Skin-Specific Expression of *ictacalcin*, a Homolog of the *S100* Genes, During Zebrafish Embryogenesis

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Full-length cDNA coding for the *ictacalcin* gene, a homolog of the *S100* genes, was isolated in zebrafish and mapped on linkage group 16 using the LN54 radiation hybrid panel. The homology and phylogenetic analyses, based on the deduced amino acid sequences, showed the orthologous relationship of *ictacalcin* genes between zebrafish and other fish species. However, *ictacalcin* genes constitute an out-group with respect to other members of the *S100* gene family. This result supports the findings that fish *ictacalcin* genes are new members of the *S100* gene family and may have evolved after the divergence of teleosts and tetrapods. The zebrafish *ictacalcin* gene was zygotically transcribed from 12 hours postfertilization onward and was stably expressed throughout adulthood. During zebrafish embryogenesis, the *ictacalcin* gene was specifically expressed in striated epidermal cells covering the entire embryo. The *ictacalcin* staining in keratinocytes of striated epithelia was absent in the cytoplasm surrounding the nuclei, but it was highly concentrated in the peripheral margin. Tissues enriched with epithelia folds, such as olfactory epithelium, hatching gland, pectoral fin buds, urogenital opening, and pharynx, showed a robust *ictacalcin* expression. The strikingly heavy staining of *ictacalcin* in the pharyngeal region provides us with an early marker to follow the pharynx formation in zebrafish embryos. *Developmental Dynamics 228:745–750, 2003.*

Key words: Ca^{2+} binding proteins; *S100* gene; *ictacalcin*; zebrafish; skin; epidermis; keratinocyte; pharynx; pectoral fin bud; urogenital opening; hatching gland; olfactory epithelium; embryogenesis

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INTRODUCTION

The *S100* genes belong to the multigenic family of low molecular weight (9 to 13 kDa) Ca^{2+}-binding proteins with two EF-hand Ca^{2+}-binding motifs. In mammals, more than a dozen of *S100* genes have been isolated, and most of them are expressed in a tissue-specific manner (for reviews, see Zimmer et al., 1995). In humans, most of the *S100* genes are clustered in an epidermis differentiation complex (EDC) located on chromosome 1q21 (Mischke et al., 1996; South et al., 1999), and preferentially expressed in the skin epithelium (Sato and Hitomi; 2002). Compared with tetrapods, only a few studies addressed the molecular structure and developmental expression of *S100* genes in teleosts (Ivanenkov et al., 1993; Bobe and Goetz, 2000). In catfish, a homolog of the *S100* gene, *ictacalcin,* had been discovered to be the most abundantly expressed sequence tag (EST) in the chemosensory (Bettini et al., 1994) and skin libraries (Karsi et al., 2002), respectively. By in situ hybridization, Porta and colleagues (1996) demonstrated that *ictacalcin* gene transcripts are highly detected in epithelial cells of the olfactory rosette, barbel, skin, and gills of adult catfish. This skin preference, showing strong expression of *ictacalcin* in catfish, promoted us to clone its ortholog in zebrafish, so as to serve as a molecular marker for skin development during embryogenesis.

