Cadherin-6 is required for zebrafish nephrogenesis during early development

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ABSTRACT We performed functional analyses of cadherin-6 (cdh6) in zebrafish nephrogenesis using antisense Morpholino oligonucleotide (MO) inhibition combined with in situ hybridization. We have cloned a zebrafish homolog (accession number AB193290) of human K-cadherin (CDH6), which showed 60–63% identity and 76–78% similarity to the human, mouse, chicken and Xenopus homologs. Whole-mount in situ hybridization showed that cdh6 is expressed in the pronephric ducts and nephron primordia in addition to the central and peripheral nervous systems. Expression of cdh6 in the pronephric ducts was first detected at 14 hours post-fertilization (hpf) and increased to 24 hpf. Embryos injected with MOs directed against cdh6 (cdh6MOs) showed developmental defects, including a small head, body axis curvature, short yolk extension and a short bent tail by 30 hpf and edema appeared in the thorax by 42 hpf. Such defects and edema became more marked by 52 hpf and most of the affected embryos died by 5 days post-fertilization. Embryos injected with cdh6MOs were subjected to in situ hybridization with probes for the pronephric markers, wt1 and pax2.1, to examine disturbed development of the anterior region of the pronephric ducts and the nephron primordia. Histological studies showed malformation of the pronephros as abnormally fused glomerulus primordia, fused or abnormally bent pronephric tubule anlagen and coarctated pronephric ducts. These results suggest that cdh6 plays pivotal roles in the development of the pronephros in zebrafish embryos.

KEY WORDS: cadherin-6, K-cadherin, zebrafish, Morpholino, pronephros

Introduction

Cell-cell interaction is a fundamental process required for development (Hynes and Lander, 1992). The cadherins are cell-surface molecules that mediate cell-cell adhesion mainly through homophilic interactions (Takeichi, 1991). Most cadherins exhibit unique expression patterns and functional studies of several cadherins have shown that these molecules play essential roles in the development of vertebrate tissues and organs (Yagi and Takeichi, 2000).

There have been a number of studies of the expression and function of cadherin-6 (K-cadherin), a member of the classical type II cadherin subfamily (Redies, 1995), in normal renal development (Cho et al., 1998; Mah et al., 2000) and in the formation of renal carcinoma (Xiang et al., 1994). Cadherin-6 has also been shown to be expressed in the mouse, chicken and Xenopus nervous systems (Inoue et al., 1997; Nakagawa and Takeichi, 1998; David and Wedlich, 2000). Liu et al. recently reported the expression of cadherin-6 in the nervous system and pronephric ducts of the zebrafish (Liu et al., 2006). However, they presented only a brief description of cadherin-6 expression in the zebrafish pronephros and there have been no reports regarding its function.

The zebrafish pronephros consists of a single glomerulus, a pronephric tubule and a pronephric duct. The pronephric primordium is first evident during early somitogenesis as a mass of intermediate mesoderm underlying the second and third somites (Kimmel et al., 1995). Growth and differentiation of the pronephric ducts follow behind somitogenesis and are completed by 24 hours post-fertilization (hpf), by which time nephron primordia have formed at the anterior tips of the pronephric ducts (Drummond et al., 1998).

The zebrafish pronephros, although more primitive than the differentiated metanephros, shares many features with this type

Abbreviations used in this paper: CDH, cadherin; hpf, hours post-fertilization; ISH, in situ hybridization; MO, Morpholino oligonucleotide.

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of kidney. For example, similar to the metanephros, the pronephros has a glomerulus lined with podocytes with foot processes that form the glomerular basement membrane together with endothelia (Drummond et al., 1998). In addition, the tubules of the pronephros have a brush-border similar in appearance to that in the polarized epithelium of the metanephric kidney (Drummond et al., 1998; Drummond, 2000).

Therefore, the zebrafish pronephros represents a valid model for the development of more complex vertebrate kidneys. The inductive events leading to the formation of the pronephros and mesonephros are thought to be quite similar to those directing formation of the metanephros (Vize et al., 1997). In fact, a number of genes involved in metanephric development are also expressed in the zebrafish pronephros: *wif1* and *pax2.1* are also expressed in the developing glomeruli, tubules and ducts of the pronephros (Kreidberg et al., 1993; Torres et al., 1995; Drummond et al., 1998; Drummond, 2000).

