Fibroblast growth factor receptor 1 in craniofacial and midbrain-hindbrain development

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I **Trokovic N**, Trokovic R, Mai P and Partanen J. Fgfr1 regulates patterning of the pharyngeal region. Genes Dev. 2003 Jan 1;17(1):141-53.

II **Trokovic N**, Trokovic R and Partanen J. Fibroblast growth factor signalling and regional specification of the pharyngeal ectoderm. Manuscript, submited to IJDB Jan 2005.

III Trokovic R, **Trokovic N**, Hernesniemi S, Pirvola U, Vogt Weisenhorn DM, Rossant J, McMahon AP, Wurst W, Partanen J. FGFR1 is independently required in both developing mid- and hindbrain for sustained response to isthmic signals. EMBO J. 2003 Apr 15;22(8):1811-23.

ABBREVIATIONS

AA	Aortic arch
A-P	Antero-posterior
AP	Alkaline phosphatase
BA	Branchial arch
BMP	Bone morphogenetic protein
CRABP-1	Cellular retinoic acid-binding protein 1
Dlx	Distal-less homeobox gene
E	Embryonic day
Ect	Ectoderm
EN	Engrailed
End	Endoderm
FGF	Fibroblast growth factor
FGFR	FGF receptor
Hox	A particular subgroup of homeobox genes
IsO	Isthmic organizer
Mb	Midbrain
Mes	Mesoderm
MHB	Midbrain-hindbrain boundary
M	Mesoderm
NCC	Neural crest cells
NBS	Nile blue sulfate
NF	Neurofilament
mRNA	Messenger ribonucleic acid
Msx	Muscle segment homeobox gene
Ngn	Neurogenin
PAX	Paired-like homeobox gene
PC	Pharyngeal cleft
PCR	Polymerase chain reaction
PP	Pharyngeal pouch
R	Rhombomere
RA	Retinoic acid
RTK	Receptor tyrosine kinase
SEF	Similar expression to *Fgf* genes
Shh	Sonic hedgehog
SPRY	Sprouty
ss	Somite stage
TUNEL	Terminal deoxynucleotidyl transferase mediated nick labeling

ABSTRACT

Craniofacial development depends on dynamic tissue interactions and their co-ordinated integration into complex structures. Elements of lower face, pharynx as well as outer and middle ear, derive from six pairs of branchial arches located around the pharyngeal endoderm. Each branchial arch contains mesodermal core surrounded by neural crest cells, and is covered by endoderm from inside and ectoderm from outside. Despite their importance and evolutionary conservation, the early processes that coordinate development and integration of different arch components are still poorly understood.

Cranial neural crest cells originate adjacent to the neural ectoderm and migrate in three streams toward arches. Migratory pattern of the neural crest cells is coupled to the segmentation of the hindbrain into rhombomeres. Traditionally, it was thought that the fate of the neural crest cells, which give rise to the skeletal elements, is determined prior to their migration, and that they play an instrumental role in the branchial arch patterning. However, recent evidence has highlighted the importance of the neural-crest independent mechanisms of pharyngeal development.

Fibroblast growth factor (*Fgf*) signalling appears to be important for multiple tissue-interactions during pharyngeal development, both within and between different germ layers. The effects of FGFs are mediated by four tyrosine kinase-type receptors, fibroblast growth factor receptors 1-4 (FGFR1-4). In the pharyngeal region, *Fgfr1* is expressed in different cellular components of the branchial arches, and may thus play multiple roles during the development of the arches and their derivatives. Mouse embryos homozygous for a null mutation in the *Fgfr1* are unable to gastrulate normally and die during early gestation. In this work, hypomorphic (partial loss-of-function) and conditional alleles of the *Fgfr1* were used to study the role of *Fgf* signaling in the pharyngeal development. The present results show that *Fgfr1* is required for the entry of neural crest cells into the second branchial arch. *Fgf* signalling has been previously implicated with the regulation of the cell migration. However,

the detailed molecular mechanisms involved are largely unclear. In many studies, possible direct effects of FGFs on the migrating cells are at the centre of the focus Presented results show that in the pharyngeal region *Fgf* signalling regulates neural crest cell migration non-cell autonomously.

Proper patterning of the pharyngeal epithelium appears to be of great importance for the interaction and integration of different branchial arch tissue components. However, only very little is know about the mechanisms responsible for the correct localization of signalling centres in the epithelium. This study highlights the importance of the ectoderm in specification of the second branchial arch. It demonstrates that *Fgfr1* is required for correct patterning of the pharyngeal ectoderm and establishment of putative signalling centre in the surface ectoderm of the presumptive second branchial arch. This ectodermal domain appears also important for development of the geniculate placode giving rise to the VIIth cranial nerve.

Another important signalling centre in the developing head is the isthmic organizer, which has been shown to regulate development of both central nervous system and craniofacial region. FGFs are signaling molecules of the isthmic organizer, which regulate patterning and growth of the posterior midbrain and anterior hindbrain. Although a lot is known on the molecular properties of the isthmic organizer, very little is known about the mechanisms responsible for the maintenance of this signalling centre. This study shows that: FGFR1 is the primary FGF receptor receiving signals from the isthmic organizer; tissue specific inactivation of *Fgfr1* in the midbrain-hindbrain, results in developmental defects in both the midbrain and hindbrain; FGFR1 is independently required in both midbrain and hindbrain for the maintenance of isthmic organizer dependent gene expression. Taken together, present studies contribute to our understanding of head formation and reveal novel functions of FGF signalling in this process.

REVIEW OF THE LITERATURE

Developmental anatomy of banchial arches

Branchial arches are transient embryonic structures characteristic to all vertebrates. In mice, there are six pairs of branchial arches, which develop around the pharyngeal foregut. They arise in antero-posterior order during early somitogenesis, between 8 and 11 days of embryonic development in the mouse (E8-11), and are numbered in this order. Only first three arches can be well discriminated, while more posterior ones are less obvious (Fig. 1A). These bud like structures are covered with the surface ectoderm from outside and pharyngeal endoderm from inside (Fig. 1B). They contain a core of paraxial mesoderm surrounded by the neural crest cells (Lumsden et al., 1991; Noden, 1986; Noden, 1988; Trainor and Tam, 1995). Endodermal pharyngeal pouches and ectodermal pharyngeal clefts separate adjacent branchial arches in areas where endodermal and ectodermal cells are in direct contact (Graham, 2001). Pharyngeal pouches appear as localized, paired evaginations of the endoderm, just prior to branchial arch formation. At the same time, pharyngeal clefts form by paired invaginations of the surface ectoderm directly overlying the pharyngeal pouches (Fig. 1A and B). Pharyngeal pouches and clefts are formed in rostral to caudal sequence and are numbered according to preceding arch.

Each fully developed branchial arch contains the basic set of structures including aortic arch, nerve, supporting cartilage rod and muscular component (Kaufman, 1999). Aortic arches are the earliest and most prominent structures seen in each branchial arch. Bilaterally symmetrical system of aortic arches develops from mesoderm in a cranio-caudal sequence. Aortic arches pass through the middle of the branchial arch core (Fig. 1B). In the symmetrical arrangement of aortic arches, the outflow from the heart is a single vessel, the ventral aorta, from which 6 pairs of aortic arches pass dorsally on either side around the pharyngeal foregut, to unite with a pair of dorsal aortas. Caudal to the heart, the paired dorsal aortas unite to form a single dorsal aorta. Muscular components of the branchial arches derive from the mesodermal core around the aortic arch (Noden, 1983; Noden, 1986), while cartilage rods that form the

skeleton of each arch derive from the neural crest cells (Couly et al., 1993; Kontges and Lumsden, 1996).

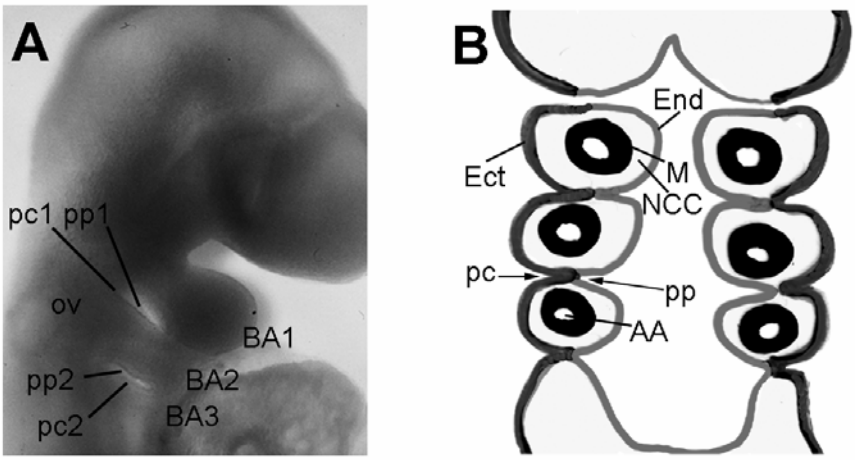

Figure 1. Branchial arches. A side view of the mouse embryo at E9.5 (A). Distinct structures in the pharyngeal region are indicated as: BA1-3, branchial arch 1-3; pc, pharyngeal cleft; pp, pharyngeal pouch; ov, otic vesicle. Schematic presentation of longitudinal section through the branchial arches (B). Aortic arches (AA), pharyngeal pouches and pharyngeal clefts, as well as branchial arch tissue components are indicated (Ect, ectoderm; End, endoderm; M, mesoderm; NCC, neural crest cells).

Cranial nerves have heterogeneous origins, arising from both neural crest cells and ectoderm. Each branichial arch is innervated by its own specific cranial nerve by E10.5. Trigeminal (Vth) nerve innervates the first arch, facial (VIIth) nerve innervates the second arch, vagal (IXth) nerve innervates the third arch and glossopharyngeal (Xth) nerve innervates the fourth arch (see Fig. 4). Cranial nerves consist of motor and sensory neurons. Motor neurons of the cranial nerves originate from the hindbrain and innervate branchial arch muscles. Sensory neurons of the cranial nerves are responsible for receiving and sending sensory information to the central nervous system. Vth sensory nerve is derived proximally from the neural crest and distally from several small placodes. VIIth, IXth and Xth sensory nerves are derived proximally from the neural crest cells and distally from the epibranchial placodes (D'Amico-Martel and Noden, 1983).

Epibranchial placodes are recognized as a series of ectodermal thickenings above pharyngeal clefts (Baker and Bronner-Fraser, 2001). They develop in antero-posterior sequence,

concomitantly with the branchial arch formation. The first epibranchial placode (geniculate) contributes to the VIIth nerve, the second epibranchial placode (petrosal) contributes to the IXth nerve, and the third epibranchial placode (nodose) contributes to the Xth nerve.

Evolutionary aspects of branchial arch development

The anatomy of the pharyngeal region is similar in all vertebrates during their embryonic development, but it gives rise to very different, specialized structures in the adult organisms (Radinsky, 1987). For example, all six pairs of aortic arches are formed in mammalian and avian embryos before the system eventually becomes simplified into the single aortic arch.

In jawless vertebrates (including lamprey and hagfish) pharyngeal region develops into gill apparatus which serve for gas exchange and filter feeding. In these organisms the embryonic arrangement of the pharyngeal region is retained throughout the life.

In jawed vertebrates, anterior portion of pharyngeal region, including the first and second branchial arch, is involved in the development of the upper and lower jaw and also gives rise to components of the middle ear apparatus (Graham, 2001). In fishes, the segmentally arranged gills form on either side of the pharynx from the third and more posterior arches. In reptiles, birds and mammals, in which lungs oxygenate the blood, pharyngeal region no longer serves a respiratory function. In these organisms, segmental arrangement of the pharyngeal region exists only during early embryogenesis, after which its constituents undergo significant modifications and further differentiation to form adult structures (Kaufman and Bard, 1999).

Branchial arch derivatives

Symmetrical arrangement of embryonic aortic arch system undergoes drastic changes in adult terrestrial vertebrates. Specific derivatives of each branchial arch with associated nerve and artery are presented in Table 1. Some structures disappear while others are strongly

reorganized. In mouse, the first and second aortic arches largely disappear, and their only derivatives are maxillary and stapedial artery respectively (Kaufman and Bard, 1999). The third arch arteries give rise to the distal part of the common carotid arteries. They also give rise to the proximal part of the internal and external carotid arteries. The terminal branches of the third arch arteries form the ophthalmic, the anterior and the middle cerebral arteries. The left fourth arch artery gives rise to the arch of aorta, and the right fourth arch artery gives rise to the brachiocephalic trunk and right subclavian artery. The fifth aortic arch is only transient structure, while the sixth aortic arch gives rise to the right and left pulmonary arteries, ductus arteriosus and pulmonary trunk.

Table1. Branchial arch (BA) derivatives

Germ layer	BA1	BA2	BA 3	BA 4 and 5
Neural crest derived skeleton	Skeleton of the maxillary arch: maxillae, palatine. pterygoid, jugal alisphenoid, incus, and squamosal Skeleton of the mandibular arch: dentary, malleus, gonial, tympanic	Stapes, styloid process, lesser horns of the hyoid and dorsoproximal hyoid body	greater horns of the hyoid, ventrodistal hyoid body	Laryngeal cartilages
Mesodermal aortic arches	Maxillary branch of carotid artery	Corticotympanic, stapedial artery	Common carotid artery	Arch of aorta subclavian artery
Mesodermal muscles	Masticatory and facial	Facial and stapedial	Stylopharyngeal and laryngeal	Pharyngeal
Nerve	Vth nerve	VIIth nerve	IXth nerve	Xth nerve
Pharyngeal cleft	External acoustic meatus and external ear	External acoustic meatus and external ear	Epithelium around ear	Epithelium around ear
Pharyngeal pouch	Middle ear, tym-panic membrane, Eustachian tube	Tonsilar clefts and crypts of palatine tonsil	Parathyroid and thumus	Ultimobranchial bodies (para-folicular cells)

Based on: Kaufman and Bard 1999.

The cartilages that develop in the pharyngeal arches from mesenchyme of neural crest origin serve the embryo as a temporary support. Some remain as cartilages, some degenerate after

bone is laid down intramembranously next to cartilage, and others are replaced by endochondral bone formation (Le Douarin and Kalcheim, 1999). Meckel's cartilage is the first arch cartilage. In primitive vertebrates, it gives rise to the bones of the upper and lower jaws. In mice, jaw bones are formed almost entirely intramembranously. Only few structures derive from the first arch cartilage including incus and malleus of the middle ear and alisphenoid, as well as the sphenomandibular ligament (Couly et al., 1993; Kontges and Lumsden, 1996; Mallo, 1998). The second arch cartilage, known as Reichert's cartilage, ossifies to form the stapes of the middle ear, the styloid process of the temporal bone, lesser horn and part of the body of the hyoid bone. It also gives rise to the styloid ligament. The third arch cartilage forms the greater horn and rest of the body of the hyoid bone, whereas the fourth and sixth arch cartilage gives rise to the laryngeal cartilages

Arch muscle components give rise to visceral muscles which are innervated by nerve fibres of specific cranial nerves. The principal arch-derived muscles are: masticatory, facial expression, pharyngeal and laryngeal muscles. Endoderm forms pharyngeal and middle ear epithelium and glandular structures including thymus, thyroid and parathyroid (Cordier and Haumont, 1980). Surface ectoderm forms epidermis and external acoustic meatus. The anterior most cleft transforms into the auditory Eustachian tube and middle ear chamber, whereas the other clefts disappear after making some important contributions to glands and lymphatic tissues in the throat region (Kaufman and Bard, 1999).

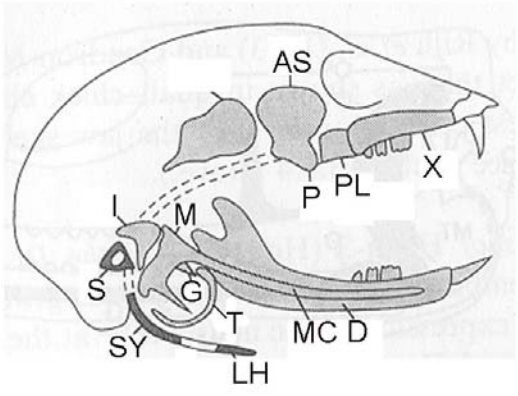

Figure 2. Skeletal derivatives of the first and second branchial arch. The first branchial arch skeletal derivatives (in grey) are indicated as: AS, alisphenoid; X, maxilla; PL, palatine; P, pterygoid; M, malleus; I, incus; G, gonial; T, tympanic ring; MC, Meckels cartilage; D, dentine. The second branchial arch skeletal derivatives (in black) are indicated as: S, stapes; SY, styloid process; LH, lesser horns of the hyoid body.

