Transgenic Overexpression of *cdx1b* Induces Metaplastic Changes of Gene Expression in Zebrafish Esophageal Squamous Epithelium

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Abstract

Cdx2 has been suggested to play an important role in Barrett's esophagus or intestinal metaplasia (IM) in the esophagus. To investigate whether transgenic overexpression of *cdx1b*, the functional equivalent of mammalian *Cdx2* in zebrafish, may lead to IM of zebrafish esophageal squamous epithelium, a transgenic zebrafish system was developed by expressing *cdx1b* gene under the control of zebrafish keratin 5 promoter (*krt5p*). Gene expression in the esophageal squamous epithelium of wild-type and transgenic zebrafish was analyzed by Affymetrix microarray and confirmed by *in situ* hybridization. Morphology, mucin expression, cell proliferation, and apoptosis were analyzed by hematoxylin & eosin (HE) staining, Periodic acid Schiff (PAS) Alcian blue staining, proliferating cell nuclear antigen (PCNA) immunohistochemical staining, and TUNEL assay as well. *cdx1b* was found to be overexpressed in the nuclei of esophageal squamous epithelial cells of the transgenic zebrafish. Ectopic expression of *cdx1b* disturbed the development of this epithelium in larval zebrafish and induced metaplastic changes in gene expression in the esophageal squamous epithelial cells of adult zebrafish, that is, up-regulation of intestinal differentiation markers and down-regulation of squamous differentiation markers. However, *cdx1b* failed to induce histological IM, or to modulate cell proliferation and apoptosis in the squamous epithelium of adult transgenic zebrafish.

Introduction

BARRETT'S ESOPHAGUS (BE) is diagnosed when evidence of intestinal metaplasia (IM) is present in human esophagus. Histopathologically, IM is characterized by replacement of normal squamous epithelium of the esophagus by intestinalized columnar epithelium. Patients with BE are at an increased risk of developing esophageal adenocarcinoma, which is now the most rapidly increasing type of cancer in Western countries. However, the molecular mechanisms of BE are not fully understood.

Transcription factors are known to play causative roles in metaplasia.^{1–3} As a Caudal-related homeobox gene that is essential for skeletal and intestinal development, *Cdx2* has been suggested to play an important role in IM (e.g., esophagus, stomach, and bile duct) and cancers (e.g., colon cancer, leukemia).^{4,5} Squamous epithelial cells of normal human esophagus

do not express *Cdx2*, while submucosal glands weakly express *Cdx2* protein in the cytoplasm. In human BE, *Cdx2* is expressed in both goblet and nongoblet cells.⁶ Many "marker" genes of BE, such as villin and sucrase-isomaltase, are known to be regulated by *Cdx2*.^{7,8} Gene expression profiling of human BE suggested that multiple genetic pathways and transcription factors, especially *Cdx2*, might play a critical role in the development of BE.⁹ Stable transfection of *Cdx2* in esophageal squamous epithelial cells caused metaplastic changes in both morphology and gene expression.¹⁰ *In vivo*, stomach-specific *Cdx2* transgenic overexpression induced IM in the mouse stomach within weeks after birth.^{11,12} Homozygous knockout of *Cdx2* was embryonically lethal, and heterozygous knockout produced colonic hamartoma with squamous epithelium appearing in the colon.¹³ These studies suggested that *Cdx2* might be a pivotal switch between intestinal columnar epithelium and squamous epithelium in the gastrointestinal tract.

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Rodents are the most commonly used animals to study BE and EAC.^{14,15} However, rodent esophagus is covered by keratinized squamous epithelium in contrast to the non-keratinized squamous epithelium in human esophagus. Such histological differences may create problems in inducing IM in rodent esophagus.^{14,16} In fact, transgenic overexpression of *Cdx2* in the esophagus failed to produce IM in mice.¹⁷

Recently, zebrafish (*Danio rerio*) has attracted much attention as a model system for human diseases due to its many favorable characteristics, including short generation time, optical transparency of embryos, prolific reproduction, external development, and easy maintenance of both the adult and the young.^{18,19} A small piece of stratified squamous epithelium was found in zebrafish upper digestive tract, and a newly cloned zebrafish keratin 5 promoter (krt5p) was capable of driving GFP expression in the squamous epithelial cells.²⁰ This system allows us to test whether transgenic overexpression of *Cdx2* may induce IM in the esophageal squamous epithelium. Zebrafish has three homeobox genes of the Caudal family, *cdx1a*, *cdx1b*, and *cdx4*.²¹ Although it was named on the basis of syntenic conservation with mammalian *Cdx1*, *cdx1b* is the functional equivalent of mammalian *Cdx2*, while *cdx1a* functions similar to mammalian *Cdx1*.^{22–24}

In this study, in order to investigate the role of *Cdx2/cdx1b* in IM of squamous epithelium, we generated *cdx1b* transgenic zebrafish lines driven by krt5p. Changes in gene expression and morphology were analyzed in the squamous epithelium of transgenic zebrafish. Our study demonstrated that transgenic overexpression of *cdx1b* disturbed the development in esophageal squamous epithelium in larval zebrafish, and it induced metaplastic changes of gene expression in adult zebrafish, that is, up-regulation of intestinal differentiation markers and down-regulation of squamous differentiation markers. However, metaplastic changes in morphology were not observed in adult zebrafish.

Materials and Methods

Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committees (IACUC) at North Carolina Central University. IACUC control number is XC-07-15-2008.

