Nkx5 genes in inner ear development and genome evolution

Doctoral Thesis

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by

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1 INTRODUCTION

1.1 Why Nkx5-1?

In the present work I attempted to unravel a part of the complicated genetic network that leads to inner ear formation. The transcription factor Nkx5-1 was the central molecule of all my investigations. Nkx5-1 is one of the earliest markers of ear development, and the phenotype of mice deficient for the Nkx5-1 gene documents its critical function in the formation of semicircular canals (Hadrys et al., 1998). Previous experiments performed in our laboratory proved strong conservation of its expression pattern, gene structure and sequence in several vertebrate species (Rinkwitz-Brandt et al., 1996; Herbrand et al., 1998; Wolff, 1997). These data, and expression of Nkx5-1 in an evolutionary conserved part of the inner ear, led me to assume that it has a conserved function in ear morphogenesis. Several animal model systems, such as mouse, chicken, medaka and zebrafish, were used to prove this hypothesis. In the course of my work several Nkx5-1 relatives were isolated from various species. Together with published data of Nkx5-1 related genes from vertebrates and invertebrates, these findings were the starting point for my attempt to decipherate the evolutionary history of the Nkx5 family.

1.2 Aims of the project

i. Characterization of the medaka Nkx5 genes (sequence, genomic structure, expression patterns) and establishing phylogenetic relationships between them and the previously described members of the Nkx5 family.

ii. Establishing the position of the Nkx5-1 gene in the genetic cascade leading to the ear formation;
   - identifying factors (other transcription factors, signaling molecules) controlling the Nkx5-1 expression in the ear,
   - identifying genes that are under Nkx5-1 control.

1.3 Summary of Results

1.3.1 Evolution of the Nkx5 family of genes - phylogenetic analysis and expression studies

The medaka fish (*Oryzias latipes*), due to its compact genome and its accessibilty for expression patterns studies, is an adequate model to study evolution of the Nkx5 family. In this project five previously identified medaka Nkx5 genes were characterized by obtaining their full genomic and coding sequences, and by analysis of their expression patterns. Sequence analysis of Nkx5 family members, including construction of phylogenetic trees using several different methods, revealed the existence of four paralogous groups of Nkx5 genes in the vertebrate genome, and only one in invertebrate species. The direct *Drosophila* homolog was also identified in the database. In medaka genome representatives of all four groups are present (in contrast to other vertebrates, were some Nkx5 genes have been lost or still await discovery), and the Nkx5-1 gene is represented by two paralogs. This situation is in line with recent hypotheses postulating genome duplications in the evolutionary history of vertebrates (reviewed by Aparicio, 2000). The obtained phylogenic, genomic linkage and published mapping data, and the comparison of expression patterns led me to propose a model of evolutionary history of this ancient group of genes (Adamska et al., manuscript in preparation).

1.3.2 Looking for genes regulating Nkx5-1 expression - mutants analysis and growth factors application

Assuming that it should be possible to identify the genes acting upstream of the Nkx5-1 gene by investigating changes in the Nkx5-1 expression in various ear mutants, I took advantage of existing mouse and zebrafish mutations. Extensive genetic screens performed with zebrafish resulted in identification of numerous mutations, many of them affecting ear formation (Felsenfeld, 1996; Mullins et al., 1994; Malicki et al., 1996; Whitfield et al., 1996; Riley and Grunwald, 1996). For example, in the FGF8 mutant, *acerebellar* (*ace*), ear placodes and vesicles are strongly diminished, and ear expression of several transcription factors is downregulated in comparison to wild type (Léger and Brand, 2000). To find out whether FGF8 can act upstream of the Nkx5-1 gene I isolated the zebrafish Nkx5-1 cDNA, and studied its expression in wild type and *ace* embryos. It appeared that zfNkx5-1 is strongly downregulated in the *ace* otic placodes and vesicles (Adamska et al., 2000). This project was performed in collaboration with Sophie Léger from Dr. Michael Brand's laboratory

(Heidelberg); additional analyzes performed in Heidelberg revealed that the otic ganglion expression domain of the Nkx5-1 gene is especially strongly affected in the *ace* ears. These results in turn encouraged me to study effects of the ectopic FGF source on expression of Nkx5-1 and other ear transcription factors, using chicken as a model system. This experiment proved that additional FGF source can activate expression of Nkx5-1, SOHo and Pax2 genes, while it has slightly downregulating effect on another ear transcription factor, Dlx5. Additionally, FGF2 sources were able to induce ectopic ear structures; this result stands in contrast with what was recently reported by Vendrell and co-workers (2000). I also studied expression of the FGF8 gene in chicken embryos and found that there is a very transient FGF8 expression domain in the anterior part of the placode, at time and place suggesting that FGF8 might regulate Nkx5-1 expression. The obtained data confirm interspecies conservation of FGF signaling in ear development, show that FGFs are not able to induce full ear program, and point at FGF8 as a potential regulator of Nkx5-1 expression (Adamska et al., manuscript submitted).

At the same time BMP2 expressing cells were implanted into chicken embryos in a manner corresponding to the FGF bead implantation; this was done together with Heike Herbrand and the results of BMP cells implantation are described in detail in her PhD thesis. I refer to the results obtained in this experiment in Discussion, as they play an important role in situating ear expressed genes in the molecular network leading to ear formation.

In the mouse and chicken ear Dlx5 pattern of expression is very similar to Nkx5-1 pattern; the Dlx5 KO mice show strong semicircular canals malformations, very much alike Nkx5-1 -/- mice (Acampora et al., 1999; Depew et al., 1999). Together with Barbara Zerega from Dr. Levi laboratory (ABC, Genova, Italy) I studied the Nkx5-1 expression in the Dlx5 KO embryos; since the Nkx5-1 expression in this mutant is unchanged in comparison to wild type, Dlx5 cannot act upstream of the Nkx5-1 in the vestibulum formation.

The Tg9257 insertion mutant was identified due to circling behavior suggesting vestibular disfunction (Ting et al., 1994). Although the mutant phenotype was analyzed in detail both anatomically and histologically, nothing was known about molecular pathways affected by the insertion. Since the Tg9257 ear phenotype is similar to both Nkx5-1 and Dlx5 knockouts, it seemed possible that either of these genes, or both, could act in the same pathway as the gene affected by Tg9257 insertion. The Nkx5-1 -/- ear, with its lateral canal affected stronger than the anterior and posterior ones, strikingly resembles the Tg9257 phenotype. The Dlx5 gene is expressed also (in addition to the inner ear) in the developing nasal structures, and Dlx5 -/- animals have short snout. I analyzed the expression patterns of Nkx5-1 genes in

Tg9257 homozygous animals to find out whether Nkx5-1 or Dlx5 are downstream of the 9257 gene and influenced by its absence. Since expression of both genes is unaffected by the mutation, the gene disrupted by insertion cannot regulate Nkx5-1 or Dlx5 expression.

1.3.3 Looking for Nkx5-1 targets - Nkx5-1 KO analysis and mRNA injections

Elements of the genetic network acting downstream of Nkx5-1 could be identified by investigating ear markers expression in the Nkx5-1 KO, or overexpressing Nkx5-1, for example by injecting the Nkx5-1 mRNA into early fish embryos and testing ear genes expression changes. To find out whether the Dlx5 gene can be responsible for Nkx5-1 expression regulation, the Nkx5-1 KO embryos were hybridized with the Dlx5 probe. The Dlx5 expression in the analyzed embryos (embryonic day 10.5) is apparently normal. This result, together with the information that in the Dlx5 KO Nkx5-1 expression is unchanged, suggests that these two genes act in parallel pathways.

One of the candidate genes for the Nkx5-1 target is BMP4, since in the Nkx5-1-/- ear it is downregulated (Silke Rinkwitz-Brand, personal communication). To find out whether Nkx5-1 is able to induce BMP4 expression, I injected zfNkx5-1 mRNA into the two cell medaka embryos. Indeed, the BMP4 domain is much stronger at the injected site, however, additional effects of the Nkx5-1 injections (enhanced apoptosis, increased lethality and developmental malformations) demand a critical view of these results; additional experiments are planned to elucidate this issue.

1.4 Vertebrate inner ear

1.4.1 Structure and function

Inner ear is one of the cranial sensory organs that are characteristic for vertebrates, and (together with advanced organs of vision and olfaction) influenced the great evolutionary success of this group. The vestibular part of ear, responsible for sensing of balance, is present in every known vertebrate phylum. In tetrapodes and jawed fishes (such as medaka or zebrafish), vestibulum is composed of utriculum, sacculum and three semicircular canals positioned in right angles one to another. In jawless fishes the vestibulum is simpler: in lamprey ear two canals are present, and in even more primitive hagfish there is only one ring-like canal (Braun, 1996). While the vestibuli of all jawed vertebrates are strikingly similar (Fig. 1), and at least some fishes can hear (Yan et al., 2000); only mammals developed cochlea, structure enabling them to precisely detect acoustic waves. It is believed that during the course of evolution the cochlea developed from the lagena, already present in fishes and

enlarged in higher vertebrates; in crocodiles and birds the lagena is lengthened and resembles the mammalian cochlea (Fig. 1) (Janiszewski, 1988).

1.4.2 Inner ear development

In all vertebrates the ear placode is induced from the ectoderm and appears as a thickening of the epithelium adjacent to the hindbrain at the level of rhombomers 5-6. The otic vesicle forms from the placode either by invagination (in higher vertebrates) or by cavitation (in fish). During the invagination process the placode forms a cup-like structure (otic cup or pit), which then closes into the vesicle; in contrast, fish placode first transforms into a solid ball of cells and subsequently a lumen forms within it. The ear is innervated by neurons of the otic (vestibulo-acoustic) ganglion; neuroblast that will form the ganglion delaminate from the antero-medial part of the vesicle. Subsequently the otocyst undergoes a series of remodelings to give rise to the complex structures of the ear (Fig. 1). In amniotes (for example mouse and chicken) these remodelings involve outgrowth of the endolymphatic duct in the dorsal direction and of cochlea in ventral direction, and evagination of the epithelial outpocketings that are subsequently forming semicircular canals. The apposing walls of outpocketings approach and contact each other, thus forming fusion plates. The formation of thin, tubular canals involves elimination of cells from the fusion plate; the mechanism of this process is unclear and seems to depend on species – in mouse involves recruitment of cells into the canal epithelium (Martin and Swanson, 1993), in chicken cell death (apoptosis) was shown to play a role (Fekete et al., 1997). In fish finger-like protrusions grow from the walls inside of the vesicle lumen, and these also meet to form fusion plates; it was shown that in zebrafish the cells in the fusion plate interdigitate and separate (Waterman and Bell, 1984).

Since the extremely complex structures of the inner ear arise from equally simple primordia, the ear development is an excellent model to study many aspects of the vertebrate development, such as the induction (Van de Water and Represa, 1991; Gallagher et al., 1996; Swanson et al., 1990), molecular genetics of organogenesis and cell differentiation (Fekete, 1996, and see Table 1), or evolutionary changes during phylogenesis (Braun, 1996). While descriptive analysis of the development of the inner ear in different vertebrates is well documented (zebrafish - Watermann and Bell, 1984; Haddon and Lewis, 1996; mouse - Anniko and Wikstrom, 1984; Martin and Swanson, 1993; chicken – Bisonette and Fekete, 1996), and many genes have been identified to be active in the developing inner ear (Torres and Giraldez, 1998; Fekete, 1999), very little is known about the interactions of the isolated genes. This project aimed at unraveling at least part of the complicated molecular network leading to the ear formation.

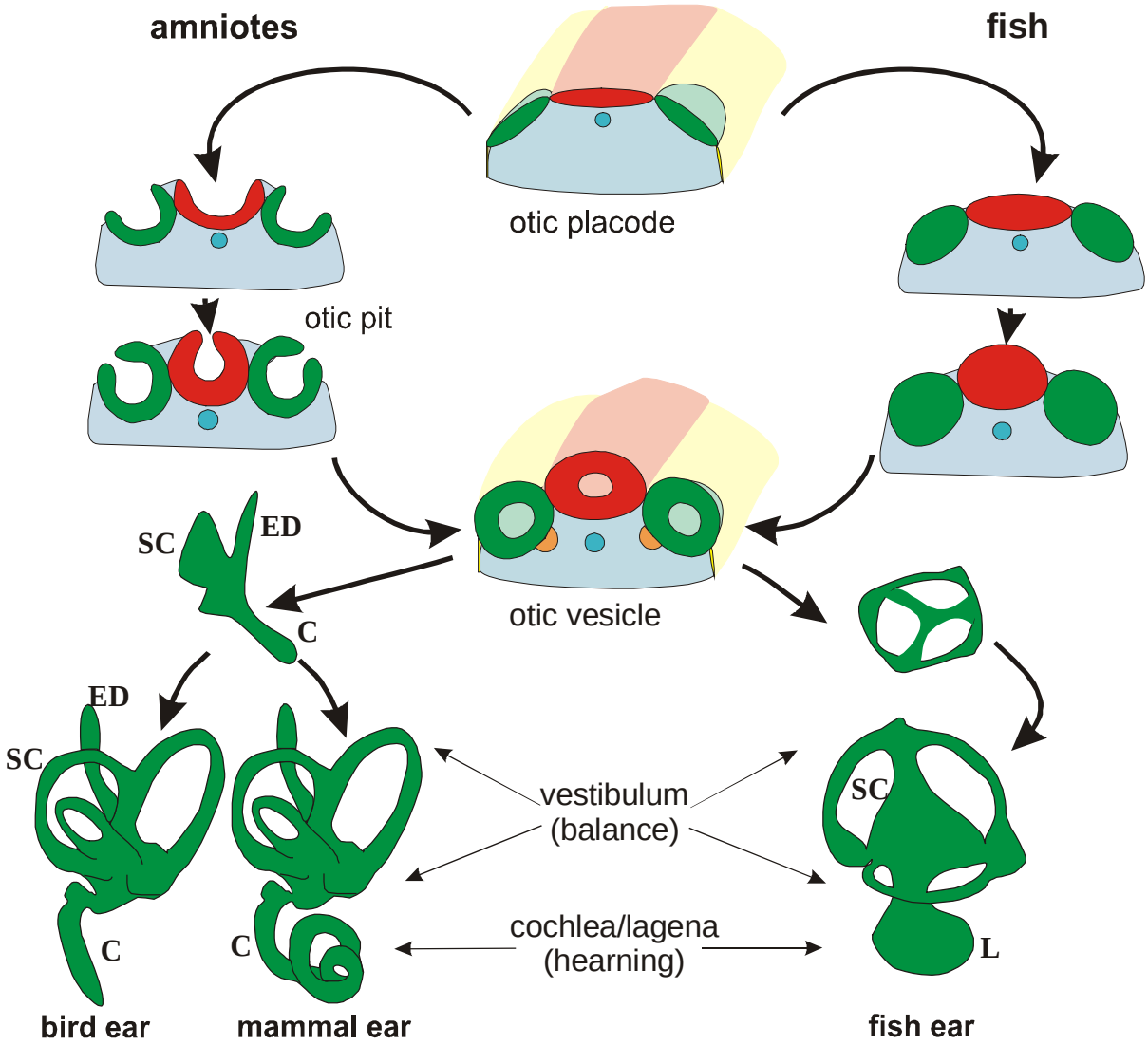

Fig. 1 Inner ear development in different vertebrates. Ear structures are green, neural plate and tube - red, otic ganglion – orange; SC - semicircular canals, ED - endolymphatic duct, C - cochlea, L – lagena; (modified from different sources).

1.5 Genes involved in the inner ear development

There are two lines of evidence showing the importance of a given gene in the ear development – first, the gene is expressed during the ear formation either in the ear itself, or in surrounding tissue, and second, functional analysis shows its necessity for a proper ear morphogenesis or function, or sufficiency to induce ear fate. The gene expression is studied by use of antisense RNA probes detecting presence of transcripts, or by antibodies directed against the gene products. The functional analysis involves ‘loss of function’ experiments, such as:

- generation of mouse strains with inactivated copy of the investigated gene (gene knock-outs, KOs);
- analysis of naturally occurring or induced mutations exhibiting ear phenotype in mouse or fish;
- application to the developing ear of antisense DNA or RNA to block the transcription; dominant negative forms of the protein, antibodies directed against the protein or the natural antagonists to inhibit normal action of the protein.

The 'gain of function' experiments – involving exogenous application of the tested molecule in its native form, can be done by:

- overexpressing the gene by injection of RNA or DNA into fish or amphibian early embryos (1 to several cell stage); injection of DNA results in mosaic embryos with localized cell clusters of ectopic expression; in contrast, injection of mRNA results in very early (few hours after injection) broad ectopic expression domain that can encompass whole embryo;
- infecting the tissue with a virus vector encoding for the protein; this is done in chicken embryos, and allows for clearly defined ectopic expression domains late in development, during organogenesis;
- application of signaling molecules either in explant culture media (explants can be derived from any model system), or in vivo in form of beads soaked in protein or clusters of cells producing the protein (in *Xenopus*, chicken or fish).

The genes with proven function in the ear development can be divided into two main groups:

i. proteins involved in cell-cell interactions and/or signal transduction pathways (signaling molecules and their receptors) – such as fibroblast growth factors (FGF) family, bone morphogenetic proteins (BMP) family, or axon guidance cues (for example netrin);
ii. transcription factors - known to be responsible for changes in other genes expression, acting via binding to specific sequences in promoters of target genes. These genes, acting downstream in genetic cascades, can themselves encode for transcription factors, or proteins involved in cell-cell interactions, and play roles in cell differentiation.

Table 1 summarizes the current information about genes controlling the inner ear development. It is important to note, that lack of the ear phenotype in mice deficient for an ear expressed gene can be due to functional redundancy of genes. If similar (related) genes are expressed in the same time/area, another member of the gene family can take over the function of the inactivated gene and rescue the phenotype. In such case a double KO (inactivating function of both genes) can prove the gene's importance in ear development.

Conversely, inactivation of genes that play roles in early development can lead to early lethality of mouse embryos and thus make it impossible to assess later function of that gene in the ear. In such cases the generation of conditional knockouts (inactivating gene in a specific place and time) allows to reveal the function of gene in the later development or specific structure (see for example Meyers et al., 1998).

1.5.1 Signaling molecules in the inner ear development

Fibroblast growth factors (FGFs) are heparin binding proteins which promote the growth and differentation of tissues of all embryonic origins in both vertebrates and invertebrates (reviewed in Mason, 1994; Yamaguchi and Rossant, 1995). The family of FGFs comprises at least 19 members (FGF1-19), acting by activation of four receptors (FGFR1-4). The complexity of signaling pathways is additionally increased by the fact that both FGF molecules and their receptors can be expressed as different splicing forms. Only FGF1, known also as acidic FGF, is able to activate all known receptor forms; other FGFs show different range of specificity towards the receptors (Ornitz et al., 1996). Nevertheless, some FGFs were shown to exert similar effects in a variety of assays (Kettunen and Thesleff, 1998). For example, FGF2 (basic FGF) and FGF8 application has been shown to lead to indistinguishable results in studies concerning tooth development (Bei and Maas, 1998; Kettunen et al., 1998). This might be due to the fact that these two molecules are able to activate the same splice variants of FGF receptors, with FGF2 having broader range of activity (Ornitz et al., 1996).

Several members of the fibroblast growth factor family (FGF2, FGF3, FGF5, and FGF8) are expressed in the vicinity of the developing ear and in the ear itself and have been implicated in all stages of the development of this organ (Represa et al., 1991; Fekete, 2000). FGF3 transcripts are present in the rhombomeres 4-6 at the time of the placode formation in mouse (Wilkinson et al., 1988, 1989) and chicken (Mahmood et al., 1995), and it was suggested that FGF3 plays a role in the induction of otic vesicle (Van De Water and Represa, 1991; Represa et al., 1991). Represa et al. (1991) showed that formation of the otic vesicle from the otic placode cultured with rhombencephalon could be blocked by antisense FGF3 oligonucleotides or antibodies directed against FGF3 protein. These results were questioned by others in light of the fact that in the FGF3 KO mice the ear vesicles formed normally. The mutant ears failed to form endolymphatic sac, which in turn led to severe deformations of both vestibular and cochlear part of the ear (Mansour et al., 1993). However, the function of FGF3 in ear induction has been proven again very recently, when Vendrell et al. (2000)

reported that injections of retrovirus expressing FGF3 in chicken led to induction of otic vesicles and increased expression of several otic genes.

Table 1 Genes necessary for normal inner ear morphogenesis; all mutant phenotypes refer to mouse KOs, except of zebrafish FGF8 mutant, *acerebellar* (*ace*), since FGF8 KO in mouse results in early lethality. TF: transcription factor; SM: signaling molecule; R: receptor; SP: structural protein; O: other

Gene	type	expression	affected part in mutant	References
Hoxa1	TF	hindbrain	whole ear	Mark et al. 1993
Kreisler	TF	hindbrain	whole ear	McKay et al. 1993
FGF3	SM	hindbrain, otic vesicle	whole ear	Mansour et al. 1993
FGF8	SM	otic vesicle	whole ear	Reifers et al. 1998
FGFR2	R	otic vesicle	whole ear	De Moerlooze et al. 2000
Fkh10	TF	otic vesicle	whole ear	Hulander et al. 1998
Eya1	O	otic placode, vesicle	whole ear	Xu et al. 1999a
Nkx5-1	TF	otic placode, vesicle	semicircular canals	Hadrys et al. 1998
Dlx5	TF	otic placode, vesicle	semicircular canals	Acampora et al. 1999
Netrin1	SM	fusion plate	semicircular canals	Salminem et al. 2000
Otx1/Otx2	TF	otic placode, vesicle	horizontal canal	Acampora et al. 1996
Prx1/Prx2	TF	otic vesicle	horizontal canal	ten Berge et al. 1998
Pax2	TF	otic placode, vesicle	cochlea, spiral ganglion	Torres et al. 1996
Rarα/Rarγ	TF/R	otic vesicle	organ of Corti, spiral ganglion	Lohnes et al. 1994
FGFR3	R	cochlea	tunnel of Corti	Colvin et al. 1996
Brn4(Pou3f4)	TF	periotic mesenchyme	cochlea	Phippard et al. 1999
TGFβ2	SM	periotic mesenchyme	spiral limbus	Sanford et al. 1997
Neurogenin 1	TF	otic placode	vestibular and spiral ganglia	Ma et al. 1998
Brn3.1	TF	hair cells	hair cells	Erkman et al. 1996
Myosin VIIa	SP	hair cells	hair cells	Self et al. 1998
Myosin VI	SP	hair cells	hair cells	Avraham et al. 1995
Myosin XV	SP	hair cells	hair cells	Probst et al. 1998

In contrast, the same paper showed that the FGF2 expressing virus did not affect any aspect of the ear development. Since FGF2 beads were able to induce ectopic ear vesicles in *Xenopus* embryos (Lombardo and Slack, 1998), it was suggested that these conflicting results might represent interspecies differences (Fekete, 2000). In mouse, rat and chicken FGF2 is expressed in the developing ear placode, otocyst, part of the rhombencephalon adjacent to the vesicle (Frenz et al., 1994; Torres and Giraldez, 1998), delaminating neuroblasts (Zhou et al., 1996) and utricular hair cells (Zheng et al., 1997). In higher vertebrates it was shown to be involved in the vestibulo-acoustic ganglion formation (Zhou et al., 1996; Hossain et al., 1996), in the induction of the otic capsule from periotic mesenchyme (Frenz et al., 1994) and proliferation of the ear epithelium in development and regeneration (Zheng et al., 1997).

FGF5 is expressed in cochlea and in the acoustic branch of the VIII ganglion in mouse (Hebert at al., 1990). IIIb isoform of FGFR2, found mainly in epithelia and activated, among other ligands, by FGF3, has been recently deactivated and the ear phenotype of generated

mice strongly resembles the FGF3 KO phenotype – in some embryos the endolyphatic duct outpocketing is missing at E10.5, and later on the whole ear is cystic in appearance (De Moerlooze et al., 2000), further confirming FGF3 function in ear development. However, due to conflicting results from different model systems, the function of particular FGFs in ear development in different species remains a disputable issue (discussed by Fekete, 2000).

Members of another family of growth factors, Bone Morphogenetic Proteins (BMP), are expressed in and around the developing ear (Wu and Oh, 1996; Oh et al., 1996), and have been recently shown to be involved in semicircular canal formation (Chang et al., 1999; Gerlach et al., 2000). In the chicken, BMP4, BMP5 and BMP7 are expressed in the otic cup and surrounding mesenchyme, later on BMP4 marks developing sensory patches of the inner ear (Wu and Oh, 1996; Oh et al., 1996). The experiments conducted in chicken embryos show that application of BMP antagonist, noggin, results in abnormal (retarded or blocked) development of the canals; this phenotype can be rescued by an additional BMP source (Chang et al., 1999; Gerlach et al., 2000).

1.5.2 Transcription factors in the inner ear development

The transcription factors involved in ear development are representatives of all main transcription factors families: nuclear receptors for retinoic acid and thyroid hormone containing zinc-finger DNA binding domain, such as RARα and Rarγ and TRα, β1 and β2; paired box containing genes as Pax2 (Torres et al., 1996), and multiple homeobox genes (reviewed in Corey and Breakefield, 1994; Torres and Giraldez, 1998; Fekete, 1999). Especially the last group has been shown to play important role in ear development, as many of these genes were deactivated ("knockouted") in mice and the resulting mutants show ear deformations (see Table 1). One of them is Nkx5-1 gene; its function during the inner ear development is a subject of the presented work.

1.6 Nkx5 genes in the ear development

1.6.1 Nkx5-1 in the mouse ear

Screening of the mouse genomic library with the NK-1 (S59) cDNA probe (Dohrmann et al. 1990) resulted in identification of two novel homeobox genes. Obviously constituting a novel subfamily of the NK family (Kim and Nirenberg, 1989; Harvey, 1996), they were designated Nkx5-1 and Nkx5-2 (Bober et al., 1994). The Nkx5-1 transcripts are detectable in the developing inner ear beginning at placode stage, and later also in central and peripheral nervous system; Nkx5-2 has a similar expression pattern with later onset (Bober et al., 1994;

Rinkwitz-Brandt et al., 1995 and 1996). The spatial and temporal expression patterns of these two genes in the developing inner ear have been described in detail by Rinkwitz-Brandt et al. (1995, 1996). The Nkx5-1 expression in the otic placode begins at E8.5, at the 10-12 somite stage, as one of first molecular markers of otic development. During invagination the Nkx5-1 transcripts distribution changes from uniform throughout the placode to concentrated in the anterior and dorsal parts of the vesicle. At the E13.5 the Nkx5-2 expression begins and its pattern is identical to that of the Nkx5-1. At E14.5, while the inner ear is almost a miniature replica of the adults one, the distribution of the Nkx5 transcripts is confined to the dorsal (now vestibular) part of the ear. Nkx5-1 and Nkx5-2 genes are clustered on chromosome 7, with the same transcriptional orientation, each of them possesses a single intron located upstream of the homeobox (Bober et al., 1994). The chicken Nkx5-1 sequence, structure and expression are very conserved as compared to mouse (Herbrand et al., 1998).

The role of the Nkx5-1 gene in the inner ear development has been demonstrated by phenotype of the Nkx5-1 -/- mice - severe malformation of the vestibular part of the ear resulting in circling behavior (Hadrys et al., 1998; Wang et al., 1998). The lateral semicircular canal is completely missing, while anterior and posterior ones are severely reduced. Interestingly, the cochleas of the mutant ears are perfectly normal (Hadrys et al., 1998; Wang et al., 1998), suggesting that these two parts of ear develop independently. This hypothesis is further supported by findings of Torres and coworkers (1996), that a KO of Pax2, transcription factor expressed in the otic vesicle in domain complementary to Nkx5-1, has a normal vestibulum and no cochlea. Additionally, expression of both of these early ear markers (Pax2 is initially expressed all over the placode) is mutually independent – in Pax2 KO Nkx5-1 is normally expressed, and vice versa (Hadrys et al., 1998).

1.6.2 Nkx5 family of genes

Several other vertebrate and invertebrate homeobox genes: human H6 (HMX1 - Stadler et al., 1992); mouse Hmx1 (Yoshiura et al., 1998); chicken GH6 and SOHo-1 (Deitcher et al., 1994; Stadler and Solursh, 1994; Kiernan et al., 1997); and two sea urchin genes: TgHbox (from *Tripneustes gratilla*, Wang et al., 1990) and SpHmx (from *Strongylocentrotus purpuratus*, Martinez and Davidson, 1997) are closely related to the Nkx5-1 and Nkx5-2 genes, the family is referred to as Nkx5 (Mennerich et al., 1999), Hmx (Wang et al., 1998) or H6 (Stadler et al., 1995). All of these genes posses highly conserved homeodomain and two other typical domains located immediately downstream of it - HmxSD1 and HmxSD2 (Yoshiura et al., 1998). Additionally, Stadler and colleagues (1995) reported isolation of short homeobox fragments of H6 (Nkx5) related genes in many species, among others *Drosophila*, lamprey

and salmon. The identified genes are expressed in partially, but not completely overlapping domains of the developing sensory organs and nervous system of vertebrate embryos (Kiernan et al., 1997; Rinkwitz-Brandt et al., 1995 and 1996; Yoshiura et al., 1998). For example, at the otic pit and early vesicle stages GH6 and SOHo-1 are coexpressed in the postero-lateral part of the ear, later on their transcripts are present in the developing semicircular canals and in the vestibular (VIIIth) ganglion, both are also expressed in the anterior part of the eye (Kiernan et al., 1997). Hmx1 is expressed in the eye (in both retina and lens), in the second branchial arch, and in the trigeminal ganglion (Yoshiura et al., 1998). Although the sequence and expression patterns of the identified genes have been analyzed in detail, no attempt to determine the phylogenetic relationships within the family was reported so far.

1.6.3 Medaka Nkx5 genes

Teleostei fishes with their small genome size have been proposed as model systems for studying vertebrate genes and regulatory elements (zebrafish - Driever et al., 1994; *Fugu rubripes* - Aparicio et al., 1995; medaka – Tanaka, 1995; Ishikawa, 2000). Pufferfish *Fugu rubripes* is known as the vertebrate with the most “compact” genome; its genes with short introns are miniature versions of the human ones. Recently published data suggest that medaka genome is in fact more similar to *Fugu* than to zebrafish, both in size (reviewed by Ishikawa, 2000) and gene number (Kurosawa et al., 1999). Additionally, medaka is much more robust and thus easier to culture than zebrafish, while small size and transparency of the embryos make the use of whole mount in situ hybridization technique equally convenient in both species.

Conservation of the Nkx5 genes in medaka (*Oryzias latipes*) genome was confirmed by Angela Wolff in her diploma project (Wolff, 1997). Screening of the medaka genomic library with a probe derived from mouse Nkx5-1 gene she isolated 13 positive phageclones; their subsequent analysis revealed presence of five different Nkx5-related genes. Two of them were identified as direct homologs of mouse Nkx5-1 and Nkx5-2 (OlNkx5-1.1 and OlNkx5-2, respectively), the third one as an additional Nkx5-1 paralog (OlNkx5-1.2), while the identity of two others remained obscure. For the first three genes only fragments corresponding to second (including homeobox) exons were isolated; in case of the two genes of unknown identity only partial homeobox sequences were known. The expression studies revealed that OlNkx5-1.1 and OlNkx5-2 are expressed in the identical ear and central nervous system domains, in a manner reminiscent of expression of their mouse and chicken orthologs

(see above: section 1.6.2): in the anterior part of the otic vesicle, otic ganglion, brain and spinal cord.

Additionally, in medaka an additional expression domain, not present in birds or rodents, was identified - the lateral line placode. The lateral line system is present in fishes, amphibian larvae and aquatic amphibia (for example *Xenopus leavis*), and is responsible for detecting the water movements close to the body surface. Interestingly, the sensory cells - hair cells - of the lateral line are identical to sensory cells of the ear. Lateral line, as other vertebrate sensory organs, develops from paired placodes; the trunk lateral line primordium forms posterior to the otic placode and migrates towards the tail, leaving behind groups of cells differentiating into sensory organs, neuromasts (Webb and Noden, 1993; Metcalfe, 1985, Fig. 2). According to Jorgensen (1989), the inner ear and the lateral line are derived from a common ancestor organ, although the common origin of these organs has been questioned by others (Northcutt, 1986). OlNkx5-1.1 and OlNkx5-2 genes, the first molecular markers expressed in both placodes, seem to confirm the close evolutionary relationship of these two organs.

