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Zebrafish *mnx* genes in endocrine and exocrine pancreas formation

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Abstract

The pancreas consists of two components, which exert distinct homeostatic function, an endocrine part that secretes hormones including insulin and an exocrine part that produces digestive enzymes. In mouse, one of the factors essential for development of the pancreas is the Mnx-class homeobox transcription factor Hb9. Genetic studies showed that *Hb9* is required for both initial morphogenesis of the pancreas as well as subsequent differentiation of insulin-producing β-cells [Nat. Genet. 23 (1999) 71; Nat. Genet. 23 (1999) 67]. To get a better understanding of what role *mnx* genes play in pancreas development, we isolated and characterized *mnx* genes in the model organism zebrafish. We found one gene with homology to *hb9* orthologs and two that display homology to the related chicken *mnr2*. Embryonic expression of the zebrafish *mnx* genes is very dynamic and is detected in derivatives of all three germ layers. Endodermal expression of *hb9* takes place in the early gut endoderm and, later, in the endocrine pancreas and the swim bladder. In addition, one of the *mnr2* genes, *mnr2a*, shows expression in an endodermal cell population that is initially intermingled with insulin-positive cells and that later becomes restricted to the exocrine pancreas. In knockdown studies using antisense morpholinos, we show that *hb9* is essential for differentiation of the insulin-producing β-cells but unlike mouse *Hb9* is not needed for early morphogenesis of the pancreas. In contrast, *mnr2a* is required during late morphogenesis of the exocrine pancreas. In summary, our data suggest a tissue-specific *mnx*-expression code in the zebrafish pancreas and they reveal a novel role of an *mnr2*-related gene.

Keywords: *hb9*; *mnr2*; *mnx*; Endocrine; Exocrine pancreas; Zebrafish; Development; Insulin; Trypsin

Introduction

The pancreas is a gland that controls many homeostatic processes in vertebrates. Its importance is emphasized by clinical conditions of pancreatic dysfunction such as diabetes or pancreatic cancer, both of which afflict a large portion of the human population. In mammals and birds, classical transplantation studies and transgenic fate mapping showed that distinct cell types with either endocrine or exocrine function develop from common precursors in primitive gut endoderm (Kawaguchi et al., 2002; reviewed by Edlund, 2002). In the adult pancreas, exocrine acinar cells secrete digestive enzymes into the gastrointestinal tract, whereas four types of endocrine cells including the insulin-producing β-cells secrete different hormones. In amniotes, endocrine cells are found in several aggregates of the four cell types, the islets, surrounded by exocrine tissue. Moreover, modern bony fish, such as zebrafish, show a similar arrangement with one or more endocrine islets surrounded by exocrine tissue suggesting that the mechanisms controlling pancreas formation are evolutionarily conserved (Biemar et al., 2001; Field et al., 2003a; Ober et al., 2003; Slack, 1995).

Studies over the last years have demonstrated that a transcriptional hierarchy plays an important role in pancreas development (Edlund, 2002). One transcription factor that is required during different stages of pancreas development is the homeodomain protein HB9 (HLXB9) (Harrison et al., 1999; Li et al., 1999). In humans, the dominantly inherited Currarino syndrome could be linked to heterozygous loss of *HB9* function (Ross et al., 1998). Patients suffering Currarino syndrome show sacral agenesis and related to endodermal functions, frequently also show colon defects and a high risk in developing diabetes. Only one *hb9* gene is known in mammals but two related genes, *hb9* and *mnr2*, were identified in chicken (Tanabe et al., 1998). Based on conservations and differences that Mnr2 and Hb9 orthologs

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share in conserved domains, it has been suggested that Hb9 and Mnr2 belong to different subfamilies of the so-called Mnx Proteins (Grapin-Botton et al., 2001; Tanabe et al., 1998; William et al., 2003). So far, only one member of the *mnr2* subfamily has been described.

Genetic studies in the mouse showed that *Hb9* is first required during initiation of pancreas morphogenesis and then for the differentiation of insulin-producing β-cells (Harrison et al., 1999; Li et al., 1999). These functions correlate with the expression of *Hb9* in the primitive gut endoderm before and during early pancreas morphogenesis, and with its subsequent expression in the endocrine lineage. Expression of *mnx* genes in developing endocrine tissue was also found in humans and chick suggesting evolutionary conserved *Hb9* functions in β-cell differentiation (Grapin-Botton et al., 2001; Hagan et al., 2000). However, functional studies that would support a role in other mammals or non-mammal systems are still lacking. *hb9*-related genes have been identified not only in vertebrates (e.g., mouse, *Xenopus* and chicken), but also in invertebrates (e.g., sea urchin, amphioxus and *Drosophila*) (Bellomonte et al., 1998; Broihier and Skeath, 2002; Ferrier et al., 2001; Odden et al., 2002; Saha et al., 1997). Interestingly, although invertebrates lack a pancreas, they still have embryonic expression of *hb9* in primitive trunk endoderm (Bellomonte et al., 1998; Broihier and Skeath, 2002; Ferrier et al., 2001; Odden et al., 2002). The relevance of this highly conserved expression remains to be yet investigated.

In addition to their endodermal functions, *mnx* genes are also essential in both vertebrates and invertebrates for motoneuron consolidation. Studies in mouse, chicken and *Drosophila* have demonstrated that *mnx* genes are required for neuronal migration, axonal outgrowth and specification of neuronal subtype identities (Arber et al., 1999; Broihier and Skeath, 2002; Odden et al., 2002; Tanabe et al., 1998; Thaler et al., 1999; William et al., 2003). The studies on neuronal functions of *mnx* genes provided first insights into the molecular function of Mnx proteins as transcriptional repressors (Broihier and Skeath, 2002; William et al., 2003).

Here, we report the identification and characterization of three *mnx* genes in zebrafish, an *hb9* ortholog and two novel *mnr2*-related genes. We show that these genes are not only expressed in evolutionarily conserved domains but also in tissues that had not been previously described for *mnx* orthologs in vertebrates. Importantly, we find non-overlapping expression of zebrafish *mnr2a* and *hb9* within the forming exocrine and endocrine pancreas, respectively. Due to the early onset of endodermal *mnr2a* expression in the exocrine pancreas, our analysis might provide new insights into timing of exocrine cell specification and morphogenic events during pancreas formation in zebrafish. Finally, we present gene knockdown studies in which the potential roles of *mnx* genes in the pancreas development are investigated, showing that zebrafish *hb9* has an essential function in endocrine cell differentiation and presenting evidence for a novel role of *mnr2a* in exocrine pancreas formation.

Material and methods

Isolation of cDNA of hb9, mnr2a and mnr2b

A cDNA fragment of zebrafish *hb9* was amplified by RT-PCR using RNA from 24 hpf embryos (degenerate primer: HB9-degATGGARAARTCNMARAAYTT, HB-R CYTGXGTYTCXGTXARCAT). Hybridization of this probe to somite stage-specific cDNA-library led to isolation of a cDNA encoding the entire open reading frame of *hb9*. Screening a genomic PAC library with the same probe under low stringency conditions led to the identification of *mnr2a*. Genomic sequences of *mnr2b* were identified in the Sanger database (clones z35724–a4290h12.q1c and z35725–a2291f05). Based on the genomic sequences, we generated PCR primers (HbKls2: 5′-CGCGTAAAACG-CAGCTATCA 3′, m2b_rev2: 5′-CCCTAATGCTACCATA-GAGT-3′) and amplified the open reading frames of *mnr2a* and *mnr2b* from a somite stage-specific cDNA. PCR fragments were cloned in pGEM-TEasy (Promega). The cDNA sequence informations on the zebrafish *mnx* genes were submitted to GenBank (NCBI accession numbers: *hb9*: AY445044, *mnr2a*: AY445045, *mnr2b*: AY445046).