Zebrafish maintenance and collection of embryos

Zebrafish were obtained from the Zebrafish International Resource Center. To generate *cdx1b* transgenic fish, AB* line was used. Fish were housed in an automatic fish housing system (Aquaneering) at 28.5°C and maintained according to the established procedures.²⁵ Stages of embryos were indicated as hour postfertilization (hpf) at 28.5°C.²⁶

Construction of the *pminiTol2-krt5p-cdx1b-EGFP* and *pDestTol2CG2-krt5p-cdx1b* plasmids

A 768-bp *cdx1b* fragment was amplified by PCR with two pairs of primers (Table 1). IMAGE clone 7907462 (Open Biosystems) containing the *cdx1b* cDNA sequence was used as the PCR template. Restriction sites (*in italic*, Table 1) were added to the forward and reverse primers, respectively, to aid the ligation process.

The reporter gene vector used in the present study, *pminiTol2-krt5p-EGFP*, was a homemade vector with krt5p representing zebrafish krt5p. The *cdx1b* PCR products without the stop codon were gel purified and cloned into the *pminiTol2-krt5p-EGFP* vector in front of the EGFP coding region at the *Bam*HI and *Age*I sites, in order to create the *pminiTol2-krt5p-cdx1b-EGFP* plasmid. The *pT3TS-Tol2* vector²⁷ was cleaved with *Xba*I and transcribed with T3 RNA polymerase using the mMessage Machine kit (Ambion), to produce capped Tol2 transposase RNA.

To generate the *pDestTol2CG2-krt5p-cdx1b* plasmid with GFP expression in the heart, multisite recombination reactions were performed as described in the Invitrogen Multisite Gateway Manual with minor modifications. The empty vector, *pDestTol2CG2*, is one vector of our vector collection named "Tol2Kit." CG2 indicates the 2nd modified version of the vector with a *cmlc2* gene promoter and the *egfp* as an effector gene. P5'E-krt5p and PME-*cdx1b* plasmids were constructed first. Equimolar amounts (20 fmol) of destination vector (*pDestTol2CG2*), P5'E-krt5p, PME-*cdx1b*, and P3'E-EGFPpA vectors were combined with TE and LR Clonase II Plus Enzyme Mix in a final volume of 10 μ L. Reactions were incubated at room temperature overnight, then treated with proteinase K for 10 min at 37°C, and 2.5 μ L was used for transformation into Subcloning Efficiency DH5a Chemically Competent *Escherichia coli* cells (Invitrogen).

Microinjection and detection of GFP expression

Transgenic zebrafish were generated by a microinjection of one cell stage fertilized AB* zebrafish eggs with a mixture of *pminiTol2-krt5p-cdx1b-EGFP* (80 μ g/mL) and Tol2 transposase RNA (4 μ g/mL),²⁷ or a mixture of *pDestTol2CG2-krt5p-cdx1b* (80 μ g/mL) and Tol2 transposase RNA (4 μ g/mL), respectively. The injected zebrafish were screened for chimeric epidermal green fluorescence at 24hpf with an Olympus MVX10 macro-view microscope. Fluorescent images were captured using a Nikon SMZ-1500 stereomicroscope. Thirty GFP-positive founder fish were raised to adulthood and were interbred. Only 10% of the F1 offspring was picked up with homogeneous green fluorescence in the nucleus of epidermal cells. Finally, three adult F1 fish were used to generate stable transgenic zebrafish line (*pminiTol2-krt5p-cdx1b-EGFP*). This transgenic zebrafish line was used for the following experiments.

In situ hybridization of tissue sections

In situ hybridization using digoxigenin-labeled riboprobes was carried out as previously described.²⁸ The plasmid DNA, *pExpress1-cdx1b* (ATCC), was linearized with proper restriction enzymes for *in vitro* transcription with T7 RNA polymerase. Sense probes were also transcribed for control experiments. The procedure of *in situ* hybridization on paraffin sections was based on the protocol of a commercial kit (IsHyb *in situ* Hybridization kit; Biochain Institute). The sections were washed and covered with cover slips for observation using an Olympus BX51 microscope.

Other templates were amplified from zebrafish total RNA by RT-PCR with T7 promoter sequence in the antisense primer. Antisense RNA probes were generated for detection of the following genes: *Fabp2*, *Fabp6*, *Cdh17*, *Vil11*, *Aqp3*, *Tp63*, *Anxa1c*, *Agr2*, *Slc15a1*, *Nkx2.2a*, and *Sox17* (Table 1). Sense probes were prepared as controls.