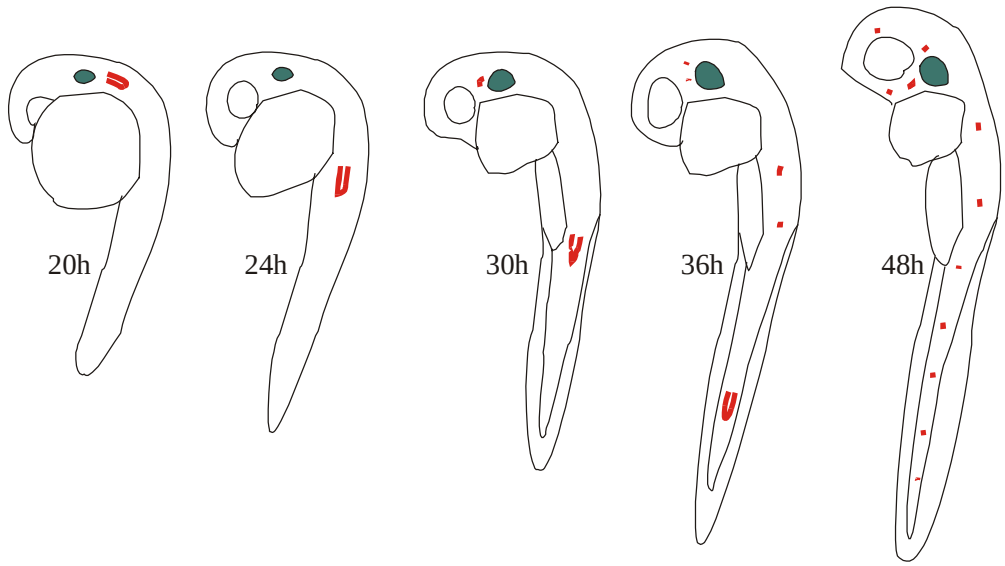

Fig. 2 Lateral line development in the zebrafish embryo. Ear vesicles are green, lateral line primordium and neuromasts - red, developmental stages are shown in hours (modified from Metcalfe, 1985).

2 RESULTS

2.1 Characterization of fish Nkx5 genes

2.1.1 Characterization of medaka Nkx5 genes

Fragments of five Nkx5-related medaka genes were isolated by Angela Wolff in her Diploma project (Wolf, 1997). Two of them, dubbed OlNkx5-1.1 and OlNkx5-1.2, were identified as putative Nkx5-1 orthologs; for both of them sequences corresponding to parts of second coding exons, encompassing homeoboxes, were obtained (605bp and 492bp, respectively). Other isolated fragments included: a 453bp fragment containing homeobox, strikingly similar to the Nkx5-2 gene (named OlNkx5-2), 208bp fragment containing homeobox equally similar to chicken GH6 and Nkx5-1 genes (isolated from Clone III), and 135bp 5' fragment of homeobox of unknown homology (Clone VIII). Table 2 summarizes the data on medaka Nkx5 genes available at the beginning of this project.

Table 2 Fragments of medaka Nkx5 genes isolated and analyzed by Angela Wolff (Wolff, 1997), and their current names used throughout the presented work.

Phage	sequenced part	similar to	corresponding to	old name	current name
I	605bp	Nkx5-1	complete second exon	OL Nkx5-1.1	OlNkx5-1.1
III	135bp	?	incomplete homeobox	Clone III	OlSOHo
IV	492bp	Nkx5-1	incomplete second exon	OL Nkx5-1.2	OlNkx5-1.2
VIII	208bp	?	homeobox	Clone VIII	OlNkx5-3
IX	462bp	Nkx5-2	homeobox	OL Nkx5-2	OlNkx5-2

To determine the homologies of the isolated medaka genes to known genes of the Nkx5 family, and thus gain insight into phylogenetic relationships within the family, I obtained full coding sequences of the identified genes. First, I performed restriction analysis of the phageclones and subsequently sequenced fragments adjacent to the previously isolated coding regions (Experimental Procedures, section 4.2.1). Interestingly, it appeared that phages III and VIII are identical, and contain two different homeobox genes (Clone III ad Clone VIII, Table 2) that are tightly linked (4.2.1.4).

Parts of the coding sequences of OlNkx5-2 and OlSOHo genes not included in the isolated phages were cloned by means of 3'RACE and 5'RACE; the intron/exon boundaries were established by PCR and RT-PCR, respectively (4.2.1). The nucleotide and aminoacid sequences of all identified genes are presented in Figures 28-31 in Experimental Procedures.

When obtained sequences were BLASTed against databanks to determine their homology to known genes, all of them proved to contain Nkx5 (Hmx) related homeodomains and two additional conserved sequences unique to this family: HmxSD1 and HmxSD2 (Yoshiura et al., 1998), see Fig. 3 for alignment of these conserved regions. Additionally, it appeared that *Drosophila* sequence CG5832 (Adams et al., 2000) is very similar to the Nkx5 genes (Fig. 3). Previously, only a short (corresponding to 34 aminoacids) fragment of homeobox of this gene has been reported by Stadler and co-workers (1995).

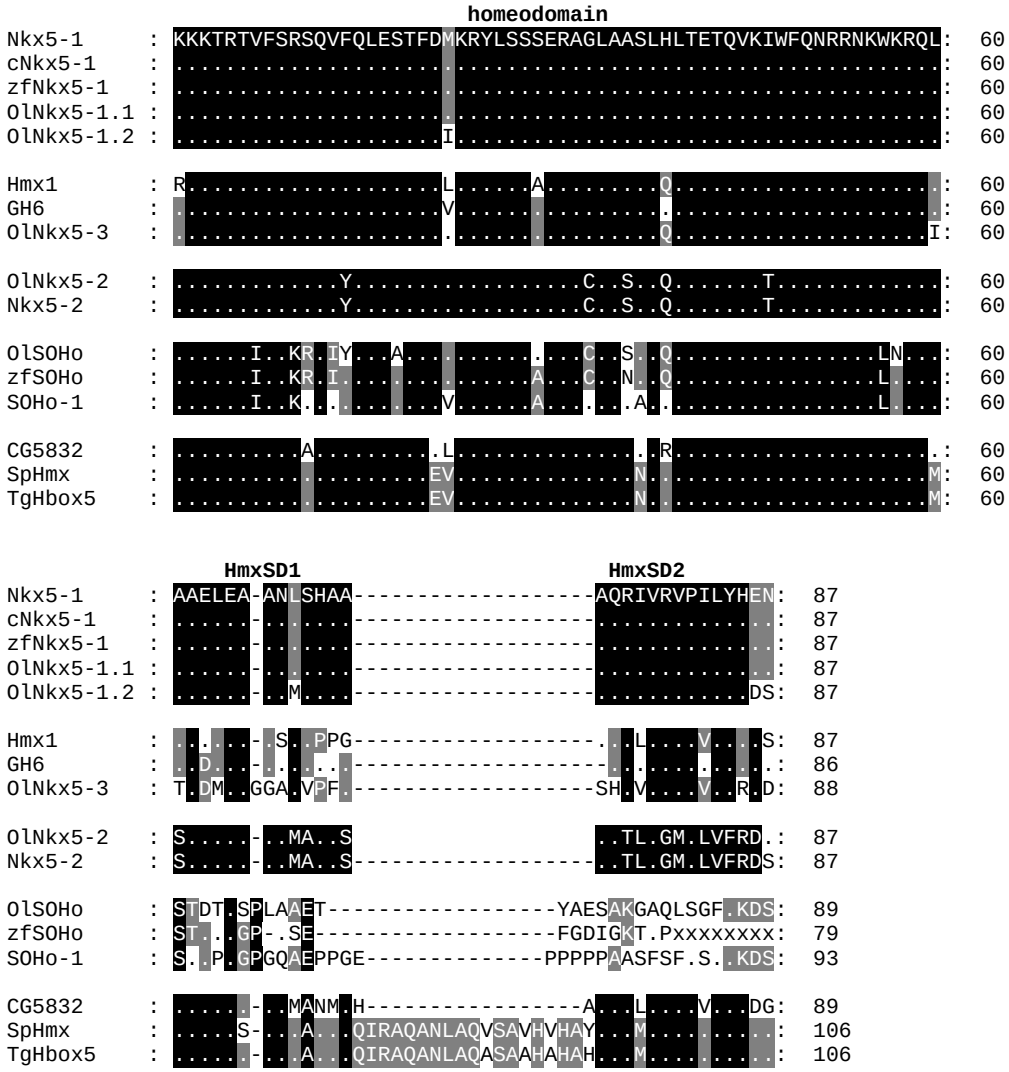

Fig. 3 Conservation of protein sequences in the Nkx5 family, dots indicate aminoacids identical to these in the Nkx5-1 fragment, dashes introduced gaps, x-unknown aminoacid. Aminoacids are shaded black if they are identical in all species in orthologous groups, and gray if in one species they are not conserved. OlNkx5-1.1, OlNkx5-1.2, OlNkx5-2, OlNkx5-3 and OlSOHo (present work) are medaka proteins, Nkx5-1 (AJ009935), Nkx5-2 (1311669) and Hmx1 (AF009614) - mouse, cNkx5-1 (Y15989), SOHo-1 (S69380) and GH6 (D. Schulte, personal communication)- chicken, zfNkx5-1 (present work) and zfSOHo (AI656291) - zebrafish, CG5832 (AAF55433) is a *Drosophila* sequence, TgHbox5 (4826981) and SpHmx (D85079) are proteins from two sea urchin species.

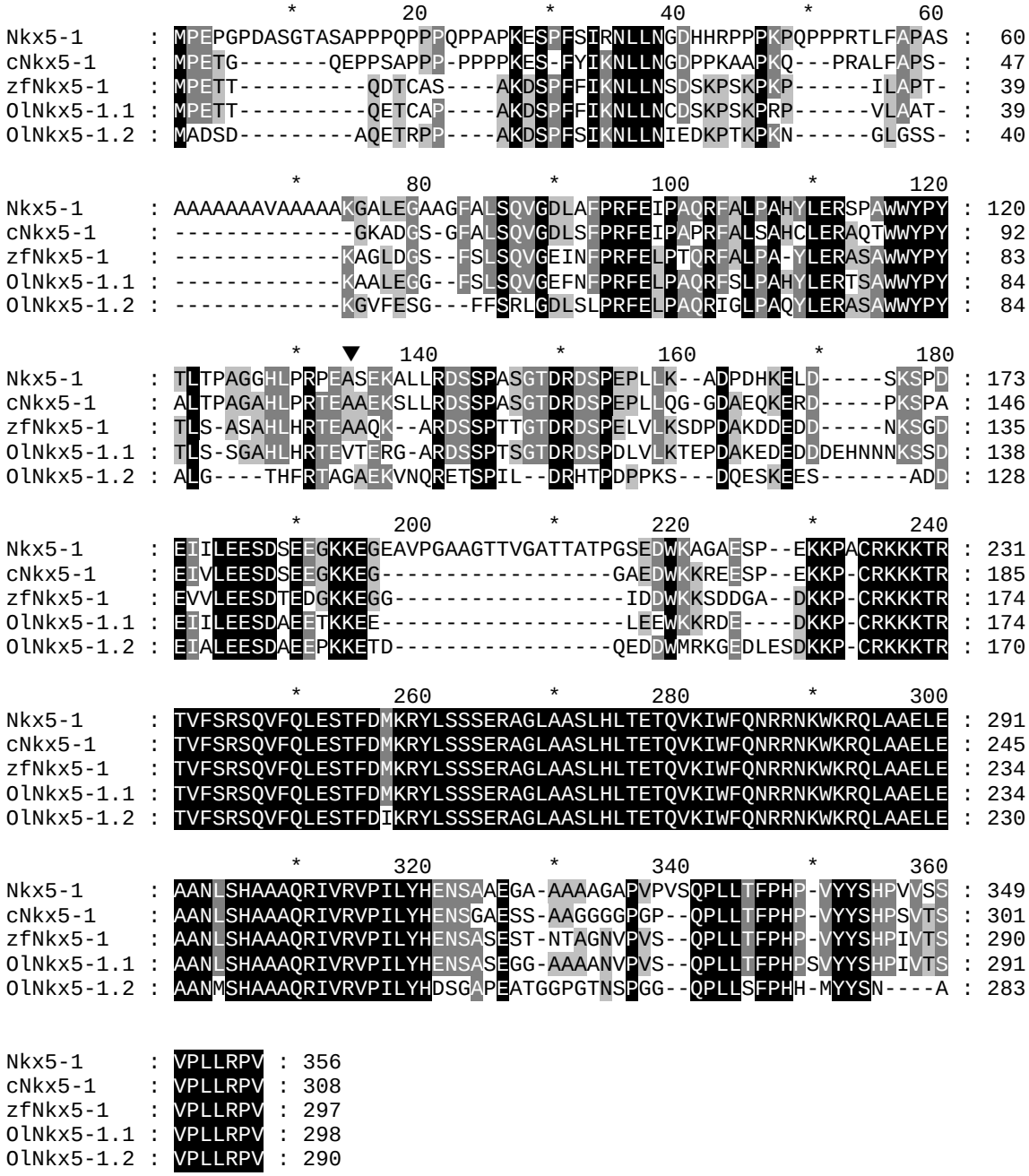

Fig. 4A Comparison of mouse (AJ009935), chicken (Y15989), zebrafish and medaka (present work) Nkx5-1 protein sequences. Aminoacids are shaded black if they are identical in all proteins, and in decreasing shade of gray if there are differences, dashes represent introduced gaps, the conserved intron position is marked by a triangle.

The comparison of full coding sequences confirmed that OlNkx5-1.1 and OlNkx5-2 constitute true orthologs of Nkx5-1 (mouse, chicken, and zebrafish, see below) and mouse Nkx5-2 genes respectively, with the homeodomains and HmxSD1 and SD2 being identical and the surrounding regions strongly conserved (Fig. 4A and 4B). The intron position is conserved between mouse and medaka Nkx5-2 genes (Fig. 4B). OlNkx5-1.2 gene, that was so far isolated exclusively in medaka, shows highest sequence similarity to Nkx5-1 genes,

with only one diverged aminoacid in the homeodomain (Fig. 4A). The position of the single intron in OlNkx5-1.1, OlNkx5-1.2, mouse and chicken Nkx5-1 is conserved, see triangle in Fig. 4A. However, Nkx5-1.2 intron is much shorter than the Nkx5-1.1 intron (184bp as compared to 627bp), which in turn is comparable to the mouse intron (600bp). While coding regions of the medaka Nkx5-1 genes show high conservation, the uncoding fragments are completely diverged. Distribution of the conserved sequences in both OlNkx5-1 genes is schematically illustrated in Fig. 5.

```
                    *        20         *        40  ▼      *        60
Nkx5-2    : VCVLGGGGAGEGWKAPACFCPDPHGPKEPSPKHHTPIPFPCLGTPKGSGGAGPAASERTP :  60
OlNkx5-2  : LCVLRGGVQRRGGLG-GLFCSD-TGHSERCT-QHQPHNFSCLGSGKGLLSGTEGLARRPH :  57

                    *        80         *       100         *       120
Nkx5-2    : FLSPSHPDFKEEKERLLPAGSPSPGPERPRDGGAERQTGAAKKKTRTVFSRSQVYQLEST : 120
OlNkx5-2  : LSQPLLQDYKEEQDRACHQMSPLSD-DRQTDG-ADKQSNSAKKKTRTVFSRSQVYQLEST : 115

                    *       140         *       160         *       180
Nkx5-2    : FDMKRYLSSSERACLASSLQLTETQVKTWFQNRRNKWKRQLSAELEAANMAHASAQTLVG : 180
OlNkx5-2  : FDMKRYLSSSERACLASSLQLTETQVKTWFQNRRNKWKRQLSAELEAANMAHASAQTLVG : 175

                    *       200         *       220
Nkx5-2    : MPLVFRDSSLLRVPVPRSLAFPAPLYYPSSNLSALPLYNLYNKLDY : 226
OlNkx5-2  : MPLVFRDNNLLRVPVARSIAFPTPLYYPGSNLPALPLYNLYNKIEY : 221
```

Fig. 4B Comparison of mouse (1311669) and medaka (present work) Nkx5-2 protein sequences. Identical aminoacids are shaded black, conservative substitutions are gray, dashes represent introduced gaps. The conserved intron position is indicated by a triangle.

Besides the direct Nkx5 homologs, a gene showing high similarity to SOHo genes (50 out of 60 aminoacids in homeodomain identical between chicken and medaka proteins, 56/60 medaka versus zebrafish, see Fig. 3 and 4C) was identified and dubbed OlSOHo. Except of the HmxSD1 and SD2 domains the rest of the sequence does not show high conservation (Fig. 4C). Interestingly, the gene was physically linked to another Nkx5-related gene, OlNkx5-3 (see section 4.2.1.4 for details). OlNkx5-3 homeodomain differs in only 2 aminoacids from Nkx5-1 homeodomain, but the similarity in regions downstream of homeodomain places it closer to mouse Hmx1 and chicken GH6 genes (Fig. 3 and 4D). In the medaka genome, OlNkx5-3 is located upstream of the OlSOHo and both genes share the same transcriptional orientation. The organization of the locus is schematically depicted in Fig. 6.

Fig. 4C Comparison of chicken (S69380) and medaka (present work) SOHo protein sequences. Identical aminoacids are shaded black, conservative substitutions are shaded gray, dashes represent introduced gaps, the intron position in OlSOHo is marked by a triangle.

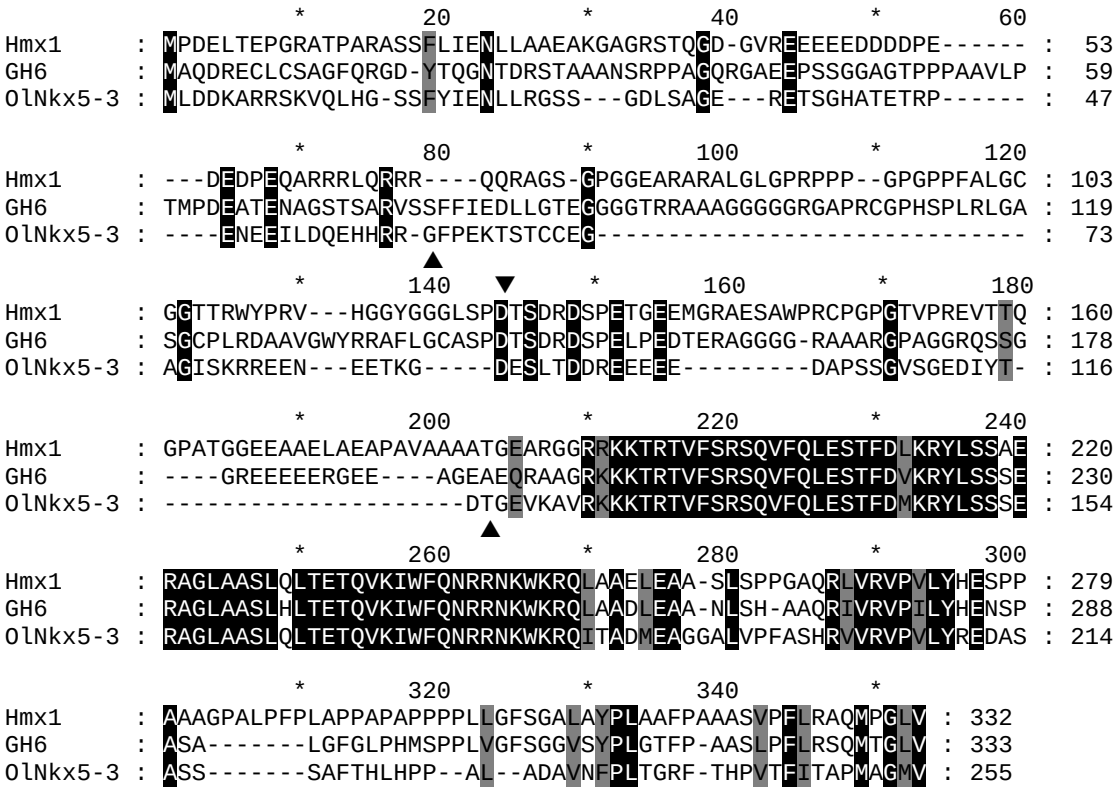

Fig. 4D Comparison of mouse Hmx1 (AF009614), chicken GH6 (D. Schulte, personal communication) and medaka Nkx5-3 (present work) protein sequences. Identical aminoacids are shaded black, conservative substitutions are shaded gray, dashes represent introduced gaps, the intron positions are marked by triangles.

OlSOHo has a single intron located in the position typical for other Nkx5 genes, while in OlNkx5-3 gene there is a second intron located immediately upstream (21bp) of the homeobox (Fig. 4D and Fig. 6). As only cDNA sequences are known for chicken GH6 and

SOHo-1, the intron/exon boundaries present in medaka OlNkx5-3 and OlSOHo genes cannot be compared to these genes. However, the mouse ortholog of OlNkx5-3, Hmx1, possesses only one intron in the typical for Nkx5 genes position (Fig. 4D, Yoshiura et al., 1998). Existence of additional introns in teleostei fishes genes, as compared to mammalian orthologs, has been described in the literature and changes in intron positions were proposed to constitute useful markers in establishing evolutionary relationships between clades (see for example Venkatesh et al., 1999 and references therein).

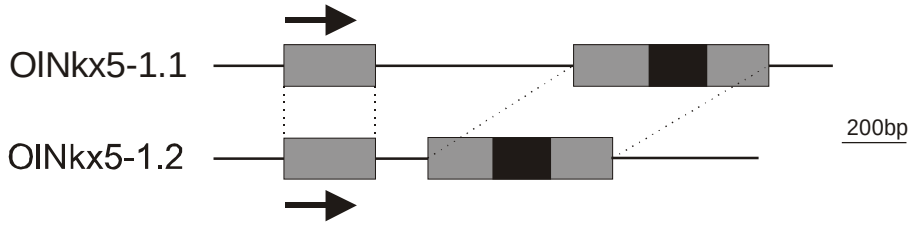

Fig. 5 Schematic representation of alignment of medaka Nkx5-1.1 and Nkx5-1.2 genes. Coding regions are shown as boxes, uncoding as lines. The OlNkx5-1.2 intron is much shorter than the OlNkx5-1.1 intron; coding parts of both exons are strongly conserved (boxes), while there is no detectable similarity in the uncoding regions. Homeoboxes are represented by black boxes, transcriptional orientation is indicated by arrows.

Fig. 6 Schematic organization of the OlNkx5-3/OlSOHo locus. Boxes indicate coding regions, homeoboxes are black, transcriptional orientation is indicated by arrows.

2.1.2 Isolation of the zebrafish Nkx5-1 cDNA

To investigate whether the Nkx5-1 gene is conserved in zebrafish, PCR analysis was performed using conserved Nkx5-1 sequences as primers (see Experimental Procedures section 4.2.2) and zebrafish genomic DNA as a template. The amplified fragment encoded 77 aminoacids from the carboxyterminus including almost complete homeodomain. The deduced protein sequence was identical to those of medaka, mouse and chicken. At the nucleotide level the corresponding regions of the medaka, mouse and chick Nkx5-1 DNA sequences displayed 85.6%, 83.5% and 83.8% identity to the zebrafish DNA sequence respectively. This strong sequence conservation indicated that direct Nkx5-1 homolog exists in the zebrafish. To get more information about a function and structure of the Nkx5-1 gene in zebrafish, the Nkx5-1 cDNA was isolated from a stage 15 – 19 hr (28^{o}C) embryonic cDNA

library, kindly provided by B. Appel (Vanderbilt). It is striking that the zebrafish Nkx5-1 protein is almost identical to medaka Nkx5-1.1 protein (Fig. 4A).

2.1.3 Phylogenetic relationships within the Nkx5 family

To determine the relationships within the Nkx5 (Hmx) family, I used the programs from the PHYLIP package (Felsenstein, 1993). First, from exhaustive (1000 replicas) bootstrap analysis the most parsimonious tree of the Nkx5 family was inferred (Fig. 7A).

Only strongly conserved, unambiguously alignable 3' fragments of the proteins (including homeodomains) were used for this analysis. The obtained tree demonstrates that there are four paralogous groups of Nkx5 (Hmx) genes in vertebrate species: '1' - mouse, chicken, zebrafish and medaka Nkx5-1, '2' - mouse and medaka Nkx5-2, '3' - mouse Hmx1, chicken GH6, medaka OlNkx5-3 and '4' - chicken and medaka (and zebrafish, not shown) SOHo. The branch lengths of the tree indicate that the genes were under different selection pressures. Nkx5-1 and Nkx5-2 proteins from species as distant as mouse and medaka are almost identical, while Hmx1/GH6/Nkx5-3 and SOHo are more diverged (see also Fig. 3 and 4 for alignments).

Additionally, the groups 1 with 3, and 2 with 4, cluster together. As can also be seen in Fig. 3, the Nkx5-2 and SOHo genes share the same aminoacid substitutions, while the homeodomains of Nkx5-3 (Hmx1) group genes are more similar to the Nkx5-1 homeodomain. *Drosophila* CG5832 and the cluster encompassing both sea urchin genes, TgHbox5 and SpHmx, branch out close to the point of separation of the two subfamilies. The invertebrate family members are closer to the Nkx5-1 and Nkx5-3 group (Fig. 3), indicating that those genes can bear more resemblance to the putative ancestral sequence. To obtain additional support for the hypothesis that Nkx5-1 with Nkx5-3 (Hmx1, GH6), and Nkx5-2 with SOHo form two subfamilies, I constructed additional phylogenetic trees basing on different methods and using different fragments of protein sequences. The Maximal Parsimony method, and methods basing on protein distances (Neighbor-Joining, Fitch-Margoliash) were applied to alignments of homeodomains, C-terminal fragments or full-length proteins. Both unrooted trees and trees rooted with related not-Nkx5 sequences were constructed (see section 4.5). Since all of them displayed essentially identical topology only one, representative tree is presented in Fig. 7B.

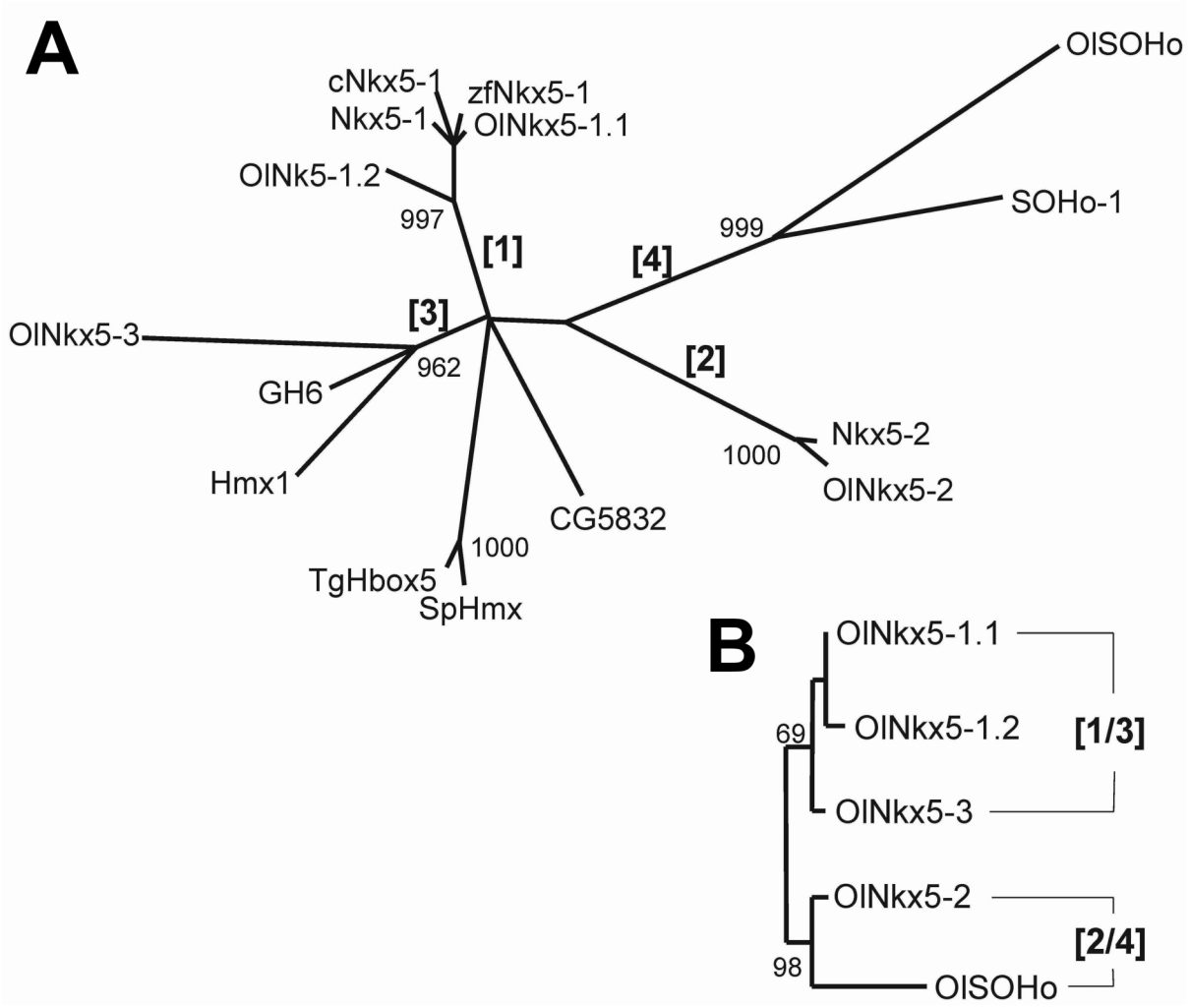

Fig. 7 Phylogenetic trees representing relationships within the Nkx5 family of genes. Branch lengths are proportional to sequence distances. OlNkx5-1.1, OlNkx5-1.2, OlNkx5-2, OlNkx5-3 and OlSOHo (present work) are medaka proteins, Nkx5-1 (AJ009935), Nkx5-2 (1311669) and Hmx1 (AF009614) - mouse, cNkx5-1 (Y15989), SOHo-1 (S69380) and GH6 (D. Schulte, personal communication)- chicken, zfNkx5-1 (present work) and zfSOHo (AI656291) - zebrafish, CG5832 (AAF55433) is a *Drosophila* sequence, TgHbox5 (4826981) and SpHmx (D85079) are proteins from two sea urchin species. Numbers in square brackets indicate identified subgroups. A) a tree obtained by the Protpars program run on 1000 bootstrap replicas; only strongly conserved C terminal parts of protein sequences, including homeodomains, were used in this analysis; distances were computed by a Protdist program; bootstrap confidence values for orthologous clusters are indicated at nodes. B) a tree computed by Protdist and Fitch programs, only homeodomain sequences of the medaka Nkx5 genes were used here, numbers at nodes indicate bootstrap values per 100 runs.

The tree can be clearly divided into two parts - one encompassing the OlNkx5-2 and OlSOHo, and the second OlNkx5-1 and OlNkx5-3. In this example only homeodomain sequences of the medaka Nkx5 proteins (as they represent all four paralogous groups found in vertebrates) were used, the protein distances were computed with the Protdist program, and the tree constructed using Fitch-Margoliash algorithm. High bootstrap values, shown at branching points, indicate that the obtained tree can be treated with high confidence (Felsenstein, 1985; Efron et al., 1996).

2.1.4 Expression of the Nkx5 genes in developing medaka embryos

Preliminary expression analysis (Wolff, 1997) shown that the OlNkx5-1.1 and OlNkx5-2 are expressed in the anterior part of the otic vesicle and in the lateral line placode at stage 24 (16 somites). As the mouse and chicken Nkx5-1 genes start to be expressed in the ear placodes (Rinkwitz-Brandt et al., 1995; Herbrand et al., 1998), and the chicken GH6 and SOHo-1 genes are expressed also in the early eye development (Kiernan et al., 1997), medaka embryos of Iwamatsu stage 18 (late neurula, after optic buds and before ear placodes formation) and older were chosen for further expression studies (Iwamatsu, 1994). Fig. 8 gives an overview on expression patterns of the Nkx5 genes in developing medaka embryos. All of the studied genes display clearly defined expression domains from stage 20 (4 somites) until 30 (34 somites), being especially prominently expressed in the developing sensory organs: eye, ear and lateral line. OlNkx5-2 expression was almost identical to the OlNkx5-1.1 expression at all investigated stages.

