Molecular Analysis and Characterization of Zebrafish Keratocan (zKera) Gene*

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Lung-Kun Yeh†§, Chia-Yang Liu†§, Chung-Liang Chien‡, Richard L. Converse*, Winston W.-Y. Kao†¶**, and I-Jong Wang†§***

From the †Department of Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei 100, Taiwan, the §Department of Ophthalmology, Chang-Gung Memorial Hospital (Linko), Chang-Gung University College of Medicine, Taoyuan 333, Taiwan, the Departments of ‡Ophthalmology and ¶*Cell Biology, Neuroscience, and Anatomy, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0838, and the Departments of **Ophthalmology and ***Obstetrics and Gynecology, National Taiwan University Hospital, Taipei 100, Taiwan.

Corneal small leucine-rich proteoglycans play a pivotal role in maintaining corneal transparency and function. In this study, we isolated and characterized the zebrafish (Danio rerio) keratocan (zKera) gene. The human keratocan sequence was used to search zebrafish homologues. The zKera full-length genomic DNA and cDNA were generated via PCR of zebrafish genomic DNA and reverse transcription-PCR of total zebrafish eye RNA, respectively. The zKera spanning 3.5 kilobase pairs consists of two exons and one intron and a TATA-less promoter. The zKera encodes 341 amino acids with 59% identity to its human counterpart and 57% identity to that of mouse keratocan. Like mouse and chick keratocan, zKera mRNA is selectively expressed in the adult cornea; however, during embryonic development, zKera mRNA is expressed in both the brain and the cornea. Interestingly, it is expressed mainly in corneal epithelium but also in the stroma. A pseudogene was proved by introducing a zKera promoter-driven enhanced green fluorescence protein reporter gene into fertilized zebrafish eggs. Using morpholino-antisense against zKera to knock down zKera resulted in a lethal phenotype due to massive caspase-dependent apoptosis, which was noted by a significant increase of active caspase-3 and caspase-8 in the developing forebrain area, including the eyes. This is different from mouse, for which keratocan-deficient mice are viable. Taken together, our data indicate that mammalian keratocan is conserved in zebrafish in terms of gene structure, expression pattern, and promoter function.

Keratocan, lumican, and mimecan/osteoglycin are extracellular keratan sulfate proteoglycans (KSPGs)* belonging to the small leucine repeat proteoglycan (SLRP) family. These molecules fold into a horseshoe-shaped structure and bind to collagen molecules, facilitate formation of uniform collagen fibril diameters and interfibrillar spacing in extracellular matrix, modulate hydration of corneal stroma, and regulate corneal transparency (1–3). Unlike lumican and mimecan, which are expressed in various tissues, keratocan gene expression is much more restricted to the cornea in adult mice (4). So far, only keratocan has been shown to be directly associated with an inherited human disease, cornea plana, that manifests itself via reduced visual acuity, a flattened corneal curvature, corneal parenchymal opacity, and a thin corneal stroma (5). It has been shown that the similar phenotype of a flattened corneal curvature, as well as a thin corneal stroma, is present in keratocan knock-out mice (6), suggesting that this mouse line can serve as a model of corneal plana.

Zebrafish is a popular vertebrate model to study the biology and molecular genetics of development (7–11). Transgenic technology is a powerful tool for studying gene functions via strategies of gain of function and/or dominant negative mutations. In the zebrafish model, tissue-specific promoters have been examined with enhanced green fluorescence protein (EGFP) reporter gene (7). In addition, the transgenic zebrafish model provides advantages (e.g. shorter embryonic development time) (72 h), and transparent embryos allow easy observation with an optical device and easier treatment schemes in comparison with transgenic mice.

In eye research, zebrafish have been proven an excellent model system for retinal development, degeneration, and glaucoma study; however, few corneal studies have been undertaken (8, 9, 12). Zebrafish have transparent corneas, implying that KSPGs may also play a significant role in regulating corneal transparency in the zebrafish similar to what has been described in mammals. In this study, we take the first step toward the possibility of using zebrafish as a model system for corneal development and disease. The ultrastructure of...
zebrafish corneal tissue is studied by transmission electron microscopy. We also characterize the zebrafish Kera gene and its expression pattern in adults and during embryonic development. To investigate, the functionality of the zebrafish Kera promoter, we have employed 1.7- and 1.3-kb genomic DNA fragments 5’ of zKera to drive the EGFP reporter gene in zebrafish via transgenic approaches. Furthermore, keratocan gene knockdown via morpholino-antisense resulted in embryonic lethality, which can be attributed in part to a significant increase in the level of caspase-dependent apoptosis in the brain.

The observation suggests that zKera may have a critical function(s) for normal zebrafish development other than serving as a regulatory molecule of extracellular matrix formation.

**EXPERIMENTAL PROCEDURES**

**Maintenance of Zebrafish**

Raising, maintaining, and spawning of adult zebrafish were performed as described in Ref. 13. Adult zebrafish and embryos were maintained at 28.5 °C on a 14-h light and 10-h dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of National Taiwan University.

