is the failure of individual components of the embryonic face to meet and fuse. However, the etiology of non-syndromic cleft lip with or without cleft palate (CL/P) is genetically complex and not well understood. Expression patterns of Bone Morphogenetic Proteins (BMPs) and antagonist Noggin suggested that there is fine control of BMP activity precisely where and when lip closure begins. *Bmp-2*, *Bmp-4* and *Bmp-7* are expressed in the zone of fusion between the frontonasal mass and maxillary prominences, and overlap regions of highest proliferation in the facial mesenchyme or are expressed in the adjacent epithelium. Noggin protein was applied to regions with high BMP expression in the zone of fusion and subsequently induced clefts as a result of reduced proliferation and outgrowth of the facial prominences. *Noggin* expression is restricted to the frontonasal mass epithelium and is downregulated at the corners of the frontonasal mass just prior to epithelial breakdown and fusion. Noggin treatment promotes epithelial survival. These experiments show for the first time that endogenous BMPs regulate two aspects of lip closure; outgrowth of facial prominences and thinning of the frontonasal epithelium prior to fusion. We also examined the well-established retinoic acid model for cleft lip to see whether BMPs were mediating this defect. Ectopic retinoic acid increases *Bmp-2* and *Bmp-4* expression prior to the induction of cleft lip in chickens. Applying BMP-2 to the face also induced clefts. These results are related to

genetic causes of non-syndromic human orofacial clefting and suggest that a loss- or gain-of-function mutation in BMP signaling molecules may be associated with cleft lip.

Introduction

The basis for cleft lip is the failure of individual components of the embryonic face (facial prominences) to meet and fuse. Fusion in the avian upper beak occurs primarily between the corners of the frontonasal mass (medial nasal prominences in humans and mice) and the maxillary prominences at the base of the nasal slit. Failure of fusion to occur results in a cleft in the upper lip extending into the nostril. The midline of the upper lip is intact, rather the cleft is lateral to the midline. Cleft lip, with or without cleft palate (CL/P), is one of the most common birth defects in humans (approximately 1:800 live births) and is genetically distinct from isolated cleft palate. Unfortunately, human nonsyndromic CL/P is difficult to study because it is due to a combination of environmental and genetic factors (Schutte and Murray, 1999). Relatively few candidate genes have been linked to nonsyndromic CL/P (Schutte and Murray, 1999). Using mouse strains with an increased liability to form cleft lip has isolated at least 2 loci that are linked to cleft lip (Juriloff et al., 2001). The transgenic mouse model has not been particularly informative for the etiology of cleft lip. The majority of orofacial clefts in mutant embryos involve the secondary palate or are midline clefts between the medial nasal prominences (Zhang et al., 1996; Schorle et al., 1996; Lohnes et al., 1994; Richman and Mitchell, 1996; Francis-West et al., 1998). For these reasons another approach is required. We have turned to the chicken model system in which we can phenocopy cleft lip in a form that resembles human cleft lip in order to identify key genetic pathways that regulate lip closure.

The fusion of apposed facial prominences is a complex process and requires 1) sufficient outgrowth in order for the prominences to obtain a critical size, 2) removal of

the epithelial seam between the apposing prominences, 3) breakthrough and invasion of the underlying mesenchyme to form the mesenchymal bridge (Wang et al., 1995) and 4) that these events occur within a certain period of development (Wang et al., 1995). The genes that regulate these individual processes are starting to be understood. The prominences proliferate at different levels and proliferation within the facial mesenchyme of each individual prominence is not uniform (McGonnell et al., 1998; Peterka and Jelinek, 1983; Patterson and Minkoff, 1985; Minkoff and Kuntz, 1977; 1978). These regional differences in proliferation as well as the direction of expansion of the facial mesenchyme (McGonnell et al., 1998) contribute to the changes in shape of the embryonic face as development proceeds. Ectopic Shh has been shown to increase the proliferation of facial mesenchyme (Hu and Helms, 1999). In its absence, using Shh antibodies, outgrowth of the facial prominences is reduced resulting in cleft lip (Hu and Helms, 1999). Shh regulates cell proliferation and cell death, thus it is not clear whether the application of Shh antibody inhibits outgrowth by suppressing mesenchymal proliferation, increasing cell death or both. Fgfs have been shown to be able to substitute for the frontonasal epithelium and support outgrowth of epithelium-stripped frontonasal mesenchyme (Richman et al., 1997). However, it isn't known whether FGF signaling is necessary for facial outgrowth.

The epithelium between fusing prominences is removed by a combination of processes: the outer peridermal cells are removed via apoptosis while the inner basal epithelial cells undergo epithelial-mesenchymal transformation (Sun et al., 2000). In the mouse palate, exposure to type I collagen appears to trigger epithelial-mesenchymal transformation (Takigawa and Shiota, 2001) perhaps through the integrin mediated

activation of integrin-linked kinase (ILK; Somasiri et al., 2001). However, the disappearance of the peridermal cells precedes transformation of basal epithelial cells and thus may be necessary for transformation to proceed. Nothing is known about the molecular regulation of apoptosis in the peridermal cells of the primary palate.

Several methods have been used to phenocopy cleft lip in chickens, including gene disruption experiments, stripping of facial ectoderm in situ (Shh, Hu and Helms, 1999) and exposure to teratogens, such as retinoic acid and cholesterol synthesis inhibitors (Young et al., 2000). However retinoic acid treatment results in complete failure of outgrowth of the frontonasal mass (Tamarin et al., 1984; Wedden and Tickle, 1986; Richman and Leon Delgado, 1995) therefore the morphology does not resemble human cleft lip. In addition few genes have been identified that specifically mediate the fusion process. Our goals were to identify genes that are expressed in the mesenchymal bridge region during the time of lip fusion (fusion occurs between stage 28.5-30; Hamburger and Hamilton, 1951; Sun et al., 2000) and then to study the function of these genes in lip fusion. Here we describe the expression of BMP's and the BMP antagonist Noggin in region of contact between the frontonasal mass and maxilla and demonstrate that BMPs are important regulators of outgrowth and of epithelial survival.

Materials and Methods

In situ hybridization

In situ hybridization was performed in wholemount as described in Shen et al., 1997 and for radioactive probe as in Rowe et al., 1991.

Bead preparation

Affigel Blue beads 150 μm in diameter were soaked in 1 mg/ml of human recombinant BMP-2 (Genetics Institute) or 1.05 mg/ml of human recombinant Noggin (Regeneron). A single BMP soaked bead was implanted in the corner of the frontonasal mass at stage 24. Noggin treated embryos had a total of 4 beads placed. There were two beads placed in the frontonasal mass and maxilla at stage 22 and 2 more beads added to the same locations at stage 26. The stage 22 and 26 embryos were separated by 24 hours. Retinoic acid beads were prepared as in Richman and Leon Delgado (1995).

Skeletal staining

Embryos were skinned and then fixed in 100% ethanol for 4 days followed by 100% Acetone for 4 days. Embryos were stained for up to 10 days in alizarin red/alcian blue solution (1 vol. 0.3% alcian blue 8GX in 70% ethanol, 1 vol. 0.1% alizarin red S in 95% ethanol, 1 vol. acetic acid, 17 vol. 70% ethanol) and then cleared in 20% glycerol/2% KOH solution followed by a series of glycerol/H_2O solutions (50%, 80%, 100%).

TUNEL: TUNEL staining was done as described in Shen et al., 1997.

BrdU staining

Embryos were treated with BrdU for 2 hours beginning 10 hours after the first 2 Noggin beads were placed. Sections were treated with 2M HCl 30 minutes, 37°C and proteinase K 5ug/mL at 37°C, 10 minutes, blocked and then incubated for 1 hour at 37°C with anti-BrdU (Becton-Dickinson, 1:30). The secondary biotinylated anti-mouse antibody (1:500, ABC kit, Vectastain) and avidin-biotin complex (ABC kit, Vectastain) were applied for 1 hour, each. Detection was performed with diaminobenzidine (DAB). Sections were counterstained with Hoechst 33258 (5 μg/ml) to visualize the nuclei. Cell counts were made on Dab stained and Hoechst stained views to calculate the percentage of proliferating cells. A paired t-test was performed to determine statistical significance.

Epithelial stripping:

Epithelium was stripped by first applying a solution of nile blue sulfate (Yang and Niswander, 1995) and using a tungsten needle.

Acridine Orange staining

Embryos were rinsed in phosphate buffered saline (PBS), put into a solution of 500 pg/ml of Acridine Orange in PBS for 10 min at room temperature, rinsed in PBS for 10 min and the face was viewed under fluorescence illumination.

RESULTS

BMPs are expressed in the region of fusion

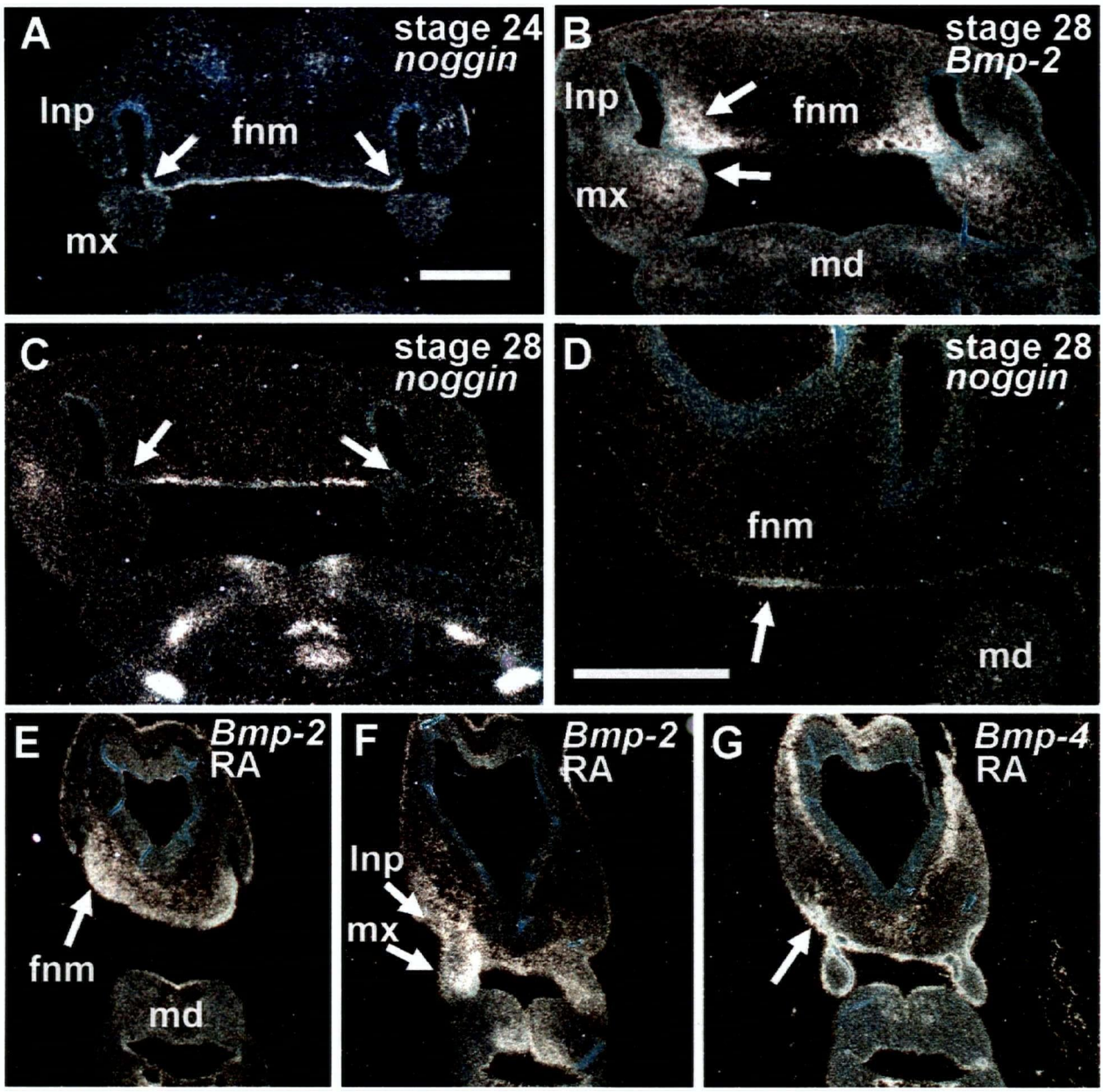
In order to identify genes and pathways that may be involved in cleft lip we looked for expression of molecules in the mesenchymal bridge region during the time of lip fusion (fusion occurs between stage 28.5-30; Hamburger and Hamilton, 1951; Sun et al., 2000). *Bmp-2*, *Bmp-4* and *Bmp-7* are expressed at high levels in the medial maxillary prominence and corner of the frontonasal mass (globular process; Fig. 4.1B; also see Fig. 3.2, 3.3, 3.4; Romanoff, 1960). Of these BMPs, *Bmp-2* is most abundant in the mesenchyme and overlaps regions of highest proliferation in the face. *Bmp-7* and *Bmp-4* are predominantly found in the overlying epithelium and my act in a paracrine manner on the mesenchyme (Fig. 3.4B,D). We also examined the expression of *noggin*, a BMP antagonist, in order to give us insight into the local control of BMP activity. *Noggin* is expressed specifically in a narrow strip of epithelium at the caudal edge of the frontonasal mass (Fig. 4.1A,D). Just prior to fusion *noggin* is down regulated at the corners of the frontonasal mass (Fig. 4.1C). These changes in expression correlate closely with the timing of fusion (Sun et al., 2000) and suggest that BMP activity is involved in the fusion of the lip.

Ectopic Noggin treatment suppress outgrowth of the facial prominences, resulting in cleft lip

We locally applied Noggin protein to the developing face in order to address whether endogenous BMPs are involved in outgrowth of facial prominences. Noggin binds all three BMPs examined here (Zimmerman et al., 1996) although the affinity for Bmp-7 is

Figure 4.1 Expression of BMP-2 and Noggin at the time of fusion of the primary palate and induction of *Bmps* with retinoic acid. **A** Expression of *noggin* extends around the corners of the frontonasal mass (arrows). **B** Expression of *Bmp*-2 in region of contact between the frontonasal mass and maxillary prominences (arrows). **C** *Noggin* transcripts are no longer present in the fusing frontonasal mass epithelium (arrows). **D** Mid-sagittal section showing a narrow strip of epithelium expressing *noggin* (arrow). **E,F,G** embryos treated with 5 mg/ml retinoic acid placed in right nasal pit and fixed 6h later. **E** Increase in expression of *Bmp-2* in right frontonasal mass. **F** deeper section through same embryo as in **E** showing upregulation in maxilla and lateral nasal prominence. **G** increased expression of *Bmp-4* in right frontonasal mass (arrow).

KEY: fnm - frontonasal mass, lnp, lateral nasal prominence, md - mandible, mx - maxilla. Scale bars = 400 μm A-C,E-G and 200 μm for D.