Mapping of mnx genes

Radiation hybrid mapping using the LN54 panel placed *mnr2a* on linkage group 9 (9.87 cR from Z4673) and *mnr2b* on linkage group 1 (0 cR from Z6177); *hb9* was placed on linkage group 7 (*unp2116*, 49.9–51 cM from the top) using the Goodfellow T52 radiation hybrid panel. Mapping data: *mnr2a* (000000012001 100010010000 001001000000 100000110000 000000100000 101100000000 000001000011 011010001); *mnr2b* (101110010100 000001220020 001001000000 001101001100 000000011010 001100000010 010011111000 001000010).

In situ analysis, RNA synthesis

Antisense RNA was synthesized from *Bam*Hl-digested hb9-pBKS plasmid using T7 RNA polymerase; *Hin*dlll-digested mnr2a-pGEM-TEasy and Sphl-digested mnr2b-pGEMT-Easy, using Sp6 RNA polymerase. Whole mount in situ analysis using DIG and Fluorescein-labeled probes were performed as described (Hauptmann and Gerster, 2000) using INT (red, Sigma I8377) or Fast Red (Roche) and NBT (blue) as substrates.

RNA and morpholino injection

For synthesis of sense RNA, we cloned cDNA of zebrafish *hb9*, *mnr2a* and *mnr2b* into pCS2+. Sense RNA was prepared from Acc651-linearized hb9-pCS2+ and mnr2a-pCS2+ using SP6 mMessage mMachine Kit (Ambion). Morpholino and sense RNA was diluted in 0,1 M KCl to concentrations of 1–8 ng/μl and 3–50 pg/μl, respectively.

One nanoliter of this dilution was injected through the chorion of 1-cell or 2-cell stage embryos. The following antisense morpholinos were used (underlined CAT corresponding to the first coding ATG):

MO^{hb9} 5′-ACCTCACAAACAGATTAACGCCTCG-3′ (seven bases 5′ of ATG),
Hb_Mo2 5′-TTTTTAGATTTCTCCATCTGGCCCA-3′,
MO^{mnr2a} 5′-TTCGACTTATCCATGAAGGCAAACT-3′,
M2a_atgMo2 5′-CCGAAAGTTCTTCGACTTATCCATG-3′,
MO^{mnr2b} 5′-GACTTTTCCATTGCAACACTTTTGT-3′,
M2b_atgMo2 5′-CCTGAAGTTCTTTGACTTTTCCATT-3′.

Results

Cloning of three mnx genes in zebrafish

To isolate zebrafish *mnx* orthologs, we performed RT-PCR reactions on embryonic zebrafish mRNA with degenerate primers specific to the highly conserved N-terminal and homeobox domains of Hb9/Mnr2. We isolated a single zebrafish cDNA fragment with homology to vertebrate *hb9* cDNAs. This fragment was used as a hybridization probe to isolate a cDNA encoding the entire open reading frame (ORF) of an *hb9* ortholog from a somite stage-specific cDNA library. Screening a genomic PAC library with the same probe in lower stringency conditions lead to the identification of a second *hb9*-related gene. Based on the genomic PAC-sequences, we generated primers that were used to amplify the entire ORF of this gene from somite stage-specific cDNA. Genomic sequences of a third *mnx* class gene, were then identified by Blast search in the Sanger database and its entire ORF was PCR-amplified from somite stage-specific cDNA (see Material and methods).

Comparisons of the deduced protein sequences with the NCBI database entries revealed that all three zebrafish proteins have the highest homology to vertebrate Mnx proteins (Figs. 1A, B). Like all vertebrate Mnx proteins, they share highly conserved N-termini, homeodomains and long stretches of acidic amino acids at the C-terminal portion (Fig. 1C and data not shown). One of these Mnx proteins, named zebrafish Hb9, is most related to vertebrate Hb9 orthologs (49% identity to human HB9, Fig. 1A). The other two, zebrafish Mnr2a and Mnr2b, show higher homology to chicken Mnr2 (50% and 54% identity compared to 32% and 33% identity to human HB9, (Fig. 1A). What specifically distinguishes the zebrafish and chicken Mnr2 proteins from Hb9 proteins are single amino acid (aa) differences within the highly conserved homeodomain and in the region flanking the homeodomain (Fig. 1C). Based on these homologies, we conclude that the isolated zebrafish genes belong to the different subfamilies of Mnx transcription factors. Radiation hybrid mapping placed the identified

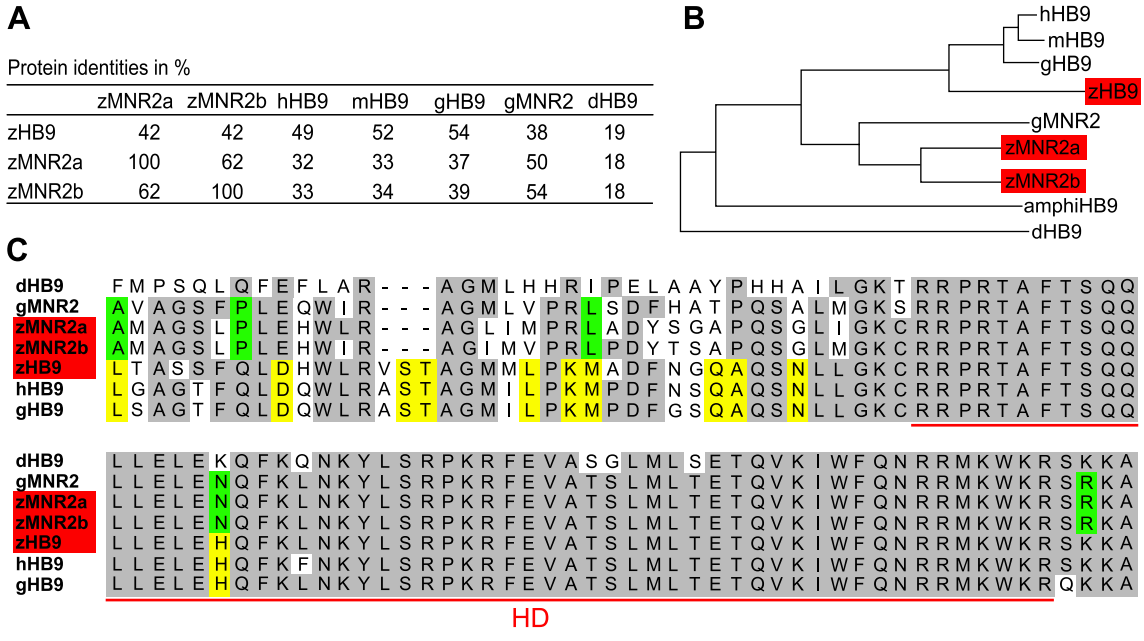

Fig. 1. Characterization of three *mnx* genes in zebrafish. Whole protein comparison (A), phylogenetic alignment (B) and sequence alignment of the homeodomain containing region (C) of MNX proteins from zebrafish (highlighted in red: zMNR2a, zMNR2b, zHB9), human (hHB9), mouse (mHB9), chicken (gHB9, gMNR2), drosophila (dHB9) and amphioxus (amphiHB9). The compared protein sequences correspond to amino acid (aa) 204–303 (B) and 243–299 (C) of hHB9. In C, the homeodomain (HD) is underlined in red; green and yellow mark those aa conserved only between MNR2 proteins or vertebrate HB9 proteins, respectively. (NCBI accession numbers: AAD41467: hHB9, AAD49613: mHB9, AAC64925: gHB, gMNR2: AAC64924, dHB9: NP_648164, amphiHB9: AAG33015).

cDNA on the linkage groups 1 (*mnr2b*), 7 (*hb9*) and 9 (*mnr2a*) confirming that three distinct genes had been isolated (see Material and methods).