**Isolation and Characterization of zKera cDNA and Genomic DNA**

Basic Local Alignment Search Tool (BLAST) analysis of the GenBank™ data base using the full-length human keratocan cDNA sequence identified a zebrafish expressed sequence tag clone encoding a putative protein sharing high sequence similarity with the human and mouse SLRP family proteins. A ~3.5-kb NotI/MluI zebrafish genomic DNA fragment containing the 5' portion of the zebrafish keratocan gene was amplified by PCR and subcloned into the pBluescript SK vector (Stratagene, La Jolla, CA). The complete nucleotide sequence of the insert was determined, using T3, T7, and reverse transcription buffer, 20 μM dNTPs, 10% of total RNA from zebrafish eyes was reverse-transcribed with a keratocan-specific primer (5’-GGAGTTGAGACATCAGGTGCTCA-3’) corresponding to a sequence in exon 2 of the zKera gene. The DNA templates were degraded by treatment with an RNase mix. A poly-dCTP tail was added to the 3’-end of the cDNAs with terminal deoxynucleotidyltransferase. Amplification of the cDNA was carried out with a second gene-specific primer (5’-TCATCAAGTGTTGAGACATCAGGTGCTCA-3’) corresponding to a sequence in the junction between exons 1 and 2 in conjunction with the abridged anchor primer (5’-GGCCACGCGTCGACCTAGTTACGAAATGCTACCAAAG-3’) provided by the manufacturer. The 34 cycles of PCR were performed at 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min followed by a 10-min extension at 72 °C at the end of these cycles. The resulting PCR products were diluted 100-fold and used as template to be reamplified with a third gene-specific primer (5’-CACAATGTGCCAAAGTGTTGAGACATC-3’) in conjunction with the universal amplification primer (5’-CUACUACUACUAGGCGCAGGCGTCCACGAC-3’). Finally, the PCR product was gel-purified, and the sequence was determined by dyeoxy sequencing. The transcription initiation site of the zKera gene was determined by a sequence comparison between genomic DNA and the 5’-RACE product.

**Generation of Transgenic Zebrafish by zKera Promoter-driven EGFP**

Genomic DNA of 1.7 and 1.3 kb from the 5’-untranslated region of the zKera gene was amplified by specific PCR primers and inserted into the multiple cloning site of pEGFP-N1 (Clontech, respectively). The recombinant plasmid was propagated in Escherichia coli DH5α and purified by the QiaGen Plasmid Purification Maxi kit (Qiagen, Hilden, Germany). Purified plasmid DNA was adjusted to 50 ng/μl in distilled water and microinjected into one-cell-stage zebrafish embryos under a dissecting microscope. The living embryos with GFP expression were observed and imaged under fluorescence microscope.

**Sequence Alignment and Phylogenetic Analysis**

The sequence alignments were performed using the ClustalW program (available on the World Wide Web). Putative transcription factor binding sites were searched using the “TFSEARCH” program (available on the World Wide Web) with a default threshold score (of 90.0). The Cladogram was constructed using TreeView software.

**Northern Blotting Hybridization**

Total RNA extracted from eye, brain, heart, liver, gut, muscle, and fin by using TRIZOL® reagent (Invitrogen) was electrophoresed in 1.3% agarose containing 2 μM formaldehyde buffered with Tris acetate-EDTA and blotted onto Magnacharge™ membrane (Osmonics, Inc., Westborough, MA) and hybridized with 32p-labeled zebrafish keratocan cDNA in a hybridization solution containing 50% formamide at 42 °C overnight as previously described (4). The excess 32P-probe was removed by stringent washing three times with 0.1× SSC and 1% SDS at 65 °C for 30 min each. Hybridization signals were detected with a PhosphorImager (Amersham Biosciences).

**RT-PCR**

RT-PCR was performed as described previously with some modifications (14). Reagents used in this procedure, unless specified, were purchased from Promega (Madison, WI). The RevertAid™ H Minus First Strand cDNA synthesis kit was purchased from Fermentas (MBI Fermentas, Hanover, MD). The zebrafish cDNA was synthesized by using 40 μl of 5X reverse transcription buffer, 20 μl of 0.1 μM dithiothreitol, 8 μl of 25 mM dNTPs, 10 μl of RNasin (40 units/μl), 10 μl of 50 mM random hexamer (Amersham Biosciences Inc.), 10 μl of avian myeloblastosis virus reverse transcriptase (9.5 units/μl), and 1 μg of heat-denatured corneal poly(A) RNA. Diethylpyrocarbonate-treated water was added to bring the final reaction volume to 200 μl, and the reaction was incubated at room temperature for 10 min, 42 °C for 90 min, 100 °C for 2 min, and 0 °C for overnight.
Zebrafish Keratocan

5 min. Each 20 μl of the above RT reactions was added to 80 μl of a PCR mixture containing the following: 8 μl of 10× PCR buffer without MgCl₂, 8 μl of 25 mM MgCl₂, 10 μl of 20 ng/μl primers, 0.5 μl of Taq polymerase (5 units/μl), and 45.5 μl of distilled H₂O. The PCR was done by using 35 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min, followed by a 15-min extension at 72°C at the end of these cycles. Primers were CCGGTCAGCGGCCTGCCAACTTTTTCAAACA (forward 5'-XhoI restriction site) and TCCCGCGGAGAGTAGTTTAACTTAAGAATC (reverse 3'-SacII restriction site). The PCR product was confirmed by an appropriate restriction enzyme digestion and analyzed by electrophoresis through a 1.5% agarose gel.

