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Function and regulation of zebrafish *nkx2.2a* during development of pancreatic islet and ducts

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Abstract

In the mouse *Nkx2.2* is expressed in the entire pancreatic anlage. Nevertheless, absence of Nkx2.2 only perturbs the development of endocrine cell types, notably beta-cells which are completely absent. In order to test the possibility that *Nkx2.2* might fulfil additional functions during pancreas development we analysed its zebrafish homologue *nkx2.2a* using gene targeting and GFP-transgenic fish lines. Our results suggest similar roles for *nkx2.2a* and *Nkx2.2* during the development of the endocrine pancreas. Morpholino-based knock-down of *nkx2.2a* leads to a reduction of alpha- and beta-cell number and an increase of *ghrelin*-producing cells but, as in mice, does not affect delta-cells. Moreover, like in the mouse, two spatially distinct promoters regulate expression of *nkx2.2a* in precursors and differentiated islet cells. In addition we found that in zebrafish *nkx2.2a* is also expressed in the anterior pancreatic bud and, later, in the differentiated pancreatic ducts. A *nkx2.2a*-transgenic line in which pancreatic GFP expression is restricted to the pancreatic ducts revealed that single GFP-positive cells leave the anterior pancreatic bud and move towards the islet where they form intercellular connections between each other. Subsequently, these cells generate the branched network of the larval pancreatic ducts. Morpholinos that block nkx2.2a function also lead to the absence of the pancreatic ducts. We observed the same phenotype in *ptf1a*-morphants that are additionally characterized by a reduced number of *nkx2.2a*-positive duct precursors. Whereas important details of the molecular program leading to the differentiation of endocrine cell types are conserved between mammals and zebrafish, our results reveal a new function for *nkx2.2a* in the development of the pancreatic ducts.
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Introduction

The pancreas is a crucial regulator of the blood glucose level and an important source for digestive enzymes. This dual role is also reflected in the organ's subdivision into an endocrine and an exocrine compartment. The endocrine pancreas releases hormones into blood-vessels and contains glucagon-producing alpha-cells, insulin-producing beta-cells, somatostatin-producing delta-cells and pancreatic polypeptide-producing PP-cells. Recently it was shown that ghrelin, a gastric peptide involved in metabolic regulation is also expressed in the endocrine pancreas (Prado et al., 2004; Wierup et al., 2002, 2004). The exocrine pancreas consists of exocrine glands called acini that synthesize and store premature forms of digestive enzymes such as trypsin or chymotrypsin and a branched network of ducts that guides them to the intestine where they become activated.

Most of our knowledge on pancreas development originates from experiments conducted in rodents. The mammalian pancreas emerges from the duodenum as a ventral and a dorsal bud that eventually fuse to form the organ (reviewed by Slack, 1995). Both buds contain precursors for all pancreatic cell types but give rise to different parts of the pancreas. The human pancreas can be subdivided in a head and a tail region referring to the proximal and the distal part of the organ. Precursors of the ventral bud give rise to the posterior part of the head, or uncinate process, whereas the dorsal bud contains precursors for the anterior part of the head and the tail.

Many transcription factors have been identified that are crucial for normal pancreas development (Edlund, 2002; Jensen, 2004; Schwitzgebel, 2001; Wilson et al., 2003). They may be classified into two groups. The first group of transcription

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factors specifies the pancreatic anlage and absence of these factors affects both the endocrine and the exocrine lineage. The second group of transcription factors is involved in the specification of pancreatic cell types from protodifferentiated precursors. Mutations in genes of this second group perturb the development of either the endocrine or the exocrine compartment. So far three transcription factors are known to belong to the first group: Pdx1, Ptf1a and Hlxb9. Pdx1 is expressed in the entire duodenum, including the pancreatic anlage, and loss of Pdx1 function leads to the formation of a rudimentary pancreas devoid of beta- and exocrine cells (Guz et al., 1995; Jonsson et al., 1994; Offield et al., 1996). Ptf1a was originally proposed to be essential only for the differentiation of the exocrine pancreas since Ptf1a-knock-out mice lack exocrine tissue but produce endocrine cell types although in some cases ectopically (Krapp et al., 1998). More recently it was shown that Ptf1a is already expressed in the pancreatic anlage where it prevents pancreatic precursors, also those of the endocrine lineage, from adopting a duodenal fate (Kawaguchi et al., 2002). Hlxb9, the third member of this group, is also expressed in the entire pancreatic anlage. While pancreatic differentiation in the dorsal bud of Hlxb9-mutant mice is completely blocked, the ventral bud displays a rather subtle phenotype and only beta-cells are affected (Guz et al., 1995; Harrison et al., 1999; Li et al., 1999).

Most transcription factors known to play significant roles in pancreas development, as for instance NeuroD, Pax6, Pax4 and Isl1, belong to the second group and are predominantly involved in the differentiation of the endocrine lineage (Jensen, 2004; Wilson et al., 2003). This second group includes also Ngn3 (Gradwohl et al., 2000) and Nkx2.2 (Sussel et al., 1998). Of special interest is *Ngn3*, the only group 2-gene so far whose loss-of-function leads to the absence of all pancreatic endocrine cell types (Gradwohl et al., 2000). Ngn3 is not co-expressed with any of the pancreatic hormones (Gradwohl et al., 2000; Schwitzgebel et al., 2000) suggesting that it induces the differentiation of pancreatic precursors into endocrine cell types but does not directly activate pancreatic hormone gene expression. Further evidence came from misexpressing Ngn3 in the pancreatic anlage before the onset of normal Ngn3 expression which led to a hypotrophic pancreas due to accelerated differentiation of endocrine cells resulting in a precocious depletion of the precursor pool (Apelqvist et al., 1999; Schwitzgebel et al., 2000).

One of the putative downstream targets of Ngn3 is Nkx2.2. Immunohistochemistry revealed that in the mouse Nkx2.2 is initially expressed in most cells of the pancreatic buds but becomes restricted to cells of the endocrine lineage during later stages (Sussel et al., 1998). In a more recent work, Chiang and Melton (2003) performed single-cell PCRs on 60 pancreatic cells obtained from mouse embryos. They were able to detect the *Nkx2.2* mRNA in each single cell, also in those expressing exocrine marker genes. This suggests a role for *Nkx2.2* similar to group 1-genes. Knock-out mice though, display an aberrant cellular composition of the endocrine pancreas whereas development of the exocrine pancreas proceeds normally. These mice are characterized by a complete lack of beta-cells,

a severe reduction of alpha- and PP-cells and an excess of ghrelin-positive cells in the islets whereas delta-cell-number, acini and overall organ morphology are unaffected (Sussel et al., 1998; Prado et al., 2004). This phenotype is more reminiscent of a group 2-gene. As a consequence, models that try to explain how pancreatic cell types are specified by a cascade of transcription factors assign different roles to Nkx2.2. Whereas some models put Nkx2.2 on top of this cascade, pointing to a significant role already in the pancreatic anlage (Chiang and Melton, 2003), others propose that its functional significance is restricted to beta-cell development (Schwitzgebel, 2001).

The transcriptional regulation of *Nkx2.2* has been characterized in detail by Watada et al. (2003). *Nkx2.2* is transcribed from three distinct promoters resulting in three mRNA isoforms with different 5′UTRs but identical coding regions. The three 5′UTRs were called exon 1a, 1b and 1c with exon 1a being the most distal one with respect to the start codon. Two promoters show a tissue-specific activity in the pancreas. Promoter 1a is exclusively active in mature endocrine cells but not in endocrine precursors. Promoter 1b activates transcription only in Ngn3-positive cells which represent pan-endocrine precursors of the pancreas, but not in cells expressing any of the pancreatic hormones. Exon 1c was not found to be expressed in the pancreas.

Many of the transcription factors known to be crucial for pancreatic development in mammals are expressed also in the zebrafish pancreas. Transcription factors such as *pdx1*, *nkx2.2a*, *pax6b* and *islet1* are expressed in the endoderm shortly before or in parallel to the onset of *insulin* expression at 12 somites (Biemar et al., 2001). *Insulin* expression is followed by *somatostatin* at 16 somites and *glucagon* at the end of somitogenesis (24 hpf) (Argenton et al., 1999; Biemar et al., 2001). The mRNAs for the first digestive enzymes are detectable at 48 hpf (Biemar et al., 2001; Yee et al., 2005). The entire exocrine pancreas and late differentiating endocrine cells are generated from *ptf1a*-positive precursors situated in the so-called anterior pancreatic bud (Field et al., 2003; Lin et al., 2004; Yee et al., 2005; Zecchin et al., 2004).

Specific antisense oligomers called morpholinos have been used to create transient loss-of-function phenotypes for group 1-transcription factors in zebrafish. The role of pdx1 during pancreas development seems to be conserved in zebrafish since it is expressed early in a defined region of the endoderm and *pdx1*-morphant embryos lack all pancreatic hormones and show no carboxypeptidase-immunoreactivity (Yee et al., 2001). Zebrafish *ptf1a* is expressed at 30 hpf in cells of the anterior pancreatic bud, after the onset of pancreatic hormone expression. In agreement with the expression data, *ptf1a*-morphants lack exocrine markers such as *trypsin* whereas their pancreatic islet develops normally (Lin et al., 2004; Zecchin et al., 2004). It is possible though that *ptf1a* is necessary for the proper development of late differentiating endocrine cells originating from the anterior pancreatic bud (Lin et al., 2004). The zebrafish homologue of mammalian *Hblx9* is called *hb9* and is expressed in the pancreas from the 10 somite stage (Wendik et al., 2004). However, blocking hb9-function only perturbs beta-cell development (Wendik et al., 2004).