TABLE 1. PCR PRIMERS USED IN THIS STUDY

Primer pair	Sequence ^a	Description	Purpose
1	5' CG GGATCC ATGTACGTGAGTTATCTCCTAGA 3' 5' CG ACCGGT CGATACTCTTCTTTGATGGACATT 3'	<i>cdx1b</i> S-primer (<i>Bam</i> HI)& As-primer (<i>Age</i> I)	Pminitol2-krt5krt5p-cdx1b-EGFP
2	5' CG GGATCC ATGTACGTGAGTTATCTCCTAGA 3' 5' CG GCGGCCGC TCATACTCTTCTTTGATGGACATT 3'	<i>cdx1b</i> S-primer (<i>Bam</i> HI)& As-primer (<i>Not</i> I)	PME-MCS
3	5' CG GCGGCCGC ATGTACGTGAGTTATCTCCTAGA 3' 5' CG GAGCTC TCAATACTCTTCTTTGATGGACATT 3'	<i>cdx1b</i> S-primer (<i>Not</i> I) & As-primer (<i>Sac</i> I)	<i>In situ</i> probe (pExpress-cdx1b), RT-PCR
4	5' ATGTCTACTTCCTTCAAAACCTTC 3' 5' TTCTGGTTGACTGTGACAGCTG 3'	<i>krt5</i> S-primer & As-primer	RT-PCR
5	5' ATGACCTTCAACGGGACCTG 3' 5' TAATACGACTCACTATAGGGAGA TTAAGCCCTCTTGAAAATCCTCT 3'	<i>Ifabp</i> S-primer & As-primer	<i>In situ</i> probe
6	5' ATGGCTTTC AACGGCAAG 3 5' TAATACGACTCACTATAGGGAGA TAAACCTTCTTGCTTGTGCG 3'	<i>Fabp6</i> S-primer& As-primer	<i>In situ</i> probe
7	5' ATGAGAGGTGCACCTTTTGCT 3' 5' TAATACGACTCACTATAGGGAGA GTTTGGATTTATGCCAAAGA 3'	<i>Cdh17</i> S-primer& As-primer	<i>In situ</i> probe
8	5' CTAGCCAAGGACATCCGC 3' 5' TAATACGACTCACTATAGGGAGA ATTGCTCATCATCTCCTTCC 3'	<i>Vil1l</i> S-primer & As-primer	<i>In situ</i> probe
9	5' ATGGGTTGGCAGAAAAGC 3' 5' TAATACGACTCACTATAGGGAGA CTGTGAAGGCCTCAAGACCT 3'	<i>Aqp3</i> S-primer & As-primer	<i>In situ</i> probe
10	5' ATGTTGTACCTGGAGACCAA 3' 5' TAATACGACTCACTATAGGGAGA ATCTCTGGTTTCCAATGTGA 3'	<i>Tp63</i> S-primer & As-primer	<i>In situ</i> probe
11	5' ATGGCCTTTTCCAGAAA 3' 5' TAATACGACTCACTATAGGGAGA GCGTCATCAATATTACGATC 3'	<i>Anxa1c</i> S-primer & As-primer	<i>In situ</i> probe
12	5' ATGCTCAAAGGATTACTGTCACTG 3' 5' TAATACGACTCACTATAGGGAGA TCACAACCTCGGTCTTCAGAAACT 3'	<i>Agr2</i> S-primer & As-primer	<i>In situ</i> probe
13	5' ATGGCAGACAAAGAGGGACAC 3' 5' TAATACGACTCACTATAGGGAGA CTAGATCT TGGTTTGCTTGATGTT 3'	<i>Slc15a1</i> S-primer & As-primer	<i>In situ</i> probe
14	5' ATGTCGTTGACCAACACAAAGAC 3' 5' TAATACGACTCACTATAGGGAGA TCACCAAGT CCACTGTTGCG 3'	<i>Nkx2.2a</i> S-primer & As-primer	<i>In situ</i> probe
15	5' ATGAGCAGTCCCGATGCG 3' 5' TAATACGACTCACTATAGGGAGA TCAAGAATTATTATAGCCGCAGTAGT 3'	<i>Sox17</i> S-primer & As-primer	<i>In situ</i> probe

^aItalic sequences are restriction sites indicated in parentheses of column "Description." Underlined sequences are T7 RNA polymerase promoter sequences.

S, sense; As, antisense.

Immunohistochemical staining

We custom synthesized four peptide antigens based on the Antigenic Determinant analysis and generated four rabbit anti-cdx1b polyclonal antibodies (Genscript Corporation). The amino acid sequences of these peptides were as follows: Peptide 1: YPPPREEWTPYGPC (AA: 66–79); Peptide 2: CGQLSPNAQRRNPYD (AA: 115–128); Peptide 3: CPPASS-GGKTRTKDK (AA: 135–148); and Peptide 4: CRRAKE-RKINKKKMQ (AA: 197–210). Additional N'-terminal or C'-terminal cysteine was added for KLH conjugation. To produce anti-cdx1b antibodies, rabbits were immunized with peptide preparation mixed with adjuvant. Antibody affinity purification and ELISA test were performed to confirm the activity and titer of antibody. These antibodies were tested by immunohistochemical staining on paraffin sections. Only the

antibody against Peptide 4 worked well and was chosen for this study.

In brief, paraffin-embedded tissue sections were deparaffinized, rehydrated, and pretreated by heating the slides for 5–10 min in 10 mM citrate buffer. Staining was performed with the ABC kit (Vector Laboratories) according to the manufacturer's instructions with a rabbit anti-cdx1b polyclonal antibody at the concentration of 20 µg/mL, or a mouse anti-proliferating cell nuclear antigen (PCNA) polyclonal antibody (1:2000; Sigma) for detection of cdx1b or PCNA. Normal serum or phosphate-buffered saline was used as a negative control, instead of the primary antibodies. Both positive and negative control slides were processed in parallel.

Proliferation index (percentage of PCNA-positive cells) was calculated by counting the number of positively stained

cells and the total number of epithelial cells on serial sections from *cdx1b* transgenic and wild-type zebrafish.