In Situ Hybridization (Whole Mount and Sectioned Corneal Tissue)

Sense and antisense digoxigenin (DIG)-labeled oligonucleotide probes were synthesized by Bio Basic Inc. The oligonucleotide sequence (5’–3’) was CTCAATCCACCTGAGACCGAAG-GTATTGAGGACGTGCTTTCTCAGTACGTATC-ATC. The embryos were sorted at the stages required for the experiment and staged according to Kimmel et al. (15). Chorions were removed by manually with Dumont Watchmaker’s Forceps No. 5. The embryos were fixed in 4% paraformaldehyde in 1× PBS overnight at 4°C, rinsed with PBS three times, transferred into 100% methanol (MeOH), and stored at −20°C until use. The embryos raised to time points beyond 24 hpf were treated with 0.003% phenylthiourea to prevent melanization. Whole mount in situ hybridization was performed according to the manufacturer’s instructions (Roche Applied Science) with some modifications. Briefly, they were treated with cold methanol and rehydrated through a descending methanol series in PBS. The samples were permeabilized by protease K treatment (25 μg/ml) for 25 min and hybridized with the appropriate probe at 65°C, followed by incubation with an anti-DIG antibody conjugated with alkaline phosphatase and stained with substrate nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate to produce purple insoluble precipitates. Furthermore, paraffin sections (5 μm thick) of adult zebrafish eyes mounted on Superfrost Plus slides (Fisher) were deparaffinized and processed for in situ hybridization using 10 mM sodium acetate, 10 mM sodium EDTA, 5 mM aminobenzamidine, and 0.1 M e-amino- n-caproic acid. After it was dialyzed exhaustively overnight against distilled water, the insoluble pellet was dissolved in 6 M urea containing 0.1 M Tris acetate solution (pH 6.0), 0.1% CHAPS, and 0.15 M NaCl (Sigma). The immuno blot of proteins from the two corneal extracts were then probed with a rabbit polyclonal antibody (0.1 μg/ml) raised against human keratocan and mouse keratocan separately. Adult zebrafish eye tissue was homogenized with lysis buffer containing 200 mM HEPES/KOH (1 ml) (pH 7.5), 200 mM sucrose (0.86 g), 50 mM KCl (0.04 g), 2.5 mM MgCl₂ (0.005 g), 10 mM dithiothreitol (100 μl) in a total 10-ml solution. To remove keratan sulfate chains, protein aliquots were incubated with 0.1 unit/ml endo-β-galactosidase (Sigma) and 1 unit/ml keratanase (Sigma) at 37°C overnight. The immunoblot of proteins were then probed with a rabbit polyclonal antibody (0.1 μg/ml) raised against zebrafish keratocan or monoclonal anti-keratan sulfite antibody (Seikagaku, Tokyo, Japan). These immunocomplexes were visualized by adding alkaline phosphatase-conjugated secondary goat anti-rabbit IgG (1:2500; Novagen) and Western Blue® stabilized substrate (Promega, Madison, WI).

Immunohistochemistry

Both zebrafish larvae and adult zebrafish corneal tissue fixed with 4% paraformaldehyde in PBS were used for immunohistochemical analysis of the keratocan protein. Deparaffinized sections (5 μm) of adult zebrafish corneal tissue were placed on slides and processed for immunohistochemistry. Before immunostaining, they were incubated with 0.1 unit/ml endo-β-galactosidase and 0.5 unit/ml keratanase at 37°C overnight. After blocking with hydrogen peroxide for 30 min, the whole mount and corneal sections were incubated with the affinity-purified primary anti-keratocan antibody (0.1 μg/ml) or monoclonal anti-keratan sulfate antibody, washed in PBS, and then incubated with a biotinylated secondary antibody (goat anti-rabbit IgG). After washing in PBS, sections were incubated with streptavidin-horseradish peroxidase (DAKO Corp., Carpente ria, CA) and then washed in PBS and incubated with the 3’3’-diaminobenzidine chromogen for 5–10 min. Negative controls were obtained by omitting the primary antibody.