Like in the mouse, also in zebrafish the precise role of nkx2.2a is currently unknown. The timing of expression of group 2-genes in the zebrafish endoderm indicates an evolutionary conserved role for these transcription factors in teleosts. Surprisingly, functional data for this group of genes in zebrafish is pending. Zebrafish *nkx2.2a* is expressed in the ventral CNS and in the pancreas (Barth and Wilson, 1995; Biemar et al., 2001). A second zebrafish *Nkx2.2* paralogue, *nkx2.2b*, is also expressed in the central nervous system but

was not detected in the endoderm (Schafer et al., 2005). At the 10 somite stage *nkx2.2a* is first detectable in the pancreatic primordium and thereafter in the pancreatic islet (Biemar et al., 2001). The early onset of *nkx2.2a* expression in the endoderm is paralleled by the onset of the expression of the group 1-gene *pdx1*. Group-2 genes like *pax6b* and *islet1* are detectable later, at 12 somites (Biemar et al., 2001). This notion is in agreement with the PCR expression data in mice (Chiang and Melton, 2003) and raises the question of the
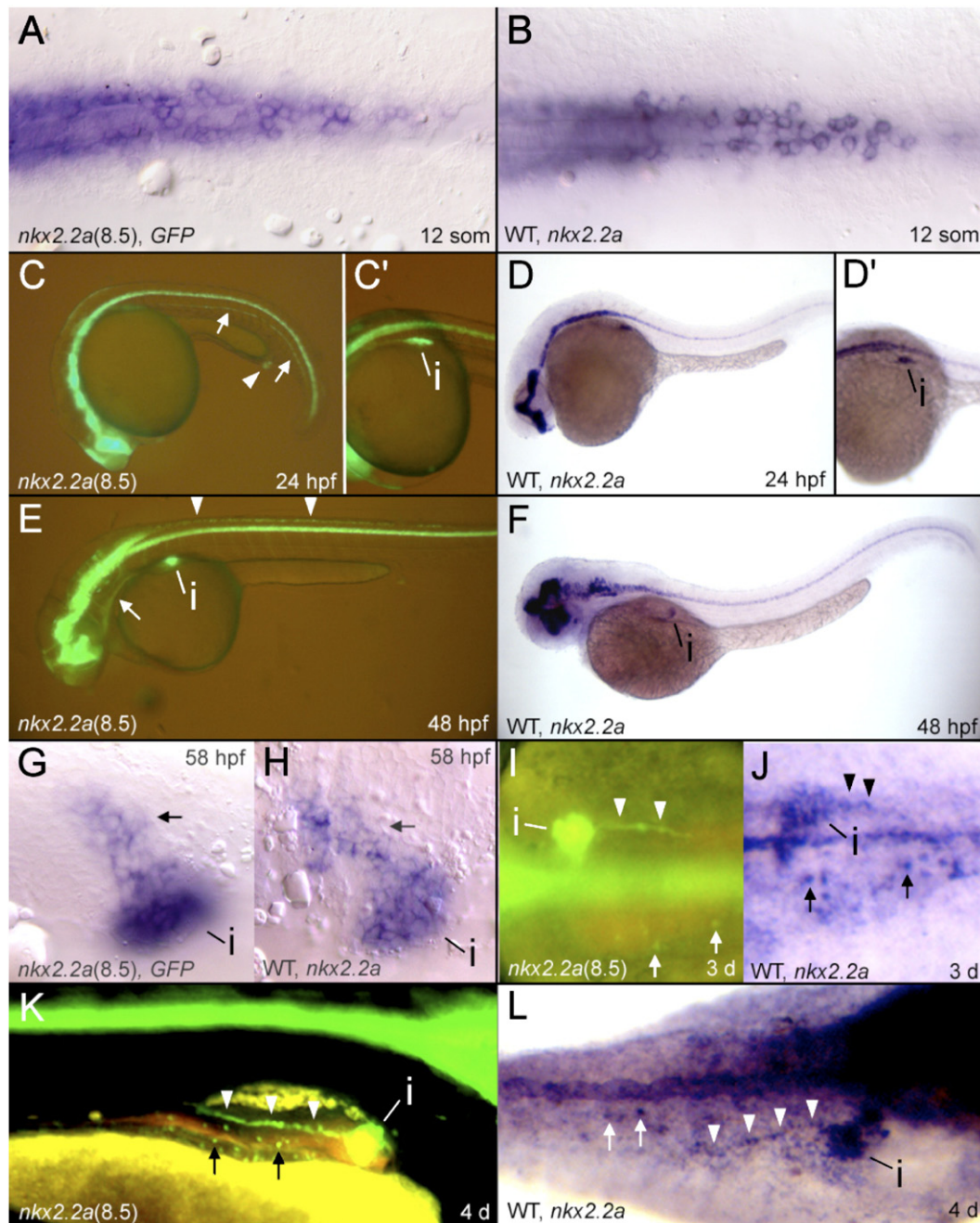

Fig. 1. Stable transgenic expression of GFP compared to *nkx2.2a* mRNA expression. (A, C, C′, E, G, I, K) GFP fluorescence or *GFP* mRNA expression in transgenic animals. (B, D, D′, F, H, J, L) *nkx2.2a* in situ hybridizations in WT zebrafish. (C–F, K) Lateral views. (L) Dorsolateral view. (I, J) Dorsal views. (A, B, G, H) Ventral views. (C′, D′) represent the same embryos shown in panels C and D respectively, focusing on pancreatic expression, dorso-lateral views. (C) Hypochord (arrows), proctodeum (arrowhead). (E) Branchial arches (arrow), dorsal spinal cord cells (arrowheads). (G, H) The arrow indicates *GFP* and *nkx2.2a* expression anterior and lateral to the pancreatic islet (i). (I–L) The pancreatic duct is indicated by arrowheads and enteroendocrine cells by arrows. i=pancreatic islet.

precise role of *nkx2.2a* also during pancreatic development in zebrafish. A detailed analysis of *nkx2.2a* expression, regulation and function during zebrafish pancreas development has led us to confirm its crucial role during endocrine development and to uncover a new function for *nkx2.2a* during the specification of the pancreatic ducts. We created stable transgenic lines expressing GFP under the control of different *nkx2.2a* promoter fragments which are valuable in vivo markers for pancreatic development. With the help of these lines and *nkx2.2a* gene targeting we revealed that *nkx2.2a* function and regulation during endocrine pancreas development are conserved between teleosts and mammals. Furthermore, we found that in zebrafish *nkx2.2a* is also expressed in precursors and differentiated cells of the pancreatic ducts and that it is essential for the proper development of this part of the exocrine pancreas in zebrafish.

Materials and methods

Animals

Zebrafish were obtained from spontaneous spawnings. The embryos were kept at 28.5 °C and staged according to Kimmel et al. (1995). For in situ hybridization embryos were fixed in 4% paraformaldehyde (PFA)/PBS. Confocal images were obtained from embryos or larvae fixed in 4% PFA/0.1% glutaraldehyde/3% sucrose in PBS (Luby-Phelps et al., 2003). For live observations transgenic embryos were anaesthetized in tricaine (Westerfield, 2000). In order to suppress pigmentation embryos were raised in 1-phenyl-2-thiourea (PTU) (Westerfield, 2000). We used the WT strains GIOTTO or UMBRIA purchased in 1994 from a local pet shop and bred in our facility for more than 6 generations. Transgenic animals used in this work belonging to the *Tg(nkx2.2a(3.5kb):GFP)* line are always homozygous whereas *Tg(nkx2.2a (8.5kb):GFP)* animals are always hemizygous.

Cloning of nkx2.2a promoter fragments

A zebrafish genomic PAC library ('Deutsches Ressourcenzentrum für Genomforschung' (RZPD), library number: 706) was screened using the primer pair NkA (5′-GGCACGAGTAATGGCTTATTTCC) and NkB (5′-TGCTGCAC-CAGTTTGACAATCC). This led to the identification of a PAC clone containing the *nkx2.2a* coding region (Accession: NM_131422 (GenBank)) (Barth and Wilson, 1995) that was used to isolate various *nkx2.2a* promoter fragments (Supplemental Fig. 1A). First, we sequenced 1.5 kb upstream of the *nkx2.2a* coding region with the help of four primers: NkB, 5′-CAACACATGAACCTG-CAGTG, 5′-AAGGACATCGAAATCGCCTC and 5′-TCTTTGCCTCCTATGTG. This region was cloned by PCR using the following primer pair: nk2.2pro5/*Xba*I (5′-TCTAGAGAAGGACTTTCCTTGATATTGTG) and NkB. We identified a *Hin*dIII-site near the 5′-end of this fragment and used it to clone an additional piece of the *nkx2.2a* promoter region. We digested the entire PAC with *Hin*dIII, cloned the resulting fragments into the pBluescript-vector (Stratagene) and screened them by PCR in order to identify a fragment whose 3′-end would overlap with the 5′-end of the already cloned 1.5 kb fragment. The primer pair used for the screening was nk2.2pro5/*Xba*I and 5′-TTTGTCCGGCCGTAGTT. We identified a 2 kb *Hin*dIII-fragment that was cloned in front of the 1.5 kb fragment resulting in the 3.5 kb piece of the *nkx2.2a* promoter that was used to

generate the *Tg(nkx2.2a(3.5kb):GFP)* line. At the 5′-end of the 3.5 kb fragment we identified an *Apa*I-site. The PAC clone was digested with *Apa*I, the resulting fragments were subcloned into pBuescript (Stratagene) and screened for the presence of a sequence identical to the 5′-end of the 3.5 kb fragment. This led to the isolation of an *Apa*I-fragment of about 20 kb, that was reduced to 5 kb using an endogenous *Xba*I site and cloned in front of the 3.5 kb promoter fragment resulting in a 8.5 kb piece of the *nkx2.2a* promoter that was used to generate the *Tg(nkx2.2a(8.5kb):GFP)* line.

For the transient expression assay promoter fragments were either cloned by cutting them out of one of the larger promoter fragments using the appropriate enzymes or by amplifying them in a PCR reaction (Supplemental Fig. 1B). Element 1 is identical to the 3′-end of the 1.5 kb promoter fragment downstream of a *Stu*I site. Element 2 is situated between an *Eco*RV and a *Sma*I site in the 3.5 kb promoter fragment. Element 3 is situated between the *Apa*I and a *Nde*I site in the 3.5 kb promoter fragment. The primer pair F2 (5′-GACTTGACTTGAA-GACGCTTGAT) and R3 (5′-TGACGGAAGAGGGTAAGTGG) was used to amplify the elements 7 and 8 together. Element 7 was amplified with F3 (5′-AAGCGCTCTTATGAGGCAAC) and R3 and element 8 was amplified with F2 and R5 (5′-TGCGACTCAGAATGTCTATGC).