Origins and organization of the branchial arch primordial tissues

Segmentation of the pharyngeal region is already present in its primordial cells before becoming morphologically expressed in the branchial arches. Neural crest cells, which give rise to majority of skeletal structures in the head, were thought to coordinate formation of the pharyngeal region (see below). Therefore, previous studies mostly concentrated on these cells. Consequently, analysis of other arch constituents was usually performed in the context of their contribution to the neural crest development.

Neural crest cells

Neural crest is vertebrate-specific cell population, which evolved soon after the split of the cephalochordates (amphioxus) and vertebrates. Neural crest cells arise at the junction between the neural plate and the surface ectoderm (Bronner-Fraser, 1995). During the process of neurulation, these cells detach form the periphery of the neural plate and migrate throughout the embryo to generate numerous derivatives including pigment cells, autonomic and sensory ganglia, and most of the facial skeleton (Le Douarin and Kalcheim, 1999). The neural crest can be divided into four main, overlapping domains (cranial, trunk, vagal and sacral, and cardiac), each forming characteristic derivatives (Table2).

Table2. Fate of the neural crest cells along the rostro-caudal axis

Neural crest cells	Origin	Cell type or structure derived
Cranial	Fore-, mid-, and hindbrain	Connective tissues, smooth muscles, pericytes Bones and cartilage of the face and neck Cranial sensory neurons and glia
Cardiac	Somite 1-3	Muscular-connective tissue wall of large arteries Septum between the aorta and pulmonary artery
Vagal and sacral	Somite 1-7 Posterior to somite 28	Parasympathetic (enteric) ganglia of the gut
Trunk	Somite 6 through the tail	Dorsal root ganaglia, Schwann cells Sensory and sympathetic gangila Adrenomedullary cells and melanocytes

Based on: Le Douarin and Kalcheim, 1999.

Neurulation and induction of neural crest cells

The acquisition of neural fate by embryonic ectodermal cells involves signalling by FGFs and attenuation of the activity of bone morphogenetic protein (BMP). In chick embryo, FGFs from medial epiblast cells promote neural fate by repression of BMP (inhibitor of neural induction), and through another pathway independent of BMP repression. But FGFs, either alone or in combination with BMP antagonists, are not sufficient to induce neural fate in prospective epidermal ectoderm of amniote embryos. High levels of WNT signals in lateral epiblast cells block the response of epiblast cells to FGFs (Wilson and Edlund, 2001). This results in expression of *Bmps,* which promote epidermal fate and repress neural fate in lateral epiblast cells.

In mouse embryo, neuroectoderm is morphologically evident as the thickened neural plate on the dorsal surface of the embryo (Colas and Schoenwolf, 2001). Following its initial formation, the neural plate changes shape dramatically, and its lateral edges elevate to form the neural folds (Fig. 3). At E 8-8.5 the neural folds fuse along the dorsal midline forming the neural tube which separates from the surface ectoderm. The neural tube generates the brain and the spinal cord (Lumsden and Krumlauf, 1996). Before formation of the neural tube, anterior neuroectoderm becomes segmented into forebrain (prosencephalon), midbrain (mesencephalon) and hindbrain (rhombencephalon). The forebrain is divided into telencephalon and diencephalon, and the hindbrain is further subdivided into eight rhombomeres (numbered from 1 to 8).

The neural crest cells are induced at the stage when lateral edges of the neural plate elevate to form the neural folds (Fig. 3). Inductive interactions between the neural and non neural ectoderm (Liem et al., 1995; Selleck and Bronner-Fraser, 1995) as well as signalling from the mesoderm (Bonstein et al., 1998; Marchant et al., 1998), are critical for the neural crest formation. Inductive signals include members of the *Bmp*, *Fgf*, and *Wnt* signalling molecule families (Ikeya et al., 1997; Kanzler et al., 2000; Knecht and Bronner-Fraser, 2002; Marchant

et al., 1998; Mayor et al., 1997). They triger expression of the early neural crest markers such as *Slug*/*Snail*, *Sox9*, *Id2*, and members of the *FoxD*, *Hox* and *Zic* families (Gavalas et al., 2001; LaBonne and Bronner-Fraser, 2000; Nakata et al., 1998; Nieto et al., 1994; Spokony et al., 2002). However, the precise function and pathways in which these molecules are involved, are still not known.

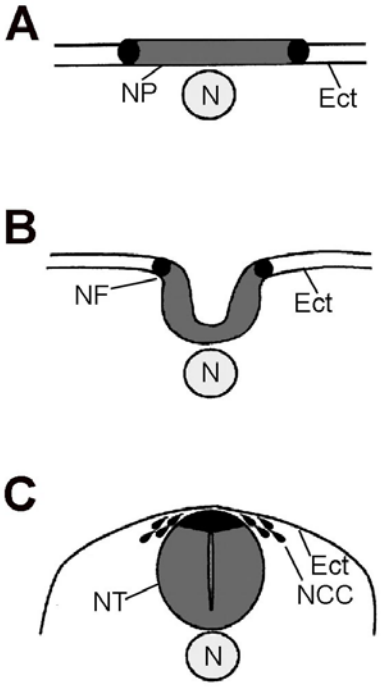

Figure 3. Neurulation and the neural crest cell induction. (A) Neural crest cell precursors (in black) between the surface ectoderm (Ect, in white) and the neural plate (NP, in grey). Notochord (N) underlines the neural plate. (B) Formation of the neural folds (NF). (C) Separation of the neural tube (NT) from the surface ectoderm and onset of neural crest cell migration (NCC).

Initiation of the neural crest cell migration

Prior to and during the neural tube closure, neural crest cells undergo epithelial to mesenchymal transition resulting in delamination and onset of migration. In order to become mesenchymal, the epithelial cells must change their shape and adhesive properties. This involves downregulation of *N-cadherin* and upregulation of *cadherin-11*, both of which code for membrane-bound proteins that mediate cell-to-cell interactions. RhoB, a small GTPase that regulates cell shape and adhesion, is also required for epithelial-mesenchymal transition of the neural crest cells (Liu and Jessell, 1998). Additionally, the transcription factor *Snail*, was implicated in induction of the neural crest delamination through repression of *E-cadherin* and upregulation of *RhoB* (Cano et al., 2000). Recent study by Zhou et al. showed that *Snail* is negatively regulated by GSK-3b (Zhou et al., 2004). They suggest that signals such as

MAPK and WNT inhibit GSK-3b, and thus in turn result in upregualtin of *Snail* and downregulation of *E-cadherin* expression.

Migratory pathways and fates of the cranial neural crest cells originating from the posterior midbrain and hindbrain

Migration of the cranial neural crest starts at the four-somite stage and is completed by the 16-somite stage. First cells migrate from the anterior hindbrain, followed by migration from the midbrain, caudal hindbrain, and diencephalon of the forebrain (Serbedzija et al., 1992). Neural crest cells populating branchial arches derive from the posterior midbrain and hindbrain (Fig. 4). They migrate ventrally in three major streams toward the arches (Birgbauer et al., 1995; Chai et al., 2000; Lumsden et al., 1991; Sechrist et al., 1993a; Trainor et al., 2002). The stream of the neural crest arising from the posterior midbrain and rhombomeres one and two, contribute to the first arch and ganglia of the Vth (trigeminal) nerve. The stream arising from rhombomere four, contribute to the second (hyoid) arch and ganglia of the VIIth (facial) nerve. The stream arising from rhombomeres six and seven, contribute to the third, fourth, and sixth branchial arches, as well as to the ganglia of the IXth (glossopharyngeal) and Xth (vagus) nerves.

No migratory neural crest cells are observed laterally to the rhombomere 3 and 5. These neural crest free zones separate adjacent crest streams. Studies in chick embryos, suggested that the majority of the neural crest cells produced by rhombomeres 3 and 5, are lost by means of apoptosis (Graham et al., 1993). However, other studies in chick and mouse, showed that DiI labelled neural crest cells from rhombomeres 3 and 5 move anteriorly and posteriorly to join the neural crest streams migrating from the adjacent even-numbered rhombomeres (Birgbauer et al., 1995; Kulesa and Fraser, 2000; Sechrist et al., 1993a; Trainor et al., 2002). These results were further confirmed by quail-chick (Couly et al., 1996; Kontges and Lumsden, 1996) and mouse-chick (Trainor et al., 2002) chimeric studies. In addition, analysis of the neural crest cell migration revealed that final destination of the neural crest

cells also depends on the timing of emigration from the neural plate (Lumsden et al., 1991; Serbedzija et al., 1992). Thus, earlier migrating neural crest cells settle more ventrally and populate the branchial arches, whereas the later migrating crest cells settle more dorsally, in the region of cranial nerve formation.

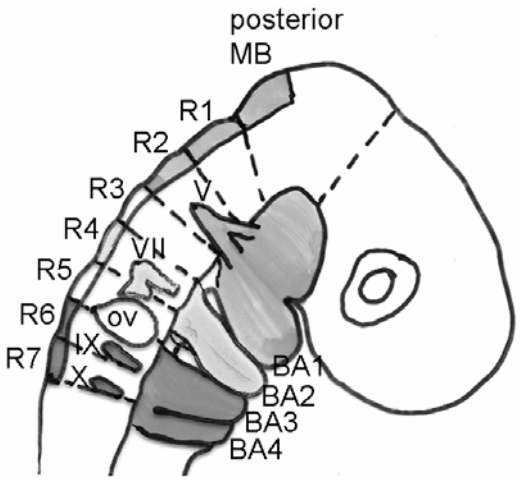

Figure 4. Origins and migratory streams of the branchial arch neural crest cells. Schematic presentation of the mouse embryo at E9.5. Neural crest cells originating from the posterior midbrain (post MB), rhombomere 1 and 2 (R1 and R2), contribute to the Vth cranial nerve and populate the first branchial arch (BA1). Neural crest cells originating from the rhombomere 4 (R4), contribute to the VIIth cranial nerve and populate the second branchial arch (BA2). Neural crest cells originating from the rhombomere 6 and 7 (R6 and R7), contribute to the IXthe and Xth cranial nerves and populate the third and fourth branchial arches (BA3 and 4). For the simplicity, migration of neural crest cells originating from rhombomeres 3 and 5 (R3 and 5) is not presented. Ov, otic vesicle.

Paraxial mesoderm

Mesoderm is generated during gastrulation, from the epiblast cells migrating through the primitive streak. Initially embryonic mesoderm becomes divided into three components, paraxial, intermediate and lateral. Cranial paraxial mesoderm is transiently segmented into seven loose aggregates, somitomeres, as observed by scaning electron microscopy (Meier and Tam, 1982; Meier and Tam, 1982). Cells of the somitomeres contribute to the branchial arches. On its migratory route from somitomeres toward branchial arches, paraxial mesoderm is co-distributed with the neural crest cells originating at the same level along the antero-posterior axis (Trainor and Tam, 1995). As revealed by cell labelling, somitomeres II and III contribute to the first branchial arch, somitomeres IV and V contribute to the second branchial arch, and somitomeres VI and VII contribute to the third branchial arch (Trainor et al., 1994).

Genes known to be expressed in the cranial paraxial mesoderm include *Mox1* (Candia et al., 1992) and *M-twist* (Wolf et al., 1991). However, these markers do not show regionally restricted expression pattern that would reflect somitomeric organization, and the existence of segmentation in the cranial mesoderm therefore remains unclear.

Ectoderm

Similar to the hindbrain, early pharyngeal surface ectoderm has been suggested to be segregated along antero-posterior axes into territories, called ectomeres (Couly et al., 1990). However, it has not yet been demonstrated that each ectomere represents a functional developmental unit and only few molecules, including *Fgf3* (Mahmood et al., 1995), were shown to be locally expressed in distinct antero-posterior stripes of the surface ectoderm prior to the branchial arch formation. Lack of the early regional markers and insufficient knowledge on origins and distribution of the pharyngeal surface ectoderm are significantly limiting our understanding of its development and function.

Endoderm

Endoderm as a layer develops during gastrulation. Initially, it gives rise to flattened sheet of the primitive gut, divided along antero-posterior axis into fore-, mid-, and hindgut (Grapin-Botton and Melton, 2000; Tam et al., 2003). Prior to the formation of the first somites, the foregut endoderm can be divided into three longitudinal zones, a medial, intermediate, and lateral zone. The medial zone gives rise to the gut roof, the intermediate zone forms the pharyngeal pouches, and the gut floor, while laterally located cells give rise to the extra embryonic endoderm. Beginning at 4 somite stage, the foregut becomes dorso-ventrally flattened. At this time, segmentally organised pharyngeal pouches form by localized invaginations of the pharyngeal endoderm at sites between presumptive branchial arches. Couly et al. showed that antero-posterior pattern within the pharyngeal endoderm are already determined at the early neurula

stage, well before the branchial pouches are formed (Couly et al., 2002). By using a microsurgical approach, defined fragments of the endoderm covering the cephalic region of the five-somite stage avian neurula were either removed or ectopically transplanted. The results of this study elegantly demonstrated that shape, proximodistal and anteroposterior polarity of the skeleton are dictated by the endoderm already at the early neurula stage. However, prior to formation of pharyngeal pouches, known markers for the early pharyngeal endoderm such as *Fgf8* (Crossley and Martin, 1998), *Pax1* (Muller et al., 1996) and *Bmp7* (Solloway et al., 1999) are expressed throughout the region. Thus, similar to the paraxial mesoderm and surface ectoderm, one difficulty with assessing early segmentation of the pharyngeal endoderm is the lack of specific regional markers.

Tissue interactions regulating neural crest segregation

Close correlation between rhombomeric organisation of the hindbrain and the patterns of the cranial neural crest cell migration has been revealed by cell labelling studies (Couly et al., 1992; Kontges and Lumsden, 1996; Lumsden et al., 1991; Sechrist et al., 1993a; Serbedzija et al., 1992). Thus, similar to the neural crest cell identity, it has been proposed that the pattern of their migration is determined before emigration form the neural tube. Along this line, Graham et al. suggested that hindbrain is responsible for establishment of the neural crest cell-free zones lateral to the rhombomeres 3 and 5 (Graham et al., 1993). They showed that signalling from the neighbouring rhombomeres induced apoptotic cell death in rhombomeres 3 and 5 through induction of *Bmp-4* and *Msx-2*. Their conclusion was that the lack of the neural crest cell generation from the rhombomeres 3 and 5 resulted in formation of the crest streams.

In contrast, observations that all rhombomeres give rise to the neural crest cells and that those from the rhombomeres 3 and 5 migrate in anterior and posterior directions, joining adjacent neural crest streams, raised the possibility that extrinsic factors play important role in the patterining of the crest cells (Birgbauer et al., 1995; Kulesa and Fraser, 1998; Sechrist et al., 1993a; Trainor et al., 2002). Accordingly, Farlie et al. proposed that regions of non-permissive mesenchyme, also called paraxial exclusion zones, inhibit neural crest migration adjacent to rhombomeres 3 and 5 (Farlie et al., 1999). Furthermore, rhombomeres 3 and 5 were suggested to be the source of these inhibitory cues (Eickholt et al., 1999). This study indicate that signalling molecule *Semaphorin-3A,* which is expressed in rhombomeres 3 and 5, and released into the adjacent mesenchume, inhibits migration of the neural crest cells between the streams. In addition, receptor tyrosine kinase *ErbB4*, expressed in rhombomeres 3 and 5, was shown to be required for establishment of the repulsive cues in the mesoderm adjacent to rhombomere 3 (Golding et al., 2000; Golding et al., 2002). Same group demonstrated that signaling from the surface ectoderm overlying rhombomere 5 maintains neural crest free zone adjacent to this segment (Golding et al., 2004).

Importantly, the time-lapse analysis suggested that the interactions between the neural crest cells are important in guidance (Kulesa and Fraser, 1998; Kulesa and Fraser, 2000). They demonstrated ability of the crest cells to migrate as individuals or as groups. Observation that some of these cells cross between adjacent streams, suggested that crest free areas, established in close proximity to the hindbrain, must be continually maintained on their migration pathway toward the branchial arches. Accordingly, *Ephrins* and their receptors (Smith et al., 1997), *Fgf2* (Kubota and Ito, 2000) and an uncharacterised chemoattractant released from the otic vesicle (Sechrist et al., 1994b) were shown to be involved in maintaining the segregation of the neural crest cell streams at the level of the branchial arches. In xenopus embryos, *ephirn B2* and its receptors *EphB1* and *EphA4* are expressed in adjacent neural crest streams and underlying mesoderm. Over-expression of either dominant-negative *Eph* receptors or wild-type *EphirnB2* caused aberrant migration of the neural crest cells, presumably because

the expression of repulsive guidance cues had been altered (Smith et al., 1997). Importantly, *Eph* receptor/*Ephrin* families were implicated in sorting mechanisms in the hindbrain, providing a key means of sharpening rhombomere boundaries already prior to neural crest cell migration (Mellitzer et al., 1999; Mellitzer et al., 1999). Kubota et al. showed *in vitro* that FGF-2 has chemotactic activity for mouse neural crest cells deriving from the midbrain (Kubota and Ito, 2000). This is one of the rare examples of the chemoattractant shown to guide migration of the branchial arch neural crest cells. Additionally, conbination of rotation and dye labelling studies demonstrated that the otic vesicle displays attractive properties during crest cell migration (Sechrist et al., 1994b). However, the nature of the otic vesicle derived chamoattractant has remained uncharacterised.