Transmission Electron Microscopy

For transmission electron microscopy, the adult fish corneal tissues were fixed in 50 mM phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 24 h at room temperature. After refixation in 1% osmium tetroxide

Preparation of an Epitope-specific Polyclonal Anti-zebrafish Keratocan Antibody

Rabbit antisera was prepared with a synthetic oligopeptide, CDNKGLKSIPVIPPYTWY, corresponding to the N-terminal amino acid residues deduced from zKera cDNA. The peptides were conjugated to keyhole limpet hemocyanin for antibody production in rabbits. The antisera were further purified through a peptide-conjugated Sulfolink gel column (Pierce) according to the manufacturer’s instructions. Fractions containing purified anti-keratocan antibody were pooled and concentrated, and the protein concentration was measured by spectrophotometry at A₂₈₀.
for 4 h at room temperature, the samples were washed in phosphate buffer, dehydrated, and embedded in Epon 812 epoxy resin. Ultrathin 50-nm sections were collected on 75-mesh copper grids and stained with uranyl acetate and lead citrate, and images were photographed with a Hitachi 7000 Transmission Electron Microscope.

Preparation and Microinjection of Morpholino-antisense Oligonucleotides

Antisense MOs (Gene Tools, Philomath, OR) were designed to target at the 5’-untranslated region or flanking region, including the initiation codon of the respective genes. The MO sequence was as follows: zeKera-MO, 5’-AATGCTACCA-GAGTACCTCCATAG. This oligonucleotide complements the sequence from −2 through +23 relative to the initiation codon. A random sequence (RS) MO served as a control for zeKera-MO: 5’-CCTCTTACCTCACTCCAAGCAGTAT-3’. This MO is offered by Gene Tools as a negative control oligonucleotide that should have no target specificity. A search of the data base did not identify any sequence similarity to known zebrafish genes with the zeKera-MO. Solutions were prepared and injected at 9–4-cell stage as described (16). Injected embryos were maintained at 28 °C until analyzed.

Apoptosis Assays

TUNEL Staining and Acridine Orange Staining—Dechorionated embryos were fixed in 4% cold paraformaldehyde in PBS for 6 h and dehydrated in a graded ethanol series (50, 70, 85, 95, 100%), followed by 20 min in acetone at −20 °C. The embryos were further permeabilized by incubating in PBS with 0.5% Triton X-100 and 0.1% sodium citrate for 15 min and in 20 μg/ml Proteinase K for 10 min. The samples were refixed with 4% paraformaldehyde before incubation with terminal deoxynucleotidyltransferase solution according to the manufacturer’s instructions (In Situ cell death assay kit) following the manufacturer’s instructions. The samples were then incubated with terminal deoxynucleotidyltransferase solution according to the manufacturer’s instructions.

Myotomal CdxA-1

C/EβP-2

GATA-1

CREB

Sox-5

CdxA

Oct-1

In Situ cell death assay kit following the manufacturer’s instructions.
Zebrafish Keratocan
detection kit (Roche Applied Science). For detection of apoptotic cells in live embryos, acridine orange (AO) was performed. For AO staining, samples were incubated in 5 μg/ml AO (Sigma) in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) for 30 min and observed by fluorescent microscopy.

Caspase-3, Caspase-8, and Mitochondrial Apoptosis—To detect active caspase enzymes and loss of mitochondrial membrane potential during apoptosis, the image-iT LIVE Green caspase-3 and -7 detection kit, image-iT LIVE Green caspase-8 detection kit, and MitoTracker Red CMXRs (Molecular Probes, Inc., Eugene, OR) were performed in live embryos according to the manufacturer’s instructions. Briefly, samples were incubated in 1× FLICA reagent working solution for 30 min and protected from light. Thereafter, samples were washed with 1× wash buffer and observed under a fluorescent microscopy.

RESULTS

Primary Structure of the zKera Gene and Comparison of Deduced Amino Acid Sequence of Zebrafish Keratocan with Other Species—We identified the zKera gene by performing a BLAST search of the publicly available zebrafish data bases against the human KERA gene. The entire DNA sequence of the zKera gene is shown in Fig. 1. As illustrated in Figs. 1 and 2A, the entire DNA sequence of the zKera gene spans ~3.5 kb (3589 bp) and contains two exons, one intron, and the promoter region. Comparing DNA sequence from the cDNA and the genomic DNA of the zKera gene, we found that exon 1 is 1373 bp and encodes an N-terminal domain and central leucine-rich repeats, and exon 2 contains 161 bp of coding sequence and 138 bp of 3’-untranslated sequence. The transcription initiation site marked +1 was determined via 5’-RACE as described under “Experimental Procedures.” The first translation initiation ATG codon is located at the 511th base from the beginning of exon 1. Therefore, exon 1 contains 511 bp of 5’-untranslated sequence. There is no TATA consensus sequence found in the ~2.2 kb of the proximal 5’-flanking region of the zKera gene, which was utilized as the promoter region. The cDNA clone (~1.9 kb) contains a 1023-bp open reading frame encoding zebrafish keratocan (341 amino acid (aa) residues). Like other SLRP core proteins (i.e. lumican, 17, miceman, 18), and keratocan (4), zebrafish keratocan shows three distinct domains: a highly conserved central leucine-rich repeat region, flanked by hypervariable N- and C-terminal regions (Fig. 1). As shown in Fig. 1, after the signal peptide (Met–His; the first 20 aa), the N-terminal region contains a consensus YE motif for protein sulfation, which indicates the possible presence of sulfotyrosine(s) in zKera (19). A central leucine-rich domain contains ten tandem repeats of the sequence LXXLXXNXX(N/L/I). In addition, another genomic DNA sequence similar to the normal zKera gene with a 724-bp deletion from bp −690 to +34 was identified by different primer pairs, suggesting that there is a pseudogene of Kera that does not have an open reading frame and is designated as zKera” in the zebrafish genome (Fig. 2, A and B). The zKera genomic DNA was sub cloned and characterized by Southern blot hybridization, PCR, and DNA sequencing (data not shown). We have isolated clones representing the full open reading frame of zebrafish keratocan. A cDNA clone encoding the keratocan open reading frame was sub cloned into