Generation of transgenic lines

Both the 3.5 kb and 8.5 kb fragment were cloned in front of a *GFP* coding sequence using the pGI cloning vector (Gilmour et al., 2002). The plasmid backbone was removed either with *Sac*I/*Kpn*I (*Tg(nkx2.2a(3.5kb):GFP)*) or *Sac*II (*Tg(nkx2.2a(8.5kb):GFP)*). The inserts were purified using the Agarose Gel DNA Extraction Kit (Roche). A solution containing 50 ng/μl DNA, 0.5% phenol red and salts (5 mM Tris pH8, 0.5 mM EDTA, 1 mM KCl (Meng et al., 1997)) was injected into WT zygotes to generate stable transgenic insertions. The injected fish were raised to adulthood, mated with WT fish and the offspring was screened for fluorescence. We found one insertion for *Tg(nkx2.2a(8.5kb): GFP)* and two independent insertions of *Tg(nkx2.2a(3.5kb):GFP)* with only one showing a fluorescence strong enough for our purposes.

Transient expression assay

In order to enhance the efficiency of transient expression, *nkx2.2a* promoter fragments were put in front of a sequence coding for a Gal4:VP16 fusion protein. This fusion protein can activate the expression of GFP in the same vector by binding to several *UAS* enhancers (Koster and Fraser, 2001). If the *nkx2.2a* promoter fragments did not contain a basal promoter, like elements 2, 3 and 8, we used an expression vector with its own basal promoter EIb (Koster and Fraser, 2001). We injected 30 ng/μl of plasmid DNA together with 0.5% phenol red and salts (Meng et al., 1997). Larvae with aberrant morphology were discarded and the number of GFP-positive cells was determined at 4 dpf.

Injection of morpholino antisense oligomers

Two *nkx2.2a* morpholinos were obtained from *Gene Tools*:

MOnk-SPLI, 5′-AGGCCGTGTACTGGTAAAACAAAGG;

MOnk-5UTR, 5′-TGGAGCATTTGATGCAGTCAAGTTG.

MOnk-SPLI is directed against the junction between the second intron and the third exon and MOnk-5UTR against a part of the 5′UTR common to both *nkx2.2a* mRNA variants and upstream of the start codon (Supplemental Fig. 1C). The morpholinos were diluted to working solutions between 0.5 and 4 mg/ml in Danieau solution (Nasevicius and Ekker, 2000). The *ptf1a* morpholinos

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Fig. 2. Sequence homology between the regulatory regions of zebrafish *nkx2.2a* and other vertebrate *Nkx2.2* genes. (A) Schematic alignment of four vertebrate *Nkx2.2* promoter regions (10 kb). Conserved sequence blocks in the zebrafish sequence are shown as black squares. The degree of identity between a given zebrafish element and the corresponding region in one of the other sequences is indicated by different colours, orange for >65% identity and blue for >85% identity. The promoter fragments used for the two transgenic lines are depicted in the zebrafish sequence. (B) Sequence alignment of the zebrafish genomic region containing elements 7 and 8 as well as a sequence identical to 5′-region of the identified *nkx2.2a* EST harbouring an alternative 5′UTR ('variant2') and the corresponding genomic sequence in mouse. Elements 7 and 8, the region corresponding to the alternative 5′UTR and a putative TATA-box are shaded in grey. The conserved E-Box motif and the HNF3 binding motif in the mouse sequence are marked in black. (C) RT-PCR to identify the two variant *nkx2.2a* transcripts at various developmental stages. Note that mRNA variant 2 is not detectable before the onset of pancreatic hormone gene expression.

ptf1a-MOa and ptf1-MOb (Zecchin et al., 2004) were injected together using a solution that contained 0.75 mg/ml of each morpholino. All morpholinos were injected into zygotes together with 0.5% phenol red and rhodamin dextran to identify successfully injected embryos.

In situ hybridization

RNA in situ hybridizations were conducted essentially as reported in Thisse et al. (1993). Digoxigenin and fluorescein-labelled antisense probes (*Roche*) were synthesized from the following cDNAs: *nkx2.2a* (Barth and Wilson, 1995), *insulin*, *glucagon*, *somatostatin* (Argenton et al., 1999), *ghrelin* (gift from Bernard Peers), *neuroD* (Korzh et al., 1998), *ptf1a* (Zecchin et al., 2004), *GFP* (pGI vector) (Gilmour et al., 2002).

Microscopy and image acquisition

GFP-expressing embryos or larvae were analysed using a stereo microscope (*Leica MZFLIII*) equipped with a UV-lamp. In situ hybridizations were examined under a *Leica DM R* microscope. All pictures were acquired with a *Leica DC 500* digital camera and contrast and brightness elaborated with *Adobe Photoshop 6.0* software. Confocal images were acquired with a *Bio-Rad radiance 2000* confocal system and elaborated with *ImageJ* software (http://www.rsb.info.nih.gov/ij/).

Sequence analysis

In order to detect conserved sequence blocks in the *nkx2.2a* promoter we compared 10 kb of genomic sequence from human, mouse and *Takifugu rubripes* (Fugu) with the zebrafish sequence. We identified the mouse and human homologues to zebrafish *nkx2.2a* in GenBank (Accession numbers: NM_131422 (zebrafish), NM_010919 (mouse) and NM_002509 (human)). The corresponding genomic sequences are located on zebrafish chromosome 17, mouse chromosome 2 and human chromosome 20. The Fugu sequence was identified by blasting the zebrafish cDNA sequence against the Fugu genomic sequences present at the "The Wellcome Trust Sanger Institute" (http://www.sanger.ac.uk). This led us to the identification of a highly similar sequence (Gene: NEWSINFRUG00000145503), on scaffold 23, that is annotated as the Fugu *nkx2.2a* homologue. In order to identify conserved sequence blocks in these 4 sequences, we analysed 10 kb immediately upstream of the start codon of the various *nkx2.2a* homologues. The sequence data were obtained from "The Wellcome Trust Sanger Institute"; conserved elements and their degree of identity were determined using the "DNA Block aligner" tool from the "European Bioinformatics Institute" (http://www.ebi.ac.uk/Wise2/dbaform.html). The *nkx2.2a* EST containing the alternative 5′UTR sequence (Accession: CN837244 (EMBL), IMAGE:7294549) was identified by blasting the *nkx2.2a* coding sequence (NM_131422) (Barth and Wilson, 1995) against a vertebrate EST database. In order to detect both *nkx2.2a* variants we performed a PCR on random hexamers-primed cDNA from whole embryos at 5 somites, 24 hpf and 3 dpf as well as from adult zebrafish pancreas using forward oligos specific for each variant 5′UTR (5′-CAGCAGTAGCGCAATATCCA (for the known 5′UTR (variant 1)) (5′-CCACTTACCCTCTTCCGTCA (for the new 5′UTR (variant 2)) and a common reverse oligo GTGGTTGCGAGCCATCTAGT.

Results

nkx2.2a:GFP transgenic embryos reveal new sites of pancreatic nkx2.2a expression

Previous studies have shown that zebrafish *nkx2.2a* is expressed in the embryonic pancreas (Barth and Wilson, 1995; Biemar et al., 2001). Nevertheless, a detailed expression analysis from somitogenesis to larval stages is missing. To this end, we generated a stable transgenic zebrafish line using the *nkx2.2a* enhancer/promoter region fused to GFP. We found

that a 8.5-kb-large fragment including the region upstream of the *nkx2.2a* start codon is sufficient to drive GFP expression in the pancreas in agreement to the *nkx2.2a* mRNA expression pattern. At 12 somites *GFP* and *nkx2.2a* expressions in the endoderm are indistinguishable (Figs. 1A, B). At 24 hpf GFP can be detected in the brain, the ventral spinal cord (Fig. 1C) and in the pancreatic islet (Fig. 1C′), tissues that also express the *nkx2.2a* mRNA (Figs. 1D and D′). In contrast to the *nkx2.2a* mRNA the transgene can also be detected in the hypochord and in a group of cells around the proctodeum (Fig. 1C). Moreover, GFP is weakly expressed by spinal motor neurons as deduced from fluorescent spinal nerves (not shown) whereas so far *nkx2.2a* has not been reported to be expressed in these cells. At 48 hpf GFP and *nkx2.2a* expression are almost identical compared to 24 hpf except for two additional sites of ectopic promoter activity which are the branchial arches and single cells in the dorsal spinal cord (Figs. 1E, F). While it is known that *nkx2.2a* is expressed in the endocrine pancreas we also detected *nkx2.2a* and *GFP* in cells anterior and lateral to the pancreatic islet at 58 hpf (Figs. 1G, H) which most likely belong to the anterior pancreatic bud. At 3 dpf GFP expression in the pancreas can be subdivided in two domains: the islet and a thin row of cells caudal and intimately attached to the islet (Fig. 1I) where also *nkx2.2a* mRNA is detectable (Fig. 1J). This row of cells becomes more prominent in 4-day-old transgenic larvae where it covers a length of about 4–5 somites (Fig. 1K). Like GFP, also *nkx2.2a* is expressed in cells forming a thin line caudal to the islet (Fig. 1L). Position, shape and its appearance at 3 dpf suggest that this row of cells represents the developing pancreatic duct. From 3 dpf we find single fluorescent cells in the intestine of transgenic larvae that later, at 4 days, cover the entire length of the gut (Figs. 1I, K). As reported recently by other authors that analysed fish lines expressing GFP under the control of *nkx2.2a* regulatory regions these cells represent enteroendocrine cells of the gut epithelium that express *nkx2.2a* (Ng et al., 2005; Figs. 1J, L).

The zebrafish nkx2.2a promoter/enhancer shares several conserved sequence blocks with other vertebrate Nkx2.2 promoters

Non-coding sequences conserved between different vertebrate classes may represent regulatory regions associated with genes crucial for embryogenesis (Woolfe et al., 2005). In order to identify potential regulatory elements in the zebrafish *nkx2.2a* promoter/enhancer we compared the zebrafish sequence to *Nkx2.2* regulatory regions of other vertebrates. The alignment depicted in Fig. 2A summarizes the results obtained by comparing the 10 kb region upstream of the start codon of the zebrafish *nkx2.2a* gene to the corresponding sequences in *Takifugu rubripes*, mouse and human. This analysis uncovered 11 sequence blocks with more than 65% identity between zebrafish and at least one of the other three vertebrate species. As expected, we found a higher number of conserved elements between zebrafish and Fugu than between zebrafish and mammals. However, element number 6 is only conserved between zebrafish and mammals. Four out of 10 elements

conserved between zebrafish and Fugu, namely elements 1, 2, 7 and 11, share over 85% sequence identity. All, except for element 11, are also present in the mammalian sequences. Two elements with a lower degree of conservation, elements 5 and 8, are present both in Fugu and mammals. Elements 3, 4, 9 and 10 are only detectable in the Fugu enhancer/promoter.