Several studies showed that tissue interactions and mechanical barriers imposed by ectoderm and endoderm may be critical for the neural crest migration in streams. For example, the otic vesicle has been proposed to represent a mechanical barrier to neural crest cells migrating from rhombomere 5 (Anderson and Meier, 1981). Another study showed that the segmental organization of the pharyngeal pouches directs neural crest cell streams into separate branchial arches (Piotrowski and Nusslein-Volhard, 2000). They demonstrated that in the van gogh (*Vgo*) mutant in zebrafish, where segmentation of the pharyngeal endoderm was absent, migrating neural crest cells initially formed distinct streams, but they fused after reaching the arches. Inhibition of *RA* signalling in the head-fold mouse embryos, results in altered morphology of the second and third pharyngeal pouches (Wendling et al., 2000). The authors suggest that this endodermal defect could impose a mechanical barrier on the neural crest cells which fail to populate the third and fourth branchial arches in the mutant embryos. Recent study by Cerny et al. demonstrated that infolding of the surface ectoderm create channels for the neural crest migration (Cerny et al., 2004). They showed that ablation of the cranial epidermis in axolotl causes fusion of the neural crest streams followed by cessation of the neural crest migration.

Taken together, these findings show that neural crest cell streaming toward the brancial arches is a result of dynamic cell- and tissue-interactions along antero-posterior and dorso-ventral axis of their migration routes. Furthermore they show that different mechanisms, including inherent information from the hindbrain, attractive and repulsive cues, signalling regulating cell survival, as well as mechanical constrains, are involved in this process.

Mechanisms of patterning in the mid- and hindbrain and the branchial arches

Early development of mid- and hindbrain

The neural crest cells which populate the branchial arches originate from and are partially patterned by the posterior mid- and hindbrain (see below). Therefore understanding the early development of the posterior mid- and hindbrain is crucial for understanding formation of the pharyngeal region.

Patterning of the hindbrain

Rhombomeric segmentation of the hindbrain is established first at the molecular level, followed by the appearance of the morphological constrictions between rhombomeres (in the period of 6-12-somite stage). Rhombomere 1 (metencephalon) is adjacent to the midbrain, while rhombomere 8 is continuous with the spinal cord. Later during development, rhombomere 1 will give rise to the cerebellum involved in processes such as motor coordination, while more posterior region of hindbrain (myelencephalon) will become the medulla oblongata.

The first genes whose expression was shown to be segmentally regulated in the hindbrain were the *Hox* genes (Wilkinson et al., 1989), the evolutionarily conserved regulators of segment identity. Four clusters of *Hox* genes (denoted a-d) are found within the vertebrate genome, on individual chromosomes. Expression of genes at the 3' ends of the *Hox* clusters precedes rhombomere formation and becomes progressively restricted such that expression boundaries coincide with the interfaces between rhombomeres. Thus, each rhombomere has

unique combination of *Hox* genes. Only exception is rhombomere 1 where *Hox* expression is missing (Fig. 5). *Hox* genes were shown to be important for establishing hindbrain segmentation, as well as specification of antero-posterior rhombomeric identities. For example, *Hoxa2,* the most anterior *Hox* gene expressed up to the rhombomere 1/2 boundary (Prince and Lumsden, 1994), was shown to be important for the specification of rhombomere 2 and 3 identity (Gavalas et al., 1997). *Hoxb1* and *Hoxb2* expressed in rhombomere 4 and rhombomere 3-7 respectively were shown to be important for specification of rhombomere 4 identity (Barrow and Capecchi, 1996; Goddard et al., 1996; Studer et al., 1996).

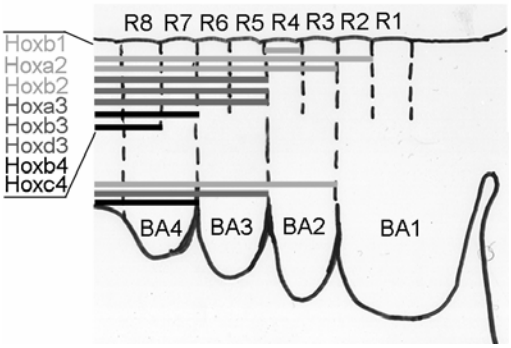

Figure 5. *Hox* gene expressions within specific rhombomeres and branchial arches in the mouse embryo at E10. Schematic presentation of rhombomeres 1-8 (R1-8) and branchial arches 1-4 (BA1-4). Correlations between *Hox* gene expression patterns in rhombomeres and branchial arches are indicated in greyscale bars.

A number of other genes were demonstrated to have expression patterns coinciding with the rhombomere boundaries. These genes fall into various categories, including transcription factors, transmembrane proteins, secreted proteins and intracellular proteins. Majority of these genes were shown to be involved in a regulatory cascade controlling hindbrain segmentation. For example ephrin receptor *EphA4*, expressed in rhombomere 3 and 5, is important for restricting cell mixing between adjacent rhombomeres (Gale et al., 1996; Smith et al., 1997). Transcription factor *Krox20*, expressed in rhombomere 3 and 5, was shown to regulate gene expression (Theil et al., 1998) and formation of these rhombomeres (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Wilkinson et al., 1989). In zebrafish, *Fgf 3* and *Fgf8* signalling from rhombomere 4 is required to establish correct segmental identity throughout the hindbrain (Maves et al., 2002; Walshe et al., 2002; Waskiewicz et al., 2002). Spatiotemporal studies of *Fgf* expression suggest that this patterning mechanism is conserved

during hindbrain development in other vertebrate classes. These studies suggest that rhombomere 4 domain of *Fgf* expression may play a role similar to other secondary centres involving *Fgf* signalling, such as the isthmic organizer (Irving and Mason, 2000).

Patterning of the posterior midbrain and rhombomere 1: the role of the isthmic organizer

Signalling centres have fundamental roles for regulation of embryonic patterning. They influence behaviour of neighbouring cells through transient and localized expression of signalling molecules. Local antero-posterior pattern of the midbrain and rhombomere 1 is generated within an unsegmented field by the graded signal from the mid- and hindbrain boundary, named isthmic organizer (Bally-Cuif et al., 1992; Martinez et al., 1991a). Tissue grafting studies first first identified the isthmus as an organizing centre, a source of a signal sufficient to induce cells in the anterior neural tube to change their fate. When transplanted into the forebrain or anterior midbrain the isthmus induced the surrounding cells to form posterior midbrain structures, and when transplanted into posterior hindbrain, it induced cerebellar differentiation characteristic for the rhombomere 1 (Gardner and Barald, 1991; Marin and Puelles, 1994; Martinez and Alvarado-Mallart, 1990; Martinez et al., 1991a; Martinez et al., 1995b). Under the control of the isthmic organizer, midbrain develops into superior and inferior colliculi, relaying visual and auditory stimuli, respectively, while rhombomere 1 forms the cerebellum (Irving and Mason, 2000; Wingate and Hatten, 1999).

Specification of the midbrain and anterior hindbrain requires expression of *Otx2* and *Gbx2* in the prospective midbrain and anterior hindbrain respectively (Broccoli et al., 1999; Millet et al., 1996; Millet et al., 1999). The border of *Otx2* and *Gbx2* expression determines the position of the isthmus (reviewed in Rhinn and Brand 2001). Subsequently, signals from the isthmic organizer are refining expression patterns of genes in the midbrain and anterior hindbrain, including expression of transcription factors *Gbx2* (Shamim et al., 1998), *Otx2* (Millet et al., 1996), *En1/2* (Davis et al., 1988) and *Pax2/5* (Puschel et al., 1992; Adams et al., 1992) (summarized in Fig6 and Joyner et al., 1996).

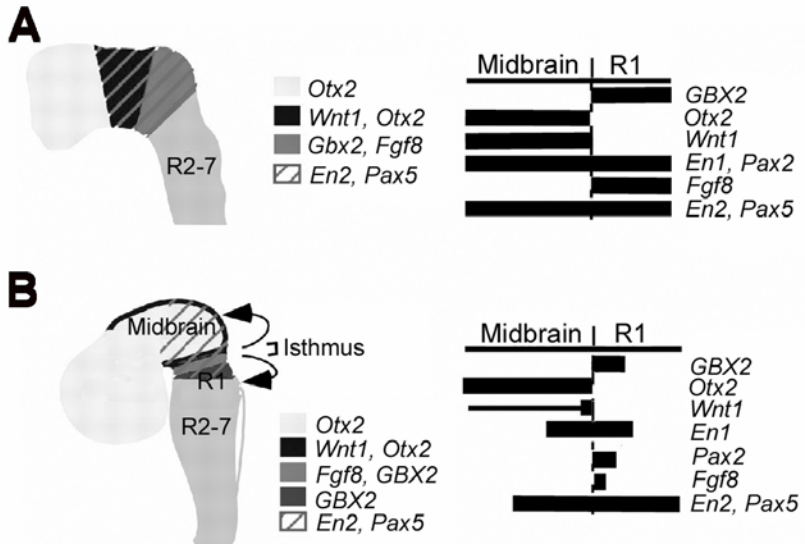

Figure 6. Early patterning in the mid- and hindbrain region. Schematic (left) and graphic (right) presentations of gene expression patterns at 5-somite stage (A) and E9.5 (B). Midbrain, isthmus and rhombomeres 1-7 (R1-7) are indicated.

Adapted from Joyner et al., 1996.

Among several signalling molecules secreted by the isthmic organizer, in particular FGF8 and WNT-1 have been implicated in the control of the mid- and hindbrain patterning (Chi et al., 2003; McMahon and Bradley, 1990; Meyers et al., 1998; Reifers et al., 1998). Initially, *Fgf8* and *Wnt1* are expressed broadly in regions of the rhombomere 1 and midbrain, respectively. By E9.5, *Fgf-8* is expressed in a ring of cells at the isthmus, the constriction between the mesencephalic vesicle, and rhombomere 1, while *Wnt-1* is expressed in a ring of cells immediately rostral to *Fgf-8* and along the dorsal midline (Fig. 6). *Fgf8* expression at the mid- and hindbrain boundary is conserved in all vertebrate classes (Christen and Slack, 1997; Heikinheimo et al., 1994; Ohuchi et al., 1994; Irving and Mason, 1999). Ectopic application of FGF8 protein can alter the regional identity of anterior midbrain and posterior hindbrain, mimicking the effects of isthmic tissue (Crossley et al., 1996; Irving and Mason, 2000; Martinez et al., 1999; Irving and Mason, 2000; Martinez et al., 1999). Null mutations of *Fgf8* in mice are embyonic lethal due to gastrulation defects. However, hypomorphic *Fgf8* mutants reveal that *Fgf8* is an essential component of the isthmus organizer, required for both cerebellum and posterior midbrain structures (Meyers et al., 1998). Similarly, zebrafish mutants in which *Fgf8* is either partially of completely inactivated also lack a cerebellum, isthmus and posterior midbrain structures (Reifers et al., 1998).

The molecular mechanism by which isthmic organizer patterns the anterior hindbrain is starting to be understood (Irving and Mason, 2000). Rhombomere 1 is the only hindbrain segment in which no *Hox* genes are expressed and it is located in the region where two distinct patterning mechanisms confront: graded signalling from the isthmus and segmentation of the hindbrain (Lumsden and Krumlauf, 1996). It has been shown that the isthmus establishes the anterior limit of *Hox* gene expression in the hindbrain and thus positions the boundary between rhombomere1 and 2 (Irving and Mason, 2000). At the molecular level it has been demonstrated that *Fgf8* from the isthmus provides a repressive signal that establishes the anterior limit of *Hoxa2* gene at the rhombomere1/2 border.

Positional identities in branchial arches

Each branchial arch within the series is a distinct unit with its own identity (inter-branchial arch identity). In addition, each arch also has sense of its own anteroposterior and dorsoventral axis (intra-branchial arch identity). Initially, positional identities of branchial arches are characterized by distinct gene expressions. For example, *Hox* genes are differentially expressed in between branchial arches, specifying inter-branchial arch identity, while *Dlx* genes are differentially expressed along dorso-ventral axis of individual branchial arches, specifying intra-branchial arch identity (Depew et al., 1999; Qiu et al., 1995; Qiu et al., 1997). Later during development, branchial arch positional identities are reflected in the formation of specific derivatives at specific sites. Consequently an important question is, which tissue posses patterning information and imposes it on other branchial arch tissue components?

Neural crest cell patterning

Prepatterning

Early studies suggested that the neural crest cells are the major players in

branchial arch patterning. According to their place of origin and time of migration, the neural crest cells follow specific migratory pathways (Birgbauer et al., 1995; Lumsden et al., 1991; Sechrist et al., 1993a; Trainor et al., 2002) and give rise to specific cranio-facial skeletal structures (Table 1), (Couly et al., 1996; Kontges and Lumsden, 1996). Chimeric grafting studies showed that after transplantations of the neural tube ectopically located neural crest cells retain identity associated with their position of origin (Kuratani and Eichele, 1993; Noden, 1983; Prince and Lumsden, 1994; Simon et al., 1995). For example, when the anterior hindbrain giving rise to the first arch neural crest cells was grafted to the rhombomere 4 teritory, emerging neural crest cells migrated into the second arch but formed first arch skeletal components (Noden, 1983). These results indicated that fates of the branchial arch neural crest cells are fixed already before their migration from the hindbrain. Moreover, Noden at al. showed that identity of the mesodermaly derived craniofacial muscles depends on the co-migrating neural crest cells, further indicating that they are the major player in arch patterning (Noden, 1986).

Observation that the neural crest cells in branchial arches express *Hox* genes characteristic for their rhombomeric origin (Fig. 5), provided clue on the molecular mechanism of the neural crest and branchial arch patterning (Hunt et al., 1991; Wilkinson et al., 1989). It was proposed that the segmental pattern of the hindbrain, encoded by *Hox* genes, is acquired and then transmitted by the neural crest cells to the branchial arches and cranial ganglia (Hunt et al., 1991). Even after grafting to a new position along the antero-posterior axis, neural crest cells were shown to retain their original set of *Hox* genes (Couly et al., 1996; Couly et al., 1998; Guthrie et al., 1992; Prince and Lumsden, 1994). Further evidence for neural crest pre-patterning and its crucial role in the arch patterning come from the analysis of *Hoxa2* mutant mice (Gendron-Maguire et al., 1993; Grammatopoulos et al., 2000; Pasqualetti et al., 2000; Rijli et al., 1993). *Hoxa2* is expressed in the neural crest of the second and more caudal arches, as well as in their rhombomeric precursors. Subsequent expression of *Hoxa2* in the surface ectoderm was thought to be imposed by the neural crest cells (Hunt et al., 1991).

Inactivation of *Hoxa2* resulted in homeotic transformations of the second arch skeletal elements into the first arch elements (Gendron-Maguire et al., 1993; Rijli et al., 1993). Consistent with this, ectopic expression of Hoxa2 in the first branchial arch resulted in its transformation into second branchial arch (Grammatopoulos et al., 2000; Pasqualetti et al., 2000). This led to the conclusion that *Hoxa2* is a selector gene that determines the fate of the pre-migratory neural crest cells destined to populate the second arch.

Plasticity

Although it was generally accepted that the neural crest cells are predetermined, some early experiments provided clues on their plasticity. For example, Noden's transplantations from the midbrain to the rhombomere 4 never resulted in skeletal structures deriving from the midbrain crest cells. Instead, they resulted in the skeletal structures deriving from the rhombomere 1 and 2 (Noden, 1983). Although neglected, this was important observation, revealing that the neural crest cells are able to change their fate. Similarly, neural fold ablations implied neural crest plasticity. For example, after ablation of rhombomere 4 neural crest, the first arch-derived crest cells repopulated the second arch and formed normal second arch skeletal structures (Couly et al., 1996). Furthermore, early and late migrating crest cells have been shown to have equivalent potential, forming structures appropriate to their new environment when transplanted into older or younger hosts (Baker et al., 1997). Accordingly, recent study demonstrated the ability of trunk neural crest cells to differentiate into skeletal structures under appropriate conditions (McGonnell and Graham, 2002).