RT-PCR and Affymetrix gene microarray

The upper digestive tract tissue from three adult zebrafish of F1 generation (3 months old) was dissected and pooled as one sample. Three *cdx1b* transgenic samples and two wild-type samples were used for microarray analysis. The total RNA was extracted using TRIzol (Invitrogen). After RNase-free DNase I treatment (Qiagen), RNA was reverse transcribed to cDNA templates using oligo dT primer and RT enzyme mix (New England Biolabs) and amplified using a Phusion™ Hotstart DNA polymerase (New England Biolabs). Primers used for RT-PCR were described in (Table 1). PCR products of keratin 5 and *cdx1b* were 400 and 768 bp, respectively.

GeneChip Zebrafish Genome Array (Affymetrix) containing 15,509 oligo probes were used to detect differential gene expression between upper digestive tract of the wild-type and *cdx1b* transgenic zebrafish. One microgram of total RNA was used for labeling and processing after quality validation. GeneChip hybridization and scanning were performed according to the Affymetrix protocols. Briefly, double-stranded cDNA containing T7 promoter (Genset) was synthesized from total RNA using the SuperScript Choice System (Invitrogen). Biotinylated cRNAs were generated from cDNAs by *in vitro* transcription and amplified by using the BioArray T7 RNA polymerase labeling kit (Enzo Diagnostics). After purification of cRNAs by GeneChip Sample Cleanup Module (Affymetrix), 15 µg of cRNA was fragmented at 94°C for 35 min. Approximately 12.5 µg of fragmented cRNA was used in a 250-µL hybridization mixture containing herring-sperm DNA (0.1 mg/mL; Promega), plus bacterial and phage cRNA controls (1.5 pM BioB, 5 pM BioC, 25 pM BioD, and 100 pM Cre) to serve as internal controls for hybridization efficiency. Aliquots (200 µL) of the mixture were hybridized to arrays for 16 h at 45°C in a GeneChip Hybridization Oven 640 (Affymetrix). Each array was washed and stained with streptavidin-phycoerythrin (Invitrogen) and amplified with biotinylated anti-streptavidin antibody (Vector Laboratories) on the GeneChip Fluidics Station 450 (Affymetrix). Arrays were scanned with the GeneArray G7 scanner (Affymetrix) to obtain image and signal intensities. Data analysis was performed using the Affymetrix MAS5 algorithm. The significance level was defined as $p < 0.05$. The gene expression data were submitted to Gene Expression Omnibus database (accession no. GSE35889).

Histological staining

Paraffin sectioning and hematoxylin & eosin (HE) staining were conducted based on standard procedures. All squamous epithelial cells in each serial section were counted and added up to represent the number of squamous epithelial cells of a fish. For Periodic acid Schiff (PAS) Alcian blue staining, paraffin sections were first dewaxed and rehydrated. After treatment with 3% acetic acid, sections were incubated with 1% Alcian blue 8Gx in acetic acid for 30 min and then washed with distilled water. Sections were further stained with 0.5% periodic acid and then Schiff's reagent according to standard procedures.

For TUNEL assay, paraffin sections were dewaxed and rehydrated. They were treated with 10 µg/mL proteinase K

for 15 min at room temperature before labeling of DNA breaks by terminal deoxynucleotidyl transferase with TDT-dNTP mix according to the protocol provided by the manufacturer (TACS™ TDT kit; R&D Systems). Apoptotic cells were visualized with DAB, and slides were counterstained with methyl green. Apoptosis index (percentage of TUNEL-positive cells) was calculated by counting the number of positively stained cells and the total number of epithelial cells in serial sections from *cdx1b* transgenic and wild-type zebrafish.

Results

Nuclear expression of *cdx1b* in squamous epithelial cells of transgenic zebrafish

In order to develop a transgenic zebrafish system for investigation on the function of *cdx1b* in IM, *cdx1b* gene was amplified by PCR. pminiTol2-krt5p-*cdx1b*-EGFP and pDest-Tol2CG2-krt5p-*cdx1b* constructs were made by cloning *cdx1b* cDNA under the control of krt5p (Fig. 1A, B). These constructs were injected into zebrafish eggs at one-cell stage.

For embryos injected with pDestTol2CG2-krt5p-*cdx1b*, approximately 60% of injected embryos expressed GFP in the heart (arrow; Fig. 1D), and were raised to the adult stage. Of 25 founder zebrafish screened, no GFP-expressing embryos were collected. For embryos injected with pminiTol2-krt5p-*cdx1b*-EGFP, approximately 70% of injected embryos expressed GFP in the periderm (arrows; Fig. 1E). Thirty GFP-positive founder fish were raised to adulthood and were interbred. Only 10% of the F1 offspring was picked up with homogeneous green fluorescence in the nucleus of epidermal cells. Finally, three adult F1 fish were used to generate stable transgenic zebrafish lines. GFP expression in the periderm was consistent with our previous study showing GFP expression in the periderm of embryos and in the esophagus and tongue of adult fish under the control of krt5p,²⁰ suggesting that the expression pattern of *cdx1b* under the control of krt5p was similar to endogenous keratin 5 gene expression. Scattered and dotted expression pattern suggested nuclear localization of *cdx1b*. The F1 generation embryos expressed GFP with a largely identical pattern without mosaicism to founder embryos, indicating germ-line transmission of the transgene (Fig. 1F, G). We chose F1 generation of pminiTol2-krt5p-*cdx1b*-EGFP transgenic zebrafish for the following experiments. GFP expression was not observed in wild-type zebrafish (Fig. 1C).

cdx1b expression in the squamous epithelium of transgenic fish was analyzed by *in situ* hybridization, immunohistochemical staining, and RT-PCR. Paraffin sections of adult zebrafish (3 months old) were hybridized with the *cdx1b* probe, and showed that *cdx1b* mRNA was uniformly expressed in the squamous epithelial cells (Fig. 2A). This expression pattern was similar to that of endogenous keratin 5.²⁰ As expected, we did not observe any *cdx1b* mRNA expression in the squamous epithelial cells of wild-type zebrafish (data not shown).