Sequence homology between a part of the zebrafish nkx2.2a promoter and the endocrine specific promoter in the mouse Nkx2.2 gene

In the mouse, *Nkx2.2* is transcribed from three different promoters resulting in mRNAs with three different 5′UTRs but
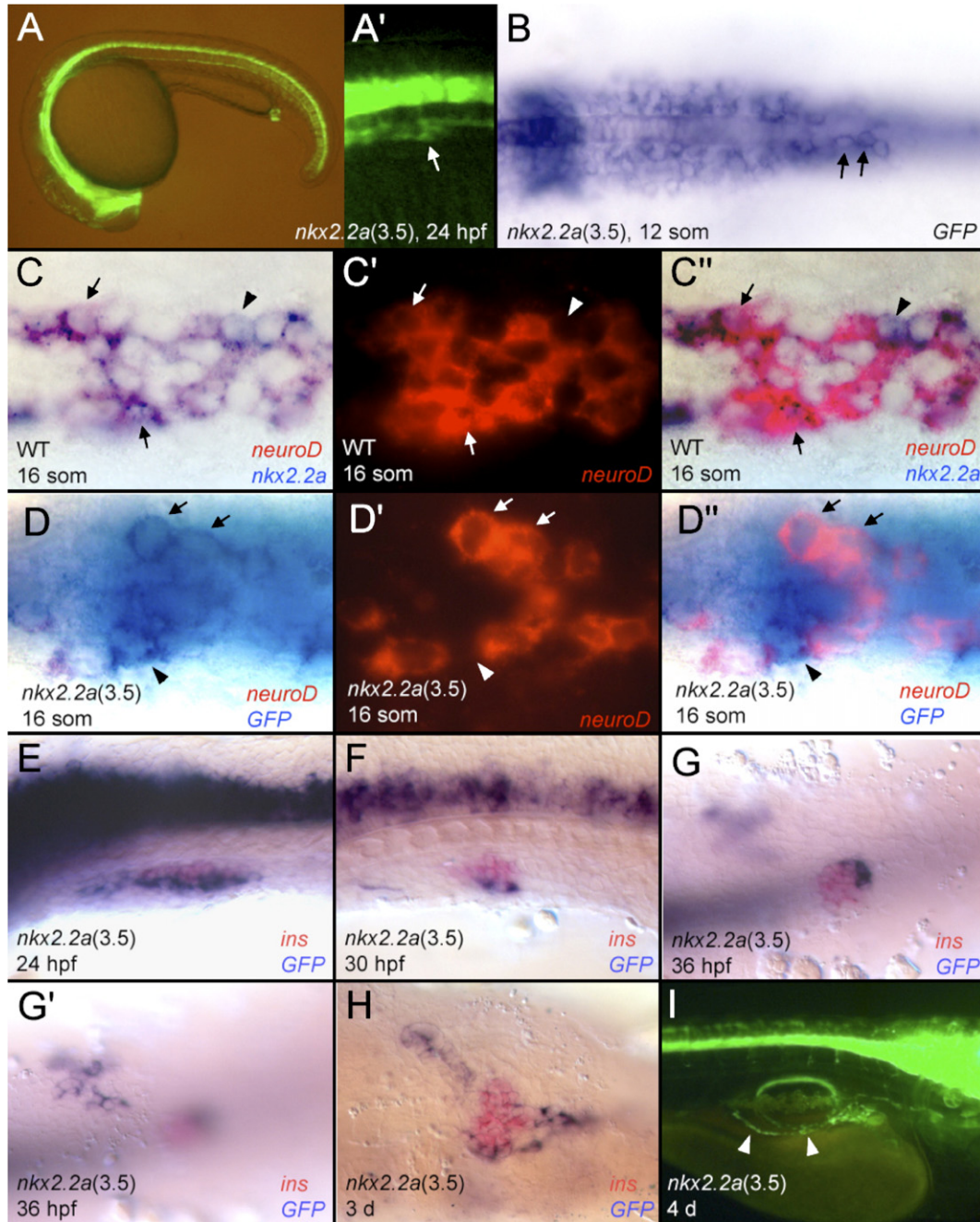

Fig. 3. GFP expression under the control of the 3.5 kb promoter fragment. (A, A′, I) GFP fluorescence. (B, D–H) *GFP* mRNA in blue. (C) *nkx2.2a* mRNA in blue. (C, D) *neuroD* mRNA in red. (E–H) *insulin* mRNA in red. (A, E, F, I) lateral views. (B–D, G, H) Ventral views. (A′) The pancreas of the same embryo shown in panel A at higher magnification (arrow). (B) *GFP* expression in the endoderm is indicated by arrows. (C, C′, C″) Same embryo, (C′) fluorescence of the *neuroD* probe, (C″) overlay of panels C and C′. (D, D′, D″) Same embryo, (D′) fluorescence of the *neuroD* probe, (D″) overlay of panels D and D′. (C, D) Cells labelled by *nkx2.2a* (C) or *GFP* (D) and *neuroD* are indicated by an arrow and cells expressing either *nkx2.2a* (C) or *GFP* (D) but not *neuroD* with an arrowhead. (E–G) Pancreatic islet showing reduction of GFP transcription. (G, G′) Two different focal planes of the same embryo, (G′) anterior pancreatic bud. (I) The pancreatic duct is highlighted by arrowheads, here the anterior is to the right.
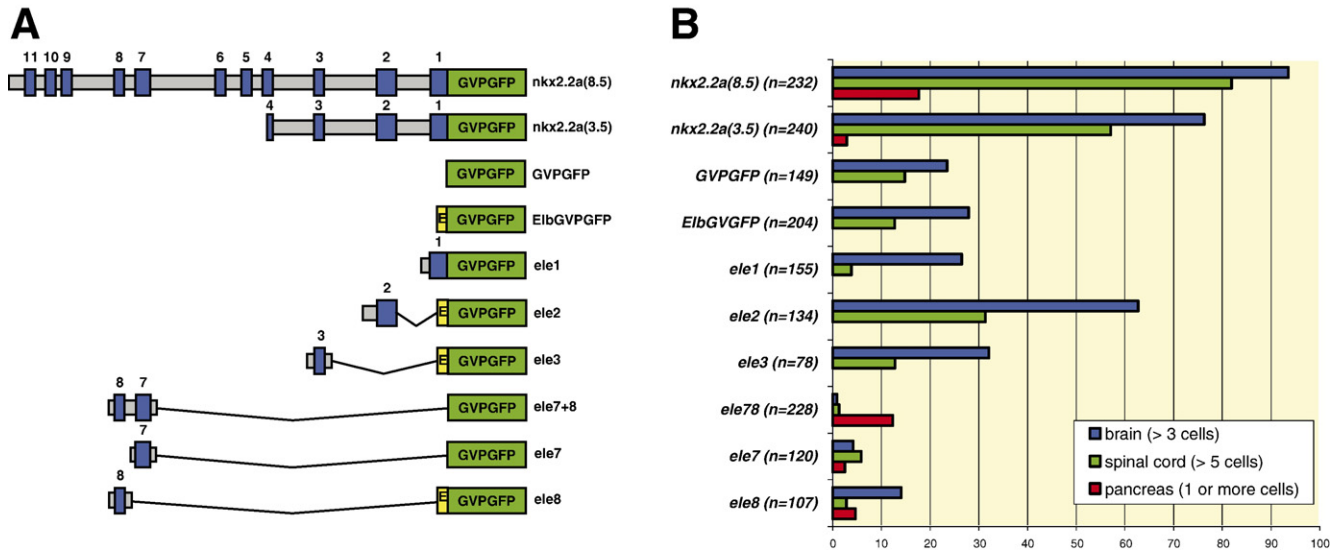
Fig. 4. Transient GFP expression under the control of various *nkx2.2a* promoter fragments in 4-day-old larvae. (A) Injected constructs. GVPGFP (green) short for Gal4:VP16UASGFP with or without a minimal promoter EIb (E, yellow). Conserved sequence blocks in the zebrafish promoter are shown in blue with the corresponding numbers. *nkx2.2a*(8.5) and *nkx2.2a*(3.5) are identical to the two promoter fragments used to generate the transgenic lines. GVPGFP and EIbGVPGFP are negative controls without any *nkx2.2a* promoter sequence. (B) Graphic representation of the numbers listed in Supplemental Table 1 in percent.

identical coding regions (Watada et al., 2003). The different initiation sites for transcription have been termed exon 1a, b and c. We found that element 7 in the zebrafish promoter is highly similar to the regulatory region upstream of exon 1a in mouse (Fig. 2B), and zebrafish element 1 is similar to the regulatory region upstream of the mouse exon 1c (not shown). However, the zebrafish sequence has no homologies with the mouse exon 1b and its upstream region.

The mouse 1a promoter contains an E-box motif and a HNF3 binding motif critical for proper promoter activity in an endocrine cell line (Watada et al., 2003). The E-box and a part of its surrounding sequence are perfectly conserved between zebrafish and mouse (Fig. 2B), indicating that element 7 might be regulated by similar factors like the endocrine specific promoter in mouse. The HNF3 motif is less well conserved (Fig. 2B) but still fulfils the criteria of a HNF3 consensus binding site that was determined for mammals (Overdier et al., 1994). We identified a putative TATA box about 100 bp downstream of element 7 raising the possibility that, like in the mouse, zebrafish *nkx2.2a* is transcribed from more than one promoter. However, the genomic sequence downstream of this TATA-Box motif does not show any homology to mouse exon 1a. Therefore, we screened an EST database for zebrafish *nkx2.2a* EST clones with 5′UTRs corresponding to the genomic sequence closely downstream of element 7. We identified one EST clone (EMBL Accession number: CN837244) whose 5′UTR is identical to a

sequence about 100 bp downstream of element 7 and 25 bp downstream of the putative TATA-Box (Fig. 2B). We termed this new *nkx2.2a* mRNA 'variant 2' and 'variant 1' the one corresponding to the sequence published by Barth and Wilson (1995). In order to determine the expression profile for both *nkx2.2a* mRNAs we performed PCRs on cDNA from whole embryos and adult pancreas (Fig. 2C). As expected, we found that *nkx2.2a* 'variant 1' expressed from early somitogenesis on and also in the adult pancreas. However, 'variant 2' is not expressed at 5 somites while it is clearly detectable at 24 hpf and 3 dpf. Like 'variant 1', also 'variant 2' is expressed in the adult zebrafish pancreas. The fact that 'variant 2' is not expressed before the onset of pancreatic hormone gene expression starting at 12 somites is consistent with the ideas that a) 'variant 2' might be homologous to mouse *Nkx2.2* transcripts starting with exon 1a, which are specifically expressed in differentiated endocrine cells but not in precursors and b) that 'variant 2' conservation throughout evolution might be connected to a crucial pancreas-specific function.