FIGURE 2. Schematic representation of the organization of the zebrafish keratocan gene A pseudogene was proved by introduction into fertilized zebrafish eggs by an enhanced green fluorescence protein (EGFP) reporter gene driven by a 1.0-kb zKera promoter region. A, the figure shows the zebrafish keratocan gene drawn to scale. The blank boxes indicate the coding region of the mRNA. The translation start and stop codons are indicated by ATG and TAA, respectively. B, for detection of the pseudogene, primers used were as follows: primer A zKera forward, 5′-ATAAGAATGCGCCGGCCGGAGGAGAGGCAGAGTAGC-3′; primer B zKera reverse, 5′-CGAGCCGCGATGCTATGATGTTAACCTAAAGAATCA-3′; primer C zKera reverse, 5′-AAATGTCTGAGTGAAGATTAACCTAACAGATCA-3′; primer D zKera reverse, 5′-CCGAATCTTATATGCACACCCTGCCCTGAG-3′. Two different sizes of PCR products from zebrafish genomic DNA were obtained simultaneously by different primer pairs: primers A and B (lane 1); primers A and C (lane 2); primers A and D (lane 3). The deleted 724 bp of DNA sequences corresponding to the 5′-flanking region revealed that it was possibly derived from different loci and had no promoter function, C, generation of transgenic zebrafish harboring zkerap1.7-EGFP SV40 (6.9 kb). It contains the 1.7-kb 5′-regulatory region of the zebrafish keratocan gene, untranslated region of exon 1 (511 bp), EGFP (~700 bp), SV40 polyadenylation signal, and pEGFP-1 vector. D, generation of transgenic zebrafish harboring zkerap1.3-EGFPbpA (6.3 kb). It contains the 1.3-kb 5′-regulatory region of the zebrafish keratocan gene, untranslated region of exon 1 (511 bp), EGFP (~700 bp), bovine growth hormone polyadenylation signal (bpA), and pBStcript-Ii vector. E, generation of transgenic zebrafish harboring zkerap1.0-EGFP SV40 (~6.2 kb, 724 bp deleted). It contains the 1.0-kb 5′-regulatory region of the zebrafish keratocan gene (690 bp deleted), untranslated region of exon 1 (477 bp), EGFP (~700 bp), SV40 polyadenylation signal, and pEGFP-1 vector. F, EGFP expression was observed under a fluorescence microscope in transgenic zebrafish after injecting linearized zkerap1.7-EGFP SV40 DNA fragment at the 72 hpf stage. G, in particular, strong EGFP expression was expressed selectively at corneal tissue at the 7 dpf stage. H, almost no EGFP expression was observed under the fluorescence microscope at the 5 dpf embryo stage in transgenic zebrafish after injecting linearized zkerap1.3-EGFPbpA DNA fragment. I, no EGFP expression was observed under the fluorescence microscope at the 5 dpf embryo stage in transgenic zebrafish after injecting linearized zkerap1.0-EGFP SV40 DNA fragment.
the pBluescript SK vector (Stratagene, La Jolla, CA). The open reading frame of the zKera gene was 1023 bp long and encoded 341 amino acid residues.

For bioinformatics analysis in the promoter region, putative transcription factor binding sites were searched using the “TFSEARCH” program (available on the World Wide Web) with threshold score (default: 95.0). The results revealed several transcription factor-binding elements in the 2.2-kb (−1700 to +510) 5′-flanking region of the zKera gene. A MyoD binding site, agacaggtgttg (−1584 to −1573), a CdxA binding site, aataata (−857 to −851), a C/EBPα binding site, atattgcaaaatga (−735 to −722), a GATA-1 binding site, agcagataaggag (−243 to −231), a GATA-X binding site, agataagaatt (−29 to +39), a CREB binding site, tgacgtct (−388 to +395), and a CdxA binding site, atataata (−443 to +449), are arranged in a sense direction to the zKera gene. One Nkx-2 binding site, cacttaa (−201 to −207), one CdxA binding site, tattaat (−442 to +448), one Sox-5 binding site, tattaat (−447 to +452), and one Oct-1 binding site, taacatctcattttg (−454 to +468), are found in an antisense direction to the zKera gene.