Several studies on correlation between *Hox* expression and neural crest plasticity, also argued against idea that neural crest fate is fixed before delamination. For example, *Hoxa2* was shown to be expressed in rhombomere 2 but not in neural crest cells derived from rhombomere 2 (Prince and Lumsden, 1994). Furthermore, it was demonstrated that *Hox* gene expression is independently regulated in the hindbrain and migrating neural crest cells. In rhombomeres 3 and 5 *Hoxa2* was shown to be regulated by *Krox20* (Nonchev et al., 1996),

and in branchial arches by *AP-2* (Maconochie et al., 1999). Consistant wiht this, Mallo and Brändlin (1997) showed that the *Hoxa2* null mutants, in which the second arch neural crest assumes first arch identities, retain a normal neuronal organization of the hindbrain. This strongly argues against neural crest cell prepatterning theory and suggests that local signals from environment are required for patterning of the neural crest cells in branchial arches.

The final proof for the neural crest plasticity came from grafting experiments in mice (Trainor and Krumlauf, 2000) and zebrafish embryos (Schilling et al., 2001). These studies revealed that the neural crest cells can change their *Hox* gene expression in the new environment, after grafting small groups of cells from the rhombencephalic levels. Consistently, Golding et al. showed that mis-migrated neural crest cells would change their *Hox* code in accordance with the new environment (Golding et al., 2000). Moreover, maintenance of normal *Hox* gene expression in the neural crest cells was demonstrated to require signalling from the paraxial mesoderm (Trainor and Krumlauf, 2000). They performed transplantation experiments from the second to the first branchial arch, and showed that the second arch neural crest cells retain *Hoxb1* expression only when transplanted in combination with the second arch mesoderm.
All together these results suggest that the neural crest cells are not irreversibly committed before migration into the arches. Furthermore, they demonstrate responsiveness of the neural crest cells to the patterning cues from environment and highlight the importance of interactions between the neural crest cells.

Early branchial arch patterning does not depend on the neural crest cells

Observation that the surface ectoderm overlying the second branchial arch still turns on its normal *Hox* gene expression, after the *Hox* expressing crest was replaced with the non-*Hox*-expressing crest (Couly et al., 1998), argued against studies highlighting the importance of the neural crest cells in the branchial arch patterning. Consistent with this, it was shown that the first arch skeletal derivatives may be transformed into the second arch derivatives only after

global expression of *Hoxa2* in the neural crest cells and surrounding tissues (Grammatopoulos et al., 2000; Pasqualetti et al., 2000).

Ablation studies in the chick by Veitch et al. were the further proof that branchial arches are not dependent upon the neural crest for their formation and patterning (Veitch et al., 1999). They demonstrated that morphologies of branchial arches, pharyngeal pouches and clefts appear normal after neural crest ablation. Furthermore, using regional molecular markers for the pharyngeal epithelium, they showed that the early branchial arch patterning is normal in the absence of the neural crest cells. This was deduced from the observation that the expression of genetic markers such as *Bmp7*, *Fgf8* and *Pax1* occurs in the same regions of the pouch endoderm whether neural crest cells immigrate or not. In concordance with this, in *Hoxa1* and *Hoxb1* compound knock-out mutants, where generation of rhombomere 4 neural crest cells is impaired, the second branchial arch forms and is patterned normally (Gavalas et al., 2001). Importantly, these findings are in accordance with the evolutionary studies according to which, pharyngeal segmentation is characteristic for chordates, while neural crest cells are characteristic only for craniates (Radinsky, 1987).

Inductive tissue interactions in the pharyngeal region, sources of patterning information

Development of branchial arches is regulated at multiple levels. Several epithelial signalling centres indicated in regulation of early pharyngeal patterning include: isthmus at the border of mid- and hindbrain, pharyngeal endoderm, pharyngeal pouches and clefts. These organizing centres are acting reiteratively. First, signalling from the isthmus and then pharyngeal endoderm are required for the early specification of the neural crest cells. Subsequently, signalling from the pharyngeal pouches and clefts is required for further patterning inside the branchial arches.

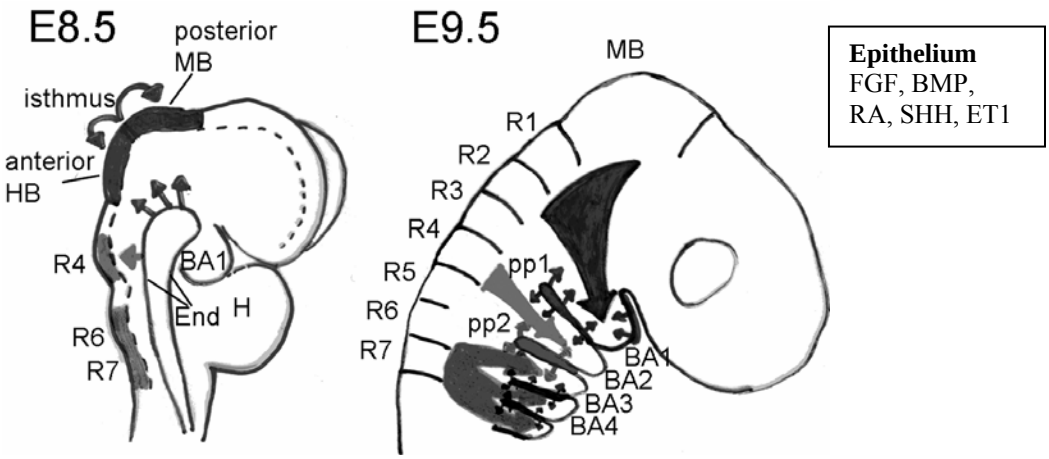

Figure7. Patterning of the branchial arches. In E8.5 mouse embryos, Isthmus, early pharyngeal endoderm and surface ectoderm (not marked) act as important sources of signalling molecules. Signalling from isthmus and endoderm is indicated by arrows. With the formation of branchial arches at E9.5 majority of signalling molecules in pharyngeal endoderm and surface ectoderm become restricted to pharyngeal pouches and clefts (small arrows). Distinct streams of neural crest cells are marked by big arrows. Some of the signalling molecules secreted by pharyngeal epithelium are indicated on the right.

Isthmic organizer

Molecular mechanism by which neuroepithlium patterns the first arch neural crest precursors was recently revealed (Irving and Mason, 1999; Trainor et al., 2002). Similar to the patterning mechanism of the rhombomere 1 (Irving and Mason, 1999), Trainor et al. showed that isthmic organizer represses expression of *Hoxa2* in the first arch neural crest cells and that this activity is mediated by *Fgf8* signalling molecule (Trainor et al., 2002). They demonstrated that presence of isthmus or *Fgf8* in the rhombomere 4 teritory will down-regulate *Hoxa2* expression in the rhombomere 4 neural crest cells and thus respecify their fate. Transplantation of isthmus together with rhombomere 1 possibly explains the results by Noden discussed above (page 29). Contradictory to these results, mutant mice in which *Fgf8* expression was eliminated in the mid- and hindbrian by the 10 somite stage have apparently normal craniofacial development (Chi et al., 2003). Thus, the function of *Fgf8* in the regulation of *Hoxa2* must be very early.

Pharyngeal endoderm

It is likely that endoderm has a primary role in patterning of the pharyngeal region. Molecular defects in the early pharyngeal endoderm of mouse embryos with impaired *RA* signalling, where suggested to be responsible for the lack of the third and more posterior branchial arches in these mutants (Quinlan et al., 2002; Wendling et al., 2000). In zebrafish *Van Gogh* (*Vgo*) mutants both pharyngeal endoderm and skeletal patterning are disrupted, although the neural tube patterning is normal (Piotrowski and Nusslein-Volhard, 2000). *Vgo* was found to act cell autonomously in the pharyngeal endoderm and influence development of the neural crest secondarily (Piotrowski et al., 2003). Importantly, this study demonstrated that *Vgo* corresponds to *Tbx1* transcription factor in mammals (Piotrowski et al., 2003). Mice heterozygous for the *Tbx1* null allele exhibit cardiovascular and craniofacial defects resembling those in DiGeorge syndrome, while *Tbx1* null mice display severe branchial arch hypoplasia (Jerome and Papioannou 2001; Lindsay et al., 2001; Merscher et al., 2001; Schinke and Izumo, 2001; Vitelli et al., 2002a). In addition to the role in patterning facial mesenchyme, pharyngeal endoderm was shown to be required for the neuronal induction in the surface ectoderm resulting in formation of the epibranchial placodes (Begbie et al., 1999). Additionally, this study revealed that inductive endodermal activity is mediated by secreted signal BMP7. Recent studies by Haworth et al. suggested that the pharyngeal endoderm has an early role in patterning the orofacial ectoderm (Haworth et al., 2004). They showed that endoderm regulates *Fgf8* expression in the precursors of the ectoderm covering proximal mandibular arch.

Importantly, distinct antero-posterior stripes of the early pharyngeal endoderm were found to send patterning cues to specify the shape and orientation of the neural crest skeletal derivatives (Couly et al., 2002; Ruhin, 2003). Transplanting small sections of quail anterior endoderm into 5-6 somite stage chick embryo resulted in duplicated skeletal elements corresponding to the axial level of origin of the grafted endoderm. For example, grafts of endoderm from underneath the anterior midbrain resulted in duplications of Meckel's

cartilage. Furthermore, changing the orientation of the grafted endoderm also changed the orientation of the duplicated skeletal elements. Although signalling molecules are still to be discovered, this study shows that prior to branchial arch formation, endoderm is required for specifying the morphology and pattern of the neural crest skeletal derivatives.

Couly et al. demonstrated that interpretation of endodermal signalling depends on the *Hox* identity of the neural crest cells (Couly et al., 2002), as *Hox* positive and *Hox* negative neural crest cells respond differently to signalling from the same endodermal fragment. This is consistent with study by Kanzler et al., showing that *Hoxa2* negatively affect the ability of neural crest cells in the second branchial arch to form skeletal elements (Kanzler et al., 1998). Thus, it is possible that *Hox* genes act to make neural crest cells competent to respond to the patterning cues from environment. This explanation provides correlation between signalling from the isthmus and pharyngeal endoderm and combines the theories of pre-patterning and plasticity.

Surface ectoderm

A paper by Shigetani et al. presents one of the rare studies on the early function of the surface ectoderm in pharyngeal patterning (Shigetani et al., 2000). This study suggested a role for the surface ectoderm in defining presumptive mandibular and premandibular regions through localized expression of *Fgf8* and *Bmp4* respectively. They showed that ectopically applied BMP4 inhibits FGF8 in the ectoderm of presumptive mandible, resulting in transformation of mandibular region into premandibular region.

BMP4 and FGF8 were implicated in regulation of expression of *Dlx* transcription factors (Ferguson et al., 2000; Luo et al., 2001; Miyama et al., 1999; Thomas et al., 2000). Importantly, inactivation of *Dlx5* and *Dlx6* in mouse embryos (Depew et al., 2002), results in transformation of the mandible into maxillae. Therefore, it is possible that FGF8 and BMP4 control proximo-distal determination of the branchial arches through regulation of *Dlx* genes

in the underlying neural crest cells (Depew et al., 1999; Qiu et al., 1995; Qiu et al., 1997). However, recent study by Ozeki et al. indicated that ET1, produced by the branchial epithelium and core mesenchyme, regulates *Dlx* expression (Ozeki et al., 2004). They showed that *ET1* knock out mice have strikingly similar phenotype to *Dlx5*/*Dlx6* null mutants. Correspondingly, expression of *Dlx5* and *Dlx6*, is significantly down-regulated in *ET1* mutants.

Pharyngeal pouches and pharyngeal clefts

Existence of a possible signalling centre in the first pharyngeal cleft/pouch was suggested by the phenotype of the *Hoxa2* knock out mouse (Gendron-Maguire et al., 1993; Rijli et al., 1993). In *Hoxa2* mutants the second branchial arch is transformed into the first arch resulting in duplicated first arch elements with the mirror image symmetry. Consistent with this theory, many signalling molecules including BMPs, FGFs and SHH are expressed by pharyngeal clefts and pouches as well as anterior epithelial border of the first branchial arch (Wall and Hogan 1995; Francis-West et al., 1998). *Fgf8*, expressed in the epithelium at the anterior and posterior border of the first branchial arch, was suggested to regulate patterning within the first arch (Trumpp et al., 1999; Tucker and Sharpe, 1999). This was based on observations that FGF8 induces expression of the anterior mesenchymal marker *Lhx6*, and the posterior mesenchymal marker *Gsc*. FGF8 was also shown to induce *Barx1,* a marker of the proximal mesenchyme (Trumpp et al., 1999). On the other hand, *Msx1,* a marker of the distal mesenchyme, was shown to be induced by epithelial signalling molecule, BMP4 (Tucker et al., 1998).

A number of individual genes have been proposed to control patterning of the branchial arches. However, it is dificult to resolve in which branchial arch tissue and at which stage certain gene is needed, what is its primary function, and in which signalling network it is involved. One reason is that same genes can be active in more than one branchial arch cell type in the same time. Furthermore, their products, signalling molecules and transcription

factors, are repeatedly used in different developmental processes during the course of arch development (including cell migration, specification, survival and differentiation). Exciting studies on the field of branchial arch development will continue to adress these issues in the years to come.

FGFs and their receptors FGFRs

FGFs

FGFs form a conserved family of secreted growth factors (Ornitz and Itoh, 2001). A wide spectrum of cellular functions such as proliferation, apoptosis, differentiation, and migration is controlled by FGFs (Yamaguchi and Rossant, 1995). In mice, FGF family consists of 22 members. Most Fgf genes are composed of three exons and two large introns, with the exception of *Fgf8* which contains at least six exons, encoding for eight isoforms (MacArthur et al., 1995). FGFs contain a conserved "core" sequence of 28 highly conserved and six identical amino acids. This sequence provides FGFs a common tertiary structure and the ability to bind heparin or heparin sulphate proteoglycans and FGF receptors (Faham et al., 1996). FGF family is subdivided into subfamilies. FGFs within a subfamily have similar receptor-binding properties and partially overlapping patterns of expression. For example, FGF8, -17, and -18 have 70-80% sequence identity, similar ligand binding properties, and are co-expressed in many tissues (Ornitz and Itoh, 2001).

FGFRs

FGF receptors (FGFRs) belong to the family of receptor tyrosine kinses (RTKs). To date four FGFR (FGFR1-4) with the same overall primary structure have been cloned (Dionne et al., 1990; Keegan et al., 1991; Lee et al., 1989; Ornitz, 2000; Partanen et al., 1991). As all other RTKs, FGFRs have a ligand binding extracellular domain, a single transmembrane region and a cytoplasmic portion containing the kinase domain. The extracellular domain consists of three immunoglobulin domains (IgI, IgII and IgIII). The ligand binding region resides in IgII

and IgIII domains. An alternative splicing event involving the exon encoding the C-terminal region of IgIII domain in FGFR1, FGFR2 and FGFR3 results in IIIb and IIIc receptor isoforms. Splice isoforms possess different expression patterns and ligand-binding specificities (Johnson and Williams, 1993; Ornitz and Itoh, 2001). IIIb isoforms of FGFRs are predominantly found in epithelial lineages and preferentially bind to FGF1, FGF3, FGF7 and FGF10 in assays in vitro. IIIc isoforms are expressed in mesenchymal lineages and bind to FGF1, FGF2, FGF4, FGF8 and FGF9.

FGFR signalling pathways

Specific recognition and interaction between receptor and ligand are prerequisites for correct intracellular signal transduction to occur. This is achieved through different binding affinities between FGFs and FGFRs. The existance of FGFR1-3 IIIb and IIIc isoforms, further adds to specificity of interactions with ligands (Ornitz et al., 1996). FGFs bind to FGFRs as monomers requiring the assistance of accessory molecules, heparin sulphate proteoglycans or heparin (Mohammadi et al., 1996; Schlessinger et al., 1995). Binding of FGF leads to dimerization and autophosphorylation of FGF receptors. Dimerization occurs both between FGF receptors of the same type, homodimerization, and between different FGF receptor types, heterodimerization (Bellot et al., 1991). FGFR autophosphorylation activates several intracellular cascades, including the RAS pathway, SRC family tyrosine kinases, phosphoinositide 3-kinase/AKT (PI3K/AKT) and the phospholipase-Cγ/protein kinase C (PLC-γ/PKC) pathway.

In the RAS pathway, the activated FGFR phosphorylates and activates two independent adaptor proteins, SHC and membrane-bound FRS2 (Kouhara et al., 1997). This creates binding sites for the GRB2 adaptor in complex with the RAS activating, nucleotide exchange factor SOS (Kouhara et al., 1997). This leads to activation of the GTPase RAS, setting off a cascade of kinases including Raf, MEK, and finally MAPK (Kolch et al., 1993; Moodie et al.,

1993; Stokoe and McCormick, 1997). MAPK translocates into the nucleus and phosphorylates and activates different transcription factors, thereby activating transcription of target genes. RAS pathway was shown to be involved in *Fgf* induced cell proliferation (LaVallee et al., 1998).