One custom polyclonal antibody of *cdx1b* worked well for immunohistochemical staining in paraffin tissue sections. *cdx1b* protein was strongly expressed in the nuclei of squamous epithelial cells of transgenic zebrafish (Fig. 2B), but not in those of wild-type zebrafish. Slight background staining in the cytoplasm and stroma was observed in both the wild-type and transgenic zebrafish. As positive controls, we observed high-level expression of *cdx1b* in intestinal epithelial cells (data not shown).

FIG. 1. Transgenic constructs and expression of *cdx1b* in zebrafish. pminiTol2-krt5p-cdx1b-EGFP plasmid (8.7 kb, **A**) and pDestTol2CG2-krt5p-cdx1b plasmid (9.3 kb, **B**) were constructed. GFP expression was observed in the heart of pDestTol2CG2-krt5p-cdx1b-injected founder embryos (**D**, arrow) and in the periderm of pminiTol2-krt5p-cdx1b-EGFP-injected founder embryos (**E**, arrow), but not in wild-type control embryos (**C**). In the stable F1 transgenic zebrafish, GFP expression was strong in some embryos (**F**), and weaker in some others (**G**). krt5p, keratin 5 promoter. Color images available online at www.liebertpub.com/zeb

Expression of *keratin 5* gene was examined by RT-PCR to determine the integrity and the specificity of RNA samples extracted from the squamous epithelium. RT-PCR of *keratin 5* and *cdx1b* confirmed transgenic expression of *cdx1b* in transgenic zebrafish. As expected, wild-type zebrafish expressed *keratin 5*, but not *cdx1b* (Fig. 2C). These RNA samples were further used in the following experiment with gene microarray. Overall, our data demonstrated that *cdx1b* was overexpressed in the nuclei of squamous epithelial cells in the *cdx1b* transgenic zebrafish.

Changes of gene expression in squamous epithelial cells induced by *cdx1b*

Using Affymetrix arrays, we examined the gene expression profile of esophageal squamous epithelium in *cdx1b* transgenic zebrafish as compared with wild-type zebrafish (Excel S1). We categorized these genes into two groups, genes up-regulated by *cdx1b* and genes down-regulated by *cdx1b*, with a cutoff value >2 or <-2 and a *p*-value <0.05 (Table 2).

FIG. 2. *cdx1b* expression in the squamous epithelium of *cdx1b* transgenic zebrafish (3-month-old, F1 generation). *cdx1b* expression was shown by *in situ* hybridization (**A**), immunohistochemical staining (**B**), and RT-PCR (**C**). Dashed lines indicate the basal membrane of squamous epithelium. Nuclear localization of *cdx1b* is shown by arrows. M, 1 kb DNA ladder; IN, intestine (positive control of *cdx1b* and negative control of *Krt5*). Scale bars: 50 μ m. Color images available online at www.liebertpub.com/zeb

- (1) Several up-regulated genes (e.g., *Fabp1b*, *Fabp6*, *Fabp2*, *Ins*, *Cdh17*, *Apob*, *Gcga*, *Gata5*, *Acat2*, and *cdx1a*) are known to be regulated by *Cdx2* or *cdx1b*.^{9,16,23,24}
- (2) Several up-regulated genes are known differentiation markers of intestinal cell lineages. For example, *Fabp2*, *Cdh17*, and *Vil1l* are known markers of columnar epithelial cells, and *Ins* is a marker of enteroendocrine cells.^{9,22,29}
- (3) Several up-regulated genes (e.g., *Fabp1b*, *Mep1a.1*, *Vil1l*, *Cdh17*, *Gata5*, *Hnf4g*, *Cdx4*, *Hnf4a*, *Selenbp1*, *Gata6*, and *Lipf*) have already been reported to be up-regulated in human BE as compared with the normal epithelium.⁹
- (4) Several down-regulated genes (e.g., *Tp63*, *Krt4*, *Pax9*, *Sox2*, *Ppl*, *Anxa1a*, *Aqp3*, *Anxa1b*, and *Anxa1c*) are known to be expressed at a lower level in human BE than in the normal epithelium.⁹
- (5) Several down-regulated genes (e.g., *Evpl*, *Krt15*) are known to be expressed in normal mammalian esophagus.^{30,31}

Differential gene expression induced by *cdx1b* was validated by *in situ* hybridization. As expected, *Fabp2* was up-regulated in the squamous epithelial cells by *cdx1b* (Fig. 3A), but not expressed in those of wild-type zebrafish (Fig. 3C). Intestinal epithelial cells of zebrafish had strong expression of *Fabp2* as positive control (Fig. 3B). Consistent with the microarray data, *Fabp6* and *Cdh17* were induced in the squamous epithelial cells by *cdx1b* (Fig. 3D, E). Similar to *Fabp2*, they were not expressed in the squamous epithelial cells of wild-type zebrafish (data not shown).

