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Original Article

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* (*cdh6*MOs) 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 *cdh6*MOs 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: *wt1* and *pax2.1* are also expressed in the developing glomerulus, 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.

TABLE 1

MALFORMATIONS INDUCED BY *CDH6* ANTISENSE MORPHOLINO OLIGONUCLEOTIDES (*CDH6*MOS)

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

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

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

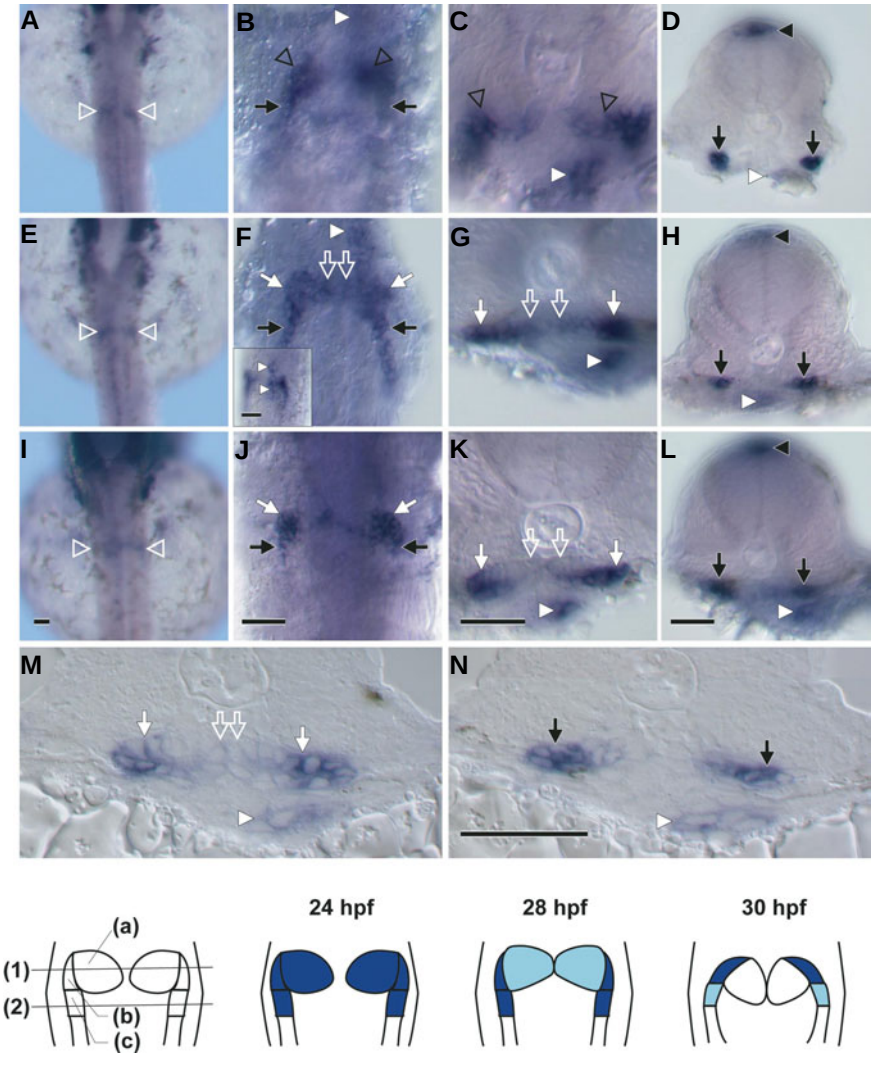

Fig. 1. *cdh6* is expressed in the pronephros during early embryogenesis. *Expression patterns in pronephric regions were determined by ISH at 24 (A–D), 28 (E–H) and 30 (I–N) hpf.* **(A,E,I)** *Dorsal views of whole-mount embryos with rostral toward the top.* **(B,F,J)** *Ventral views around the pronephric region with rostral toward the top.* **(C,G,K)** *Transverse sections of embryos at the nephron primordium region (Section level is 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 found 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*, *cdh6*MO-1 and *cdh6*MO-2, to induce targeted knockdown of *cdh6* function. Briefly, aliquots of about 8 nl of *cdh6*MOs 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 *cdh6*MOs, only those fulfilling both of these conditions were used for further experiments.

