Fibroblast growth factor signalling and regional specification of the pharyngeal ectoderm

NINA TROKOVIC, RAS TROKOVIC and JUHA PARTANEN*
Institute of Biotechnology, Viikki Biocenter, University of Helsinki, Finland

ABSTRACT  Branchial arch development involves dynamic interactions between neural crest cells as well as ectodermal, endodermal and mesodermal cell populations. Despite their importance and evolutionary conservation, the intercellular interactions guiding the early development of the branchial arches are still poorly understood. We have here studied fibroblast growth factor (FGF) signalling in early pharyngeal development. In mice homozygous for a hypomorphic allele of Fgfr1, neural crest cells migrating from the hindbrain mostly fail to enter the second branchial arch. This defect is non-cell-autonomous suggesting that Fgfr1 provides a permissive environment for neural crest cell migration. Here we demonstrate localized down-regulation of the expression of the FGF responsive gene, Sprouty1 in the epithelium covering the presumptive second branchial arch of hypomorphic Fgfr1 mutants. This appears to result in a failure to establish an ectodermal signalling center expressing Fgf3 and Fgf15. We also studied differentiation of the ectoderm in the second branchial arch region. Development of the geniculate placode as well as the VIIth cranial ganglion is affected in Fgfr1 hypomorphs. Our results suggest that Fgfr1 is important for localized signalling in the pharyngeal ectoderm and consequently for normal tissue interactions in the developing second branchial arch.

KEY WORDS: FGF signalling, fgfr1, patterning, mouse embryo, branchial arch, epibranchial placode, cranial nerve

Introduction

Six pairs of branchial arches form around pharyngeal foregut of developing mouse embryos. These bud like structures contain a core of paraxial mesoderm and aortic arch artery, which are surrounded by neural crest cells. The arches are covered with surface ectoderm from outside and pharyngeal endoderm from inside. Ectodermal pharyngeal clefts and endodermal pharyngeal pouches separate branchial arches in areas where ectodermal and endodermal cells are in direct contact.

Branchial arches arise in antero-posterior order between 8-11 days of embryonic development in the mouse, after which their cell types rearrange and further differentiate into terminal structures of head and neck regions. Neural crest cells form skeletal and connective structures, cranial paraxial mesoderm forms craniofacial muscles and endothelium, endoderm forms pharyngeal and middle ear epithelium and glandular structures while ectoderm forms epidermis and external acoustic meatus. Ectoderm also forms thickenings, called epibranchial placodes, which give rise to some of the sensory neurons of the cranial ganglia (Kaufman and Bard, 1999). Each arch also has its own identity and contributes to specific structures. For example, the second branchial arch contributes to the stapes of the middle ear, the styloid process of the temporal bone, the lesser horns of the hyoid bone, the facial nerve, muscles of facial expression and the stapedial artery.

Majority of earlier studies have highlighted the importance of the neural crest in the craniofacial patterning (Noden, 1983, 1988). Cranial neural crest cells, which originate from the dorsal part of the posterior midbrain and rhombomeres of the hindbrain, migrate ventrally in three distinct streams toward the branchial arches (Lumsden et al., 1991, Serbedzija et al., 1992). Early migrating neural crest cells populate the arches while late migrating crest cells form sensory nerve ganglia. Neural crest cells interact with other tissues and were thought to be the major player coordinating the integration of tissues into specific structures (Noden, 1983, Kontges and Lumsden, 1996). It was suggested that the segmental pattern of the hindbrain, encoded by Hox genes, is transmitted by neural crest cells to the branchial arches and cranial ganglia (Hunt et al., 1991). However, more recent data indicated that the initial development of these structures does not

Abbreviations used in this paper: fgf, fibroblast growth factor; fgfr, fibroblast growth factor receptor.