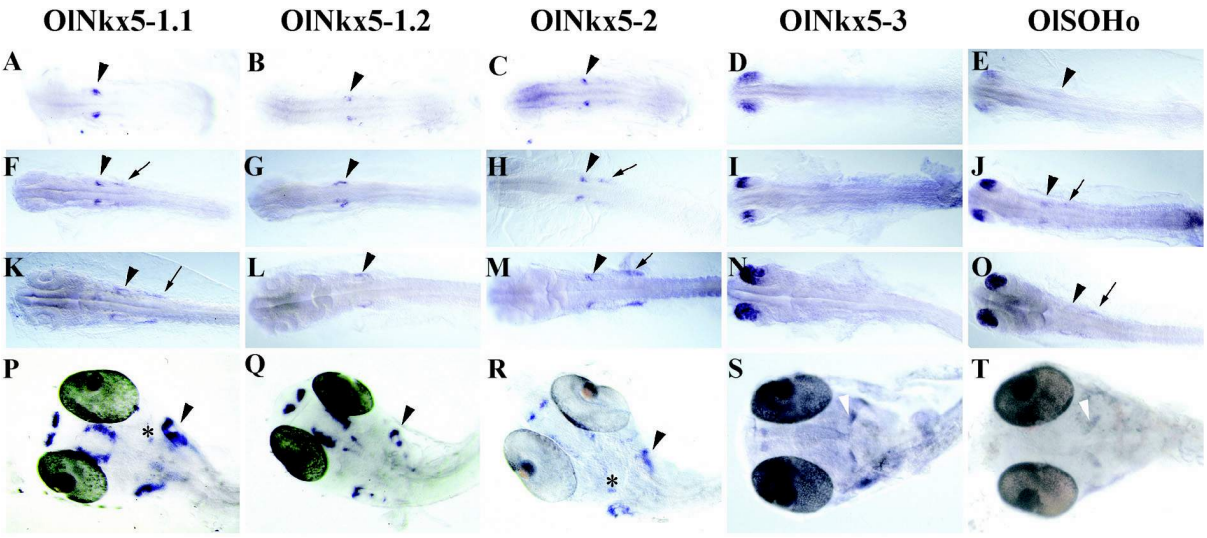

Fig. 8 Expression patterns of Nkx5 family members in medaka embryos; gene names are above the columns, black arrowheads indicate otic expression, arrows - lateral line, white arrowheads - branchial arches; asterisks - otic ganglion. A-E st. 20, F-J st. 22, K-O st. 24, P-R st. 29, S-T st. 30; anterior is to the left, A-O are dorsal views, P-T ventral views.

2.1.4.1 Eye expression

Two of the five analyzed genes, OlNkx5-3 and OlSOHo, showed distinct expression domains in the developing eye. OlNkx5-3 and OlSOHo eye expression is first detectable at stage 18, when transcripts of both genes are present all over the optic buds (see Fig. 9A for Nkx5-3 expression, SOHo expression is identical at this stage, not shown). During stage 20 expression domains of both genes shift and start marking the anterior part of the eye (compare figures 8DE versus IJ, and 9A versus B). The two genes are expressed in both the

retinas and lenses (see Fig. 9I for a section of st. 24 medaka eye). The expression pattern of OlNkx5-3 and OlSOHo in medaka eye is very similar to GH6 and SOHo-1 expression in the chicken eye (Deitcher et al., 1994; Stadler and Solursh, 1994).

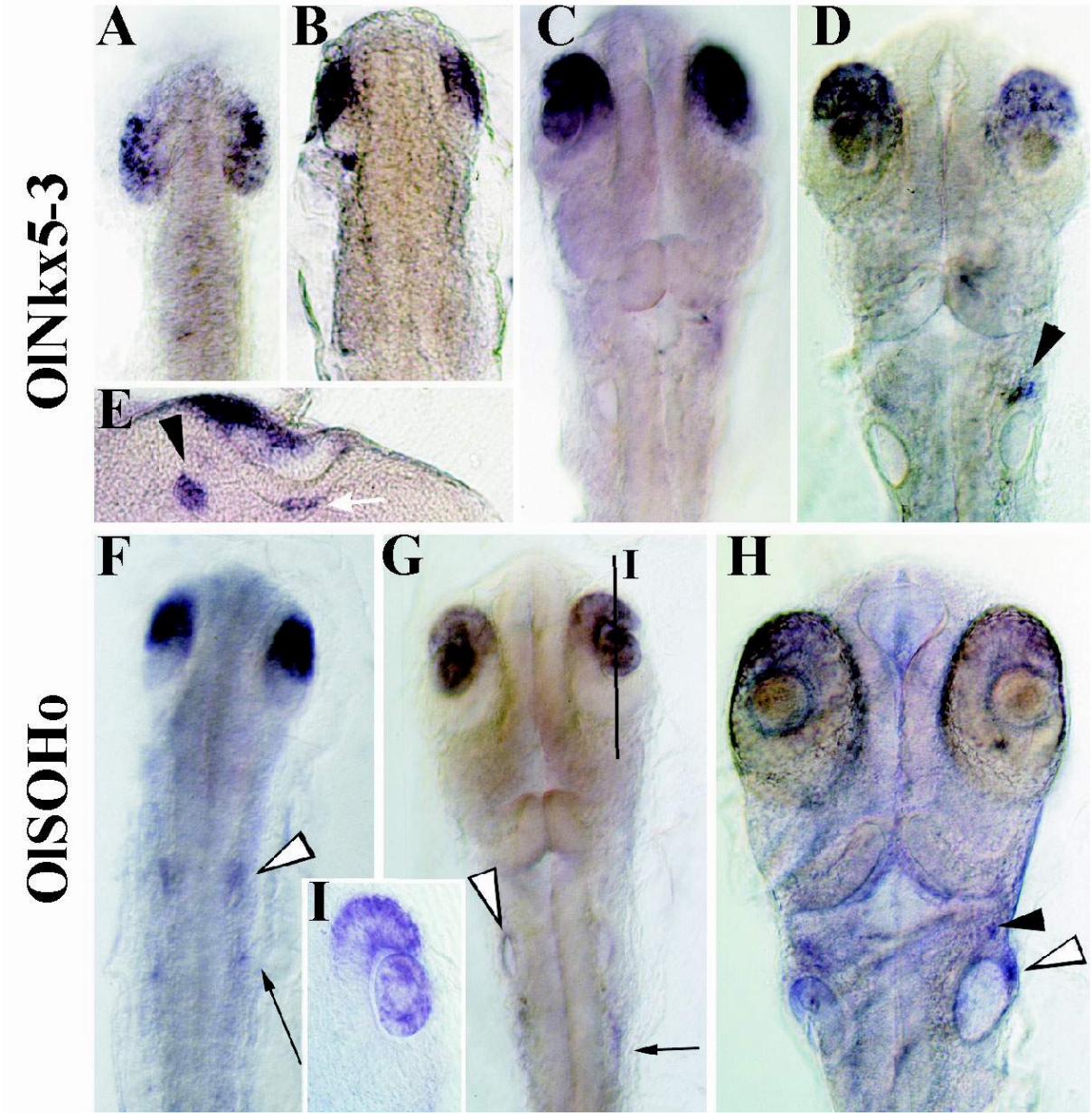

Fig. 9 Expression of the OlNkx5-3 (top panel, A-E) and OlSOHo (bottom panel, F-I) in medaka embryos. St. 18 (A), st. 20 (B), st. 22 (F), st. 24 (C, G and I, section through the eye), st. 27 (D), st. 28 (H), st. 29, section through the ear area (E). Black arrowheads indicate otic ganglion, white arrowheads - ear expression, white arrow - cells adjacent to the ear and expressingOlNkx5-3, thin black arrows - lateral line primordium.

2.1.4.2 Ear expression

Expression of all but one (OlNkx5-3) of the analyzed genes was detected in the developing ear. Beginning with stage 20, Nkx5-1.1, Nkx5-1.2, Nkx5-2 and SOHo mark the otic placode, and all of them continue to be expressed in the otic vesicle (see Fig. 8, black arrowheads, and

9, white arrowheads). Interestingly, while OlNkx5-1.1 and OlNkx5-2 transcripts show similar localization to their vertebrate orthologs - always marking the anterior part of the ear vesicle, the expression of OlNkx5-1.2 is different. OlNkx5-1.2 is initially expressed in the posterior part of the otic vesicle (st. 22), then transiently in its lateral part (st. 24), and later (st. 26 and later) it overlaps with the OlNkx5-1.1 and OlNkx5-2 expression in the anterior part of the vesicle. The comparison of OlNkx5-1.1 and OlNkx5-1.2 expression in the ear is presented in Fig. 10.

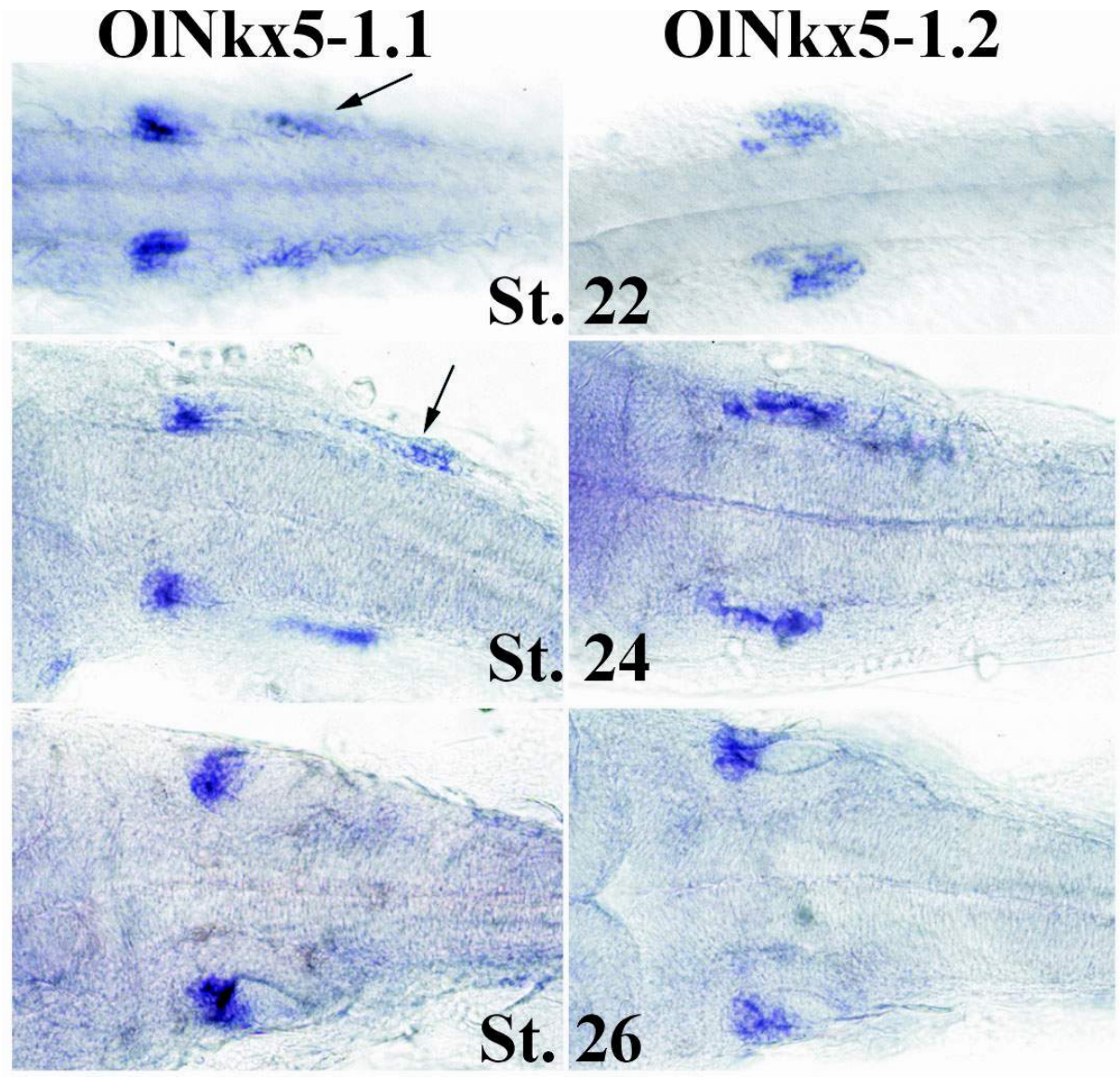

Fig. 10 OlNkx5-1 genes expression in the developing medaka ear and lateral line; stages are indicated below embryos, gene names above the columns, arrows point at the trunk lateral line primordium. All pictures are dorsal views, anterior is to the left.

OlSOHo expression in the ear is relatively weak in comparison to its eye domain and concentrates in the anterior part of the ear (Fig. 9 F-H). This is surprising, since in the

chicken ear the SOHo-1 expression domain is initially complementary to Nkx5-1 domain (Kiernan et al., 1997, see also Fig. 17 in section 2.2.2), and then shifts more anterior, exactly as the OlNkx5-1.2 domain in the medaka otic vesicle. OlNkx5-3 expression is not detectable in the otic vesicle itself, but at stage 29 a small group of cells adjacent to the medial wall of the ear expresses OlNkx5-3, see section in Fig. 9E.

2.1.4.3 Otic ganglion expression

Fig. 11 illustrates OlNkx5-1.1 expression in the neuroblasts delaminating from the otic vesicle to form the vestibular ganglion. All medaka Nkx5 genes, except of OlNkx5-1.2, are expressed in the developing ganglion, starting at around st. 27 (Fig. 8, asterisks, and Fig. 9, black arrowheads). The ganglion expression persists up to at least stage 30, see section in Fig. 9E for OlNkx5-3 and Fig. 11E for the OlNkx5-1.1 expression. Figure 11E illustrates also that the OlPax2 domain (red) is complementary to the OlNkx5-1.1 domain (blue) within the otic vesicle, as it is also the case in mouse ear vesicle (Rinkwitz-Brandt et al., 1996).

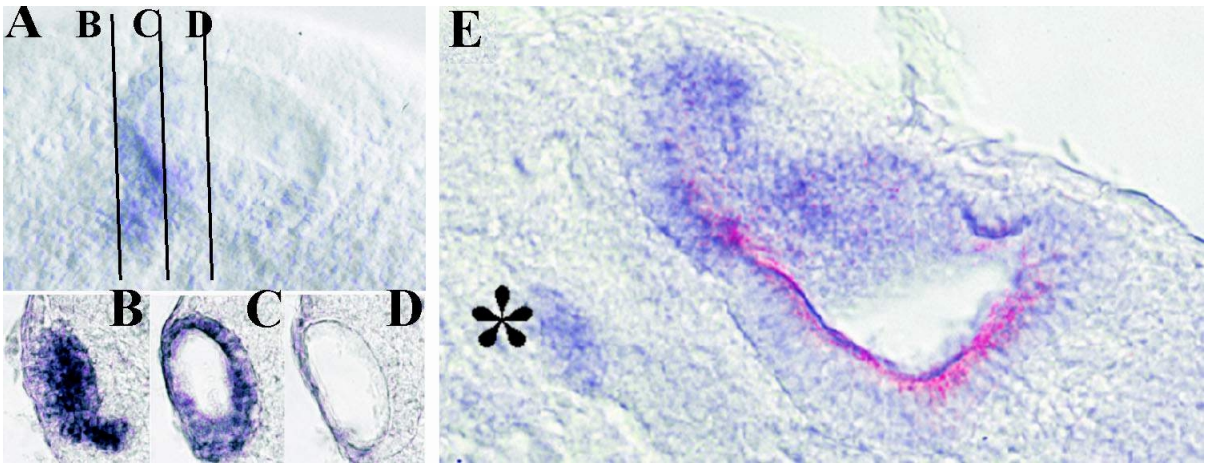

Fig. 11 OlNkx5-1.1 is expressed in the delaminating neuroblasts forming otic ganglion and in the anterior part of the otocyst; A-D stage 27, thin lines on A indicate levels of sections on B-D; E - section of the st. 30 ear, double stained with OlNkx5-1.1 probe (blue) and OlPax2 probe (red), asterisk marks otic ganglion. In A and E anterior is to the left.

2.1.4.4 Lateral line expression

At stage 22 (9 somites) OlNkx5-1.1, OlNkx5-2 and OlSOHo genes start to be expressed in the lateral line primordium (see arrows in Fig. 8, 9 and 10). The trunk lateral line primordium migrates from its place of origin (just posterior to the ear) towards the tail (Metcalfe et al., 1985; see Fig. 2). At the same time the head lateral line placode extends in opposite direction, towards the front of the head. The lateral line expression is detectable in the migrating primordia of the head and trunk lateral line until stage 29, when the trunk lateral line

primordium reaches tip of the tail (Fig 12 and results not shown). In contrast to OlNkx5-1.1 and OlNkx5-2, OlSOHo is expressed only weakly in the lateral line, and OlNkx5-3 transcripts are not detected there at all (Fig. 8 and 9).

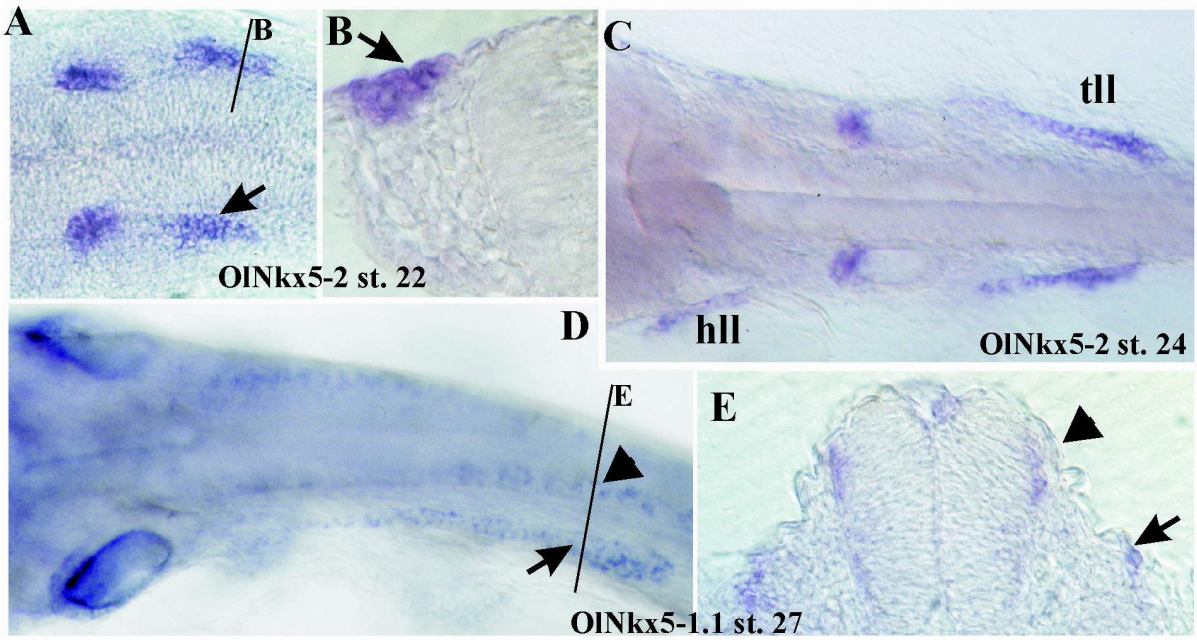

Fig. 12 OlNkx5-1.1 and OlNkx5-2 expression in the lateral line anlage. Thin lines in A and D indicate levels of sections (B, E), arrows point at the lateral line, arrowheads at spinal cord OlNkx5-1.1 domain, hll - head lateral line, tll - trunk lateral line. Embryos are positioned with anterior to the left, dorsal views. Gene names and stages are indicated.

2.1.4.5 Other expression domains

Except of strong expression in the developing sensory organs, the medaka Nkx5 genes are expressed in few other domains that were not analyzed in detail. As shown in Fig. 13, OlNkx5-1 is distinctly expressed in two domains in the ventral part of the medaka brain. OlNkx5-1.2 and OlNkx5-2 are coexpressed in both these domains (Fig. 8P-R), while OlNkx5-3 is weakly expressed in the more rostral one (Fig. 8S). Beginning at stage 30 Nkx5-3 and SOHo transcripts are detectable in the branchial arches (Fig. 8ST, white arrowheads). OlNkx5-1.1 and OlNkx5-2 are also expressed in the spinal cord (Fig 12DE, black arrowheads).

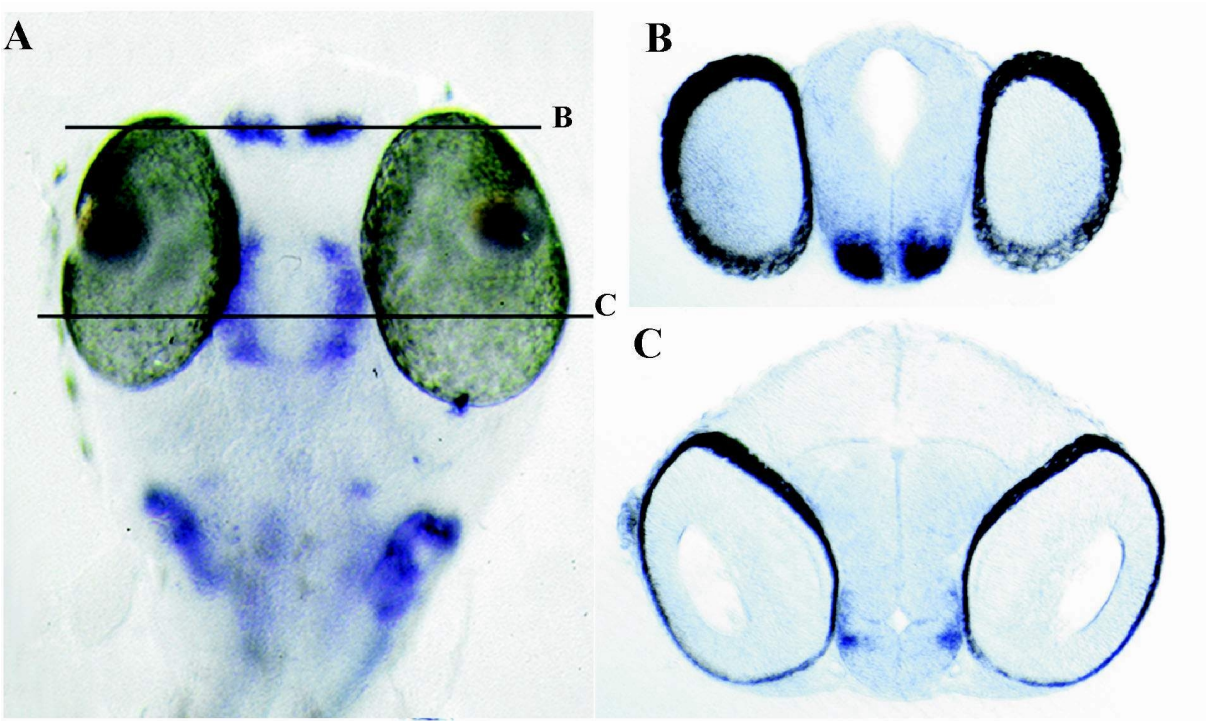

Fig. 13 OlNkx5-1.1 expression in st. 29 medaka brain; thin lines indicate sections levels. OlNkx5-1.2 and OlNkx5-2 brain expression at this stage is undistinguishable from OlNkx5-1.1. OlNkx5-3 is expressed only in the more rostral domain (compare Fig. 8). The dark color in the retina is natural pigmentation.

2.1.5 zfNkx5-1 expression

Expression of zebrafish Nkx5-1 was analyzed using a cDNA probe and whole mount in situ hybridization procedures on embryos from different stages, starting with the 11 hours embryos, prior to ear placode formation, to 30 hours embryos (late otic vesicle). The onset of expression was estimated to take place at the 4 somites stage (11.3h of development). The only regions expressing zfNkx5-1 at this developmental stage are the lateral line placodes. A few hours later, at the 14 somites stage (16h), zfNkx5-1 starts to be expressed in the otic placode. At much later stages (28h) zfNkx5-1 also starts to be expressed in distinct domains of the developing brain, in pattern corresponding to OlNkx5-1 and OlNkx5-2 expression domains in medaka brain. The spatial and temporal distribution of zfNkx5-1 transcripts in representative zebrafish embryos is illustrated in Fig. 14. The trunk expression domain of zfNkx5-1 (Fig. 14 E-G) strikingly follows the position of migrating lateral line primordium as described by Metcalfe (1985) and shown on Fig. 2. Thus, Nkx5-1 expression in zebrafish embryos strongly resembles the expression pattern of OlNkx5-1 and OlNkx5-2 genes in medaka (compare Fig. 12C and 14C), with the only difference that the lateral line placode expression appears few hours earlier than the otic expression (compare Fig. 8A and 14A).

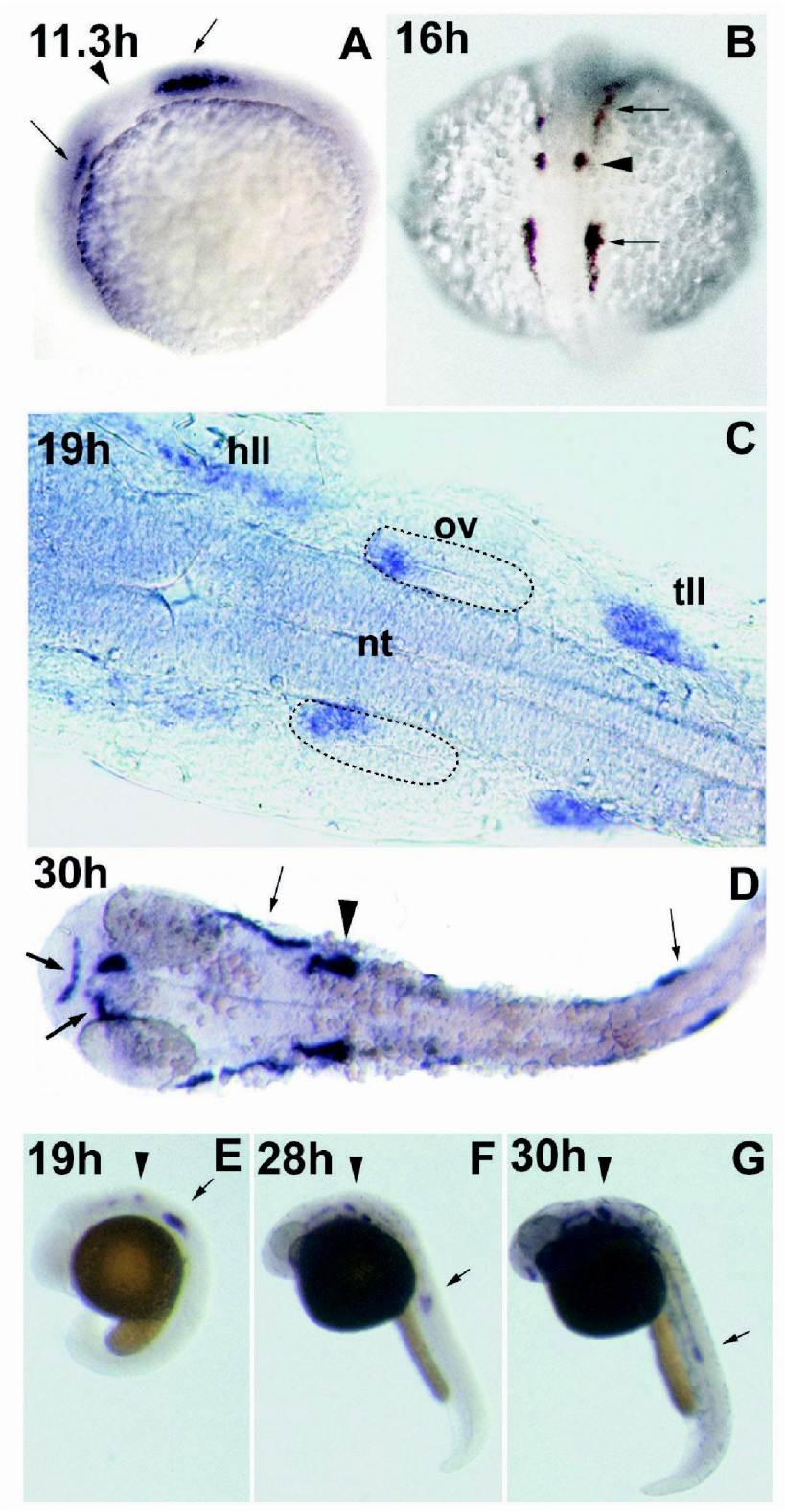

Fig. 14 Zebrafish Nkx5-1 expression in the otic vesicle, lateral line primordium and distinct brain regions. A - 11.3h of development (4 somites), B - 16h (14 somites), C - a higher magnification of a 19h old embryo, otic vesicle is outlined, D - 30h embryo, E–G - Nkx5-1 expression in the primordium of the trunk lateral line (arrow) correlates with its caudal migration during ongoing development (19, 28 and 30h respectively). Otic placodes and vesicles are indicated by arrowheads, lateral line by thin arrows, brain domains by thick arrows, head and trunk lateral lines - hll and tll, respectively, otic vesicle - ov, neural tube - nt; the dark brown color of the yolk is due to unspecific background. Fig. A is a kind gift from S. Léger.

2.2 FGFs control the inner ear development

2.2.1 Nkx5-1 expression in the ear depends on FGF8 signaling

An FGF8 inactivating zebrafish mutation, *acerebellar* (*ace*), results in severe defects of the midbrain and hindbrain and general diminishment of the otic placode, as indicated by changes in Pax2.1 expression in this region (Reifers et al., 1998). Therefore, I wanted to investigate whether Nkx5-1 gene activity in the ear depends on the FGF8 signal. As shown in Fig. 15, Nkx5-1 expression in the otic placode (19h of development, arrowheads) is dramatically reduced in the *ace* mutant (Fig. 15D) as compared to wild type fish (Fig. 15A). In contrast, Nkx5-1 expression in the lateral line seems to be completely unaffected at 19h and at later stages of development (arrows in Fig. 15). The later appearing brain expression domains remained unchanged in the *ace* mutant (data not shown). Since Nkx5-1 transcripts are present in otocysts of the *ace* mutants, FGF8 signal is not absolutely necessary for Nkx5-1 gene activation in the developing ear, but the level of Nkx5-1 expression is always strongly reduced in the *ace* ears.

In wild type zebrafish embryos Nkx5-1 is expressed in the neuroblasts delaminating from the otic vesicle to form the otic (VIIIth) ganglion. Figures 15C and F illustrate that whereas Nkx5-1 is highly expressed in the otic ganglion of the wild type zebrafish embryos, almost no expression can be seen in the developing VIIIth ganglion of the ace mutants. Despite the lack of Nkx5-1 expression, ganglion cells still seem to be morphologically discernible (asterisk in Fig. 15F). At the same time, Nkx5-1 expression in the otocyst is still present. This finding might suggest that FGF8 might directly influence ganglion formation. This hypothesis is further supported by the fact that in zebrafish FGF8 is expressed in the otic vesicle at the time point and area that coincides with the neuroblast delamination from the otic vesicle (Reifers et al., 1998; Léger and Brand, 2000).

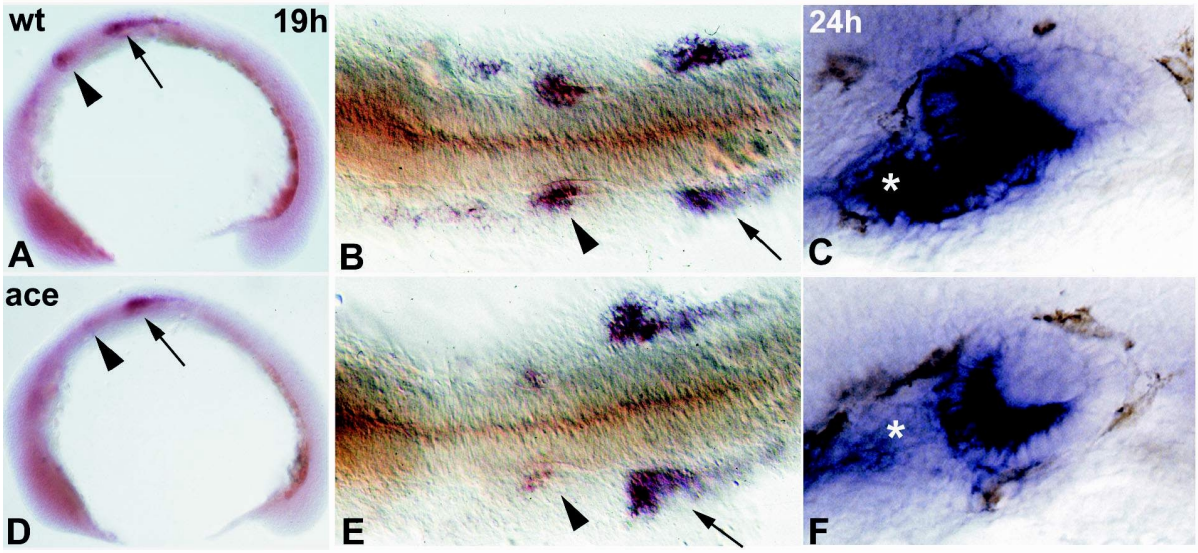

Fig. 15 Nkx5-1 expression is affected in the ears of the FGF8 zebrafish mutant, ace. A and D demonstrate overviews of wild type and ace embryos respectively, B and E show higher magnifications in a dorsal view of the otic and trunk lateral line areas of embryos presented in A and D respectively; note the reduction of the Nkx5-1 expression in the ear, but not lateral line, of the ace mutant as compared to the wild type embryo. C (wild type) and F (ace mutant) - lateral views at the ear region of whole mount embryos illustrate lack of Nkx5-1 expression in the developing vestibulo-acoustic ganglion (asterisk) of the ace mutant. The anterior is to the left, ear is marked by arrowhead, lateral line by thin arrow. Pictures C and F are a kind gift from S. Léger.