Many types of cadherin appear to be important for kidney morphogenesis. In zebrafish, cadherin-17 (cdh17) is expressed specifically in the posterior portion of the pronephric ducts during embryonic development and knockdown of cdh17 disrupts the normal formation of the posterior portion of the pronephric ducts (Horsfield et al., 2002). We have isolated a zebrafish cadherin that is orthologous to human K-cadherin (CDH6), which we characterized as zebrafish cadherin-6 (cdh6). Here, we report the results of functional analysis of *cdh6* in zebrafish embryos especially in the pronephros and our findings indicating that it plays an essential role in the development of the pronephros.

**Results**

**Zebrafish cdh6 expressed in the pronephros**

In addition to its expression in the central and peripheral nervous systems, zebrafish *cdh6* (DDBJ no. AB193290) was expressed in the pronephros during the early stages of development. We analyzed the expression pattern of *cdh6* in pronephric regions by whole-mount *in situ* hybridization at 24, 28 and 30 hpf (Fig. 1). At 24 hpf, the zebrafish pronephros consists of pronephric ducts and nephron primordia. About 4 h later, at ~28 hpf, the nephron primordia of both sides come into close

**Table 1**

MALFORMATIONS INDUCED BY *CDH6* ANTISENSE MORPHOLINO OLGONUCLEOTIDES (*CDH6*MOs)

<table>
<thead>
<tr>
<th>Morpholino</th>
<th>Developmental defects at 30 hpf</th>
<th>Edematous embryos at 52 hpf</th>
<th>Survival of embryos until 5 dpf</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>cdh6</em>MO-1 (2 mg/ml)</td>
<td>70.5%</td>
<td>71.5%</td>
<td>27.0%</td>
<td>200</td>
</tr>
<tr>
<td>(1 mg/ml)</td>
<td>33.9%</td>
<td>36.6%</td>
<td>61.6%</td>
<td>112</td>
</tr>
<tr>
<td><em>cdh6</em>MO-2 (2 mg/ml)</td>
<td>50.1%</td>
<td>53.9%</td>
<td>43.8%</td>
<td>89</td>
</tr>
<tr>
<td>(1 mg/ml)</td>
<td>20.5%</td>
<td>20.5%</td>
<td>78.2%</td>
<td>78</td>
</tr>
<tr>
<td>Negative control MO (2 mg/ml)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>94.0%</td>
<td>200</td>
</tr>
<tr>
<td>(1 mg/ml)</td>
<td>0.0%</td>
<td>0.0%</td>
<td>95.0%</td>
<td>100</td>
</tr>
</tbody>
</table>

n: Number of embryos counted as viable at 6 hpf. Success of injections was determined by monitoring fluorescent labeling of the Morpholinos.

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**Fig. 1. cdh6 is expressed in the pronephros during early embryogenesis.** Expression patterns in pronephric regions were determined by ISH at 24 (A–D), 28 (E–H) and 30 (I–N) hpf. (A,E,I) Dorsal views of whole-mount embryos with rostral toward the top. (B,F,J) Ventral views around the pronephric region with rostral toward the top. (C,G,K) Transverse sections of embryos at the nephron primordium region (Section level 1 indicated by (1) in the schematic illustration below). (D,H,L) Transverse sections of embryos at the pronephric duct region (Section level 2 in the illustration below). (M,N) Transverse histological sections 6 µm thick at the same regions as Fig. 2 K and L, respectively. Open white arrowheads, pronephros; open black arrowheads, nephron primordia; open white arrows, glomerular primordia; white arrows, tubule primordia; black arrows, anterior region of the pronephric ducts; closed white arrowheads, organ primordia of the digestive system; closed black arrowheads, dorsal spinal cord. Illustrations at 24, 28 and 30 hpf indicate *cdh6* expression (light blue, weak expression): (a) glomerular primordium, (b) tubule primordium, (c) anterior region of the pronephric duct (at 24 hpf, (a) and (b) indicate nephron primordium). Scale bars, 30 µm.
Contact and differentiate into the tubular and glomerular primordia.

By 24 hpf, cdh6 was detected in the anterior region of the pronephric duct and nephron primordium (Fig. 1A–D). By 28 hpf, cdh6 expression in the glomerular primordium became weaker than in the other positive regions and was not found at 30 hpf (Fig. 1E–L). Expression of cdh6 was detected in the tubule primordium until 30 hpf (Fig. 1E–L). Its expression was also noted in the pronephric duct around 28 hpf, which became weaker by 30 hpf (Fig. 1E–L).