What are then the tissues and signals responsible for patterning of the pharyngeal region? Cranial paraxial mesoderm is the other mesenchymal cell type of the branchial arches. It fills the core of the arch and differentiates into muscular structures (Meier and Tam, 1982). On its migratory route toward brancial arches, paraxial mesoderm is co-distributed with the neural crest cells originating at the same level along the antero-posterior axis. Studies on the craniofacial muscles indicated that this tissue functions passively in the head patterning, receiving instructive signals from the neural tube and the neural crest (Noden, 1983, 1986, Trainor et al., 1994, Kontges and Lumsden, 1996). However, more recent studies demonstrated that the paraxial mesoderm is a source of permissive signals important for maintaining expression of Hox genes in the neural crest (Trainor and Krumlauf, 2000) and suggested that it could be involved in regulation of the neural crest cell migration (Trainor et al., 2002a).

Recent studies have indicated importance of the endoderm in the pharyngeal patterning. Localized invaginations of the pharyngeal endoderm at sites between presumptive branchial arches, form segmentally organised pharyngeal pouches. They are important for directing the neural crest cell streams into separate branchial arches (Piotrowski and Nusslein-Volhard, 2000) as well as for induction of the epibranchial placodes (Begbie et al., 1999). Furthermore, recent analysis in chick (Couly et al., 2002) demonstrated that distinct stripes of the foregut endoderm send differential instructive signals to skeletogenic neural crest cells along the antero-posterior axis. Same study revealed that this antero-posterior regionalization of the foregut endoderm exists already at a stage before formation of the pharyngeal pouches.

Much less is known about early developmental function of the cranial surface ectoderm. Its inductive role was reported only at the later stage of the craniofacial development, during skeletal differentiation of the neural crest cells (Bee and Thorogood, 1980, Tucker and Sharpe, 1999). During embryonic development, cranial surface ectoderm forms antero-posterior series of the pharyngeal clefts and the epibranchial placodes. Already prior to formation of these segmentally organized structures, cranial ectoderm becomes regionalised into discrete domains known as ectomeres (Couly and Le Douarin, 1990). Although their function is not defined, distinct ectomeres coincide with the underlying streams of the migratory neural crest cells. Along this line, signaling from the surface ectoderm was suggested to be involved in regulation of the neural crest cell migration (Golding et al., 2002, Trainor et al., 2002a).

Growth factors of Wnt, Fgf and Tgf-β families as well as Shh are signalling molecules which are expressed at different stages of the pharyngeal development. The fibroblast growth factors (FGFs) are encoded by 22 Fgf gene family members in the mouse. Numerous studies showed that FGFs are repeatedly involved in instructive signaling during embryonic develop-
ment, for instance in regional specification and patterning of the hindbrain (Marin and Charnay, 2000, Waskiewicz et al., 2002) and branchial arch area (Tucker and Sharpe, 1999, Shigetani et al., 2000, Trainor et al., 2002b, David et al., 2002).

The effects of FGFs are mediated by four tyrosine kinase-type receptors, FGFR1-FGFR4. Fgfr1 null mutants are unable to gastrulate normally and die early during gestation (Deng et al., 1994, Yamaguchi et al., 1994). However, mice homozygous for a hypomorphic (partial loss-of-function) Fgfr1 allele survive till birth (Partanen et al., 1998). Analysis of hypomorphic Fgfr1 mutants revealed defects in formation of the second branchial arch and in skeletal structures deriving from the first and second arch neural crest. We demonstrated that in hypomorphic Fgfr1 mutants neural crest cells migrating from rhombomere four level mostly fail to enter the second branchial arch. This neural crest migration defect is non-cell-autonomous and thus Fgfr1 appears to be necessary for development of a permissive environment for neural crest cell migration into the second branchial arch (Trokovic et al., 2003a).

Here we further define molecular and cellular nature of the branchial arch defect in hypomorphic Fgfr1 mutants. We confirm our previous finding that Fgfr1 is needed to establish appropriate patterns of gene expression in the cranial ectoderm overlying the presumptive second branchial arch and show that differentiation of this domain is affected in hypomorphic Fgfr1 mutants. Localized expression patterns of Fgf3 and Fgf15 imply existence of a putative signalling centre in the surface ectoderm of the second branchial arch. Together, our results suggest that FGFR1 regulates establishment of this signalling center, which is required for normal integration and differentiation of the second branchial arch cell types.