Complex and dynamic early embryonic expression of zebrafish hb9/mnr2 genes

As a first step to gain insights into the function of zebrafish *mnx* genes, we determined their expression between 1 cell stage and 3 days post fertilization (dpf) in whole mount in situ studies. During the first 24-h post fertilization (hpf), *hb9* and *mnr2b* show dynamic expression patterns in derivatives of all three germ layers of the embryo, while *mnr2a* expression is restricted to the neuroectoderm (Fig. 2).

Expression of zebrafish *hb9* and *mnr2b* could be first detected at 90% epiboly in axial mesoderm and slightly later in endodermal cells with a restriction to the vegetal half of the embryo (Figs. 2F, G, K and L). While *hb9* and *mnr2b* expression appears to overlap in the endoderm, these genes are expressed in different parts of the axial midline, with *hb9* expression in the notochord (Figs. 2K, L) and *mnr2b* in a population of tailbud cells (Figs. 2F–J). After gastrulation, *hb9* and *mnr2b* transcripts are also detected in laterally positioned mesoderm (Figs. 2G, H, L and M). During somitogenesis, all three zebrafish genes start to be expressed in two rows of cells in the ventral portion of the spinal cord (Figs. 2B, C, D, I and N; early expression is only shown for *mnr2a*). In late segmentation, expression of *hb9* and *mnr2b* in lateral mesoderm and gut decreases from anterior to posterior and, with the exception of the anterior *hb9* expression domain, it was not detected in these tissues after 20 somite stage (Figs. 2D, I and N). Differently, older embryos show an increasing number of *hb9*- and *mnr2b*-, as well as *mnr2a*-expressing cells in the ventral spinal cord. From the 15-somite stage onwards, *hb9* shows additional expression in a single row of cells ventral to the notochord (the hypochord) and in a broader region of the tail mesoderm underlying the tail notochord (Figs. 2N, O). During later embryonic development, axial *hb9* and *mnr2b* expression shifts posterior toward cells of the newly formed tail mesoderm (Fig. 3F) and after 36 hpf no *mnx* gene expression was detectable in mesoderm structures.

Expression of hb9 in the endocrine pancreas and in the swim bladder

Although, the endodermal *hb9* expression overall decreases during segmentation, an anterior cell population shows increased levels of *hb9* expression after the 10 somite stages (Fig. 3A). This anterior endodermal expression initially spreads over a region covering several somites but forms a single cluster positioned at somite levels 3–4

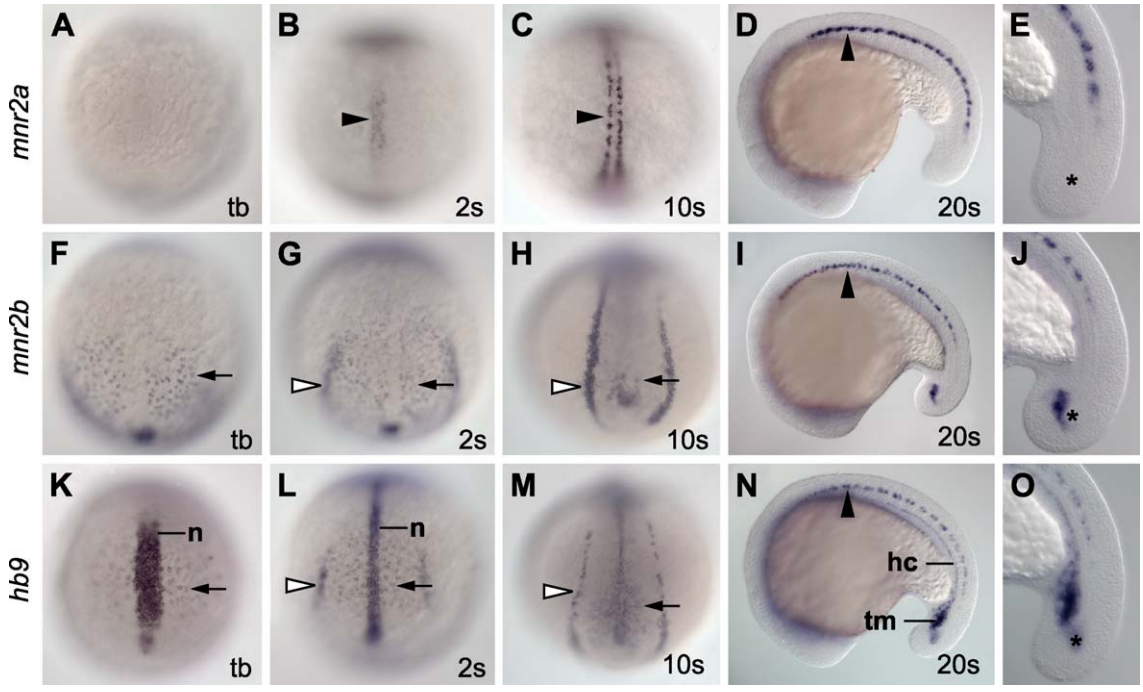

Fig. 2. Expression of zebrafish *mnx* genes in early embryogenesis. Expression of *mnr2a* (A–E), *mnr2b* (F–J) and *hb9* (K–O) in whole embryos at tailbud stage (A, F, K), two somite stage (B, G, L), 10 somite stage (C, H, M) and at 20 somite stage (D, I, N). (E, J, O) Higher magnification of the tail region of 20-somite stage embryos. Embryos are shown from dorsoposterior with anterior up (A–C, F–H, K–M) or from lateral with dorsal up and anterior to the left (D, E, I, J, N, O). Indicated are expression domains in the ventral spinal cord (black arrowhead), lateral positioned mesoderm (white arrowhead), endoderm cells (arrow), notochord (n), hypochord (hc), ventral tail mesoderm (tm) and tailbud (asterisk). Note the complementary axial expression of *hb9* and *mnr2b* in notochord and tail bud.