Since zebrafish has three intestinal cell lineages, columnar epithelial cells, goblet cells, and enteroendocrine cells,²² expression of marker genes of these lineages was examined in *cdx1b* transgenic and wild-type zebrafish. *Agr2* was chosen as a marker gene of intestinal goblet cells, *Nkx2.2a* as a marker gene of enteroendocrine cells, and *Slc15a1* as a marker gene of columnar epithelial cells.^{22,32,33} All of these genes were known to be regulated by *Cdx2* or *cdx1b*.^{22,24} *In situ* hybridization demonstrated overexpression of *Agr2*, *Nkx2.2a*, and *Slc15a1* in the squamous epithelial cells of *cdx1b* transgenic zebrafish (Fig. 3F, G, H), but not in wild-type zebrafish (data not shown). When *Sox17*, another crucial transcription factor for endoderm development,³⁴ was examined by *in situ* hybridization, we found overexpression of *Sox17* in the squamous epithelial cells of *cdx1b* transgenic zebrafish (Fig. 3I), but not in wild-type zebrafish. These results demonstrated that *cdx1b* transgene functioned properly in inducing the expression of intestinal marker genes, which is consistent with its role in IM.

Morphology of squamous epithelium in *cdx1b* transgenic zebrafish

HE staining was performed on the serial sections of larval zebrafish. In wild-type zebrafish at 7 and 14 days post-fertilization (dpf), the esophageal squamous epithelium was composed of basal, parabasal, superficial, and dead cell layers (Fig. 4A, C). In ~50% *cdx1b* transgenic zebrafish at 7 dpf, this epithelium was featured by small cells without distinct features of basal, parabasal or superficial layers, and lack of dead cells on the surface (Fig. 4B). In the zebrafish that survived at 14 dpf, this epithelium showed no obvious abnormality. The number of squamous epithelial cells in the

transgenic zebrafish was significantly less than that in the wild-type zebrafish at 7 dpf. However, no significant difference was observed between transgenic and wild-type zebrafish at 14 dpf (Fig. 4C, D, M). This is probably due to the death of the transgenic zebrafish with severe abnormalities before 14 dpf. No significant change was observed with HE staining in adult *cdx1b* transgenic zebrafish as compared with wild-type zebrafish (Fig. 4E, F). The squamous epithelium was further stained with PAS Alcian blue to identify acidic mucins that characterize intestinal phenotype. No significant change was found with PAS Alcian blue staining in adult *cdx1b* transgenic zebrafish as compared with wild-type zebrafish (Fig. 4Q, R).

Immunohistochemical staining using an anti-PCNA antibody was performed to analyze cell proliferation (Fig. 4G–L), and the percentage of PCNA-positive cells was counted in the squamous epithelium of both *cdx1b* transgenic and wild-type zebrafish (Fig. 4N). No significant difference in the proliferation index was observed between wild-type and transgenic zebrafish at these time points. We failed to detect apoptotic cells in the squamous epithelium at 7 and 14 dpf with the method of TUNEL. In adult fish, only very few apoptotic cells were detected (Fig. 4O, P). These data suggested that transgenic overexpression of *cdx1b* interfered with morphogenesis of zebrafish esophageal squamous epithelium at the early stage. It is very likely that early death of those zebrafish with severe abnormalities does not allow us to observe histological phenotypes in cell proliferation, apoptosis, or metaplasia later on.

Discussion

Many previous studies by others and us have strongly suggested *Cdx2* as a crucial gene in IM of the esophagus.^{5,10,12,16} In this study, we developed tissue-specific transgenic zebrafish to investigate the functional role of *cdx1b*, the functional equivalent of mammalian *Cdx2*, in IM of squamous epithelium. We found that transgenic overexpression of *cdx1b* up-regulated intestinal differentiation markers and down-regulated squamous differentiation markers in the squamous epithelium. However, histological IM was not observed.

Cdx2 gene is a master regulator in the posterior endoderm and is essential for the establishment of intestinal identity.³⁵ Ectopic expression of *Cdx2* has been detected in the IM of the gastrointestinal tract, such as gastric mucosa, biliary mucosa in intraductal papillary neoplasia of the liver associated with hepatolithiasis, Barrett's epithelium, and inflammatory esophageal mucosa.^{36–38} Similar to *Cdx2*, *cdx1b* plays important roles in regulating proliferation and differentiation of various intestinal cell lineages. Antisense morpholino oligonucleotide mediated knockdown, and overexpression analyses revealed that zebrafish *cdx1b* regulated the expression of several downstream factors involved in early endoderm development.²³ *Fabp2* and *Slc15a1* are terminal differentiation markers for intestinal columnar epithelial cells and required for absorptive enterocyte function.²² *Agr2* is a secreted protein that is produced by goblet cells and plays a specialized role in intestinal mucus production.³⁹ Loss of *Nkx2.2a* expression within the developing gut mucosa affects differentiation toward the enteroendocrine lineage.⁴⁰ *Cdh17* is an intestine-specific cell adhesion molecule that is ectopically expressed in the metaplastic mucosa.²⁹ As