Results

Expression of Fgf-s, Fgfr-s and FGF responsive genes in the pharyngeal region

To get insight into Fgf signalling in the pharyngeal region, we first analysed expression of receptors Fgfr1-3 ligands Fgf3, Fgf8, Fgf15, Fgf17 and Fgf18 and downstream targets Spry1 and Spry4 in the developing branchial arches. We focused our studies on mouse embryos at 7-9-somite stage, the time point prior to appearance of morphological defect in the second branchial arch of Fgfr1 hypomorphs. For summary of the results, see Table 1.

Table 1

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<tr>
<th>Gene</th>
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<tr>
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*Fgf3 signal appears in endoderm shortly after expression in ectoderm.
+ low expression signal; ++, strong expression signal; -, absent expression signal; BA1, first branchial arch; BA2, second branchial arch; Ect, ectoderm; End, endoderm; M, mesenchyme; R4-6, rhombomere 4-6.
We found that \textit{Fgfr1} is broadly expressed in the pharyngeal region. \textit{Fgfr1} transcripts were detected in all the cell types of the branchial arches (Fig. 1 A,B,C). \textit{Fgfr2} was co-expressed with \textit{Fgfr1} in this domain, but at a significantly lower level (Fig. 1 D,E,F). Expression of \textit{Fgfr3} was not detected in the presumptive second branchial arch region (Fig. 1 G,H,I), while in the first branchial arch \textit{Fgfr3} was detected at low level in all cell types. Co-expression of \textit{Fgfr1}, \textit{Fgfr2} and \textit{Fgfr3} imply that they may have to some extent redundant roles in the branchial arch region.

Broad expression of \textit{Fgfr1} and \textit{Fgfr2} in the branchial arch region suggests that Fgf signaling specificity depends on the localized expression of Fgf ligands. We studied expression patterns of \textit{Fgfr3}, \textit{Fgf8}, \textit{Fgf15}, \textit{Fgf17} and \textit{Fgf18} genes in 7-9-somite stage embryos. We did not detect expression of \textit{Fgf}17 and \textit{Fgf18} in the pharyngeal region (data not shown). \textit{Fgf8} was found to be broadly expressed in the ectoderm and endoderm of the branchial arch area (Fig. 2 G,I). This observation is consistent with the previous studies of \textit{Fgf}8 expression pattern (Wall and Hogan, 1995). \textit{Fgf3} (Fig. 2A; Mahmood et al., 1996) and \textit{Fgf15} (Fig. 2C; McWhirter et al., 1997) were first detected at 8-somite stage in the pharyngeal region where their expression was found to be more restricted to the presumptive second arch area. \textit{Fgfr3} was detected in neuroectoderm and surface ectoderm. \textit{Fgfr3} expression in rhombomeres 4-6 continued in the surface ectodermal domain involving the otic placode and the presumptive second branchial arch region. \textit{Fgf15} was detected throughout the hindbrain except in the rhombomere 3. \textit{Fgf15} expression extended ventrally from the hindbrain toward the presumptive second branchial arch in both ectoderm and endoderm (Fig. 2E). Similar to \textit{Fgfr3}, surface ectodermal expression of \textit{Fgf15} included the otic placode and the presumptive second arch regions. Neither \textit{Fgfr3} nor \textit{Fgf15} transcripts were detected in the mesenchymal cells. Spatial and temporal expression patterns of \textit{Fgfr3} and \textit{Fgf15} suggest that they are important specifically for the second branchial arch formation and that there could be some functional redundancy between them.

We also analysed expression of the downstream targets of Fgf signalling, \textit{Sprouty1} (\textit{Spry1}) and \textit{Sprouty4} (\textit{Spry4}), in 7-9-somite stage embryos. Whole mount \textit{in situ} hybridization revealed broad expression of \textit{Spry1} and \textit{Spry4} in the pharyngeal region (Fig. 3 A,G; Minowada et al., 1999). \textit{Spry1} displayed graded expression with the highest expression at the level of rhombomere 4. To detect expression of \textit{Spry1} at the cellular level, whole mount \textit{in situ} hybridization treated embryos were sectioned in transverse plane with a vibratome. Expression of \textit{Spry1} was found both in ectodermal, endodermal and mesenchymal cells of the branchial arches (Fig. 3 A,B,C). \textit{Spry4} was detected in mesenchyme and at a lower level in the pharyngeal endoderm (Fig. 3 G,H). Expression patterns of \textit{Spry1} and \textit{Spry4} correlate with broad \textit{Fgf}8 expression in the pharyngeal epithelium and suggest that FGF signalling is active in all the pharyngeal cell types.

