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Cloning, expression, and functional characterization of zebrafish *Mist1*

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Abstract

The basic helix–loop–helix (bHLH) protein Mist1 is an important exocrine pancreas transcriptional factor expressed in the acinar cells of mammals. In the present study, we cloned the homologous *Mist1* cDNA encoding a predicted protein of 184 amino acids in zebrafish. The typical bHLH domain of zebrafish Mist1 shares high identity with that of its orthologs in mouse, rat, and human. Expression analysis revealed that *Mist1* maternal transcripts are distinct in the very beginning of embryogenesis and that endogenous *Mist1* is chronologically expressed in polster, hatching gland, hindbrain and appears exclusively in the pancreas from 72 hpf onward. Knockdown of *Mist1* conditionally causes mild morphological defects in embryos. In MO-treated embryos, midbrain–hindbrain boundary is missing and exocrine pancreas is significantly reduced and disorganized. These results suggest that *Mist1* functions in an evolutionary conserved way as a key transcriptional regulator specific for exocrine pancreas development in zebrafish.

Keywords: *Mist1*; Basic helix–loop–helix (bHLH); Exocrine pancreas; Development; Zebrafish

Most of the transcription factors known as essential for pancreas development or maintenance are endocrine-specific, along with relatively elaborated molecular mechanisms and signaling pathways involved in endocrine differentiation and maintenance. By contrast, much fewer genes have been identified concerning regulation of exocrine pancreas development. Mist1, a class II basic helix–loop–helix (bHLH) protein which has been identified in mouse, rat, and human, is an important transcriptional factor necessary for exocrine pancreas development [1–5]. *Mist1* has been found expressed in secretory epithelial cells of various tissues including the pancreas, salivary gland, chief cells of the stomach, seminal vesicle, lacrimal gland, and mammary gland [6,7]. Although Mist1 can form heterodimer complexes with class I bHLH factors, the preferred DNA binding complex is a Mist1 homodimer [8–10]. Mist1/DNA interactions can either activate or repress transcription of target genes, depending on different cellular contexts [8,9]. During mouse pancreatic development, *Mist1* is first expressed at E10.5 in the primitive foregut that will develop into exocrine pancreatic precursors and later becomes restricted to the acinar cells at high levels [1,4]. While *ptf1a*, another essential exocrine pancreas transcription factor, is known to be required for acinar cell determination, *Mist1* is crucial for complete maturation and identity maintenance of acinar cells [4,10–14]. *Mist1*-null mice, outwardly normal with proper exocrine lineage differentiation, exhibit extensive acinar cell disorganization and loss of cellular polarity, accompanied by molecular changes and progressively developing lesions characteristic of pancreatic injury. Inhibition of *Mist1* transcriptional activity also leads to activation of duct-specific genes (*cytokeratin 19*, *cytokeratin 20*) in the original acinar cells, representing an acinar-to-ductal phenotype transition. Moreover, analysis of signaling pathways controlling the regulated exocytosis suggests functional defects in this event of *Mist1*-null mice [4,9,10,14,15]. Studies using *Mist1*-null and dominant-negative mouse models are underway to determine additional targets of *Mist1* and further elucidate the regulated circuits of exocrine differentiation and maintenance.

Zebrafish has become an important model organism for studies of vertebrate development in recent years. In view

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of its different pancreas patterning from that of mouse [16,17], research on pancreas development in zebrafish may uncover some novel mechanisms absent in mouse. In this study, we cloned the full-length cDNA of zebrafish *Mist1*, analyzed its expression pattern and explored its function *in vivo* by gene-specific knockdown approach.

Materials and methods

Fish and embryos' maintenance. Fish were raised and maintained under standard laboratory conditions at 28.5 °C, as described by Westerfield [18]. Embryos used in whole-mount *in situ* hybridization were raised in 0.003% PTU (Sigma) to prevent pigment formation. Morphological features and hours post-fertilization (hpf) were used to define the developmental stages of the embryos [19].