A 3.5 kb fragment of the proximal nkx2.2a promoter region is active in pancreatic precursors and the pancreatic duct but not in beta cells

In the mouse, transgenic lines with just 3.5 kb of the *Nkx2.2* promoter/enhancer did not show reporter gene

Fig. 5. Confocal analysis of pancreatic duct morphogenesis in fixed *nkx2.2a:GFP* transgenic zebrafish. (A–C) Confocal images of a *nkx2.2a*(8.5) larva. (D) WT embryo. (E–K) Confocal images of *nkx2.2a*(3.5) embryos and larvae. (A–C) Lateral view, anterior to the right. (A) Fluorescent pancreatic duct (B) transmission, showing the acinar cells of the pancreatic tail (arrowheads) (C) overlay. (D) Ventral view, double in situ hybridization of *nkx2.2a* (blue) and *ptf1a* (red). *nkx2.2a*-positive cells of the anterior pancreatic bud are marked by an arrow. (E–I) Ventral views, anterior to the left. Different fixed individuals. The intrapancreatic duct is highlighted by arrowheads. The position of the pancreatic islet in panel E is indicated by a dashed circle. Arrows in panels G and H indicate filopodia. (J–K) Lateral view, anterior to the right. Arrows in panels J and K indicate first order branches. Scale bars: A–C and J 50 μm, D–I and K 25 μm.
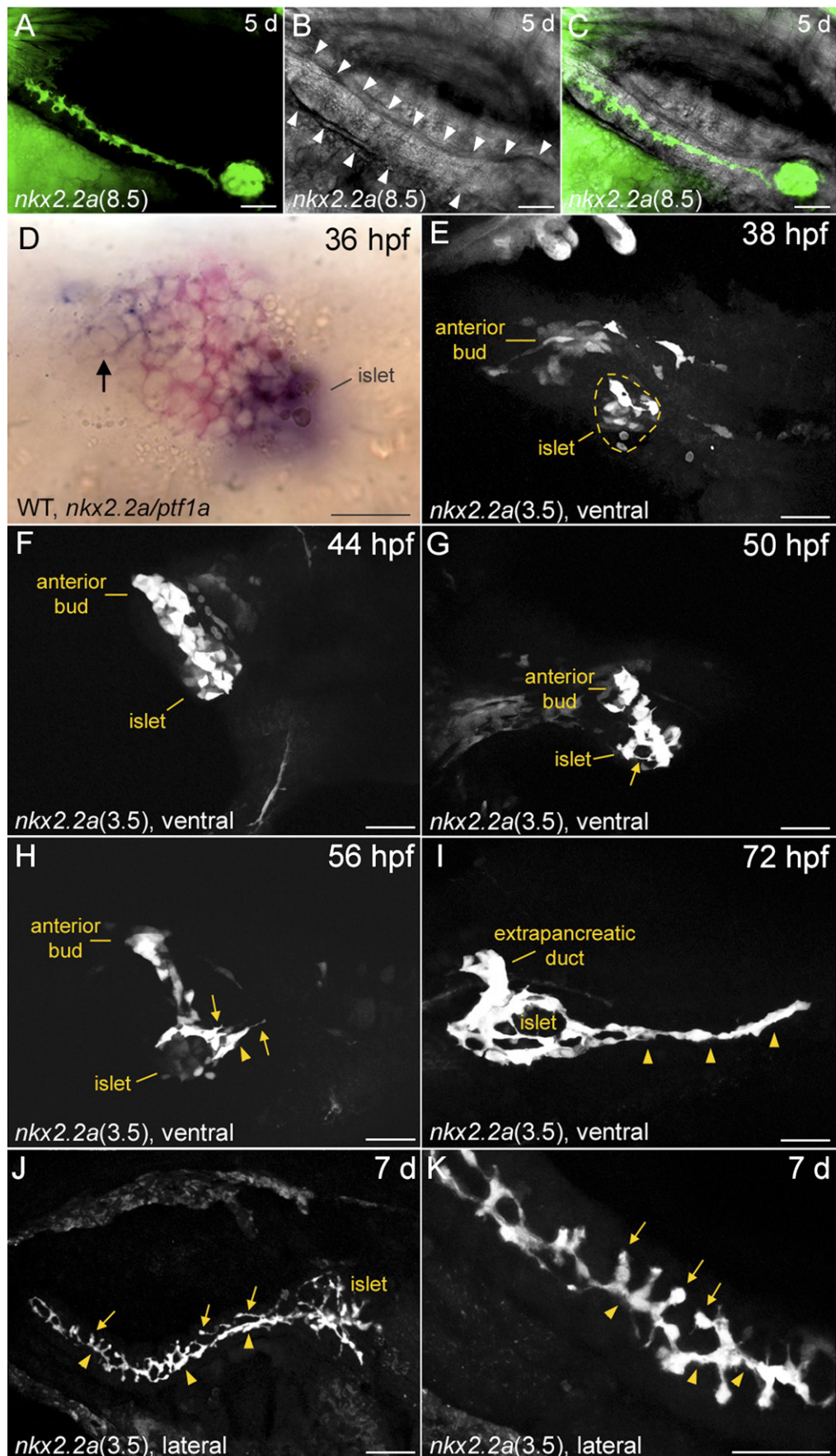
expression in the pancreas (Watada et al., 2003). This indicated that exons 1a and 1b, not included in this transgene, were necessary to activate gene expression both in pancreatic precursors and differentiated islet cells. Given the co-linearity of the mouse and zebrafish regulatory regions (see the order of conserved sequence blocks in Fig. 2A) we asked whether a 3.5-kb-large fragment of the zebrafish *nkx2.2a* promoter would also lack any pancreatic activity. To this purpose we generated a second transgenic line in which GFP was under the control of 3.5 kb of the *nkx2.2a* promoter/enhancer, including only elements 1, 2, 3 and a part of element 4 (*nkx2.2a*(3.5)) (Fig. 3A). The overall expression of GFP at 24 hpf resembles the one observed in transgenic animals expressing GFP under the control of the 8.5 kb fragment except for fluorescence in the posterior notochord and some single GFP-positive cells in the head (Fig. 3A). However, while GFP under the control of the 8.5 kb *nkx2.2a* promoter/enhancer is strongly expressed in the islet (Fig. 1C), only faint fluorescence can be seen in the pancreas when GFP is regulated by the shorter promoter (Fig. 3A′), indicating that crucial activator sequences reside upstream of the 3.5 kb promoter fragment.

Since some fluorescence remains in the islet when *GFP* is driven by the shorter promoter fragment, we performed in situ hybridizations against *GFP* to uncover the nature of these GFP-expressing cells. *GFP* expression in the endoderm starts at 12 somites (Fig. 3B). In order to determine whether the 3.5 kb fragment is active in pancreatic precursors we examined *nkx2.2a* and *GFP* expression in parallel to *neuroD* expression at 16 somites. We find almost identical expression patterns for *nkx2.2a* and *neuroD* at this stage (Figs. 3C, C′, C″). However, there are a few *nkx2.2a*-positive cells that do not seem to express *neuroD*. Also in the *nkx2.2a*(3.5) transgenics most cells coexpress *GFP* and *neuroD* but, as for *nkx2.2a* expression, there are some cells that express the transgene without being labelled by the *neuroD* probe (Figs. 3D, D′, D″). Double in situ hybridizations for *GFP* and *insulin* at later stages of development reveal that *GFP*-positive cells are different from differentiated beta-cells (Figs. 3E–H). This suggests that the regulatory elements driving *nkx2.2a* expression in differentiated beta-cells are not included in the 3.5-kb promoter. At 30 hpf only few cells in the pancreas express *GFP* whereas the vast majority or even all beta-cells do not express the transgene (Fig. 3F). Later during development we find *GFP*-positive cells anterior and lateral to the islet at 36 hpf (Figs. 3G, G′) indicating that the 3.5 kb promoter fragment is also active in the anterior pancreatic bud. At the same stage there are still only few *GFP*-positive cells near the pancreatic islet (Fig. 3G). At 3 dpf beta-cells are still devoid of *GFP* mRNA, while *GFP* is expressed in pancreatic duct precursors (Fig. 3H). Strong GFP-fluorescence can be detected in the pancreatic ducts at 4 dpf, whereas only faint fluorescence can be seen in the islet (Fig. 3I). These findings suggest that the shorter promoter fragment contains all regulatory elements necessary for proper *nkx2.2a* expression in endocrine precursors as well as differentiated cells of the pancreatic ducts while it is silent in differentiated islet cells.

Elements 7 and 8 are sufficient to activate expression in the pancreas

Given the sequence homology between element 7 and the mouse promoter 1a the genomic region containing elements 7 and 8 is a good candidate for harbouring the activator sequences for expression in mature islet cells. To this end we analysed reporter gene expression under the control of various *nkx2.2a* promoter fragments in a transient approach that makes use of a plasmid containing a chimeric Gal4:VP16 protein (Koster and Fraser, 2001). This protein is working as an amplifier between the promoter/enhancer to be tested and the *GFP* which is under the control of several *UAS* enhancer elements, the binding sites for Gal4, on the same plasmid. We injected elements 7 and 8 either separately or together and also elements 1, 2 and 3 in order to identify the specific enhancers for endocrine precursors, pancreatic ducts and neural tissue. The promoter constructs as well as positive and negative controls are shown in Fig. 4A. Injection of the *Gal4: VP16GFP* plasmid, either promoter-less or driven by a minimal one, resulted in strong fluorescence in the yolk with some labelled cells mostly in muscles and CNS, very rarely in the pancreas (Fig. 4B and Supplemental Table 1). We decided to perform all further analyses at 4 dpf, because the pancreas is easily identifiable on the right side of the larvae. Moreover, strong fluorescence from the yolk made it difficult to detect fluorescent pancreatic cells during earlier stages. Injected larvae were analysed for fluorescent cells in the brain, the spinal cord and the pancreas. The numbers are summarized graphically in Fig. 4B and as raw data in Supplemental Table 1; examples for larvae with transient GFP-expression are given in Supplemental Fig 2. The data sets represent a summary of 2 to 5 independent experiments for each construct, except for the two negative controls that were tested only once.