**\textit{Fgfr1} regulates gene expression in the epithelium of the presumptive second branchial arch**

In the previous study we showed that \textit{Fgfr1} expression in the pharyngeal ectoderm overlying the developing second branchial arch

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**Fig. 3. Expression patterns of \textit{Spry1} and \textit{Spry4} in the pharyngeal region of wild-type \textit{Gfgr1}n7/n7 embryos.** Whole-mount RNA \textit{in situ} hybridization at 7-somite stage with \textit{Spry1} \textit{(A,D)} and at 8-somite stage with \textit{Spry4} \textit{(G,J)}. \textit{Spry1} and \textit{Spry4} are expressed broadly in the pharyngeal region of control embryos \textit{(A,G)}. In mutants \textit{(D,J)}, pharyngeal expression of \textit{Spry1} and \textit{Spry4} appears normal in the first branchial arch but down-regulated posterior to it (indicated by arrows in \textit{(D,I)}). Expression of \textit{Spry1}, \textit{(B,E)} and their closeups in \textit{(C,F),} and \textit{Spry4} \textit{(H,J)} in wild-type \textit{(B,C,H)} and mutant embryos \textit{(E,F,J)}, was examined on transverse vibratome sections through the presumptive second arch (levels of sections are indicated in \textit{(A,E)}). Note that only ectodermal expression of \textit{Spry1} is affected in \textit{Gfgr1}n7/n7 embryos (indicated by arrowhead in \textit{(E,F)}): BA1, first branchial arch; Ect, ectoderm; End, endoderm; H, heart; M, mesenchyme; R4, rhombomere 4.
was affected in embryos homozygous for a hypomorphic \textit{Fgfr1\textsuperscript{n7/n7}} allele, which expresses reduced levels of the \textit{Fgfr1} transcript (Partanen \textit{et al.}, 1998, Trokovic \textit{et al.}, 2003a). Down-regulation of \textit{Fgf3} was observed already at a stage prior to neural crest entry into the arch (Trokovic \textit{et al.}, 2003a). To confirm this result and to further understand the role of \textit{Fgfr1} in the branchial region, we analyzed additional pharyngeal epithelial markers in normal and \textit{Fgfr1\textsuperscript{n7/n7}} embryos at 7-9-somite stage.

\textit{Fgf8} expression, detected by whole mount \textit{in situ} hybridization (Fig. 2. G-J), was unchanged in the \textit{Fgfr1\textsuperscript{n7/n7}} mutants compared to control embryos. Similar to \textit{Fgf3} (Fig. 2 A,B; Trokovic \textit{et al.}, 2003a), \textit{Fgf15} expression was affected in the \textit{Fgfr1\textsuperscript{n7/n7}} embryos compared to wild-type (Fig. 2 C,D). \textit{Section in situ} hybridization revealed that ectodermal as well as endodermal expression is strongly down-regulated in mutant embryos compared with wild-type (Fig. 2 E,F).