FGF signal transduction also involves activation of the SRC kinase pathway, which in turn stimulates increases in the level of *Myc* (LaVallee et al., 1998). Inhibition of *Myc* expression was shown to severely compromise the cells' migratory potential. Furthermore, SRC-induced tyrosine phosphorylation of cortactin was shown to decrease ability of cortactin to cross-link actin and to enhance migratory potential of the cells (Boilly et al., 2000; LaVallee et al., 1998; Liu et al., 1999).

In response to FGF stimulation, FRS2α and Gab1 associate indirectly via Grb2 resulting in tyrosine phosphorylation of Gab1 and activation of the PI3-kinase pathway (Ong et al., 2001). One of the best characterized targets of PI3K lipid products is the protein kinase AKT. It mediates many PI3K-regulated biological responses, including the inhibition of apoptosis (Vanhaesebroeck and Alessi, 2000). Activated AKT has been shown to phosphorylate the pro-apoptosis BCL2 family member BAD, Caspase 9, FKHRL1, and IκB kinase, preventing apoptosis and possibly leading to endothelial cell survival (Vanhaesebroeck and Alessi, 2000).

In the PLCγ pathway, autophosphorylation of FGFR at Y766 recruits PLCγ to the FGFR for activation. This elicits phosphoinositol hydrolysis and metabolism, PKC activation, and Ca^{2+} mobilization (Mohammadi et al., 1992; Peters et al., 1992). The intracellular Ca^{2+} concentration modulates the activity of numerous proteins. In FGFR-1 Y766F mutants tyrosine 766 is replaced by a phenylalanine residue, resulting in FGFR1 which is unable to bind PLC-γ. Partanen et al. showed that this mutation leads to alterations in antero-posterior

patterning of the vertebral column, with transformations occurring exclusively in the posterior direction (Partanen et al., 1998). Their results suggest that a signal starting at phosphorylated Y766 plays a role in the negative regulation of FGFR1 activity in vivo. Cross et al. suggested that FGFR-1 mediated cytoskeletal reorganisation is dependent upon PLC-γ pathway (Cross et al., 2000), as cells expressing FGFR-1 Y766F fail to form stress fibres, needed for the cell shape-changes.

FGF responsive genes

Downstream transcriptional targets of the FGF signalling include *Sef* and members of *Sprouty* (*Spry*) and *ETS* gene families. FGF signaling is both necessary and sufficient to control their expression. In addition, some of these molecules are feedback regulators of FGF signalling. SPRY is a conserved protein that was originally identified as an antagonist of FGF-dependent tracheal development in Drosophila (Casci et al., 1999) and was subsequently shown to function as a general inhibitor of receptor tyrosine kinase (RTK) signalling (Furthauer et al., 2001). Four mammalian homologues (SPRY1, 2, 3 and 4) have been identified (de Maximy et al., 1999; Hacohen et al., 1998; Minowada et al., 1999; Tefft et al., 1999). Sprouty proteins function as FGF-induced feedback inhibitors (de Maximy et al., 1999; Minowada et al., 1999). SPRYs are intracellular proteins associated with the inner surface of the plasma membrane. Concerning the mechanisms involved in the actions of SPRYs, studies have shown that SPRY1 and SPRY2 inhibit the activation of the Erk pathway in response to FGF signaling (Impagnatiello et al., 2001). Decreased activation of Ras (Casci et al., 1999; Lee et al., 2001) or Raf (Yusoff et al., 2002) has been reported to be responsible for the inhibition of Erk activation.

SEF (similar expression to *Fgf* genes) was identified in zebrafish as an inhibitor of RAS/MAPK-mediated FGF signaling (Furthauer et al., 2002; Tsang et al., 2002). SEF has a putative signal peptide and a putative transmembrane domain and thus is believed to be a transmembrane protein. SEF has been identified in other vertebrates and thus is thought to be

a conserved inhibitor of FGF signaling (Furthauer et al., 2002; Kawakami et al., 2003; Lin et al., 2002; Niehrs and Meinhardt, 2002; Preger et al., 2004; Tsang et al., 2002). Vertebrate Sef is expressed in highly restricted patterns in early stages of embryos, and its expression pattern is similar to the expression patterns of *Fgf* genes such as *Fgf3, Fgf8, and Fgf17* and *sprouty* members such as *Spry2* and *Spry4* (Furthauer et al., 2002; Kawakami et al., 2003; Lin et al., 2002; Tsang et al., 2002).

Pea3 and *Erm* are defined by the presence of an evolutionarily conserved Ets domain that mediates DNA binding (de Launoit et al., 1997; Sharrocks et al., 1997). FGF signaling is both necessary and sufficient for their expression (Firnberg and Neubuser, 2002; Kawakami et al., 2003; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). They are present at regions of FGF signaling in several developmental contexts, and are thought to be general transcriptional targets of FGF signaling (Chotteau-Lelievre et al., 1997; Raible and Brand, 2001; Roehl and Nusslein-Volhard, 2001). Additionally, *Pea3* and *Erm* were shown to activate *Fgf* signalling in zebrafish (Chotteau-Lelievre et al., 2001).

FGFs and FGFRs in mid- and hindbrain development

Fgf8 expression in the isthmus is conserved in all vertebrate classes (Crossley and Martin, 1995; Crossley et al., 1996; Furthauer et al., 1997; Heikinheimo et al., 1994; Ohuchi et al., 1994; Reifers et al., 1998). *Fgf17* and *Fgf18* (Maruoka et al., 1998) were detected in the mid- and hindbrain boundary, after initiation of *Fgf8*. In the mid and hindbrain region, *Fgf8* is required for the isthmic organizer activity and for cell survival (Chi et al., 2003; Irving and Mason, 2000; Meyers et al., 1998; Reifers et al., 1998). Inactivation of *Fgf17* in the mouse, results only in mild cerebellar defects, probably caused by reduced cell proliferation of the cerebellum precursors (Xu et al., 2000). However, mice homozygous for the *Fgf17* null allele and heterozygous for the *Fgf8* null allele have more severe phenotype, implying redundancy between *Fgf8* and *Fgf17* in the mid- and hindbrain development.

Detailed analysis of *Fgfr1-4* expression at early stages of neural development (E8.5-10) has not been reported in mouse embryos. At the corresponding stage in chick embryos, *Fgfr1* is expressed throughout the neural tube. *Fgfr2* and *Fgfr3* are expressed in the anterior midbrain and throughout the hindbrain, with the exception of most anterior portion corresponding to rhombomere 1 (Walshe and Mason, 2000; Wilke et al., 1997; Yamaguchi et al., 1992). *Fgfr4* was not detected within developing neural tube (Marcelle et al., 1994). Consistent with results from chick, in zebrafish *Fgfr1* is the only *Fgfr* expressed at the mid- and hindbrain boundary (Scholpp et al., 2004). Several genetically engineered mouse models affecting FGFRs have been generated (Table 3). However, defects in the mid- and hindbrain region have not been reported in these mutants.

FGFs and FGFRs in branchial arch development

In mouse embryo, *Fgf3*, *Fgf4, Fgf8* and *Fgf15* have been detected in the developing branchial arches. *Fgf3* (Mahmood et al., 1996; Wilkinson et al., 1988) and *Fgf15* (McWhirter et al., 1997) are expressed in the anterior epithelial margins of the second and third branchial arch. In addition, *Fgf3* was detected in prospective otic ectoderm (Mahmood et al., 1996; Wilkinson et al., 1988). *Fgf8* (Heikinheimo et al., 1994; Crossley and Martin, 1995) and *Fgf4* (Niswander and Martin, 1992) are expressed at the anterior and posterior epithelial borders of all branchial arches. Furthermore, expression of *Fgf-s* in the pharyngeal region was found to be conserved in chick and *Xenopus* (Christen and Slack, 1997; Lombardo et al., 1998; Ohuchi et al., 1994; Shamim and Mason, 1999; Vogel et al., 1996).

Gene inactivations of *Fgf3, Fgf4, Fgf8* and *Fgf15* did not add to our understanding of their role in the branchial arch development. Inactivations of *Fgf4* (Feldman et al., 1995) and *Fgf8* (Minowada et al., 1999) result in early embryonic lethality, before branchial arch formation. In contrast, *Fgf3* knockout mice show only mild phenotypic alterations in the middle ear and tail (Mansour et al., 1993), and *Fgf15*-deficient mice embryos (E9.5-E12.5) appear normal (Wright et al., 2004).

Fgf8 from the isthmic organizer patterns the first branchial arch neural crest cells, through regulation of *Hox*-gene expression (Trainor et al., 2002). Tissue-specific gene inactivation of *Fgf8* in the ectoderm of the first branchial arch demonstrated that *Fgf8* is required for survival and patterning of the neural crest cells in the mandibular arch of mouse embryo (Trumpp et al., 1999). Several studies have implicated *Fgf8* signalling defects in DiGeorge syndrome, showing that strongly reduced expression of *Fgf8* affects both the development of the branchial arches and cardiac structures (Meyers et al., 1998; Abu-Issa et al., 2002; Frank et al., 2002).

Data on *Fgfr1-4* expression in the pharyngeal region comes from studies in chick embryos (Walshe and Mason, 2000; Wilke et al., 1997). *Fgfr1* is expressed in ectoderm, endoderm and more weakly in mesenchyme of all branchial arches. Furthermore, expression is higher in the first and second branchial arch compared with posterior arches. Similarly, *Fgfr2* signal is stronger in the first two branchial arches. At the tissue level, it is present in ectoderm and at lower level in medial pharyngeal endoderm. However, Fgfr2 is absent in endoderm of the pharyngeal pouches. *Fgfr3* expression is restricted to posterior first and anterior second branchial arch. In contrast to *Fgfr2*, *Fgfr3* is detected only in the mesenchyme and pouch endoderm.

Genetic manipulations of *Fgfr1* and *Fgfr2* demonstrate their role in craniofacial development (see Table 3). Mice homozygous for hypomorphic alleles of the *Fgfr1* gene have cleft palate, and reduced pinna of the outer ear (Partanen et al., 1998) as well as inner ear defects (Pirvola et al., 2002). It was suggested that reduced size of the second branchial arch could affect craniofacial development in the *Fgfr1* hypomorphs, but the mechanism of this defect remained unknown (Partanen et al., 1998). Inactivation of the *Fgfr2(IIIb)* function results in a thinner mandible and cleft palate (De Moerlooze et al., 2000). Recently, Rice et al. demonstrated that cleft palate in *Fgfr2(IIIb)* mutants is caused by disrupted epithelial-

mesenchymal interactions resulting in decreased cell proliferation in both epithelium and mesenchyme (Rice et al., 2004). In addition, *Fgfr-2(IIIb)* was shown to be critical for tooth (De Moerlooze et al. 2000) and the inner ear development (Pirvola, 2000).

Table 3. Phenotypes of *Fgfr1-4* transgenic mice

Gene	Mutation and defects	Refference
Fgfr1	*Fgfr1* null mutants have gastrulation defects, die at E8.5-9.5 mesodermal pattering is affected, somites do not form	(Yamaguchi et al., 1994) (Deng et al., 1994)
	Fgfr1IIIb-/- mutants have defect in tail development, do not have other obvious defects	(Partanen et al., 1998)
	Fgfr1 chimeras reveal defective migration of mesodermal cells through the primitive streak	(Ciruna et al., 1997)
	Fgfr1 hypomorphs die neonataly; have craniofacial, somite and limb defects, and abnormalities in A-P patterning	(Partanen et al., 1998)
Fgfr2	A null mutation of *Fgfr2* results in peri-implantation lethality at E4.5	(Arman et al., 1998)
	Embryos with a homozygous hypomorphic *Fgfr2* allele die by E10.5 with no limb buds and defective placenta.	(Xu et al., 1998)
	Inactivation of the *Fgfr2(IIIb)* function results in craniofacial and inner ear defects	(De Moerlooze et al., 2000)
Fgfr3	A null mutation of *Fgfr3* results in skeletal dysplasia of the long bones and an inner ear defect	(Colvin et al., 1996) (Deng et al., 1996)
Fgfr4	*Fgfr4*-deficient mice show no apparent phenotype but *Fgfr3/Fgfr4* double null mutants demonstrate a late lung defect not found in the single receptor-deficient mice	(Weinstein et al., 1998)

FGF signalling in human genetic disorders

In humans, mutations of *Fgfr1, Fgf2* and *Fgfr3* result in dwarfing chondrodysplasia syndromes and craniosynostosis syndromes characterized by premature fusion of the cranial sutures (reviewed by Wilkie et al., 2001; Ornitz and Marie, 2002). Craniosynostosis syndromes include Apert syndrome, Crouzon syndrome, Pfeiffer syndrome, Jackson-Weiss

syndrome, and a non-syndromic craniosynostosis. Most of these syndromes are associated with dominant, gain-of-function mutations, affecting the highly conserved extracellular FGFR lignad binding domain. Some of these mutations result in ligand-independent dimerization of FGFRs constitutively activating the receptor, other mutations prolong the duration of FGFR signalling or alter ligand-binding specificity. Studies on the cellular processes controlled by FGF signalling suggest that it enhances suture closure by regulating the balance among skeletal cell growth, differentiation and apoptosis.

More recently, FGF signalling in the pharyngeal region has been associated with the DiGeorge syndrome. *Fgf8* hypomorphic mice phenocopy DiGeorge syndrome (Abu-Issa et al., 2002; Frank et al., 2002), which is characterized by cardiac outflow tract anomalies, hypoplasia of the thymus and parathyroid glands, cleft palate and facial dysmorphogenesis, and is attributed to abnormal development of the pharyngeal arches and pouches. As many of the structures affected in patients with DiGeorge syndrome are derived from the neural crest cells in branchial arches, it is likely that FGF8 produced by the pharyngeal epithelial cells regulates development of the neural crest cells. *Tbx1* is the major candidate gene for DiGeorge syndrome (Jerome and Papioannou 2001; Lindsay et al., 2001; Merscher et al., 2001; Schinke and Izumo, 2001; Vitelli et al., 2002a). It was shown that *Tbx1* interact genetically with *Fgf8*, as double heterozygous *Tbx1*+/-; *Fgf8*+/- mutants reveal higher penetrance of aortic arch artery and thymic defects than Tbx1+/-;Fgf8+/+ mutants (Vitelli et al., 2002b). Furthermore, this study showed that *Fgf8* is not detected in the pharyngeal endoderm of *Tbx1* null mutants, implying that *Tbx1* is required for *Fgf8* expression in the endoderm. Recent study by Macatee et al., demonstrated that ectodermal and endodermal *Fgf8* domains in pharyngeal region have discrete functional roles (Macatee et al., 2003). Specific inactivation of *Fgf8* in pharyngeal ectoderm resulted in vascular defects, while inactivation of *Fgf8* in the third and fourth branchial arch ectoderm and endoderm resulted in pharyngeal gland and aortic valve defects characteristic for DiGeorge syndrome.

AIMS OF THE STUDY

The aim of this study was to investigate function of FGFR1 in branchial arch, midbrain and hindbrain development. Specifically the aims were:

1. to characterise craniofacial defects in the hypomorphic *Fgfr1* mutants

2. to define when and where FGFR1 is required during branchial arch development

3. to describe cellular and molecular mechanisms by which FGFR1 regulates branchial arch development

4. to study if FGFR1 is required for mid- and hindbrain development

5. to describe mechanism by which FGFR1 regulates mid- and hindbrain development

MATERIALS AND METHODS

Mouse strains

Ffr1^{n15YF} Hypomorphic *Fgfr1* allele *Fgfr1^{n15YF}*, has a *neo*-cassette insertion in intron 15 (Partanen et al., 1998). As a result, the amount of full-length *Fgfr1* transcripts produced by *Fgfr1^{n15YF}* allele is only ~10% of the amount produced by the wild-type Fgfr1 allele.

Fgfr1^{n7} Hypomorphic *Fgfr1* allele *Fgfr1^{n7}*, has a *neo*-cassette insertion in intron 7 (Partanen et al., 1998). As a result, the amount of full-length *Fgfr1* transcripts produced by *Fgfr1^{n7}* allele is only ~20% of the amount produced by the wild-type Fgfr1 allele.

Fgfr1^{17} The *neo*-cassette in *Fgfr1^{n7}* allele is flanked by loxP sites, which allow excision of the cassette by the Cre recombinase.This results in the *Fgfr1^{17}* allele, which is functionally a wild-type allele.