With regard to the CNS, our data suggest that element 2 is the main neural enhancer amongst the elements tested. About one quarter of all larvae injected with either one of the negative controls had more than 3 fluorescent cells in the brain whereas both positive controls resulted in over 75% of larvae with more than 3 GFP-positive cells in the brain. Only element 2 was able to induce GFP expression at a level similar to that of the positive controls. For the spinal cord we found that after injection of the negative controls less than 15% of all larvae expressed GFP in more than 5 spinal cord cells whereas the positive controls induced expression in 57.1% and 81.9% of all larvae. As for the brain, only the injection of element 2 resulted in a significant amount of larvae expressing GFP in more than 5 cells of the spinal cord. However, this fraction of 31.4% was considerably smaller compared to the positive controls. This indicates that element 2 is capable of activating the reporter in neural tissue even though it seems to be a weaker enhancer when compared to the positive controls.

Pancreatic GFP expression was observed in less than 1% of the negative controls. In contrast, 17.7% of the positive controls injected with the entire 8.5 kb promoter showed fluorescence in the pancreas. As injection of both elements

Table 1
Analysis of *nkx2.2a*- and *ptf1a*-morphant embryos

	Insulin reduced 32 hpf %	*Glucagon* reduced 32 hpf %	*Somatostatin* reduced 32 hpf %	*Ghrelin* increased 48 hpf %	Duct (GFP) absent 4 dpf %
MOnk-SPLI	10/25 (40.0)	22/31 (71.0)	5/25 (20.0)	–	8/40 (20.0)
MOnk-5UTR	9/18 (50.0)	8/20 (40.0)	0/26	26/52 (50.0)	9/37 (24.3)
MOnk-5UTR/MOnk-SPLI	31/38 (81.6)	15/25 (60.0)	5/28 (17.9)	–	34/79 (43.0)
All *nkx2.2a* morpholinos	50/81 (61.7)	45/76 (59.2)	10/79 (12.7)	26/52 (50.0)	51/156 (32.7)
MOptf1a-5UTR/ MOptf1a-ATG	–	–	–	–	29/44 (65.9)

Absolute numbers and percentages are given for each single morpholino or combination of morpholinos. Morphant embryos are scored as "reduced" or "increased" expression when their staining area was unambiguously (>25%) smaller or larger than the controls respectively. Examples are given in Fig. 6.

together resulted in 12.2% of all larvae with positive cells in the pancreas, we conclude that elements 7 and 8 were sufficient to confer transgenic expression to the pancreas. Since our assay did not allow us to discriminate between GFP expression in different pancreatic cell types, we can not rule out the possibility that elements 7 and 8 could be active also in cells outside the endocrine pancreas. The injection of element 7 alone led to fluorescent cells in the pancreas in only 2.5% of all analysed larvae whereas element 8 showed activity in the pancreas of 4.7% of all larvae. These findings suggest synergy between elements 7 and 8. Positive controls injected with the 3.5 kb promoter exhibited pancreatic GFP expression in only 2.9% of all cases. This promoter fragment includes elements 1, 2 and 3 which when injected separately from each other did not displayed any significant activity in the pancreas. Therefore, the position of the pancreas specific activator sequences in the 3.5 kb promoter fragment remains elusive.

Duct morphogenesis in nkx2.2a:GFP transgenic zebrafish

In 5-day-old larvae of the *nkx2.2a(8.5kb):GFP* line GFP is expressed in the islet and a row of cells in the centre of the acinar tissue forming the pancreatic tail (Figs. 5A–C). This row of cells represents the main intrapancreatic duct as described recently by Yee et al. (2005). After 36 hpf the transgenic line containing 3.5 kb of the zebrafish *nkx2.2a* promoter/enhancer expresses GFP almost exclusively in cells of the pancreatic duct or its precursors. Therefore we decided to perform our analysis of early duct morphogenesis using individuals belonging to this line. In order to increase the resolution of this analysis we examined GFP expression in fixed embryos and larvae (Figs. 5E–K). First, we wanted to confirm *nkx2.2a*-expression in the anterior pancreatic bud by determining its expression with respect to *ptf1a* (Lin et al., 2004; Zecchin et al., 2004). From 36 hpf we detected a group of *nkx2.2a*-positive cells separated from the pancreatic islet (Fig. 5D). These cells are situated in the same region of cells expressing *ptf1a* (Fig. 5D) suggesting that these *nkx2.2a*-expressing cells are part of the anterior pancreatic bud. In embryos of the *nkx2.2a*(3.5) line fluorescence in the anterior pancreatic bud is detectable from 38 hpf (Fig. 5E). Except for one or two cells, fluorescence is weak in the anterior bud. Fluorescence can also be seen in the islet, but only few cells are strongly labelled, possibly those that continue to express the GFP mRNA at 36 hpf (Fig. 3G). At 44 hpf, cells of the anterior pancreatic bud strongly express GFP and some of them have already reached the islet (Fig. 5F). Six hours later, at 50 hpf, GFP-positive cells near the islet have formed filopodia-like connections which might indicate a premigratory stage (Fig. 5G). Subsequently (56 hpf), as deduced from the orientation of their filopodia, some of these cells start to migrate in a caudal direction (Fig. 5H), where the main pancreatic duct will form. We also find GFP-positive cells between the pancreatic islet and the gut tube (Figs. 5F–H)
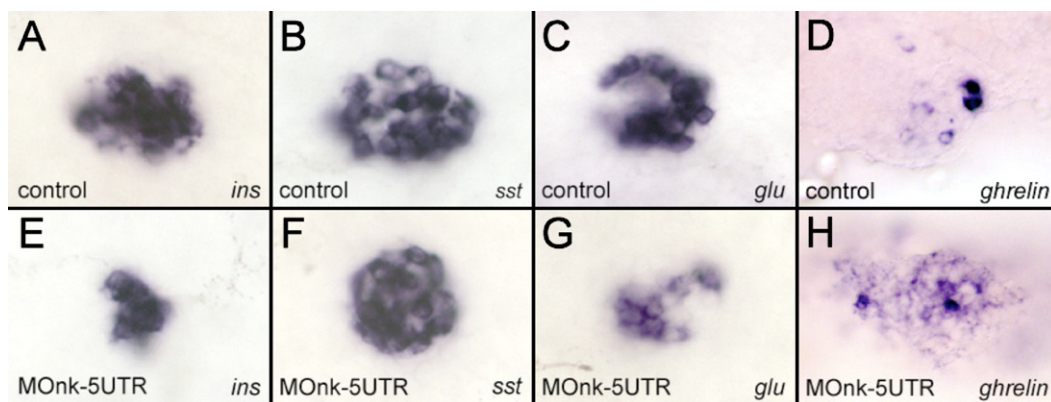

Fig. 6. The endocrine pancreas in *nkx2.2a*-morphant embryos. Ventral views. (A–C, E–G) 32 hpf. (D, H) 48 hpf. Pancreatic hormone expression in control embryos (A–D) and representative MOnk-5UTR injected embryos (E–H). (A, E) *Insulin*, (B, F) *somatostatin*, (C, G) *glucagon*, (D, H) *ghrelin*. Using ImageJ software we could calculate that *insulin* expression in panel E is 52% less than control (A), while *glucagon* expression in panel G is 43% less than control (H). In panel H, *ghrelin* expression levels are 60% more than control (D).

representing precursors for the anteromedial or extrapancreatic duct (Fig. 5I). At 3 dpf a network of GFP-expressing cells surrounds the pancreatic islet and fluorescent cells are also situated in the pancreatic tail (Fig. 5I). Duct morphogenesis is completed at day 7 (Fig. 5J), when the appearance of first order branches in the caudal part indicates that the ductal system of the larval pancreas is functional (Fig. 5K).

In order to confirm that cell migration takes place during the formation of the pancreatic ducts, we performed time-lapse analysis of GFP-positive cells in *nkx2.2a*(3.5) transgenic embryos (Supplemental movies 1 and 2). Our analysis reveals that initially GFP-positive cells from the anterior pancreatic bud migrate towards the islet and that later fluorescent cells start to migrate caudally to form the intrapancreatic duct.

nkx2.2a is needed for the proper development of alpha- and beta-cells and the pancreatic ducts

The role of *nkx2.2a* during pancreas development in zebrafish was assayed by injecting morpholino antisense-oligomers directed either against the part of the 5′-UTR common to both *nkx2.2a* transcripts (MOnk-5UTR) or the junction between intron 2 and exon 3 of the *nkx2.2a* premature mRNA (MOnk-SPLI) (Supplemental Fig. 1C). Injecting either 3 µg/µl of MOnk-5UTR or 1.5 µg/µl of MOnk-SPLI resulted in a similar enlargement of a distinct part of the brain at 32 hpf leaving the overall morphology of the embryo unchanged (Supplemental Figs. 3A, B, D, E, G, H). At 4 dpf the morphant larvae are smaller compared to the controls (not shown) and display edema in the heart region and reduced jaws (Supplemental Figs. 3C, F, I). The same phenotype was obtained by injecting 2.5 µg/µl of MOnk-5UTR together with 0.5 µg/µl MOnk-SPLI (not shown). In order to determine whether MOnk-5UTR could block efficiently the translation of *nkx2.2a* transcripts we coinjected the morpholino with a messenger consisting of the *nkx2.2a* 5′UTR fused to *GFP*. The mRNA alone resulted in bright fluorescence until day 4 (Supple-

mental Figs. 3J, K) whereas coinjection of 3 µg/µl MOnk-5UTR led to a complete absence of fluorescence or, in a few cases, to a strong reduction (Supplemental Figs. 3L, M).