Cloning of the zebrafish Mist1 cDNA. To gain the cDNA sequence of zebrafish *Mist1*, a BLAST search with mouse *Mist1* cDNA sequence was performed in zebrafish genomic sequence project database (http://www.ensembl.org/Danio_rerio/blastview). *Mist1* cDNA was cloned by PCR on cDNA of 24 hpf embryos (forward primer: 5′-GTGAGTCAAGT ATGAAGTCCAAAGG-3′, reverse primer: 5′-CATGAGCGTAAGTAA GTGATGACAG-3′). The PCR conditions were 96 °C for 2 min, a touchdown program of 35 cycles beginning with 94 °C, 30 s; 64 °C, 30 s; 72 °C, 45 s and a 0.5 °C reduction of annealing temperature in every cycle, and a final extension at 72 °C for 8 min. 5′ RACE using the 5′ full RACE Core Set (TaKaRa) and 3′ RACE using the FirstChoice RLM-RACE kit (Ambion) were employed to recover the missing 5′ and 3′ ends. Finally, the full-length *Mist1* cDNA was amplified by PCR (forward primer: 5′-ACAACTTATTATCGCTCTTCGCAAGG-3′, reverse primer: 5′-ATGG TTTTCAGAATTGCACCCCTG-3′) for verification. All the PCR products were cloned into pGEM-T easy vector (Promega) and sequenced.

Sequencing and bioinformatics analysis of zebrafish Mist1. Sequencing was carried out by Invitrogen Biotechnology Corporation (Shanghai) and Shanghai Sangon Corporation. The zebrafish *Mist1* nucleotide sequence, exon–intron organization, amino acid sequence and conserved domain were analyzed online with NCBI blast server 2.0 and zebrafish whole genomic sequence project database (http://www.ensembl.org/Danio_rerio/blastview). Multiple alignments, the phylogenetic tree and their graphic presentations were generated online with ClustalW version 1.83 (http://www.ebi.ac.uk/clustalw).

RT-PCR analysis. Total RNAs were isolated from zebrafish embryos at different developmental stages using Trizol agent (Invitrogen). Reverse transcription (RT) of RNA was performed with ReverTra Ace (improved M-MLV reverse transcriptase) (TOYOBO). A pair of specific primers spanning the intron between the first and second exons in the genomic sequence (forward: 5′-ATCGCTCTTCGCAAGGCTCTTATC-3′, reverse: 5′-AGGCATTGTTGAGTTTGTGCATCC-3′) was used to amplify a 384 bp *Mist1* transcript fragment by RT-PCR. PCR conditions were carried out in 35 cycles of 94 °C, 30 s; 64 °C, 30 s; 72 °C, 1 min. As an endogenous control, a 475 bp sequence of β-actin cDNA was amplified by RT-PCR using two specific primers (forward: 5′-ATGGATGATGAAATTGCCG CAC-3′, reverse: 5′-ACCATCACCAGAGTCCATCACG-3′).

Morpholino, mRNA, and microinjection. The sequences of *Mist1*-MO and a standard control oligo (Gene Tools, LLC) were designed as 5′-TT GACCCCTTTCCTTTGGACTTCAT-3′ and 5′-CCTCTTACCTCAGTT ACAATTTATA-3′. To test the knockdown effectiveness of *Mist1*-MO, the *Mist1*–GFP construct was generated by fusing a 462 bp fragment of *Mist1*, which contains a 36 bp 5′ UTR and its adjacent coding sequence encoding the first 142 amino acids, in-frame into vector pEGFP-N2. The coding sequence of zebrafish *Mist1* was cloned into the expression vector pXT7 that has 5′ and 3′ UTR from *Xenopus β-globin* gene [20]. Capped mRNA was synthesized *in vitro* using *Sma*I linearized plasmid DNA and T7 promoter. We directly microinjected about 4–8 ng of MO into each embryo at 1–4 cell stages as described by Nasevicius and Ekker [21]. For coinjection of mRNA with the MO, the capped mRNA was diluted in 0.1 M KCl to an appropriate concentration and injected before MO.