Fgfr1^{flox} In conditional *Fgfr1* null allele (*Fgfr1^{flox}*) the exons 8-15 encoding the transmembrane domain, juxtamembrane domain and most of the tyrosine kinase domain of FGFR1 are flanked by two loxP sites (Trokovic et al., 2003). *Fgfr1^{flox}* allele is functionally equal to a wild-type allele.

Fgfr1$^{\Delta flox}$ Cre recombination of *Fgfr1^{flox}* allele results in excision of exons 8-15 generating *Fgfr1* null allele, *Fgfr1$^{\Delta flox}$*.

Wnt1-Cre *Wnt1-Cre* is a transgene containing *Cre* gene under the control of *Wnt1* promoter. *Cre* expression is driven by *Wnt1* promoter in the dorsal neural tube where the neural crest precursors are situated (Danielian et al., 1998).

En1-Cre — *En1-Cre* mice express the Cre-recombinase under the *En1* locus, in the midbrain and rhombomere 1 (Kimmel et al., 2000).

Z/AP — *Z/AP* double-reporter transgene contains ubiquitous promoter driving expression of *lacZ* and human alkaline phosphatase (Lobe et al., 1999). *LacZ* is followed by polyadenylation signal which stops transcription and disables expression of human alkaline phosphatase. The region comprising *LacZ* and polyadenylation signal is flanked with loxP sites recognized by Cre recombinase. Cre-mediated excision of this loxP flanked region results in expression of human alkaline phosphatase.

Tie1^{lcz} — *Tie1^{lcz}* is a reporter transgene containing *lacZ* gene under the control of *Tie1* promoter (Puri et al., 1995). *LacZ* expression is driven by *Tie1* promoter in the endothelial cells.

Pgk-Cre — *Pgk-Cre* transgene ubiquitously drives *Cre* expression (Lallemand et al., 1998).

Mice and genotyping

Analyses of mice and embryos carrying *Fgfr1^{n7}*, *Fgfr1^{n15YF}*, *Fgfr1^{flox}* and *Fgfr1 $^{\Delta flox}$* alleles, as well as the *Pkg-Cre, En1-Cre, Wnt1-Cre*, *Tie1^{lcZ}* and *Z/AP* transgenes were carried out in outbreed (ICR) background. Embryonic age was estimated by counting the somites or considering noon of the day of a vaginal plug as E0.5. Mice and embryos were genotyped by polymerase chain reaction (PCR) analysis of DNA (Article I).

Oligonucleotide primer pairs used for detection of distinct alleles are listed in Table 4. The *Z/AP* and *Tie^{lcZ}* alleles were detected by β-galactosidase staining (Lobe et al., 1999).

Table 4. Primers used for genotyping

Allele	Upstream primer 5' - 3'	Downstream prime 5' - 3'	Article
Fgfr1	CCCCATCCCATTTCCTTACCT	TTCTGGTGTGTCTGAAAACAGCT	I, II, III
Fgfr1^{n7},	AATAGGTCCCTCGACGGTATC	CTGGGTCAGTGTGGACAGTGT	I, II, III
Fgfr1flox			I, III
Fgfr1^{15YF},	AATAGGTCCCTCGACGGTATC	TAGTAGTCGGCACTGTTTGGA	III
Fgfr1$^{\Delta}$flox			I, III
Pgk-Cre	ATTCTCCCACCGTCAGTACG	CGTTTTCTGAGCATACCTGGA	III
Wnt-Cre,	ATTCTCCCACCGTCAGTACG	CGTTTTCTGAGCATACCTGGA	I, III
En1-Cre			I, III

Crosses performed to obtain transgenic mouse lines and embryos used in this study are indicated in Tables 5 and 6, respectively.

Table 5. Generation of transgenic mouse line

Cross		Generated mouse line	Article
Fgfr1$^{flox/+}$	*Fgfr1$^{flox/+}$*	*Fgfr1$^{fox/flox}$*	I, III
Pgk-Cre/+	*Fgfr1$^{flox/+}$*	*Fgfr1$^{\Delta flox/+}$*	I, III
Wnt1-Cre/+	*Fgfr1$^{n7/+}$*	*Wnt1-Cre/+; Fgfr1$^{n7/+}$*	I, III
Wnt1-Cre/+	*Fgfr1$^{flox/flox}$*	*Wnt1-Cre/+; Fgfr1$^{flox/+}$*	I, III
Wnt1-Cre/+	*Fgfr1$^{\Delta flox/+}$*	*Wnt1-Cre/+; Fgfr1$^{\Delta flox/+}$*	I, III
En1-Cre/+	*Fgfr1$^{flox/flox}$*	*En1-Cre/+; Fgfr1$^{flox/+}$*	I, III
En1-Cre/+	*Fgfr1$^{\Delta flox/+}$*	*En1-Cre/+; Fgfr1$^{\Delta flox/+}$*	I, III
Tie$^{lcZ/+}$	*Fgfr1$^{n7/+}$*	*Tie$^{lcZ/+}$; Fgfr1$^{n7/+}$*	I

Table 6. Generation of transgenic mouse embryos

Cross		Generated mouse embryo	Article
Fgfr1$^{n7/+}$	*Fgfr1$^{n7/+}$*	*Fgfr1$^{n7/n7}$*	I, II, III
Fgfr1$^{n15YF/+}$	*Fgfr1$^{n15YF/+}$*	*Fgfr1$^{n15YF/n15YF}$*	III
Wnt1-Cre/+; Fgfr1$^{n7/+}$	*Fgfr1$^{n7/+}$*	*Wnt1-Cre/+; Fgfr1$^{n7/n7}$*	I
Wnt1-Cre/+; Fgfr1$^{flox/+}$	*Fgfr1$^{flox/flox}$*	*Wnt1-Cre/+; Fgfr1$^{flox/flox}$*	I, III
Wnt1-Cre/+; Fgfr1$^{\Delta flox/+}$	*Fgfr1$^{flox/flox}$*	*Wnt1-Cre/+; Fgfr1$^{\Delta flox/flox}$*	I, III
En1-Cre/+; Fgfr1$^{flox/+}$	*Fgfr1$^{flox/flox}$*	*En1-Cre/+; Fgfr1$^{flox/flox}$*	I, III
En1-Cre/+; Fgfr1$^{\Delta flox/+}$	*Fgfr1$^{flox/flox}$*	*En1-Cre/+; Fgfr1$^{\Delta flox/flox}$*	I, III
En1-Cre/+	*Z/AP/+*	*En1-Cre/+; Z/AP/+*	I, III
Wnt1-Cre/+	*Z/AP/+*	*Wnt1-Cre/+; Z/AP/+*	I, III
Tie$^{lacZ/+}$; Fgfr1$^{n7/+}$	*Fgfr1$^{n7/+}$*	*Tie$^{lacZ/+}$; Fgfr1$^{n7/n7}$*	I

Riboprobes and antibodies

Riboprobes and antibodies used in this study are listed in Tables 7 and 8.

Table 7. Antisense riboprobes for in situ hybridization

Probe	Reference	Article
Ap2		I
Bmp4	(Tucker et al., 1998)	I
Bmp7	IMAGE 5121825	II
Crabp1	IMAGE 468821	I
Dlx1	(McGuinness et al., 1996)	I
Dlx2	(Porteus et al., 1992)	I
Dlx5	(Liu et al., 1997)	I
Dopamine-β-hydroxylase	a gift from Wolfgang Wurst	III
En1	(Davis and Joyner, 1988)	III
En2	(Davis and Joyner, 1988)	III
Erm	IMAGE 3674281	II
EphA4	(Gilardi-Hebenstreit et al., 1993)	I
EphB2	IMAGE 4983886	I
EphB3	IMAGE 1110951	I
EphrinA5	Agift form David Wilkinson	III
Fgf3	(Peters et al., 1993)	I, II
Fgf8	(Crossley and Martin, 1995)	I, II, III
Fgf15	(McWhirter et al., 1997)	II, III
Fgfr1$^{\Delta Flox}$	bp 1152-1724 of NM 010206; (Trokovic et al., 2003)	I, II, III
Fgfr2	a gift from Alka Mansukhani	I, II, III
Fgfr3	(Wilkinson et al., 1988)	I, II
Ggx2	a gift from Wolfgang Wurst	III
Hoxa2	a gift from Mario Capecchi	I, III
Hoxb1		I
Hoxb2		I
Hoxd4		I
Krox20	(Nieto et al., 1991)	I
Msx1	(Jowett et al., 1993)	I
Ngn2	IMAGE 2922473	II
Otx2	(Acampora et al., 1997)	III
Pax1	IMAGE 1327502	I, II,
Pax2	a gift from Gregory Dressler	III
PB-cadherin	Clone ID:: UI-M-BH1-akr-h-03-0-UI	III
Sox10	IMAGE 4165363	II
Spry1	a gift from Seppo Vainio	II,III
Spry4	a gift from Seppo Vainio	II
VachT	a gift from Wolfgang Wurst	III
Wnt1	(McMahon and Bradley, 1990)	III

Table 8. Antibodies

Antibody	Description / Source	Article
α -calbindin	Rabbit-α-Calbidin (Swant cat. CB38)	III
α -TH	Rabbit-α-Tyrosine Hydroxylsase (Chemicon AB152)	III
α -NF	Mouse monoclonal α–neurofilament (Sigma N-5139)	II, III
α -ChAT	Rabbit-α-Choline Acetyltransferase (Chemicon, AB5042)	III

Experimental methods

The experimental methods used in this study are listed in Table 9. The description of each method is found in the original publication.

Table 9. Experimental methods used in this stu

Methods	Reference	Article
Skeletal analysis		I
Radioactive *in situ* on sections	(Wilkinson and Green, 1990)	I, II, III
Whole-mount *in situ*	(Henrique et al., 1995)	I, II, III
Nile blue sulfate (NBS) staining		I, III
Terminal deoxynucleotidyl transferase mediated nick end labelling (TUNEL) analysis	In Situ Cell Death Detection Kit (Roche, cat. 1684 795)	I, III
ß-galactosidase	(Lobe et al., 1999)	I, III
Alkaline phosphatase		I, III
Hematoxylin-eosin staining		I, III
Semi-thin sections		III
Toluidine blue		III
NADPH-Diaphorase		III
AChE histochemistry		III
Behaviour studies (rotarod and stationary beam assay)		III

RESULTS AND DISCUSSION

Expression of *Fgfr-s* in the pharyngeal region (I)

Mice homozygous for the hypomorphic alleles of the *Fgfr1* gene, $Fgfr1^{n7}$ and $Fgfr1^{n15}$ die neonatally and display craniofacial defects, including cleft palate and reduced size of the pinna of the outer ear (Partanen et al., 1998). In addition, his study implied that the craniofacial defects in *Fgfr1* hypomorphs are caused by an early defect in formation of the second branchial arch. Based on expression studies, *Fgf* signalling was implicated in the epithelial-mesenchymal interactions in the branchial arches (Francis-West et al., 1998; Wall and Hogan, 1995). In the developing mouse, *Fgf3*, *Fgf4, Fgf8* and *Fgf15* were detected in the pharyngeal epithelium (Crossley and Martin, 1995; Heikinheimo et al., 1994; Mahmood et al., 1996; McWhirter et al., 1997; Niswander and Martin, 1992; Wilkinson et al., 1988), but data on expression of *Fgfr-s* was incomplete. To study the *Fgf* signalling in the branchial arches, we first analysed expression of *Fgfr1, Fgfr2* and *Fgfr3* at E9.5. We found that, *Fgfr1* is uniformly expressed in different branchial arches, both in mesenchyme (neural crest cells and mesoderm) and epithelia (ectoderm and endoderm). At this time, *Fgfr2* is strongly expressed in the surface ectoderm of the first and second branchial arches and at lower level in the pharyngeal mesenchyme (Fig. 8 and I, Supplementary data). These observations were similar to reports on *Fgfr* expression in chick (Walshe and Mason, 2000; Wilke et al., 1997). However, in contrast to expression in chick, we detected low level of *Fgfr2* in the pharyngeal pouch endoderm (Fig. 8F, arrowhead). Furthermore, we could not detect expression of *Fgfr3* in the branchial arches (Fig. 8 and I, Supplementary data). Co-expression of *Fgfr1* and *Fgfr2* in branchial arches implies that they may have to some extent redundant roles in the branchial arch region.

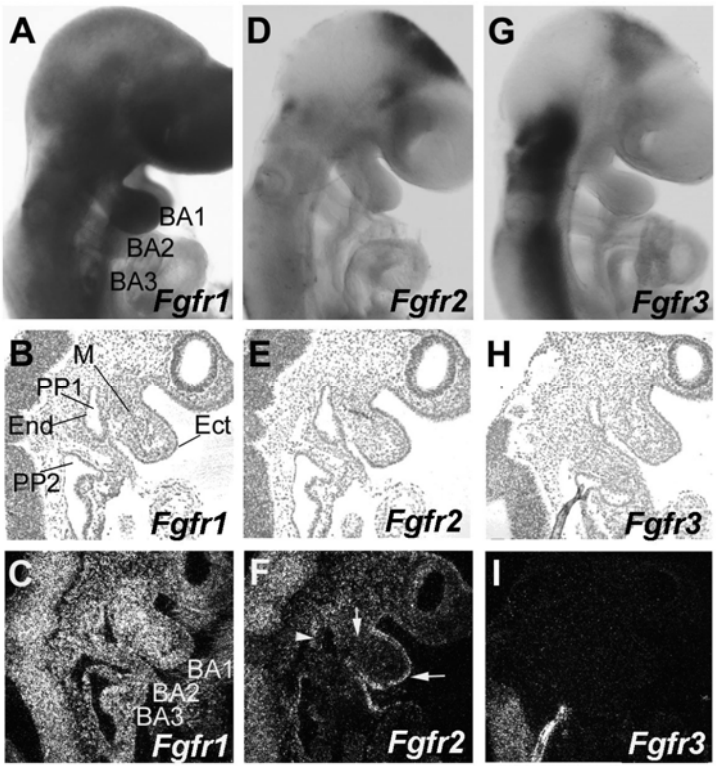

Figure 8. Expression of *Fgfr1-3* in the branchial arches. Whole mount in situ hybridization with *Fgfr1-3* probes at E9 (A,D,G). Radioactive in situ hybridization analysis using *Fgfr1*, *Fgfr2*, and *Fgfr3* riboprobes on adjacent sagittal serial sections at E9.5 (B,C,E,F,H,I). Bright field images (B,E,H) correspond to dark field images (C,F,I). BA1-3, branchial arch 1-3; Ect, ectoderm; End, endoderm; M, mesenchyme; PP1-3, pharyngeal pouch 1-3.

In this study we did not look at the expression patterns of different *Fgfr* splice isoforms. Importantly, wealth of data on *Fgfr* expressions in other regions showed that *IIIb* isoforms are expressed mainly in epithelia and the *IIIc* isoforms in mesenchyme, as well as that the splice isoforms dramatically differ in ligand specificity (Ornitz and Itoh, 2001). For example during limb bud development, FGF10 in the mesenchyme interacts with the FGFR2(IIIb) which is expressed in the surface ectoderm, while FGF8 in the ectoderm interacts with the mesodermally expressed FGFR2(IIIc) (Xu et al., 1998). FGF signalling within the same tissue was also observed. For example in the inner ear, FGFR2(IIIb) and FGF10 interaction operates within the epithelium (Pirvola et al., 2000).

***Fgfr1* is required for the normal craniofacial development (I)**

Second branchial arch defects in hypomorphic *Fgfr1* mutants

In order to further characterise the craniofacial defects in the hypomorphic *Fgfr1* mutants we decided to use the $Fgfr1^{n7/n7}$ mutants in our studies, because the $Fgfr1^{n15/n15}$ embryos are often growth-retarded and therefore difficult to compare with the wild-type littermates. First we

performed detailed analysis of the branchial arch morphology with the electron-microscopy in E9.5-E10.5 *Fgfr1*$^{n7/n7}$ mutants and control embryos (I, Fig.2). We have observed variable deficiencies in the second branchial arch of the mutants, ranging from the almost completely missing structure to the well formed distal part of the arch. The proximal part of the second branchial arch was always strongly affected. Morphologies of the first and the third branchial arches were normal in the *Fgfr1*$^{n7/n7}$ embryos. To visualize the endothelial cells of the aortic arches in the *Fgfr1* hypomoprhs, we generated *Fgfr1*$^{n7/n7}$; *Tie1*$^{lcZ/+}$ embryos. β–Galactosidase staining of the *Fgfr1*$^{n7/n7}$; *Tie1*$^{lcZ/+}$ embryos revealed specific defect in the development of the second aortic arch (I, Fig. 2).