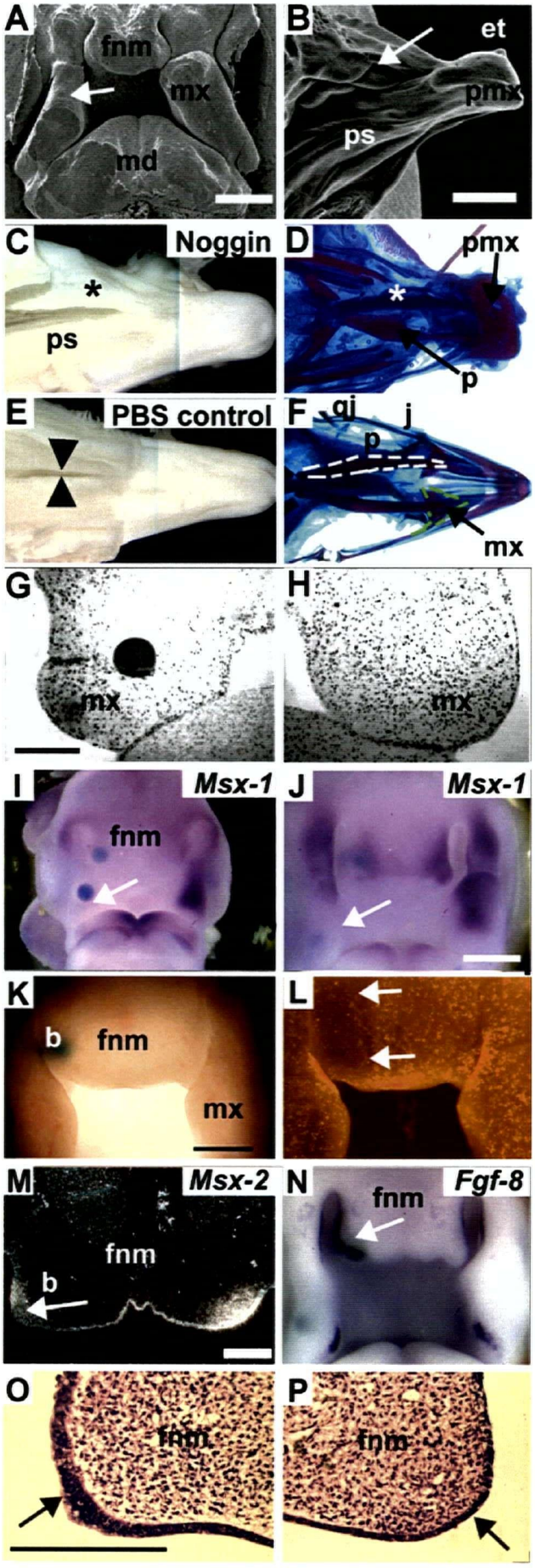

A
stage 24
noggin
lnp
fnm
mx
B
stage 28
Bmp-2
lnp
fnm
mx
md
C
stage 28
noggin
D
stage 28
noggin
fnm
md
E
Bmp-2
RA
fnm
md
F
Bmp-2
RA
lnp
mx
G
Bmp-4
RA

10,000 fold less than for Bmp-4. Noggin treatment reduces activity of several BMPs at once thereby avoiding potential redundancy in BMP activity. We had determined that the placement of 4 beads over a 24 hour period produced extremely reliable clefts. Noggin treatment leads to unilateral clefts in the primary palate and deficiencies in the secondary palate (stage 30-38, N = 20/27, Fig. 4.2B,C) while control embryos are normal (N= 4/4; Fig. 4.2E,F). Prior to extension of the beak (stage 30), the chicken cleft closely resembles that of human; a gap between the frontonasal mass and the maxillary/lateral nasal prominences extends into the nasal pit (data not shown). The size of the maxilla on the treated side is initially reduced compared to the contralateral side and control embryos (Fig. 4.2A) whereas the globular process is only mildly affected. The clefts are therefore due to the inability of the frontonasal mass and maxillary prominences to make contact. Endogenous BMPs thus regulate outgrowth of the facial prominences.

Skeletal abnormalities are similar to those found in human cleft lip

In order to find addition evidence that our chicken clefts resemble those of humans we examined the skeletal abnormalities in our Noggin treated embryos. The skeletal phenotype fits with the early changes in morphology in the maxillary prominence. On the side of treatment, the maxillary and nasal bones are completely absent while the palatine bone is either very thin or absent (10/10; Fig. 4.2B-D). Other derivatives of the maxilla are less affected (jugal, quadratojugal and pterygoid). In human cleft lip there are deficiencies in the maxillary bone often necessitating bone grafts (Eppley and Sadove, 2000). The palatine bones are also commonly deficient in human cleft palate, similar to our phenotype. All frontonasal mass derivatives form normally in Noggin treated

Figure 4.2 Effects of Noggin on facial morphogenesis. A A frontal SEM view of a stage 28 embryo, 12h after the second set of beads was implanted. The decreased size of the maxilla on the treated side is evident (arrow). **B** a side view of the stage 36 beak showing a disruption in the normally smooth edge (arrow). The absence of palatal shelves can be seen on the treated side. The beak has extended fully and is capped with an egg tooth. **C,D** surface and cleared inferior views of a specimen with a deficient palate. Asterisk indicates absent palatal shelf and underlying palatine bone. **E,F** Control embryo with normal size gap between palatal shelves. White dashed lines outline palatine bone, green dashed line outlines maxilla. **G,H** Treated and control maxillary prominences, respectively, of BrdU labelled embryo showing decreased proliferation around bead 6h after bead implantation. **I** Decrease in expression in the frontonasal mass and maxillary prominence (arrow) 6h after the first set of beads compared to contralateral side (stage 24) and **J** 12h after the second set of beads (stage 28). Expression in frontonasal mass is partially restored on treated side, however expression in maxilla remains low (arrow). **K** Bright field view of embryo stained with acridine orange, 12h after second set of beads, stage 28 **L** Fewer dying cells are seen on left side of frontonasal mass (arrows) overlapping the position of the bead. **M** Decrease in expression in epithelium and mesenchyme 6h after the first set of beads (arrow). **N** Increase in size of expression domain near bead, 12h after second set of beads (arrow). **O** Treated side of stage 28 embryo 12h after second set of beads with thicker frontonasal mass epithelium (arrow). **P** Non-treated side of same embryo as in O with thin layer of epithelium. **Scale bars** = 0.5 mm for A,I,J,K,L; 1mm for B; 200 μm for G,H,M,O,P. KEY: b - bead, et - egg tooth, fnm - frontonasal mass, j - jugal, qj - quadratojugal, lnp - lateral nasal prominence, md - mandible, mx - maxilla, p - palatine, pmx - premaxilla, ps - palatal shelf.


A
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M
Msx-2
fnm
b
N
fnm
Fgf-8
O
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P
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embryos (premaxilla, prenasal cartilage and egg tooth, Fig. 4.2B,D). Similarly, non-syndromic CL/P in humans does not affect the nasal septum and the premaxilla. Thus, the noggin-induced clefts are a close phenocopy of human CL/P.

Noggin treatment suppresses proliferation of the facial mesenchyme

The reduced growth of the maxillary and frontonasal mass prominences may potentially be due to either decreased cell proliferation or increased cell death. There is no increase in programmed cell death (stage 28-29, 7/7; data not shown) therefore reduced proliferation is a more plausible explanation. The maxillary prominences would be particularly affected because rate of proliferation remains high for a longer period of time than in the frontonasal mass (Minkoff and Kuntz 1977, 1978). Proliferation is significantly decreased 12h after treatment (stage 24, Fig. 4.2G,H; N = 3, 31% reduction, P = 0.043, frontonasal mass; 39% reduction, P = 0.002, maxilla). This data indicates that endogenous BMP positively control proliferation of the facial mesenchyme.

***Msx-1* expression is reduced by Noggin**

We looked for gene expression changes that are related to the decrease in cell proliferation and examined *Msx-1*. There are two other lines of evidence supporting a role for *Msx-1* in cell proliferation in the face. First, areas with highest expression of *Msx-1* overlap regions with relatively higher proliferation (McGonnell et al., 1998; Mina et al., 1995). Secondly, a reanalysis of the cleft palate of *Msx-1* knockout mice shows a decrease in proliferation in palatal mesenchyme (Chen et al., 2001). *Msx-1* is down regulated in the maxillary and frontonasal mass mesenchyme 6h after the first beads are

implanted (3/3, Fig. 4.2I). The second set of beads maintains the down regulation of *Msx-1* in the maxillary prominence (3/3; Fig. 4.2J) however, *Msx-1* expression is restored somewhat in the frontonasal mass. There is no change in embryos treated with PBS-soaked beads (6/6). The relative difference in *Msx-1* expression at stage 28 between the maxilla and frontonasal mass correlates with the greater suppression of proliferation in the maxillary prominence. We conclude that Msx-1 is downstream of endogenous BMPs in facial mesenchyme and may be a downstream mediator of BMP-induced proliferation.

Noggin maintains survival of the frontonasal epithelium

Noggin expression in the corners of the frontonasal epithelium is downregulated prior to the induction of apoptosis (Fig. 4.1A,C), suggesting that elevated BMP activity may regulate epithelial thinning. Survival should be enhanced in Noggin-treated embryos. Acridine Orange staining was used to preferentially label dying epithelial cells in the primary palate. Noggin treatment leads to a qualitative reduction in staining near the bead (6/7; Fig. 4.2K,L).

Potential downstream targets of BMPs associated epithelial cell survival

Accompanying the inhibition of cell death, there is also down regulation of *Msx-2* expression in the epithelium (Fig. 4.2M; 5/5). The increased cell survival is evident in the thickened epithelium on the treated side (3/4, Fig. 4.2O) and is accompanied by an expansion of *Fgf-8* expression (Fig. 4.2N, 6/7). Noggin also induces an expansion of the apical ectodermal ridge in limb buds (Pizette and Niswander, 1999). There are no changes in *Shh* expression (data not shown). Our data shows that the epithelium is

responding to decreased BMP signalling in the mesenchyme by increased *Fgf-8* expression and increased survival of periderm cells.

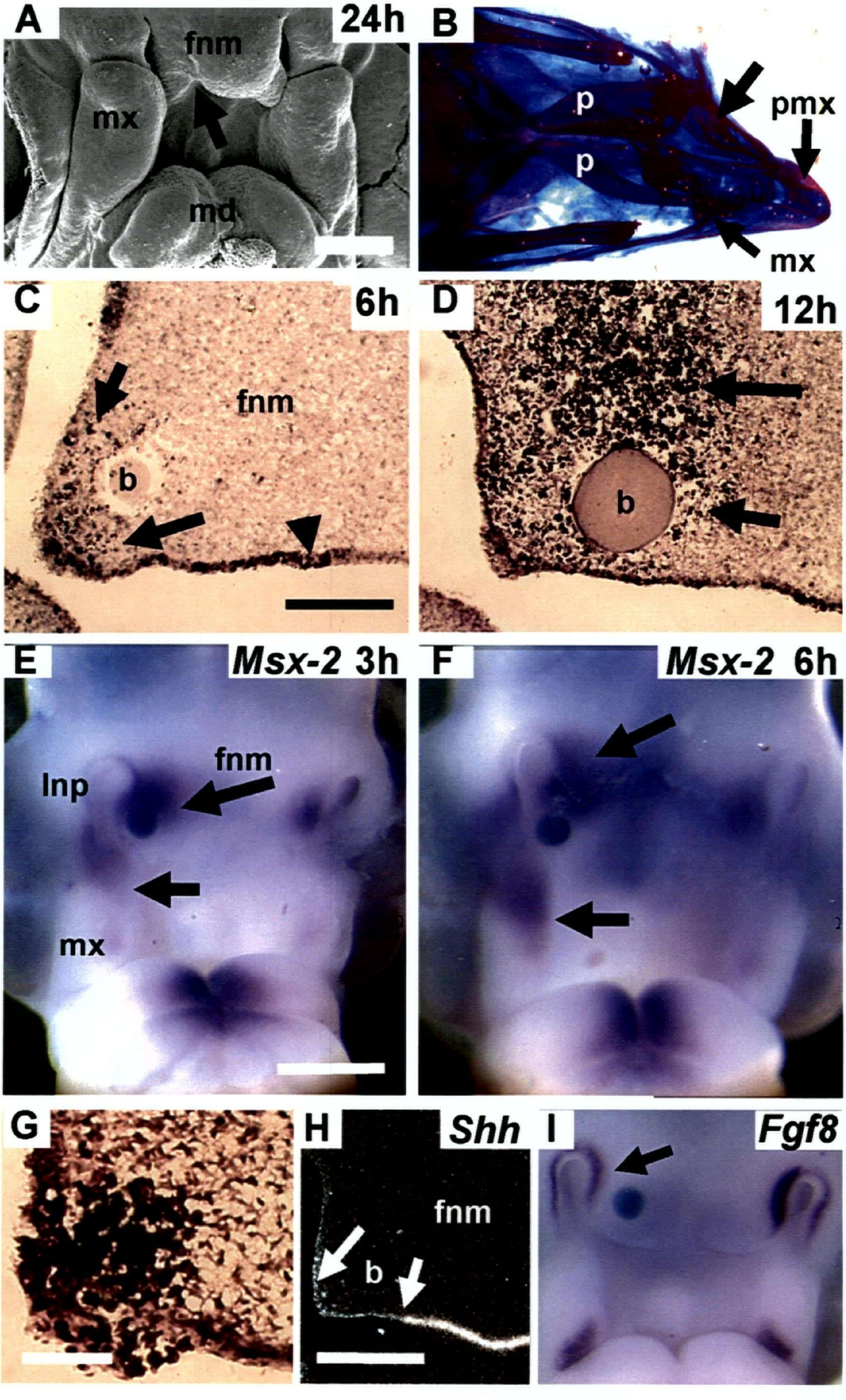
Retinoic acid upregulates *Bmp-2* and *Bmp-4* expression

Our data with Noggin treatment suggests that BMPs are mediating outgrowth of the facial prominences. We wished to see whether BMPs are mediating cleft lip in another model system with selective inhibition of outgrowth - embryos treated with retinoic acid (RA). Irreversible changes occur by 16 hours (Richman and Leon Delgado, 1995) therefore we looked for changes in gene expression that take place at a shorter interval. Contrary to what we expected, the expression of *Bmp-2* and *Bmp-4* is rapidly up regulated in the frontonasal mass, maxilla and lateral nasal prominences adjacent to the bead (3/3, Fig. 4.1E-G). Expression of *Bmp-7* and *noggin* is unchanged following retinoic acid treatment (data not shown). This is the earliest reported change in expression specifically in all facial prominences affected by RA (Brown et al., 1997; Shen et al., 1997; Helms et al., 1997). Therefore, increased BMP levels may mediate RA-induced inhibition of outgrowth in the maxillary and frontonasal mass prominences.

Ectopic BMP-2 application induces clefts that resemble defects induced by Noggin

Based on the results with RA we wished to test in a direct way whether increased levels of BMP inhibit outgrowth. BMP-2 treated embryos had reduced outgrowth of the frontonasal mass (Fig. 4.3A, 3/3) ultimately resulting in a cleft in the upper beak (7/8). Despite the early effects on the morphology of the frontonasal mass, the frontonasal mass derivatives form in all specimens (Fig. 4.3B premaxilla, prenasal cartilage, nasal septum

Figure 4.3 Effects of BMP-2 on facial morphogenesis. A SEM showing the reduction in size of the frontonasal mass (arrow). **B** Cleared specimen showing loss of the maxillary bone and distal tip of the palatine bone (large arrow). There is a notch in the side of the beak coinciding with the lost maxillary bone. **C** TUNEL stained section with increase in staining between the bead and the lateral edge of the frontonasal mass (arrows) and no staining medial to the bead. This embryo represents the highest amount of cell death in the mesenchyme at this time point. Epithelial cell death extends medially (arrowhead). **D** Peak TUNEL staining in mesenchyme but not extending into the midline. **E, F** Upregulation of expression in frontonasal mass and maxillary prominence (arrows). **G** Increase in cell death in the mesenchyme adjacent to stripped epithelium, epithelium has partially regrown 10h after stripping. **H** Decrease in expression of *Shh* near the bead. **I** Downregulation of expression in the nasal slit epithelium compared to the contralateral side (arrow). **Scale bars** = 0.5 mm for A,C-H,J,K; 50 μm for I. **KEY:** b - bead, fnm - frontonasal mass, lnp - lateral nasal prominence, md - mandible, mx - maxilla, p-palatine, pmx - premaxilla.


A
24h
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B
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C
6h
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D
12h
b
E
Msx-2 3h
fnm
lnp
mx
F
Msx-2 6h
G
H
Shh
fnm
b
I
Fgf8

and egg tooth 8/8). Surprisingly, the skeletal defects are similar to Noggin treated embryos. The maxilla and the distal end of the palatine bone are usually absent (Fig. 4.3B; 7/8). Beads soaked in PBS or BMP-2 protein diluent affected neither outgrowth of the globular process nor craniofacial morphology (5/5).