Whole-mount in situ hybridization. Wild-type and MO-injected embryos of various stages were fixed in 4% paraformaldehyde and processed for whole-mount *in situ* hybridization. The *Mist1*-T easy plasmid containing 700 bp of *Mist1* was linearized by *Nco*I and transcribed *in vitro* by T7 for digoxigenin (DIG) (Roche)-labeled antisense RNA probe. Other antisense RNA probes used include *ptf1a* [13], *trypsin* [22], and *insulin* (containing the first 437 bp of zebrafish *insulin* mRNA (GenBank Accession No. AF036326)). Whole-mount *in situ* hybridization was performed as described by Westerfield [18]. Images of embryos were acquired from SZX12 microscope (Olympus) using a E995 digital camera (Nikon) or DP70 CCD digital camera (Olympus) and processed by Adobe PhotoShop software.

Results and discussion

Cloning and nucleotide sequence analysis of full-length zebrafish Mist1 cDNA

A 1069 bp sequence was finally gained by PCR and verified by sequencing. It was therefore termed full length zebrafish *Mist1* cDNA and has been deposited in GenBank under the Accession No. DQ995273.

Similar to the gene organization of mouse and rat *Mist1* [2,3], the zebrafish *Mist1* gene consists of two exons separated by a 987 bp intron with the entire coding region situated in the second exon, while the 5′ UTR is separated by the intron and dispersed on the two exons. This consistent genomic structure pattern of *Mist1* orthologs indicates that they probably function in similar ways in different species to regulate transcription.