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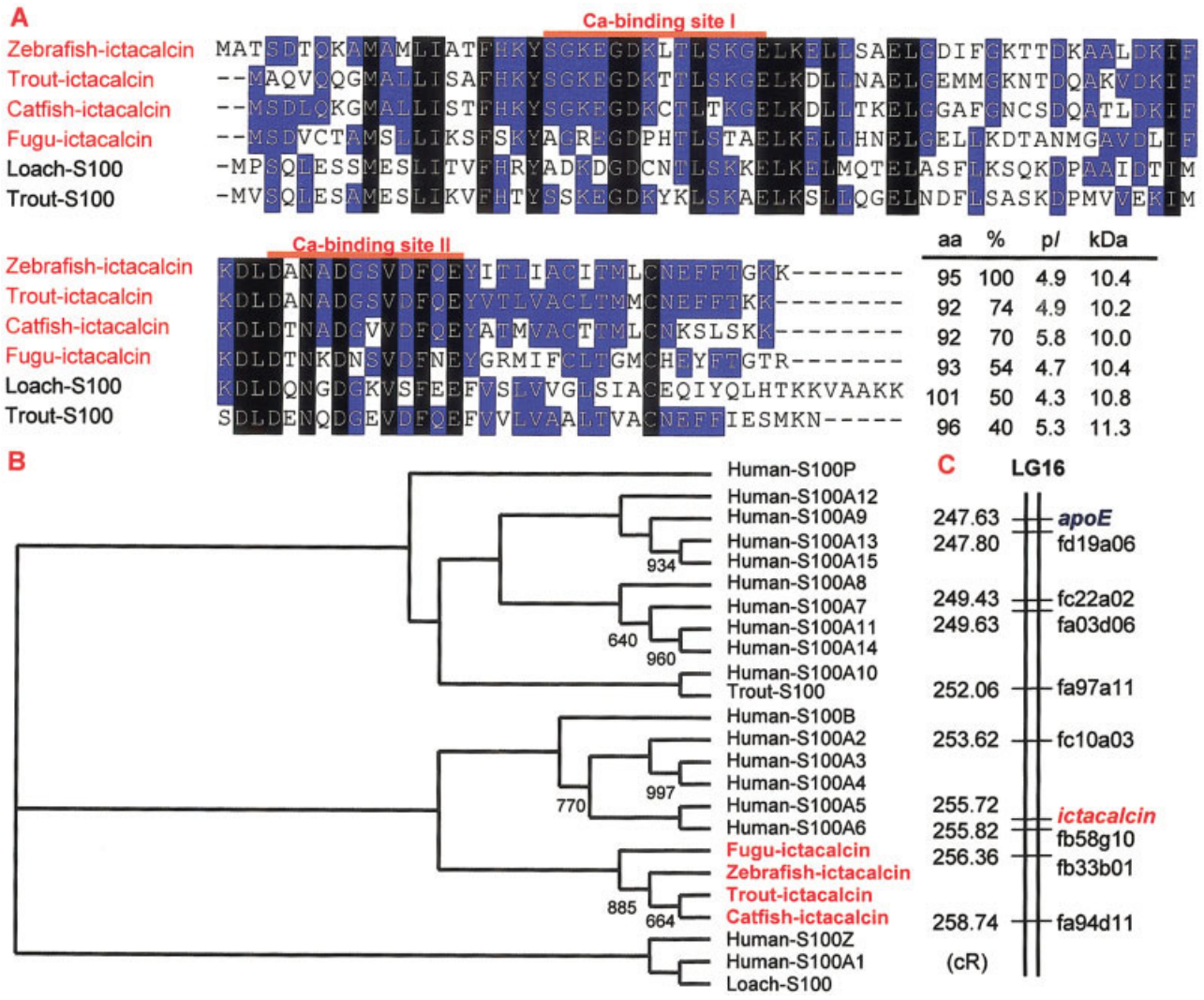

Fig. 1. **A:** Alignment of amino acid sequences between zebrafish *ictacalcin* and other fish *S100* genes. Residues conserved throughout the entire *S100* gene family are highlighted in black; residues conserved in at least 50% of the family members are highlighted in blue. Predicted Ca^{2+} binding motifs of the N- and C-terminal EF-hand are marked by red horizontal lines. The residue number (aa), identity (%), predicted isoelectrical point (p*I*), and calculated molecular weight (kDa) of the amino acid sequences are indicated in the lower-right corner. Fish *ictacalcin* genes are highlighted in red color. **B:** An un-rooted phylogenetic tree of *S100* genes based on amino acid sequences. The tree was constructed by using the neighbor-joining method and the CLUSTAL W program. Bootstrap values below 500 (based on 1,000 iterations) have been deleted. Sources of human *S100* genes: *S100A1* (NP_006262), *S100A2* (P29034), *S100A3* (XP_040006), *S100A4* (NP_777020), *S100A5* (XP_001343), *S100A6* (XP_040007), *S100A7* (XP_048124), *S100A8* (XP_086400), *S100A9* (B31848), *S100A10* (XP_001468), *S100A11* (P31949), *S100A12* (JC4712), *S100A13* (Q99584), *S100A14* (O60417), *S100A15* (Q9HCY8), *S100B* (P04271), *S100P* (S24146), and *S100Z* (AAL30893). Sources of fish *S100* genes: trout *S100* (AF077613), loach *S100* (S35985), catfish *ictacalcin* (AAB52610), fugu *ictacalcin* (AL834856), trout *ictacalcin* (CA378383), and zebrafish *ictacalcin* (AY233453). Fish *ictacalcin* genes are highlighted in red color. **C:** Map position of the zebrafish *ictacalcin* gene based on radiation hybrid mapping. Zebrafish *ictacalcin* gene is highlighted in red color. Mapped gene and placement expressed sequence tag markers are highlighted in blue and black colors, respectively, for orientation.