2.2.2 Ectopic FGF2 and FGF8 sources influence ear development in chicken

The data obtained by *ace* mutant analysis suggested that FGF8 could play a role in ear patterning. Since in zebrafish ear lack of the FGF8 signal results in downregulation of Pax2.1 and Nkx5-1 expression, I wanted to investigate whether opposite situation, i.e. aplication of ectopic FGF sources, could lead to upregulation of these ear markers. Additionally, defects in otic ganglion formation in the *ace* ears, and other data from experiments with different FGFs (Hossain et al., 1996) suggested that FGFs might play a role in the VIIIth ganglion development. In this experiments I chose chicken as a model system, and implanted FGF soaked heparin coated beads close to the developing ear placode. I decided to use FGF2 soaked beads, since it was shown to influence the ear formation in *Xenopus* embryos (Lombardo and Slack, 1998). Additionally, FGF2 activates broader range of FGF receptor forms than FGF8 (Ornitz et al., 1996) and thus should be able to mimic FGF8 function.

The FGF2 soaked beads were implanted in positions anterior and posterior to the developing ear placode of HH stage 10-11 (10-14 somites) chicken embryos, and incubated for at least 24 hours. In most cases the beads were implanted on the right side of the embryo, so the left ear constituted the internal control for each implantation. The operated embryos, along with

control embryos (with implanted PBS soaked beads) were submitted to in situ hybridization with probes for ear-expressed transcription factors: cNkx5-1, SOHo-1, cPax2 and cDlx5.

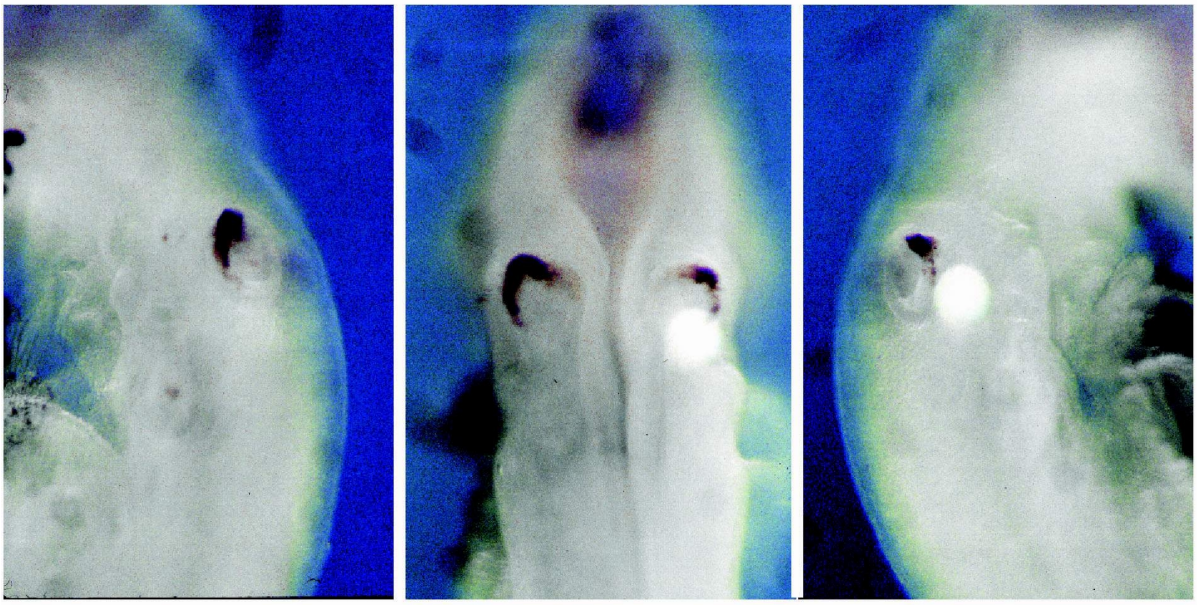

Fig. 16 Nkx5-1 expression in chicken otic vesicle after implantation of PBS soaked heparin coated acrylic bead. Left, middle and right panels show the left (control) side of the embryo, dorsal view, and right (operated) side, respectively. Note that expression of Nkx5-1 in the right ear is slightly downregulated, this effect of operation trauma was never observed when FGF containing beads were implanted.

2.2.2.1 Ectopic FGF2 sources lead to changes in expression pattern of otic specific genes

The implantation of PBS beads did not cause any strong changes in gene expression patterns or size of the ear vesicle (Fig. 16 and see below). However, in the embryos with ectopic FGF2 source close to the developing ear, expression domains of cNkx5-1, SOHo-1 and cPax2 were visibly enlarged in most of the analyzed cases, see Fig. 17 and 18AB for examples. On the contrary, ear expression of the cDlx5 gene seemed to be unchanged, or even slightly downregulated (Fig. 18CD). However, the extent of upregulation differed strongly between embryos, and additionally the FGF-influenced ears seemed to be bigger than the control (left) ones. To definitely prove that FGF sources influenced gene expression patterns, a method allowing to objectively assess the change of the expression of analyzed genes was developed (see Experimental Procedures 4.3.4). Shortly, the hybridized embryos were vibratome cut, the images of the serial sections were captured and processed in a computer (see Fig. 34 in Experimental Procedures) to obtain values describing relative change of expression between left (control) and right (subjected to ectopic FGF2 source) vesicle. The values were summed up for several ($6 \leq n \leq 14$) embryos belonging to each group (depending on bead position and analyzed marker gene), and are presented in Fig. 19.

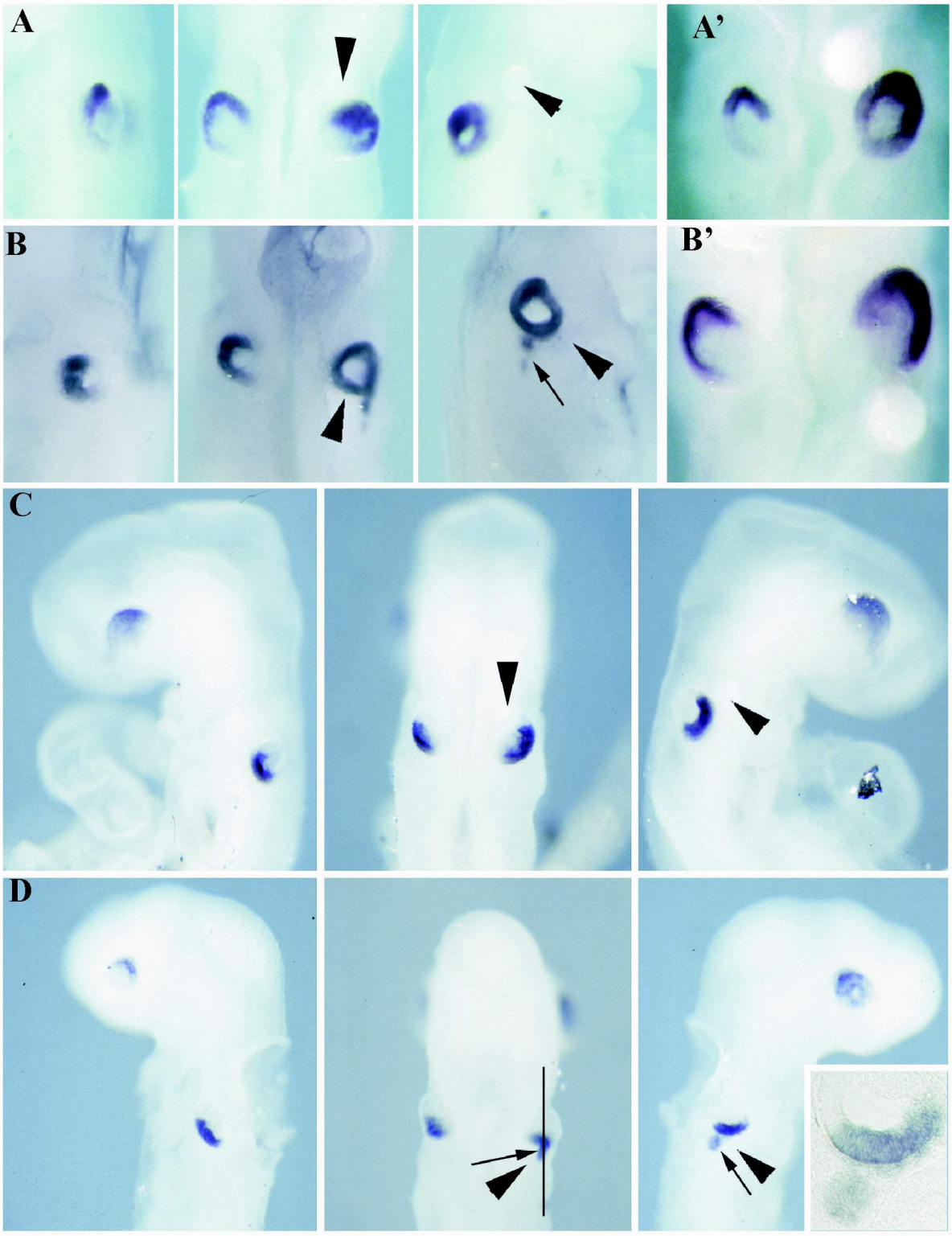

Fig. 17 Expression of cNkx5-1 and SOHo-1 genes in representative embryos with ectopic FGF2 source. FGF2 soaked beads were implanted at HH stage 10 anterior (A and C) or posterior (B and D) to the developing ear placode, embryos incubated for 24 hours and hybridized with cNkx5-1 (A and B) or SOHo (C and D) probe. Additional pictures in first two rows (A´ and B´) show Nkx5-1 expression after bead implantation at HH stage 15 anterior (A´) and posterior (B´) to the developing vesicles and 24 hours incubation. Left panel shows unoperated (left) side of the embryo, middle panel the dorsal view on both otic vesicles and the right panel the operated (right) side. Arrowheads indicate the position of FGF2 beads, arrows point at the ectopic expression domains. Thin black line in D indicates the level of section shown on inset.

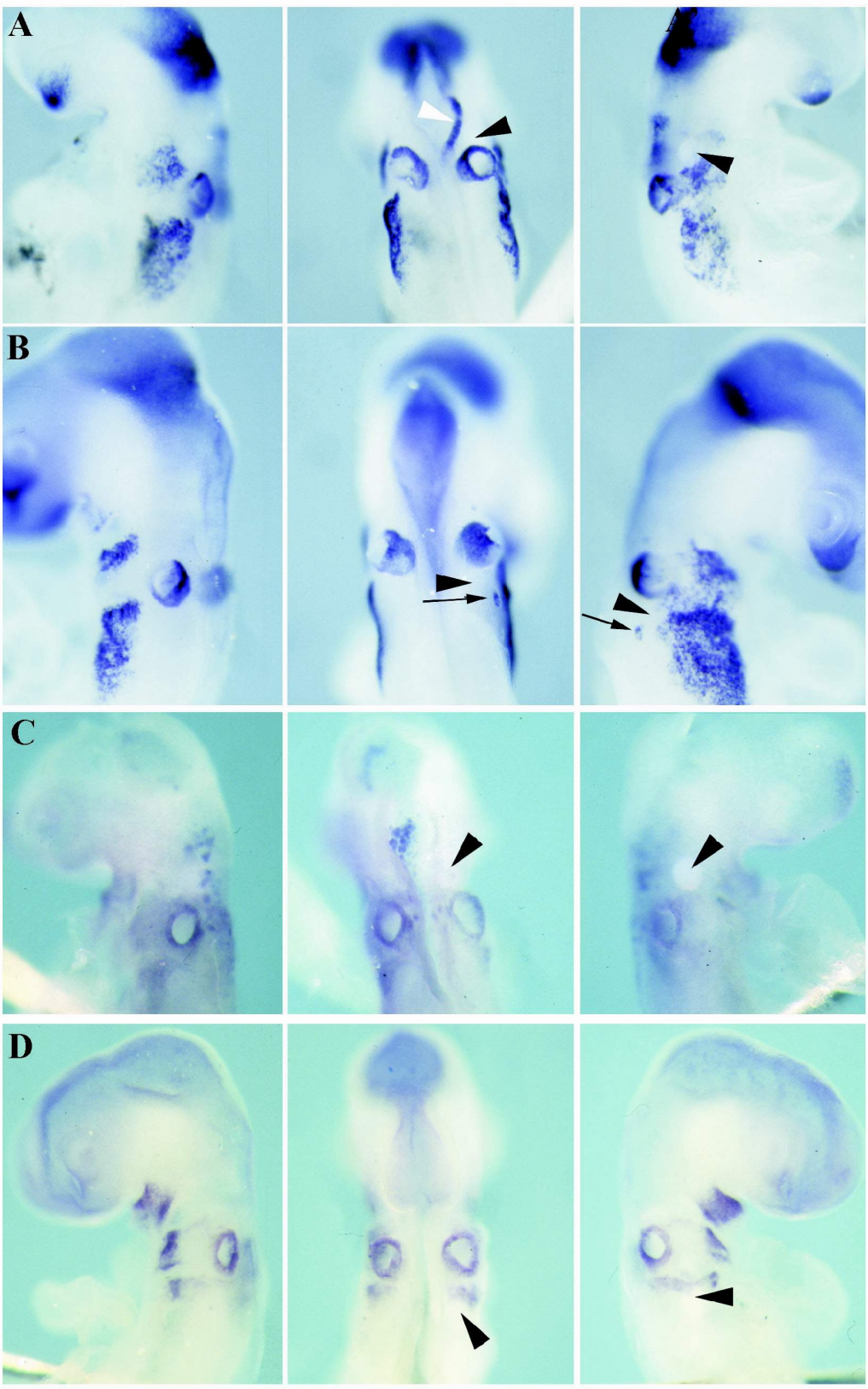

Fig. 18 Expression of cPax2 (A and B) and cDlx5 (C and D) genes after FGF2 bead implantation at HH stage 10 and 24 hours incubation. Left panel shows the control (left) side of the representative embryo, middle panel the dorsal view, right panel the operated side. Black arrowheads indicate bead position, white arrowhead in A an ectopic cPax2 brain domain, arrows in B point at the ectopic structure caudal to the otic vesicle expressing cPax2. Note the extended cPax2 expression in the branchial region (A and B).

Changes observed in the signal intensity and the size of expression domains were not simply due to the bigger size of the operated vesicle, as the presented values are normalized to the FGF-influenced vesicle size (Fig. 19). The same procedure carried out for control embryos, either with PBS soaked beads or not operated, proved that there were no significant differences between left and right ears not subjected to FGF treatment (not shown).

As shown in the histogram (Fig. 19) the anteriorly located FGF2 beads led to stronger and broader cNkx5-1 and SOHo-1 expression (see also Fig. 17A and C respectively). The effect of posteriorly applied beads on gene expression was comparable to the anterior ones in case of Nkx5-1 (Fig. 17B) but was less pronounced for SOHo-1 expression (Fig. 17D). The activation of cNkx5-1 gene by the FGF signal could be achieved during relatively long time period of the otic development up to around HH stage 16. Fig. 17A´and B´ illustrate enlargement of cNkx5-1 expression domain after anterior (Fig. 17A´) or posterior (Fig. 17B´) FGF2 bead implantation at HH stage 15 and 24 hours incubation.

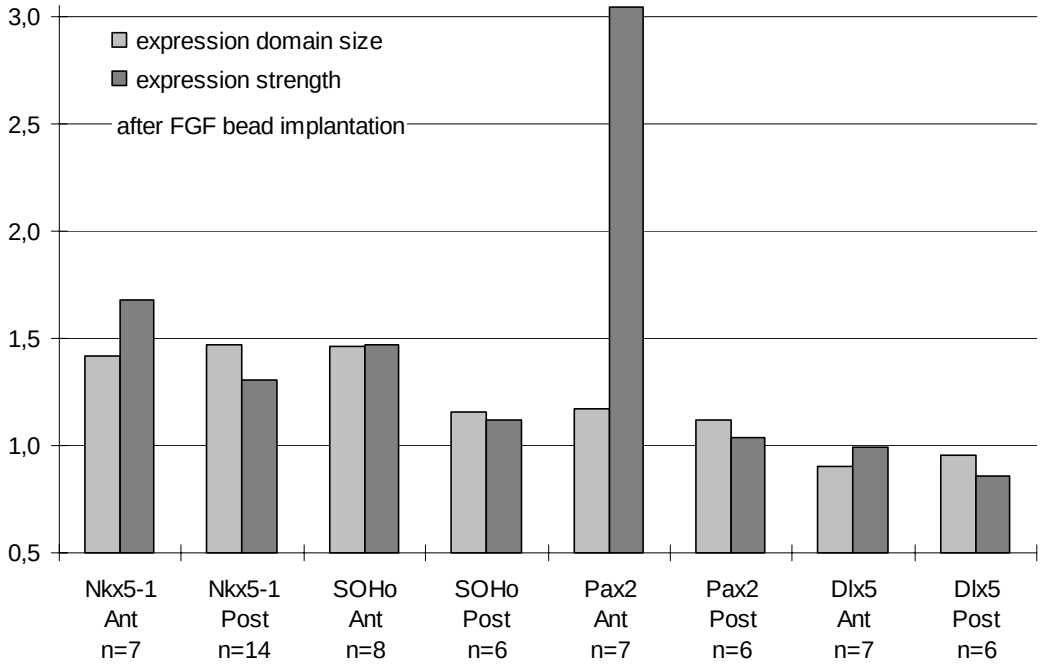

Fig. 19 Effects of FGF2 bead implantation on gene expression. The beads were implanted at the placode stage (HH 10), embryos incubated for 24 hours and hybridized with cNkx5-1, SOHo, cPax2 and cDlx5 probes. Columns represent change of the size of the entire expression domain (light gray) and the strength of expression domain (dark gray) of the right (operated) vesicle in relation to the left (control) vesicle. For description of method used to obtain depicted values see Experimental Procedures 4.3.4. Ant = anterior FGF2 bead, Post = posterior FGF2 bead, n = number of embryos in a given group.

cPax2 expression behaved similarly to cNkx5-1 and SOHo-1 after anterior bead implantation, showing an enlargement of the expression domain and higher signal intensity (Fig. 18A and

19). In contrast, posterior ectopic FGF source did not result in strong changes of expression (Fig. 18B and 19). In addition to the ear effects, both anterior and posterior beads influenced other cPax2 expression domains. For example an extension of the cPax2 expression in the branchial region can be clearly recognized in Fig. 18B whereas ectopic cPax2 expression in the hindbrain is indicated in Fig. 18A (white arrowhead). The histogram indicates as well that cDlx5 expression domain size and strength were slightly downregulated after the operation (Fig. 19, compare Fig. 18CD).

Additionally, I analyzed changes in cBMP4 expression after the FGF2 beads implantation. BMP4 was postulated to play a role during the patterning of the otocyst and later in the determination of the sensory areas of the otocyst (Wu and Oh, 1996), and recently in the semicircular canals formation (Chang et al., 1999; Gerlach et al., 2000). Furthermore, implantation of BMP2 expressing cells close to developing ear placodes in chicken resulted in upregulation of the cNkx5-1 expression (Herbrand, Adamska and Bober, unpublished results). However, ectopic FGF2 source induced no changes of cBMP4 expression in the ear vesicle or surrounding mesenchyme (n=12; data not shown)

2.2.2.2 FGF2 acts as proliferative factor for ear tissue

As mentioned above, in most analyzed embryos the FGF influenced ears seemed to be bigger than the control one. To judge whether the increase was statistically significant, the embryos were grouped according to the bead position, not depending on the probe used for hybridization; that allowed to compare relatively big numbers of embryos (29 embryos with anterior FGF2 bead, 32 embryos with posterior FGF2 bead and 21 control embryos). As expected, the operated vesicles were significantly ($p \leq 0.03$) bigger than the unoperated controls (Fig. 20). FGF2 beads applied posterior to the otic placode (n=32) showed more pronounced effect on the size of the operated vesicles (about 20% increase) than the beads placed anterior to the placode (n=29, 10% increase). The control embryos (n=21) did not show any changes of the vesicle size (Fig. 20). The statistical analysis was performed by M. Adamski.

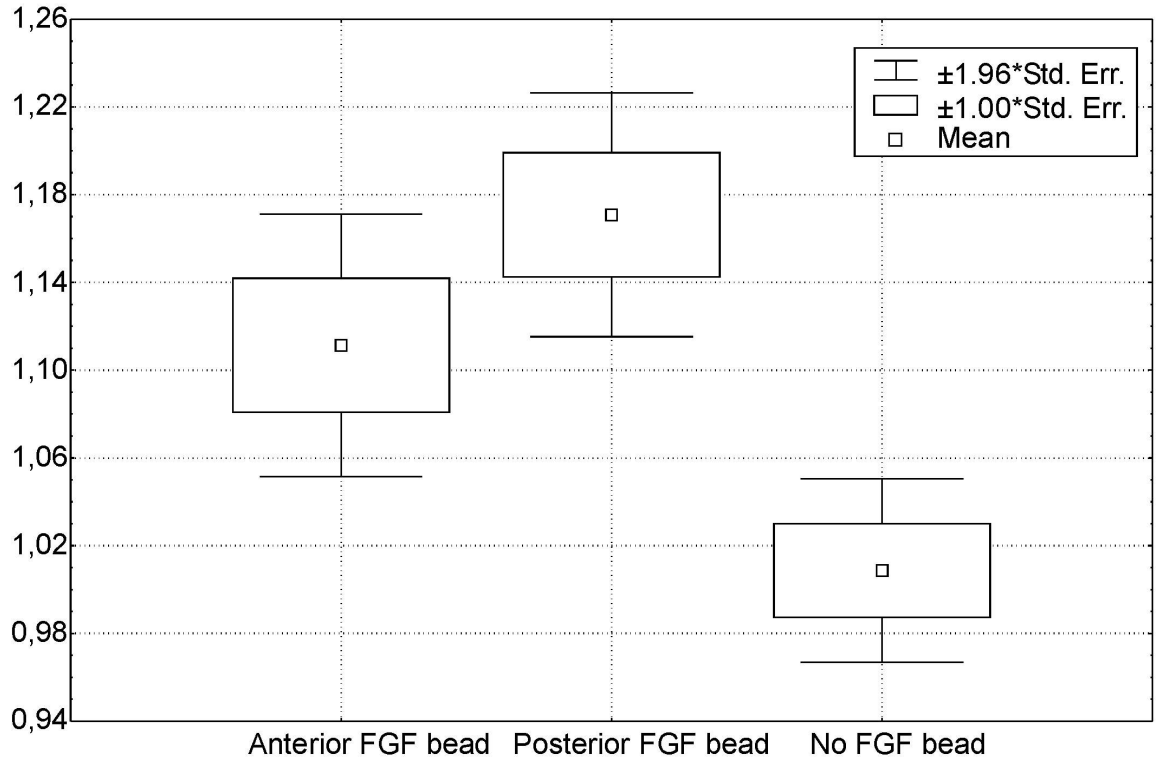

Fig. 20 Changes of otic vesicle sizes after ectopic FGF2 source implantation. Depicted values represent relative size of the right (FGF influenced) vesicles as compared to the left ones; the data were obtained from 29 embryos with FGF2 bead implanted anterior to the developing ear, 32 embryos with a posterior FGF2 bead and 21 control (with PBS bead or not operated) embryos.

2.2.2.3 FGF2 induces ectopic structures of otic identity

The posterior FGF2 beads resulted in some cases in induction of ectopic areas positive for cNkx5-1 (3 out of 29), SOHo-1 (4 out of 17) or cPax2 (1 out of 16) but not for the cDlx5 gene (n=18; Fig. 17B, D and 18B). Such ectopic structures were never observed in control embryos or in operated embryos which received FGF2 beads anterior to the otic placode (n=60). A relatively low incidence of the ectopic induction may be due to a specific position required for the source of FGF bead. In all cases where ectopic gene areas were observed the bead was placed very close to the vesicle, immediately below its ventro-caudal aspect (see a representative example in Fig. 21A). The morphology of the induced cells was of epithelial character. As shown in Fig. 21B, the ectopic cNkx5-1 expressing cells displayed columnar shape characteristic for otic epithelium and different from the surrounding mesenchyme and adjacent cuboidal epidermal cells. In some cases the induced cells appeared to directly migrate out of the otic vesicle as in the example in Fig. 21A. The epithelial morphology and ectopic gene expression domains persisted up to 2 days after the operation (Fig. 21C-E), when small vesicle-like structures could be observed (Fig. 21D, E). However, they were no more visible when the incubation time was prolonged to 3 days.

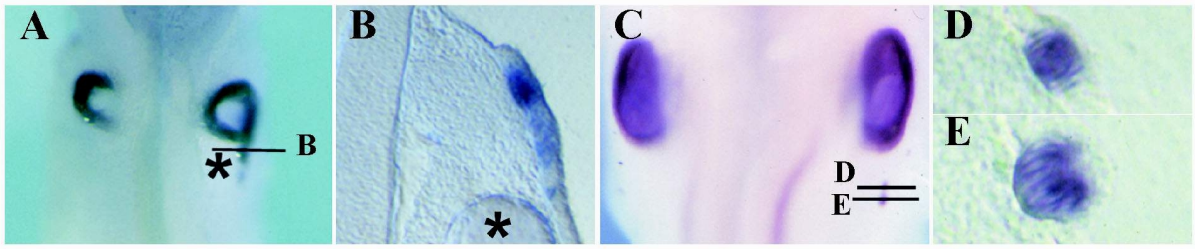

Fig. 21 Posterior FGF2 beads induce ectopic cNkx5-1 positive structures caudal to the otic vesicle. FGF2 beads were implanted posterior to the otic placode (HH 10) and the embryos incubated for 24 hours (A, B) or for 2 days (C-E). The embryos were hybridized with a cNkx5-1 probe. A and C show dorsal views of the operated embryos, the FGF bead is marked by asterisk in A and B. The planes of transversal sections presented in B, D and E are indicated by black lines in A and C.

2.2.2.4 FGF8 is transiently expressed in the otic placode

Although the FGF8 inactivation clearly affects ear development of the zebrafish mutant *ace*, and FGF8 expression pattern has been studied for several species (Crossley and Martin, 1995; Reifers et al., 1998), its transcripts were never reported to be present in early ear development. This could either be explained by the fact that FGF8 expressed in regions adjacent to the otic placode (for example branchial region) influences ear formation, or that the FGF8 is expressed in the otic placode for a very short time, and its domain was overlooked by researchers. Indeed, it appears that the second case is true at least for chicken embryos. I performed whole mount in situ hybridization with the cFGF8 probe on 5-16 somites chicken embryos, at the time when the ear placodes develop. As described for mouse embryos (Crossley and Martin, 1995) at 5-8 somites cFGF8 is expressed in the nasal placode, midbrain-hindbrain boundary, pharyngeal endoderm and branchial region. At 9 somites additional weak domain appears in the ectoderm adjacent to the hindbrain, where ear placodes are forming (not shown). In older, 11-14 somites embryos, this domain is stronger, and corresponds to the anterior part of the placode, (Fig. 22A-F). Ear domain is not present in older embryos; otic placodes of 15-16 somites embryos are completely devoid of FGF8 transcripts (Fig. 22G-I).

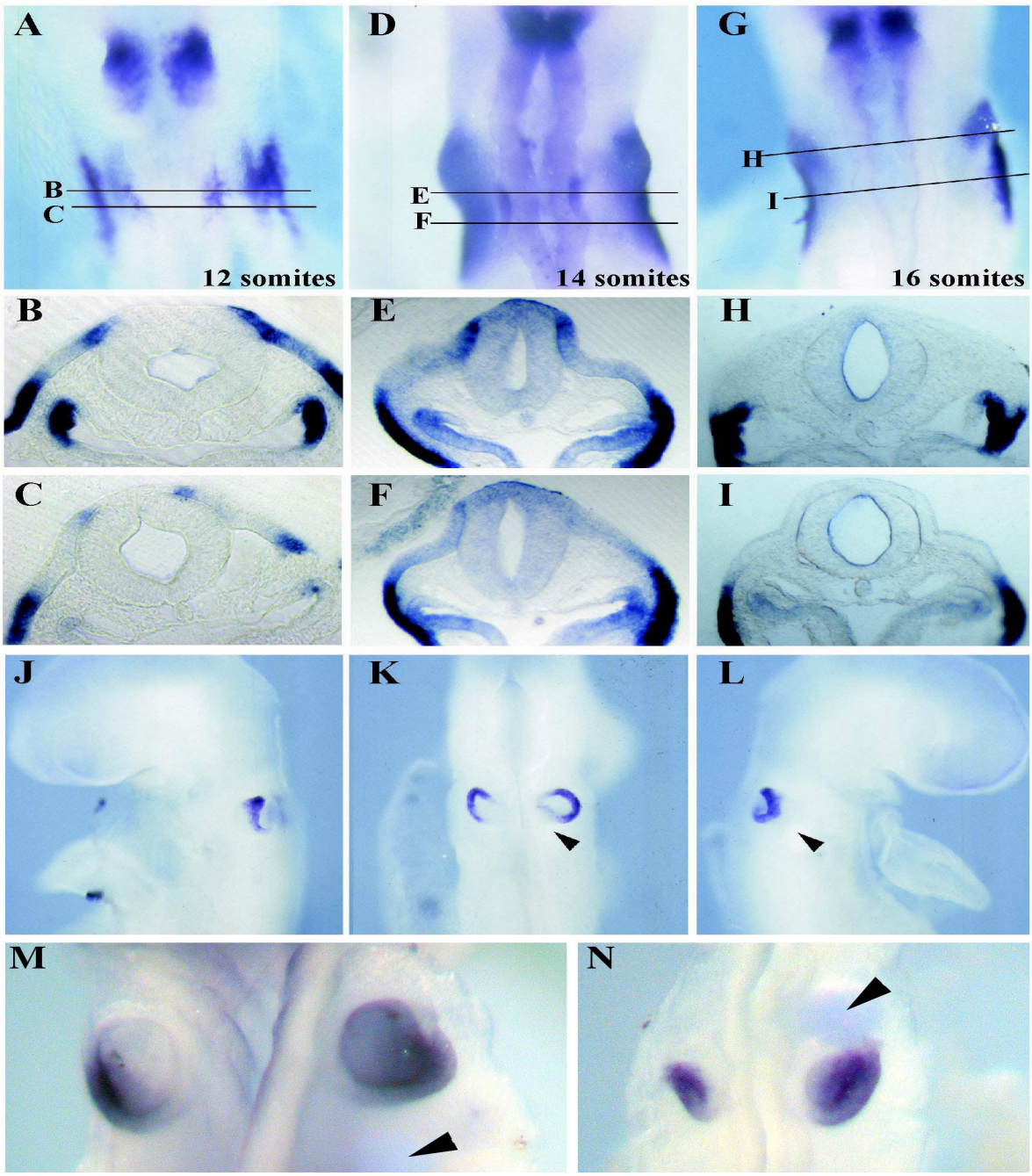

Fig. 22 cFGF8 is transiently expressed in the ear placode and affects cNkx5-1 expression. cFGF8 hybridized embryos of 12 somite (A-C), 14 somite (D-F) and 16 somite (G-I) stages are shown as dorsal views (A, D and G) and corresponding transversal sections as indicated. An example of cNkx5-1 expression after implantation of FGF8 soaked bead at HH 10 and 24 hours incubation is presented in J-L. Left, dorsal and right views of the operated embryo are presented respectively. Two SOHo-1 stained embryos which received posterior (M) and anterior (N) FGF8 soaked beads are shown in the lowest panel, arrowheads indicate bead positions.

2.2.2.5 FGF8 can exert similar effects as FGF2

To find out whether ectopic FGF8 sources can cause similar effects as FGF2 sources, FGF8 soaked beads were implanted as described above for FGF2 beads. The embryos were incubated for 24 hours and hybridized with cNkx5-1 or SOHo-1 probes. Representative

embryos are shown in Fig. 22J-N. On the operated side the cNkx5-1 and SOH0-1 expression domains are visibly stronger and broader, and the vesicles are bigger than the control (left) ones. Computer analysis of the Nkx5-1 stained embryos revealed that the right vesicles (subjected to FGF8 treatment) are in average 10% bigger and the cNkx5-1 expression domains are 70% bigger and 3 times stronger than the left ones (n=5), exactly as in the case of FGF2 bead implantation. Thus FGF8 is not only transiently expressed in the otic placode but can also influence its growth and patterning.