In the \textit{Fgfr1\textsuperscript{n7/n7}} embryos, expression of \textit{Spry1} and \textit{Spry4} appeared normal in the first branchial arch, but was down-regulated in the pharyngeal region around the presumptive second branchial arch, as shown by whole mount \textit{in situ} hybridization (Fig. 3 A,D,G,I). Wild-type and \textit{Fgfr1\textsuperscript{n7/n7}} embryos, labelled with \textit{Spry1} and \textit{Spry4}, were sectioned in the transverse plane through the presumptive second branchial arch (Fig. 3 B,C,E,F,H,J). Already at 7-somite stage, strong reduction of \textit{Spry1} transcripts was detected in the ectoderm of the presumptive second branchial arch in the \textit{Fgfr1\textsuperscript{n7/n7}} embryos compared with controls (Fig. 3 B,C,E,F). This was the earliest molecular change which we were able to detect in the \textit{Fgfr1\textsuperscript{n7/n7}} embryos. Interestingly, expression of \textit{Spry1} (Fig. 3 B,C,E,F), as well as \textit{Spry4} (Fig. 3 H,J), was detected both in the pharyngeal endoderm and mesenchymal of
the Fgfr1<sup>n7/n7</sup> mutants. Molecular changes in the epithelium of the presumptive second branchial arch in the Fgfr1<sup>p27n7</sup> embryos imply that Fgfr1 is needed to establish appropriate patterns of gene expression in this domain.

**Impaired differentiation of the geniculate placode in Fgfr1<sup>n7/n7</sup> embryos**

To further investigate the potential role of Fgfr1 in the pharyngeal ectoderm we analyzed its differentiation in the Fgfr1<sup>n7/n7</sup> embryos. Surface ectoderm at distinct sites in the proximal region of the branchial arches gives rise to the epibranchial placodes and undergoes neurogenesis, revealed by Neurogenin 2 (Ngn2) expression (Sommer et al., 1996). We studied formation of the first (geniculate) epibranchial placode, which is related to the second branchial arch. We detected significantly lower number of Ngn2 positive cells in the geniculate placode of E9.5-10.5 Fgfr1<sup>n7/n7</sup> embryos compared with control embryos (Fig. 4 A-D). The degree of Ngn2 down-regulation correlated with the severity of the second branchial arch defect in the Fgfr1<sup>p27n7</sup> embryos. Expression of Ngn2 in the petrosal and nodose epibranchial placodes, related to the third and fourth branchial arches respectively, appeared normal in the mutants (Fig. 4 A,B,C,D).

Geniculate placode gives rise to neurons of the VIIth cranial nerve, protruding their axonal processes toward rhombomere four and the second branchial arch (see below) is not caused by the primary defect in neural crest cells. At E10.5 Sox10 expression pattern in the Fgfr1<sup>p27n7</sup> embryos revealed failure of the facial nerve to innervate the second branchial arch (Fig. 4 G,H). This defect probably reflects lack of normal cues in the second branchial arch of the Fgfr1<sup>p27n7</sup> mutants, which are needed for the neuronal innervation of the arch.

Bmp7 from the endoderm was shown to be the signal needed for induction of the epibranchial placodes in the ectoderm (Begbie et al., 1999). Thus, defect in the endoderm could lead to defect in formation of the geniculate placode observed in the Fgfr1<sup>n7/n7</sup> embryos. In order to address this possibility, we looked at the expression of Bmp7 in 13-somite stage control and mutant embryos (Fig. 4 I-L). We observed similar patterns of Bmp7 expression in wild-type and the Fgfr1<sup>p27n7</sup> embryos. Consistent with this we have detected Pax1 expression in the pharyngeal endoderm of both mutant and wild-type embryos at 10-somite stage (Fig. 4 M,N). These results suggest that the placodal ectoderm in the Fgfr1<sup>n7/n7</sup> embryos receives inductive signal from the endoderm comparable to control embryos and that defect in geniculate placode formation is not caused by a defect in the endoderm.

Neural cells from the geniculate placode migrate inward and give rise to distal ganglia of VIIth cranial nerve, protruding their axonal processes toward rhombomere four and the second branchial arch (D’Amico-Martel and Noden, 1983). Using neurofilament staining at E10.5, we revealed deficient development of VIIth cranial nerve in Fgfr1<sup>p27n7</sup> embryos (Fig. 5 A,B). Consistent with observed defect in formation of the geniculate placode. In the mutant embryos the VIIth cranial ganglion and its axonal projections were disorganized and reduced and bridging was seen between VIIth and IXth cranial nerves (arrow in Fig. 5 B).