RESULTS AND DISCUSSION

Isolation of Zebrafish *ictacalcin* cDNA

Through BLAST and keyword searches of the zebrafish EST database, we found more than 100 EST clones sharing high nucleotide identities to catfish *ictacalcin* gene. This high frequency of *ictacalcin* EST clones indicates that it is a highly abundant transcript in zebrafish. We also identified two potential EST clones corresponding to fugu (AL834856) and trout (CA378383) *ictacalcin* homologs by mining EST database. The putative fish ictacalcin polypeptides consisted of 92- to 95-amino acid residues, acidic pH of 4.7–5.8, and low molecular weight of around 10 kDa (Fig. 1A). This low molecular weight and acidic pH of fish ictacalcin proteins are consistent with other members of the S100 protein family. Homology analyses revealed zebrafish *ictacalcin* gene shares 54–74% amino acid identity with other fish *ictacalcin* genes, whereas it shares only 20–53% identity with other known *S100* genes (Fig. 1A). When *ictacalcin* and fish *S100* genes are aligned, we found the two EF-hand Ca^{2+}-binding motifs (N-termi-

nal motif, S^{21} to E^{36} and C-terminal motif, D^{61} to E^{74}) are highly conserved, whereas the amino acid residues outside the two Ca^{2+}-binding motifs are less conserved (Fig. 1A). Compared with trout and loach *S100* genes, which showed apparent orthologous relationship to human *S100A10* and human *S100A1* genes, respectively (Fig. 1B), the tree topology did not ambiguously support an orthologous relationship of fish *ictacalcin* to any member of the human *S100* genes (Fig. 1B). We further explored the possibility that *ictacalcin* orthologs exist in nonfish vertebrates. However, no obvious *ictacalcin* ortholog was discovered in tetrapods in our BLAST search. This result strongly supports the hypothesis that fish *ictacalcin* genes are new members of the vertebrate *S100* gene family and probably evolved after the divergence of the fish and tetrapod lineages.

In human, most of the *S100* genes are clustered within a 2-Mb region at chromosome band 1q21 called the EDC (Mischke et al., 1996; South et al., 1999). By using the LN54 radiation hybrid panel, we have mapped the zebrafish *ictacalcin* gene to LG16, 8.2 cR (118 kb/cR) downstream from the *apoE* gene (Fig. 1C). Of interest, we found one placement marker, fa94d11, located 3 cR downstream from the *ictacalcin* gene that shows high amino acid identity to the human *S100A10* gene. Further studies on the genomic structure and chromosomal localization of the zebrafish *S100* genes will provide insights into the evolution of the *S100* gene family in vertebrates.

Spatiotemporal Expression Patterns of *ictacalcin*

By reverse transcriptase-polymerase chain reaction (RT-PCR), we found that *ictacalcin* gene expression was not detectable in the cleavage-stage embryos. The zygotic transcripts of the *ictacalcin* gene were initially detected at the mid-somitogenesis stage (12 hours postfertilization, hpf) and remained expressed throughout the adult stage (data not shown). We assayed the spatial expression of the *ictacalcin* gene by whole-mount in situ hybridization (WISH) during embryonic development from 9 to 72 hpf. In a negative control experiment with sense riboprobe for *ictacalcin*, we did not find any positive staining signal (data not shown). The tentative expression patterns of *ictacalcin* were observed as follows.