Amino acid sequence and bioinformatics analysis of zebrafish Mist1

The 1069 bp zebrafish *Mist1* cDNA contains an ORF of 555 bp spanning the 137th to 691th nucleotides, encoding a putative protein of 184 amino acids. Although there is obvious distinction in the overall lengths of the putative zebrafish Mist1 protein (GenBank Accession No. NP_001071120) and its mammalian orthologs, the bHLH domain of zebrafish Mist1 shares 77%, 76%, and 73% identity with that of mouse (GenBank Accession No. NP_034930), rat (GenBank Accession No. NP_036995), and human (GenBank Accession No. NP_803238) orthologs, respectively, and when it comes to the HLH region, the identity reaches as high as 90% in all three comparisons (Fig. 1). In addition, a phylogenetic tree was constructed by aligning zebrafish Mist1 with the three mammalian orthologs and some other published bHLH proteins in zebrafish (Supplementary Fig. 1). Results of the phylogenetic analysis revealed that zebrafish Mist1 significantly differs from its mammalian orthologs, indicating a relatively distant relationship resulted from evolution between the two groups. However, zebrafish Mist1 still exhibits clear distinction from other bHLH subfamilies. The similarity in conserved protein domain, as well as in gene structure, is probably the basis of functional consistency.

```
mouse          MKTKNRPPRRRTP---MQDTEATPGEQTPDRPQSGSGGSELTKGLRSRTARASG-GRGEV 56
rat            MKTKNRPPRRRTP---MQDAEATPGEQTPDRSQSGSGASEVTKGLRSRTARASG-TRAEV 56
human          MKTKNRPPRRRAP---VQDTEATPGEGTPDGSLPNPG-PEPAKGLRSRPARAAARAPGEG 56
zebrafish      MKSKGKGSKRGRQSWAEQDDPDFTLETEPGSSEQEGSEASVRLGGSWRTLRGEGHSRTRV 60
               **:*.: .:*       **    . *  *. .    . ..   *   *. *. .    .

mouse          -SRRR---QGSGGRRENSVQRRLESNERERQRMHKLNNAFQALREVIPHVRADKKLSKIE 112
rat            -SRRR---QGSSSRRENSVQRRLESNERERQRMHKLNNAFQALREVIPHVRADKKLSKIE 112
human          -RRRRPGPSGPGGRRDSSIQRRLESNERERQRMHKLNNAFQALREVIPHVRADKKLSKIE 115
zebrafish      GARRR---RQHGNTKERNVRR-LESNERERQRMHKLNNAFQALREAIPHVKTEKKLSKIE 116
                 ***      .. :: .::* **********************.****:::*******

mouse          TLTLAKNYIKSLTATILTMSSSRLPGLEAPGPAPGPKLYQHYHHQQQQQQQQQQVAGAML 172
rat            TLTLAKNYIKSLTATILTMSSSRLPGLEAPGPAPGPKLYQHYHHQQQQQQQQQQVAGAVL 172
human          TLTLAKNYIKSLTATILTMSSSRLPGLEGP----GPKLYQHY-------QQQQQVAGGAL 164
zebrafish      TLTLAKNYIKALTTIILGMSNGCLPDAESQMEANASRLLQCY-------QQHLREEG--- 166
               **********:**: ** **.. **. *.     ..:* * *       **: :  *  :

mouse          GVTEDQPQGHLQRYSTQIHSFREGS 197
rat            GVTEDQPQGHLQRYSTQIHSFREGS 197
human          GATEAQPQGHLQRYSTQIHSFREGT 189
zebrafish      -------EEDLSNYLNHIHSFSQDS 184
                     : .*..* .:**** :.:
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Fig. 1. Alignment of zebrafish Mist1 (GenBank Accession No. NP_001071120) with mouse Mist1 (GenBank Accession No. NP_034930), rat Mist1 (GenBank Accession No. NP_036995), and human Mist1 (GenBank Accession No. NP_803238). Identical residues are denoted by "*", conserved substitutions by ":", semi-conserved substitutions by ".". Note that high identity appears in the conserved bHLH domain (indicated in gray).