TABLE 2. DIFFERENTIAL GENE EXPRESSION INDUCED BY TRANSGENIC OVEREXPRESSION OF *CDX1B* IN ZEBRAFISH ESOPHAGUS

Affymetrix probe ID	Gene title	Gene symbol	p-Value (TG vs. C; <0.05)	Fold change (TG vs. C)	Comment ^a
<i>Genes up-regulated by cdx1b</i>					
Dr.24261.1.S1_at	Fatty acid binding protein 1b	<i>fabp1b</i>	4.13E-08	709.024	Cdx1, Cdx2, BE
Dr.18599.1.S1_at	Fatty acid binding protein 6, ileal (gastrotropin)	<i>fabp6</i>	2.57E-06	235.958	Cdx2
Dr.8143.1.S1_at	Fatty acid binding protein 2, intestinal	<i>fabp2</i>	3.32E-05	53.208	cdx1b
Dr.25237.1.A1_at	Meprin A, alpha.1	<i>mep1a.1</i>	9.581E-05	50.9836	BE
Dr.11838.1.S1_at	Preproinsulin	<i>ins</i>	9.02E-05	45.0255	Cdx2
Dr.6348.1.S1_at	Villin 1 like	<i>vil1l</i>	9.58E-06	44.5119	BE
Dr.3609.1.S1_at	Cadherin 17, LI cadherin (liver-intestine)	<i>cdh17</i>	6.0E-04	26.4975	BE, Cdx2
Dr.4002.1.A1_at	Apolipoprotein B	<i>apob</i>	1.53E-04	20.2748	Cdx1, Cdx2
Dr.10728.1.S1_at	Glucagon a	<i>gcga</i>	1.19E-04	19.0772	Cdx2
Dr.8100.1.S1_at	GATA-binding protein 5	<i>gata5</i>	3.37E-04	18.0811	BE, cdx1b
Dr.1835.1.S1_at	Hepatocyte nuclear factor 4, alpha	<i>hnf4a</i>	5.20E-04	15.2341	BE
Dr.22420.1.A1_at	Hepatocyte nuclear factor 4, gamma	<i>hnf4g</i>	7.71E-04	10.149	BE
Dr.11836.1.S1_at	Caudal type homeo box transcription factor 4	<i>cdx4</i>	1.38E-03	5.14949	BE
Dr.1835.1.S2_at	Hepatocyte nuclear factor 4, alpha	<i>hnf4a</i>	2.83E-03	3.25735	BE
Dr.18416.1.A1_at	Selenium binding protein 1	<i>selenbp1</i>	1.30E-02	2.56039	BE
Dr.813.1.S1_at	Acetyl-CoA acetyltransferase 2	<i>acat2</i>	1.19E-03	2.52665	Cdx2
Dr.8293.1.S1_at	GATA-binding protein 6	<i>gata6</i>	6.46E-03	2.51302	BE
Dr.4249.1.S1_at	Lipase, gastric	<i>lipf</i>	6.16E-03	2.26852	BE
DrAffx.1.50.S1_at	Caudal type homeo box transcription factor 1a	<i>cdx1a</i>	2.02E-02	2.00519	cdx1b
<i>Genes down-regulated by cdx1b</i>					
Dr.19227.3.S1_a_at	Tumor protein p63	<i>tp63</i>	2.53E-03	-2.05879	NE
Dr.4387.1.S1_at	Keratin 4	<i>krt4</i>	7.38E-04	-2.22835	NE
Dr.5577.1.A1_at	Envoplakin	<i>evpl</i>	4.52E-04	-2.62801	NE ^b
Dr.1467.2.S1_a_at	Paired box gene 9	<i>pax9</i>	1.48E-02	-3.24033	NE
Dr.5379.1.A1_at	SRY-box containing gene 2	<i>sox2</i>	5.76E-03	-3.36879	NE
Dr.24923.1.S1_at	PERP, TP53 apoptosis effector	<i>perp</i>	2.65E-04	-3.39104	NE
DrAffx.2.82.S1_at	SRY-box containing gene 2	<i>sox2</i>	9.68E-03	-3.53427	NE
Dr.9761.1.S1_at	Periplakin	<i>ppl</i>	2.51E-03	-4.21447	NE
Dr.17133.1.S1_at	PERP, TP53 apoptosis effector	<i>perp</i>	1.41E-03	-6.30637	NE
Dr.25556.1.S1_at	Keratin 15	<i>krt15</i>	1.55E-02	-6.47597	NE ^b
Dr.59.1.S1_at	Annexin A1a	<i>anxa1a</i>	6.03E-03	-6.66634	NE
Dr.922.1.S1_at	Aquaporin 3	<i>aqp3</i>	4.68E-02	-7.1635	NE
Dr.1434.1.S1_at	Keratin 5	<i>krt5</i>	3.03E-02	-7.77776	NE
Dr.19227.1.S1_at	Tumor protein p63	<i>tp63</i>	5.69E-04	-8.23421	NE
Dr.1190.1.S1_at	Annexin A1b	<i>anxa1b</i>	4.80E-02	-8.87754	NE
Dr.4833.1.S1_at	Annexin A1c	<i>anxa1c</i>	2.36E-02	-14.9514	NE

^a“BE” indicated that this gene is known to be over-expressed in human Barrett’s esophagus. “NE” indicated that this gene is known to be expressed in the normal human esophagus more than Barrett’s esophagus.^{9,16} “Cdx2” indicated regulation of this gene by mammalian *Cdx2*, “Cdx1” by mammalian *Cdx1*, and “cdx1b” by zebrafish *cdx1b*, respectively.