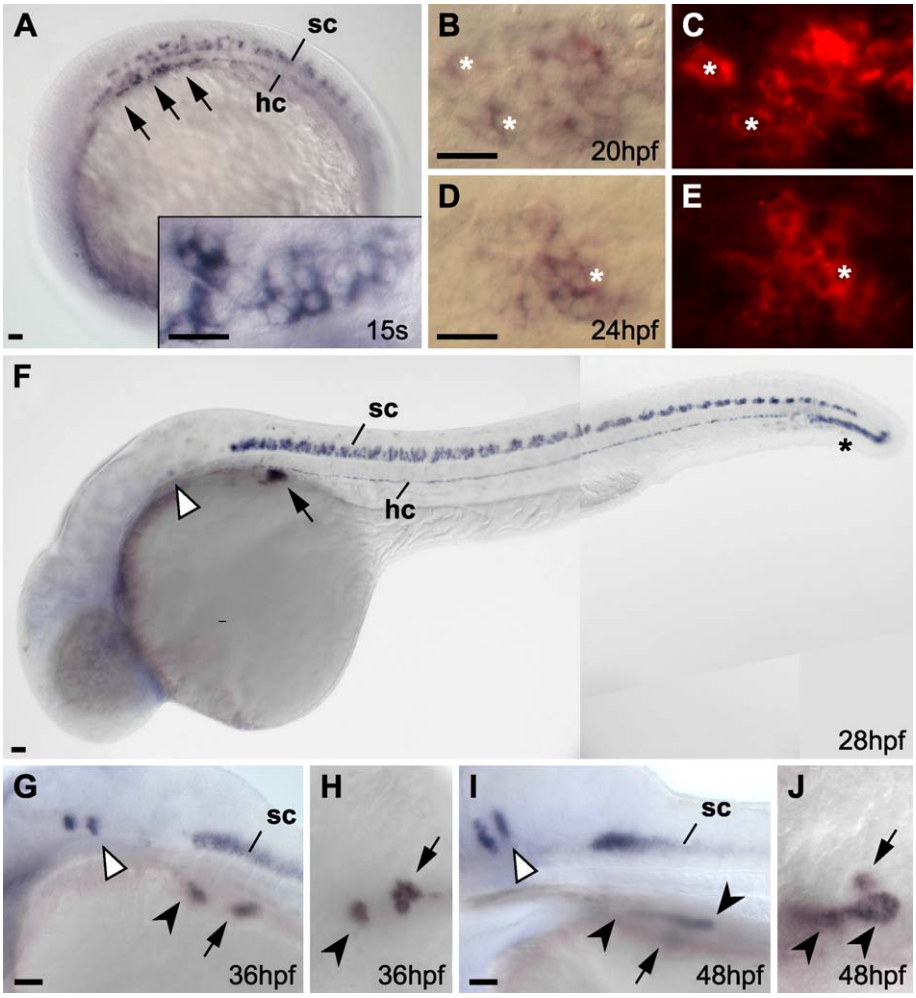

Fig. 3. Tissue-specific expression of *hb9* in endocrine pancreas. Expression of *hb9* in purple at 15 somite (A), 20 hpf (B, C), 24 hpf (D, E), 28 hpf (F), 36 hpf (G, H) and 48 hpf (I, J). Between 15 somite stage (inlay in A shows a dorsal view of the same embryo in a higher magnification) and 24 hpf endodermal *hb9* expression changes from an elongated mono-layered domain to a condensed structure underlying the hypochord (hc). (B–E) Bright field (B, D) and fluorescence (C, E) images of double in situ stains for *hb9* (NBT/BCIP) and insulin (Fast Red) show expression of insulin in most *hb9*-expressing cells; asterisks mark examples for double-labeled cells. Further indicated are *hb9* expression domains in the spinal cord (sc), rhombomeres 5 and 6 (white arrowheads) and the swim bladder (black arrowheads). Embryos are shown from a lateral (A, F, G, I) or dorsal view (B, C, D, E, H, J) with anterior to the left. Scale bars correspond to 20 μm.

after 24 hpf (Figs. 3A–E). This dynamic *hb9* expression resembles that of genes, which mark the early-forming endocrine pancreas (Biemar et al., 2001; Milewski et al., 1998). To further explore if *hb9* expression is specific to endocrine tissue, we performed double in situ analysis with *hb9* (in blue) and the β-cell-marker insulin (in red) (Milewski et al., 1998). We found that *hb9* expression overlaps with that of insulin, demonstrating that zebrafish *hb9* is expressed in the endocrine lineage (Figs. 3B–E). During later development, the pancreatic *hb9* expression remains restricted to the endocrine tissue, which becomes positioned on the right side of the embryo after 36 hpf (Figs. 3F, H, J). Around 36 hpf additional endodermal *hb9* expression is established anteriorly to the endocrine pancreas (Figs. 3G, H). Until 48 hpf, this *hb9*-domain elongated posteriorly forming a sac-like morphology (Figs. 3I, J). In sections, this tissue was identified as the swim bladder (Field et al., 2003b; Ober et al., 2003) (data not shown). In summary, these data demonstrate that *hb9* is expressed in the developing endocrine pancreas and suggest that *hb9* expression may be the first specific marker for the swim bladder.

mnr2a is expressed in the exocrine pancreas

Expression of *mnr2a* from 24 hpf on, is not only found in neural tissue but also in a few endodermal cells underlying the notochord at the level of the most anterior somites (Fig. 4A). During further development, endodermal *mnr2a*-expressing cells increase in number, and after 72 hpf, form an elongated structure on the right side of the embryo (Figs. 4B, C). The late *mnr2a* expression resembles that of the exocrine marker *trypsin* (Fig. 4K, in red) (Biemar et al., 2001), which suggests that *mnr2a* might be specifically expressed in the exocrine lineage. To explore this possibility and to determine

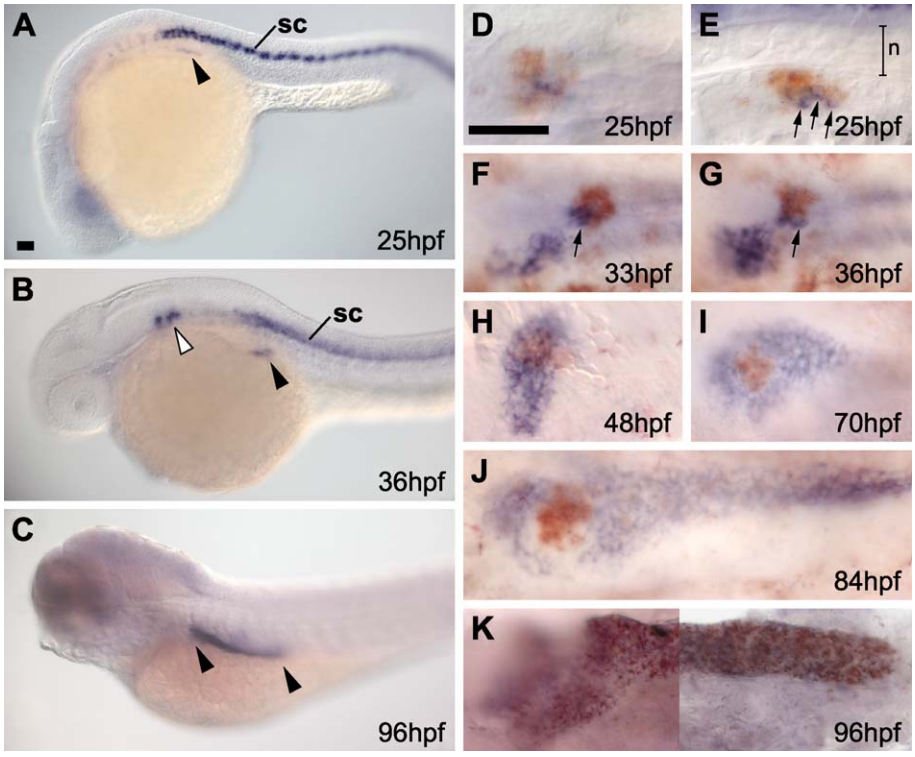

Fig. 4. Expression of *mnr2a* in the exocrine pancreas. (A–C) Expression of *mnr2a* at 25 hpf (A), 36 hpf (B) and 96 hpf (C). Indicated are *mnr2a* expression domains in the endoderm (black arrowhead), in rhombomeres 5, 6 (white arrowhead) and in the spinal cord (sc). (D–J) Double labeling of *mnr2a* (purple) and insulin (red) expression at 25 hpf (D, E), 33 hpf (F), 36 hpf (G), 48 hpf (H), 70 hpf (I) and 84 hpf (J). Arrows in E, F and G mark *mnr2a*-positive close to insulin-expressing cells, note that *mnr2a* and insulin are expressed in different cells; the bar marks the position of the notochord (n). (K) Overlapping expression of *mnr2a* (blue) and *trypsin* (red) at 96 hpf. Embryos are shown from lateral (A–C, E) or dorsal (D, F–K) with anterior to the left. Scale bars correspond to 50 μm.