The VIIth cranial ganglion, derived from the otic vesicle, is positioned next to the VIIth ganglion. In order to distinguish between these two ganglia we have looked at Fgf10 expression (Pirvola et al., 2000). At E10.5, similar pattern of Fgf10 expression was detected in Fgfr1<sup>p27n7</sup> and wild-type embryos (Fig. 5 C,D). VIIth ganglion appeared normal on the histological sections of E12.5 Fgfr1<sup>p27n7</sup> embryos, whereas the VIIth ganglion was drastically reduced (Fig. 5 E,F). Thus our results suggest that an ectodermal defect in the differentiation of the geniculate placode leads to abnormal development of the VIIth ganglion.

**Discussion**

FGF signalling appears to be important for multiple tissue-interactions during pharyngeal development, both within and be-
between different germ layers. Our results demonstrate that general reduction in the signalling intensity of one of the FGF receptors (Fgfr1) leads to a molecular defect in a specific domain of ectoderm overlying the presumptive second branchial arch. Signalling from this region appears to be important for development of both the second branchial arch and geniculate plaque.

In the hypomorphic Fgfr1<sup>n7/n7</sup> embryos, rhombomere 4 derived neural crest cells mostly fail to enter the second branchial arch. This defect is non-cell-autonomous, as a neural crest cell – specific inactivation of Fgfr1 does not result in early second branchial arch defects, suggesting that Fgfr1 is providing permissive environment for the neural crest cell migration (Trokoiv et al., 2003a). To address the primary target tissue requiring FGFR1 signalling, we analysed expression of known target genes of FGF signalling. We found that already at 8-somite stage Fgfr1<sup>n7/n7</sup> embryos display down-regulation of Spry1 in the ectoderm of the presumptive second branchial arch. This is the first defect observed in these mutants. Thereafter, pharyngeal epithelial markers Fgf15 (in both ectoderm and endoderm) and Fgf3 (in the ectoderm) are down-regulated within a narrow window of time. We propose that Fgfr1 is important for expression of the ectodermal signalling molecules and consequently for the normal patterning of other branchial arch components. We can not completely rule out the possibility that the changes in the ectodermal gene-expression in the Fgfr1<sup>n7/n7</sup> mutants are caused by a defect in another tissue, for example endoderm. However, the normal level of Pax1 and Bmp7 transcripts in the pharyngeal endoderm of the Fgfr1<sup>n7/n7</sup> mutants at E8.5 suggest that the endoderm is correctly patterned in the hypomorphic Fgfr1 mutants at this stage. Ectoderm and endoderm – specific Fgfr1 inactivation can be expected to provide a definitive answer to the question of target tissue specificity.

In addition to changes in the gene-expression, we demonstrated that this specific domain of ectoderm fails to differentiate normally in Fgfr1<sup>n7/n7</sup> embryos. Cranial nerves have heterogeneous origin, arising from both neural crest cells and ectodermal placodes. In the Fgfr1<sup>n7/n7</sup> embryos development of the Vth cranial nerve is affected. We observed fewer Ngn2 expressing cells in the geniculate placode of the Fgfr1<sup>n7/n7</sup> embryos, whereas expression of Sox10, marking the neuronal crest cells, was normal at E9.5. These results imply that cranial nerve defects in the Fgfr1<sup>n7/n7</sup> mutants are initiated in its ectodermal component.

Pharyngeal endoderm has been shown to be the source of the inductive signal Bmp7, which is necessary and sufficient for induction of neurogenesis in epibranchial placodes (Begbie et al., 1999). In the Fgfr1<sup>n7/n7</sup> embryos Bmp7 is normally expressed in the pharyngeal endoderm. Thus, we suggest that Fgfr1 is required in the surface ectoderm of the future second arch well before neurogenesis in the geniculate placode is initiated by Bmp7 from the pharyngeal endoderm.

What could be the FGF ligand activating FGF1 in the ectoderm? Based on the expression analyses performed by us and others, FGF8 appears to be one possible candidate. Fgfr1 is expressed both in pharyngeal ectoderm and endoderm and FGF8 could thus activate FGF1 in either autocrine or paracrine fashion. However, Fgfr1 expression is widespread in the pharyngeal region. Therefore, additional factors must contribute to the localized activation of Fgfr1 in the ectoderm overlying the presumptive second branchial arch.