Analysis of zKera Promoter Activity in Transgenic Zebrafish—To confirm the zKera gene that we isolated is a functional gene, we prepared two reporter gene constructs, zKera pr1.7-EGFP-SV40 and zKera pr1.3-EGFP-bpA, for microinjection into the one-cell stage of fertilized zebrafish eggs. Live transgenic EGFP-positive embryos were screened and selected under a fluorescent microscope. Eighty-two percent of injected embryos continued developing, with 93% of them hatching and over 75% surviving beyond 12 days postfertilization (dpf). In transgenic zebrafish, 33.25% (n = 411) of the 1.7-kb zKera promoter fragment-injected embryos expressed the EGFP transgene restricted to the eye (Fig. 2, C, F, and G), whereas the 1.3-kb promoter fragment of the zKera gene-injected embryos shows nearly undetectable low levels of EGFP expression in all zebrafish (n = 401) (Fig. 2, D and H). The deletion of the 5′ 0.4 kb of sequence had an effect on the efficiency of EGFP expression and was likely to contain regulatory elements of importance. On the other hand, the second zKera gene similar to the normal zKera gene with a 724-bp deletion (Fig. 2, A, B, and E) could not drive EGFP cDNA expression in transgenic zebrafish (Fig. 2, E and I), suggesting that this is a zKera pseudogene.

For comparison, multiple alignment analysis of the predicted amino acid sequences of zKera with that of other species was shown in Fig. 3A. To examine the evolutionary relationship between different species, we conducted a phylogenetic tree based on unspliced primary sequences of the zKera (Fig. 3B). The predicted amino acid sequences of these proteins showed a high homology to known Keratocan protein of other species. zKera exhibited a 59% amino acid sequence identity to human keratocan while exhibiting 57% amino acid sequence identity to mouse keratocan.

Expression of the mRNA Encoding the Zebrafish Keratocan—Northern blot hybridization was performed to compare the relative amounts of zKera mRNA in the adult zebrafish cornea and other tissues. Electrophoretically separated total RNA from

chicken, bovine, and puffer fish keratocan are aligned. B, phylogenetic tree of keratocan proteins from different species. The tree was constructed with ClustalW.
various parts of adult zebrafish tissue were transferred to membranes and probed with zKera cDNA. Ethidium bromide staining of 28 and 18 S rRNA served as loading controls. Fig. 4A showed that zKera mRNA is a ~1.9-kilobase transcript selectively expressed in eye tissue, with no hybridization signal present in other tissues (Fig. 4A). Interestingly, the intense hybridization signal derived from total RNA extracts from eyes indicated that the zKera gene was specifically expressed in the eye tissue at high levels. To further confirm the tissue distribution of zKera, RT-PCR analysis was carried out using template cDNA synthesized from total RNA, and a PCR product ~1 kb in size was found specifically in the eye (Fig. 4B).

**Spatial Distribution of mRNA Encoding the Keratocan Protein in the Zebrafish**—In order to determine the spatial pattern of keratocan expression during zebrafish development, whole mount in situ hybridization with antisense riboprobes was performed. Whole mount in situ revealed that zKera mRNA was expressed in the eyes and the major subdivisions of the embryonic central nervous system, including the fore-, mid-, and hindbrain, and the anterior spinal cord at 2 dpf (Fig. 5, A–C). The expression pattern of zKera mRNA at 3 dpf was similar to the pattern at 2 dpf. Interestingly, there is a specific localization pattern expressed in the corneal epithelial layer in the adult zebrafish (Fig. 5, E and G). The corresponding sense riboprobes showed negligible staining in samples serving as negative controls (Fig. 5, D, F, and H).

**Western Blotting and Immunohistochemistry**—To detect zebrafish keratocan, we generated an affinity-purified antibody against a synthetic peptide corresponding to the 18 N-terminal amino acid residues deduced from the zKera cDNA. To compare zebrafish keratocan with mammalian keratocan, total lysates from human, mouse, and zebrafish eyes, respectively, were treated with or without endo-β-galactosidase and keratanase and then subjected to Western blot analysis. Fig. 6 shows a smearing pattern (lanes 1, 3, and 7) in human, mouse, and eye tissue without endo-β-galactosidase digestion and one band (lanes 2, 4, and 8) with endo-β-galactosidase digestion. In addition, adult zebrafish corneal proteins extracted by lysis buffer and probed with the antibody against keratan sulfate showed the similar smearing pattern without keratanase digestion (lane 5), whereas the smearing pattern disappeared after treatment with keratanase and proved to have keratan sulfate chains (lanes 6 and 9). Fish samples treated with endo-β-galactosidase (lane 8) and with keratanase (lane 9) showed the similar pattern. These data demonstrate that the zKera protein isolated from the eyes appears as a KSPG, which is similar to those of humans and mice.
to show that zebrafish embryos. Western blot and RT-PCR were carried out (6). This led us to examine the effects of keratocan depletion in exhibit a flattened corneal phenotype and a thin corneal stroma.

The layer of epithelium is thicker than that of stroma. The epithelium contains only 3–4 cell layers. The endothelium comprises mainly in the epithelial layer. No immunoreactivity was detected in the negative control sample (data not shown).