2.2.2.6 FGF treatment results in an enlargement of the vestibulo-acustic ganglion

To find out whether re-patterning of the ear vesicle by an ectopic FGF source might lead to subsequent morphological changes, embryos which received FGF2 beads at the HH stage 10 were incubated for 3 days after bead implantation. Interestingly, embryos with posteriorly implanted beads (n=7) developed completely normal ears as did control embryos with PBS soaked beads. Fig. 23A shows an embryo hybridized with the cNkx5-1 after the posterior bead implantation. As revealed on transversal sections (see a representative example in Fig. 23B) ears on both sides looked fairly normal. The vestibulo-acoustic ganglion was also forming normally at the medio-ventral side of the developing inner ear as indicated by the dotted line in Fig. 23B. In contrast, the vesicles which received anterior FGF beads displayed significant morphological changes (Fig. 23C,D). The right operated ears of such embryos (4 out of 6) appeared shorter in their longitudinal axis in comparison to the left, unoperated side (Fig. 23C). Transversal sections revealed that the affected ears were flattened in their medio-lateral axes resulting in a strong constriction of the inner ear lumen (see Fig. 23D). Strikingly, the vestibulo-acoustic ganglion appeared much bigger on the affected side (compare areas marked by dotted lines in Fig. 23D). To prove that the enlarged area is built in fact by additional ganglionic cells, a ganglion specific probe was hybridized to the operated embryos. For this purpose a cDNA fragment corresponding to approximately 400bp of the 3´untranslated region of the chicken NSCL-2 mRNA has been generated (see Experimental Procedures). Transcripts for this gene mark specifically ganglion forming cells beginning at E3 of chick development (not shown). In 11 out of 14 operated embryos analyzed for NSCL-2 expression a clearly enlarged NSCL-2 positive ganglionic domain was observed. Representative examples of two different embryos are shown on sections in Fig. 23E, F. Implantation of FGF8 soaked beads resulted in comparable results (n = 5, data not shown).

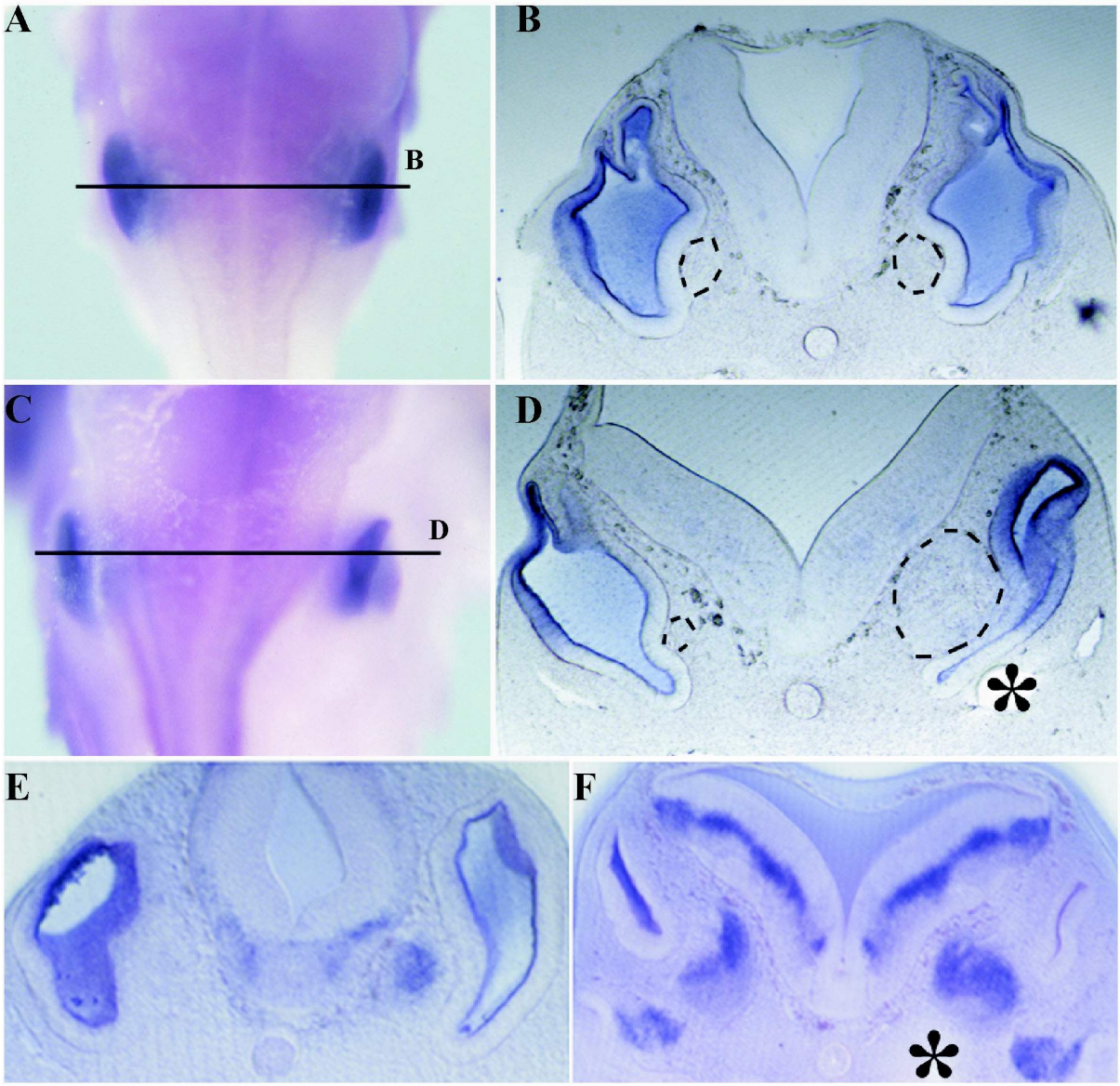

Fig. 23 Anterior FGF2 beads lead to lasting morphological changes in the inner ear and to enlargement of the vestibulo-acoustic ganglion. FGF2 beads were implanted at HH 10 and operated embryos incubated for 3 days and hybridized with cNkx5-1 (A-D) or cNSCL-2 probe (E,F). A and C show dorsal views of embryos with posterior (A) and anterior (C) FGF2 bead. Levels of sections shown in B and D are indicated by black lines in A and C, the ganglion is marked by dotted lines and the beads by asterisks. Sections in E and F show examples of two different embryos which received anteterior FGF2 beads and show an increased ganglionic expression domain of cNSCL-2 on the operated side.

2.3 Establishing epistatic relationships between Nkx5-1 and other regulatory genes

2.3.1 Nkx5-1 and Dlx5 expression is mutually independent

The Dlx5 gene, encoding for a Distal-less-related homeodomain transcription factor, is expressed during early embryonic development in otic and olfactory placodes, branchial arches, restricted brain regions, and limb buds. The KO mice for the Dlx5 gene die shortly after birth, and display a number of craniofacial abnormalities, among others severe malformations of the vestibular organ (Acampora et al., 1999; Depew et al., 1999). Since the semicircular canals deformations strikingly resemble Nkx5-1 KO phenotype, I decided to test whether these two transcription factors do not regulate each other expression. I first analyzed in detail the timing and pattern of Nkx5-1 and Dlx5 expression in the developing ear (Fig. 24A-E). The Dlx5 expression precedes the Nkx5-1 expression in the region where otic placode forms, as Dlx5 transcripts are detectable in this area at least 24 hours earlier (Fig. 24 A-C). Later on the domains completely overlap, with the only difference that the developing endolymphatic duct expresses Dlx5, but not Nkx5-1 (compare Fig. 24 J versus N and P versus R). Thus, since Dlx5 is expressed earlier and in broader domain, it would be more likely that the Dlx5 is an Nkx5-1 regulator than vice versa. Together with B. Zerega I hybridized Dlx5 KO embryos of two different stages (E9.5, E11.0) with the Nkx5-1 probe. The analysis of stained embryos proved that the expression pattern of the Nkx5-1 gene in the Dlx5 KO embryos is unchanged in comparison to wild type and heterozygous littermates (Zerega and Adamska, unpublished observations). I subsequently hybridized 10.5 days old Nkx5-1 mouse embryos with the Dlx5 probe. The embryos are presented in Fig. 24FG, which clearly shows that the expression of Dlx5 gene is normal in the Nkx5-1 KO embryos, and thus that the function of Nkx5-1 is not required for the Dlx5 expression. The presented data suggest that expression of Nkx5-1 and Dlx5 is mutually independent.

2.3.2 Disruption of Tg9257 does not influence expression of Nkx5-1 and Dlx5

The Tg9257 mouse strain was generated by random insertion of human amylase gene. Heterozygote animals displayed an abnormal, circling behavior suggesting vestibular dysfunction (Ting et al., 1994). On the original C57Bl/6J background the inner ears of +/- animals exhibited reduction of horizontal (lateral) semicircular canal, that could account for the circling. In addition, a shortened nasal bone was apparent. Homozygosity resulted in

prenatal lethality (Ting et al., 1994). In contrast, on the mixed C57BL/6J x DBA/2J background Tg9257 homozygotes were viable but showed abnormal development of the three neurosensory systems and their associated bony structures:

i. eye: microphtalmia or anophtalmia, dysmorphic lenses recognizable at E10.5,
ii. ear: the lateral canal hypoplastic or missing already at E14.5,
iii. nose: anomalies in both nasomaxillary structures and neuroepithelium (Griffith et al., 1999).

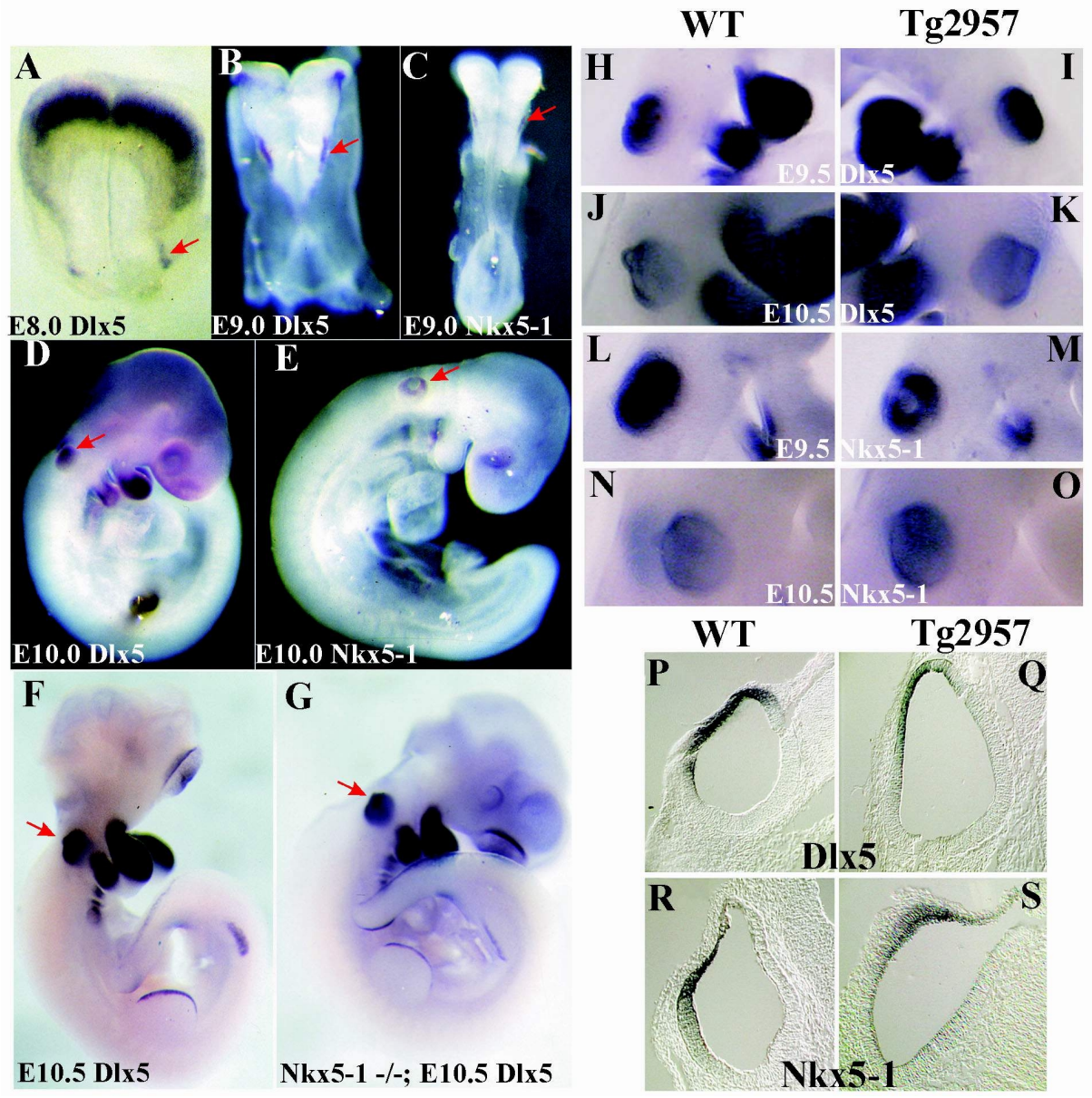

Fig. 24 A-F wild type embryos hybridized with Nkx5-1 or Dlx5 probe, stage and probe are indicated, arrows mark otic area. G - Nkx5-1 -/- littermate of embryo shown on F, note that the ear expression ofDlx5 is normal. H-O - Tg9257 mutants and their wild type littermates hybridized with Nkx5-1 or Dlx5 probe. P-S sections of E10.5 Tg9257 mutant and wild type ears; note that both Nkx5-1 and Dlx5 are normally expressed in the Tg9257 mutant ears

Since the Tg9257 insertion results in ear phenotype resembling the Nkx5-1 and Dlx5 KOs phenotype, and additionally the Tg9257 nasal structures are affected similarly as in case of the Dlx5 KO, it seemed likely that the observed defects might be due to lack of expression of one of these genes. If the early expression of any of these transcription factors was changed in the mutant, one might conclude that the affected gene's expression is regulated by the gene disrupted by the Tg9257 insertion. On the other hand, normal expression of the analyzed genes would suggest that they are either upstream, or act in a parallel pathway to the pathway in which Tg9257 is involved. I analyzed the ear expression of both transcription factors in homozygous Tg9257 embryos at different developmental stages in comparison to their wild type and heterozygous littermates. Analysis of embryos at E9.5, when ear and nasal structures are in the placodal stage, should in principle result in detection of primary expression changes, while older embryos (E10.5 - advanced otic vesicle stage and endolymphatic duct formation; E11.5 - well advanced nasal development) are more likely to reveal secondary differences. The Pax2 gene, as expressed in complementary manner to Nkx5-1 and Dlx5, and not involved in the semicircular canals development, was used as a control marker. As shown in Fig. 24H-S, expression patterns of Nkx5-1 and Dlx5 genes in the developing ears are normal in the Tg9257 embryos in all analyzed stages. The Dlx5 expression pattern in the nasal regions is also normal (not shown). The cryo-sectioning of the mutant ears confirms normal position and strength of the expression domains (Fig. 24P-S). As expected, the Pax2 expression analyzed at day 9.5 was also completely normal (not shown).

2.3.3 Nkx5-1 overexpression in medaka results in upregulation of BMP4 and enhanced apoptosis

Whole mount in situ hybridization of the Nkx5-1 KO embryos with the BMP4 probe showed that the BMP4 ear domains are weaker in the KO embryos, what suggests that BMP4 could be a target of the Nkx5-1 transcription factor (Silke Rinkwitz-Brandt, personal communication). To find out whether ectopic Nkx5-1 expression can lead to upregulation of BMP4, I overexpressed Nkx5-1 protein by injecting Nkx5-1 mRNA into two- to four-cell medaka embryos. Injection of mRNA into such early embryos results in ectopic expression of injected factor in approximately half of the embryo, usually on left or right side. The successfully injected cells can be visualized if the tested RNA is co-injected with a lineage-tracer, for example mRNA encoding for Green Fluorescent Protein. To allow differentiation between the endogenous and injected Nkx5-1, the mRNA was synthesized on zebrafish cDNA template. To establish Nkx5-1 mRNA concentration eliciting a specific response, a series of concentrations was injected first.

Approximately 500pl of zfNkx5-1 mRNA solution was injected into one cell of two to four cell medaka embryo. The concentration ranged from 5ng/µl up to 500ng/µl. The Nkx5-1 mRNA was co-injected with 100ng/µl GFP RNA, to visualize the injected area. After 5 hours the injected embryos were examined under the fluorescence microscope. Of the injected embryos about 95% expressed GFP, the other embryos were omitted in further analysis. Initially, scoring for morphological changes was performed 54 hours after injections, when normally developing embryos reach stage 26 (22 somites, two otoliths easily recognizable in the otic vesicle, pigmented retina). As control for morphological changes uninjected embryos and embryos injected with RNA encoding for GFP in concentration of 2µg/µl were used. This control showed that the injection procedure itself is not causing any significant increase of developmental deformations. However, the Nkx5-1 mRNA injection caused increase of death rate and a number of head and body deformations (Fig. 25AB). The deformations were sometimes confined to the ear, ranging from slightly smaller ear on the injected side, single otolith, up to no ear at the injected side at all; see example in Fig. 25A. In other cases they were more general: smaller or no eye, cyclopia, lack of entire head, only body-like structure instead of embryo, an example is shown in Fig. 25B. The percentage of deformed embryos depending on concentration of injected mRNA, and compared to control embryos, is shown in Table 3.

Table 3 Embryonic malformations caused by injection of Nkx5-1 mRNA into medaka embryos; def. = deformation

	mRNA	not injected	GFP 2µg/µl	zfNkx5-1				
	concentration			500ng/µl	250ng/µl	50ng/µl	12.5ng/µl	5ng/µl
number of embryos	analyzed	479	127	46	62	288	72	86
	dead	1.7%	1.6%	87%	32%	16%	12.5%	0
	general def.	0.4%	0.8%	6%	11%	11%	10%	0
	ear def.	0.6%	1.6%	4%	35%	11%	6%	0

For further analysis concentration of 50ng/µl was used, and 100 embryos were hybridized with ear marker genes to find out whether the ear malformations were correlated with decrease of expression of early ear expressed genes. For this analysis embryos of stage 20-22 (24-36h after injection) were used. The whole mount in situ hybridization of the injected embryos has shown that not only genes belonging to the Nkx5 family (OlNkx5-1.1, OlNkx5-1.2, OlNkx5-2) but also OlPax2 are downregulated by the Nkx5-1 overexpression (see representative examples in Fig. 25C-F). In the most severely affected cases ear domain was completely missing on one side (Fig. 25E). Summarizing, the early and ectopic expression of

Nkx5-1 resulted in unspecific (= not confined only to inner ear) developmental malformations and downregulation of several genes expression.

Another series of experiments was conducted to find out whether the increased lethality and deformations might be due to enhanced cell death in response to expression of Nkx5-1 in the ectopic place and time. I also wanted to know whether this effect is specific to Nkx5-1 or could be elicited by early overexpression of any other transcription factor. The apoptosis was analyzed by a TUNEL reaction, and as a control GFP mRNA and mRNA encoding for another transcription factor, belonging to paired-box family Pax3, was used. As apoptosis program in response to specific stimuli can be triggered in *Xenopus* or zebrafish embryos as early as during gastrulation (Hensey and Gautier, 1997; Ikegami et al., 1999), injected embryos in mid-late gastrula stage were fixed and submitted to whole mount TUNEL protocol, modified after Yager et al. (1997). To visualize the apoptotic cells in the context of injected cells (cells expressing co-injected GFP mRNA) the rhodamine labeled antibodies were used for detection of the apoptotic nuclei. Therefore the injected cells could be seen as green, and apoptotic nuclei as red. The red labeled cells could be easily counted under the microscope. Table 4 shows a comparison between number of apoptotic cells in not injected, GFP, Pax3 and Nkx5-1 mRNA injected gastrula-stage embryos. While injection of high concentrations of GFP or Pax3 mRNA did not cause increase in number of apoptotic cells, even relatively low concentrations of Nkx5-1 mRNA resulted in significant induction of apoptosis (Fig. 25G-J and Table 4).

Table 4 Dependence of total number of apoptotic cells per gastrula stage embryos on concentration and type of injected mRNA. conc. = concentration, n. = number

mRNA →	**not injected**	**GFP**	**Pax3**		**Nkx5-1**	
conc. [µg/µl] →	-	2	0.1	0.5	0.05	0.1
n. of embryos →	16	18	10	39	13	14
n. of apoptotic cells ↓	percent of embryos with n. apoptotic cells					
0	81	78	80	82	30	0
1-2	6	22	20	13	8	0
3-5	13	0	0	5	15	0
6-10	0	0	0	0	30	0
15-25	0	0	0	0	15	32
50-100	0	0	0	0	0	28
>100	0	0	0	0	0	50

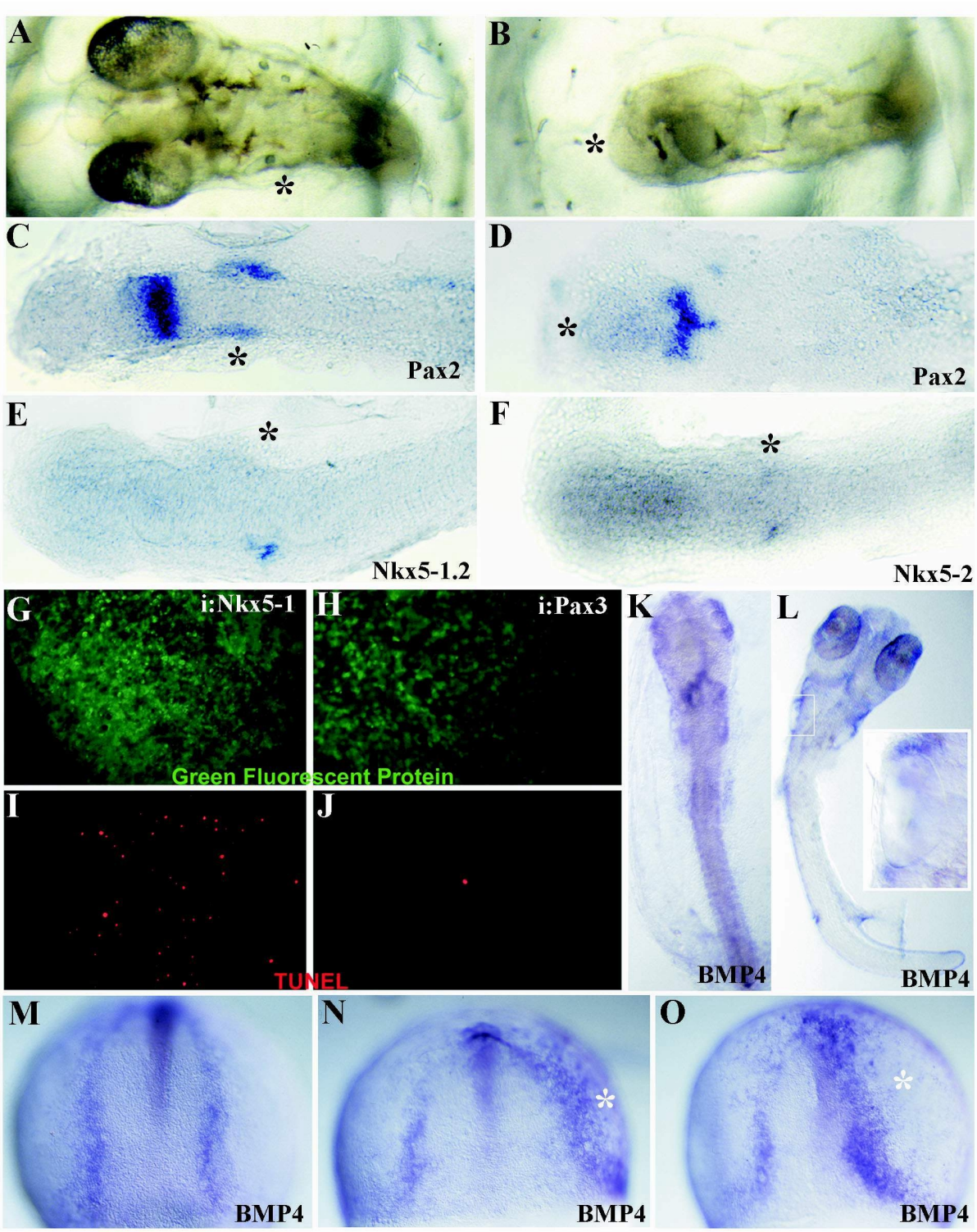

Fig. 25 Effects of injections of the Nkx5-1 mRNA into medaka embryos. A-B - living embryos 3 days after injection; C-F- embryos hybridized with ear marker probes 24-36 hours after injection; G-J middle gastrula stage embryos assayed for apoptosis (GI and HJ are pictures of the same area photographed in different light conditions); K (st. 24) and L (st. 28) - BMP4 expression in normal medaka embryos, enlarged ear vesicle is shown on insert; M-O - BMP4 expression at late gastrula stage, M- uninjected embryo, N, O - embryos injected with Nkx5-1 mRNA. A-F, G/I and N-O embryos were injected with 50ng/ml zfNkx5-1 mRNA and 100ng/ml GFP mRNA, H/J embryo was injected with 500ng/ml Pax3 mRNA and 100ng/ml GFP, K-M embryos were not injected; asterisks indicate the more affected areas; probes or assay types are marked below embryos.

To find out whether Nkx5-1 overexpression causes upregulation of the endogenous BMP4 expression I first cloned a BMP4 probe suitable for whole mount in situ hybridization and studied the normal expression of this gene. The medaka BMP4 probe was cloned by means of PCR amplification, see Experimental Procedures 4.2.3 for details. In short, a 120bp fragment was amplified with a method described by Basler et al. (1993) using degenerated primers complementary to conserved sequences of all TGFβ family members and medaka genomic DNA as a template. The obtained sequence was used to design a pair of nested primers, subsequently used in 3'RACE reaction. The amplified 1kb 3' fragment was cloned into pGemT vector and used for hybridization. The sequence of the fragment is shown in Fig. 33 in the Experimental Procedures section. As its zebrafish homolog (Nikaido et al., 1997), medaka BMP4 is initially expressed in extraembryonic area, and later in the eye, ear, heart, anal area, developing fins and other domains (Fig. 25K-M and not shown).

Having established that the expression of BMP4 in medaka embryos is conserved as compared to other vertebrates (Nikaido et al., 1997; Wu and Oh, 1996), I could further analyze its changes upon Nkx5-1 overexpression. 77 uninjected embryos and 116 embryos injected with 50ng/µl of zfNkx5-1 mRNA were fixed at late gastrula stage and hybridized with BMP4 antisense probe. All uninjected embryos and 51 (44%) of the injected displayed normal, symmetrical expression of BMP4 in the blastoderm surrounding embryonic body. In further 32 of the injected embryos (28%) BMP4 on one side was visibly stronger, and in 33 (28%) the stronger domain extended towards the embryonic body (Fig. 25M-O). Additionally, on the side of the embryo with stronger BMP4 expression morphology of the cells was clearly apoptotic (rounded cells detaching from the surrounding tissue), and this side sometimes was reduced in size, showing clearly that the stronger and ectopic BMP4 domain overlaps with the area overexpressing Nkx5-1. Therefore it can be concluded that Nkx5-1 overexpression in early medaka embryos causes upregulation of BMP4.

3 DISCUSSION

3.1 Evolution of the Nkx5 family

3.1.1 There are four paralogous Nkx5 subfamilies in the vertebrates

To get insight into evolutionary history of the Nkx5 family of transcription factors, I analyzed sequences and expression patterns of the medaka Nkx5 genes. The sequence comparison, together with extensive analysis of phylogenetic relationships within the entire Nkx5 family revealed existence of four paralogous groups in the vertebrate genome: Nkx5-1, Nkx5-2, Nkx5-3 (including Hmx1 and GH6) and SOHo. In medaka, representatives of all four groups are present (with one gene, Nkx5-1, represented by two paralogs, see 3.1.4). In other vertebrates analyzed so far less genes have been identified: Nkx5-1, Nkx5-2 and Hmx1 (Nkx5-3) in mouse, Nkx5-1, SOHo-1 and GH6 (Nkx5-3) in chicken, and Nkx5-1 and SOHo in zebrafish.

Since the Nkx5 related genes have been found in sea urchins and *Drosophila*, the ancestral gene must have existed in the common ancestor of Proto- and Deuterostomia. In the fully sequenced *Drosophila* genome the only gene bearing high similarity to the Nkx5 genes is CG5832. Two sea urchin Nkx5 proteins, TgHbox5 from *Tripneustes gratilla*, and SpHmx from *Strongylocentrotus purpuratus*, have been described so far, and they as well might be the only Nkx5-like genes present in the primitive Deuterostomia genome. Similar relations of gene number in invertebrates and vertebartes have been described for multiple gene families. While only a single Pax258 gene is present in invertebrates as *Drosophila* (Fu and Noll, 1997), amphioxus (Kozmik et al., 1999) and ascidian *Halocynthia* (Wada et al., 1998), in the vertebrates at least three members of this family have been identified per species (see for example Pfeffer et al., 1998). Dlx family is represented by a single distalless gene in *Drosophila* genome; in the tunicate *Ciona intestinalis* two genes, Dll-A and Dll-B have been identified, and in the mammalian genome three Dlx clusters, each composed of two genes, are present (Stock et al., 1996).

3.1.2 Vertebrate Nkx5 genes are organized in two clusters of two genes each

In mouse, Nkx5-1 and Nkx5-2 genes are clustered on chromosome 7, with the same transcriptional orientation, and Nkx5-1 located upstream of Nkx5-2 (Bober et al., 1994). In medaka genome OlNkx5-3 and OlSOHo are tightly clustered on one chromosome, lying only 1kb apart and sharing the same transcriptional orientation. OlNkx5-3 is located upstream of

OlSOHo, and this situation strikingly resembles the mouse Nkx5-1 + Nkx5-2 cluster. Interestingly, in the constructed trees the Nkx5-2 and SOHo genes on the one side and Nkx5-1 and Nkx5-3 genes on the other side form two groups, thus indicating existence of two subfamilies in the vertebrate Nkx5 family of genes. It is striking, that while the upstream located genes (Nkx5-1 and Nkx5-3) and downstream located genes (Nkx5-2 and SOHo) share sequence similarities, the genes clustered in genome share similar expression patterns.
In mouse Nkx5-1 and Nkx5-2 are expressed in the same ear and brain domains, with Nkx5-2 detectable slightly later. In medaka, OlSOHo and OlNkx5-3 expression is identical in the eye, vestibular ganglion and branchial arches. Additionally, OlSOHo is weakly expressed in the otic vesicle and lateral line, and OlNkx5-3 in brain. Whether both clusters are still continuous in all contemporary vertebrates remains unknown, but coexpression of GH6 and SOHo-1 in chicken and OlNkx5-1.1 and OlNkx5-2 in medaka might suggest that they share common regulatory elements. OlNkx5-1.1 and OlNkx5-2 expression starts at the same time point and their expression patterns are practically indistinguishable. Both genes are expressed not only in the anterior part of the ear and in two brain domains, as are their mouse and chicken homologs, but also in the lateral line primordium, as is zebrafish Nkx5-1. Chicken GH6 and SOHo-1 expression patterns are strikingly similar to each other; both genes are coexpressed in the anterior part of the eye, in the vestibular (VIIIth) ganglion and in the ear (Kiernan et al., 1997). In contrast, the mouse GH6 homolog, Hmx1, is not expressed in the ear vesicle (Yoshiura et al., 1998). It remains unclear whether SOHo ortholog exists in mammalian genome, or whether it was lost in course of evolution. For the zebrafish SOHo gene only partial sequence is known, and no expression information has been published up to now.

3.1.3 Nkx5 genes in vertebrates could arise by a tandem duplication followed by a chromosome duplication

The genomic organization, sequence homologies and expression patterns similarities, suggest a model of evolutionary history of the family (Fig. 26). As it seems unlikely that the two strikingly similar clusters (Nkx5-1 + Nkx5-2 and OlNkx5-3 + OlSOHo) arose independently, it can be assumed that an ancestral cluster existed. It is plausible that the tandem duplication of the single ”Nkx5” gene in the ancestor of all vertebrates resulted in formation of the Nkx5-1/Nkx5-3 + Nkx5-2/SOHo cluster, and that event was followed by duplication of entire cluster giving rise to Nkx5-1+Nkx5-2, and Nkx5-3+SOHo clusters.