In summary, our results shed light on the molecular basis of intercellular interactions in the pharyngeal region (Fig. 6). We show that signalling through Fgfr1 is needed for establishment of a local signalling center revealed by Fgf3 and Fgf15 expression. Perhaps similar to other secondary organizing centers involving FGFs, such as the isthmus, the anterior neural ridge and hindbrain (Marin and Charnay, 2000), we propose organizing role for
FGF signalling in the ectoderm covering the presumptive second arch. However, in contrast to many of the well-studied secondary organizers this ectodermal domain appears to be only a transient source of signals, Fgf3 and Fgf15 being expressed only at 8-12-somite stage. Interestingly, this domain appears to be at the same axial level as rhombomere 4, which has been shown to be a source of FGF signals patterning the surrounding rhombomeres (Maves et al., 2002, Walshe et al., 2002). Rhombomere 4 might also induce Fgf3 and Fgf15 expression in the overlying ectoderm, but the other tissues are possible sources of such signals as well (Fig. 6A). In addition to the ectoderm itself, this signalling center in the pharyngeal ectoderm may have impact also on other cell types, including neural crest, pharyngeal endoderm as well as aortic arches. We suggest that defective development and integration of distinct tissues in the second branchial arch region (failure of neural crest cells to populate the second arch, fusion of the first and the second pharyngeal pouch, disintegrated second aortic arch, deficient geniculate epibranchial placode and VIIth cranial nerve) are probably all related to the defect in the pharyngeal ectodermal signalling center in the Fgfr1<sup>n7</sup>n7 mutants.

Materials and Methods

Embryos
Analyses of mice carrying hypomorphic Fgf1 allele (Fgf1<sup>n7</sup>; Partanen et al., 1998) were carried out in outbred (ICR) background. Embryonic age was estimated by counting somites. Wild-type allele was detected by upstream 5'-CCCCATCCCATTTCTTCTACCT-3' and downstream 5'-TCCTGTGTGTCTGAAACAGCT-3' oligonucleotide primers (145 bp product). Fgfr1<sup>n7</sup> allele was detected by upstream 5'-AATAGGTCCCTCGACGGTATC-3' and downstream 3'-CTGGGTCAGTGTGGACAGTGT-5' primers (166 bp product). Embryos for in situ hybridization were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) at 4°C overnight.

In situ hybridization
Whole-mount RNA in situ hybridization was performed as described by (Henrique et al., 1995). For vibratome sectioning stained embryos were embedded into gelatine-albumin (0.45% gelatin, 25%albumin, 20% sucrose) fixed with 2.5% glutaraldehyde and cut at 20 μm in the transverse plane. In situ hybridisation on paraffin-embedded tissue sections, cut at 7μm, was performed using 35S labelled riboprobes as described (Wilkinson and Green 1990).

Antisense riboprobes were as follows: Fgfr1 (Trokovic et al., 2003b), Fgfr3 (Peters et al., 1993), Fgf3 (Wilkinson et al., 1988), Fgf8 (Crosby and Martin, 1995), Fgf10 (Bellusci et al., 1997), Fgf15 (McWhirter et al., 1997), Spry1 and Spry4 (gift from Seppo Vainio), Pax1 (IMAGE 1327502), Erm (IMAGE 3674281), Bmp7 (IMAGE 5121825), Sox10 (IMAGE 4165363) and Ngn2 (IMAGE 2922473). On average five and at least three mutants and same number of littermate controls were hybridized with each probe.

Anti-neurofilament Immunohistochemistry
Whole mount neurofilament staining with monoclonal anti-neurofilament antibody (Sigma N-5139) was performed according to combination of procedures by (Dent et al., 1989; LeMotte et al., 1989).

Acknowledgements
We thank Eija Kolunen and Päivi Hannuksela for expert technical assistance. This work was supported by the Academy of Finland, the Sigrid Juselius foundation, Biocentrum Helsinki and Viikki Graduate School in Biosciences (N.T., R.T.).

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Received: February 2005
Reviewed by Referees: May 2005
Modified by Authors and Accepted for Publication: July 2005