how *mnr2a* cells behave relatively to the endocrine pancreas, we performed a series of double in situ stains with *mnr2a* (in blue) and *insulin* (in red) probes at different time points between 25 hpf and 4 dpf (Figs. 4D–J). At 25 hpf, few *mnr2a* cells are found in the field of *insulin*-expressing cells (Figs. 4D, E). During the next hours, a small number of *mnr2a*-positive cells remain associated with insulin-expressing cells, while at 33 hpf, a second domain of *mnr2a* expression is established on the left side of the embryo slightly anterior and ventral to the insulin-positive cells (Figs. 4G, H). Until 48 hpf, the left *mnr2a* expression extends to the right side of the embryo where it appears to merge with the medial *mnr2a* expression (Fig. 4H) and after 70 hpf *mnr2a* is found on the right side of the embryo in a domain surrounding the β-cells (Figs. 4I, J). Double in situ stains for *mnr2a* (in blue) and *trypsin* (in red) at 4 dpf show overlapping expression of these genes confirming that late *mnr2a* expression is specific to the exocrine pancreas (Fig. 4K).

Distinct roles of mnr2a and hb9 in exocrine and endocrine pancreas formation

To determine the developmental roles of zebrafish *mnx* genes, we injected antisense morpholinos to knockdown their functions and analyzed the resulting phenotypes. For our analysis, we used two morpholinos for each *mnx* gene targeting different sequences around the start regions of the mRNAs. As each morpholino pair for a specific *mnx* mRNA induced very similar phenotypes, all the detailed studies reported here were performed with one morpholino for each mnx gene (MO^{mnr2a}, MO^{mnr2b}, MO^{hb9}).

Our expression studies demonstrated that zebrafish *mnx* genes are transcribed in complex patterns in different tissues suggesting that they may have different functions. In this study, we specifically focused on the roles of the *mnx* genes during pancreas formation. Since we find that injection of MO^{mnr2a} and MO^{hb9}, but not injection of MO^{mnr2b} resulted in loss of pancreas tissue, as assayed by insulin and *trypsin* expression (Fig. 6J), we mainly concentrated our analysis on the roles of *hb9* and *mnr2a* during pancreas formation.

The specificity of morpholinos is commonly tested by co-injection of morpholino and corresponding mRNA to determine if the RNA can rescue morpholino-induced phenotypes. However, for *mnx* genes such rescue experiments were not feasible for several reasons. First, *mnx* RNA injection results in strongly affected embryos that lack anterior head structures or fail to complete gastrulation movements, demonstrating that Mnx proteins have instructive functions (Table 1, data not shown). Second, the

Table 1
Efficient block of *mnx* activities by injection of gene-specific morpholinos

Injection	No. of embryos	Wild type (%)	Small head (%)	No. eyes (%)	Epib. def. (%)	Dead (%)
3 pg *hb9*	40	10	45	40	3	3
10 pg *hb9*	37	–	22	22	30	27
3 pg *hb9* + 2 ng MO^{hb9}	40	88	10	3	–	–
10 pg *hb9* + 2 ng MO^{hb9}	47	96	–	–	–	4
3 pg *hb9* + 2 ng MO^{mnr2a}	52	6	29	63	2	–
15 pg *mnr2a*	152	41	24	26	3	6
50 pg *mnr2a*	120	6	17	15	24	38
50 pg *mnr2a* + 1 ng MO^{mnr2a}	19	68	32	–	–	–
50 pg *mnr2a* + 4 ng MO^{mnr2a}	15	93	–	–	–	7
50 pg *mnr2a* + 1 ng MO^{hb9}	27	7	41	22	26	4

One cell stage embryos were injected with indicated amounts of *hb9* or *mnr2a* RNA or with different combinations of *hb9* or *mnr2a* RNA and indicated amounts of MO^{hb9} or MO^{mnr2a} and the resulting phenotypes were analyzed at 30 hpf. Phenotypes of the injected embryos were classified according to reduction of eye and head size (small head), loss of eyes and anterior head structure (no eyes, see B), epiboly defects (epib. def.) or embryonic death (dead).

morpholino-injected embryos do not show any phenotypes that could be considered for rescue experiments before 15 hpf, that is, during the period when injected RNA is known to be stable. However, we used this approach as a test for the efficiency with which morpholinos block injected *mnx* mRNA. While injection of 3–50 pg *hb9* or *mnr2a* alone affected developing embryos with almost 100% penetrance, only very few embryos showed abnormal morphology when the mRNAs were co-injected with the corresponding morpholinos. In control experiments, embryos co-injected with *hb9* mRNA and MO^{mnr2a} or *mnr2a* mRNA and MO^{hb9} were indistinguishable from embryos injected with each mRNA alone. Similar specificity was observed when *mnr2b* and/or MO^{mnr2b} were co-injected with *hb9*- or *mnr2a*-specific reagents (Table 1). These results show that the morpholinos used in our experiments efficiently block translation of the corresponding *mnx* mRNA.

For the knockdown studies, the embryos were injected with 1–8 ng morpholino, raised for 20 h to 4 days and then analyzed for the expression of different pancreatic markers. Since *hb9* and *mnr2b* expressions appear to overlap in the portion of the gut endoderm that supposedly originates the pancreas, we injected MO^{mnr2b} and MO^{hb9} to test for potential redundant functions of these genes during early steps of pancreas development. However, we find that expression levels of several pancreas marker such as *pdx1* (Figs. 5A–D), marking the anlagen of pancreas and gut at 20 hpf, *islet1* (Figs. 5E–H) and *hb9* (Figs. 5I–L), presumably both marking endocrine cells at 36 hpf, in *mnr2b*/*hb9*-double morphants is very similar to that in un-injected embryos or in embryos injected with MO^{hb9} alone (Biemar et al., 2001; Korzh et