The expression of *insulin*, *somatostatin*, *glucagon* and *ghrelin* was examined at 32 hpf or 48 hpf respectively and the numbers are summarized in Table 1. The injection of each *nkx2.2a*-morpholino on its own as well as a mixture of both resulted in a reduced expression of *insulin* and *glucagon* while expression of *somatostatin* was unaffected (Figs. 6A– C, E–G). This is in good agreement with what has been reported for the *Nkx2.2* knock-out mice (Sussel et al., 1998) although the morphant phenotype is weaker compared to the mouse knock-out that is completely devoid of beta-cells. An important detail of the mutant phenotype in mouse is the increase of ghrelin-expressing cells in the mutant pancreas (Prado et al., 2004). So far, there were no data on *ghrelin* expression in zebrafish. Our initial analysis revealed that at 48 hpf *ghrelin* is expressed in the zebrafish pancreas. *Ghrelin* expression is often restricted to 2 or 3 cells strongly labelled and sometimes surrounded by some additional cells with very low expression (Fig. 6D). Analysis of *nkx2.2a*-morphant embryos at 48 hpf show a considerable increase in the number of *ghrelin*-expressing cells in half of the embryos injected with MOnk-5UTR (Fig. 6H). Additional *ghrelin*-positive cells are always only weakly labelled by the *in situ* probe and their occurrence is not restricted to the principle islet.

Next we injected the morpholinos into eggs from *nkx2.2a* (3.5):GFP transgenic parents and screened the 4-dpf-old morphants for the presence of the intrapancreatic duct. We found that the duct was absent in 24.3% of all MOnk-5UTR-injected larvae and in 20% of all MOnk-SPLI-injected larvae (Figs. 7A, B) (Table 1). The injection of both *nkx2.2a*-morpholinos together resulted in 43% larvae without an intrapancreatic duct. This indicates a role for *nkx2.2a* during the differentiation of the pancreatic ducts. A closer examination of morphant larvae with no intrapancreatic duct revealed that
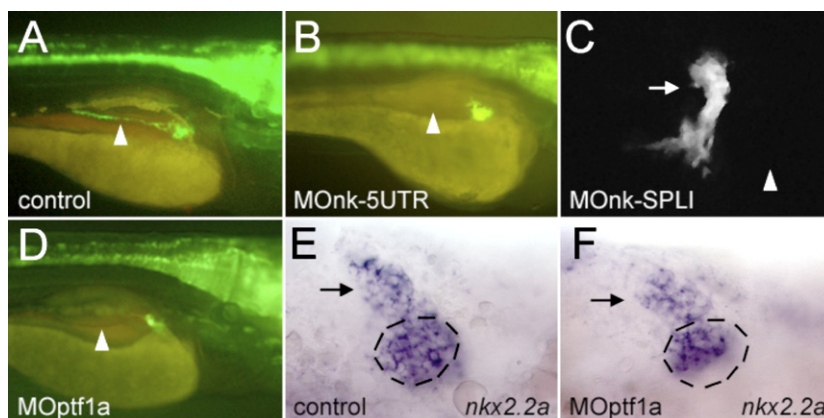

Fig. 7. The development of the pancreatic ducts in *nkx2.2a*- and *ptf1a*-morphants. (A, B, D) Lateral views, anterior to the right. (C, E, F) Ventral views, anterior to the left. (A, E) Control embryos. (B, C) *nkx2.2a*-morphant larvae and (D, F) *ptf1a*-morphants. (A–D) GFP fluorescence in larvae of the *nkx2.2(3.5):GFP* line at 4 dpf. (A–D) The position of the intrapancreatic duct, which is lacking in panels B–D is indicated by an arrowhead. (C) Confocal image of a *nkx2.2a*-morphant larva where the extrapancreatic duct is marked by an arrow. A rudimentary duct is pointing in an anterior direction. (E, F) 48 hpf, putative precursors of the extrapancreatic duct express *nkx2.2a* (arrow) whereas there is a reduced number of *nkx2.2a*-expressing cells in the medial portion of the *nkx2.2a* expression domain (this area is marked by a dashed circle).

the extrapancreatic duct was present (Fig. 7C) suggesting a differential requirement for *nkx2.2a* during the formation of intra- and extrapancreatic duct. As mentioned above, *nkx2.2a* is co-expressed with *ptf1a* in cells of the anterior pancreatic bud but follows *ptf1a* expression in these cells, suggesting that *ptf1a* might be upstream of *nkx2.2a*. To verify this possibility we injected *ptf1a*-morpholinos into wild-type and *nkx2.2a(3.5): GFP* zygotes. At 4 dpf 66% of these *ptf1a*-morphants failed to develop an intrapancreatic duct (Fig. 7D). Moreover, almost 68% (19, *n* = 28) of the *ptf1a*-morphants show an aberrant pancreatic *nkx2.2a* expression pattern at 48 hpf (Figs. 7E, F) whereas *ptf1a* expression is reduced in only 3.6% (1, *n* = 28) of all *nkx2.2a*-morphants at the same stage. In *ptf1a*-morphant embryos the *nkx2.2a* transcript is present in the putative extrapancreatic duct but the number of *nkx2.2a*-positive cells closer to the midline is reduced compared to control embryos. Since it was shown that the main islet develops normally in *ptf1a*-morphants (Lin et al., 2004; Zecchin et al., 2004) we conclude that the loss of *ptf1a* function affects either formation or proper migration of *nkx2.2a*-expressing precursors of the intrapancreatic duct.

Discussion

The detailed analysis of *nkx2.2a* expression, regulation and function during pancreas development in zebrafish revealed an evolutionary conserved role during endocrine differentiation and a formerly unknown involvement of *nkx2.2a* in the specification of the pancreatic ducts in zebrafish.

Immunohistochemistry in mice (Sussel et al., 1998) and expression data from zebrafish (Biemar et al., 2001) suggest that *nkx2.2a* expression is restricted to cells of the endocrine lineage. More recently, a PCR-based analysis in mice detected *Nkx2.2* in all analysed cells including those expressing exocrine markers (Chiang and Melton, 2003). Our results partially confirm the latter findings since zebrafish *nkx2.2a* is expressed in precursors and differentiated cells of the pancreatic ducts. However, the PCR data of Chiang and Melton (2003) indicate that *Nkx2.2* is also co-expressed along with acinar cell markers like *carboxypeptidase A*. These cells are most likely exocrine cells that have not completed their morphological differentiation yet, since neither in mice nor in zebrafish Nkx2.2 is reported to be expressed in acinar cells. As digestive enzymes of the zebrafish pancreas are expressed before the completion of exocrine tissue morphogenesis (Yee et al., 2005) it could be possible that some *nkx2.2a*-positive cells in the anterior pancreatic bud also express acinar markers before the differentiation of the pancreatic ducts. This issue has not been addressed, yet.

Data on gene function during the development of the zebrafish pancreas are scarce when compared to mammals and, as mentioned before, the analysis of a few loss-of-function phenotypes has uncovered both conserved and divergent aspects of gene function. Our results suggest that the function of Nkx2.2 during the development of the endocrine pancreas is largely conserved between teleosts and mammals. As in the mouse (Prado et al., 2004; Sussel et al., 1998), blocking of *nkx2.2a*-function in zebrafish affects alpha- and beta-cells but

not delta-cells and leads to an increase in *ghrelin*-positive cells. An important difference between the mouse knock-outs and the zebrafish morphants is the severity of the observed phenotype. Whereas the mouse mutants are completely devoid of beta-cells we only detected a reduction in beta-cell number. This may be explained by the fact that the morpholinos do not completely block *nkx2.2a* function, even though MOnk-5UTR can efficiently block translation of *nkx2.2a:GFP* hybrid mRNA. Interestingly, the increase of *ghrelin*-positive cells is more evident than the reduction of alpha- and beta-cell number. This might suggest that there is a stronger involvement of *nkx2.2a* in the specification of endocrine cells originating from the anterior pancreatic bud. This hypothesis is further strengthened by the fact that early expression of pancreatic hormones in zebrafish has some particularities when compared to mammals. One example is the requirement for *ptf1a* that in zebrafish is not expressed before 30 hpf, a time point when all pancreatic hormones are already detectable in the embryonic pancreas (Lin et al., 2004; Zecchin et al., 2004).

Like in the mouse, loss of *nkx2.2a* function in zebrafish has distinct effects on different pancreatic cell type. Whereas alpha- and beta-cells are reduced in number, delta-cells are not affected and *ghrelin*-positive cells increase in number. This may be explained either by the existence of a heterogeneous group of precursors already at the onset of *nkx2.2a* expression at 10 somites or by a differential requirement for *nkx2.2a* during the terminal differentiation of endocrine cell types. The latter explanation is more likely since expression data in zebrafish does not support the existence of differently specified precursors early during pancreas development as shown for *nkx2.2a* and *neuroD* expression at 16 somites. However, it is noteworthy that we find both *nkx2.2a*-positive and *GFP*-positive cells that do not seem to express *neuroD* at 16 somites. This can be most easily explained by *nkx2.2a* being activated slightly earlier than *neuroD* in these cells. In zebrafish *nkx2.2a* seems to precede also the expression of other genes known to be expressed in pancreatic precursors like *pax6b* and *islet-1* (Biemar et al., 2001). Alternatively, cells that do not appear to express *neuroD* but only *nkx2.2a* may express *neuroD* at a level below the sensitivity of our in situ protocol.

It has been proposed that the increase of *ghrelin*-positive cells in the mouse mutant reflects a misspecification of endocrine precursors (Prado et al., 2004) where beta-cells change their fate and start to express *ghrelin* instead. This would also explain the phenotype observed in *nkx2.2a* morphants but further analysis of *ghrelin* expression and function in zebrafish is needed to clarify this point.

In addition to gene function also gene regulation is conserved between zebrafish and mouse *Nkx2.2* orthologues. Like in the mouse, distinct promoters regulate transcription of *nkx2.2a* in pancreatic precursors and differentiated endocrine cells. However, while in the mouse *Nkx2.2* gene there are three different promoters (Watada et al., 2003), we could find only two in zebrafish. We were not able to identify a region in the zebrafish promoter showing sequence homology to mouse exon 1b, that in the mouse is expressed in *Ngn3*-positive endocrine precursors (Watada et al., 2003). Our data suggest that the

functional homologue to the regulatory region that drives the expression of mouse exon 1b is situated in the first 3.5 kb of the promoter, but we were not able to map this region more precisely. It is possible that, in our transient analysis, at 4 dpf the number of endocrine precursors is too low to be easily labelled by the reporter gene. Alternatively, endocrine precursors may not express *nkx2.2a* as long as they remain silent but only when they are about to differentiate which might be a rather rare event.