To determine the arrangement of cdh6-positive cells in detail, frozen sections of 30-hpf embryos stained for cdh6 were made for histological studies (Fig. 1M, N). Cells showing positive staining for cdh6 were detected clearly in the pronephric tubule primordia and pronephric duct (Fig. 1M, N). In addition, cdh6 expression was detected in the organ primordia of the digestive system (closed white arrowheads in Fig. 1B–D, F–H, K–N) and in the dorsal spinal cord (closed black arrowheads in Fig. 1D, H, L) throughout the experimental period.

Zebrafish cdh6 antisense Morpholino oligonucleotides induced embryo malformation

To determine the role of cdh6, we used two different antisense Morpholino oligonucleotides (MOs) complementary to the translational initiation site of cdh6, cdh6MO-1 and cdh6MO-2, to induce targeted knockdown of cdh6 function. Briefly, aliquots of about 8 nl of cdh6MOs were microinjected into embryos at the 1-cell stage at a concentration of 1 or 2 mg/ml. At 6 hpf, MO-injected embryos were examined for viability and for fluorescence labeling corresponding to Morpholino distributed throughout the embryos. To determine the effects of cdh6MOs, only those fulfilling both of these conditions were used for further experiments.

By 24 hpf, embryos injected with cdh6MOs showed developmental defects, including small head, small eyes, abnormal body axis curvature, short yolk extension and a short bent tail. These abnormal phenotypes were obvious by 30 hpf (Table 1; Fig. 2C, E).

As cdh17-knockdown embryos showed a disturbance in the cloaca region (Horsfield et al., 2002), we also examined the phenotype of the cloaca in whole fish. No abnormalities were observed in the posterior portion of the pronephric duct or cloaca in either control or cdh6MO-injected embryos at 30 or 52 hpf (Fig. 2H, J, L, N).

Following the abnormal development in cdh6MO-injected embryos, areas of edema appeared in the thorax around 42 hpf,
which became more marked at 52 hpf (Table 1; Fig. 2D, F, M). Embryos injected with cdh6MO-1 or cdh6MO-2 showed virtually the same phenotypes and similar percentages of abnormalities at 30 and 52 hpf (Table 1). Most of the embryos with MO injections died by 5 days post-fertilization (dpf) (Table 1).

Zebrafish cdh6 antisense morpholino oligonucleotides disrupted normal nephrogenesis

To investigate the disturbed regions in detail, the cdh6MO-injected embryos were subjected to in situ hybridization with probes for the pronephric markers, wt1 and pax2.1.

wt1 is expressed in glomerular primordia and is limited to the podocytes in mature fish (Drummond et al., 1998). In the present study, wt1 was expressed in the glomerular primordia at 30 hpf in embryos injected with control MO (Fig. 3A). Both of the glomerular anlagen were symmetrical and showed a contacted arrangement. In contrast, the cdh6MO-injected embryos showed quite different phenotypes; in some cases, glomerular primordia of both sides showed abnormal fusion (Fig. 3B), while in others the glomerular primordium showed asymmetric expression and localization (Fig. 3C).

For quantitative evaluation of the abnormalities in pronephros development, we also stained for pax2.1, which is known to be expressed in the pronephric tubule primordia and the pronephric ducts (Drummond et al., 1998) (Fig. 3E). At 30 hpf, the expression patterns of embryos injected with cdh6MO-1 or cdh6MO-2 could be classified into four types: (1) expression in the pronephros resembling that in negative controls (class N), (2) both pronephric tubule primordia fused (class F) (Fig. 3F), (3) either or both of the pronephric tubule primordia curved abnormally (class CV) (Fig. 3G) or (4) coarctation found in the pronephric ducts (class CA) (Fig. 3F). Embryos showing two expression phenotypes (as in Fig. 3F) were counted once for each phenotype. The results were as follows: cdh6MO-1: F 16.0% CV 20.8% CA 12.3% N 58.5%. cdh6MO-2: F 10.9% CV 16.3% CA 6.5% N 79.3% (Table 2). To confirm specificity of the cdh6MOs, we performed RNA rescue experiments using cdh6 mRNA; 94–95% of the RNA-rescued embryos showed no abnormalities throughout the whole body and were categorized as showing class N phenotype (Table 2; Fig. 3D, H). As the percentages of affected embryos at 30 and 52 hpf were almost the same, we stained injected embryos for pax2.1 at 30 hpf.