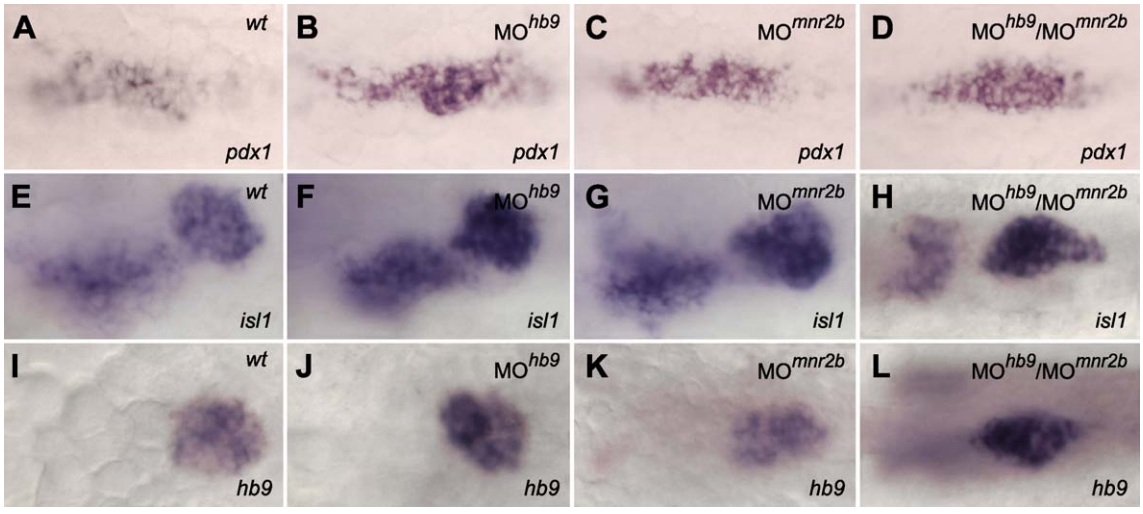

Fig. 5. *mnr2b* and *hb9* are not required for the initiation of pancreas formation. Similar expression of *pdx1* at 20 hpf (A–D), and *isl1* (E–H) and *hb9* (I–L) at 36 hpf in wild type embryos (A, E, I) and in embryos injected with 4 ng MO^{hb9} (B, F, J) or 4 ng MO^{mnr2b} (C, G, K) or 4 ng MO^{hb9} and 4 ng MO^{mnr2b} (D, H, L) (unchanged expression levels were found for A: $n = 15/15$, B: $n = 16/16$, C: $n = 19/19$, D: $n = 15/16$, E: $n = 11/11$, F: $n = 9/11$, G: $n = 13/13$, H: $n = 10/13$, I: $n = 20/20$, J: $n = 12/15$, K: $n = 12/12$, L: $n = 21/23$). Slightly reduced expression levels were found in few *hb9* morphants (F: $n = 2/11$, J: $n = 3/15$) and *hb9*-*mnr2b* double morphants (D: $n = 1/16$, H: $n = 3/13$, L: $n = 2/23$). Notably, 2 of the uninjected embryos and 3 *hb9* morphants showed split *pdx1* expression, while expression levels were not changed. All embryos are shown from the ventral with anterior to the right. Expression of *isl1* (E–H) marks prospective endocrine pancreas cells (right expression domain) and a population of cells that remains to be determined (left expression domain). Also note that the expression levels of *pdx1*, *isl1* (right domain only) and *hb9* are similar in morphants and wild types, while the endocrine expression domains of *isl1* and *hb9* are more condensed and extended along the anterior–posterior axis in *hb9/mnr2b* double morphants (H, L) than in the wild types (E, I) and single morphants (F, G, J, K). Scale bars correspond to 50 μm.

al., 1993; Milewski et al., 1998). Notably, the pattern of marker-gene expression in the *mnr2b*/*hb9*-double morphants appears more condensed when compared to the un-injected embryos or the single morphants. This suggests that *mnr2b* might be involved in the regulation of pancreas morphogenesis but that it is not required for the differentiation of pancreatic cells.

Our analyses of *hb9* and *mnr2a* function revealed that injection of MO^{hb9} consistently resulted in a strong reduction or loss of the insulin expression at 3–4 dpf without having major effects on the expression of *trypsin* (Fig. 6). In contrast, in embryos injected with 2–8 ng MO^{mnr2a}, the *trypsin* expression domain was frequently strongly reduced in size or had abnormal morphology with the posterior extension lacking at 4 dpf (Figs. 6F, J and not shown). In these embryos, the number of insulin-expressing cells was normal or appeared even slightly increased (Figs. 6C, F, I and J). Although these data suggest that *mnr2a* is required for exocrine pancreas formation, we noted that reduced *trypsin* expression was most frequently seen in embryos with a smaller body and head size, thus in embryos that appear delayed in development (Figs. 6C, F, I and J). Since expression of *trypsin* or other known exocrine markers is not detected before 3 dpf (Biemar et al., 2001), the lack of marker expression in the analyzed embryos could be due to morpholino-induced delay in embryonic development. However, we observed the same reduction in *trypsin* expression in MO^{mnr2a}-injected embryos at 3.5–5 dpf suggesting that the *mnr2a*-morphant phenotype is not due to morpholino-induced delay in development (Fig. 6J). To assess the consequence of *mnr2a* knockdown at earlier stages of pancreas development, we looked at the expression of *mnr2a* itself in morpholino-induced embryos. While no differences in *mnr2a* expression could be seen in control and MO^{mnr2a}-injected embryos at 54 hpf, the embryos showed a reduction in *mnr2a* expression similar to that of *trypsin* at 3.5 dpf (Figs. 6G–J). In summary, these data suggest a role for *hb9* in early β-cell differentiation and a novel role for a *mnr2*-related gene in late morphogenesis of the exocrine pancreas in zebrafish.

Discussion

Here, we present the isolation and characterization of three novel *mnx* class homeobox genes from zebrafish, namely *hb9*, *mnr2a* and *mnr2b*. We describe the temporal and spatial expression of these genes and present evidence, that two of them, *hb9* and *mnr2a*, are required for endocrine and exocrine pancreas development, respectively.