These results demonstrate that *Fgfr1* is required for the formation of the second branchial arch and its aortic arch. Importantly, our results and those from others (I, Supplementary data), (Yamaguchi et al., 1992), showed that *Fgfr1* is expressed throughout pharyngeal region in E9.5 mouse embryos, indicating its role in formation of all branchial arches. Having in mind that in the hypomorphic *Fgfr1* mutants *Fgfr1* is only partially inactivated, it is possible that the first and the third branchial arches require lower activity of the *Fgfr1* than the second arch. Similarly, the proximal part of the second branchial arch could be more sensitive to reduction in the *Fgfr1* then the distal part of the arch. Additionally, *Fgfr2* is expressed throughout pharyngeal region at E9.5 (Fig. 8 and I, Supplementary data). Therefore, *Fgfr2* could also be involved in the branchial arch development, and it could exhibit redundancy with the *Fgfr1*.

Craniofacial skeletal deficiencies in hypomorphic *Fgfr1* mutants

Analysis of the skeletal preparations of the newborn hypomorphic *Fgfr1* mutants revealed abnormalities in structures deriving from the proximal part of the first branchial arch (palatine, pterygoid, squamosum, alisphenoid and incus), structures deriving from the distal part of the first arch (malleus, tympanic and gonial), structures deriving from the proximal

part of the second arch (styloid and stape), and structures deriving from the distal part of the second arch (lesser horns of the hyoid) (I, Fig.1 and Table 1).

Styloid process and stapes were strongly affected or missing in the *Fgfr1*$^{n7/n7}$ mutants. This could be explained by the early defect in the second branchial arch formation, as the proximal part of the arch was always strongly affected in the *Fgfr1*$^{n7/n7}$ embryos. Lesser horn of the hyoid bone was either only mildly affected or appeared normal in mutants, implicating that even small population of the neural crest cells in the remaining second arch is enough to form this structure. Surprisingly, skeletal preparations of newborn *Fgfr1*$^{n7/n7}$ mice also showed abnormalities in several first arch derived skeletal elements. Consistent with expression studies, this could reflect a function of *Fgfr1* in the later development of the first arch. Alternatively, abnormal development of the second branchial arch may disrupt presumptive patterning center between the first and the second branchial arch, leading secondarily to defect in the first arch (Rijli et al., 1993). In 80% of the *Fgfr1*$^{n7/n7}$ mice the lesser horns of the hyoid bone and pterygoid processes were positioned abnormally laterally, and the palatal shelves were open, while in 20% of mutants these elements appeared normal. Defects in palatine, pterygoid and lesser horn of the hyoid bone always appear together, indicating their correlation.

Role of *Fgfr1* in the development of the second branchal arch (I, II)

Fgfr1 indirectly regulates migration of the neural crest cells

Generation, migration and survival of neural crest cells in hypomorphic Fgfr1 embryos

Normal development of *Fgfr(IIIb)-/-* mouse mutants (Partanen et al., 1998) suggests that it is the *Fgfr1(IIIc)* isoform, which is required for the craniofacial development. As *Fgfr1(IIIc)* is mainly expressed in mesenchymal tissues (Ornitz et al., 2001), the branchial arch defect in *Fgfr1* hypomorphs could be caused by defect in the mesenchymal neural crest cells. Consistent with this theory, neural crest cells were thought to be a major player during

branchial arch development (numerous studies including: Clouthier et al., 1998; Gavalas et al., 1998; Kurihara et al., 1994; Qiu et al., 1995; Qiu et al., 1997). Furthermore, *Fgf* signalling has been implied in cellular processes regulating neural crest cell generation (Mayor et al., 1997), neural crest patterning and survival (Trumpp et al., 1999; Tucker and Sharpe, 1999), and neural crest migration (Kubota and Ito, 2000). Therefore, in order to understand the cellular mechanism of the second branchial arch defect in the *Fgfr1*$^{n7/n7}$ embryos, we first focused our studies on the neural crest cells.

Second branchial arch neural crest cells originate from the rhombomere 4 in the hindbrain. Consequently, rhombomere 4 specification defect could affect development of the second branchial arch. To analyse the antero-posterior patterning of the hindbrain in the hypomorphic *Fgfr1* embryos, we studied expression of the several hindbrain regional markers by the whole mount in situ hybridisation. We detected similar patterns of *Krox20*, *Hoxd4*, *Hoxb1* and *EphA4* in the mutant and control embryos at E8.5-E9 (I, Fig. 3). These results suggested that the antero-posterior patterning of the hindbrain is normal in the hypomorphic *Fgfr1* mutants.

We next analysed whether rhombomere 4 neural crest cells were generated properly and whether they migrated normally toward the second branchial arch in the *Fgfr1*$^{n7/n7}$ mutants. *Crabp1, Ap2* and *Hoxa2* riboprobes were used to detect neural crest cells in E9.0-E9.5 *Fgfr1*$^{n7/n7}$ and wild-type embryos (I, Fig. 4). A stream of neural crest cells was found to originate from the rhombomere 4 region and migrate ventrally toward the second branchial arch in the *Fgfr1*$^{n7/n7}$ and control embryos. Thus, generation and initial migration of the rhombomere 4 neural crest cells are normal in the *Fgfr1*$^{n7/n7}$ embryos. However, in contrast to the wild type embryos, majority of the rhombomere 4 derived neural crest cells in *Fgfr1* mutants fail to enter the second branchial arch and instead accumulate proximal to it. Thus, *Fgfr1* is needed for migration of the neural crest cells into the second branchial arch.

To analyse whether apoptosis of the neural crest cells contributed to the second branchial arch defect in the *Fgfr1*$^{n7/n7}$ embryos, we performed whole mount Nile blue staining (at E9-E9.5) and TUNEL on tissue sections (at E9.5). We did not detect increase in the cell death inside the second branchial arch of *Fgfr1*$^{n7/n7}$ mutants (I, Fig. 6). This suggested that the early defect in the second branchial arch formation in the *Fgfr1*$^{n7/n7}$ embryos is not caused by apoptotic cell death. However, increased neural crest cell death was detected proximal to the second branchial arch in E9.5 *Fgfr1*$^{n7/n7}$ embryos. Because this is the region where the neural crest cells accumulate in the *Fgfr1*$^{n7/n7}$ embryos, it is possible that they do not receive appropriate cues from the environment and are therefore depleted by apoptosis.

Dlx1, Dlx2, *Dlx5* and *Msx1* probes were used to analyse the proximo-distal patterning of the neural crest cells populating the second branchial arch of E9.5-E10.5 *Fgfr1*$^{n7/n7}$ embryos (I, Fig. 5). Appropriate gene-expressions were observed at E9.5, suggesting that the neural crest cells populating the second arch of the *Fgfr1*$^{n7/n7}$ embryos are patterned correctly along the proximo-distal axis. At E10.5 *Dlx1* and *Dlx2* were almost diminished in the proximal second branchial arch. Probable explanation for this is progressive decrease in number of the neural crest cells due to the migration defect and cell death. Alternatively, this could reflect a later role of *Fgfr1* in neural crest differentiation.

Neural crest cell specific inactivation of the Fgfr1

Based on expression data in the pharyngeal region, it is possible that FGFs expressed in the branchial arch epithelia directly attract the neural crest cells into the arch through activation of FGFR1 (Fig.7 and I, Supplementary data). Along this line, *FGF2 and FGF8* were shown to induce chemotactic migration of mesencephalic neural crest cells (Kubota and Ito, 2000).

To understand whether *Fgfr1* is required cell-autonomously for the neural crest cell migration we took advantage of the Cre recombination technique. We used the *Wnt1-Cre* mice expressing *Cre* under the *Wnt1* promoter that is active in the neural crest precursors (Danielian et al., 1998). First we analysed stage and tissue specificity of Cre activity with the

Z/AP reporter allele (Lobe et al., 1999). Analysis of the *Wnt1-Cre/+*; *Z/AP /+* embryos at E8.5 revealed efficient recombination of the *Z/AP* reporter allele in the migrating neural crest cells, before their entry to the second branchial arch (I, Fig. 7).

Our first strategy was to inactivate *Fgfr1* specifically in the neural crest cells. We crossed the $Fgfr1^{flox}$ mice (III) with the *Wnt1-Cre* mice to generate the *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ embryos. Cre-mediated recombination of the $Fgfr1^{flox}$ deletes the transmembrane and most of the intracellular region encoding exons, resulting in the inactive $Fgfr1^{\Delta flox}$ allele (schematically presented in I, Fig. 7). In the *Wnt1-Cre* allele, the *Cre* expression is driven under the *Wnt1* promoter throughout the dorsal neural tube. In the *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ embryos *Fgfr1* will be inactivated also in the neural crest cells, as they derive from cells in the dorsal neural tube. To check for the inactivation of the $Fgfr1^{flox}$ allele, we carried out *in situ* hybridization analyses of *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ embryos with a *Fgfr1* RNA probe. Using tissue sections, in situ hybridization was performed with the *Fgfr1* probe containing exonic sequences between the loxP sites in the $Fgfr1^{flox}$ allele. In the pharyngeal region of mutant embryos, the *Fgfr1* signal was detected in pharyngeal endoderm, ectoderm and mesoderm, whereas it was absent from the neural crest cells (I, Fig. 7). This result clearly showed that the *Fgfr1* was efficiently inactivated in the neural crest cells of the *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ embryos at E9.5. But to our surprise, the second branchial arch was normally formed in these embryos (I, Fig. 7). We also generated the *Wnt1-Cre/+;* $Fgfr1^{\Delta flox/flox}$ embryos, carrying one conditional and one null allele of *Fgfr1*. In these mice a single Cre mediated recombination event is enough to inactivate *Fgfr1* function in the target cell. Similar to the *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ embryos, development of the second branchial arch was still normal in the *Wnt1-Cre/+;* $Fgfr1^{\Delta Flox/Flox}$ embryos (data not shown).

The *Wnt1-Cre/+;* $Fgfr1^{flox/flox}$ and *Wnt1-Cre/+;* $Fgfr1^{\Delta flox/flox}$ mice die neonataly of unknown cause. Beside cleft palate and abnormally laterally positioned pterygoid processes and lesser

horns of the hyoid bones, other skeletal derivatives of the branchial arches form normally in the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ and *Wnt1-Cre/+; Fgfr1*$^{\Delta flox/flox}$ mice (I, Fig. 7, Table 1 and Supplementary data). Defects in palatine, pterygoid and lesser horns of the hyoid bone were stronger than in the *Fgfr1*$^{n7/n7}$ mice, providing additional proof for the complete inactivation of the *Fgfr1* in the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice.

Neural crest cell specific rescue of the hypomorphic Fgfr1 allele

In order to understand whether *Fgfr1* is required cell-autonomously for the neural crest cell migration, our second approach was to rescue the hypomorphic *Fgfr1*^{n7} allele specifically in the neural crest cells of the *Fgfr1*$^{n7/n7}$ embryos. For this purpose, we crossed *Wnt1-Cre/+* and *Fgfr1*$^{n7/n7}$ mice to generated the *Wnt1-Cre/+; Fgfr1*$^{n7/n7}$ embryos. In the dorsal neural tube cells where Cre is active, recombination will result in excision of the *neo* cassette from the hypomorphic *Fgfr1*^{n7} allele and its conversion into the *Fgfr1*^{l7} allele functionally equal to a wild-type allele (schematically presented in I, Fig. 7). Similar to the *Fgfr1*$^{n7/n7}$ embryos, analysis of the *Wnt1-Cre/+; Fgfr1*$^{n7/n7}$ embryos revealed the second branchial arch specific defect (I, Fig. 7). Therefore, rescue of the hypomorphic *Fgfr1*^{n7} allele specifically in the neural crest cells failed to rescue the second branchial arch defect in the *Fgfr1*$^{n7/n7}$ embryos. This is consistent with observations in the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ embryos. Based on these results, we concluded that *Fgfr1* is required non-cell-autonomously for the neural crest cell migration into the second branchial arch.

Similar to *Fgfr1*$^{n7/n7}$ mice, *Wnt1-Cre/+; Fgfr1*$^{n7/n7}$ mice die neonatally and have defects in the branchial arch skeletal derivatives. However, the palatal shelves are closed and lesser horns of the hyoid bone and the pterygoid processes are normally oriented in the *Wnt1-Cre/+; Fgfr1*$^{n7/n7}$ mice (I, Fig. 7 and Supplementary data). Together, analysis of skeletal structures in the *Fgfr1*$^{n7/n7}$, *Wnt1-Cre/+; Fgfr1*$^{n7/n7}$ and *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice imply correlation between defects in palatine, pterygoid and lesser horns of the hyoid bone, and demonstrate their independency on branchial arch development.

Early patterning of pharyngeal ectoderm is affected in the *Fgfr1* mutants

A study by Veitch et al. showed that early segmentation and patterning of the pharyngeal epithelium are independent on the neural crest cells (Veitch et al., 1999). Thus, to further understand the nature of the second branchial arch defect in the *Fgfr1* hypomorphs, we have analysed pharyngeal epithelial patterning in the $Fgfr1^{n7/n7}$ mutants. For this purpose, we have used epithelial markers *Fgf3* and *Fgf8*, expressed in pharyngeal clefts and pouches, as well as *Bmp4* and *Pax1*, specifically expressed in pharyngeal pouches. Our results revealed fusion of the first and the second pharyngeal pouches and abnormal patterning of the epithelium surrounding the second branchial arch in the $Fgfr1^{n7/n7}$ embryos at E9 (I, Fig. 8).

Our studies on the *Fgfr1-3* expression patterns at E8.5, prior to migration of the neural crest cells into the second branchial arch, demonstrated that *Fgfr1* is broadly expressed in the pharyngeal region in all cell types (II, Fig. 1 and Table 1). *Fgfr2* is co-expressed with *Fgfr1* in this domain, but at the significantly lower level. Expression of *Fgfr3* was not detected in the presumptive second branchial arch region, while in the first branchial arch *Fgfr3* was detected at low level in all cell types.

In the light of studies showing that pharyngeal epithelium is crucial for the branchial arch formation (Piotrowski and Nusslein-Volhard, 2000; Wendling et al., 2000), we asked whether epithelium is the primary domain of *Fgfr1* activity in the pharyngeal region? First, we studied the timing of the onset of the neural crest cell migration defect in the *Fgfr1* hypomorphs. Neural crest cells expressing *Crabp1* normally start to populate the second branchial arch at 10-somite stage. At this stage we could not detect *Crabp1* positive cells in the second branchial arch of the $Fgfr1^{n7/n7}$ embryos. However, at 8-somite stage, before the influx of the neural crest cells into the second arch, similar expression of *Crabp1* has been detected in the $Fgfr1^{n7/n7}$ mutants and the wild type embryos (I, Fig. 9). These results reveal that the neural crest cell migration defects in the hypomorphic *Fgfr1* mutants appear at the onset of the

neural crest influx into the second branchial arch.

We then analysed whether defect in the pharyngeal epithelium precedes defect in the neural crest cells. For this purpose, we compared expression of pharyngeal epithelial marker *Fgf3* in 8-9-somite stage *Fgfr1*$^{n7/n7}$ mutants and control embryos. At this stage normal expression of *Fgf3* in the pharyngeal region is located in the surface ectoderm of the presumptive second branchial arch. However, this domain of *Fgf3* expression was strongly downregulated in the *Fgfr1*$^{n7/n7}$ mutants (I, Fig. 9 and II, Fig. 2). These results show that defects in the pharyngeal epithelium of the *Fgfr1*$^{n7/n7}$ mutants precede defects in the migratory neural crest cells. This suggests that, *Fgfr1* is required for development of a permissive environment for the neural crest cell migration into the second branchial arch.

Next, we wanted to analyze whether pharyngeal endoderm is also affected in the *Fgfr1* hypomorphs and which tissue is the primary target of the *Fgfr1* signalling. For this purpose, we studied expressions of *Fgf8*, *Fgf15*, *Spry1, Spry4* and *Erm* in the pharyngeal epithelium of *Fgfr1*$^{n7/n7}$ mutant and wild-type embryos at 8-10-somite stage. Normally, *Fgf8*, *Spry1, Spry4* and *Erm* are expressed broadly in the pharyngeal region, whereas *Fgf15* is localized to the epithelium of the presumptive second branchial arch (II, Fig. 2 and 3), similar to *Fgf3*. In *Fgfr1* hypomorphs, specific down-regulation of *Spry1* in the ectoderm of the second branchial arch was detected already at 8-somite stage (II, Fig. 3). Subsequently, we observed specific down-regulation of *Fgf15*, *Spry1*, *Spry4* and *Erm* in both ectoderm and endoderm of the presumptive second branchial arch in 9-10-somite stage *Fgfr1*$^{n7/n7}$ embryos (II, Fig. 2 and 3). In contrast, expression of *Spry1, Spry4* and *Erm* in the pharyngeal mesenchyme, as well as in all tissue-components of the first branchial arch appeared normal in *Fgfr1*$^{n7/n7}$ embryos.