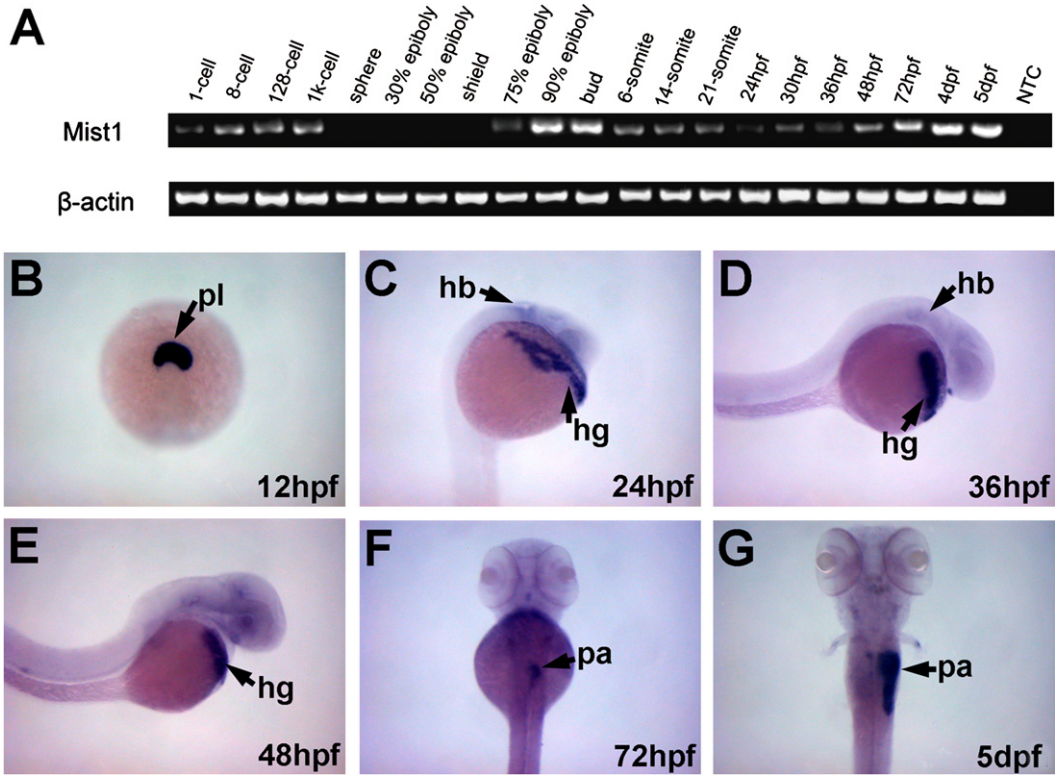

Fig. 2. Expression pattern of *Mist1* during zebrafish embryogenesis. (A) RT-PCR results of *Mist1* expression at various stages from 1-cell to 5 dpf. The second panel shows expression data of zebrafish *β-actin* as an endogenous positive control. The negative control is designated as NTC (no-template control). (B–G) Whole-mount *in situ* hybridization results of Mist1 expression at stages from 12 hpf to 5 dpf. (B) Ventral view; (C–E) lateral views; (F,G) dorsal views. (B) 12 hpf; (C) 24 hpf; (D) 36 hpf; (E) 48 hpf; (F) 72 hpf; (G) 5 dpf. *Abbreviations:* pl, polster; hb, hindbrain; hg, hatching gland; pa, pancreas.

Expression pattern of zebrafish Mist1 during embryo development

The temporal expression of *Mist1* during zebrafish development was first examined by RT-PCR analysis. Results have revealed the dynamic trend of *Mist1* expression from 1-cell embryo to 5 dpf larva. As shown in Fig. 2A, distinct expression is observed at very early stages from 1-cell (0.2 hpf) to 1k-cell (3 hpf), representing positive maternal transcripts. After that, expression is absent and

does not appear as endogenous transcripts until 75% epiboly (8 hpf). Then, expression dramatically increases to an outstandingly high level at 90% epiboly (9 hpf) and bud (10 hpf) stages. From 6-somite (12 hpf) on, expression declines and is maintained at a moderate level at the following few stages. A rise occurs again at 48 hpf, and finally results in a significant amount of transcripts at 5 dpf, the latest developmental stage examined.

To further determine the spatiotemporal expression pattern of *Mist1* during zebrafish development, we performed whole-mount *in situ* hybridization on embryos at different stages. The displayed expression profile can be characterized into two phases. During the first phase by 72 hpf, *Mist1* is first substantially expressed in the polster, the rudiment of hatching gland, at 12 hpf (Fig. 2B). Later at 24 hpf, notable signals are observed in the more developed hatching gland, along with weak signals in the hindbrain (Fig. 2C). When development enters the 36 hpf stage, expression, although still restricted to the hatching gland, is withdrawing to the lateral sides and becomes almost undetectable in the hindbrain (Fig. 2D). Embryos at 48 hpf maintain fading expression in the hatching gland (Fig. 2E). The second phase is characteristic of *Mist1* exclusive expression in the pancreas, with 72 hpf as the outset (Fig. 2F), after which the specific signals grow stronger and remain restricted to the pancreas at a high level in the 5 dpf larva (Fig. 2G).