By 24 hpf, embryos injected with *cdh6*MOs 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 *cdh6*MO-injected embryos at 30 or 52 hpf (Fig. 2H, J, L, N).

Following the abnormal development in *cdh6*MO-injected embryos, areas of edema appeared in the thorax around 42 hpf,

TABLE 2

PRONEPHROS DISTURBED BY *CDH6* ANTISENSE MORPHOLINO OLIGONUCLEOTIDES (*CDH6*MOS)

Injection	Phenotypes revealed by ISH	n
No injection	N:100%	30
Negative control Morpholino	N:100%	50
*cdh6*MO-1	F:16.0% CV:20.8% CA:12.3% N:58.5%	106
*cdh6*MO-2	F:10.9% CV:16.3% CA:6.5% N:79.3%	92
*cdh6*MO-1 + *cdh6* mRNA	F:2.9% CV:4.4% CA: 1.5% N:94.1%	68
*cdh6*MO-2 + *cdh6* mRNA	F:1.8% CV:3.8% CA: 1.8% N:94.6%	56

Abbreviations: N, almost normal; F, both pronephric tubule primordia fused; CV, pronephric tubule primordia curved abnormally; CA, coarctated pronephric ducts

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

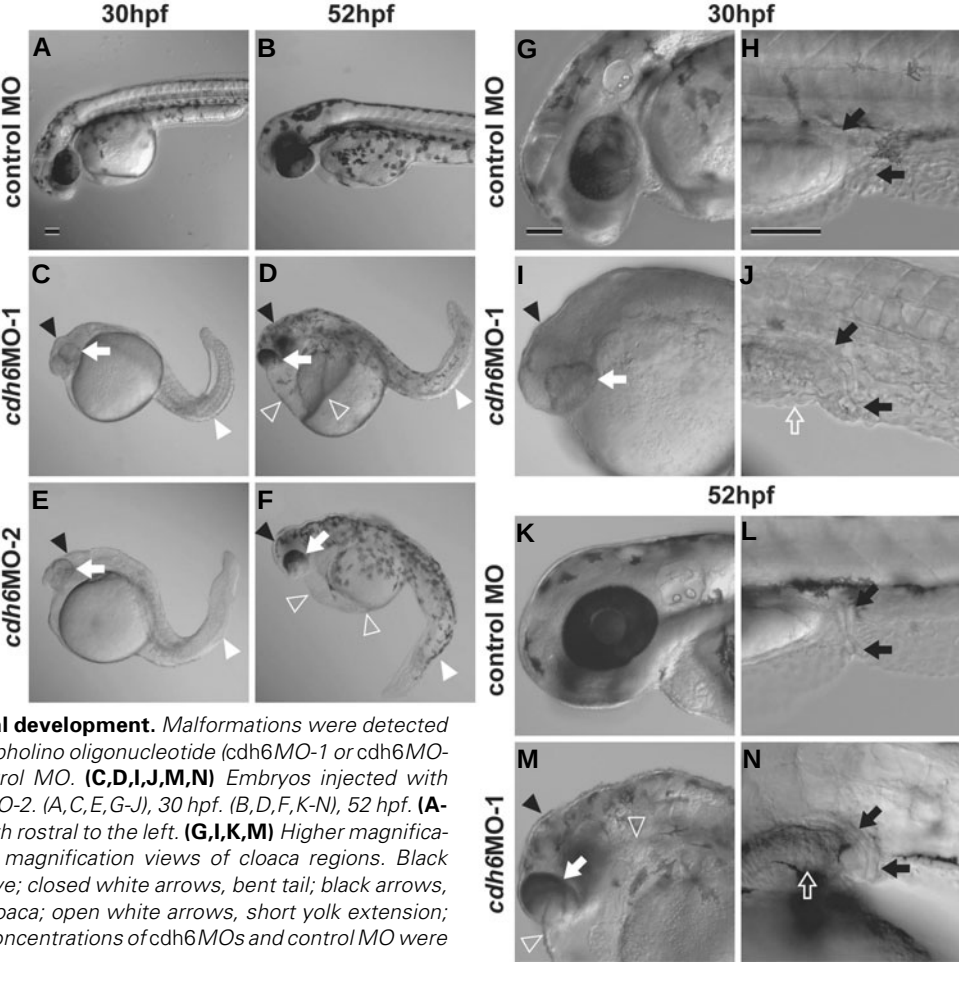

Fig. 2. Zebrafish *cdh6* is necessary for normal development. *Malformations were detected in zebrafish injected with a* cdh6 *antisense Morpholino oligonucleotide (*cdh6*MO-1 or* cdh6*MO-2).* **(A,B,G,H,K,L)** *Embryos injected with control MO.* **(C,D,I,J,M,N)** *Embryos injected with* cdh6*MO-1.* **(E,F)** *Embryos injected with* cdh6*MO-2. (A,C,E,G-J), 30 hpf. (B,D,F,K-N), 52 hpf.* **(A-F)** *Lateral views of whole-mount specimens with rostral to the left.* **(G,I,K,M)** *Higher magnification views of head regions.* **(H,J,L,N)** *Higher magnification views of cloaca regions. Black arrowheads, small head; white arrows, small eye; closed white arrows, bent tail; black arrows, posterior portion of the pronephric duct and cloaca; open white arrows, short yolk extension; open white arrowheads, edema in the thorax. Concentrations of* cdh6*MOs and control MO were 2 mg/ml. Scale bars, 200 μm.*

which became more marked at 52 hpf (Table 1; Fig. 2D, F, M). Embryos injected with *cdh6*MO-1 or *cdh6*MO-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 *cdh6*MOs-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 *cdh6*MO-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 *cdh6*MO-1 or *cdh6*MO-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: *cdh6*MO-1; F 16.0% CV 20.8% CA 12.3% N 58.5%. *cdh6*MO-2; F 10.9% CV 16.3% CA 6.5% N 79.3% (Table 2). To confirm specificity of the *cdh6*MOs, 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 *cdh6*MO-1 showed a disturbed appearance. The cells in the pronephros were disorganized and had an abnormal structure (Fig. 4D–F).