In addition to the investigation at the organ level, we studied the effects of knockdown of cdh6 expression on the cells. The cell arrangement in cdh6-positive regions of the pronephros was examined in frozen sections (Fig. 4). Epithelial cells of glomerular primordia, tubule primordia and pronephric duct regions in control embryos showed well-ordered cellular arrangements (Fig. 4A–C). However, the epithelial cells in each region of the embryos affected by cdh6MO-1 showed a disturbed appearance. The cells in the pronephros were disorganized and had an abnormal structure (Fig. 4D–F).

Discussion

cdh6 is expressed in the glomerular and pronephric tubule primordia and the anterior region of the pronephric ducts

Zebrafish cdh6 was expressed in the glomerular and pronephric tubule primordia and in the anterior region of the pronephric ducts. We examined the expression pattern of cdh6 in the pronephros in detail. During zebrafish nephrogenesis, cdh6 was expressed in nephron primordia and in the anterior region of the pronephric ducts from 14 hpf, at the beginning of nephrogenesis, to 24 hpf. From 24 to 30 hpf, cdh6 was expressed in the glomerular and the pronephric tubule primordia and in the anterior region of the pronephric ducts. In zebrafish, the stages from 24 to 36 hpf are important for proper pronephros development, as normal nephron primordia differentiate into glomerular and pronephric tubule primordia during this stage (Drummond et al., 1998).

Cadherin-6 is expressed in the developing nervous system and kidney in mammals and has been suggested to play important roles in the formation of epithe-
Zebrafish cdh6 is important for the proper formation of the glomerulus, the pronephric tubules and the anterior region of the pronephric ducts during early embryogenesis.

Based on the following observations, we concluded that zebrafish cdh6 is required for correct formation of the glomerulus, the pronephric tubules and the anterior portion of the pronephric ducts during early embryogenesis.

First, we observed abnormal pronephra in cdh6 knockdown zebrafish embryos. That is, whole-mount specimens showed fused or abnormally curved pronephric tubule primordia, fused abnormal glomerular primordia and coarctated pronephric ducts. Examination of histological sections revealed disturbance of the epithelial tissue; the glomerular and pronephric tubule primordia, as well as the anterior region of the pronephric ducts, were disrupted and the epithelial cells showed a disordered arrangement.

Second, cdh6 knockdown embryos showed obvious edema in the thorax at 52 hpf. Filtration at the pronephra is thought to begin between 40 and 48 hpf and the edema observed in these embryos was thought to be caused by abnormal filtration (Drummond et al., 1998). Pronephric mutants have been reported to develop kidney cysts and edema (Drummond et al., 1998; Horsfield et al., 2002). We consider that abnormalities in filtration would be lethal in zebrafish, which have only a single nephron. Therefore, in contrast to CDH6-knockout mice (Mah et al., 2000), which are both viable and fertile, cdh6 is necessary for the normal development and survival of zebrafish.

cdh6 and cdh17 seem to regulate development of the different parts of the pronephros during early embryogenesis.

During development, cdh6 and cdh17 are expressed in different regions of the pronephros, suggesting that these proteins have mutually supplementary roles in the morphogenesis of the pronephros. While cdh6 is expressed in the nephron primordia and the anterior portion of the pronephric ducts, cdh17 is expressed in the posterior portion of the pronephric ducts in the pronephros. On the other hand, the transcription factors wt1, pax2.1 and sim1 are thought to have regulatory effects specific to different parts of the zebrafish kidney (Serluca and Fishman, 2001). These factors seem to have some relationships to the cadherins. The spatial and temporal relationship between sim1 and cdh17 expression—with the timing of sim1 expression being slightly ahead of that of cdh17—suggests that sim1 may regulate the expression of cdh17 (Horsfield et al., 2002). On the other hand, cdh6 is expressed in the anterior region of the pronephros, which is positive for wt1 and pax2.1 in early embryogenesis and cdh6-knockdown mutants showed malformation in this area. Although these genes are first expressed in the intermediate mesoderm (Drummond et al., 1998), it is possible that wt1 and/or pax2.1 act upstream or downstream of cdh6. Further studies are
necessary to determine the factors involved in regulation of \textit{cdh6} and \textit{cdh17} expression during early nephrogenesis.