Examination of the temporal and spatial expression pattern of zebrafish *Mist1* provides some clues as to the protein's functions in development. Expressions early in the hatching gland and later in the pancreas are both typical, given the characteristic expression of *Mist1* in secretory cells of various tissues. However, *Mist1* is also found expressed in the hindbrain, which is not detected in mouse and rat but also present in human [1,5]. This may imply some specific roles *Mist1* plays in zebrafish, which will probably bring to light some underlying pathway shared by zebrafish and human in the CNS development that *Mist1* is involved in hence making the further study of *Mist1* in zebrafish more intriguing. Here, we can recall that zebrafish *ptf1a*, another exocrine-specific transcription factor, is also expressed in hindbrain, earlier than *Mist1* at 12-somite (15 hpf) [13]. As *ptf1a* is thought to be upstream of *Mist1* in mouse, the successive expression of *ptf1a* and *Mist1* in the CNS supports the same relationship in zebrafish. Interestingly, notable *Mist1* maternal transcripts are also detected by RT-PCR in the very initial developmental stages, which have never been reported before in other species. This finding will possibly lead to exploration of *Mist1* function in the outset of embryogenesis, and further research is needed to determine whether *Mist1* is implicated in some unknown signaling pathways and elucidate the specified mechanism.

Knockdown of Mist1 conditionally causes mild morphological defects in embryos

To explore the particular role of *Mist1* in zebrafish development, we designed a specific MO to target endogenous *Mist1* mRNA. Before direct microinjection, MO was first coinjected with *Mist1*–pEGFP-N2 plasmid which encodes a Mist1–GFP fusion protein. Inhibited fluorescence resulted from coinjection has proved the knockdown efficiency of *Mist1*-MO (Supplementary Fig. 2A and B).

After injected with 8 ng of MO per embryo, nearly half of the embryos exhibit smaller bowing head, dropsy in the hindbrain and moderate level of developmental delay (Supplementary Fig. 2C and D) (embryos injected with the control oligo at the same dose resemble the wild-type ones and display no morphological abnormity). Furthermore, when lower dosages of MO are applied, smaller percentages of defects are observed, suggesting a dose-dependent knockdown effect of *Mist1*-MO. However, these mild defects are far from lethal, for all the MO-injected embryos are alive when recorded at 10 dpf.

In *Mist1*-null mice, extensive disorganization of exocrine tissue and acinar-to-ductal metaplasia are the primary defects, while no overt phenotypic or growth abnormality is shown [4,9,10,14,15]. Compared to *Mist1*-null mice, the phenotypic changes in some of the MO-treated zebrafish embryos are perhaps due to *Mist1*'s participation in regulation of the CNS development and the even earlier initial period of development as we previously assumed based on *Mist1*'s early expression. Yet the spare capacity of exocrine pancreas still works to ensure the growth and development, though a little lagged, in the face of the compromised exocrine function.

The midbrain–hindbrain boundary and exocrine pancreas are affected in MO-treated embryos