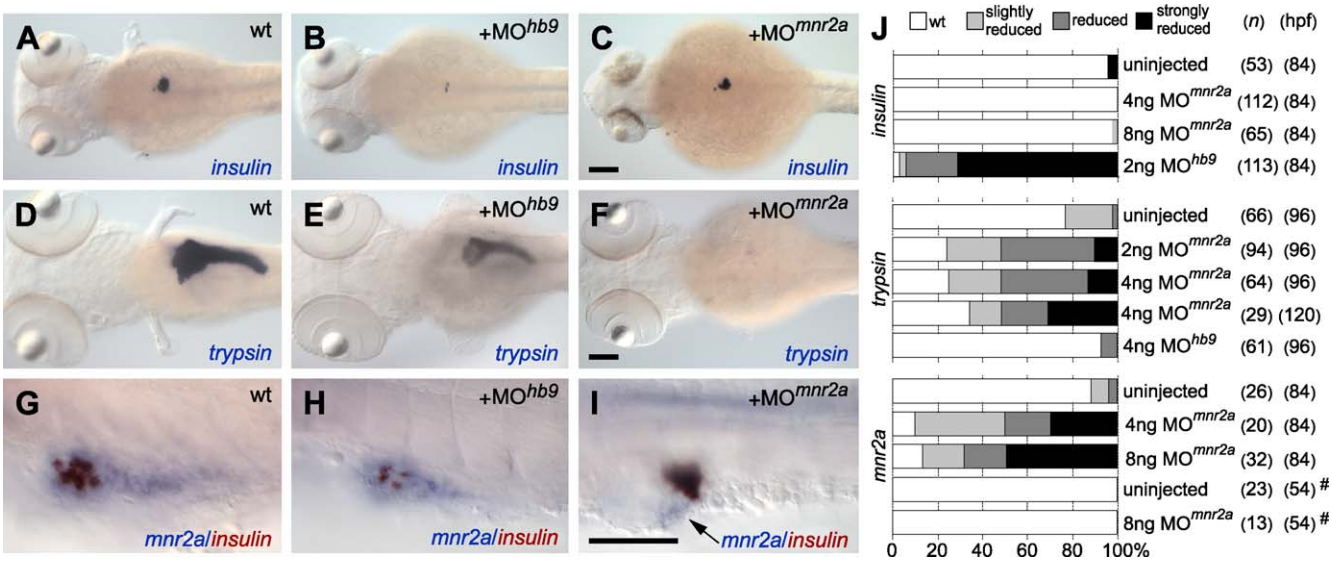

Fig. 6. *hb9* and *mnr2a* are differentially required in endocrine and exocrine pancreas formation. Expression of insulin at 3.5 dpf (A–C), *trypsin* at 4 dpf (D–F), and *mnr2a* (purple) and insulin (red) at 3.5 dpf in higher magnification (G–I) in wild type embryos (A, D, G) and in embryos injected with 2 ng MO^{hb9} (B, E, H) or 4 ng MO^{mnr2a} (C, F, I). (G–I) Expression of insulin is strongly reduced in embryos injected with MO^{hb9} (B, H) but not in embryos injection of MO^{mnr2a} (C, I). In contrast, expression of *trypsin* is strongly reduced after injection of MO^{mnr2a} (F) but is not changed after injection of MO^{hb9} (E). Similarly, *mnr2a* expression is only reduced in MO^{mnr2a}-injected embryos but not in MO^{hb9}-injected embryos (I); arrow marks *mnr2a* expression. Embryos are shown from dorsal (A–F) or in a higher magnification from lateral with anterior left (G–I). (J) Summary of morpholino experiments. Indicated numbers of embryos (*n*) were injected with the indicated type and amount of morpholino and analyzed for expression of insulin, *trypsin* or *mnr2a* at indicated stages (hpf). The phenotypes of the embryos were classified relative to the expression level of the marker genes in the un-injected embryos: average wild type level (wt), reduced up to 50% (slightly reduced), reduction up to 10% (reduced), reduced to less than 10% or missing (strongly reduced). While injection of MO^{hb9} or MO^{mnr2a} could result in total loss of insulin or *trypsin* expression, respectively, we did not find MO^{mnr2a} embryos entirely lacking endoderm *mnr2a* expression. Note that the un-injected embryos frequently show different levels of *mnr2a* and *trypsin* expression representing natural variations found in zebrafish. (#) All analyzed embryos showed similar level of expression and since only few *mnr2a*-positive cells can be detected at 54 hpf, the presence of *mnr2a*-expressing cells was classified as 100%. Scale bars correspond to 100 μm.

Three mnx genes in zebrafish

The identification of two *mnx* genes in chick, but only one in mammals, led to the speculation that *hb9* and *mnr2* genes must have been present in a common ancestor of vertebrates but that the *mnr2* ortholog was lost in mammals (Tanabe et al., 1998; William et al., 2003). Now, we provide evidence for this hypothesis by showing that genes of both *mnx* subfamilies exist in zebrafish. The presence of two *mnr2*-related genes in zebrafish could be a result of the partial genome duplication that occurred in teleost but not in other vertebrates (Postlethwait et al., 2000; Woods et al., 2000). Consistent with this idea is the finding that the *mnr2* genes map to linkage groups (*mnr2a*: LG9, *mnr2b*: LG1) that contain duplicated chromosome segments (Woods et al., 2000). Concerning the evolution of *mnx* genes, it is interesting to note that expression of *mnr2b* is more similar to that of *hb9* than to that of *mnr2a*. The related expression of *hb9* and *mnr2b* in early endoderm, axial mesoderm, lateral mesoderm and in ventral spinal cord supports the idea of a common origin of *mnr2* and *hb9* genes while the differences in *mnr2a* and *mnr2b* expression suggest, consistently with the 'subfunctionalisation hypothesis' (Force et al., 1999), a fast evolution in the regulation of these genes.

Conserved expression of hb9 in the forming pancreas

Our expression analysis revealed a complex and dynamic regulation of zebrafish *mnx* genes in endoderm and in the forming pancreas (Fig. 7). Shortly after gastrulation until late somite stages *mnr2b* and *hb9* show a broad expression in endodermal cells. Different from the expression of pan-endoderm marker *sox17* in cells underlying head and trunk structures, the *hb9* and *mnr2b* expression at this stage is restricted to the endoderm of the forming trunk, thus providing new markers to study early patterning of the endoderm in zebrafish. Most *mnx* genes show expression in the posterior endoderm that will form the gut, suggesting that *mnr2b* and *hb9* transiently mark early gut progenitors that in mice and chicken will also form the pancreas (Bellomonte et al., 1998; Broihier and Skeath, 2002; Grapin-Botton et al., 2001; Harrison et al., 1999; Li et al., 1999; Odden et al., 2002). After 10-somite stage, *hb9* expression is maintained in endodermal cells underlying the first somites. Since *hb9* expression later overlaps with that of the β-cell marker insulin, this suggests that *hb9* expression marks progenitors of the β-cells similar to mouse *Hb9*. Consistent with the early onset of β-cell differentiation in zebrafish, we find continuous expression of zebrafish *hb9* in the endocrine pancreas from 10-somite stage onwards. In its onset and early temporal and spatial pattern, zebrafish *hb9* expression is very similar to that reported for the early pancreas marker *pdx1* and *nkx2.2* which could also indicate overlapping activities of *hb9* and these factors during initial steps of pancreas formation (Biemar et al., 2001; Milewski et al., 1998). In summary, this suggests a conserved expression of *mnx* genes in the progenitors of the pancreas and later in differentiating β-cells. Consistent with the idea of a conserved transcriptional regulation of *hb9* in vertebrates, *hb9* genes from zebrafish and mammals show highly conserved sequence motives in their promoter regions (D.M., unpublished results; B. Peers, personal communication).

Zebrafish hb9 is required for β-cell differentiation

To determine if the conserved regulation of *mnx* genes correlates with a conserved function in pancreas development, we analyzed pancreas development in morpholino-

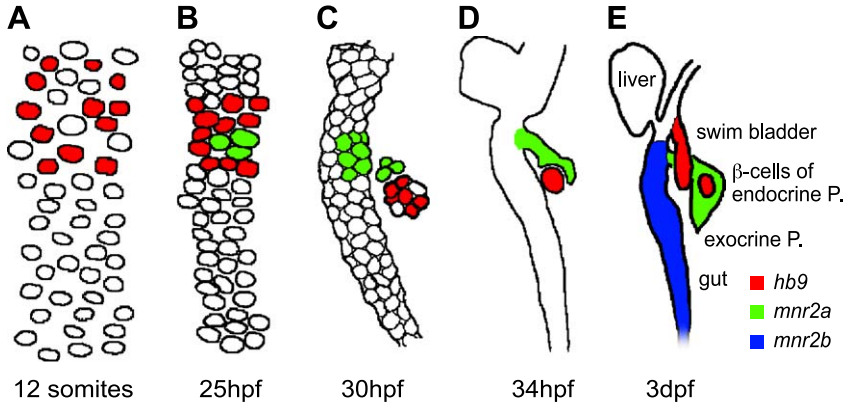

Fig. 7. Summary of *mnx*-gene expression during zebrafish pancreas development. (A) At 12-somite stage, *hb9* is expressed in the progenitors of the endocrine pancreas positioned in the anterior gut. (B) Until 25 hpf, expression of *hb9* is mainly restricted to β-cells where *hb9* is required for the initiation of insulin expression. At 25 hpf endodermal expression of *mnr2a* starts in few cells intermingled with the endocrine pancreas. (C) Until 32 hpf, the islet is formed as a separate structure right to the gut tube with *hb9* expression inside the islet and *mnr2a* expressing cells positioned adjacent to the islet. In addition, a second domain of *mnr2a* expression is established in the gut tube possibly marking the progenitors of the exocrine pancreatic bud. After 34 hpf, *mnr2a* expression expands toward the islet. (D) After 36 hpf, a single domain of *mnr2a* expression marks the progenitors of the exocrine pancreas. (E) After 3 dpf, *mnr2a* is expressed in differentiated exocrine cells and *mnr2a* function is required for the proper morphogenesis of the posterior exocrine extension. At this stage, *hb9* marks a population of endocrine cells and the swim bladder, and *mnr2b* (blue) is expressed in the posterior gut (data not shown).