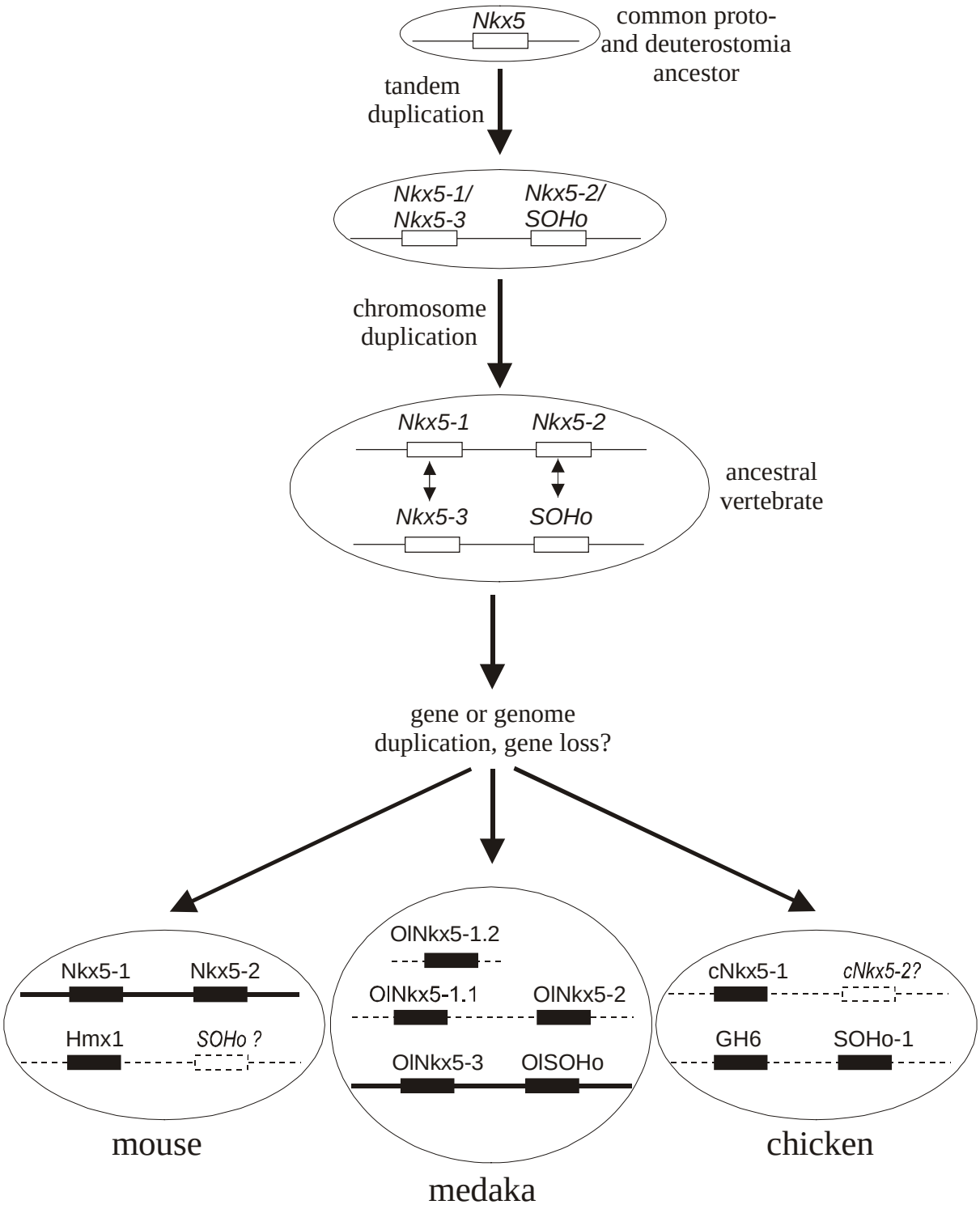

Fig. 26 Proposed evolution events in the Nkx5 family. Putative ancestral genes are shown as empty boxes (*names in italics*), identified genes as black boxes, putative genes that might have been lost as dashed boxes (their names in *italics* and marked by question marks); thick lines indicate known linkage of sequences.

It is believed that during the vertebrate evolution gene number was increased by a factor of 4 as compared to invertebrates (see for example Holland and Garcia-Fernàndez, 1996 for a review on Hox genes), although the mechanism of this amplification remains controversial;

both entire genome duplication, and partial duplication events were proposed (Ohno, 1970; Sidow, 1996; Amores et al., 1998; Hughes, 1999; Skrabanek and Wolfe, 1998; Ruddle, 1997). The view that entire chromosomes or their parts were duplicated is supported by the existence of paralogous mouse and human chromosome segments bearing members of gene families; one of best studied examples, apart from the Hox clusters, are neurotrophins and Trk receptor genes (Hallböök, 1999). Accordingly, an additional line of evidence supporting the presented model comes from chromosomal mapping. Nkx5-1/Nkx5-2 gene cluster was mapped to mouse chromosome 7 (Bober et al., 1994), and Hmx1 is present at chromosome 5 (Yoshiura et al., 1998). There are several other groups of paralogous genes present on this pair of chromosomes, for example: Gabrb-1 and Gabra-2 on chromosome 5, and Gabra-4 and Gabrb-3 on 7 (Danciger et al., 1993); interleukin 4 on chromosome 5 and interleukin 6 on 7 (Sutherland et al., 1988); Zp-2 on chromosome 7 and Zp-3 on 5 (Lunsford et al., 1990), suggesting that at least parts of these two chromosomes are derived from a common ancestral segment. These data indicate that the two Nkx5 clusters arose probably by a duplication event of a big genome fragment, possibly entire chromosome. Since the Nkx5 genes are clustered in two, and not four, pairs, as would be implied by the "x4" model, either they are a result of a partial duplication event, or other clusters have been lost.

3.1.4 OlNkx5-1.2 might be a result of an additional genome duplication in the fish lineage

Interestingly, in medaka not four, but five Nkx5 genes are present. The additional gene is OlNkx5-1.2, highly similar to vertebrate Nkx5-1 genes. Its coding region and intron position are reminiscent of the OlNkx5-1.1 gene, the strongly conserved medaka Nkx5-1 ortholog, but the uncoding regions bear no detectable similarities, suggesting that the duplication took place relatively long time ago. On line with this finding, the OlNkx5-1.2 expression pattern also differs from the OlNkx5-1.1 (which is identical to zfNkx5-1) – OlNkx5-1.2 is not expressed in the lateral line primordium, and in the otic vesicle its expression domain is initially complementary to the OlNkx5-1.1 domain. Thus the two medaka Nkx5-1 genes do not only differ in sequence, but must also have different regulatory elements. This situation could be explained by an additional (and ancient) duplication event in the medaka genome evolutionary history. Indeed, it was recently suggested that in the ray-finned fish lineage (leading to *Fugu*, zebrafish and medaka) another genome duplication event happened after separation of the line leading to tetrapods (Amores at al., 1998; Meyer and Schartl, 1999). As in case of the genome duplications leading to formation of vertebrate genome, this additional duplication of *entire* genome has to be treated as a hypothesis only, but the fact that *part* of

the teleostei fishes genome is duplicated is unarguable (see Aparicio, 2000 for review). Judging from sequence and expression pattern divergence, the Nkx5-1.2 must be a result of such an ancient duplication. This situation is reminiscent of existence of two paralogous Pax2 genes: Pax2.1 and Pax2.2 in zebrafish (Pfeffer et al., 1998). While Pax2.1 expression closely resembles Pax2 expression in mouse, the Pax2.2 transcripts are absent in the nephric system, and in other domains the Pax2.2 onset of expression is delayed. The Nkx5-1.2 and Pax2.2 sequence and expression divergence from their more conservative counterparts is in line with the "semiconservative model" of gene evolution. This model, first proposed by Ohno (1970), and named by Ruddle (1997); states that after a duplication event one of the pair of genes retains the attributes of the precursor, while the second is free to diverge.

The proposed model of Nkx5 genes evolution leads to several questions. For example, is there a SOHo gene in mammals or Nkx5-2 in birds? In primitive chordates, ascidians or lancelet, is there only one Nkx5 gene or more? Could it be a single cluster, similar to Nkx5-1+Nkx5-2? What could be expression patterns of such genes? Are they expressed in organs of balance or vision, or both? How many Nkx5 genes could be found in hagfish and lampreys, phyla that probably underwent less genome duplications that the "true" vertebrates? Where are these genes expressed?

3.2 FGF function in ear development

3.2.1 FGFs in ear induction

The embryonic induction, considered a central process of the development, has been studied by experimental biologists long before any molecules involved were identified, and the inner ear development has been one of the favorite objects of these studies. From classical grafting experiments it was long known that the neural tube and mesoderm play important roles in the otic induction (reviewed by Van De Water and Represa, 1991, and Torres and Giraldez, 1998). The analysis of mouse mutants with strongly deformed ears, in which the ear vesicles develop further away from the hindbrain than in the wild type situation confirms this hypothesis (Kreisler – McKay et al., 1993; Hoxa1 – Mark et al., 1993). However, the main question - the exact nature of the molecules acting as otic inducers - remains open. FGFs are diffusible factors expressed in the region of the otic placode formation, and they are considered to be the best candidate molecules for being the inducing factors (see Introduction 1.5.1). Especially FGF3, expressed in the developing hindbrain prior to and during formation

of the otic placodes and vesicles, was a subject of both experimental analysis and many speculations. The speculations based on reports showing that interfering with FGF3 expression in chicken by application of antisense oligonucleotides or antibodies results in blockade of the vesicle formation (Represa et al., 1991). However, in the FGF3 KO mice the otic vesicles form normally (Mansour et al., 1993).

Nevertheless, in both experimental systems only the formation of the vesicles, and not the original induction of the otic placodes, was assessed. More recently, FGF3 (but not FGF2) was proved to be sufficient to induce ectopic ear placodes and vesicles from broad area of ectoderm surrounding the ear in the chicken embryos (Vendrell et al., 2000). On the other hand, in *Xenopus* FGF2 was shown to be able to induce ectopic otic vesicles (Lombardo and Slack, 1998). These conflicting results were ascribed to species differences (Vendrell et al., 2000; Fekete, 2000).

The presented data (part 2.2.2.3) show that the ectopic FGF2 sources implanted close to the developing ear placode (i.e. in the competent area) in the chicken embryos are able to induce ectopic ear structures, although at a lower frequency than in *Xenopus*. Thus, not only *Xenopus*, but also the chicken tissues are competent to respond to the FGF2 signal by forming additional ear structures. The low frequency can be due to timing of the experiment – in my experiment the beads were implanted at the time point (over HH 10; 10 somites) when the endogenous otic placode induction process is already well advanced (Groves and Bronner-Fraser, 2000). In contrast, implantations in *Xenopus* embryos were performed at much earlier (neurula) stage (Lombardo and Slack, 1998). It is known that the competence of ectodermal tissues to form placodes decreases with time (Gallagher et al., 1996). The reason why Vendrell and co-workers were unable to induce ectopic placodes using FGF2 virus remains unclear. Many FGFs are interchangeable in biological assays (Kettunen et al., 1998; Bei and Maas, 1998), and KOs of both FGF2 and FGF3 in mouse develop normal ear placodes. Therefore, it cannot be concluded which FGF plays the major role in the ear placode induction in any of the studied species (*Xenopus*, chicken or mouse), and what is their functional redundancy in vivo. However, it can be concluded that both FGF2 and FGF3 are able to induce at least part of the ear program (see below) in frogs and birds.

3.2.2 FGFs in ear patterning

Surprisingly, the FGF2 beads were not able to upregulate all of the investigated ear markers, suggesting that the observed changes in the endogenous vesicles were not due to generalized ear fate “overinduction”, but rather that the applied factor influenced expression of particular genes. While the cNkx5-1, SOHo-1 and cPax2 were strongly upregulated in the FGF-

influenced ears; the cDlx5 and cBMP4 expression was slightly downregulated. Similarly, in none of the analyzed embryos ectopic ear structures expressing cDlx5 were found. The different response of the analyzed markers towards the FGFs is especially interesting in light of the expression patterns and mutant phenotypes of the mouse knockouts of the Nkx5-1, Dlx5 and Pax2 genes. Of the three early otic placode markers Nkx5-1expression is detectable as the last. During the invagination the expression domains segregate and while Pax2 is expressed in the ventro-medial part of the vesicle, Nkx5-1 and Dlx5 transcripts are present in the dorso-lateral part. Accordingly, the Nkx5-1 and Dlx5 KOs show severe vestibular malformations (Hadrys et al., 1998; Wang et al., 1998; Acampora et al., 1999; Depew et al., 1999), while Pax2 inactivation leads to lack of the cochlea (Torres et al., 1996). The ear expression of these transcription factors is mutually independent (Nkx5-1/Pax2: Hadrys et al., 1998; Nkx5-1/Dlx5: Acampora et al., 1999; this work part 2.3.1, and Zerega and Adamska, unpublished results). The presented data (2.2.2.1) suggest that Nkx5-1 and Pax2 expression depends on the FGF signaling, while Dlx5 does not.

As in case of the otic induction, a question arising here is which particular FGF is normally responsible for regulation of expression of the ear genes. One hint comes from the expression pattern of the FGF8 gene described in part 2.2.2.4. A short pulse of cFGF8 expression at the anterior pole of the placode precedes localization of the cNkx5-1 transcripts in this region. One might speculate, that while FGF3 and/or FGF2 expression in the hindbrain (Frenz et al., 1994; Mahmood et al., 1995) is responsible for early homogenous expression of Pax2 in the placode, the FGF8 in the anterior part of the placode is responsible for establishing gradient of Nkx5-1 expression in the otic pit/vesicle. The medial shifting of the Pax2 domain, on the other hand, would require additional environmental cues. Indeed, it was shown that while cNkx5-1 expression in the transplanted chick ear vesicles is not depending on the surrounding tissues, cPax2 requires correct environment to be expressed in a proper fashion (Herbrand et al., 1998). Importance of the FGF8 in otic vesicle patterning is supported by the *acerebellar* (*ace*) zebrafish mutant analysis. In the *ace* otic placodes and vesicles both zfNkx5-1 (2.2.1) and zfPax2.1 (Reifers et al. 1998; Léger and Brand, 2000) are downregulated. It can be therefore concluded that while the definite proof of in vivo functions of particular FGFs is still missing, the FGF8 is a good candidate for a molecule patterning the otic placode/vesicle, and especially for regulating Nkx5-1 gradiental expression.

3.2.3 FGFs in otic ganglion development

Previously, FGF2 was implicated in the otic ganglion development (Hossain et al., 1996, 1997; Brumwell et al., 2000). In the in vitro cultures of explanted otocysts it supported migration and differentiation of the neuroblasts, but only during the first 2 days of culture; further on it led to degeneration of the ganglion (Hossain et al., 1996). Interestingly, another growth factor, BDNF, was able to rescue the ganglionic cells and accelerate their growth if added to otocysts pre-treated with FGF2 (Hossain et al., 1997). This promotion of the BDNF role by FGF2 is probably due to the fact that FGF2 upregulates the expression of BDNF receptor, TrkB (Brumwell, 2000). Thus, the FGF2 in chicken is not a sufficient factor for the complete ganglion formation, but seems to play an important function in its early development. Although FGF2 is expressed in the ventral part of the vesicle (Torres and Giraldez, 1998), it is hard to conclude whether it is the FGF responsible for the ganglion formation in vivo. It is also possible that in the culture it is just taking over a function of another member of the FGF family, especially considering the fact that the FGF2 KO mice do not have any problems with otic ganglion formation (Dono et al., 1998).

The analysis of the zebrafish *ace* mutant suggests a role for FGF8 in the formation of the VIIIth (otic) ganglion. As shown in section 2.2.1, the zfNkx5-1 expression in the VIIIth ganglion is absent in the *ace* ear. Since FGF8 was shown to be present in the region where the neuroblast delaminate to form the otic ganglion in zebrafish (Reifers et al., 1998), and recently in chicken (Hidalgo-Sanchez et al., 2000), it might be in fact the FGF involved in the ganglion development. As shown by Ornitz et al. (1996), FGF2 is able to activate all receptors activated by FGF8, and thus the results of experiments using FGF2 (Hossain et al., 1996, 1997 and the data presented in section 2.2.2.6) might in fact reflect endogenous function of FGF8, especially considering the specific expression pattern of the FGF8 gene.

3.3 FGFs versus BMPs in ear formation

Traditionally, the roles of FGFs and BMPs have been viewed as opposite, with FGFs acting as survival and proliferative factors maintaining tissues in undifferentiated state, and BMPs promoting differentiation and programmed cell death. Only balanced action of both types of factors can result in proper morphogenesis. The often cited example of such relations is limb development (Merino et al., 1998; Niswander and Martin, 1993). Similarly, during lung bud morphogenesis FGF10 induces proliferation and chemotaxis of endoderm and both these effects can be inhibited by BMP4 (Weaver at al., 2000). BMP2 and 4 have been shown to

counteract induction of tooth development genes by FGF (Neubuser et al., 1997), and in *Xenopus* embryo eFGF inhibits erythropoiesis induced by BMP4 (Xu et al., 1999b).

On the other hand, the last report shows that although BMP and FGF exert opposing effects, they both can activate the same genes: while BMP4 activates GATA-2, transcription factor promoting erythropoiesis, and PV.1 which inhibits it, FGF induces only PV.1 expression (Xu et al., 1999b). Thus, while some transcription factors expression is under control of only one of the signaling molecules, others can be activated by both. In early chick development both FGF8 and BMP4 can induce msx1 expression, and both factors interact in specification and maintaining the border of the neural plate (Streit and Stern, 1999). During the tooth development Msx1 can be activated by several FGFs (FGF1, 2 and 8) and BMP4, but the two pathways are distinct, since BMP expression cannot be induced by FGF, nor vice versa (Bei and Maas, 1998).

It is well documented that development of several organs requires action of both kinds of factors. For example, BMP2 and FGF4 applied together are able to induce rhythmically contractile multicellular vesicles from non-precardiac mesoderm, but none of the factors alone can exert such an effect (Lough et al., 1996). Synergistic action of FGF2 and BMP7 was also shown in development of metanephric mesenchyme (Dudley et al., 1999).

Application of a BMP inhibitor, noggin, results in defects in semicircular canals development, proving that BMPs are necessary for normal vestibulum development (Gerlach et al., 2000; Chang et al., 1999). To find out which genes are regulated by BMPs, a similar experiment as in case of FGF beads, but with BMP2 expressing cells instead, was performed (Herbrand, Adamska and Bober, unpublished). Interestingly, while FGF source strongly upregulates cNkx5-1 and cPax2, and slightly downregulates cDlx5 expression in the ear, implantation of BMP2 producing cells in vicinity of the developing ear placode results in strong upregulation of cNkx5-1 and cDlx5, and downregulation of cPax2 expression. Indeed, mouse Dlx5 has been shown to be a BMP-inducible gene (Miyama et al., 1999).

Summarizing, Dlx5 and Pax2 respond differently, and Nkx5-1 in the same way to FGF and BMP signals; Dlx5, the only transcription factor not upregulated by FGF, is positively regulated by BMP. This can be viewed as an additional example of cooperation of BMP and FGF signaling in the vertebrate development. Interfering with FGF or BMP signaling results in blocking of the otic development, but neither FGFs, nor BMPs alone are sufficient to induce a full ear program (Represa et al., 1991; Gerlach et al., 2000; Chang et al., 1999; Herbrand, Adamska and Bober, unpublished, and section 2.2.2 of the presented work). All of the analyzed ear markers were upregulated by either (or both) of the tested signaling

molecules; and thus it is possible that the combination of both factors would be able to elicit full ear program in a competent tissue.

3.4 Nkx5-1 and Dlx5 in ear formation

While ear phenotypes of Nkx5-1 and Dlx5 KO mutant mice are strikingly similar (Hadrys et al., 1998; Wang et al., 1998; Acampora et al., 1999; Depew et al., 1999), the expression of both genes is mutually independent (2.3.1, Zerega, Adamska and Bober, unpublished). Therefore, it might be concluded that the two transcription factors act in parallel pathways. On the other hand, the lack of mutual expression regulation does not exclude a possibility that the two proteins interact physically, and thus the deficiency for any of them results in developmental deformations. Basing on expression patterns, Yoshiura et al. (1998) proposed that Dlx and Hmx (Nkx5) factors interact in the areas where their expression overlap – for example in the developing branchial arches. What is more, Msx1 and Msx2 proteins, products of genes related to the Nkx family genes, were shown to interact in vitro with Dlx2 and Dlx5 proteins by forming heterodimers (Zhang et al., 1997). Thus, it is possible that the similarity of the mutant phenotypes reflects necessity of synergistic action of the two genes. It is worth to mention here that the BMP4 expression domain seems to be similarly affected in the Dlx5 KO ear, as it is in the Nkx5-1 knockout (Rinkwitz, Zerega, Adamska and Bober, unpublished).

3.5 Is Nkx5-1 a BMP4 regulator?

To investigate results of Nkx5-1 overexpression, mRNA encoding for zfNkx5-1 was injected into early medaka embryos (section 2.3.3). The experiment resulted in:

- enhancement of apoptosis in the middle gastrula stage embryos;
- upregulation of BMP4 expression (tested at late gastrula stage);
- general deformations of the injected embryos characterized by reduction of organs, especially anterior structures;
- strong reduction of expression domains of ear marker genes;
- increased death rate of embryos, presumably secondary to other defects.

This complex phenotype makes it difficult to conclude whether any of the observed features reflect physiological functions of the Nkx5-1 transcription factor. It is important to note that the observed malformations were not simply due to the injection trauma, since control injections of an order of magnitude higher concentrations of GFP or Pax3 mRNA never led to

comparable results. The explanation of the obtained results could follow one of three scenarios, not necessarily excluding one another.

i. *Nkx5-1 is positively regulating BMP4 expression in the ear*, early overexpression of Nkx5-1 leads to strong upregulation of the BMP4; all other effects are secondary and due to the BMP4 overexpression. Zebrafish embryos overexpressing BMP4 upon BMP4 mRNA injections are strongly ventralized (Neave et al., 1997), and it is striking that the medaka embryos with most severe phenotype appear strongly ventralized (Fig. 25B and D). BMPs, and especially BMP4, have been implicated in the apoptosis processes during development of several vertebrate organs (see for example Marazzi et al., 1997, and references therein). However, in normally developing early embryos (gastrula or neurula stage) the BMP4 expression domain does not overlap with any "apoptosis domain". Therefore, while the BMP4 upregulation in the Nkx5-1 injected medaka embryos, taken together with downregulation of BMP4 in Nkx5-1 -/- otic vesicle suggest that Nkx5-1 transcription factor is a BMP4 activator, this hypothesis can still be treated only as "working hypothesis".

ii. *The enhanced apoptosis upon Nkx5-1 overexpression reflects its physiological function in inducing programmed cell death during development*. The strongly enhanced apoptosis most probably accounts for the deformations and lethality of the injected embryos; reduction of the expression domains of tested genes could simply be due to reduction of the size of the Nkx5-1 expressing part of the body. Indeed, programmed cell death is an important process in the ear development (Fekete et al., 1997), and the "apoptosis domains" partially, but not completely overlap with the expression of Nkx5-1 (Fekete et al., 1997 and Sanz et al., 1997). It is plausible that while Nkx5-1 expression alone is inducing apoptosis, there are other factors in the ear that protect the cells from entering the apoptosis pathway. These factors might be absent in the early embryos and thus Nkx5-1 overexpression results in dramatic increase of cell death.

iii. *Nkx5-1 is a repressor of transcription, and in the early overexpression experiment it acts as nonspecific repressor*. Nkx5-1 contains a strongly conserved repression domain (approximately aminoacids 28-45 on alignment in Fig. 4A) – *eh1* region (Smith and Jaynes, 1996) upstream of the homeodomain. If the repression function of the Nkx5-1 protein were true, it would have to activate the BMP4 expression indirectly (via repressing its repressor?).

It should be possible to differentiate between these three explanations by overexpressing Nkx5-1 in more physiological conditions. A retrovirus carrying Nkx5-1 coding sequence was

already constructed by D. Schulte and will be used for infection of chicken otic area. The upregulation of BMP4 domain in the ear area upon Nkx5-1 overexpression would further support the function of Nkx5-1 as a BMP4 positive regulator. It is worth mentioning that a function of other members of the Nkx5 family, namely GH6 and SOHo-1, in regulating the BMP expression, was proposed (Kiernan et al., 1997). Also, a knockout of related Nkx2.3 gene results in downregulation of the BMP2 and BMP4 expression in mouse intestine (Pabst et al., 1999).

3.6 Conclusions

In the presented work I attempted to establish the phylogenetic relationships within the Nkx5 family of genes, and to place the Nkx5-1 gene in the molecular pathway leading to the inner ear formation. The phylogentic analysis led me to propose a model of Nkx5 evolution (Fig. 26). The expression studies performed in fish embryos show strong conservation of the expression patterns of Nkx5 genes in sensory organs (eye, ear, lateral line) of different vertebrate species, suggesting conserved function of Nkx5 genes in the development of placode-derived sensory organs.

Basing on expression pattern and functional analysis, FGF8 seems to be a good candidate to be an Nkx5-1 expression regulator. Additionaly, the FGF bead implantation experiments provide strong evidence for importance of FGF signaling in all stages of the ear development. The FGF2 soaked beads are able to induce ectopic ear structures in chicken, showing interspecies conservation of the pathways involved in the ear induction. Differential regulation of ear markers genes by FGFs suggests a function of FGFs in the ear patterning. The interspiecies conservation of FGF pathways was recently questioned, and on the other hand it was suggested that FGFs alone are able to induce the full program of the ear development (Vendrell et al., 2000; Fekete, 2000). Therefore, the findings presented here contribute to the ongoing discussion on FGFs functions in the ear development. Interestingly, the Dlx5 gene, not upregulated by the FGF signaling, is strongly overexpressed upon BMP2 stimulation (Herbrand, Adamska and Bober, unpublished), and thus the inner ear development is another example of FGF/BMP cooperation in vertebrate organogenesis.

The expression studies on Tg9257 mutant embryos proved that the gene disrupted by this insertion is not necessary for proper expression of Nkx5-1 and Dlx5 genes. However, once the gene is isolated it would be easier to position it in the genetic network of the ear formation.

The function of Nkx5-1 transcription factor as BMP4 activator remains to be proven in other system. Infecting otic area of the chicken embryo with a retrovirus encoding for Nkx5-1 would eliminate a problem of expressing Nkx5-1 in time and part of the embryo where it is normally never expressed, and provide further support for the hypothesis.

The relations between individual genes established in the presented project are schematically depicted in Fig. 27. Although there is no doubt that the relations established here are only a little piece of the great puzzle of the vertebrate ear development, I believe that the results of this project help to position the Nkx5-1 gene in the molecular network leading to the ear formation. They should also place this gene in context of the evolutionary history of the vertebrate genome.

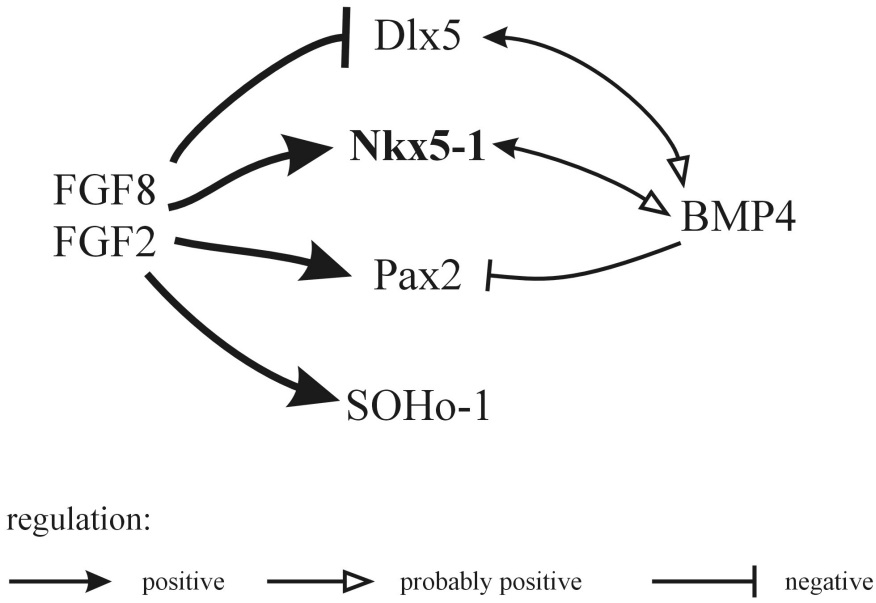

Fig. 27 Position of the Nkx5-1 gene in the molecular network leading to inner ear formation. The relations shown were established in the present work (thick line), or in collaborative projects (thin line).

4 EXPERIMENTAL PROCEDURES

4.1 General molecular biology techniques

All procedures of molecular cloning were performed according to standard protocols (Sambrook et al., 1989; Promega, 1996). Composition of solutions and protocols used during the presented project are provided below only if they differ from available in the references or manufacturers instructions. Clones and PCR products were sequenced with Perkin-Elmer ABI 310 automatic sequencer.

4.2 Experiments in fish system

Medakas were cultivated, reared and staged according to Iwamatsu (1994). Wild type zebrafish embryos were a kind gift from Reinhard Koester (Pasadena). The *ace* mutants were provided by Sophie Léger (Heidelberg).

4.2.1 Characterization of medaka Nkx5 genes

4.2.1.1 Clone I: OlNkx5-1.1

Fig. 28 illustrates sequence of 2kb XhoI/NotI subclone encompassing full coding sequence of medaka homolog of the Nkx5-1 gene. Angela Wolff obtained sequence corresponding to 2nd coding exon (1kb KpnI fragment and part of the XhoI/NotI fragment up to stop codon). For expression studies she used the homeobox-containing 1kb KpnI fragment. To obtain full sequence of the OlNkx5-1 gene I subcloned and sequenced two additional fragments of the XhoI/NotI clone: 0.4kb KpnI fragment that appeared to contain complete coding 5' end of the gene, and 1kb SacII fragment, containing 3' end of the gene. The SacII fragment was subsequently used in expression studies, as a no-homeobox containing specific probe.