To specify the regulating role *Mist1* plays in zebrafish development, whole-mount *in situ* hybridization was performed with exocrine pancreas markers *ptf1a* and *trypsin*, using MO-treated embryos from 24 hpf to 5 dpf.

Expression domains of *ptf1a* are almost identical in treated and wild-type embryos at 24 hpf, both mainly located in the hindbrain (data not shown). However, in the MO-injected embryos at 48 hpf, compared to wild-type embryos, expression is completely missing at the midbrain–hindbrain boundary and retina, only present in the hindbrain (Fig. 3A and B). At 72 hpf, while *ptf1a* expression in wild-type embryos begins to fade in the retina and specifically label exocrine pancreas at the right side of the midline, expression in MO-treated embryos considerably increases in the retina and in some of them the marked cells presumed to be exocrine pancreas are positioned at both sides of the midline, indicating disrupted organization of exocrine cells (Fig. 3C–F). Expression in exocrine pancreas is notably reduced in MO-injected embryos at 4 and 5 dpf, some with more distinct segregation of two clusters of pancreatic cells than at 72 hpf (Fig. 3G–L). Change in expression pattern of another exocrine marker *trypsin* is similar to that of *ptf1a*, except that *trypsin* is not expressed in the retina of wild-type embryos (Fig. 4A–D).

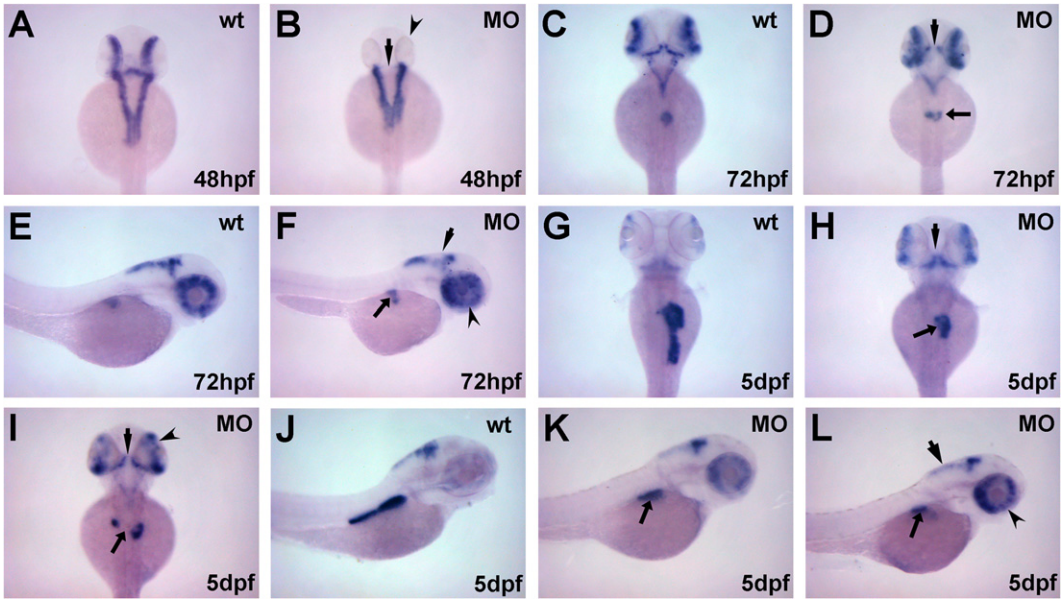

Fig. 3. Expression pattern of *ptf1a* is altered in MO-treated embryos. (A,C,G) Dorsal views of wild-type embryos; (E,J) lateral views of wild-type embryos; (B, D, H, I) dorsal views of MO-treated embryos; (F,K,L) lateral views of MO-treated embryos. (A,B) 48 hpf; (C–F) 72 hpf; (G–L) 5 dpf. The midbrain–hindbrain boundary, retina, and exocrine pancreas are designated with sharp-headed arrow, arrowhead, and blunt-headed arrow, respectively.

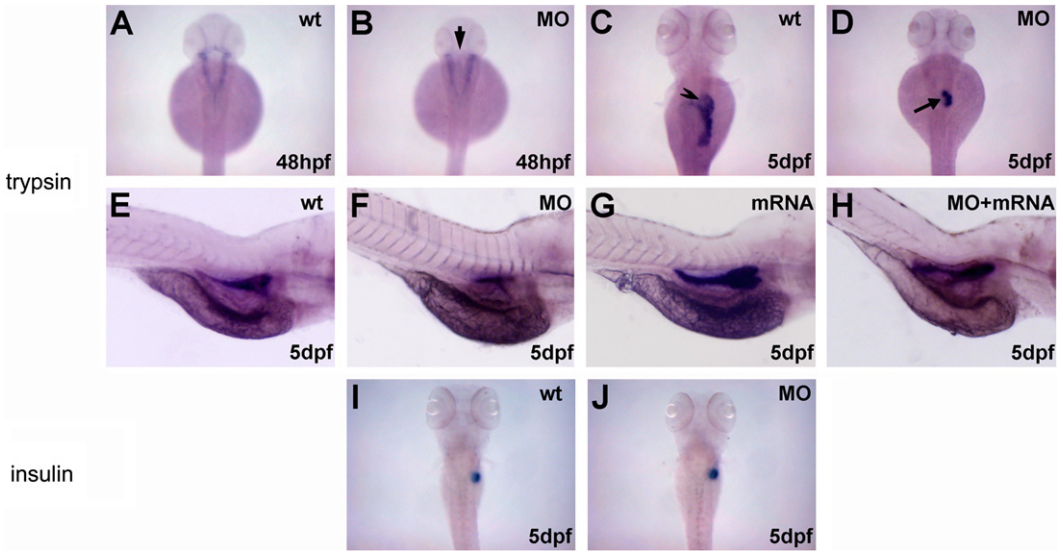

Fig. 4. (A–D) Expression pattern of *trypsin* is also affected in MO-treated embryos. (A,C) Dorsal views of wild-type embryos; (B,D) dorsal views of MO-treated embryos. (A,B) 48 hpf; (C,D) 5 dpf. The midbrain–hindbrain boundary and exocrine pancreas are designated with sharp-headed arrow and blunt-headed arrow, respectively. Note that in (C) there is a negative hole (arrowhead) in the head of the expression domain in pancreas, representing the endocrine islet. (E–J) *Mist1*-MO specifically represses exocrine pancreas development. (E–H) *Mist1* mRNA can rescue the knockdown effect in MO-injected embryos. All are lateral views with anterior to the right. (E) Wild-type embryo; (F) MO-injected embryo; (G) mRNA-injected embryo; (H) MO-mRNA-coinjected embryo. (I,J) *Insulin* expression remains unaffected in MO-treated embryos. Both are dorsal views. (E) Wild-type embryo; (F) MO-treated embryo.

According to the data stated above, combined with *Mist1* expression profile, it can be inferred that *Mist1* is involved in the normal development of midbrain–hindbrain boundary and retina through its early expression and is essential for exocrine cells development and organization in later stages, and that the lagged expression of *ptf1a* in the retina is consistent with the represented developmental delay of the MO-treated embryo.