injected embryos. So far, pancreatic *mnx* functions were only analyzed in mouse where *Hb9* was shown to function during initiation of pancreas development and during differentiation of β-cells. Our morpholino knockdown analyses suggest that *mnx* requirements are slightly different in mouse and fish. Specifically, neither injection of MO^{hb9} or MO^{mnr2b} nor co-injection of MO^{hb9} and MO^{mnr2b} resulted in a reduced number of pancreas cells as revealed by the unchanged expression levels of *pdx1*, *islet1*, and *hb9*. While we cannot exclude the possibility of a partial phenotype due to incomplete block of protein synthesis in the morphants, this suggests that the early endodermal expression of *mnr2b* and *hb9* genes is not required for initiation of pancreas formation. Notably, early pancreas morphogenesis is different in zebrafish and mouse. The mammalian pancreas forms from a ventral and a dorsal epithelial protrusion that are under different molecular control. Since only initiation of the dorsal protrusion requires *Hb9* function, the molecular control of zebrafish endocrine pancreas formation could be similar to that of the ventral pancreatic bud in mouse (Harrison et al., 1999; Li et al., 1999). In zebrafish, the endocrine pancreas progenitors are induced in a scattered fashion within a sheet of non-epithelial endodermal cells and subsequently these cells aggregate to from a single islet (Biemar et al., 2001; Figs. 3, 7). Thus, the different requirements of early *mnx* function in fish and mouse may also reflect the different requirement to initiate formation of an epithelial bud.

We find that block of *hb9* function results in the reduction or loss of insulin expression. This is different from the situation in *Hb9* mutant mice that fail to express late β-cell markers, but show only a reduction of 20% concerning the expression of insulin. Since insulin is one of the earliest markers for differentiating β-cells, this suggests an essential role of zebrafish *hb9* during an initial step in β-cell differentiation. Thus, while *hb9* is required for the formation of β-cells in mouse and fish, *hb9* appears to function at an earlier step in β-cell maturation in zebrafish than in mouse.

A novel role of mnx genes in exocrine pancreas development

While the endodermal expression of *hb9* appears to be conserved in vertebrates, we found *mnr2a* to be active in a tissue that has not been described to express *mnx* genes, the exocrine pancreas. Specifically, we show that *mnr2a* marks two populations of endodermal cells, one positioned close to insulin-expressing islet cells that starts to express *mnr2a* at 24 hpf and a second that starts to express *mnr2a* after 30 hfp. Recent studies that used transgenic zebrafish embryos with endoderm-specific GFP expression showed that the exocrine pancreas is formed from an endodermal protrusion that is first detectable after 34 hpf (Field et al., 2003a; Ober et al., 2003). Very similar to the second domain of *mnr2a* expression, this protrusion extends from the gut on the left side of the embryo into the direction of the islet, then migrates around the islet and, after 70 hpf, form a posterior extension characteristic for the exocrine pancreas in zebrafish. Together with the overlapping expression of *trypsin* and *mnr2a* after 3 dpf, this suggests that *mnr2a* marks cells of the forming exocrine tissue as well as a population of endodermal cells that have not been described yet. While the early *mnr2a*-positive cells are associated with the islet (see also Fig. 7), their position left to islet at 30 hpf is different from those of the known endocrine cell types (Biemar et al., 2001; Milewski et al., 1998). Due to the extension of the left *mnr2a* expression domain in direction of the islet, we were not able to distinguish the two populations of *mnr2a* cells after 36 hpf. While we cannot exclude the possibility that different cells express *mnr2a* at 30 hfp and at later stages, the overlapping expression of *mnr2a* and *trypsin* after 3 dpf could also indicate that all *mnr2a* cells contribute to exocrine tissue. Fate map studies will now be required to analyze if the early *mnr2a*-positive cells contribute to endocrine or exocrine tissue. Notably, the position of the early *mnr2a*-positive cells at 33–36 hpf correlates with that of insulin-expressing cells located outside the islet at 76 hpf that have been described in a recent study (Field et al., 2003a). There is evidence that these β-cells could be formed from bipotential precursors of endocrine and exocrine cells of the duct that build the exocrine pancreas together with the enzyme-producing acini (Field et al., 2003a; Yee et al., 2001). One interesting possibility is that the *mnr2a* cells mark the progenitors of these duct cells.

Finally, we show that knockdown of *mnr2a* results in reduced size and/or abnormal morphology of the exocrine pancreas defects. While our expression analysis suggest that *mnr2a* is one of the earliest marker for exocrine tissue in zebrafish, *mnr2a* function appears not to be required during the early outgrowth of the exocrine progenitors, as revealed by unchanged expression of *mnr2a* in MO^{mnr2a}-injected embryos. The variable exocrine pancreas defects in the *mnr2a* morphants were only found after 3 dpf, thus at the time when the exocrine pancreas rapidly increases in size and the cells start to produce digestive enzymes such as trypsin. This suggests a novel role of a *mnx* genes in late exocrine pancreas morphogenesis and might correlate *mnr2a* functions with the proliferation of late exocrine progenitor cells and/or with the differentiation of acinar cells. The late role of *mnr2a* limits the use of morpholinos, as the cellular concentration of MO^{mnr2a} might not be sufficient to efficiently block *mnr2a* translation in the proliferating exocrine cells after 3 dpf. Similar dilution effects were reported in other morpholino studies (Draper et al., 2001; Yee et al., 2001) and might account for the variable expressivity of the *mnr2a*-morphant phenotype. Thus, more detailed studies on the function of *mnr2a* in the exocrine pancreas might require the identification of *mnr2a* mutants.

Conclusions

So far, mouse was the only organism where *mnx* gene function had been analyzed in the context of pancreas

development. Now, we present data that suggest differences in the function of *mnx* genes in zebrafish and mice indicating that pancreas development could also be differently regulated in other vertebrates. While Mnx proteins might have different activities in mouse and zebrafish, it is also possible that factors with partially redundant functions differently compensate for loss of conserved *mnx* gene function in these organisms. Since mouse *hb9* is differently required in the dorsal and the ventral pancreatic lobe, it was already suggested that *hb9* and *pdx1* could have overlapping activities during initiation of pancreas morphogenesis (Li et al., 1999). Similarly, *mnr2a* function in the exocrine pancreas, that does not express *mnx* genes in mouse, could be compensated by *pdx1* or *ptf1a/p48* encoding a basic helix-loop-helix factor required for exocrine pancreas development in mouse and zebrafish (Krapp et al., 1998; F. Argenton and M. Pack, personal communication). Interaction of these genes could also explain the relative weak pancreas phenotype that we found in *mnr2a* morphants. Comparative studies on the interactions of such transcription factors in mouse and fish will help to gain a better understanding of the genetic control underlying pancreas development in vertebrates.

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