Ectopic BMP-2 expands regions of apoptosis and Msx-2 expression in the frontonasal mesenchyme

We were curious about the effects on maxillary derivatives and the lack of effect of BMP on the frontonasal mass derivatives especially because the early deficit is localized to the frontonasal mass. In the frontonasal mass, apoptosis peaks 12h-post bead implantation and is predominantly in the lateral mesenchyme (Fig. 4.3C,D; Table 4.1D; 7/7 have > 100 dead cells in lateral mesenchyme). Cell death does not extend into the central region where cartilage will differentiate (7/7 have < 5 dead cells). This accounts for the normal development of frontonasal mass derivatives. Apoptosis does however extend into the maxilla (6h post bead placement, 4/6 show increased cell death; 2/4 had 5-10 dead cells; 2/4 had 10-20 dead cells) indicating that the BMP-2 protein is able to diffuse across from the frontonasal mass. The absence of maxillary bones appears to be due to increased cell death in the maxilla. BMP-soaked beads implanted directly into the maxilla delete maxillary derivatives and cause clefts similar to embryos with beads placed into the frontonasal mass (4/4; data not shown).

We examined expression of *Msx-2*, a gene that is induced by BMPs (Vainio et al., 1993; Barlow and Francis-West, 1997; Wang et al., 1999; Ekanayake and Hall, 1997) and can

Table 4.1 Number of TUNEL positive cells in BMP-2 treated embryos

Table 4.1A: FNM corner epithelium								
Number of cells	Treated side (N = number of embryos)				Untreated side (N = number of embryos)			
	4.5 hours	6 hours	12 hours	24 hours	4.5 hours	6 hours	12 hours	24 hours
0-5	3	0	0	0	4	6	5	4
5-10	1	1	0	4	0	0	2	1
10-50	0	5	7	2	0	0	0	1
50-100	0	0	0	0	0	0	0	0
> 100	0	0	0	0	0	0	0	0

Table 4.1B: FNM caudal epithelium								
Number of cells	Treated side (N = number of embryos)				Untreated side (N = number of embryos)			
	4.5 hours	6 hours	12 hours	24 hours	4.5 hours	6 hours	12 hours	24 hours
0-5	2	0	0	3	4	6	6	5
5-10	2	0	0	3	0	0	1	0
10-50	0	6	7	0	0	0	0	1
50-100	0	0	0	0	0	0	0	0
> 100	0	0	0	0	0	0	0	0

Table 4.1C: FNM corner mesenchyme (distal region of prominence)								
Number of cells	Treated side (N = number of embryos)				Untreated side (N = number of embryos)			
	4.5 hours	6 hours	12 hours	24 hours	4.5 hours	6 hours	12 hours	24 hours
0-5	1	0	0	0	4	4	2	3
5-10	2	0	0	6	0	1	3	3
10-50	1	6	1	0	0	1	2	0
50-100	0	0	6	0	0	0	0	0
> 100	0	0	0	0	0	0	0	0

Table 4.1D: Lateral third of FNM mesenchyme and future mesenchymal bridge region						
Number of cells	Treated side (N = number of embryos)			Untreated side (N = number of embryos)		
	4.5 hours	12 hours	24 hours	4.5 hours	12 hours	24 hours
0-5	0	0	0	6	2	4
5-10	0	0	0	0	5	2
10-50	6	0	0	0	0	0
50-100	0	0	0	0	0	0
> 100	0	7	6	0	0	0

increase programmed cell death (Graham et al., 1994), and find that expression is expanded in the lateral third of the frontonasal mass and extends into the maxillary prominence (3h 6/6, Fig. 4.3E; 6 hours, 3/3, Fig. 4.3F). We show that up regulation of *Msx-2* precedes BMP induced cell death and that the spatial distribution of cell death correlates with the skeletal defects.

Frontonasal mass epithelium maintains mesenchymal survival

There appears to be a sequential relationship between the cell death in the epithelium and mesenchyme. Programmed cell death in the epithelium occurs following a lag of 4-5 hr (Table 4.1). By 6-7h it is apparent that the frontonasal mass epithelium is particularly sensitive to BMP (Fig. 4.3C; Table 4.1). At the same time point, mesenchyme medial and directly contacting the bead lacks TUNEL positive cells (Fig. 4.3C, 6/6 have <5 dead cells medial to the bead). It is possible that initial cell death in the epithelium promotes cell death in the mesenchyme. To test this we stripped frontonasal mass ectoderm. Programmed cell death is increased on the side that was stripped (5/5; Fig. 4.3G; no increase in control embryos, 3/3). Embryos that are left to grow following epithelial stripping develop cleft lip due to reduced outgrowth of the globlular process (Hu and Helms, 1999). Here we show that reduced outgrowth is in part due to mesenchymal cell death.

BMPs suppress *Shh* and *Fgf-8* expression

The effects of exogenous BMPs on epithelium and subsequently on mesenchymal cell death are similar to the effect of epithelial removal. The BMP experiment however,

allows us to study the molecular changes in the epithelium that may be important in maintaining cell survival in the mesenchyme. BMP treatment may decrease expression of genes in the epithelium that encourage outgrowth and survival. SHH and FGF-*8* have been shown to promote cell survival in the head and limb bud (Ahlgren and Bronner-Fraser, 1999; Sanz-Ezquerro et al., 2000; Crossly et al. 1995; Trumpp et al., 1999). SHH in the frontonasal mass epithelium may also be promoting outgrowth of facial mesenchyme (Hu and Helms, 1999). In our study, *Shh* and *Fgf-8* are down regulated by BMP-2 prior to the induction of programmed cell death in the epithelium (*Shh 6/6,* 3h post bead implantation, Fig. 4.3H; *Fgf-8* 3/3, 3-6h after bead implantation, Fig. 4.3I). The loss of epithelial maintenance factors precedes and may facilitate epithelial apoptosis. The frontonasal epithelium is required for mesenchymal outgrowth (Wedden, 1987; Richman and Tickle, 1989; 1992; Hu and Helms, 1999) but our findings suggest that it is also required for mesenchymal survival.

Discussion

Concentration dependant effects of BMPs in the face

BMPs are versatile molecules that regulate many biological processes, including cell proliferation (positively or negatively), cell differentiation and apoptosis in a concentration and stage specific manner. In the face, we have shown that endogenous BMPs regulate proliferation of the facial mesenchyme and apoptosis in the frontonasal mass epithelium. Noggin application to face suppressed proliferation of the mesenchyme by as much as 39%. *Msx-1* was absent from the maxillary prominence suggesting that most of the BMPs were being antagonized. These findings indicate that BMPs act with other genes to regulate proliferation. These candidates genes include SHH and FGFs, which are not downregulated following Noggin treatment. Previous experiments where BMP-2 soaked beads were placed in the maxillary prominence did not cause clefts (Barlow and Francis-West, 1997). The reasons may be: 1) the protein concentrations were less than those used here, 2) the position of the bead was more posterior. Thus programmed cell death occurred near the bead, far from the region that fuses with the frontonasal mass (Barlow and Francis-West, 1997).

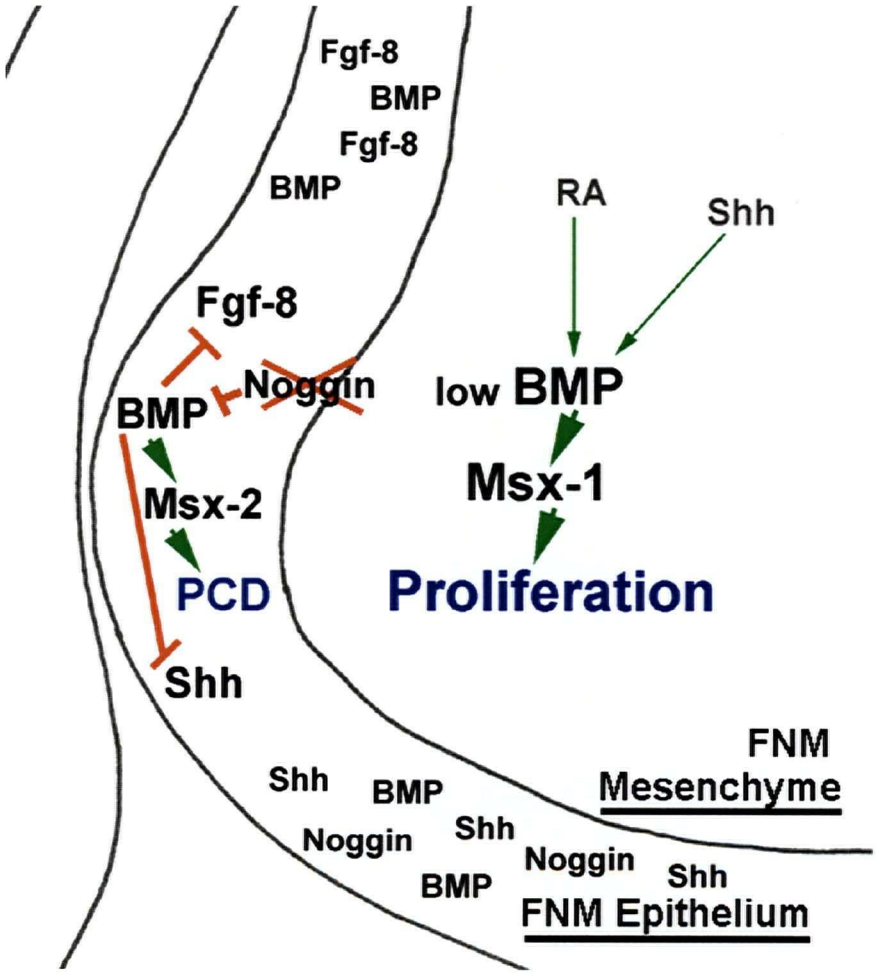
Despite close proximity with the mesenchyme, the epithelium undergoes cell death. This may be due to higher levels of BMPs in the epithelium as compared to the mesenchyme. The mesenchyme expresses *Bmp-2*, while the epithelium expressed *Bmp-2*, *Bmp-4* and *Bmp-7*. It isn't known whether this difference in expression levels translates into differences in protein bioactivity however regional differences in *Msx-1* and *Msx-2* suggests this. BMPs induce cell proliferation al low levels and apoptosis at high levels,

which appear to be mediated by Msx-1 and Msx-2 respectively. Thus at low levels BMPs induce *Msx-1* expression and at high levels induce both *Msx1* and *Msx-2* expression. Msx-2 must override the proliferative effects of Msx-1 in regions of co-expression. Thus, in the frontonasal mass *Msx-1* is expressed widely in the mesenchyme relative to the restricted region of *Msx-2* expression in the immediate corner of the prominence. Alternatively, the epithelium may just be more sensitive to apoptosis as we showed in our study and thus there may not be any differences in BMP levels between the epithelium and mesenchyme.

Interplay between cell death and cell survival factors prior to fusion

The epithelial seam normally expresses cell death (*Bmp-2, Bmp-4, Bmp-7, Msx-2*) and cell survival factors (*Fgf-8, Shh, noggin*) and the expression levels, with the exception of *noggin,* remain constant as the prominences contact each other (Figure 4.4). The ratio between BMP and FGF activities determines the balance between cell survival and cell death (Buckland et al., 1998). SHH should also be included in this equation as ectopic SHH can also inhibit BMP regulated apoptosis in the interdigital tissue (Sanz-Ezquerro, 2000). It is necessary to carefully orchestrate cell death and cell survival signals in order to facilitate fusion of the lip. When the decision is made to induce apoptosis, the localized downregulation of *noggin* allows the embryo to tip the balance towards cell death rather than survival (Fig. 4.4). Different signals may operate to control BMP activity in maxillary epithelium since *Fgf-8* and *Noggin* are not expressed here.

Figure 4.4 Model of BMP function and pathways in the frontonasal mass. In the frontonasal mass mesenchyme, low BMP levels (only *Bmp-2* is expressed) regulate proliferation, which may be mediated through Msx-1. RA and Shh may act upstream of Bmp-2. In the frontonasal mass epithelium, high BMP levels (*Bmp-2*, *Bmp-4* and *Bmp-7* are expressed) regulate apoptosis in the epithelium, which may be mediated through Msx-2. This apoptotic signal is antagonized by Noggin in the caudal epithelium and balanced by survival signals, such as Shh and Fgf-8 in the caudal and lateral epithelium, respectively. Prior to fusion, BMP inhibtion is removed from the corner of the frontonasal mass epithelium (noggin is downregulated) and thus the balance is shifted to favor cell death.


Fgf-8
BMP
Fgf-8
BMP
RA
Shh
Fgf-8
Noggin
low BMP
BMP
Msx-2
Msx-1
PCD
Proliferation
Shh
FNM
Mesenchyme
Shh
BMP
Noggin
Shh
Noggin
Shh
BMP
FNM Epithelium

BMPs are downstream of RA

RA induces cellular effects by binding to its receptors that are transcription factors, and altering gene transcription. These downstream targets of RA thus mediate RA's biological functions. In the chicken, elevated RA levels reduces outgrowth of facial prominences that give rise to the upper beak. Several genes have been identified whose transcription is altered following RA treatment, including *Shh* (Helms et al., 1997), *FHF-4* (Muñoz-Sanjuán *et al.*, 2001), *Msx-1* and *Msx-2* (Brown et al., 1997) and AP-2 (Shen et al., 1997). SHH has been shown to be particularly important since in inhibition results in clefts (Hu and Helms, 1999). However in RA treated embryos, *Shh* is downregulated very late, 30 hours after treatment leaving open the possibility that other genes are mediating this change in expression. Other genes downstream of RA may also be in part altered through BMP overexpression. We have shown that ectopic BMP-2 can suppress *Shh* expression. The fact that high RA concentrations inhibit *Msx-1* and *Msx-2* expression is somewhat perplexing since BMPs upregulate *Msx-1* and *Msx-2* expression in the face (Barlow and Francis-West, 1997; Wang et al., 1999; Ekanake and Hall, 1997). At low RA concentrations, RA is able to upregulate *Msx-1* and *Msx-2* expression (J. Hui, personal communication) and may be acting through BMPs. However, at high concentrations perhaps RA-RAR complexes are able to bind to Msx promoter regions and prevent Smad binding. Whatever the case, high RA levels can clearly over-ride BMP induced transcription of Msx genes.

Bmp-2 (and *Bmp-4*) however is upregulated in all the facial prominences of the upper beak. In addition, this upregulation is very rapid and thus may be an early response gene.

By 6 hours increased *Bmp-2* transcription is very evident. We did not examine the earliest time at which *Bmp-2* transcription is altered. Finally, we replicated this facial defect by apply ectopic BMP-2 protein to the mesenchyme where *Bmp-2* is upregulated. One mechanism by which BMP-2 may mediate RA's suppression of outgrowth is by increasing cell death. *Bmp-4* and *Bmp-7* have been shown to mediate RA-induced cell death in the limb (Rodriguez-Leon, 1999).