^bGenes expressed in normal mammalian esophagus.^{30,31}

a crucial transcription factor for endoderm formation, *Sox17* regulates endodermal gene expression such as *Gata5* and *Gata6*,³⁴ which are known to be differentially expressed in human BE.⁹ By examining expression of these genes with microarray and *in situ* hybridization, we aimed at determining how *cdx1b* may cause metaplastic changes in the squamous epithelium of *cdx1b* transgenic zebrafish. Indeed, the over-expression of *Agr2*, *Nkx2.2a*, *Fabp2*, *Slc15a1*, *Cdh17*, and *Sox17* in the squamous epithelial cells of *cdx1b* transgenic zebrafish was observed. Moreover, *cdx1a*, another intestine-specific homeobox gene, was induced in the squamous epithelium of *cdx1b* transgenic zebrafish. This is consistent with the induction of *Cdx1* in the stomach of *Cdx2* transgenic mice.⁴¹ In fact, *Cdx1* was found to be strongly expressed in human BE and a rat BE model through a regulatory mechanism similar to *Cdx2*.^{42–44} Transfection of *Cdx1* into cultured esophageal epithelial cells induced expression of *Cdx2* protein.⁴² Using an organotypic

culturing method, *Cdx1* and *c-myc* have been shown to cooperate to induce mucin production and changes in keratin expression.⁴⁵ Our data suggested that *cdx1b* activated a broad program of intestinal differentiation in the squamous epithelium of transgenic zebrafish by inducing a variety of intestinal genes. Such metaplastic changes in gene expression may be an intermediate step, leading to IM of the squamous epithelium.

On the other hand, many squamous differentiation markers were down-regulated by *cdx1b*. These data suggested that *cdx1b* might play dual roles in the development of IM, inducing intestinal differentiation and interfering with squamous differentiation. These data were consistent with our previous *in vitro* cell culture study in which stable transfection of *Cdx2* into human esophageal squamous epithelial cells caused up-regulation of intestinal genes and down-regulation of squamous genes.¹⁰

Ectopic expression of *cdx1b* in squamous epithelial cells resulted in developmental abnormality of the esophageal

FIG. 3. Induction of intestinal marker genes in the squamous epithelium of *cdx1b* transgenic zebrafish. *Fabp2* was expressed in the squamous epithelium of *cdx1b* transgenic zebrafish (3-month-old, F1 generation) as detected by *in situ* hybridization (A). As a positive control, intestine expressed *Fabp2* as well (B). Wild-type zebrafish esophagus did not express *Fabp2* (C). Similarly, *Fabp6*, *Cdh17*, *Agr2*, *Slc15a1*, *Nkx2.2a*, and *Sox17* were induced by *cdx1b* (D–I). Expression patterns of these genes in positive control (intestine) and negative control (wild-type zebrafish) were similar to those of *Fabp2* (data not shown). Dashed lines indicate the basal membrane of squamous epithelium. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/zeb

FIG. 4. Histopathology of the squamous epithelium of *cdx1b* transgenic zebrafish. The esophageal squamous epithelial cells in transgenic zebrafish at 7 days postfertilization (dpf) were disorganized, smaller, and less than those in wild-type zebrafish (A, B, M). No significant change was observed in *cdx1b* transgenic zebrafish at 14 dpf and adulthood (C–F). Significant changes in cell proliferation (G–L, N), cell apoptosis (O, P), or PAS Alcian blue staining (Q, R) were not observed in transgenic fish at 7 dpf, 14 dpf, and adulthood. Dashed lines indicate the basal membrane of squamous epithelium. Scale bars for (A–D, G–J): 20 μ m; Scale bars for (E, F, K, L, O–R): 50 μ m. PAS, periodic acid Schiff. Color images available online at www.liebertpub.com/zeb

epithelium, that is, small and disorganized cells without dead cells on the surface. Most transgenic zebrafish with strong green fluorescence in epidermal cells could not survive for approximately 3 weeks. *Cdx1b* transgene may cause death by insertional disruption of other vital genes. Transgenic overexpression of *Cdx1b* may also disturb the normal differentiation program, weaken the barrier function, and, thus, cause the death of transgenic fish. Similarly, high expressers of *K14-Cdx2* transgenic mouse died early according to a recent study by Dr. John P. Lynch's group at UPenn.¹⁷ The transgenic zebrafish with relative weak fluorescence survived to adulthood, and these zebrafish failed to show significant changes of morphology in the esophageal epithelium. This observation was consistent with that in *K14-Cdx2* transgenic mouse, which also failed to produce IM in the esophagus.¹⁷ These data suggested that a higher expression level of *cdx1b* and probably cooperation of other genetic factors might be necessary to induce IM. A recently published study of *IL-1 β* transgenic mice also showed ectopic induction of *Cdx2* and failed to produce IM in esophageal epithelium.⁴⁶ In fact, our previous microarray study on human BE indicated multiple factors, and pathways might interact with each other in driving the development of BE.⁹ In addition to gain of intestinal transcription factors, loss of squamous transcription factors (e.g., *P63*, *Sox2*) may be needed for the development of BE as well.¹⁶ It is known that embryonic esophageal epithelium of *p63*-deficient mice appeared columnar and contained both ciliated and goblet-like cells.⁴⁷ Hypomorphic *Sox2* mice developed metaplastic changes in morphology and gene expression in the esophagus.⁴⁸

In summary, we developed *cdx1b* transgenic zebrafish to examine the functional role of *cdx1b* in IM *in vivo*. Transgenic overexpression of *cdx1b* in the squamous epithelial cells induced metaplastic changes in gene expression, but failed to produce histological IM. It is likely that a higher expression level of *cdx1b* and probably cooperation of other genetic factors are required for the induction of IM. To our knowledge, this study represents the first zebrafish system that studies the mechanisms of IM *in vivo*.

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Disclosure Statement

The authors have declared that no competing interests exist.

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