There is a proposed mechanism that can explain the segregation of pancreatic cells. As previously described, *ptf1a* is essential for the determination of exocrine cells, while *Mist1*, downstream of *ptf1a*, is responsible for organization and maintenance of differentiated exocrine cells. Thus, these two patches of cells are likely to be fully differentiated exocrine cells with obvious disorganization owing to loss of *Mist1* function. Based upon this common function required

for normal maintenance and organization of exocrine pancreas between zebrafish and mammalian *Mist1*, we can conclude that *Mist1* works in an evolutionary conserved way to regulate exocrine pancreas development.

Mist1-MO specifically represses exocrine pancreas development

Mist1 mRNA was synthesized for rescue assay of MO effect in order to verify the specificity of *Mist1*-MO to *Mist1* expression. After *Mist1* mRNA is injected alone, expression domain of *trypsin* is obviously enlarged and signals are stronger in embryos at 5 dpf compared to that of the wild-type, which ensures the over-expression effect of *Mist1* mRNA (Fig. 4E and G). Then, when coinjection of *Mist1*-MO and mRNA is applied, inhibited *trypsin* expression in MO-treated embryos at 5 dpf is successfully rescued to a comparable level with that of the wild-type (Fig. 4E, F, and H). Therefore, it is evident that *Mist1*-MO specifically blocks translation of *Mist1* mRNA and thus leads to abnormal development of exocrine pancreas.

Expression pattern of the endocrine-specific marker *insulin* was also tested. It has turned out that *insulin* is expressed in almost the same pattern in MO-injected and wild-type embryos (Fig. 4I and J). Previous research showed that the pancreas derives from two distinct anlagen (posterodorsal and anteroventral) that fuse subsequently. Both in the temporal and the spatial senses, transcription factors that specifically regulate exocrine pancreas development are not to place influence on the endocrine pancreas [16]. The unaffected *insulin* expression in MO-treated embryos once again justifies the specificity of *Mist1* and independent developmental processes of exocrine and endocrine pancreas.

Recently in zebrafish, the timing and mechanism of acinar and ductal differentiation and morphogenesis have been well characterized [17] and a transgenic line that displays highly specific exocrine pancreas expression of GFP has been fully developed [23]. Genetic analyses in zebrafish offer the opportunity to identify genes and signal pathways that selectively regulate exocrine pancreas and perhaps to explore additional roles of transcription factors that mouse lacks. Screens to identify such mutants will help to find *Mist1*'s target and related genes and gain insights into the detailed molecular pathways *Mist1* is involved in during exocrine pancreas development.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.055.

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