BMP pathway association with human cleft lip

We conclude that the 2 physiological roles of BMP during primary palate formation are 1) to control proliferation and outgrowth of the facial prominences; and 2) to thin the epithelium within the contact region via apoptosis. The question then arises, are BMP signalling pathways affected in mammalian orofacial clefts? Msx-1 and Msx-2 genes have been linked to orofacial clefting. van den Boogaard et al. (2000) reported that haploinsufficiency of human *MSX-1*, is correlated with cleft lip and palate. *Msx-1* knockout mice have cleft palate but no cleft lip (Satokata and Maas, 1994) whereas *Msx-2* knockout mice have calvarial defects but no clefts (Satokata et al., 2000). The double knockout of *Msx-1* and *Msx-2* has bilateral cleft lip and palate (Maas, personnel communication). We predict that *Msx-1/Msx-2* -/- embryos will have decrease in programmed cell death in the epithelium (due to lack of Msx-2, our Noggin data) and this will accompany decreased mesenchymal proliferation (due to lack of Msx-1; Chen et al., 2001). It will be interesting to see whether the double knockout embryos have a persistent epithelial seam in the primary palate. The data on Msx knockouts associates a part of the BMP pathway with clefts of the primary and secondary palate, however we are the first to demonstrate that endogenous BMPs are required for fusion. Our findings

predict that elevated BMP activity may also cause clefting in humans. Directed overexpression of BMP-4 in the epithelium with a keratin promoter, which results in cleft palate (Blessing et al., 1993). It is not clear in the paper whether cleft lip is also observed in these trangenic mice. A loss-of-function mutation in the BMP antagonists would lead to increased BMP signalling. We identify for the first time, several critical roles for BMP signaling in fusion of the primary palate - by directly altering BMP activity we are able to phenocopy cleft lip. We suggest that a gain- or loss-of- function mutation in a BMP signaling molecule could be associated with human cleft lip.

Contributions to this chapter

The preceding chapter represents a manuscript that was submitted to the journal Science. Due to the space restraints in the journal, it is written in a very concise manner. These findings are presently being re-written for submission to the journal Development.

The scientific ideas from the initiation to the completion of this chapter were solely mine. The majority of the work presented in this chapter, data analysis and interpretation of results was done myself. Dr. Richman did the skeletal analysis of stage 38 heads and assisted with the TUNEL data analysis. I received technical assistance from Kathy Fu, who did the whole-mount in situs, TUNEL assay and BrdU labeling. Dr. Richman assisted in the writing of this manuscript and in the assembling the accompanying figures. Special thanks to Mary MacDonald, Sonia Donati, Kathy Fu, Jenny Hui, Jessica Kim and Mya Tuong for counting the BrdU labelled cells.

CHAPTER V

The type IA and type IB Bone morphogenetic protein receptors (BMPR) have similar functions in regulating chondrogenesis and feather formation in the chicken embryo

Summary

The facial skeleton is unique because it is neural crest derived. We have identified the expression of BMPs, BMP receptors and noggin during the different phases of cartilage morphogenesis in the face. We sought to examine the role of the type IA or type IB receptors' signaling. Our approach was to express dominant-negative (dn) and constitutively active (ca) BMP type I receptors prior to and during chondrogenesis. Ectopic expression of a dominant-negative form of BMPR-IB unilaterally in the face resulted in thinner, shorter cartilage elements and deviation of the upper and lower beaks towards the injection site. The phenotypes of caBMPR-IA and caBMPR-IB were similar - deviations of the beak away from the injection site caused by an overgrowth and fusion of cartilage elements. The embryos injected with ca viruses also lacked feather germs over much of the head and about 50% of specimens did not form an egg tooth. Both the ca viruses lead to ectopic expression of *Msx-1* and *Msx-2* in infected regions and upregulation *Bmp-2* and *Bmp-4* in adjacent regions. We conclude that the type IA and type IB receptors play similar roles in regulating cartilage and feather formation in the skull.

Introduction

BMPs were originally purified on the basis of their ability to induce local formation of ectopic bone and cartilage when implanted under the skin or into the muscle of rats (Urist 1965; Urist et al., 1979). Since their discovery, the BMPs have been shown to be important signaling molecules in a range of developmental processes including apoptosis, chondrogenesis and embryo patterning (Hogan, 1996). In the developing chicken face, *Bmp-2*, *Bmp-4* and *Bmp-7* are expressed in both the ectoderm and mesenchyme (Francis-West et al., 1994; Wall and Hogan, 1995; Wang et al., 1999; Ashique et al. submitted) in specific patterns. The addition of exogenous BMP-2, BMP-4 and BMP-7 protein to the developing chick face induces the formation of supernumerary bones in the maxillary region and secondary growth sites branching off of Meckel's cartilage (Barlow and Francis-West, 1997; Wang et al., 1999). These data argue for an important role for endogenous members of the BMP family in facial development but the mechanism of signal transduction is not clear.

BMPs bind to two type I receptors (IA also known as Alk-3, Brk-1; IB also known as Alk6, Brk2) and one type II receptor (Liu et al., 1995). Ligand binding can form heterotetrameric complexes with the type I and II transmembrane serine-threonine kinases (ten Dijke et al., 1996; Yamashita et al., 1994). The type I receptor phosphorylates downstream targets including members of the Smad family (Smad 1, 5, 8; Heldin et al., 1997; Kretzschmar and Massagué, 1998; Hoodless and Wrana, 1998). Despite sequence divergence between *Bmp-4* and *Bmp-7*, both of these growth factors share the same receptors (Koenig et al., 1994; ten Dijke et al., 1994). Other members of

the BMP family, growth/differentiation factors (GDF5, GDF7) also bind to BMP receptors (Yamashita et al., 1996; Nishitoh et al., 1996). Thus there is some promiscuity in ligand binding characteristics of BMP receptors.

Expression patterns of *BmprIA* and *BmprIB* in the developing craniofacial complex have been described and there are considerable areas of overlap (Ashique et al., submitted). However, in a small subset of tissues, the type IB receptor is expressed at high levels suggesting that the BMPR-IB receptor may have unique functions in craniofacial development. Knock-out mice lacking *BmprIA* die early in development prior to gastrulation (Mishina et al., 1995) confirming that widespread expression of IA receptor is necessary for early stages of embryogenesis. In contrast, the phenotypes of *BmprIB -/-* mice are much more limited. Mice lacking the cartilage-specific isoform of *BmprIB* do not form the distal elements of the foot and handplate (Baur et al., 2000). A similar distal limb truncation has been reported in mice lacking exon 1 of *BmprIB* (Yi et al., 2000). The lack of abnormalities in the rest of the embryo suggests that the type IA receptor can take over for the IB receptor during differentiation of the craniofacial skeleton, most of the axial skeleton and long bones of the appendicular skeleton. It seems likely, therefore that the type I BMP receptors share similar functions in the differentiation of craniofacial skeletal tissue.

Another approach used to determine the redundant or unique functions of BMP receptors, has been to express constitutively active (ca) or dominant negative (dn) forms of BMPR-IA and BMPR-IB in the chicken limb bud. The ca receptors can simulate Smad1

phosphorylation in the absence of a BMP ligand (Hoodless et al., 1996; Kretzschmar et al., 1997) whereas dn receptor constructs will bind ligand but are unable to phosphorylate downstream targets (Zou and Niswander, 1996). Retroviruses expressing mutated forms of BMPR-IA and BMPR-IB have been injected into the limb bud region of chick embryos (Zou and Niswander, 1996; Kawakami et al., 1996; Yokouchi et al., 1996; Zou et al., 1997). Infection of stage 20 limb buds showed that both ca receptors lead to increased chondrogenesis indicating that at this stage of development they have overlapping functions. In contrast injections done before stage 17 gave different results for the ca IA and caIB receptors. The caBMPRIA delays endochondral bone formation with no apoptosis detected whereas caBMPR-IB virus gave rise to thin limbs due to increased programmed cell death (Zou et al., 1997). Work from two different labs showed that dn BMPRIA gave no phenotype (Zou and Niswander, 1996: Kawakami et al., 1996). These studies suggest that the type IB receptor is involved in the cell death pathway whereas the type IA receptor mediates chondrocyte differentiation (Zou et al., 1996).

The facial skeleton differs from the appendicular or axial skeleton in that most of the mesenchyme is derived from cranial neural crest (Köntges and Lumsden, 1996; Couly et al., 1993; Le Lièvre, 1978; Noden, 1978) cells rather than paraxial mesoderm. The difference in embryonic origin may mean that BMP receptors function differently in the face compared to elsewhere in the body. The three facial prominences, frontonasal mass, lateral nasal prominence and mandibular prominence differentiate into cartilage between stage 24 and 28 (Matovinovic and Richman, 1997). The frontonasal mass gives rise to the

interorbital septum, prenasal cartilage, premaxilla and egg tooth (Wedden, 1987; Richman and Tickle, 1989; 1992; Richman et al., 1997), the lateral nasal prominence forms the nasal chonchae and the paired mandibular prominences form Meckel's cartilage and all the mandibular bones.

The present study addresses the function of type IA and type IB *Bmprs* in facial development by targeted viral misexpression of constitutively active or dominant negative BMP receptors. We compared the phenotype of embryos at several stages to determine whether there are differences in function of the type IA or type IB receptors during craniofacial development. We find that in most cases the phenotypes were similar and therefore our data suggests the type I receptors have similar roles in chondrogenesis and feather formation.

Materials and Methods

Chicken Embryos

Fertilized white leghorn chick eggs were obtained from Coastline Chicks, Abbotsford, B.C. and specific-pathogen-free Hyline chick fertilized eggs were obtained from Hyvac, Iowa.

In Situ Hybridization probes

The cDNAs were kindly provided by the following individuals: RCASBP *pol* (L. Niswander), chick *Msx-1* and *Msx-2* (S.E. Wedden), chick type II collagen (P. Brickell; Devlin et al., 1988), chick *BmprIA*, chick *BmprIB* (L. Niswander; Zou and Niswander, 1996; Zou *et al.*, 1997), chick *Bmp-2* and *Bmp-4* (P. Brickell; Francis-West et al., 1994), and chick *Bmp-7* (B. Houston; Houston *et al.*, 1994). Antisense riboprobes labelled with [^{35}S]-UTP were prepared as described in Rowe *et al.* (1991).

Virus Infection

Replication competent RCASBP virus was grown up using methods described in Morgan and Fekete (1997). RCASBP (A) virus containing the following genes were injected into embryos: human wild type (wt) BMPR-1A, human dominant-negative (DnA) BMPR-1A, human constitutively-active (CaA) BMPR-1A (human alk-3 cDNAs originally provided by P. ten Dijke), chick wtBMPR-1B, chick dnBMPR-1B (DnB), chick caBMPR-1B (CaB, Zou and Niswander, 1996; all BMP receptor viruses generously provided by L. Niswander). Human placental alkaline phosphatase (PLAP) cloned into RCASBP (E) was provided by S. Hughes. A Picospritzer (General Valve Corp.) with electrolytically pulled needles was used

to deliver the virus to the embryo. In stage 15 and 20 embryos the vitelline membrane was torn prior to injection.

A third group of virus infected embryos were incubated until they reached stage 38 and then skeletal morphology was assessed. Approximately 50% of embryos used for the characterization of the stage 38 phenotype were pathogen-free. There was no difference in the morphology between pathogen-free and pathogen-containing chick embryos.

Skeletal staining

Stage 38 embryos were fixed in 10% neutral-buffered formal saline or 5% tricloroacetic acid (TCA) for at least 24 hours. The eyes and skin were then removed, and specimen was washed in water for two days. Specimens were stained for a week in alcian blue solution for one week (10mg alcian blue 8GX (BDH), 70ml absolute alcohol and 30 ml glacial acetic acid (BDH)), and then destained in the above solution minus the alcian blue for 4 days. De-staining was continued in 75% alcohol, 50% alcohol, 25% alcohol, distilled water (2x) for two hour each and then 0.5% potassium hydroxide (KOH) until soft tissue was nearly transparent. KOH solution was changed regularly. Specimens were then stained for bone with fresh KOH solution containing approximately 0.005% alizarin red for 24h. De-staining was done using 25%, 50% and 75% glycerol in 1% KOH for 5-7 days each and then 100% glycerol for storage.

Results

Targeting viral spread in the face.

Retrovirus injections have not been done in the face previously. Therefore, it was necessary to test various injection sites in order to find several that gave reproducible viral spread. The RCASBP retrovirus containing a PLAP (placental lactogen alkaline phosphatase) insert was injected into the mesenchyme at multiple sites and stages (Fig. 5.1). Histochemical detection of alkaline phosphatase activity indicates extent of virus spread. Injection sites that resulted in unilateral viral spread were of particular importance allowing us to compare phenotypic effects with the contralateral side of the face/head. Two sites (II and V) gave predominantly unilateral viral spread and were chosen for subsequent injections using mutant BMP receptor constructs (Table 5.1). When injected into the right of the mesencephalon at stage 10 (site II), there is heavy infection of the right side of the face including the frontonasal mass, mandible, and maxilla at stage 28 or 96h post-injection (Fig. 5.2 and Table 5.1). Injections caudal to site II, right of the mid-hind brain junction (site IV), resulted in similar viral spread and thus were grouped together. Virus suspension injected at stage 14 into the presumptive maxilla/lateral nasal prominence (right side dorsal to the eye; site V) show similar virus spread but less infection to the mandible (Table 5.1). Thus, late injection allowed us to selectively infect the upper face and injection at stage 10 lead to infection of both the upper and lower facial primordia. Viruses used were replication-competent and therefore are capable of spreading throughout tissue. We detected minimal virus spread across the midline of the frontonasal mass or mandibular prominences 4-5 days post-injection (Fig.

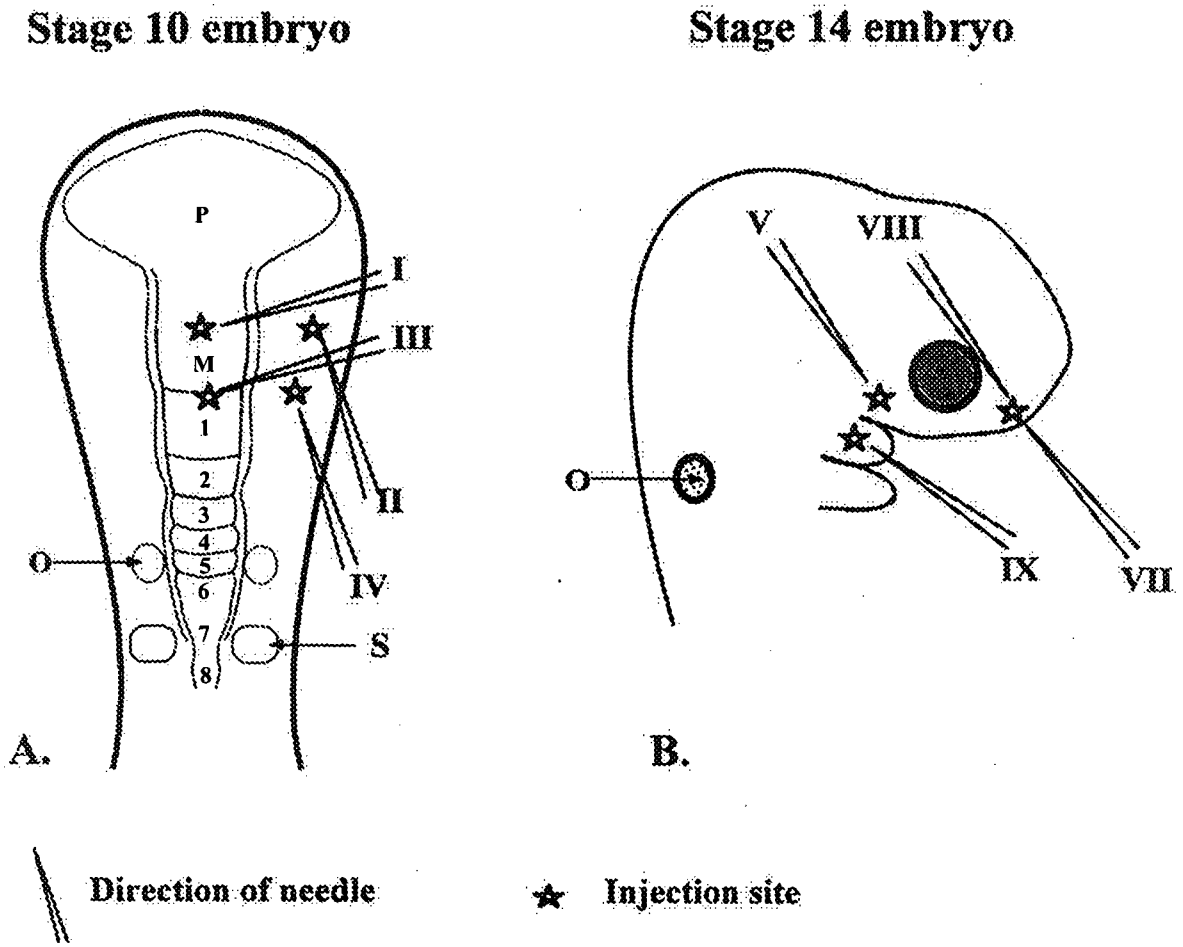


Figure 5.1 Targeting virus to the developing facial promiences. Multiple areas were chosen for retroviral injection in order to determine sites that gave reproducible infection. The sites used for the present study were II, IV (A) and V (B). These gave primarily unilateral spread of the virus. The direction of the needle was found to be important in reproducing the viral spread.