Our results suggest that the surface ectoderm of the presumptive second branichial arch is the primary target for *Fgfr1* signalling. Also pharyngeal endoderm is affected. However, we do not now whether gene expression in the pharyngeal endoderm is directly regulated by *Fgfr1*, or indirectly through its interaction with the ectoderm. Localized expression patterns of *Fgf3*

and -*15*, imply existence of the local signalling centre in the surface ectoderm of the second branchial arch. We suggest that *Fgfr1* has a role in the early patterning of the pharyngeal ectoderm and establishment of this signalling centre.

Impaired differentiation of the geniculate placode and VIIth cranial nerve in hypomorphic *Fgfr1* embryos

In order to further investigate the role of the *Fgfr1* in the pharyngeal ectoderm we analyzed neuronal differentiation of the pharyngeal ectoderm in the *Fgfr1* hypomorphs. Neurofilament staining at E10.5 revealed specific deficiencies in the VIIth cranial nerve of $Fgfr1^{n7/n7}$ embryos (II, Fig. 5). Cranial nerves have heterogeneous origin, arising from both neural crest cells and ectodermal placodes. In order to examine the nature of the neuronal defect in the *Fgfr1* hypomorphs, we first studied formation of the epibranchial placodes in the *Fgfr1* hypomorphs at E9.5-10.5, using *Ngn2* probe as a molecular marker. In mutants, we observed localized downregulation of *Ngn2*, revealing specific defect in formation of the geniculate placode, related to the second branchial arch (II, Fig. 4). Next, we analyzed the neurogenic neural crest. We demonstrated that the marker for the neurogenic neural crest cells, *Sox10*, is normally expressed in the $Fgfr1^{n7/n7}$ embryos at E9 (II, Fig. 4). These results suggest that in mice with general reduction of *Fgfr1* signaling, deficient development of the geniculate placode and VIIth cranial nerve is primarily caused by defect in ectoderm.

Begbie et al. showed that pharyngeal endoderm induces formation of the epibranchial placodes through secretion of the signalling molecule *Bmp7* (Begbie et al., 1999). To understand whether defect in formation of the geniculate placode is caused by the defect in the endoderm, we studied expression of *Bmp7*. Similar pattern of *Bmp7* expression was observed in 13-somite stage $Fgfr1^{n7/n7}$ mutant and wild-type embryos (II, Fig. 4). This result implies that the placodal ectoderm in the $Fgfr1^{n7/n7}$ mutants receives inductive signal from the endoderm comparable to the normal embryos.

We suggest that general reduction of FGFR1 signalling leads to a local defect in the competency of ectoderm (corresponding to the prospective geniculate placode) to respond to the inductive signal from the endoderm. This is consistent with our molecular studies suggesting that *Fgfr1* is required for the localized gene-expression in pharyngeal ectoderm well before neurogenesis in the geniculate placode is initiated. Signals from a putative signalling centre in the presumptive second branchial arch region, including FGF3 and FGF15, might be important for the interaction between ectoderm and underlying endoderm that is in turn critical for the proper tissue integrations. Our results suggest that this local signalling centre fails to form normally in the mouse embryos with reduced signalling intensity of the FGFR1, because of the patterning defect in the pharyngeal surface ectoderm. Consequently, the neural crest migration, formation of the second branchial arch and its innervation are affected in these mutants (II, Fig. 6).

Role of the *Fgfr1* in the development of the mid- and hindbrain development (III)

Expression of *Fgfr1* and *Fgfr2* during early development of the mid- and hindbrain

Genetic loss-of-function studies both in zebrafish and mouse (Meyers et al., 1998; Reifers et al., 1998; Xu et al., 2000), have demonstrated the importance of *Fgf-s*, and *Fgf8* in particular, in the development of the midbrain–hindbrain region. However, the direct target tissues and the receptors of FGF signals were poorly understood. Studies with mice carrying null mutations in each of the *Fgfr* genes have suggested that two of these, *Fgfr1* and *Fgfr2*, carry out the majority of FGF receptor functions during early embryonic development. To study *Fgf* signaling in midbrain-hindbrain development, we first analyzed the expression of *Fgfr1* and *Fgfr2*. At a late head-fold stage (E 7.5), around the stage when the isthmic organizer is induced, expression of both genes is detected in the head folds (III, Fig. 1). At E8.5–9.5, widespread *Fgfr1* expression was observed in the developing central nervous system, including the midbrain–hindbrain region. However, no *Fgfr2* expression was detected at the midbrain–hindbrain boundary in the anterior rhombomere 1 or posterior midbrain (III, Fig. 1).

Tissue-specific inactivation of *Fgfr1* in the midbrain-hindbrain region

To study the role of *Fgfr1* in the isthmic organizer, we wanted to inactivate *Fgfr1* specifically in the neuroepithelium of the mid- and hindbrain after their regional specification. For this purpose, we have used *En1-Cre* mice (Kimmel et al., 2000). To characterize the Cre activity expressed in the *En-Cre* allele, we crossed *En-Cre* mice with *Z/AP* reporter mice. Cre activity and specific recombination in the mid- and hindbrain region of the *En1-Cre*/+; *Z/AP*/+ embryos was observed already at 8 somite stage (III, Fig. 2). Next, we crossed the $Fgfr1^{flox/flox}$ mice with the *En1-Cre* mice to inactivate *Fgfr1* specifically in the mid-and hindbrain (schematically presented in III, Fig. 2). At 10 somite stage the midbrain and the entire rhombomere 1 of the *En1-Cre*/+; $Fgfr1^{flox/flox}$ embryos, were negative for the *Fgfr1* signal (III, Fig. 2 and Supplementary data). The majority of *En1-Cre*/+; $Fgfr1^{flox/flox}$ mice survived till adulthood, but they had ataxia (impaired motor coordination) demonstrated by behavioral tests, including stationary beam and rotarod assays (III, Table 1). Consistent with the impaired motor coordination, severe defects were observed in the cerebellar structures in adult *En1-Cre*/+; $Fgfr1^{flox/flox}$ mice. The vermis of the cerebellum was completely absent and foliation of the cerebellar hemispheres was abnormal (III, Fig. 3). In addition, extensive deletions including the entire inferior colliculi were also evident in the posterior midbrain.

We also generated *En1-Cre/+;* $Fgfr1^{\Delta flox/flox}$ mutants carrying one conditional and one null allele of *Fgfr1*. These mice had same defects as *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice, further confirming successful inactivation of *Fgfr1* by *En-Cre* transgene. Consistently, analysis of the brains of the newborn mice homozygous for the hypomorphic *Fgfr1* alleles, $Fgfr1^{n7}$ and $Fgfr1^{n15YF}$ (Partanen et al., 1998), revealed almost complete absence of the cerebellar vermis and partial deletions of the inferior colliculi of the midbrain (III, Fig. 3). As expected, the phenotype of the hypomorphic *Fgfr1* mutants, expressing only 10-20% of the wild-type *Fgfr1* mRNA levels, was similar but less severe than the phenotype of the *En1-Cre/+;* $Fgfr1^{flox/flox}$ mice.

Fgfr1 is required for the expression of isthmic organizer dependent genes

Next, we wanted to understand the mechanism through which *Fgfr 1* regulates development of the mid- and hindbrain. First we analysed expression patterns of the mid-hindbrain regional markers, *Otx2, En2* and *Fgf8.* Similar expressions were detected in *En1-Cre*/+; $Fgfr1^{flox/flox}$ and wild-type embryos at E9.5-10.5. *Otx2* was detected in the midbrain, *En2* in posterior midbrain and anterior hindbrain, and *Fgf8* in the anterior hindbrain (III, Fig. 4). Therefore, these results demonstrated that the isthmic organizer is present and correctly positioned in the E9.5-E10.5 *En1-Cre*/+; $Fgfr1^{flox/flox}$ embryos. In contrast, expression of genes thought to depend on isthmic signals, including *Sprouty1* and *Pax2*, were clearly affected in the *En1-Cre*/+; $Fgfr1^{flox/flox}$ embryos at E9.5-10 (III, Fig. 4 and Supplementary data). *Sprouty1* was completely abolished from the isthmic domain. Expression of *Pax2* at the mid- and hindbrain boundary was decreased already at E9.5 and virtually absent at E10. *Wnt1* was downregulated in the posterior midbrain. These results demonstrate that FGFR1 is involved in maintaining expression of isthmus-dependent genes and they confirm our suggestion that FGFR1 is the primary FGF receptor receiving isthmic signals.

Tissue-specific inactivation of *Fgfr1* in the midbrain

We wanted to understand whether both midbrain and hindbrain are direct targets of *Fgf* signalling. Therefore we decided to inactivate *Fgfr1* specifically in the midbrain, using the *Wnt1-Cre* transgene. First we analyzed the patterns of Cre activity in *Wnt1-Cre/+* mice, by crossing them with the *Z/AP/+* reporter mouse line. The *Z/AP* allele was observed to be recombined efficiently and specifically in the midbrain of the *Wnt1-Cre/+; Z/AP/+* embryos already at E8.5. As expected, Cre activity was absent from the rhombomere 1 (III, Fig. 2). We next generated *Wnt1-Cre*/+; $Fgfr1^{flox/flox}$ embryos to inactivate the $Fgfr1^{flox}$ allele specificaly in the midbrain (schematically presented in III, Fig. 2). At E9.5, *Fgfr1* expression signal could not be detected in the midbrain of the *Wnt1-Cre*/+; $Fgfr1^{flox/flox}$ embryos, whereas abundant *Fgfr1* signal was detected in the rhombomere 1 (III, Fig. 2).

Analysis of the brains of the newborn *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mice revealed deletion of the inferior colliculi of the midbrain, reminiscent of the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice (III, Fig. 5). Development of the dorsal cerebellum was also abnormal. However, in contrast to the *En1-Cre/+; Fgfr1*$^{flox/flox}$ mice, the vermis was not completely missing although it was severely malformed in the *Wnt1-Cre/+; Fgfr1*$^{flox/flox}$ mutants (III, Fig. 5). Comparable phenotype was observed in newborn *Wnt1-Cre/+; Fgfr1*$^{\Delta flox/flox}$ mice.

Fgfr1 is independently required in both midbrain and hindbrain

Our next aim was to understand whether *Fgfr1* directly regulates gene expressions on both sides of the isthmic organizer. Therefore, we analysed the expression of isthmic genes *Otx2*, *En2* and *Fgf8* in the *Wnt1-Cre*/+; *Fgfr1*$^{flox/flox}$ and wild-type embryos at E9.5-10.5. Our results show that in the midbrain specific *Wnt1-Cre*/+; *Fgfr1*$^{flox/flox}$ mutants the isthmic organizer forms and is correctly positioned, similar to the *En1-Cre*/+; *Fgfr1*$^{flox/flox}$ mutants (III, Fig. 6). However, expression of isthmus regulated genes was abnormal specifically in the midbrain of *Wnt1-Cre*/+; *Fgfr1*$^{flox/flox}$ mutants (III, Fig. 6). *Sprouty1*, although still normally expressed in the hindbrain, was markedly downregulated in the midbrain in E9.5 *Wnt1-Cre*/+; *Fgfr1*$^{flox/flox}$ mutants. Similarly, *Pax2* was downregulated specifically in the midbrain by E10 in *Wnt1-Cre*/+; *Fgfr1*$^{flox/flox}$ mutants. Thus, our results show that *Fgfr1* is independently required in both midbrain and hindbrain for the maintenance of isthmic dependent gene expression.

CONCLUSIONS AND FUTURE PROSPECTS

In this study we have analysed roles of *Fgfr1* in the early craniofacial and mid- and hindbrain development. Using a hypomorphic *Fgfr1* allele, we show that *Fgfr1* is necessary for formation of the proximal region of the second branchial arch. We demonstrate that perturbation of *Fgfr1* function leads into a failure in neural crest cell entry into the second branchial arch. Both rescue of the hypomorphic *Fgfr1* allele and inactivation of a conditional *Fgfr1* allele specifically in neural crest cells suggest that *Fgfr1* regulates the entry of neural crest cells into the second branchial arch non-cell-autonomously (I). Furthermore, we demonstrate that the first molecular defect in hypomorphic *Fgfr1* mutants is localized down-regulation of gene expression in the surface ectoderm of presumptive second branchial arch (I and II). Additionally, ectoderm associated with the second arch fails to respond to the neurogenic signal from endoderm, resulting in defective formation of the geniculate epibranchial placode in *Fgfr1* hypomorphs (II). Thus, our results strongly suggest that *Fgfr1* is primarily needed for the patterning of the pharyngeal ectoderm.

Together with other studies, our results suggest that *Fgf* signaling is involved in different signalling centers that regulate formation of the pharyngeal region. *Fgf* signalling from the isthmic organizer was shown to regulate patterning of the posterior midbrain and anterior hindbrain as well as the neural crest cells deriving from this region (Trainor et al., 2002). Our studies of *Fgf* signalling in the mid- and hindbrain region, demonstrate that FGFR1 is the primary FGF receptor receiving signals from the isthmic organizer, and that it has direct functions on both sides of the organizer (III). Furthermore, we propose existence of FGF signalling centre in the ectoderm covering presumptive second branchial arch (II). Based on our results *Fgfr-1* is required for establishment of this putative signalling centre (II), in contrast to the isthmic organizer where *Fgfr1* is required for its maintenance (III). Signalling from overlying ectoderm appears to be important for development of both the second branchial arch and geniculate placode. One possible source of inductive FGF signals is rhombomere 4. In zebra fish and mice, rhombomere 4/5 has been shown to be a transient

source of FGF signals required for patterning of the hindbrain and induction of the otic placode (Alvarez et al., 2003; Leger and Brand, 2002; Maroon et al., 2002; Mahmood et al., 1996; Maves et al., 2002; Walshe et al., 2002; Wright and Mansour, 2003). Being at the same axial level as the second branchial arch, rhombomere 4 is also potential inducer of the signalling centre in ectoderm overlying presumptive second branchial arch. Once the branchial arches are formed, new transient epithelial signalling centres appear between arches and coordinate their outgrowth and differentiation. Our results propose role of *Fgfr1* also in these signalling centres (I).

This study allows us to present a model of how *Fgfr1* contributes to the formation of the second branchial arch (II). *Fgfr1* is primarily needed for the patterning of the pharyngeal ectoderm and formation of a local signalling centre in ectoderm overlying the presumptive second branchial arch. Subsequent interactions between pharyngeal ectoderm and endoderm ensure proper integration of the second branchial arch cell types and formation of the geniculate placode. This will result in complex anatomy of the second branchial arch, surrounded by pharyngeal clefts and pouches, and innervated by VIIth cranial nerve.

To further confirm presumptive role of *Fgfr1* in regionalization of the pharyngeal ectoderm, expression of additional regional markers should be analyzed. One way to test our model would be to rescue *Fgfr1* hypomorph mutant and/or to inactivate *Fgfr1* specifically in the ectoderm of the second branchial arch. Alternatively, mutant embryos could be cultured and grafted with the wild-type pharyngeal ectoderm or with beads of FGF3 and/or FGF15 close to the second branchial arch to see whether they can rescue the phenotype. Co-expression of *Fgfr1-3* suggests that they may have to some extent redundant roles in the pharyngeal region. Therefore, getting the complete picture of the *Fgf* signalling in the pharyngeal region requires generation of transgenic mice with different combinations of mutated *Fgfr* genes. Newborn *Wnt1-Cre*/+; $Fgfr1^{flox/flox}$mice have midfacial defects. One posibility is that they are caused by defect in the neural crest cell migration and or survival. As neural and head development are

interdependent (Schneider et al., 2001), the clefting in the midfacial region may also be linked to defect in the brain development.

From the broader perspective, our study supports view that the formation of different branchial arches rely on distinct developmental mechanisms and that the patterning of the proximal and distal parts of the branchial arches are separately regulated. It also emphasizes importance of the pharyngeal ectoderm in formation of the branchial arches and patterning of the neural crest cells. This study again demonstrates that the same signalling molecules are repeatedly used in tissue interactions at different points during development.

Understanding the genetic programs and tissue interactions that direct branchial arch patterning are critical when considering evolution during craniofacial morphogenesis in craniates. Due to recent findings on branchial arch development, the evolutionary studies will redirect focus of their interest from the neural crest cells toward pharyngeal epithelium as the main source of changes in patterning of craniofacial structures. Furthermore, these studies are crucial for understanding and curing diseases which are caused by defects during pharyngeal development. More emphasis will be put on changes in the patterning of the endoderm and ectoderm for an explanation of why these defects occur.

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