Materials and Methods

\textbf{Care of fish and obtaining zebrafish embryos}

Fish were purchased from a local pet shop and used as “wild-type.” These wild-type zebrafish were maintained and the embryos were obtained as described previously (Westerfield, 1995). They were staged in hours post-fertilization (hpf) at the standard temperature of 28.5°C. With the exception of the antisense Morpholino experiment, embryos for whole-mount \textit{in situ} hybridization were raised from 18 hpf throughout development in 0.003% 1-phenyl-2-thiourea (PTU; Wako Pure Chemical Ind., Osaka, Japan) to reduce pigmentation.

\textbf{Cloning of cadherin genes}

We screened a zebrafish cDNA library for molecules with homology to cadherin, which yielded a number of cadherin and protocadherin genes including \textit{cdh6} (Murakami \textit{et al.}, 2006). Zebrafish \textit{wtf} and \textit{pax2.1} (GenBank: \textit{wtf}, AY028627; \textit{pax2.1}, AF067534) were cloned by PCR using a zebrafish CDNA library and specific primers:

- \textit{wtf}: forward ATTTGCTCTGCTCCTGAAAGTCCTC reverse GGAACACAGTGTGTATTGCGACC
- \textit{pax2.1}: forward GCTGGCCTGTCCTTAATATGAGC reverse CAGGAAATAGTTCAAGTGGTGCC

\textbf{In situ hybridization (ISH)}

Digoxigenin-labeled RNA probes were synthesized using the cDNA clones as templates. Staged zebrafish embryos were stained by \textit{whole-mount ISH} as described previously (Weinberg \textit{et al.}, 1996; Bellipanni \textit{et al.}, 2000).

\textbf{Microinjection of cadherin-6 morpholinos and mRNA}

Fluorescein-labeled antisense Morpholino oligonucleotides (MOs) were purchased from Gene Tools (Philomath, OR, USA). These MOs were designed complementary to the 5' sequence near the translational initiation site of \textit{cdh6} (\textit{cdh6}MOs with the sequences: \textit{cdh6}MO-1, 5'-AACAGTTACATCCAACTCGCATC-3' (targets 5' sequence spanning the start codon, italicized); and \textit{cdh6}MO-2, 5'-ATCCTATCTGGACAAATGGTGCC-3' (directed against the sequence 5' of the UTR to the start codon). The negative control MO had the sequence: 5'-CCTTTAAAAATATTTATCTG-3' (Capped \textit{cdh6} mRNA was synthesized from \textit{cdh6}cDNA subcloned in the pCS2+ vector as described previously (Bellipanni \textit{et al.}, 2000). \textit{cdh6}MO alone (1 or 2 mg/ml) or with \textit{cdh6} mRNA (75 µg/ml) were injected into the blastomere of 1-cell embryos or into the yolk using an IM-300 microinjector (Narishige, Tokyo, Japan). Success of injection was determined by monitoring GFP fluorescence labeling of the Morpholino.

\textbf{Histological sections}

Zebrafish embryos stained by ISH for \textit{cdh6}, \textit{wtf} and \textit{pax2.1} were embedded in OCT compound (Miles Inc. Diagnostic Division, Elkhart, IN, USA) and snap-frozen in liquid nitrogen. Sections 5 or 6 µm thick were cut and mounted on glass slides for microscopy. In some cases, sections of 1–2 somite-thick were cut using a razor blade without freezing.

\textbf{Microscopy and image processing}

Embryos were mounted on 1.5% agarose plates with pit cast with 0.5-mm glass beads and viewed under an Olympus SZX-12 dissection microscope. A Nikon E-1000 compound microscope was used for examination of dissected embryos and histological sections.

Microscopic images were recorded with a C5810 chilled 3CCD camera (Hamamatsu Photonics, Shizuoka, Japan), a Spot RT SE6 Monochrome cooled CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA) or Olympus E-330 digital SLR camera and processed with Adobe Photoshop on a Macintosh personal computer to match colors to the real images. “Extended-focus” pictures were composed for thick specimens of whole- and flat-mount embryos by taking a series of 2–5 images of the specimen by shifting the focus manually. Each image was placed as an individual layer on a single Photoshop image. Out-of-focus areas in each layer were masked out to reveal the regions of interest of the specimen in-focus in the Photoshop image.

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\textbf{References}


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