Key: M - mesencephalon; O - otic pit; P - prosencephalon; S - somite; 1-8 - rhombomeres 1 through 8.

Figure 5.2 Histochemical detection of alkaline phosphatase activity in chicken embryos infected with the RCASBP/alkaline phosphatase retrovirus. Purple stain shows the extent of viral spread. Viral suspension injected into the mesenchyme to the right of the mesencephalon at stage 10 resulted in infection of all facial prominences. The embryowas fixed at stage 28, 96 hour following injection. Note how the virus has not spread extensively across the midline of the frontonasal mass or mandibular prominences.

Key: F - frontonasal mass; Mx - maxilla; M_D - mandible. Scale bar = 1mm.

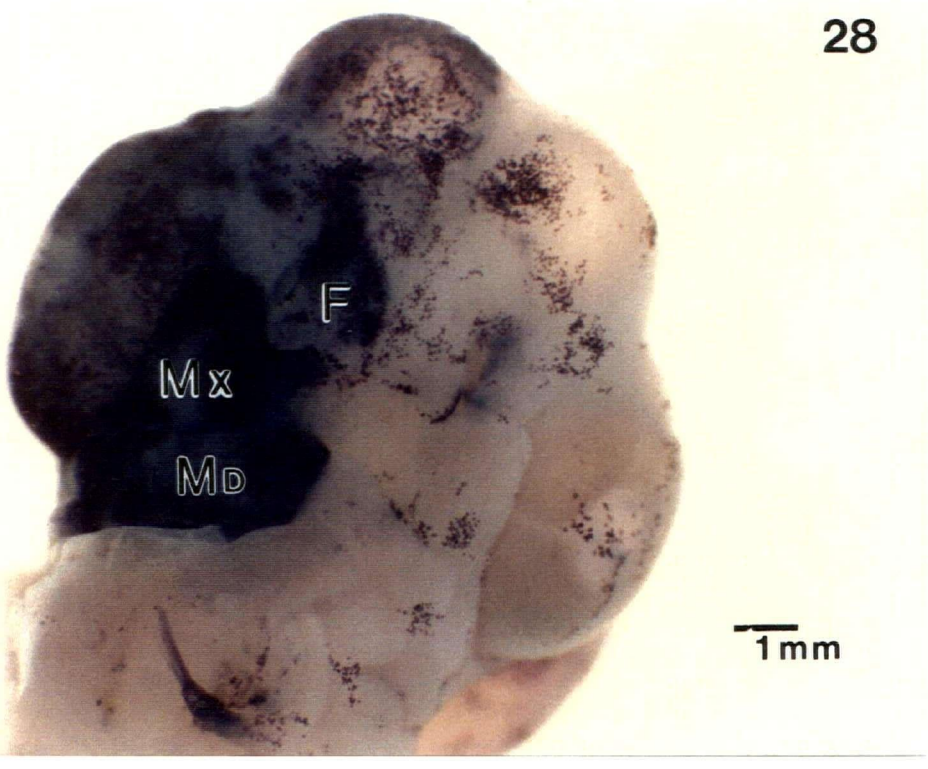

28
F
Mx
MD
1mm

Table 5.1 Alkaline phosphatase activity showing viral spread following injection at multiple sites and stages with RCASBP/E retrovirus containing an alkaline phosphatase insert.

Injection Site	Stage	N	Unilateral		Bilateral	No expression
			Upper Facial Prominences (FNM, Mx, LNP)	All Facial Prominences (FNM, Mx, LNP, Md)		
I: center of the midbrain	9 10	4 3		1	2 3	1
II: adjacent to the midbrain into mesenchyme	10 10/11	2 4		1 3	 1	1
III: center of the mid-hindbrain junction	10 11	2 5			2 5	
IV: adjacent to the mid-hindbrain junction into mesenchyme	10	3		2	1	
V: just candal to eye	14/15	6	4	1		1
VI: adjacent to the midbrain into mesenchyme	13	11	2	4	5	
VII: into nasal pit	14/15	3			3	
VIII: just rostral to eye	14/15 16	3 1			3 1	
IX: 1st arch	14/15	2			2	

5.2). There are some differences in the degree of infection or intensity of alkaline phosphatase staining which is likely due to variation in infection efficiency within individual injection. Generally however, embryos examined for alkaline phosphatase activity 5-6 days post infection showed more intense staining and increased spread throughout the primordia than those embryos analyzed 3 days post infection. There were never any malformations of the face produced in embryos injected with the PLAP-containing virus indicating that the virus itself is not affecting morphogenesis (data not shown).

We also checked viral spread in tissue sections (Table 5.2). A riboprobe made to the *pol* region of the RCASBP virus was used to detect viral expression in injected embryos. These results show that at stage 24 (72 hrs post injection) there are foci of viral expression within the mesenchyme but by stage 29 much more of the mesenchyme on the right side of the head is expressing the virus (Fig. 5.6A). The *pol* probe shows the virus location. Sections hybridized to the *BmpR-IB* probe show only the position of the virus if exposed for a short period (6 days, Fig. 5.6B). The endogenous *BmpR-IB* signal does not normally develop for another 10-15 days. There was no difference in the extent of virus spread between the various viral constructs.

DnBMPR-IB virus leads to decrease in the size of cartilage condensations

The uniform, bilateral production of developmental signals results in a symmetrical body pattern. We unilaterally altered BMP signaling in order to disrupt this symmetry and compare the phenotype resulting from ectopic BMP receptor expression with the normal

Table 5.2 Analysis of viral spread within the times in sections using a riboprobe made to the *pol* region of the RCASBP virus.

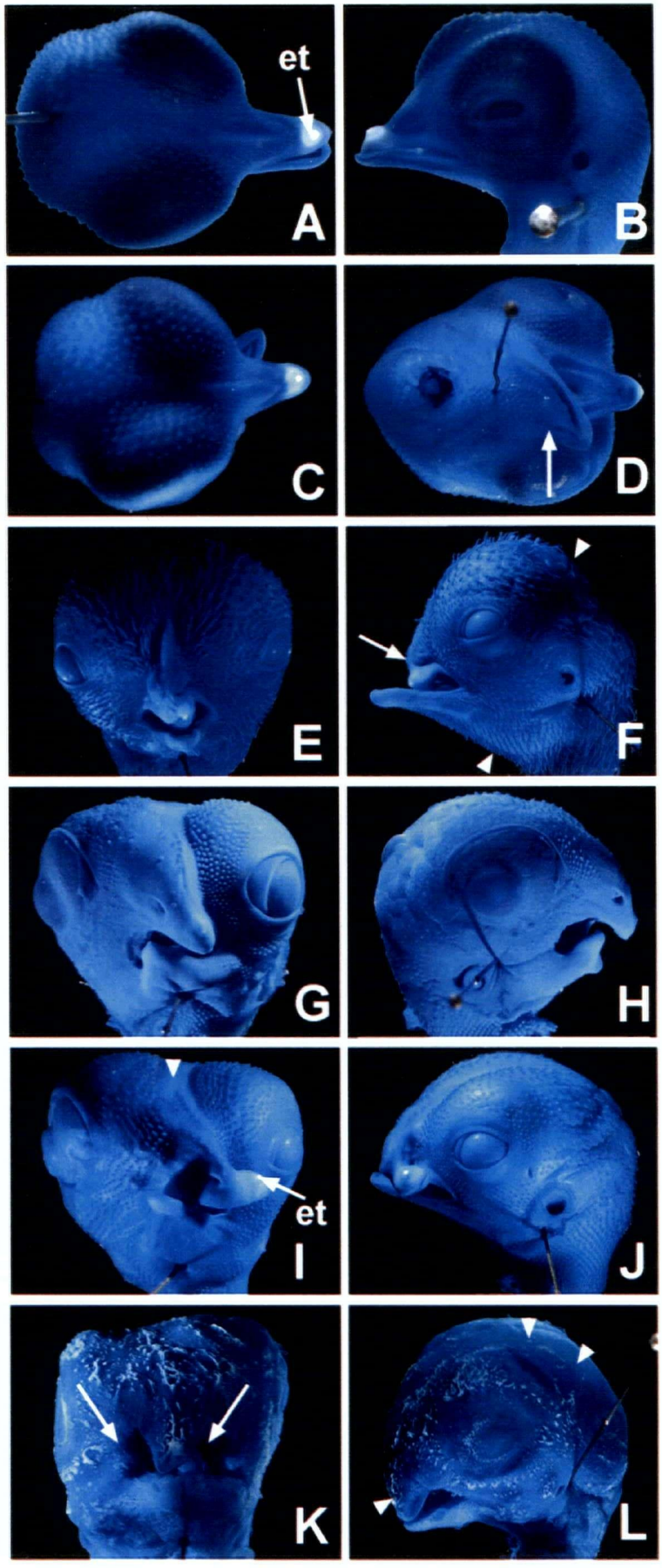
Injection Site	Virus	Stage	N	Unilateral		Bilateral	No expression
				Upper Facial Prominences (FNM, Mx, LNP)	All Facial Prominences (FNM, Mx, LNP, Md)		
II	Dn-B	10	5		4	1	0
V	Ca-B	14/15	4	3		1	0

contralateral craniofacial region. In order to determine the role of the endogenous BMPs in craniofacial skeletogenesis we used two dn virus constructs; dnBMPR-IA and dn BMPR-IB. There was no phenotype produced with the dnBMPR-IA in any embryo. These results are similar to those reported in the limb bud (Zou and Niswander, 1996; Kawakami et al., 1996).

In contrast to the dn-BMPRIA virus, embryos injected with dnBMPR-IB virus had a phenotype. Injection of dominant negative dn-BMPR-IB at stage 10 results in deviation of both lower and upper beaks towards the injection site (Fig. 5.3C,D; Table 5.3). Straight extension of lower beak is prevented because the infected side of Meckel's cartilage is shorter and thinner than the contralateral side (Fig. 5.4D,E,F). Approximately half of the embryos have a short segment of cartilage missing from the centre of Meckel's cartilage on the infected side (Fig. 5.4D). Components of hypobranchial complex are normal. The prenasal cartilage and distal half of the nasal septum in the upper beak is shorter and deviated to the side of injection. The cartilage differentiation is not as advanced as non-infected cartilage judging by alcian blue staining (Fig. 5.5 A,B,C). Similar defects were observed in the upper beak of embryos injected at stage 14. Truncations in the upper beak are also seen which is likely due to virus crossing the midline and affecting the entire FNM region (Fig. 5.3E,F and 5.4G,H,I). The lower beak was unaffected, consistent with the virus being targeted to the upper beak.

Figure 5.3 Surface morphology of heads following injection of retroviruses containing BMP receptor constructs. (A,B) wtBMPR-IB injected at stage 14 has nearly normal beak development except for a mild deviation of the upper beak towards the left. (C,D) dnBMPR-IB injected at stage 10 on left side resulted in lower beak deviation towards the left (arrow) and normal extension of upper beak. (E,F) dnBMPR-IB injected at stage 14 led to shortening of upper beak (arrow). Note presence of feather germs on head (arrowheads). (G,H) caBMPR-IB injected at stage 10 on right side. The lower beak is curved to right and upper beak is much thicker than normal. The egg tooth is small and there are many patches of ectoderm have no feather development (arrowheads). (I,J) caBMPR-IB injected at stage 14. The upper beak is curved severely to the left and lower beak is normal. Note denuded areas of ectoderm lacking feather germs (arrowheads). (K,L) caBMPR-IB injected at stage 14. The second phenotype was general spread of virus causing thickening of upper and lower beak cartilages. The upper beak has a bilateral partial cleft or notching of the tomium (arrows). The egg tooth is absent in this specimen (arrowhead) as are all feather germs. Keratinization has occurred randomly on the ectoderm instead of in the feathers.

Key: ET - egg tooth.


et
A
B
C
D
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et
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K
L

Figure 5.4 Morphology of the chondrocranium in cleared specimens. Photographs in A,G,M are superior views while those in B,D,J are inferior views. (A,B,C) wtBMPR-IB injected at stage 14. Mild curvature of the prenasal cartilage towards the right seen in superior and inferior views. (D,E,F) dnBMPR-IB injected at stage 10 on the right. Meckel's cartilage is thinner and has a small dehiscence on the right side causing the mandible to curve towards the right. There is a sharp bend in the cartilage on the left side (arrow). (G,H,I) dnBMPR-IB injected at stage 14. Prenasal cartilage is shorter and curved inferiorly (arrow) and the lower beak has extended normally. (J,K,L) caBMPR-IB injected at stage 10 on the right side. Extensive viral spread throughout the head has lead to the formation of many ectopic nodules and thickening of Meckel's cartilage. (M,N,O) caBMPR-IB injected at stage 14. The upper beak is curved sharply towards the left side (arrow) and is much thicker than normal. Meckel's cartilage has extended normally.

Key: PNC - prenasal cartilage; MC - Meckel's cartilage; NC - nasal cartilage

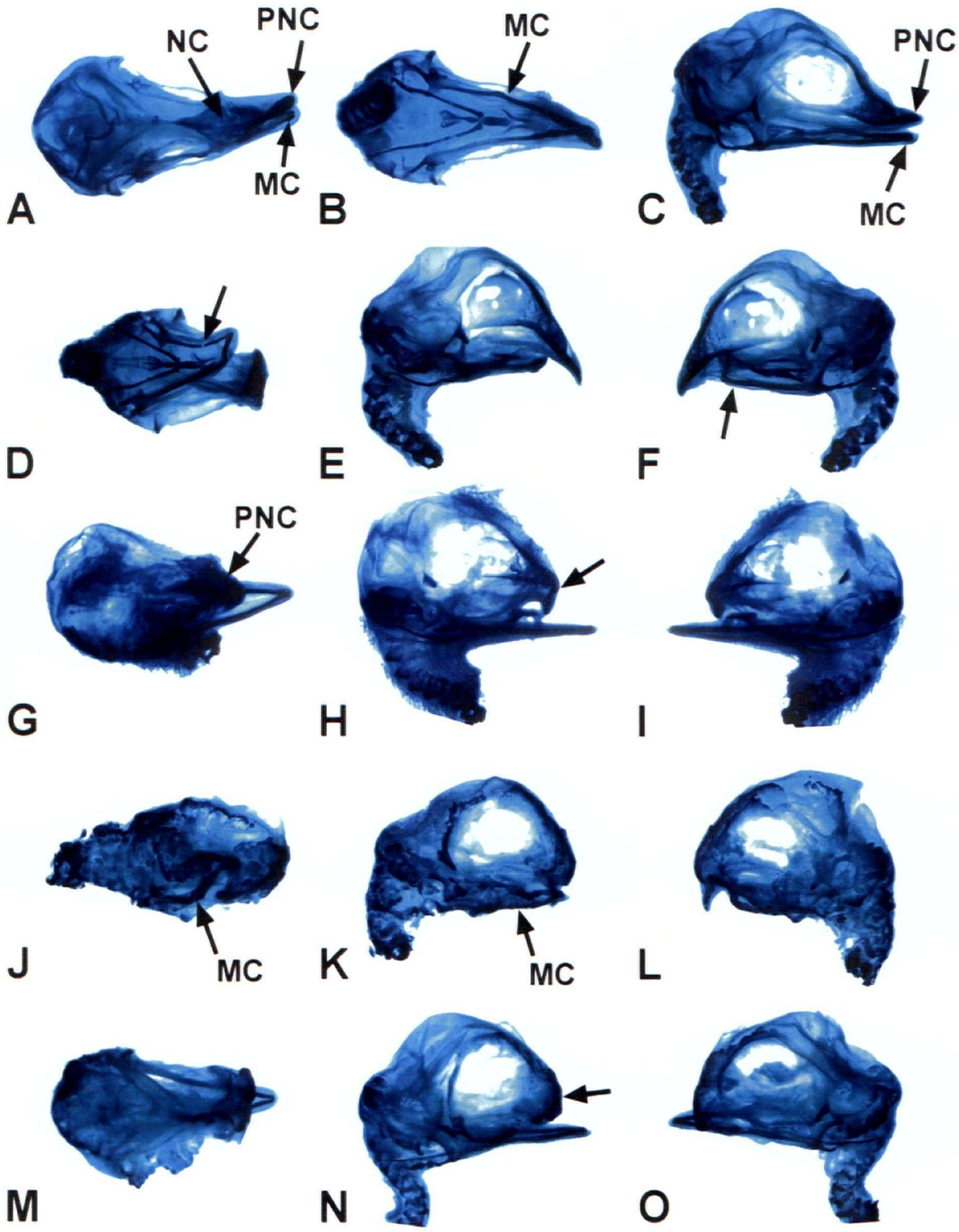

NC
PNC
MC
A
MC
B
PNC
MC
C
D
E
F
PNC
G
H
I
MC
J
MC
K
L
M
N
O

Figure 5.5. Effects of BMPR viruses on cartilage differentiation.