To identify the exon-intron boundaries two pairs of primers matching putative coding regions flanking putative introns were designed basing on similarities to homologous sequences in chicken and mouse (primer sequences are provided in section 4.8.2 and marked in Fig. 28). The RT PCR was carried out using mRNA isolated from 1-3 days old medaka embryos as a template. For isolation of the RNA IN VITROGEN Micro-Fast-Track kit was used according to the manufacturer instruction. In both cases the amplification yielded single bands; their subsequent direct sequencing resulted in identification of the splicing site.

```
         *        20         *        40         *        60         *        80
CTCGAGGGCTCTGTTTACTATCTTTATTATTTTTTTCATTCCACCCCCTTGCATAGAAGGACTTGCGCGCTGTCGCGCTC
         *       100         *       120         *       140         *       160
CACGGGAGAAATTCACAGAAAAACTGGGACTCCTCTGAGCCAGGATATTCTCCGGCTGAAGGTGAGCTTCGCTTTTCCCC
         *       180         *       200         *       220         *       240
CTCCTTCCCCCTCCCCAAACTCCTGCTTCCGTGGTTGTCGGTGCTCAGACCACAGTCACAGCGATGCCAGAGACAACGCA
                                                                 M  P  E  T  T  Q
         *       260         *       280         *       300         *       320
AGAGACGTGCGCTCCGGCCAAGGATTCTCCCTTCTTCATTAAGAACCTACTGAACTGTGACAGCAAACCATCCAAACCCA
  E  T  C  A  P  A  K  D  S  P  F  F  I  K  N  L  L  N  C  D  S  K  P  S  K  P
         *       340         *       360         *       380         *       400
GGCCGGTACTGGCTGCGACTAAGGCGGCCCTAGAGGGAGGATTCTCCCTTTCCCAAGTCGGAGAGTTCAACTTCCCTCGC
 R  P  V  L  A  A  T  K  A  A  L  E  G  G  F  S  L  S  Q  V  G  E  F  N  F  P  R
         *       420         *       440         *       460      KpnI*       480
TTTGAGCTGCCTGCGCAAAGGTTCAGTCTACCGGCTCACTACTTGGAGCGCACATCGGCGTGGTGGTACCCGTACACGCT
 F  E  L  P  A  Q  R  F  S  L  P  A  H  Y  L  E  R  T  S  A  W  W  Y  P  Y  T  L
         *       500         *       520         *       540         *       560
CAGCTCAGGAGCGCATCTTCACAGAACGGAAGGTAAAAGGCTTTAATCCACTTGAAATATYAAATGCAATGCCAAAAAGT
  S  S  G  A  H  L  H  R  T  E
         *       580         *       600         *       620         *       640
AGTATTTGGTTCCTATATAAATGTGCAAACTTGCAGAGCTTTTTGCGTTTTTGTGACGGTAAAAAAACGATCTAATGTCG
         *       660         *       680         *       700         *       720
TAATTTGACTTAAGGCTTAAAATAAATATGGAGCTATTTTGTTATTCCACATTGGATGGGATAATGGATCAATAAAGTGT
         *       740         *       760         *       780         *       800
GCATAAAATGAAGCAGACATCATGAAATCATTGGGTGAGGGCGATGATGCGCAGCGCGCCTGTGATTTGTGTGTTGGCGC
         *       820         *       840         *       860         *       880
TCACGCAGCACATCTTCACCCTTAAGGTTGGTCACTTGACAACCTTTTGAACGCGCACTGGCACATTCTCAGTGCACTAT
         *       900         *       920         *       940         *       960
ACAACACGCACATTTATTTTAAAATAAGTAGCATGATTAAAGTTTGTTGCATAAAAGAAAACTANTTTTATTTAGGACAG
         *       980         *      1000         *      1020         *      1040
AATTTTTTAATTTTATAGTTTGAGATTTACTGCCAATCCACCCCTGAAATTGTTTTGAAGACTTTATTTGGCGCTCATTT
         *      1060         *      1080         *      1100         *      1120
GTGCGCCCTTTTACTTTACTCGAAAATAATCTCGTTTGGCTCCAATAATTTTCCAAACACTCCTTGCTTATGACTGAGTG
         *      1140         *      1160         *      1180         *      1200
TCTCTTCTAAATGTTTCCGTCACCGAGAGACCAGGAGCCAGGGACTCCTCTCCAACCTCGGGCACAGACAGAGACTCTCC
                  V  T  E  R  P  G  A  R  D  S  S  P  T  S  G  T  D  R  D  S  P
         *      1220         *      1240         *      1260         *      1280
AGATCTGGTACTCAAAACCGAACCAGACGCCAAAGAAGACGAGGATGACGATGAGCACAACAACAACAAGAGCAGCGACG
  D  L  V  L  K  T  E  P  D  A  K  E  D  E  D  D  D  E  H  N  N  N  K  S  S  D
         *      1300         *      1320         *      1340         *      1360
AGATCATCCTGGAGGAAAGCGACGCCGAGGAGACCAAAAAAGAGGAGCTGGAGGAGTGGAAGAAGAGGGATGAGGATAAG
E  I  I  L  E  E  S  D  A  E  E  T  K  K  E  E  L  E  E  W  K  K  R  D  E  D  K
         *      1380         *      1400         *      1420         *      1440
AAGCCGTGTCGCAAGAAGAAGACGCGCACGGTTTTCTCCCGGAGCCAGGTCTTCCAGCTGGAGTCCACCTTCGACATGAA
 K  P  C  R  K  K  K  T  R  T  V  F  S  R  S  Q  V  F  Q  L  E  S  T  F  D  M  K
   KpnI  *      1460         *      1480         *      1500         *      1520
GCGGTACCTGAGCAGCTCTGAGCGCGCGGGCCTGGCGGCGTCTCTCCATCTCACAGAGACTCAGGTGAAGATCTGGTTTC
  R  Y  L  S  S  S  E  R  A  G  L  A  A  S  L  H  L  T  E  T  Q  V  K  I  W  F
         *      1540         *      1560         *      1580      SacII    1600
AAAACAGAAGGAACAAGTGGAAGCGGCAGCTGGCCGCAGAACTGGAGGCTGCCAACCTCAGCCACGCCGCGGCGCAGAGG
 Q  N  R  R  N  K  W  K  R  Q  L  A  A  E  L  E  A  A  N  L  S  H  A  A  A  Q  R
         *      1620         *      1640         *      1660         *      1680
ATAGTCCGCGTCCCCATCCTTTACCACGAGAACTCGGCCTCAGAGGGCGGGGCTGCCGCTGCCAACGTGCCCGTGAGCCA
 I  V  R  V  P  I  L  Y  H  E  N  S  A  S  E  G  G  A  A  A  A  N  V  P  V  S  Q
         *      1700         *      1720         *      1740         *      1760
GCCGCTGCTCACTTTTCCTCACCCCAGTGTGTATTACTCCCACCCCATCGTCACATCTGTGCCGTTGCTTAGACCGGTTT
  P  L  L  T  F  P  H  P  S  V  Y  Y  S  H  P  I  V  T  S  V  P  L  L  R  P  V
         *      1780         *      1800         *      1820         *      1840
GAAAAATGGAACAGGGTTTTGTTGGTGTCCTGATTAAAAAAATATAAAGAAACTAAATAGAATAAATGAAACCATATTTT
*
         *      1860         *      1880         *      1900         *      1920
TTTTGATGACACTGTGCGACTAAAGACACTAAAAGAAACANAGAAAGACTGTTTTAATATTTCAAAAACGTTTCCCNTAC
         *      1940         *      1960
AAGATGTATTATTATTATTATTATATTATTATATTATTAT : 1963
```

Fig. 28 Genomic sequence of the OlNkx5-1.1 gene. Coding sequences are marked yellow, primers used for RT PCR red, important restriction sites green, homeodomain is blue.

4.2.1.2 Clone IV: OlNkx5-1.2

The analysis was performed as described above for OlNkx5-1.1: fragments containing putative coding sequences were sequenced, the specific probe not containig homeobox was subcloned, and the intron-exon boundaries identified with two pairs of primers (section 4.8.2 and Fig. 29).

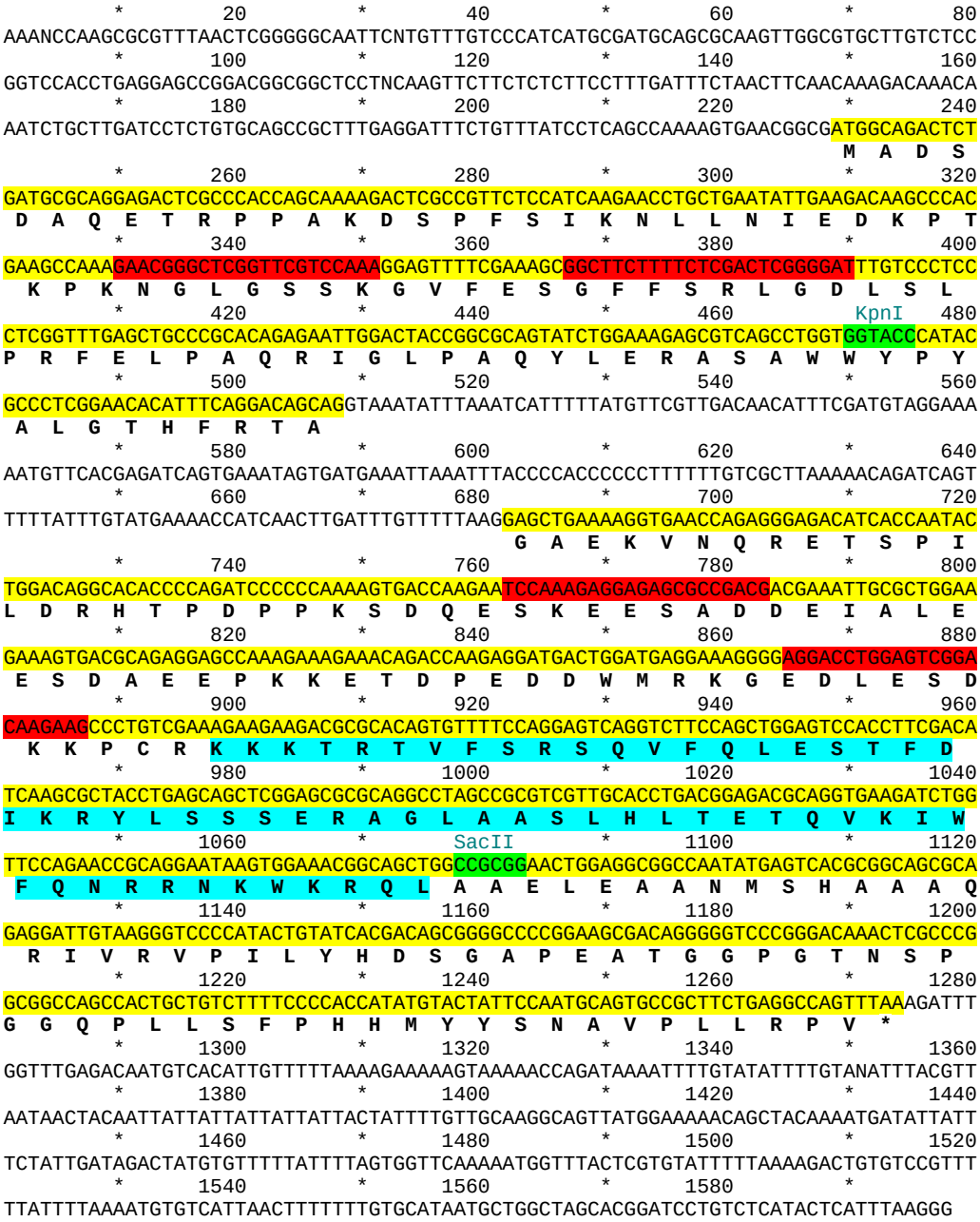

Fig. 29 Genomic sequence of the OlNkx5-1.2 gene. Coding sequences are marked yellow, primers used for RT PCR red, important restriction sites green, homeodomain is blue.

4.2.1.3 Clone IX: OlNkx5-2

The organization of 5kb Clone IX containing 453bp SacI/XmaI homeobox encompassing fragment was revealed by standard procedures of molecular cloning. 1800bp SacI fragment, hybridizing with the 453bp radioactive probe on the Southern blot, was used for subsequent analysis. The specific probe for expression studies was subcloned from this fragment by removing sequence upstream of the MluI site. Since the region upstream of the homeobox was missing in the identified genomic clone, 5'RACE procedure was carried out, using GIBCO BRL systems according to manufacturer's protocol. The PCR reactions with 3 nested primers (section 4.8.2 and Fig. 30) and cloning in pGemT vector resulted in isolation of several overlapping fragments, however even the longest (300bp) fragment did not include the initiation codon. The intron position was established by PCR using genomic DNA as a template (Fig. 30 and section 4.8.2). The single amplified band, 600bp long indicated existence of 400bp long intron. Direct sequencing of the fragment revealed that the intron position is perfectly conserved between mouse and medaka (Fig 4B).

```
         *       20        *       40        *       60        *       80
TTATGTGTCCTCAGAGGAGGAGTGCAGCGGCGGGGAGGACTCGGCGGACTGTTCTGCTCAGACACGGGTCACAGCGAGCG
 L  C  V  L  R  G  G  V  Q  R  R  G  G  L  G  G  L  F  C  S  D  T  G  H  S  E  R
         *      100        *        ▼        *      140        *      160
CTGCACGCAGCACCAACCCCACAACTTCTCCTGCCTAGGTTCTGGAAAAGGACTTCTTTCCGGGACTGAGGGGCTCGCCC
  C  T  Q  H  Q  P  H  N  F  S  C  L  G  S  G  K  G  L  L  S  G  T  E  G  L  A
         *      180        *      200        *      220        *      240
GGCGGCCGCACCTGTCCCAGCCTCTGCTGCAGGATTATAAGGAGGAGCAGGACCGAGCATGCCATCAAATGTCACCGCTG
R  R  P  H  L  S  Q  P  L  L  Q  D  Y  K  E  E  Q  D  R  A  C  H  Q  M  S  P  L
         *      260        *      280        *      300        *      320
TCGGACGACAGGCAGACAGACGGTGCAGACAAGCAGAGCAACTCGGCCAAGAAGAAGACGCGCACGGTTTTCTCCCGGAG
 S  D  D  R  Q  T  D  G  A  D  K  Q  S  N  S  A  K  K  K  T  R  T  V  F  S  R  S
         *      340        *      360        *      380        *      400
TCAGGTGTACCAGCTGGAGTCCACCTTCGACATGAAGCGGTACCTGAGCAGCTCGGAGCGGGCCTGCTTGGCCTCCAGCC
  Q  V  Y  Q  L  E  S  T  F  D  M  K  R  Y  L  S  S  S  E  R  A  C  L  A  S  S
         *      420        *      440        *      460        *      480
TGCAGCTGACGGAGACTCAAGTCAAGACGTGGTTCCAGAACAGAAGGAACAAATGGAAAAGGCAGCTCTCTGCGGAACTG
L  Q  L  T  E  T  Q  V  K  T  W  F  Q  N  R  R  N  K  W  K  R  Q  L  S  A  E  L
         *        MluI     *      520        *      540        *      560
GAGGCGGCCAACATGGCCCACGCGTCCGCACAGACATTAGTGGGGATGCCGCTGGTTTTCAGAGATAACAACCTACTGCG
 E  A  A  N  M  A  H  A  S  A  Q  T  L  V  G  M  P  L  V  F  R  D  N  N  L  L  R
         *      580        *      600       XmaI     620        *      640
CGTACCTGTTGCCCGCTCCATCGCCTTCCCGACGCCCCTCTACTACCCGGGGAGCAACCTTCCAGCTCTACCTTTATACA
  V  P  V  A  R  S  I  A  F  P  T  P  L  Y  Y  P  G  S  N  L  P  A  L  P  L  Y
         *      660        *      680        *      700        *      720
ACCTGTACAACAAAATCGAGTACTGACTTTACCACTGCAGTCCACAACACAAGACAGCATTCTCTTAAGTGTCTCCACAT
N  L  Y  N  K  I  E  Y  *
         *      740        *      760        *      780        *      800
AACCCTTGTATATTAATTGTTATTTGTTGGAATAACTCAATTATGCAAAAAAAAAAACCTGTTACGTTTCTTTAAAAAAAA
         *      820        *      840        *      860        *      880
AGAAGCAATTAAGCCTGCCATGNTATAATTCTAAATATCACTTTTGTTACTTTCATTTNCTTTATCCAACAATATTTGAC
         *      900        *      920        *      940        *      960
TTTCCTCTACATTTCATTTAAAAAGTGATTGGGAGTCAAAATATGGNAAAGATGAAATATACNAATGGATTTGGCTGACA
         *      980        *     1000        *     1020        *     1040
CTAAAAATNCATGTGACTCTGAAAGTTTTTTCCNTACGGGGAAAAACATTTTNTTTTATNCNCNCAAATATATAACATAT
         *     1060        *     1080        *     1100
ANTNTGTTTTNTTTTNCTGGTAATTTAAAGTTCTTCAATTNGTACCNCCTTCCATTAAAATTTTTCT : 1107
```

Fig. 30 Sequence of the OlNkx5-2 gene. Coding sequences are marked yellow, primers used for 5'RACE red, the cDNA sequence is in italic; important restriction sites are green, homeodomain is blue. Intron position is indicated by a triangle.

4.2.1.4 Clones III and VIII: OlNkx5-3 and OlSOHo

Isolated by A. Wolff 1kb NotI fragment of Clone III contained part of homeobox, displaying some similarity to Nkx5-2 homeobox. Complete sequencing of this fragment revealed that it encoded for sequences similar to N-terminal sequences of chicken SOHo-1 gene. To identify intron-exon boundaries RT PCR with a pair of primers was carried out as described for the OlNkx5-1.1 gene, and the resulting single product was directly sequenced. To obtain the missing 3' fragment of homeobox and sequences downstream of it, 3'RACE reaction was carried out with GIBCO BRL system. The amplification reaction with a pair of nested primers resulted in isolation of a single band that was cloned into pGemT vector; it included an in frame stop codon and thus most probably encompassed whole 3' end of the OlSOHo cDNA. The obtained sequence is presented in Fig. 31.

Isolated by A. Wolff 700bp BamHI fragment, containing homeobox bearing high similarity to Nkx5-1 and GH6 homeoboxes, was a part of 4kb XbaI fragment of Clone VIII. The sequences downstream of homeobox were obtained by sequencing the 4kb fragment, and the intron-exon boundaries identified by RT PCR with a pair of primers matching putative coding regions. Sequencing of the obtained PCR product revealed that the gene (dubbed OlNkx5-3) contains two introns, with the second one in untypical for Nkx5 genes position just upstream of the homeobox. The complete sequence is illustrated in Fig. 31.

Additionally, it appeared that 700bp BamHI fragment of Clone III, analyzed by Martin Kreusler, was identical to the corresponding fragment of Clone VIII. It suggested that the clones III and VIII are identical, and thus that the two genes are linked in medaka genome. To establish the relative orientation of the genes I performed PCR reaction using the phage VIII lysate (prepared by A. Wolff) as a template, and four primers (section 4.8.2) in four combinations. The primer positions are marked in Fig. 31. Only one set of primers yielded a product (1kb), suggesting that the OlNkx5-3 gene is located upstream of the OlSOHo gene, and that the genes share the same transcriptional orientation. The fragment was subsequently directly sequenced. The sequence of the OlNkx5-3 + OlSOHo locus is presented in Fig. 31.

```
         *        20         *        40         *        60         *        80
ACATGAAAAATTGAGCAAAACGCTTTGTAAAAATATAATAAAACATCTACCAGGCTAATCACGACAAAAATTCAACCAAC
         *       100         *       120         *       140         *       160
TTTTTTCGAGTTTTTTGGTTTGTTTTGTTTTATCTCAGGGGTTTCATAATTAAAATGATGGTATAAAACTTTAAAACCAA
         *       180         *       200         *       220         *       240
ATGTTGCGCGTAAACAGACACGTTTCTTTACTCTTACTCTTTGTTTTGGGTGTTTTGTCTTTTTTATTTCATCACCAAGG
         *       260         *       280         *       300         *       320
GAAAAAAAGAAAAGAAAAATAACTGAAGTCCTAAAACAAACAAAAAACAGAGAATTTGTGTTAACGTTTGTGTCTTTAAT
         *       340         *       360         *       380         *       400
TGGTCAGCACAGGCTTCCACAGCTCGGCGTAAACAGAGGGGTGCAGGCTCCATGTGTAACCAATAGGCGGTACTGTGCCA
         *       420         *       440         *       460         *       480
TTCTTCATGACGCTTCCTCCATCTCAGCCCTGAGGACAGCGCTGTGTCTCTGCTTTGGTTTGCTCCCTAACTGACCTAAA
         *       500         *       520         *       540         *       560
TCCAGCATCTCTGCGCTCCCCGAGGCAGACGTCCTCAGAGGCTCCGAGGAGCAGCGCGGACACTCGCGCACCCCTTTTTC
         *       580         *       600         *       620         *       640
CTCTTCTGGACCAAATCTGCGCCTTCTGACAGCAGCTAAAGCCTGAAACATACCCCAACTGGACCCTTTGGAGCAGCAGC
         *       660         *       680         *       700         *       720
GGGCCGTCCTGGCACCGAACTCCGACATGGTTTTACGCGGAGGAGACGAGAAGCGGGAGTGAGCTCAGAACATCCACCGC
         *       740         *       760         *       780         *       800
TATTGGGATTTTATTTTGGAAACGAAGGGGAGGGGGGGGGAGGGGGTCACGATGTTGGATGATAAAGCGCGCAGATCGAA
                                                    M  L  D  D  K  A  R  R  S  K
         *       820         *       840         *       860         *       880
GGTTCAACTCCACGGGTCATCTTTTTACATCGAGAATTTGCTGCGGGGCTCCAGCGGGGATTTGTCCGCCGGGGAGCGCG
  V  Q  L  H  G  S  S  F  Y  I  E  N  L  L  R  G  S  S  G  D  L  S  A  G  E  R
                  900         *       920         *       940         *       960
AGACTTCCGGACACGCAACGGAAACGAGACCAGAAAACGAGGAAATTTTGGACCAGGAGCATCACCGAAGAGGTTCACTC
E  T  S  G  H  A  T  E  T  R  P  E  N  E  E  I  L  D  Q  E  H  H  R  R
         *       980         *      1000         *      1020         *      1040
GACTTCCTATTTATCTTTTTCTCAAATTTGACTTAAAAAATAAAAAAAATCATAAAGTTTCACGATTTATCAGCAGCACA
         *      1060         *      1080         *      1100         *      1120
ACATTTTTTTGATCCCACAACAAGTGACATTTTTAGACTTTTTGGTCTAAAAATCCGTGCTCCGTGCGTAAACTCTCCAT
         *      1140         *      1160         *      1180         *      1200
CTATCAAAACAAATCCCCCCAAACTTTCTTACTCTCCAAACAGATTTGTAAATGGTGGAAAAGGAAACGAGCATGCGCGC
         *      1220         *      1240         *      1260         *      1280
ACGCTCAAACCCAAATGTGCAAGAAATGAAGCGAGATTTCCCCTCCAATGTCAATGCTTGGTTTCAGGTTTTCCAGAGAA
                                                                  G  F  P  E  K
         *      1300         *      1320         *      1340         *      1360
GACAAGCACATGCTGTGAAGGAGCTGCAGGGATCAGCAAGCGGCGGGAGGAGAACGAGGAGACGAAAGGAGACGAGAGTT
  T  S  T  C  C  E  G  A  A  G  I  S  K  R  R  E  E  N  E  E  T  K  G  D  E  S
         *      1380         *      1400         *      1420         *      1440
TGACAGATGACAGAGAGGAGGAGGAGGAAGATGCGCCTTCCTCCGGCGTCTCTGGAGAGGACATCTATACTGATACAGGT
L  T  D  D  R  E  E  E  E  E  D  A  P  S  S  G  V  S  G  E  D  I  Y  T  D  T
         *        BamHI      *      1480         *      1500         *      1520
GGCATAAATACCCATATTGGATCCTTTTTAGAAAAATAAATTAATAAATAAAAATCAATAAGTTTAAAAAAAGATATTGA
         *      1540         *      1560         *      1580         *      1600
ATCTTTTTGAATCAAATCTTATTTTTAAAGTAAGTTTATAAACTCTACTCATCTCGCTCCTAGGTGAGGTCAAAGCCGTG
                                                          G  E  V  K  A  V
         *      1620         *      1640         *      1660         *      1680
CGTAAAAAGAAGACGCGCACGGTGTTCAGCCGCAGCCAGGTCTTCCAGCTGGAGTCCACCTTCGACATGAAACGCTACCT
 R  K  K  K  T  R  T  V  F  S  R  S  Q  V  F  Q  L  E  S  T  F  D  M  K  R  Y  L
         *      1700         *      1720         *      1740         *      1760
GAGCAGCTCGGAACGGGCCGGCCTGGCCGCCTCGCTGCAGCTCACAGAGACCCAGGTGAAGATCTGGTTCCAGAACCGCA
  S  S  S  E  R  A  G  L  A  A  S  L  Q  L  T  E  T  Q  V  K  I  W  F  Q  N  R
         *      1780         *      1800         *      1820         *      1840
GGAACAAGTGGAAGCGCCAGATCACGGCGGACATGGAGGCCGGAGGCGCGCTCGTCCCCTTCGCATCCCACAGAGTCGTC
R  N  K  W  K  R  Q  I  T  A  D  M  E  A  G  G  A  L  V  P  F  A  S  H  R  V  V
         *      1860         *      1880         *      1900         *      1920
CGAGTCCCCGTGCTGTACCGCGAGGACGCGTCCGCATCTTCATCCGCCTTCACTCATCTGCATCCTCCAGCGCTCGCGGA
 R  V  P  V  L  Y  R  E  D  A  S  A  S  S  S  A  F  T  H  L  H  P  P  A  L  A  D
         *      1940         *      1960         *      1980         *      2000
CGCTGTGAACTTTCCCCTGACTGGTCGCTTCACCCATCCGGTGACGTTTATAACAGCACCCATGGCAGGAATGGTGTGAC
  A  V  N  F  P  L  T  G  R  F  T  H  P  V  T  F  I  T  A  P  M  A  G  M  V  *
         *      2020         *      2040         *      2060         *      2080
TCCAGACAGACTTTTCAAGAAATCAATAAAAAAAATTGCTGCAAAATATTTGGTTTCTATGGGTTTTACGCACTGGAGATG
         *      2100         *      2120         *      2140         *      2160
ATCCATTGCTGCATTAAAGTGAATGATTACTGTCTGACAAAGAGTTCCAGGTCATCCGTCGCCAAGGTGACCCAACAGTT
         *      2180         *      2200         *      2220         *      2240
CTAGAGTTTCTCATTAATGAATCTTCAAGAGTTTACAAAATAGTAATGACTGTGCACTTGTCTTCTCAGGGCTGCGCATA
         *      2260         *      2280         *      2300         *      2320
TTTCCAACTTTATTGTACAAATACTTTTTTTCACACAAAACTTCCACAGAAATGAGCCCCTTTTATGCAAAAACAATCAA
         *      2340         *      2360         *      2380         *      2400
GAGGTGCGCAAAGTGACGCAAAAGAAAACACTGTGAAGAACTTCGTTCCATTTCTTTCCCAAATTAGATTACTGACTTCA
         *      2420         *      2440         *      2460         *      2480
AAAACTCTCTTTTCCTATAAAATGATAGGAATCTGTACCTATTGCCTTTCTTAAACATACAACTGGTTTCTGCACATTCA
         *      2500         *      2520         *      2540         *      2560
GAATTGCTTAAAAGATATTGTCCAGCAGCTTTCATTTAACGCATTTTACTAATTACTTTTGACACTTTTGTGTTGACATG
         *      2580         *      2600         *      2620         *      2640
TGAATAAATTCATGCACAAACTTCTTTTGCATTTGCGTTGTAATGTTGAAATCTTTAAAGTTTGGTGTTTTAGAGTAGGA
```

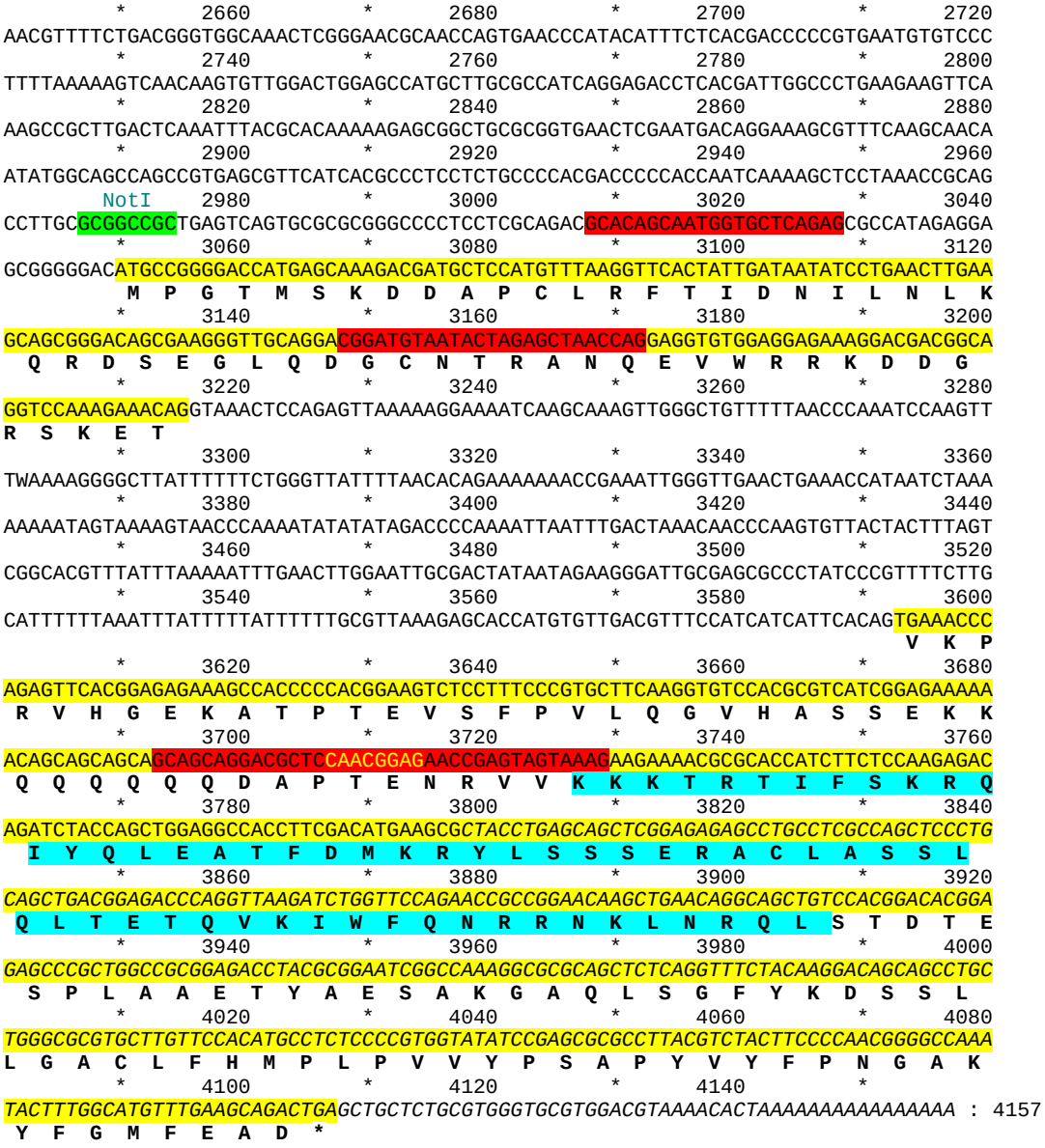

Fig. 31 Sequence of the OlNkx5-3 + OlSOHo locus. Coding sequences are marked yellow, primers red, the cDNA sequence obtained by 3'RACE is in italics; important restriction sites green, homeodomain is blue.

4.2.2 Isolation of the zebrafish Nkx5-1 cDNA

230bp fragments of zebrafish Nkx5-1 homolog was amplified using genomic DNA as a template and degenerated primers designed to match conserved regions of medaka, mouse and chicken Nkx5-1 gene (section 4.4.1). The fragment was directly sequenced and subsequently cloned into the pGemT vector. To isolate the zebrafish homolog of the Nkx5-1 gene a cDNA library constructed from polyA+ embryonic zebrafish RNA (15-19 hr, 28^{o}C and kindly provided by Dr. B. Appel (Vanderbilt) was screened using the 230bp amplified zfNkx5-1 fragment as a probe. A single positive cDNA clone was identified and contained a complete coding zebrafish Nkx5-1 cDNA sequence as revealed by sequencing (Fig. 32).

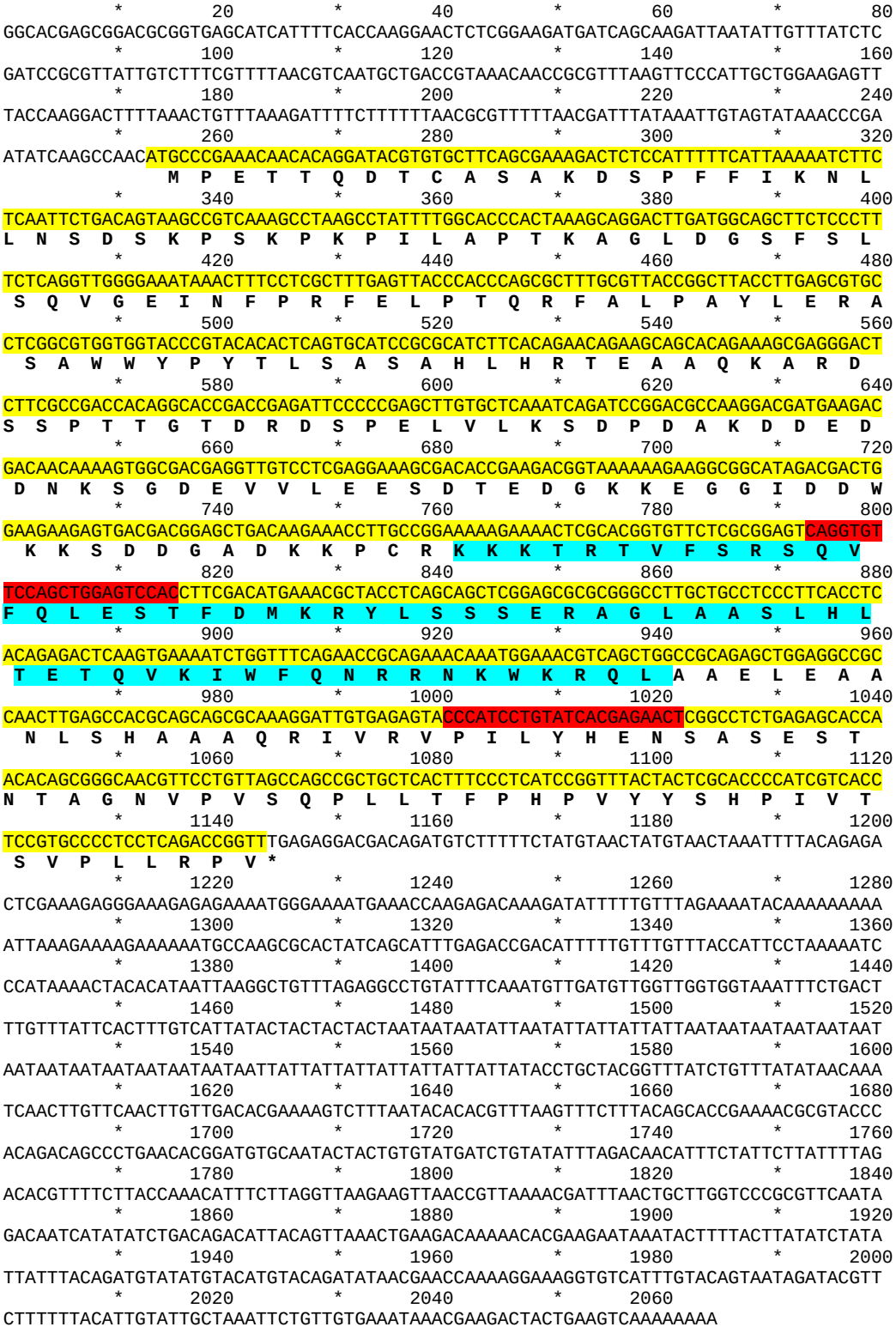

Fig. 32 zfNkx5-1 cDNA sequence. Coding sequence is marked yellow, primers are red, homeodomain is blue.