Ultrastructure of Adult Zebrafish Corneas—Electron microscopic examination was performed to analyze the ultrastructure of zebrafish cornea as shown in histograms (Fig. 7G). The layer of epithelium is thicker than that of stroma. The epithelium contains only 3–4 cell layers. The endothelium comprises mainly in the epithelial layer.

Caspase-dependent Apoptosis Triggered by zKera Morpholino Injection—Keratocan knock-out mice exhibit a flattened corneal phenotype and a thin corneal stroma (6). This led us to examine the effects of keratocan depletion in zebrafish embryos. Western blot and RT-PCR were carried out to show that zKera protein and mRNA decreased after and a few scattered in the zebrafish body (Fig. 8C). Few apoptotic cells were found in RS-MO embryos (Fig. 8D). AO staining, a membrane-permeable aromatic derivative resulting fluorescent in acidic lysosomal vesicles for detecting apoptotic cells, showed increased levels of cell death, as determined by TUNEL assays and acridine orange staining on embryos derived from fertilized eggs injected with keratocan-specific morpholinos (Figs. 8C and 9A). This indicates that zKera is indispensable for the normal development of zebrafish.

Caspase enzymes participate in a series of reactions that are triggered in response to proapoptotic signals and result in apoptosis. Based on a fluorescent inhibitor of caspases (FLICA™) methodology, we found that caspase-3-positive cells (Fig. 9E) and caspase-8-positive cells (Fig. 9G) were significantly increased in zKera-MO injected group in 3-dpf embryos. However, apoptotic cells were not detected by MitoTracker Red CMXRos staining due to no significant loss of mitochondrial membrane potential (Fig. 9C). Therefore, depletion of keratocan protein induces apoptosis through activated caspase-3 and -8 in zebrafish embryos. Taken together, our findings suggest that the phenotype caused by keratocan depletion can be attributed at least in part to a significant increase in the level of caspase-dependent apoptosis.

DISCUSSION

In this study, we isolated the zebrafish keratocan gene and examined its expression during development and in adults. Analysis of genomic clones suggests that the zebrafish genes in general do not contain introns. Zebrafish keratocan shared 59%
Like mouse keratocan, zebrafish keratocan has all of the structural features of SLRPs (i.e. a central domain of leucine-rich repeats flanked by N- and C-terminal domains with conserved cysteines) (20). Zebrafish keratocan gene contains 10 leucine-rich repeats in tandem and four and two cysteine residues at the amino and carboxyl terminus, respectively. From deduced amino acid sequences (Fig. 3A), we found that there are four possible sites (resides 95, 169, 224, and 262) for N-linked glycosylation in the domain of leucine-rich repeat among species other than zebrafish. In zebrafish keratocan, residues 95 and 262 in the deduced amino acid sequence of zKera contain the consensus NX(S/T) sequences for N-linked glycosylation. However, other amino acids substituted asparagines in residues 169 and 224. Therefore, only two potential N-linked glycosylation sites are available in zKera.

Decorin, biglycan, and epiphycan have GAG attachment sites, which consist of a Ser-Gly pair preceded by acidic amino acid residues at the N-terminal end proximal to the cysteine-rich region (21, 22). In contrast, lumican, keratocan, and fibromodulin have sulfotyrosine residues at the N-terminal end of the mature core proteins. The sulfated tyrosine may affect interactions with cationic domains of other extracellular matrix components and/or cell surface proteins. In zKera, residue 34 is the only putative tyrosine followed by an acidic amino acid (Glu) near the N terminus, a consensus sequence for tyrosine sulfation and there is no consensus sequence for an N-linked glycosylation. However, other amino acids substituted asparagines in residues 169 and 224. Therefore, only two potential N-linked glycosylation sites are available in zKera.

Amino acid identity with human keratocan and 57% amino acid identity with mouse keratocan. Interestingly, the size and structure of zKera is not similar to the mouse gene. Moreover, like mouse and bovine keratocan gene, the zKera promoter does not contain a conventional TATA box in the proximal promoter region; as a result, zKera is a TATA-less gene (4). Unlike the TATA-less promoters of housekeeping genes, the zKera promoter does not have a GC-rich sequence. The transcription factor binding motif in the 5'-flanking region of the zKera gene indicated several consensus-binding sites for various transcription factors. The identification of the 1.7-kb regulatory elements of the zKera gene is an important first step in elucidating regulatory mechanisms of keratocan during zebrafish development. Therefore, a functional analysis is needed to further determine cis-elements that contribute to zKera gene regulation.
keratocan. The expression of keratan sulfate-keratocan in the fish stroma may preserve its function for a role in collagen fibrillogenesis, as has been suggested for mammals (e.g. mice and human beings). Moreover, keratocan is significantly expressed by zebrafish corneal epithelium, which contains little extracellular matrix and keratin sulfate if there is any. Thus, the zebrafish keratocan may have not yet defined functions other than serving as a regulatory molecule of collagen fibrillogenesis. Therefore, expression of keratocan may serve as a specific marker of corneal stromal cells, and the application of a zKera promoter to drive heterologous transgenes in mice can be useful for studies of for corneal cell biology studies. It is of interest to note that, in the zebrafish 2–3 dpf larvae stage, keratocan is widely expressed in the brain in addition to in ocular tissue, whereas its expression is restricted to the corneal epithelial layer and stromal layer in the adult. Therefore, the zKera promoter is invaluable in preparing transgenic zebrafish lines to elucidate the mechanisms controlling corneal gene expression. In the present study, we identified a 1.7-kb zKera promoter and successfully generated transgenic zebrafish that showed a tissue-specific EGFP expression pattern that is similar to that of Kera. This promoter drove EGFP expression from embryonic through adult stages, suggesting that transcription factor binding sites within this 1.7-kb region are sufficient to confer cornea-specific expression in zebrafish.