A, stage 38 dn-BMPR-IB injected embryo showing lack of Alcian Blue staining of Meckel's cartilage. B, right side enlargement of Meckel's cartilage showing one small region with normal Alcian Blue staining (arrow). C, normal staining of left Meckel's cartilage with Alcian Blue. D, frontal section of stage 32 embryo injected at stage 15 into the presumptive maxillary region with ca-BMPR-IB, hybridized to *pol* probe. Black silver grains (red arrow) indicate expression of virus primarily on left side of embryo. E, near adjacent section stained with alcian blue to show the cartilage. F, higher power of nasal septal cartilage showing closely packed chondrocytes in disorganized matrix.

Scale bars = 200 μm for D,E; 50 μm for F; 100 μm for B,C; 2mm for A.

Key: e - eye, ios - interorbital septum, mc - Meckel's cartilage.

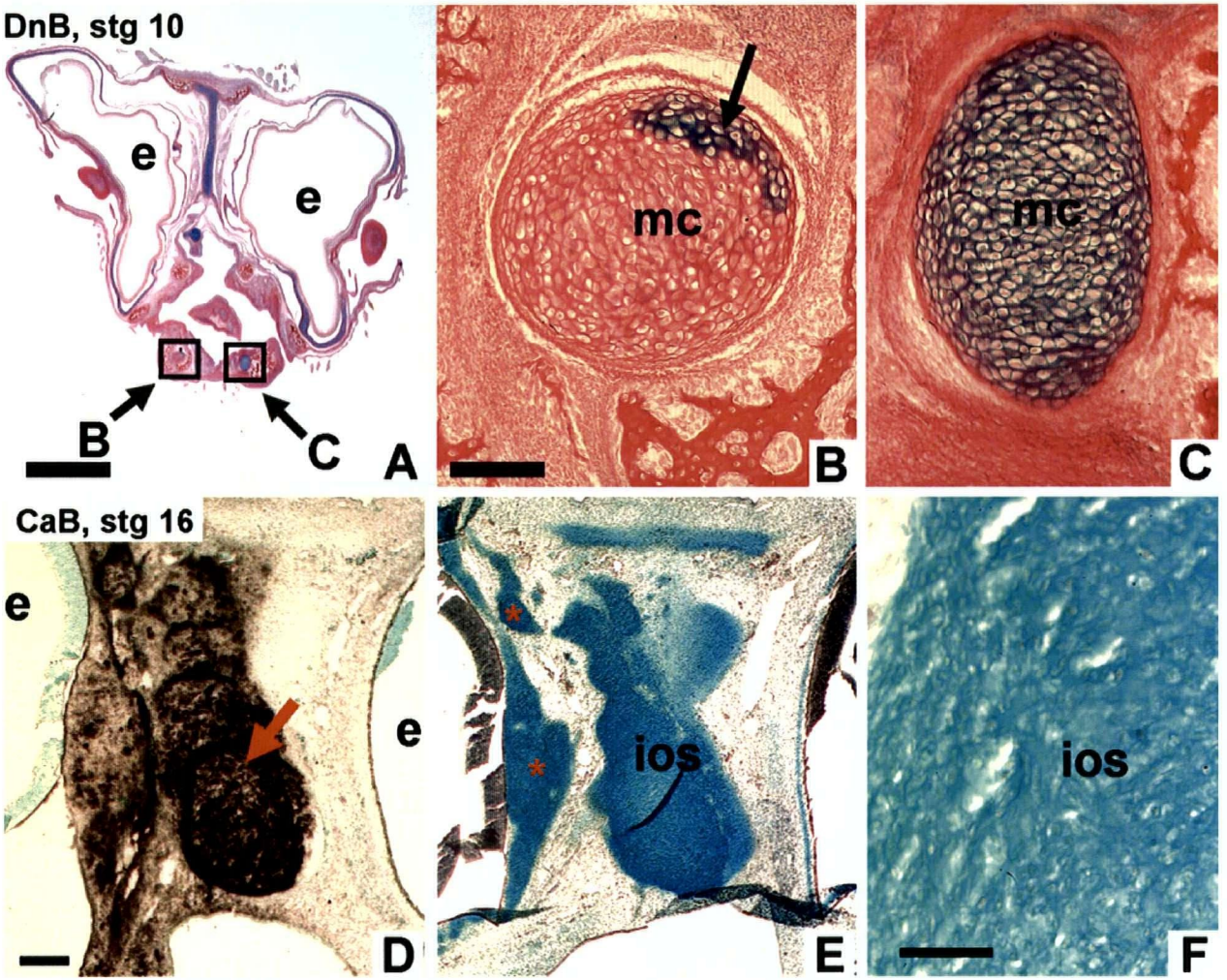

DnB, stg 10
e
e
B
C
A
mc
B
mc
C
CaB, stg 16
e
e
D
*
*
ios
E
ios
F

Table 5.3 Morphology of virus injected embryos.

Virus/Site of Injection	# Defects	Upper Beak	Lower Beak	Cleft	Enlarged Head	Suppression of Feather Formation	Suppression of Egg Tooth
wtBMRR-IB II+IV	7/10	6 mild (+)	2 mild (-)	0	0	0	0
V	14/15	10 mild (+)	5 mild (+)	0	0	0	0
dnBMPR-IB II+IV	10/13	5 mild (-), 1 short	10 mild-severe (-)	0	0	0	0
V	16/23	13 mild (-), 7 short	4 moderate (-)	3	0	0	0
caBMPR-IB II+IV	11/14	6 severe (+)	7 moderate (-)	1	6	6	0
V	15/15	12 severe (+)	6 mild (-)	3	5	5	2
caBMPR-IA II+IV	11/13	6 severe (+)	5 moderate (-)	1	4	4	0
V	12/16	7 severe (+)	1 mild (-), 2 mild (+)	0	8	8	5

(+) deviation away from side of injection

(-) deviation towards side of injection

CaBMPR-IA and caBMPR-IB produce enlarged cartilage

In general, the wtBMPR-IB virus produced a similar but much less severe phenotype as the caBMPR-IB infected embryos, therefore these results will not be described in detail. Injection of wtBMPR-IB virus particles, at stage 10 and at stage 14, results in a mild deviation of the upper beak away from the side of injection (Fig. 5.3A,B). Cleared embryo indicates a deviation of the prenasal cartilage at the nasal septum and a mild increase in the chondrogenic region on the injected side of the nasal septum (Fig. 5.4A,B,C).

In order to compare the potential functions of the type I receptors, caBMPRIA and caBMPRIB viruses were injected into the facial region. The effects of the two constitutively active type I viruses are very similar. There were two phenotypes produced with the embryos injected with the constitutively active viruses; one was due to an infection limited to the beak and the other phenotype was due to widespread infection of the entire head. Embryos with primarily beak infections have either very severe curvature of the upper beak away from the side of the injection or straight but thickened upper beaks (Fig. 5.3G,H). In no instance is the upper beak deviated towards the side of the injection. Cleared specimens indicate that cartilage elements are thickened, abnormally shaped and often fused together. There is hypertrophy of the prenasal cartilage, nasal septum, interorbital septum, and orbital rim on the side of the injection (Fig. 5.4J,K,L). The prenasal cartilage is occasionally fused with the nasal septum (data not shown). In contrast, embryos with deviated lower beaks, the deviation is nearly always deviated towards the side of the injection (Fig. 5.3G,H). The cleared specimens reveal that Meckel's cartilage is thicker and often bent on the injected side (Fig. 5.4J,K,L). The

deviation is therefore not due to lack of cartilage but rather because growth is not directed rostrally on the injected side resulting in tension on the opposite side of the mandible. The milder defects are predominantly observed when injections are done at stage 14/15, and either do not effect the mandible at all or only mildly (Fig. 5.3I,J, and 5.4 M,N,O).

Embryos with more widespread infection had enlarged heads compared to control embryos at similar stages (Fig. 5.3K,L). The earlier in development the infections are done the more severe the disruption of morphology. Virtually no individual cartilage elements could be identified in the embryos injected at stage 10 (data not shown, less severe defects are seen in Fig. 5.4J,K,L). Despite the severe impact the virus has on chondrogenesis a recognizable upper and lower beak still form (Fig. 5.3K,L).

Both ca viruses lead to a substantial increase in cartilage. Because of this similarity, only embryos infected with ca-BMPR-IB were further examined. Irregular shaped cartilages was the result of expansion in the cartilage elements and formation of ectopic condensations; the onsets of which are relatively rapid. By the time cartilage had differentiated (3-4 days after injection), the virus had disrupted the smooth outline of the cartilage (Fig. 5.5E). There are numerous closely packed chondrocytes in a disorganized matrix (Fig. 5.5F). There are also many extra condensations that coalesce to form large irregular masses of cartilage (Fig. 5.5E). The locations of the disruption in cartilage are closely correlated to the spread of the virus (Fig. 5.5D).

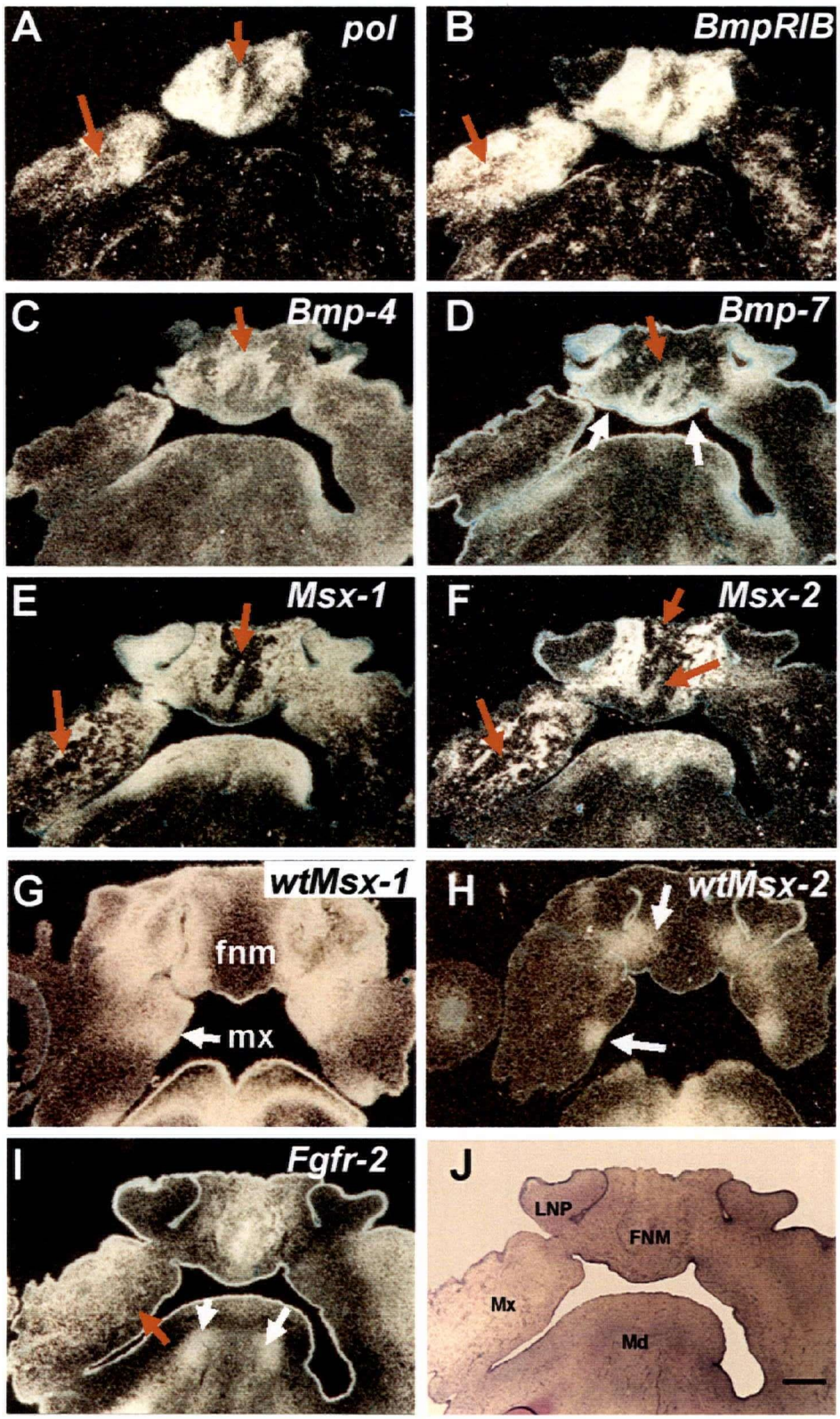
Constitutively active BMPR-IB leads to ectopic expression of Bmp-4, Bmp-7, Fgfr-2, Msx-1 and Msx-2

In order to understand the molecular basis by which BMP signaling controls facial skeletogenesis we examined the expression of several genes associated with cartilage formation. Surprisingly, in dn-BMPR-IB infected embryos we did not observe any expression changes in the genes examined (data not shown). Because of the similarities in phenotypes between the two ca-BMP type I receptors we performed gene expression studies only in ca-BMPR-IB injected embryos.

Muscle specific homeobox containing genes (*Msx-1* and *Msx-2*) are expressed in the facial prominences in discrete patterns from stages 24-28, prior to fusion of the facial prominences (Nishikawa et al., 1994; Mina et al., 1995; Barlow and Francis-West, 1997). After fusion occurs, at stage 29, *Msx-1* is expressed around the entire nasal passage (in the lateral edges of the frontonasal mass and nearly the entire the lateral nasal prominences), medial edges of the maxillary prominences and cranial mandibular regions (Fig. 5.6G). At stage 29, *Msx-2* is expressed below the nasal passage (lateral corners of the frontonasal mass and medial region of the lateral nasal prominence) and in a small patch of mesenchyme just below the budding palatal shelves (Fig. 5.6H). Neither of the *Msx* genes are normally expressed in the center of the frontonasal mass. In embryos infected with caBMPR-IB, *Msx1* and *Msx-2* transcripts are ectopically expressed in the center of the frontonasal mass (Fig. 5.6E,F). This expression closely overlaps regions where ca-BMPR-IB is localized. Therefore, *Msx-1* and *Msx-2* are downstream of BMPR-IB.