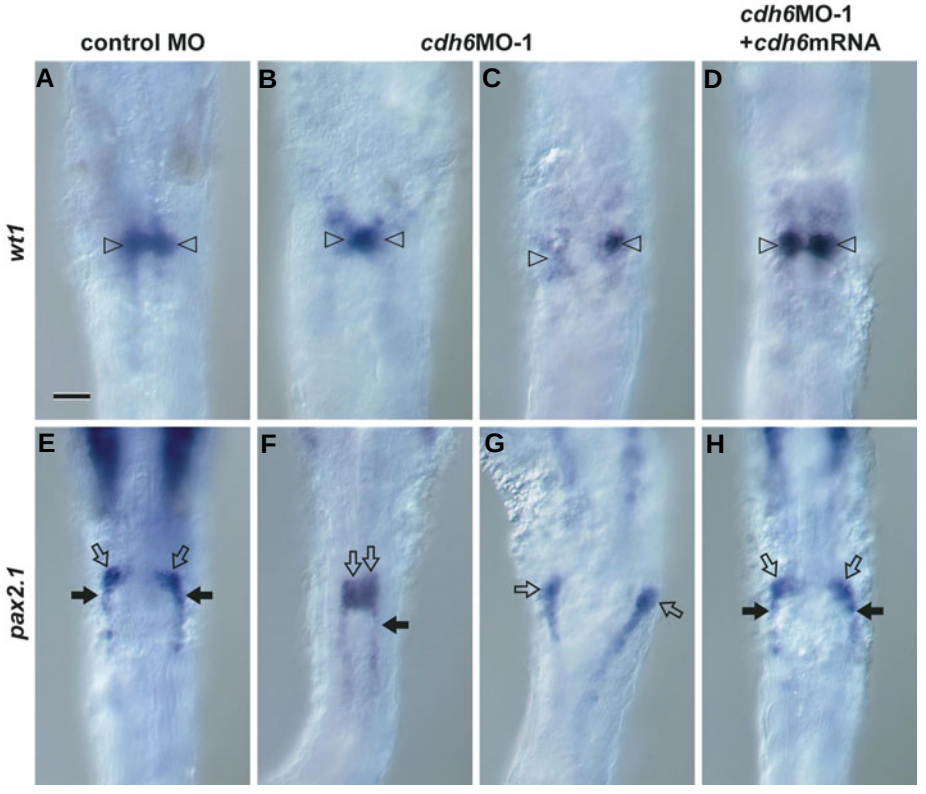

Fig. 3. Expression of *cdh6* is required for normal pronephros formation. *(A–H), Ventral views of the pronephric regions with rostral at the top at 30 hpf.* **(A,E)** *Embryos injected with control MO.* **(B,C,F,G)** *Embryos injected with* cdh6 *antisense MO (*cdh6*MO-1).* **(D,H)** *Embryos injected with* cdh6*MO-1 and* cdh6*mRNA.* **(A–D)** *Stained by* in situ *hybridization for* wt1 *to show glomerular primordia. Open arrowheads, glomerular primordia.* **(E–H)** *Stained for* pax2.1 *to show tubule primordia and pronephric ducts. Open arrows, tubule primordia; closed arrows, pronephric ducts. Concentrations of* cdh6*MO-1 and control MO were 1 mg/ml. Scale bar, 50 μm.*