Our analysis revealed that element 2 probably corresponds to the neural enhancer in the *nkx2.2a* promoter, since it is the only element tested that resulted in a significantly stronger activation of the transgene in the nervous system when compared to the negative controls. However, element 2 seems to be a somewhat weaker enhancer when compared to the full promoter, which indicates that there are additional activators for the CNS that regulate *nkx2.2a*. A comparison of the corresponding 10 kb regions of zebrafish *nkx2.2a* and *nkx2.2b* revealed that the only conserved sequence block between these two paralogues corresponds to element 2 (not shown). This is in good agreement with the fact that *nkx2.2b* is specific for the CNS (Schafer et al., 2005).

The promoter sequence that regulates transcription in differentiated endocrine cells is well conserved between mouse and zebrafish. This region includes elements 7 and 8 and is both sufficient and necessary for the expression of the reporter in the pancreatic islet. In the mouse a small fragment containing only the E-Box and a closely situated HNF3 binding site is sufficient to obtain full promoter activity in a beta-cell line (Watada et al., 2003). Whereas the E-box motif and its surrounding base pairs are perfectly conserved between zebrafish and mouse, the HNF3-binding site is only partially conserved in element 7. However, this partial conservation still matches the consensus sequence determined for mammalian HNF3-binding sites (Overdier et al., 1994). Since injection of element 7 alone did not lead to a strong activation of the reporter gene in the zebrafish pancreas, it is likely that either the real HNF3-binding site or the one for another crucial co-factor is situated in element 8. Even though the numbers of larvae with GFP-positive cells in the pancreas are similar for the construct harbouring both elements and the *nkx2.2a*(8.5) positive control, we cannot rule out the possibility that other regulatory elements located between 3.5kb and 8.5kb control the pancreatic specific expression of *nkx2.2a*. Moreover, further analyses are needed to finally confirm the cell-type specificity of transient expression triggered by elements 7 and 8 in the pancreas.

The analysis of the *nkx2.2a(3.5):GFP* line revealed a group of cells that express the transgene in close vicinity to the pancreatic islet before the emergence of the anterior pancreatic bud. Since we obtained only one transgenic line with a sufficiently strong GFP-expression we can not rule out completely the possibility that transgenic expression is modified by regulatory elements close to the integration site. However, at 36 hpf there is a small distinct group of GFP-expressing cells in the pancreas, clearly different from beta-, alpha- or delta-cells as these endocrine cell types are much more abundant. Likewise we did not observe coexpression of *GFP* and *ghrelin* in the

pancreas of *nkx2.2a*(3.5) transgenic animals (not shown). It is more likely that these cells are identical to *mnr2a*-positive cells observed in the zebrafish islet at around 30 hpf (Wendik et al., 2004). The exact nature and function of these cells remain elusive but one possibility is that they give rise to later differentiating endocrine cells and represent precursors set aside after the first phase of pancreatic cell differentiation as proposed by Wendik et al. (2004). We conclude that *nkx2.2a* function and, more important, transcription in the endocrine pancreas is conserved between mammals and zebrafish. This indicates that the identification of both upstream activators and downstream targets of *nkx2.2a* in zebrafish will substantially contribute to our understanding of beta-cell differentiation, an issue of significant medical impact.

A description of the development of the zebrafish exocrine pancreas, including the pancreatic ducts, was recently given by Yee et al. (2005). This study unravelled the time-course of cellular differentiation and morphogenesis. However, nature and behaviour of precursor cells generating the pancreatic ducts are still unknown. Here we provide a molecular and structural description of these precursors. The pancreatic ducts originate from *nkx2.2a*-positive cells that are a sub-population of *ptf1a*-expressing cells in the anterior pancreatic bud. These *nkx2.2a*-positive cells are first detectable at 36 hpf when they start to move towards the pancreatic islet. Once arrived at the islet they form intercellular connections or filopodia and start to move in a caudal direction. This leads to a network of cells that surround the pancreatic islet at 3 dpf and a row of single interconnected cells in the centre of the pancreatic tail. After 3 dpf these cells differentiate into the pancreatic ducts, a process that is fully completed at 7 dpf.

Pancreas development has been described classically as a process of branching morphogenesis where a pre-existing epithelium buds off, proliferates and forms a branched structure of primitive pancreatic ducts (Pictet et al., 1972; Slack, 1995). These primitive pancreatic ducts are thought to contain precursors for endocrine and acinar cells. Recently it has been pointed out that this description might be inexact since cells situated in the mammalian pancreatic buds are not a polarized epithelium but merely a tightly packed mass of cells without a lumen and without any apico-basal polarity (Hogan and Kolodziej, 2002). Later, at a relatively advanced stage of morphogenesis, some of these cells become polarized resulting in the formation of multiple microlumens that eventually fuse to give rise to a tube (Hogan and Kolodziej, 2002; Jensen, 2004). This means that lumen formation is a secondary process in pancreas morphogenesis. Our analysis of early duct formation in zebrafish using transgenic animals supports this latter notion. We do not observe any signs of continuous expansion and folding of an epithelium generating the pancreatic ducts but rather single precursor cells that are initially not connected between each other and do not seem to be polarized. Moreover, our results suggest that there is no such thing like a primitive duct that generates acinar cells in zebrafish. Zebrafish acinar cell markers like *trypsin* are already expressed at 48 hpf, well before the pancreatic ducts can be identified morphologically (Yee et al., 2005). This is in
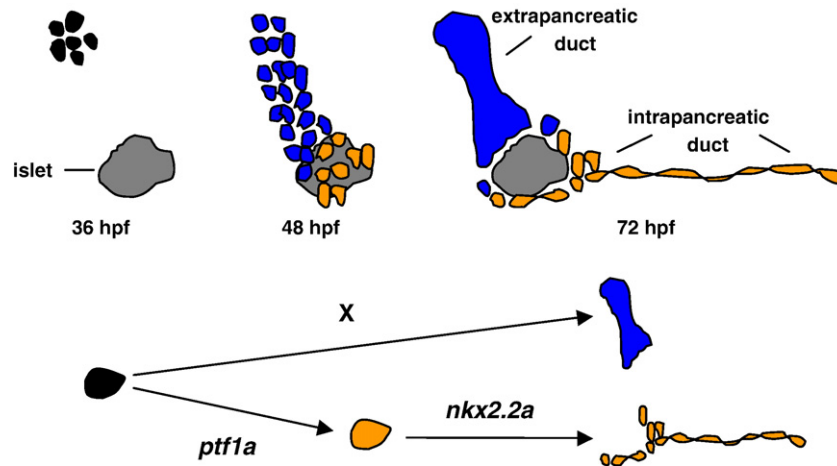
Fig. 8. A model for the differentiation of the pancreatic ducts in zebrafish. Schematic drawing of the distribution of different *nkx2.2a*-positive cell groups at 36 hpf, 48 hpf and 72 hpf. Common precursors for the pancreatic ducts are coloured in red. At 48 hpf the precursor group has split into one group that gives rise to the extrapancreatic duct (brown) and a second that gives rise to the intrapancreatic duct (orange). Only the latter one depends on *ptf1a* function. A model for the development of the pancreatic ducts is given at the bottom with x representing unknown factors involved in extrapancreatic duct development. See text for details.

agreement with our observation that at 48 hpf the precursor population of the pancreatic ducts can be identified while duct morphogenesis is still incomplete.

We found that *nkx2.2a* is not only expressed in the pancreatic ducts but also crucial for their differentiation. A significant fraction of *nkx2.2a*-morphant larvae did not form an intrapancreatic duct at 4 dpf. One obvious limitation of the morpholino technology is that gene function can not be blocked permanently. The degradation of the morpholinos over time may explain the large portion of injected larvae with a normal duct. Nevertheless, our results suggest an involvement of nkx2.2a in the development of the exocrine pancreas. Exocrine development was found to be normal in *Nkx2.2* knock-out mice (Sussel et al., 1998) but since only acinar markers were considered in this study the ductal phenotype remains to be examined. The timing of *nkx2.2a* expression with respect to *ptf1a* as well as the analysis of *ptf1a*-morphants suggests that the activation of *nkx2.2a* in precursors of the pancreatic ducts or their proper migration depends on *ptf1a*. Our results, however, indicate a difference in the molecular programs that lead to the differentiation of the extrapancreatic and the intrapancreatic duct. While the number of *nkx2.2a*-positive cells in a medial position around the islet is reduced in *ptf1a*-morphants, at 48 hpf *nkx2.2a* expression in the anterior bud is not abolished and the extrapancreatic duct is always present in both *ptf1a*- (not shown) and *nkx2.2a*-morphants. This, of course, does not exclude a more subtle but still abnormal phenotype present in the extrapancreatic ducts of morphants. However, additional observations, as for instance *pdx1* expression in the zebrafish pancreas, are in favour for the presence of two molecular programs. Whereas *pdx1* is expressed by cells of the extrapancreatic duct (Field et al., 2003) so far it has not been found in the intrapancreatic duct. Moreover, a recently published collection of zebrafish lines with a defective exocrine pancreas contains mutants in which the extrapancreatic duct is present whereas the intrapancreatic duct is severely affected (Yee et al., 2005).

Our results suggest a model for duct morphogenesis in which duct precursors are specified from *ptf1a*-positive cells situated in the anterior pancreatic bud (Fig. 8). Regulatory elements that drive *nkx2.2a* expression in precursors and, later, in differentiated ducts are situated in the first 3.5 kb of the *nkx2.2a* gene promoter. Duct morphogenesis starts when unpolarized cells from the anterior pancreatic bud start to migrate and form a cellular network surrounding the pancreatic islet as well as a row of single cells in the pancreatic tail. Subsequently, these cells initiate the morphogenetic program that finally leads to the formation of the pancreatic ducts. Different molecular cues seem to guide the formation of extra- and intrapancreatic ducts since only a subset of *nkx2.2a*-positive cells emerging from the anterior pancreatic bud depend on *ptf1a* function (orange cells in Fig. 8). Furthermore, even though *nkx2.2a* is expressed by all duct cells it is only necessary for the differentiation of the intrapancreatic duct. This could be explained by the existence of unknown factors that work in parallel to *nkx2.2a* during the formation of the extrapancreatic duct whereas no such parallel pathway is present during the generation of the intrapancreatic duct.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.01.024.

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