Figure 5.6 Expression of genes in adjacent frontal sections of a stage 29 embryo injected with caBMPR-IB at stage 14. The expression in the mandibular prominence is normal for all genes but virus has changed gene expression in the frontonasal mass and maxillary prominences on the right side of the embryo. A, high levels of virus expression are seen in the right frontonasal mass and right superior maxillary prominence (red arrows). There is very little viral spread to the mandibular prominence. B, the expression of *BmprIB* is similar to the *pol* gene and no endogenous *BmpRIB* expression is visible yet in mandible. C, *Bmp-4* is not normally expressed at high levels in the stage 29 facial mesenchyme but is considerably increased in the frontonasal mass (red arrow). D, *Bmp-7* is normally expressed along the caudal edge of the frontonsasal mass (white arrows) but in this specimen there is additional expression in the center of the prominence (red arrows). E, expression of *Msx-1* extends medially in the frontonasal mass and is spread in patches throughout the maxillary prominence on the right side (red arrows). F, expression of *Msx-2* in the right maxillary prominence is present in small, disconnected areas of mesenchyme and there is an ectopic expression domain in the center of the frontonasal mass (red arrows). G, endogenous expression of *Msx-1* at stage 29 with high expression in medial maxillary prominences, lateral nasal prominences and lateral edges of the frontonasal mass. H, endogenous expressions of *Msx-2* in a discrete medial patch in the maxillary prominences and lateral edges of frontonasal mass (white arrows). I, *Fgfr-2* is expressed in Meckel's cartilage condensations (white arrows). In the frontonasal mass expression in the midline cartilage condensation does not have well-defined borders. In the right maxillary prominence there is an expansion of the lateral expression into medial regions (red arrow). J, toluidine blue stained section from same specimen as in A-F,I. Scale bar = 200 μm.

Key: FNM - frontonasal mass; LNP - lateral nasal prominence; Md - mandibular prominence; Mx - maxillary prominence.


A
pol
B
BmpRIB
C
Bmp-4
D
Bmp-7
E
Msx-1
F
Msx-2
G
wtMsx-1
fnm
mx
H
wtMsx-2
I
Fgfr-2
J
LNP
FNM
Mx
Md

In order to determine whether there is a feedback loop between BMPR-IB signaling and BMP ligands we examined the expression of *Bmp-4* and *Bmp-7*. Both ligands are normally expressed along the caudal edge of the frontonasal mass mesenchyme (along with *Bmp-2*) but not in the central frontonasal mass (see chapter 2). The ca-BMPR-IB virus induces an ectopic region of *Bmp-4* and *Bmp-7* expression in the center of the frontonasal mass at stages 28 (data not shown) and 29 (Fig. 5.6C,D). The regions of ectopic expression did not overlap with the location of virus infection or regions of ectopic *Msx-1* and *Msx-2* expression, but rather was expressed in adjacent tissue where little (or no) infection was found.

We next examined the expression of *Fgfr-2,* a gene that is also expressed in facial mesenchyme and at high levels in stage 28 condensing cartilage (Wilke et al., 1997). We find that at stage 29, *Fgfr2* continues to be expressed in chondrogenic regions in the centers of the frontonasal mass and paired-mandibular prominences. In ca-BMPR-IB infected frontonasal mass, the edge of *Fgfr2* expression is very irregular and extends outside the chondrogenic region (3/3, Fig. 5.6I).

CaBMPR-IA and caBMPR-IB activity suppresses feather and egg tooth formation

Infection of the craniofacial region with caBMPR-IA or caBMPR-IB resulted in retardation of feather formation (Fig. 5.3G,H,I,J) or complete elimination of the feathers covering the head (Fig. 5.3K,L). Embryos with nearly complete absence of feathers generally had enlarged heads, widespread changes in cartilage morphology and white granular keritinizations on the surface of the skin (Fig. 5.3K,L; Table 5.3). The egg tooth is also an

ectodermal specialization and is reduced in size or absent in 50% of embryos with no cranial feathers (Fig. 5.3K,L; Table 5.3).

Discussion

Overlapping and unique function of type IA and type IB receptors in regulating the size of cartilage

Expression of dnBMPRIA had no effect on cartilage differentiation whereas dnBMPRIB decreased the size of cartilage condensations. The dn type I receptor constructs are not designed to inhibit endogenous BMP signaling from a particular receptor type. Rather, the mutation is designed to inhibit all BMP signaling. Following infection, the mutation in the ATP binding domain still allows dimerization with the type II receptor and ligand binding. This abundant mutant complex would compete for available BMP ligands and subsequently inhibit BMP signaling. Therefore, in theory, similar defects should be observed with each dn receptor type but defects were only observed in dn-BMPR-IB infected embryos. I attribute this to the BMPR-IB receptor having greater receptor affinity for BMP-4, BMP-7 (ten Dijke et al., 1994; Nohno et al., 1995; Rosenzweig et al., 1995; and GDF-5, Nishitoh et al., 1996) than BMPR-IA.

No phenotype was also observed when dnBMPRIA was injected into the chicken limb bud (Kawakami et al., 1996; Zou et al., 1997) and in cultures of sternal cartilage (Enomoto-Iwamoto et al., 1998). A different dn-BMPR-IA construct, which lacks the entire cytoplasmic Ser/Thr kinase domain, inhibited interdigital BMP-induced apoptosis but only partially inhibited digit formation (Yokouchi et al., 1996). This construct appears to be a better inhibitor (than that used by us and Kawakami et al., 1996; Zou et al., 1997). Perhaps in the absence of the large cytoplasmic domain, dn-BMPR-IA can dimerize with BMPR-II receptors more easily and therefore partially inhibit BMP

signaling. Differences in ligand affinity between BMPR-IA and BMPR-IB may explain why this construct is able to inhibit apoptosis but not chondrogenesis. BMPR-IB is expressed in the distal phalages (Zou et al., 1997) and regulates chondrogenesis in this region (Baur et al., 2000; Yi et al., 2000). However, BMPR-IB is neither expressed in the interdigital mesenchyme (Zou et al., 1997) nor does it regulate apoptosis (Baur et al., 2000; Yi et al., 2000). Dn-BMPR-IB must be able to better compete for BMP ligands with whichever BMP receptor mediates BMP-induced apoptosis, which awaits identification. Now that the entire human genome has been sequenced we can search the database to determine how many BMP type I related receptors exist. A new BMP type I receptor was recently identified in zebrafish (Alk8; Bauer et al., 2001; Mintzer et al., 2001).

Under the conditions used here we do not entirely prevent the early stages of chondrogenesis with dnBMPRIB as has been seen in vitro (Kawakami et al., 1996; Zou et al., 1997). The difference may be that we infect only a proportion of the prechondrogenic cells allowing a smaller condensation to form. In some locations we observed a gap in the cartilage (Fig. 5.4D) suggesting that cells in this region were unable to organize into a condensation and thus failed to differentiate. The retroviral system causes very high expression of dnBMPRIB. Despite this, the ectopic dn-BMPR-IB was unable to completely inhibit chondrogenesis as was observed with overexpression of Noggin (Capdevilla and Johnson, 1998). These differences suggest that the endogenous type IB receptor is very abundant within cartilage condensations. The BMPRIB knockouts indicate that in vivo the role of the BMPRIB is much more limited than our dominant

negative virus experiments would suggest (Baur et al., 2000; Yi et al., 2000). Almost all the embryo develops normally with the exception of the absence of metacarpals and metatarsals.

Ca-type I receptors have been used to identify similarities and differences between BMPR-IA and BMPR-IB signaling. One difference that is known is that ca-BMPR-IB can induce apoptosis while ca-BMPR-IA suppresses cartilage differentiation (Zou et al., 1997). A function that my results suggest is that both receptors share the ability to promote neural crest derived cartilage formation. This similarity may either be due to the receptors sharing similar functions or due to the induction of the other BMP receptor (Zou et al., 1997). Our studies may better explain why the type I receptors have similar functions. We showed that ca-BMPR-IB induces *Bmp-4* and *Bmp-7* expression. The elevated BMPs would then be able to induce localized BMP activity. Others have shown that retrovirus-mediated overexpression of BMP proteins (Duprez et al., 1996a) or exogenous BMP-4 (Buckland et al., 1998) and BMP-7 (Macias et al., 1997) applied to the early cartilage rudiments greatly increases the size of cartilage in the limb. The interesting feature of our finding is that the BMPs were upregulated in adjacent regions of low or no infection suggesting that the feedback loop involves neighbouring, and not infected cells. Thus, cells stimulated by BMPs (or activated receptor) stimulate neighboring cells to produce *Bmp-4* and *Bmp-7* in a paracrine mechanism. The upregulation of *Bmp-4* and *Bmp-7* may be part of the mechanism by which chondrogenesis is promoted in embryos infected with constitutively active BMP receptors. Since the ectopic *Bmp-4* and *Bmp-7* are able to activate either of the BMP receptors this would prevent us from being able to identify specific signaling functions using

the ca-BMPR-IB receptor mutant. It is not known if ca-BMPR-IA also increases the expression of BMP ligands

We show that ca-BMPR-IB induces cartilage formation in ectopic regions. A possible mechanism to explain this observation may be ca-BMPR-IB increases cartilage formation through recruitment of non-chondrogenic precursors. Similar results were reported in the limb (Zou et al., 1997). These limb studies also showed that caBMPRIB virus increases the proliferation of chondrocytes. We did not examine proliferation in the facial cartilage but increased proliferation of chondrocytes may further explain why, in addition to ectopic cartilage, the individual cartilage elements were also much larger then usual. The expression of *BmpR-IB* in normally condensing and differentiating cartilage (chapter 2) suggests that the receptor plays a role in the proliferation of chondrocytes.

Msx genes are down stream of the BMPR-IB receptor

The Msx-1 and Msx-2 genes are homeobox containing transcription regulators involved in cell proliferation (Odelberg et al., 2000; Chen et al., 2001) and apoptosis (Ferrari et al., 1998), respectively. Both Msx genes are upregulated by BMPs. We are the first to show a relationship between BMP receptor signaling and *Msx-1*, *Msx-2* gene expression. We identified that both Msx genes are downstream of BMPR-IB signaling. It is not known whether BMPR-IA can also regulate the expression of one or both Msx genes. Ectopic application of BMPs in two separate regions of the face induces both Msx genes (Barlow and Francis-West, 1997). Midline regions express BMPR-IA but not BMPR-IB while lateral regions express both BMPR-IA and BMPR-IB. Thus both type I receptor types can regulate

Msx gene expression. Examination of *Msx-1* and *Msx-2* gene expression following ca-BMPR-IA infection may help to determine whether Msx genes are downstream of both type I BMP receptors or only downstream of BMPR-IB.

Msx-1, Msx-2 and Fgfr2 are associated with chondrogenesis

Msx-1 overexpression is associated with cellular proliferation (Chen et al., 2001) and dedifferentiation (Odelberg et al., 2000). Msx-1 is expressed at the edges of cartilage condensations and cartilage elements (Mina Mina et al., 1995) suggesting that Msx-1 may be involved in the proliferation of chondrocytes. The ectopic overexpression of Msx-1 has not been used to examine the effect of elevated Msx-1 protein in cartilage morphogenesis. However, the MSX-1 knockout mouse has reduced size of cartilage elements (Satokata and Maas, 1994). BMPs therefore may partially regulate the size of cartilage elements through the Msx-1gene. In my study, ectopic Msx-1 expression may be associated with increased cartilage formation by stimulating proliferation.

Msx-2 associated with cell death in early development. Overexpression of Msx-2 stimulates ectopic apoptosis in the neural crest cells (Takahashi et al., 1998) and limb bud (Ferrari et al., 1998). Msx-2 knockout mice also have abnormal cartilage and endochondral bone formation. Msx-2 mutants have fewer resting, proliferative and hypertrophic chondrocytes (Satokata et al., 2000) suggesting that Msx-2 is required for chondrocyte proliferation and formation. Thus, the increased *Msx-2* expression that we observed following ca-BMPR-IB infection may promote the formation of chondrocytes. Msx-2 has very different functions

during skeletogenesis than in early development. A proliferative role for Msx-2 is also observed in the calvariae of transgenic mice that overexpress Msx-2 (Liu et al., 1999).

I also detected an increase in *Fgfr2* expression in response to increased BMPR-IB activity. Several lines of evidence suggest that both gain-of-function (reviewed in Webster and Donoghue, 1998) and loss-of-function mutations in FGF signaling (Celli et al., 1998) cause skeletal malformations in the skull and limb. The skeletal alterations observed in these embryos may be secondary to defects earlier in development. Examining the effects of FGFs directly on cartilage or chondrocytes would better address FGF function. In vitro, FGF-2 increased cartilage formation from facial mesenchyme (Richman and Crosby, 1990 and stimulated greater proliferation of porcine ear chondrocytes (Arévalo-Silva et al., 2001). In mouse, FGF4 can induce digit bifurcation (Ngo-Muller and Muneoka et al., 2000). Thus, ca-BMPR-IB infected tissue may stimulate cartilage formation through the upregulation of *Fgfr2*, and increased FGF signaling.

Increased BMP signaling inhibits formation of ectodermal specializations

The ectopic expression of caBMPR-IA or IB reduces or eliminates feather formation and instead causes random keratin deposites in the epithelium. A similar result was obtained when BMP-2 was applied to the healing epidermis of a lamb fetus. A scar formed due to increased keratinization (Stelnicki et al., 1998). In an avian model, the application of BMP-4 suppresses differentiation of feather buds (Noramly and Morgan, 1998; Jung et al., 1998). Our data confirms that both BMP type I receptors are able to mediate this response. Overexpression of Noggin however results in abnormally large feather buds due to fusion of

adjacent placodes (Noramly and Morgan, 1998). We did not observe any change in feather morphology using our dn-BMPR-IB construct, which may not be able to sufficiently suppress BMP activity. The *notch* receptor and its ligands, *delta* and *serrate* are expressed in a dynamic fashion during patterning of the feather array and differentiation of the feather buds (Crowe et al., 1998). Misexpression of Delta-1 leads to a loss of feathers in virally infected skin and, contrary to what we would expect, inhibition of *Bmp-2*, *Bmp-4* and *Bmp-7* expression (Crowe et al., 1998). The loss of BMP ligand expression in feather buds is likely secondary to the absence of bud formation. Since similar phenotypes are produced with caBMPR-I and Delta-1 viruses it is possible that the two molecular pathways share common downstream signaling events.

Similar to feather formation, the BMPs are expressed in a dynamic pattern during egg tooth formation (see chapter 2). The egg tooth is a keratinization at the tip of the upper beak that the chick uses to break through the eggshell upon hatching. The pattern of BMP expression suggests that BMP signaling is involved in egg tooth morphogenesis. Since elevated BMP activity increases keratinization (our findings, Fig. 5.3K,L and Stelnicki et al., 1998) we did not anticipate that increased BMP signaling would inhibit egg tooth formation. However, the effects of BMPs are highly dependent on concentration. For example, BMPs regulate outgrowth in the limb (Capdevilla and Johnson, 1998) and proliferation in face (my findings, chapter 4) mesenchyme but at high concentrations they induce cell death (Buckland et al., 1998; Ekanayake and Hall, 1997; Barlow and Francis-West, 1997; my findings, chapter 4). Inhibiting endogenous BMPs will determine whether they are indeed involved in morphogenesis of the egg tooth. We

did observe defects in egg tooth dn-BMPR-IB infected embryos (Fig. 5.3E). This observation was missed in preliminary analysis of our data because the defect was mild. A stronger inhibitor, like Noggin, may be more informative and generate a more obvious defect.

Evolutionary implications

The BMPs may be the most highly conserved family of proteins in evolution with homologs that predate the evolution emergence of bone and cartilage. After the divergence of vertebrates and invertebrates the BMPs may have assumed the role for normal formation of skeletal elements as homologous genes are found in all mammals. Although some variation exists, the chondrocranial pattern is essentially conserved across the vertebrate taxa (de Beer, 1937). Specification of the primary form of the facial bony skeleton occurs epigenetically (Bosma 1976). Therefore, it has been suggested that much of the phylogenetic diversity of skull form can be accounted for by minor quantitative heterochronic shifts in the rate and timing of developmental events that regulate chondrocranial form (Alberch and Alberch, 1981; Wake 1980). The BMPs may be the genetic control resulting in phylogenetic variations of chondrocranial morphogenesis between and among species.