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-

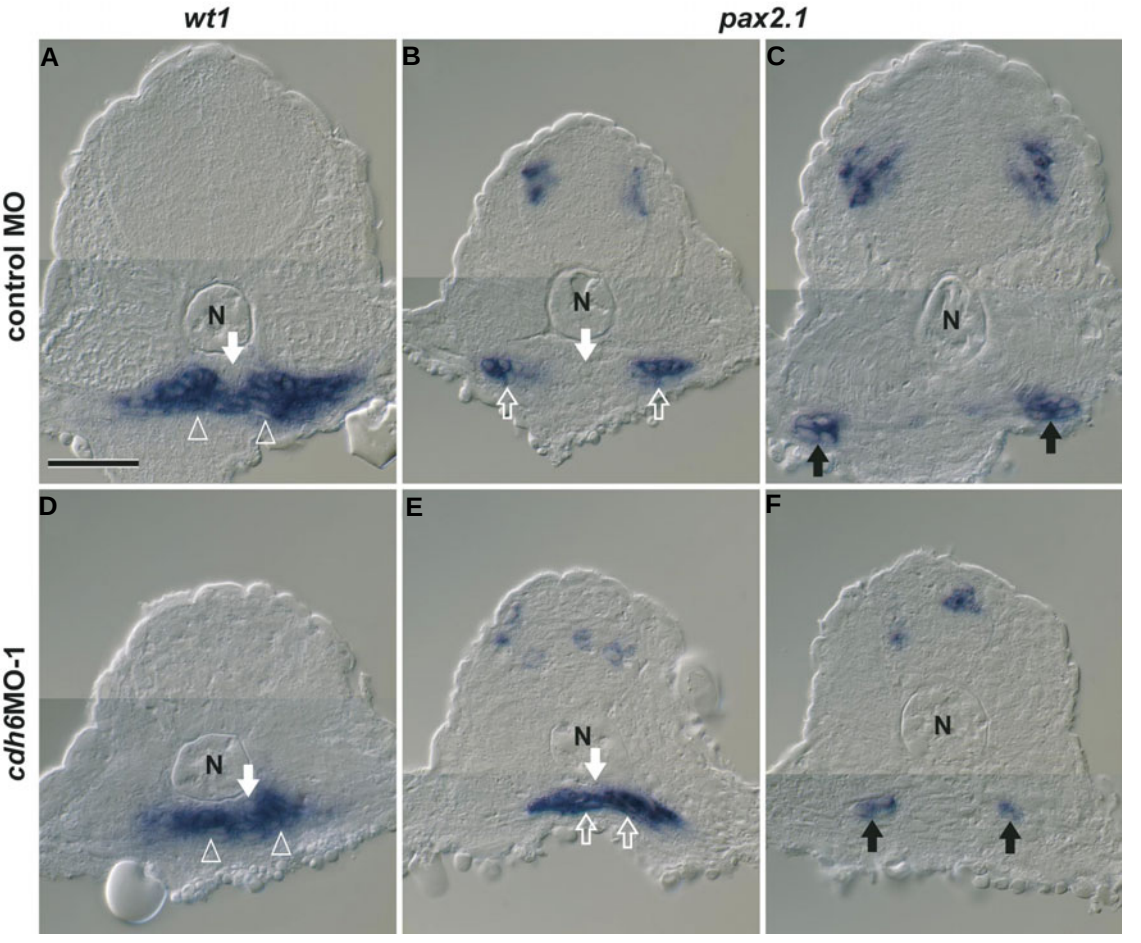

Fig. 4. Epithelial cells of affected embryos were deformed by *cdh6* MOs. *(A–F), Transverse histological sections of 5 μm thick at 30 hpf.* **(A–C)** *Embryos injected with control MO.* **(D–F)** *Embryos injected with* cdh6 *antisense MO* (cdh6*MO-1).* **(A,D)** *Stained by* in situ *hybridization for* wt1 *to show glomerular primordia.* **(B,C,E,F)** *Stained for* pax2.1 *to show tubule primordia and pronephric ducts. Open white arrowheads, glomerular primordia; open white arrows, tubule primordia; closed black arrows, pronephric ducts; closed white arrows, dorsal aorta; N, notochord. Concentrations of* cdh6*MO-1 and control MO were 1 mg/ml. Scale bar, 20 μm.*

lial cells in the latter (Cho *et al.*, 1998; Inoue *et al.*, 1998).

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, *chd17* 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 *cdh6* and *cdh17* expression during early nephrogenesis.

Materials and Methods

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 *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.

Cloning of zebrafish genes

We screened a zebrafish cDNA library for molecules with homology to cadherin, which yielded a number of cadherin and protocadherin genes including *cdh6* (Murakami *et al.*, 2006). Zebrafish *wt1* and *pax2.1* (GenBank: *wt1*, AY028627; *pax2.1*, AF067534) were cloned by PCR using a zebrafish cDNA library and specific primers:

wt1	forward	ATTTGCTCTGCTCCTGAAAGTCCTC
	reverse	GGAAACACAGTTGTTTATTGGCACC
pax2.1	forward	GCTTGCGGTCCCTTAAATATGTAGC
	reverse	CAGGAAATAGTTCAGTGATGGTGCC

In situ *hybridization (ISH)*

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

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 *cdh6* (*cdh6*MOs) with the sequences: *cdh6*MO-1, 5'-AAGAAGTACAATCCAAGTCCTCATC-3' (targets 5' sequence spanning the start codon, italicized); and *cdh6*MO-2, 5'-ATCCTATCTGCCAAAGTTACAGAGC-3' (directed against the sequence 5' of the UTR to the start codon). The negative control MO had the sequence: 5'-CCTCTTACCTCAGTTACAATTTATA-3'. Capped *cdh6* mRNA was synthesized from *cdh6* cDNA subcloned in the pCS2+ vector as described previously (Bellipanni *et al.*, 2000).

*cdh6*MO alone (1 or 2 mg/ml) or with *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.

Histological sections

Zebrafish embryos stained by ISH for *cdh6*, *wt1* and *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.

Microscopy and image processing

Embryos were mounted on 1.5% agarose plates with pits 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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