In this study, we showed that the Kera gene is expressed by corneal epithelial cells and stromal cells in adult zebrafish. The expression pattern of the zebrafish keratocan is much different from the expression pattern in the stromal layer in other species. Therefore, we can predict that the corneal epithelial cells in zebrafish may be more important in the maintenance of corneal transparency and structure. These findings also reveal an as yet unknown role of keratocan in maintaining corneal physiology. Further investigation needs to focus on the function of keratocan in corneal epithelial cells for a better understanding of the molecular basis of corneal epithelial development.

In humans, mutations of the human KERA gene are associated with cornea plana (CNA2) in which the forward convex curvature of cornea is flattened, leading to a decrease in light refraction (5). In our previous study (6), Kera knock-out mice presented a thinner corneal stroma and a narrower cornea-iris angle of the anterior segment in comparison with the wild type littermates. For zebrafish, we show that antisense morpholino oligonucleotides are capable of down-regulating the expression of the zKera mRNA and reduction of zKera protein synthesis, which manifests with enhanced apoptosis and embryonic lethality (Fig. 8, B and C). Several investigators have reported that nonspecific effects of morpholino may induce widespread cell death and neural degeneration (16, 25, 26, 28). To prevent nonspecific effects of morpholino, embryos transfected with...
control oligonucleotides (RS-MO) and wild type embryos were prepared in our study. No significant morphological difference could be recognized between zKera-MO and RS-MO embryos at early developmental stages; a significantly higher mortality rate was noticed in the zKera-MO group (58.23%, 658 of 1130) than RS-MO embryos (30.64%, 201 of 656) and the wild type group (27.52%, 287 of 1043). Moreover, significant increases of apoptotic signals were prominent in the anterior part of the body, particularly in the eye and head of zKera-MO-injected embryos, whereas very few apoptotic signals were found in either RS-MO-injected or wild type embryos. This may be responsible for the increased mortality rate in zKera-MO-injected embryos. In addition, apoptosis induced by zKera-MO injection went through the caspase-dependent pathway by the evidence of the activation of caspase-3 and caspase-8 enzymes in injected zebrafish embryos. In contrast, mice keratocan knock-out exhibited thin corneas, since keratocan was primarily found in corneal stroma of mammals (6). Besides species differences, there are alternate explanations as to why zKera morpholino injection does not have a phenotype similar to that of keratocan knock-out mice. First, the zKera morpholino may not persist at concentrations higher enough to sustain continued inhibition of zKera core protein synthesis in adult zebrafish. Furthermore, the keratocan may not have a key role in regulating collagen fibrillogenesis for the formation of stromal extracellular matrix. This hypothesis is substantiated by our finding that there was no significant difference in corneal thickness between these groups by transmission electron microscopy study (zKera-MO versus RS-MO versus wild type; data not shown).

It is plausible to hypothesize that keratocan may have a function in cell survival by preventing apoptosis during embryonic development of zebrafish. “Gene sharing” has been proposed to explain the adaptation of gene usage by various species during evolution (29, 32–35). For example, Piitagorsky et al. (36, 37) studied lens crystallins and proposed the concept of “gene sharing” by which proteins can evolve new functions by changes in gene regulation in the absence of or before gene duplication without a change in protein structure. Other examples, such as Drosophila rhodopsin and xanthine oxidoreductase, also show that gene sharing is consistent with changes in gene regulation being an evolutionary driving force for innovation of protein function (24, 27, 37). Our results showed that the expression of zebrafish keratocan is not only in eyes but also in brains during development. Thus, it is possible that the loss and/or reduction of keratocan may have a detrimental impact on cell survival in the brain during development through the effect of gene sharing. As expected, brain cells may be sensitive to the decrease of keratocan, because the effect of “gene sharing” in the brain cells and a reduction of keratocan concentration may cause apoptosis in zKera-MO embryos, but this small change has little effect on corneal morphogenesis. This phenomenon is very different from previous studies in mice and suggests a novel role of keratocan; it has not yet been shown whether such cell survival functions also extend to other members of the SLRP family (e.g., lumican in zebrafish development).

Collectively, these results show that the zebrafish Kera gene can serve as a specific marker for eye tissue and promise the potential use of the Kera promoter to drive heterologous transgenes in fish for studies in corneal biology. They also lead to the discovery of a novel function of the zKera in zebrafish.

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