4.2.3 Isolation of medaka BMP4

The suitable for in situ hybridization procedures medaka BMP4 fragment was isolated as follows. (1) Degenerate primers suitable for amplification of conserved 120bp fragment of all members of the TGFβ family (Basler et al., 1993) and genomic DNA as a template were used

in a PCR reaction; cycle parameters were: (5’ 95°C, 1’ 33°C, 2’ 72°C) x1; (1’ 95°C, 1’ 33°C, 2’ 72°C) x5; (40" 95°C, 1’ 50°C, 1’ 72°C) x40; 10’ 72°C. (2) The 120bp fragments were gel purified, digested with EcoRI and BamHI enzymes and cloned into suitable pBluescript KS vector. (3) 49 randomly picked clones were sequenced, what revealed existence of 6 types of clones, BLAST searches revealed their constituted medaka homologs of Veg1, GDF6, IGF, BMP4 and BMP7 (two different clones) (4) 2 nested primers suitable for 3’RACE procedure were designed for the obtained BMP4 sequence (4.8.3) and together with GIBCO BRL system for 3’RACE used for amplification of the 3’ends according to GIBCO protocol. (5) The amplified fragments were cloned into pGemT vector and sequenced. The nucleotide and aminoacids sequences are presented in Fig. 33.

```
         *        20          *        40          *        60          *        80
GCACCTCCTGGTTACCAGGCCTATTACTGCCACGGAGAGTGCCCTTTTCCGCTGGCAGATCATCTGAACTCAACTAACCA
 A  P  P  G  Y  Q  A  Y  Y  C  H  G  E  C  P  F  P  L  A  D  H  L  N  S  T  N  H
         *       100          *       120          *       140          *       160
CGCCATCGTGCAGACACTGGTGAACTCTGTGAACAACAACATTCCCAAGGCCTGCTGCGTACCAACAGAGCTCAGCGCCA
  A  I  V  Q  T  L  V  N  S  V  N  N  N  I  P  K  A  C  C  V  P  T  E  L  S  A
         *       180          *       200          *       220          *       240
TCTCAATGCTATACCTAGACGAATATGACAAGGTGGTCCTAAAAAACTACCAGGAAATGGTAGTGGAAGGCTGCGGCTGC
I  S  M  L  Y  L  D  E  Y  D  K  V  V  L  K  N  Y  Q  E  M  V  V  E  G  C  G  C
         *       260          *       280          *       300          *       320
CGCTGACCCACAAACTGAAAGCCTCTTCATACTCCGCGGGAGCAGAGAAATGTAATCTCCCAGTCGTGCAAGAGCAATTG
 R  *
         *       340          *       360          *       380          *       400
TTGCATTTCTTTTCTCAAGTTCTCCCAGCTTGTTCCCAACTCAACTTTTAAGCAGAACTTTGTATTTTGTGCAAAGGTGT
         *       420          *       440          *       460          *       480
TTAATGATTGGTTGATTTGAAGTGGGCGGGGTTAATGCAGCTCTGTCTGTAGGTGCAAATTGACTGAATGGCATTATCTG
         *       500          *       520          *       540          *       560
ATGGACCATGTCGTAAAAACAATCGGTTGACATTCTGAAAAAGTCTTTTATGGCCGATTGGTTTCCACTGTTGAAGGAAG
         *       580          *       600          *       620          *       640
ACCCTGCTCTAAAAACGACAAATCCAACATGATTCTTCCTTTCCTTCTCCTCAATGAACTGCATTTTCACTTTTTCATTA
         *       660          *       680          *       700          *       720
TATATTGTGTAATGTCACAGCAGGAGGACCCAATCAGGACTTCCAATAAGTTTAGTTTCAAAATATCCTGGATTTCTTTC
         *       740          *       760          *       780          *       800
ATCTACCTTGKGAAGCAGAAAAAATTGTCCTGTTCCCGTGGAGTATGAAGACCTTTTTTTTAGAAAAGAAAAAAAACCTG
         *       820          *       840          *       860          *       880
TCATTTGGACTGGCAACAAACAAAGCAGAAACAATTCCACCCAAAAACATTTACCATTATTTATAACTTCTATGATCTGT
         *       900          *       920          *       940          *       960
ATGTTTGTCTCACTTTTTTCCCTTTTATCATACATTTTGGCAAAATATATATATTTATATCTACATATTAAAGACAAATT
         *       980          *      1000          *      1020          *      1040
GAGCCCTTATTTTAAAACNAAAGCTGTACAACTTTTCATAATGTACNTTAGATTGTTTTTTTGTTTTTTTATGGAGAAAA
         *      1060          *      1080          *
TAGTAAAAACAAAGTAAAGTTTATTTACTGGTGAAAAAAAAAAAAAAAAAAAA
```

Fig. 33 Partial OlBMP4 cDNA sequence. Coding sequence is marked yellow, primers used in 3’RACE are red.

4.2.4 Expression studies

In order to analyze the expression patterns of the isolated medaka and zebrafish genes, whole mount in situ hybridization procedures were carried out using digoxigenine labeled antisense RNA probes, alkaline phosphatase coupled antibodies and NBT/BCIP or BM Purple substrate. In double OlNkx5-1.1/Pax2 staining the OlPax2 probe (Koester et al., 1997) was labeled with fluoresceine and detected with Fast Red substrate (protocol 4.7.1.4). For RNA synthesis following fragments were used: OlNkx5-1.1 (1kb) and OlNkx5-1.2 (700bp) -

3'fragments, not including homeoboxes and mostly composed of uncoding regions; OlNkx5-2 - 450bp including homeobox and 1450 bp of the 3' region downstream of homeobox, probably partially untranscribed - both probes hybridized to the same domains, the latter one giving stronger signal/lower background and was thus used in most cases; OlNkx5-3 – 0.7kb and 4kb full length genomic fragment (gave identical results); OlSOHo - 1kb fragment comprising of exon 1, intron and fragment of exon 2 including 2/3 of homeobox; OlBMP4 – 1kb 3'end of cDNA, zfNkx5-1 – 2kb full length cDNA probe. The restriction sites used for linearization of plasmids and RNA polymerases for synthesis of the antisense probes are indicated in Table 5.

Stained embryos were cleared in glycerol and photographed under light microscope, sections were made on a vibratome or criotome as described previously (Herbrand et al., 1998). The pictures were processed in Adobe Photoshop program.

Table 5. Templates for antisense probes used in whole mount in situ procedures.

gene	fragment, cloned with (clone no)	size (kb)	vector	antisense probe:	
zfNkx5-1	complete cDNA	2	pBluescript SK	EcoRI	T7
OlNkx5-1.1	genomic 3'end, SacII	1	pBluescript KS	KpnI	T7
OlNkx5-1.2	genomic 3'end, SacII/KpnI	0.7	pBluescript KS	NdeI	T3
OlNkx5-2	genomic 3'end, SacI/MluI (16)	1.45	pBluescript KS	XhoI	T7
OlNkx5-2	genomic 3'end, SacI/MluI (17)	1.45	pBluescript KS	SacI	T3
OlNkx5-3	complete genomic, XbaI	4	pBluescript KS	NotI	T3
OlNkx5-3	genomic with homeobox, BamHI	0.7	pBluescript KS	ApaI	T7
OlSOHo	genomic with partial homeobox, NotI	1	pBluescript KS	ApaI	T7
OlBMP4	cDNA 3'end (18)	1	pGemT	NcoI	SP6

4.2.5 Nkx5-1 mRNA injections

Capped mRNAs for injections were transcribed from linearised plasmids using mMESSAGE mMACHINE Kit (Ambion). The concentration was tested both spectrophotometrically and on agarose gel in comparison to standards, and the injections were performed as described by Koester et al., 1997.

4.3 Experiments in chicken system

4.3.1 Bead preparation

Heparin coated acrylic beads (Sigma) were extensively washed in PBS and soaked for 2 hours at room temperature in humidified chamber in 1mg/ml solution of FGF2 or FGF8 in

PBS/0,2% bovine serum albumin. Before implantation beads were washed in PBS. As controls, PBS soaked beads were used.

4.3.2 Embryo preparation and bead implantation

Fertilized chick (*Gallus gallus*) eggs were incubated at 37.8°C and 50% humidity. Eggs were windowed, several drops of Ringer solution added and embryos visualized by sub-blastodermal injection of India Ink (Pelican Fount, 1:15 in Ringer). Embryos were staged according to Hamburger and Hamilton (1951). Embryos with 10-14 somites (HH stage 10-11) were chosen for implantation and as not operated controls, one batch of experiments was performed with embryos stage 14-15 (20-25 somites). The vitelline membrane was dissected away from the desired region of the embryo and a slit was made by a tungsten needle in a position anterior or posterior to the otic placode/vesicle. The bead was added to the embryo using a pipette and maneuvered to the slit with a tungsten needle. In most cases the bead was implanted on the right side of the embryo, however in few cases the operation was performed on the left side what gave the same result (not shown). Windows in operated eggs were sealed with tape and the eggs were incubated for additional 24 hours, two or three days. After incubation embryos viability and bead position were visually assessed (except for the 2 and 3 days incubation time where bead position could be visualized only after processing and sectioning, see below). Healthy looking embryos with beads close to the vesicle (located closer than a vesicle diameter away from the vesicle) were fixed in 4% PFA/PBS overnight at 4°C. Such embryos constituted about 75% of the operated embryos.

4.3.3 Whole-mount in situ hybridization

Whole mount in situ hybridization experiments and vibratome sectioning were performed according to protocol 4.7.1.3. Antisense RNA probes were generated as described; cNkx5-1 and cPax2, Herbrand et al., 1998; cDlx5, Pera et al., 1999; SOHo; Kiernan et al., 1997; cBMP4, Wu and Oh, 1996; cFGF8, Crossley et al., 1996. cNSCL-2 probe was PCR-cloned using cDNA from 4 days old chicken embryos as a template and primers matching published (AF109012) sequence (section 4.8.4). The 0.4kb fragment corresponding to 3'UTR was cloned into pGemT vector (Promega), and antisense probe generated by linearizing the vector with Not I and transcription with T7 polimerase.

4.3.4 Computer analysis

To asses changes in otic vesicle size and level of gene expression after FGF bead implantation the embryos were submitted to computer image analysis. Only embryos with

bead implanted on the right side at stage 10-11 and incubated for 24 hours were used for this analysis. Embryos were grouped according to the bead position (anterior and posterior) and probe used for whole mount in situ hybridization. As controls either not operated embryos of the same age, or embryos with PBS beads were used. Sizes of embryos' ear vesicles, expression domains and strength of expression were evaluated as exemplified in Fig. 34. For each embryo all images of the serial sections through both ear vesicles were captured directly from the microscope using a SeeScan camera. Compound pictures were prepared in Adobe Photoshop program, left and right vesicle areas were manually identified (outlined) on each picture (Fig. 34A). Whole area except vesicles on each picture was erased (filled white) and the pictures were converted to 255 gray levels format (Fig. 34B).

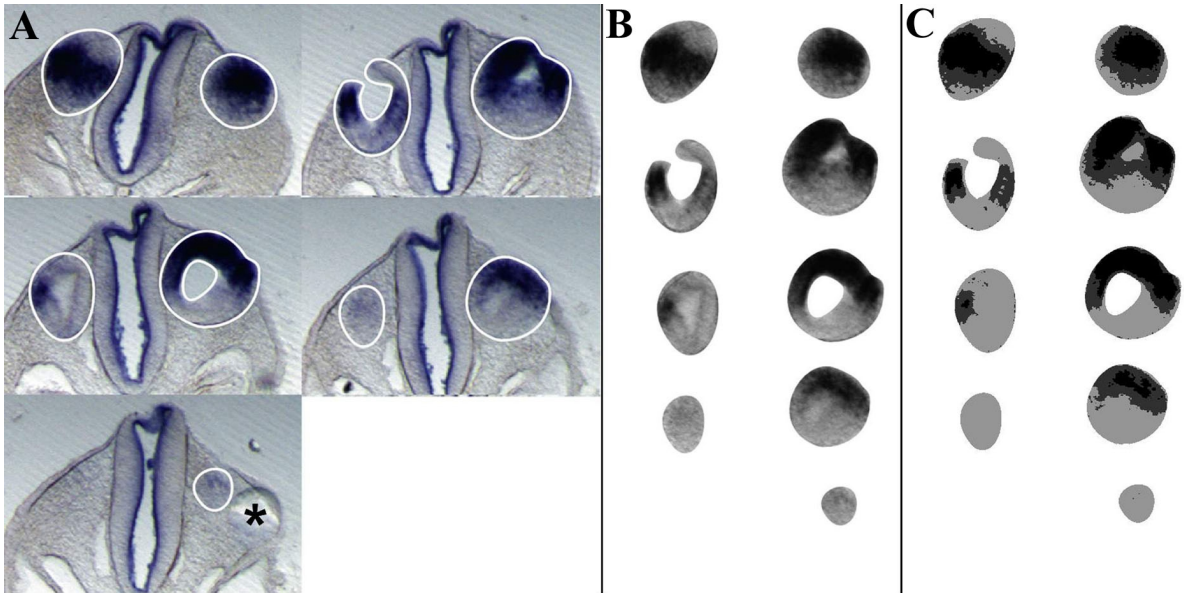

Fig. 34 An example of image processing employed for comparison of ear size and expression changes after FGF2 bead implantation. The bead was implanted posterior to the developing ear (asterisk); after 24 hours of incubation the embryo was hybridized with cNkx5-1 probe and vibratome cut. The compound picture was made from serial sections captured directly from the microscope and the ear vesicles manually identified on each section (A). All area except the ear was erased, images of left and right vesicles separated and the image desaturated (B). Brightness levels were reduced to four values, indicating strength of the expression (C).

Pictures brightness levels were reduced (posterized) to four levels: 0 – black, 85 – dark gray, 170 – light gray and 255 – white (Fig. 34C). Brightness levels acquired following characteristics: 255, background; 0 ~ 170, vesicle area; 0 ~ 85, expression domain area; 0, strong expression area. Pixels belonging to background were neglected. Pixels belonging to other levels were counted for left and right vesicle separately, and summed up to obtain six parameters describing every group; they were dubbed V_L, V_R, E_L, E_R, S_L, S_R (0 ~ 170, otic

Vesicle size; 0 ~ 85, Expression domain size and 0, Strong expression domain size of the Left and Right vesicle, respectively).

To compare the sizes of the left and right ear vesicle the embryos were grouped according to the bead position, not depending on the probe used for hybridization. That allowed comparing relatively big numbers of embryos (29 embryos with anterior FGF2 bead, 32 embryos with posterior FGF2 bead and 21 control embryos). The change of vesicle size (VS) was obtained by dividing V_R by V_L:

$$VS = \sum V_R / \sum V_L$$

Statistical tests used to evaluate the differences in means between analyzed groups proved that the differences between mean sizes of control and FGF2 treated vesicles are statistically significant with p-level less then 0.03 (M. Adamski, personal communication).

To assess change of expression domain size and strength of expression embryos were grouped according to bead position and a marker tested. Change of expression domain size (DS) was calculated by dividing ratio E_R to V_R by ratio E_L to V_L.

$$DS = \frac{\sum E_R}{\sum V_R} \Big/ \frac{\sum E_L}{\sum V_L}$$

Change of expression strength (ES) was calculated by dividing ratio S_R to E_R by ratio S_L to E_L.

$$ES = \frac{\sum S_R}{\sum E_R} \Big/ \frac{\sum S_L}{\sum E_L}$$

4.4 Experiments in mouse system

The wholemount in situ hybridization with antisense probes (Nkx5-1 and Pax2, Rinkwitz-Brandt et al., 1996; Dlx5, Acampora et al., 1999) was carried out according to protocol 4.7.1.3.

4.5 Phylogenetic analysis

To analyze phylogenetic relationships between the isolated genes and known Nkx5 family members, BLAST searches were first performed using nucleotide and deduced aminoacid sequences. The identified homologous sequences were drawn from the GenBank (CG5832, AAF55433; SpHmx, D85079; TgHbox5, 4826981; Nkx5-1, AJ009935; Nkx5-2, 1311669; Hmx1, AF009614; cNkx5-1, Y15989; SOHo-1, S69380; zfNkx5-1, AF288211; zfSOHo, AI658291). The complete cDNA sequence of chicken GH6 gene was kindly provided by

Dorothea Schulte (Harvard Medical School). Since sequence downstream of homeodomain is not known for the zebrafish SOHo, it was omitted in the analysis. The human ortholog of mouse Hmx1 gene, H6 (HMX1), was also omitted since the sequence shown in Yoshiura et al. (1998) does not agree with the one in the GenBank. The obtained sequences were aligned in ClustalW program at http://pbil.ibcp.fr/NPSA, and alignment edited in GeneDoc program (http://www.cris.com/~ketchup/genedoc.shtml); only unambiguously alignable C terminal fragments (including homeodomains) were used in further analysis. Subsequently phylogenetic trees were constructed basing on maximal parsimony (Protpars) and protein distances (Protdist and Fitch or Neighbor), using the PHYLIP package (Felsenstein 1993; http://evolution.genetics.washington.edu/phylip). All methods, including construction of unrooted trees and trees rooted with related sequences (mouse proteins Nkx2-5, NM_008700 and Msx1, NM_010835, and *Cenorhabditis elegans* C39E6.3, T29739) resulted in trees with essentially identical topologies, and thus only representative trees are shown in the Results section. The confidence values for obtained trees were computed by bootstrapping (PHYLIP package).

4.6 Fine reagents and kits

4.6.1 Embryo staining

All reagents from Roche, Promega or Sigma:
SP6, T7 and T3 Polymerases with reaction buffers, Ribonucleotide Mix containig DIG-11-UTP or Fluoresceine labeled UTP, Placental Ribonuclease Inhibitor, RNase free DNAse I, RNase A, Alkaline Phosphatase coupled anti-digoxigenin and aniti-fluorescein antibodies, BM Purple AP Substrate or NBT and BCIP, Fast Red tablets

4.6.2 FGF beads implantation

Heparin immobilized on acrylic beads (Sigma), recombinant human FGF2 (bFGF) and recombinant mouse FGF8 isoform b (R&D Systems)

4.6.3 PCR and cloning products

Takara ExTaq, Gibco BRL 3´and 5´RACE Systems, QiaGen Kits for nucleic acid isolation and purification.

4.6.4 Kits for injections experiments

mMessage mMachine (Ambion), ApopTag Red (Oncor)

4.7 Embryo staining protocols

4.7.1 Whole mount in situ hybridization

(modified after D. Wilkinson and J. Wittbrodt)

4.7.1.1 Probe synthesis

i. Add to Eppendorf tube: 1µg linearized template, 2µl DIG-mix, transcription buffer, 50U placental ribonuclease inhibitor, 10U polymerase; sterile distilled H_2O up to 20µl, incubate 2h at 37°C.
ii. Remove 1µl aliquot and run on agarose gel; the RNA band should be 10-fold more intense than plasmid band indicating 10µg of probe synthesized.
iii. Add 1-2µl DNAse I and incubate 15-30 minutes at 37°C.
iv. Add 100µl TE, 10µl 4M LiCl, 300µl EtOH, mix and incubate 30 min. to overnight at -20°C.
v. Spin 15 minutes at maximal speed in microfuge, wash pellet with 80% EtOH and air-dry.
vi. Redissolve in 100µl TE including 1-2 µl placental ribonuclease inhibotor and run 5µl on agarose gel to check integrity of probe.
vii. Use 1-5µl of probe per 100µl of hybridization mix.

4.7.1.2 Embryo preparation:

Mouse and chicken – dissect in PBS, fix overnight at 4°C, rocking in 4% PFA in PBS.
Medaka – fix 4 h at room temperature, remove chorion with fine forceps
Wash each time 5 min, rocking at room temperature: 2x PBT, 25%, 50%, 75% MeOH/PBT, 2x 100% MeOH; store at -20°C in MeOH.

4.7.1.3 Mouse and chicken staining

4.7.1.3.1 Solutions

Prehybridization mix: 50% formamide, 5xSSC pH 4.5 (citric acid to adjust pH), 50µg/ml tRNA, 1% SDS, 50µg/ml heparin
Solution 1: 50% formamide, 5x SSC pH 4.5, 1% SDS
Solution 2: 0.5 M NaCl, 10mM TrisHCl pH 7.5, 0.1% Tween-20
Solution 3: 50% formamide, 2x SSC pH 4.5

10x TBS: 8g NaCl, 0.2g KCl, 25ml 1M TrisHCl, add H_2O to 100ml

TBST: 1xTBS, 0.1% (1% for post-antibody washes) Tween-20, 2mM levamisole

NTMT: 100mM NaCl, 100mM TrisHCl pH 9.5, 50mM MgCl2, 0.1% Tween-20

4.7.1.3.2 Embryo powder

Homogenize E12.5-14.5 mouse, or E6 chicken embryos in minimum volume of PBS, add 4 volumes of ice-cold acetone, mix and incubate on ice 30 min; spin at 10Kxg for 10 minutes, remove supernatant; wash pellet with ice-cold acetone and spin again; spread pellet on filter paper, air-dry and grind; store at 4°C in air-tight tube.

4.7.1.3.3 Antibody preabsorption

Heat at 70°C, 30 minutes 3mg of embryo powder in 0.5ml TBST; cool on ice, add 5µl sheep serum and 1µl anti-dig antibody; shake gently at 4°C 1-2h; spin at 4°C 10 minutes, dilute the supernatant to 2ml with 1% sheep serum in TBST.

4.7.1.3.4 Staining

All washes are rocking except of proteinase treatment, and 5 minutes unless otherwise stated.

Room temperature washes in 24 or 6 well tissue culture dish:

i. 75%, 50%, 25% MeOH/PBT, 2x PBT
ii. 6% hydrogen peroxide in PBT, 1 hour
iii. PBT 3 times
iv. 10µg/ml proteinase K in PBT, 15 minutes for mouse embryos E9.5 and chicken E3, shorter for younger and longer for older embryos
v. 2mg/ml glycine in PBT
vi. 2x PBT
vii. 0.2% glutarladehyde / 4% PFA in PBT, 20 minutes
viii. 2x PBT

70°C washes in 2ml Eppendorf tubes, waterbath:

ix. prehybridization mix, 1 hour
x. hybridization mix, overnight
xi. 2x solution 1, 30 minutes
xii. 1:1 solution 1 : solution 2, 10 minutes

Room temperature washes:

xiii. 3x solution 2

37°C washes:

xiv. 2x 100 µg/ml RNase A in solution 2, 30 minutes

Room temperature washes:

xv. solution 2

xvi. solution 3

65°C washes:

xvii. 2x solution 3, 30 minutes

Room temperature washes:

xviii. 3x TBST

xix. 10% sheep serum in TBST, 60-90 min.,

4°C wash:

xx. preabsorbed antibody overnight

Room temperature washes in 24 or 6 well tissue culture dish:

xxi. 3x TBST

xxii. 5x TBST 1h

4°C wash:

xxiii. TBST overnight

Room temperature washes:

xxiv. 3x NTMT, 10 minutes

xxv. Staining in darkness with BM Purple, or 4.5µl NBT and 3.5µl BCIP per ml NTMT; up to 48 hours

xxvi. 2x TBST

4.7.1.4 Medaka and zebrafish double stainig

4.7.1.4.1 Solutions

Prehybridization mix: 50% formamide, 5xSSC pH 4.5 (citric acid to adjust pH), 50µg/ml tRNA, 50µg/ml heparin, 0.1% Tween 20

Solution 1: 50% formamide, 2xSSC pH 4.5, 0.1% Tween 20

Solution 2: 2xSSC pH 4.5, 0.1% Tween 20

Solution 3: 0.2xSSC pH 4.5, 0.1% Tween 20

4.7.1.4.2 Antibody preabsorbtion:

Fix embryos in 4% PFA, wash with PBT and store in MeOH at -20°C. Rehydrate 1ml of embryos in PBT, transfer to 2ml Eppendorf tube and homogenize with a pestle, adjust volume to 1ml, add 10µl antibody, incubate shaking overnight at 4°C. Spin down, filter

supernatant 0.2µm, resuspend and sterile filter again; combine filtered antibody solutions, add 0.5ml sheep serum and fill with PBT to 20ml. Store at 4°C.

4.7.1.4.3 Staining

All washes are rocking except of proteinase treatment, and 5 minutes unless otherwise stated.

Room temperature washes in 24 or 6 well tissue culture dish:

i. 75%, 50%, 25% MeOH/PBT, 2x PBT
ii. 10µg/ml proteinase K in PBT, from 1 minute for gastrula stage embryos to 15 minutes for stage 30 medaka, and 30h zebrafish embryos
iii. 2x 2mg/ml glycine in PBT
iv. 4% paraformaldehyde in PBT, 20 minutes
v. 5x PBT

60°C washes in 2ml Eppendorf tubes, waterbath:

vi. prehybridization mix, 1 hour
vii. hybridization mix including digoxigenin and fluorescein labeled probes, overnight
viii. 2x solution 1, 30 minutes
ix. solution 2, 15 minutes
x. 2x solution 3, 30 minutes

Room temperature washes:

xi. 10% sheep serum in PBT, 60-90 min.,
xii. 200µl preabsorbed antibody 2 hours

Room temperature washes in 6 well tissue culture dish:

xiii. 6x 10 minutes PBT

4°C wash:

xiv. overnight PBT

Room temperature washes:

xv. 3x NTMT, 10 minutes
xvi. Staining in darkness with BM Purple, or 4.5µl NBT and 3.5µl BCIP per ml NTMT up to 48 hours
xvii. 3x PBT
xviii. 2x 10 minutes 0.1M glycine/HCl, pH 2.2/0.1% Tween 20
xix. 4x PBT

In 2ml Eppendorf tube:

xx. 1-2 hours in 200µl pre-absorbed anti-fluorescein antibody

In six-well dish:

xxi. 6x 10 minutes PBT

xxii. 2x 0.1 M TrisCl, pH 8.2/0.1% Tween 20 (RSB solution)

xxiii. Staining in darkness with Fast Red tablets (1 per 2ml RSB, sterile filtered 0.2µm), solution has to be replaced while orange

xxiv. 3x PBT

4.7.2 TUNEL protocol for gastrula-stage medaka embryos

Performed with Oncor ApopTag Red Kit, modified from manufacturer protocol for sections and Yager et al., 1997.

i. Fix embryos in 4% PFA in PBS, 4-6h at room temperature, or overnight at 4°C, rocking.

ii. Dechorionate in 4% PFA.

iii. Wash 5x 5 minutes in PBT, rocking.

iv. Treat with 10 µg/ml with proteinase K in PBT, 1 minute.

v. Wash 2x 5 min. 2mg/ml glycine, rocking.

vi. Fix in 4% PFA in PBS, at room temperature, rocking, 20 min.

vii. Wash 5x 5 min. in PBT, rocking.

viii. Equilibrate in humidified chamber in equilibration buffer, room temperature.

ix. Perform reaction in enzyme working solution, at 37°C, in humidified chamber.

x. Wash 6x 10 min. in PBT.

xi. Incubate with anti-dig antibody in humidified chamber in darkness, 30 min..

xii. Wash 3x 10 minutes in PBT, at room temperature, and overnight at 4°C.

xiii. If embryos were co-injected with GFP mRNA, view GFP green fluorescence with blue light, and rhodamine red fluorescence with yellow light, take picture of red fluorescence first as it fades much faster.

4.8 Primers

4.8.1 Zebrafish Nkx5-1 cloning

Nkx5-1 I: CAGGTCTTCCAGCTNGAGTCCAC

Nkx5-1 II: AGTTCTCGTGGTANAGGATGGG

4.8.2 Medaka Nkx5 genes sequencing and RT-PCR

OlNkx5-1.2 RT PCR

RT1: GAACGGGCTCGGTTCGTCCAAA

RT2: GGCTTCTTTTCTCGACTCGGGGAT

RT3: CTTCTTGTCCGACTCCAGGTCCT

RT4: CGTCGGCGCTCTCCTCTTTGGA

OlNkx5-1.1 RT PCR

RT5: GAGGATTCTCCCTTTCCCAAGT

RT6: GAGAGTTCAACTTCCCTCGCTT

RT7: GTTGTTGTGCTCATCGTCATCC

RT8: GCTGCTCTTGTTGTTGTTGGTGC

OlNkx5-2 5'RACE

1: GACGACAGGCAGACAGACGGTG

2: CAGACAAGCAGAGCAACTCGGCCAAG

3: CAAATGTCACCGCTGTCGGAC

OlNkx5-2 intron position

5´: GTGTCCTCAGAGGAGGAGTGC

3´: CTTATAATCCTGCAGCAGAGG

OlSOHo and OlNkx5-3 analysis:

5-4 3': CAACGGAGAACCGAGTAGTAAAG

5-4 5': CGGATGTAATACTAGAGCTAACCAG

5-4 back: GTCCTTGTAGAAACCTGAGAGCTG

Position A: GCTGTGAACTTTCCCCTGACTG

Position B: GCAGCAGGACGCTCCAACGGAG

Position C: GCACAGCAATGGTGCTCAGAG

5-3 RT1: ATGTTGGATGATAAAGCGCGC

5-3 RT2: TACGCACGGCTTTGACCTCCAC

4.8.3 Medaka BMP4 cloning

Bmp4: TCCTGGTTACCAGGCCTATTAC

Bmp42: CACGGAGAGTGCCCTTTTCCGCTG

4.8.4 Chicken NSCL-2 cloning

forward: TAAGCAGAAAGTGTAAAATG

backward: AGAAAAGAAGTCGAGAAACAAATG

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6 APPENDIX

6.1 Abbreviations

A	Adenine
ace	*acerebellar*
bp	base pair
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenetic Protein
BSA	Bovine Serum Albumine
C	Cytosine
cDNA	DNA complementary to RNA
Dig, DIG	Digoxigenin
E	Embryonic day
EtOH	Ethanol
FGF	Fibroblast Growth Factor
FGFR	Fibroblast Growth Factor Receptor
G	Guanine
GFP	Green Fluorescent Protein
HH	Hamburger-Hamilton (stage)
h	hour
kb	kilo base pairs
KO	Knock Out
MeOH	Methanol
min.	minute
mRNA	messenger RNA
Ol	*Oryzias latipes* (medaka)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PFA	Paraformaldehyde
RACE	Rapid Amplification of cDNA Ends
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
St., st.	Stage
T	Thymine
TGF	Transforming Growth Factor
TUNEL	Terminal deoxynucleotidyl transferase mediated UTP Nick End Labeling
U	Uracil
wt, WT	wild type
zf	zebrafish

6.2 Curriculum Vitae

Personal Data

Family name	Adamska
Given name	Maja Anna
Citizenship	Polish
Born	18 December 1973 in Zabrze, Slaskie Woivodship, Poland
Maiden name	Siara
Married	09.07.1994, to Marcin Adamski

Education

09.1980 – 06.1988	Primary School No. 66, Katowice, Poland
09.1988 – 06.1991	Adam Mickiewicz III Grammar School Katowice, Poland (accomplished education with distninction)

Master degree studies

10.1991 - 05.1996	Jagiellonian University in Cracow, Department of Biology and Earth Sciences
02.1994 - 06.1994	University of Wolverhampton, Tempus Exchange
09.05.1996	Master of Sciences Degree (diploma with distinction) Scientific supervisor: Prof. Szczepan Bilinski Research subject: Insect oogenesis

Ph.D. studies

10.1996 – 09.1997	Jagiellonian University in Cracow, Department of Biology and Earth Sciences, Institute of Zoology
10.1997 – 09.2000	Boehringer Ingelheim Fellowship Research subject: Molecular biology of inner ear development Scientific supervisor: Dr. Eva Bober Department of Cell and Molecular Biology, Institute of Biochemistry and Biotechnology, Technical University Braunschweig Carolo-Wilhelmina, Germany
Since 10.2000	Research Position Scientific supervisor: Prof. Thomas Braun Institute of Physiological Chemistry, University of Halle-Wittenberg, Germany

6.3 Publications and Presentations

Published

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