BMP mediated morphological changes have been reported that result in features resembling other animals or related species (Zou and Niswander, 1996; King et al., 1994). In our caBMP receptor injected embryos, two features are particularly striking. First, instead of the normally occurring long and narrow beak found in chicks, our

(bilaterally infected) caBMP receptor injected embryos had a much wider, thicker, rounded beak. This resulting beak displayed features much more characteristic of other birds such as parrots or budgies. Birds have wide variations in beak shape and this may be regulated by BMPs. Secondly, it was reported that infection of chick limbs with dnBMPR-IB resulted in a partial conversion of scales to feathers on the dorsal surface of the foot (Zho and Niswander, 1996). It appears that BMPs regulate a binary decision between the choice of scales or feathers. Our embryos with widespread caBMP receptor infection had complete absence of feathers and instead have patches of keratin. Perhaps other birds that lack feathers on their head and instead display keratinizations patches on the surface of their skin, such as turkeys, have elevated BMP signaling in the head.

Other intriguing genetic changes that result in morphological features resembling other animals or species have also been reported to be associated with BMPs and are worth mentioning. Zou and Niswander (1996) found that infection of chick limbs with dnBMPR-IB inhibited interdigital apoptosis resulting in a webbed chick foot resembling that seen in ducks. However, *Bmp-2*, *Bmp-4* and *Bmp-7* were all detected in the duck interdigit (Laufer et al., 1997). The interdigit region of ducks may not express the BMP receptor or downstream gene involved in BMP-induced apoptosis. Moreover, in the limb, BMPs regulate digit number (Drossopoulou et al., 2000) and digit patterning (Dahn and Fallon, 2000), therefore they could potentially regulate differences in digit formation among distinct species. Finally, compared to normal mice the *se* mouse (which has a mutation in the BMP5 gene) has a shorter nose, wider skull and shorter ears, resembling voles rather than house mice (King et al., 1994). Very short external ears are the most

characteristic feature of the *se* mouse. Could BMP-5 regulate morphological differences in ear size observed between chickens, mice, and rabbits? In situ hybridization studies determined that *Bmp-5* is the only BMP gene that is expressed at high levels in the condensations of the developing ear (King et al., 1996). Further studies should help clarify the importance of BMP-5 in regulating region-specific morphological features in higher animals. BMP signaling has been identified as far back as in *c. elegans* and *Drosophila*, and thus the alteration in BMP function and activity may be a mechanism employed during evolutionary progression over time.

We conclude from these experiments that the embryo can use either the type IA or type IB receptor for chondrogenesis and formation of ectodermal specializations. In vivo however, there may be a preference for using one receptor over another in certain locations and at certain stages of development. BMP signaling is very important during embryogenesis; therefore it is necessary to build some redundancy into the system. We have demonstrated several overlapping functions of the type I receptors.

Contributions to this chapter

The preceding chapter represents a portion of a manuscript that has been submitted to the International Journal of Developmental Biology. The scientific ideas initiating these studies were Dr. Richman's and I completed the study. Most of the introduction for this chapter was taken from our manuscript, which Dr. Richman wrote. I wrote the rest of the chapter. The majority of the work presented in this chapter, data analysis and

intrepretations were done by me. I received technical assistance from Sandra Babich in growing the virus and Steve Ritchie in assembling some of the figures.

CHAPTER VI

General Discussion and Future Directions

In this thesis I set forth to identify candidate genes associated with cleft lip and to test the function of these candidate genes in normal and abnormal facial development. These studies were undertaken at the organ (bead implantation experiments, viral expression experiments), tissue (epithelial stripping experiment) and molecular levels (changes in expression following bead implantation). First, the expression of BMP signaling molecules was described during most of the critical stages of craniofacial development in order to identify the locations where endogenous BMP signaling occurs. Second, functional experiments were performed that identified roles for BMP signaling in primary palate formation and the differentiation of cartilage, bone, feathers and egg teeth.

The expression studies in Chapter III are entirely descriptive. The presence of mRNA transcripts does not necessarily mean that protein is made – gene expression does not translate into gene function or bioactivity. Despite this, expression studies are an absolutely essential preliminary step in identifying where BMP signaling may potentially occur. The genes examined here are those protein products found extracellularly (BMP ligands, secreted antagonist) or at the cell surface (BMP receptor). Although my studies were quite extensive, there are numerous other BMP ligands, antagonists and receptors. All the main BMP receptors were examined with the caveat that there are two isoforms of the type IB receptor. Mice were identified to have two BMPR-IB mRNA isoforms derived from different promoters (Baur et al., 2000). It is not known if this applies to chickens, but my study would have detected all isoforms since the entire gene was used

as a probe. There may be other BMP receptors. For example, a new BMP type I receptor (Alk8) was recently identified in zebrafish (Bauer et al., 2001; Mintzer et al., 2001) and a pseudoreceptor, BAMBI, (BMP and activin membrane-bound inhibitor) was identified in *Xenopus* (Onichtchouk et al., 1999) and mice (Grotewold et al., 2001), which inhibits BMP signaling by binding to BMP type I receptors. Since the whole human genome is now sequenced we can search this database for other possible BMP type I receptors, as was done for FGF receptors (J. Rossant, personal communication). There are also numerous BMP antagonists (described in Chapter 2) that might modulate BMP signaling in the face. In addition to what I reported in Chapter 2, the expression of *chordin* was also examined. *Chordin* is ubiquitously expressed in the all head regions similar to what was reported in the mouse (Scott et al., 2001). I also found that the expression of *tolloid* (Reynolds et al., 2000), a metalloproteinase that cleaves Chordin, is highly expressed in the face mesenchyme but was absent in the neuroepithelium. I did not examine the numerous intracellular BMP signaling molecules, Smads for example, which remain to be investigated in the face. *Smad 1* and *Smad 5* are highly expressed in the mouse facial mesenchyme (Dick et al., 1998; Flanders et al., 2001).

Our expression studies were examined at numerous phases of facial morphogenesis; neural crest migration, initiation of facial prominences, outgrowth of the facial prominences, fusion, differentiation and skeletogenesis. This data was used as a springboard for our functional studies, which concentrated on the later phases mentioned.

I examined the involvement of BMPs in primary palate closure using two methods: 1) retrovirus mediated overexpression of mutant BMP type I receptors, and 2) the ectopic application of recombinant BMP-2 and Noggin proteins. The retrovirus mediated approach resulted in less consistent clefts (Table 5.3). We attribute this to not being able to consistently infect the facial prominences in the region of fusion and perhaps differences in the extent of viral spread. Approximately 10-15% of embryos examined prior to fusion localized virus expression in the corners of the frontonasal mass and maxillary prominences, which is the percentage of embryos that exhibited clefts. Clefts were observed in embryos infected with dn-BMPR-IB and ca-BMPR-IB, but not with dn-BMPR-IA or ca-BMPR-IA. I attribute clefts in dn-BMPR-IB infected embryos to the BMPR-IB receptor having greater receptor affinity than BMPR-IA (Nohno et al., 1995; Rosenzweig et al., 1995), and therefore dn-BMPR-IB is better able to compete for available ligands. Phenotypic differences were observed with the two ca type I receptors. Clefts were only observed with the ca-BMPR-IB virus, once again in approximately 10-15% of infected embryos. A difference between the two ca type I receptors is that ca-BMPR-IB can stimulate apoptosis in infected cells (Zou et al., 1997). It is possible that ca BMPR-IB increased apoptosis therefore reducing outgrowth of the facial prominences leading to clefts. Despite the lack of control of viral infection and spread, these preliminary data supported a role for BMP signaling in primary palate closure.

I found the bead-mediated application of ectopic protein to be a better method of targeting the regions of fusion and subsequently our results were much more consistent. For example, Noggin protein was implanted into the frontonasal mass and maxillary

mesenchyme in regions where high BMP expression was identified. These regions also corresponded with areas of highest cell proliferation. By applying ectopic Noggin I identified that endogenous BMPs regulate proliferation of the facial mesenchyme. Similarly, by targeting Noggin to near the epithelial surface I found that BMPs regulate epithelial survival. Combined with my gene expression studies, this finding suggests that epithelial apoptosis, normally during fusion, occurs by the downregulation of *noggin*. Using this method I was also able to examine gene interactions downstream of BMPs in the face.

At the molecular level, BMPs interact with FGFs and SHH in many different organisms and in many regions of the developing embryo. In the avian embryo there are many examples of mutations that affect both limb and face development (Schneider et al., 1999). This may be unexpected since these regions of the body are structurally quite dissimilar, have separate evolutionary origins, and have different embryonic origins. I find that BMPs act upstream of and define the region of *fgf-8* expression in the face, similar to what is known in the limb apical ectodermal ridge (Pizette and Niswander, 1999). I find that elevated BMP activity also suppresses *Shh* expression in the facial ectoderm. Application of ectopic BMP protein to the limb also suppresses *Shh* expression in the limb mesenchyme (Zhang et al., 2000). However, the face is different from the limb as *Shh* is expressed in the ectoderm rather than in the mesenchyme (Helms et al., 1997) and we showed that ectopic BMP-2 applied to the mesenchyme suppresses *Shh* expression in the ectoderm. I also showed that inhibiting endogenous BMP activity, with ectopic Noggin, does not alter *Shh* expression suggesting that BMPs are not required to

maintain *Shh* expression. Other similarities involve the *Msx-1* and *Msx-2* genes. Ectopic BMP application increases *Msx-1* and *Msx-2* expression in the face (Barlow and Francis-West, 1997; Wang et al., 1999) and limb (Merino et al., 1999; Ganan et al., 1996). *Msx-1* and *Msx-2* have also been shown to be downstream of endogenous BMP signaling in the limb (Pizette and Niswander, 1999; Msx-2 only, Zou and Niswander, 1996). I am the first to identify this endogenous relationship in the face.

There are some differences in the signaling pathways that operate in the face and limb. The expression of *noggin* in the frontonasal epithelium is a feature unique to the face. No BMP antagonist has yet been localized to the limb apical ectoderm ridge (AER) or any other domain of the limb epithelium. In the limb, ectopic SHH upregulates *noggin* (Pizette and Niswander, 1999) and *gremlin* (Zuniga et al., 2000) expression but this relationship is localized to the limb mesenchyme. In the face, *Shh* overlaps with *noggin* expression in the frontonasal epithelium. Although I did not study this relationship, my findings suggest that Noggin may act to prevent BMPs from suppressing *Shh* expression.

The genes upstream of BMP signaling in the face were not extensively examined. However, in the limb it has been shown that SHH (Laufer et al., 1994; Yang et al., 1997), FGF-4 (Laufer et al., 1994) and Noggin (Capdevila and Johnson, 1998) can upregulate *BMP* ligand expression. By applying exogenous SHH and FGF-8, or antagonists to these proteins, we can better understand the upstream genes that regulate BMP expression. In this manner we can sort out gene hierarchy in the face. SHH may act partially through BMPs since Hu and Helms, (1999) reported that ectopic SHH protein upregulates *Bmp-2*

expression. If this is true the simultaneous application of SHH and Noggin would suppress SHH-induced proliferation and ectopic beak formation.

I touch on upstream signaling using our mutant viral constructs and showed the BMP signaling upregulates its own, BMP ligand (*Bmp-4*, *Bmp-7*), expression (also seen in the tooth, Vainio et al., 1993). I also show that exogenous RA acts upstream of BMPs, and upregulates *Bmp-2* and *Bmp-4*. RA induces apoptosis in the face (Shen et al., 1997; McGonnell et al., 1998) and based on this data, I hypothesized that BMPs mediate RA-induced apoptosis. I proposed to simultaneously apply RA and Noggin to the face. If BMPs mediate RA-induced apoptosis Noggin would inhibit the BMP-mediated apoptosis, and perhaps even prevent the RA induced cleft. Unfortunately, this experiment was performed in the interdigital region of the limb and published in Nature Cell Biology (Rodriguez-Leon, 1999) before I could begin the experiments. Programmed cell death normally occurs between the digits, however, when RA is applied, apoptosis is increased and interdigital tissue regression is accelerated. RA upregulates *Bmp-7* and *Bmp-4* in the limb. While I did see an upregulation of *Bmp-2* and *Bmp-4* immediately following RA treatment, I did not detect any change in *Bmp-7* expression in the face. *Bmp-7* may not be downstream of RA in the face. Upregulation of *Bmp-2* and *Bmp-4* is the earliest reported change in gene expression following RA treament (Munoz-Sanjuan et al., 2001; Shen et al., 1997; Helms et al., 1997; Brown et al., 1997) therefore, BMPs may be an important early response genes in RA-induced cleft lip. Instead of performing the Noggin/RA cleft rescue experiment, I decided to focus on examining the endogenous role of BMP signaling during craniofacial development.

Retrovirus mediated overexpression of mutant BMP receptors was also used in attempts to identify biological differences between the type IA and IB receptor signalling. The effects of altered BMP signaling was examined on facial skeletogenesis. Although I reported some interesting functions of ca-BMP receptors on cartilage formation, I did not observe any obvious differences between the two type I receptors. Zou et al, (1997) found distinct roles for these ca-BMP receptors in limb skeletogenesis. The BMPR-IB was shown to be involved in the initiation of cartilage formation, while BMPR-IA regulated chondrocyte differentiation. My virus work is very preliminary and requires additional investigation in order to decipher the functional differences between BMPR-IA and BMPR-IB in facial cartilage morphogenesis. An unexplored area of investigation is the roles of BMP type I receptor signalling on intramembraneous bone formation. Since the maxillary skeletal derivatives form by intramembraneous bone formation, in contrast to the limb where endochondral bones form, there is potential to identify some novel functions for BMP signaling. In order to address this, I injected the virus constructs specifically into the maxillary prominence. Whole heads and sections from infected embryos were stained for bone. Dr. Richman analyzed this data, which was not included in this thesis but can be found in the manuscript described in Chapter V.

My studies raise many new questions about BMP function. There are numerous other regions in the face that express BMPs, suggesting potential BMP function, but were beyond the scope of this thesis. For example, prior to tongue papillae formation BMPs are expressed in the epithelium and mesenchyme. Expression and functional analysis

right at the time of papillae initiation will help us to better understand the function of BMPs during papillae formation. The restricted expression of *Bmp-7* and *BmpR-IB* in the olfactory epithelium, and *chordin* in the respiratory epithelium suggests a possible role for BMPs in specifying the nasal epithelium. BMP signaling molecules are also expressed in the pharyngeal membrane, prior to its breakdown, during nasal pit epithelium differentiation and egg tooth morphogenesis. Little or nothing is known about the involvement of BMP signaling in the development of these structures. Our expression studies provided the basis from which numerous hypotheses can be formed. In addition, my differential display work suggests that there are numerous genes that may mediate RA teratogenicity in the face. Screening a cDNA library with the clones I isolated may identify novel genes in facial morphogenesis. My protein application work can also be further examined.

In conclusion my thesis work has identified many new functions for BMPs in craniofacial morphogenesis. The most significant contribution to science this thesis offers is the work involving avian primary palate formation. These findings raise many important questions. The most significant being: are BMP signaling molecules associated with human clefting? BMP may have a central role in human primary palate closure as my data identifies interactions with molecules upstream (RA, Lammer et al., 1985; RARα, Chenevix-Trench et al., 1992) and downstream (Msx-1; van den Boogaard et al., 2000) of BMPs whom have been associated with human dysmorphogenesis. Since I showed that both gain-of-function and loss-of-function experiments induce cleft lip, numerous BMP

signaling molecules need to be added to the list of candidate genes that may cause human, non-syndromic orofacial clefting.

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