Expression of *ictacalcin* in Envelope Cells and Keratinocytes

Our understanding on skin development in fish can be largely attributed to the expression of the *keratin* genes (Imboden et al., 1997; Schaffeld et al., 1998, 2003; Conrad et al., 1998; Martorana et al., 2001). According to the expression sites, *keratin* genes can be divided into E (epidermal keratinocyte)-type and S (simple epithelia)-type (Conrad et al., 1998; Schaffeld et al., 1998). We found that the expression of *ictacalcin* is similar to E-type *keratin*, because the *ictacalcin* staining is detected solely in striated epithelial cells covering the entire embryo. Initially, *ictacalcin* transcripts were detected weakly and diffusely in ectoderm-derived epidermis around 12 hpf (data not shown), which coincided well with the RT-PCR result. By 18 hpf, *ictacalcin* transcripts were detected diffusely in the epidermis (Fig. 2A), but they were up-regulated in envelope cells scattered on the surface of the yolk sac (Fig. 2A), yolk extension (Fig. 2B) and trunk (Fig. 2C). From 24 hpf onward, *ictacalcin* expression was sharply down-regulated in envelope cells. However, an up-regulated expression of *ictacalcin* was noticed in the keratinocytes (Fig. 2C), including those in the cephalic epidermis (Fig. 2D), tail fin folds epidermis (Fig. 2E), and mucosal epithelium (Fig. 2F,G) when development proceeded. In teleosts, the polygonal-shape keratinocytes are often adjacent to each other in tight junctions, to form the impermeable epithelial sheets (Mittal and Whitear, 1979; Fristrom, 1988). High-power magnification revealed that the *ictacalcin* staining was gradually concentrated in the peripheral domain of the hexagonal-shape keratinocytes, whereas most of the cytoplasm and nucleus were absent from *ictacalcin* staining by 48 hpf (Fig. 2D,F). This mesh-like appearance of *ictacalcin* staining is distinct from that of E-type *keratin*, which showed a concentrated expression in the cytoplasm surrounding the nuclei of keratinocytes (Imboden et al., 1997; Gong et al., 2002). By 72 hpf, the epithelial cells covering the entire embryo, are all *ictacalcin*-positive except for the neuromasts deposited in the cephalic and trunk lateral lines (Fig. 3J).

Expression of *ictacalcin* in Olfactory Epithelium

The expression of *ictacalcin* in the olfactory placodes was initially activated around 18 hpf. From 24 hpf onward, *ictacalcin* transcripts were robustly detected in the entire olfactory epithelium (Fig. 2H,I), especially in the epithelial cells surrounding the opening of the nasal cavity (Fig. 2J). Similar to *pvalb3a* (Hsiao et al., 2002), also a member of the EF-hand Ca^{2+} binding protein gene family, a down-regulated expression of *ictacalcin* was apparent in the olfactory epithelium from 48 hpf onward. By 72 hpf, only the peripheral domain of the nasal cavity was positive for *ictacalcin* staining (Fig. 2K).

Expression of *ictacalcin* in Hatching Gland, Pectoral Fin Buds, and Urogenital Opening

During zebrafish embryogenesis, *ictacalcin* was also preferentially expressed in tissues enriched with epithelial folds such as the hatching gland, pectoral fin buds, urogenital opening, and pharynx. From 24 (Fig. 2L) to 36 hpf (Fig. 2M), we noticed that the entire hatching gland, arranged in a hemicircle shape, was heavily stained for *ictacalcin* transcripts. However, the *ictacalcin* expression in hatching gland was very transient, because its transcripts were completely abolished after hatching out (52 hpf). The onset of pectoral fin buds outgrowth begins around 31 hpf in zebrafish (Monnot et al., 1999), and we noticed that the *ictacalcin* expression was coincidental with the outgrowth of pectoral fin buds. By 36 hpf, *ictacalcin*

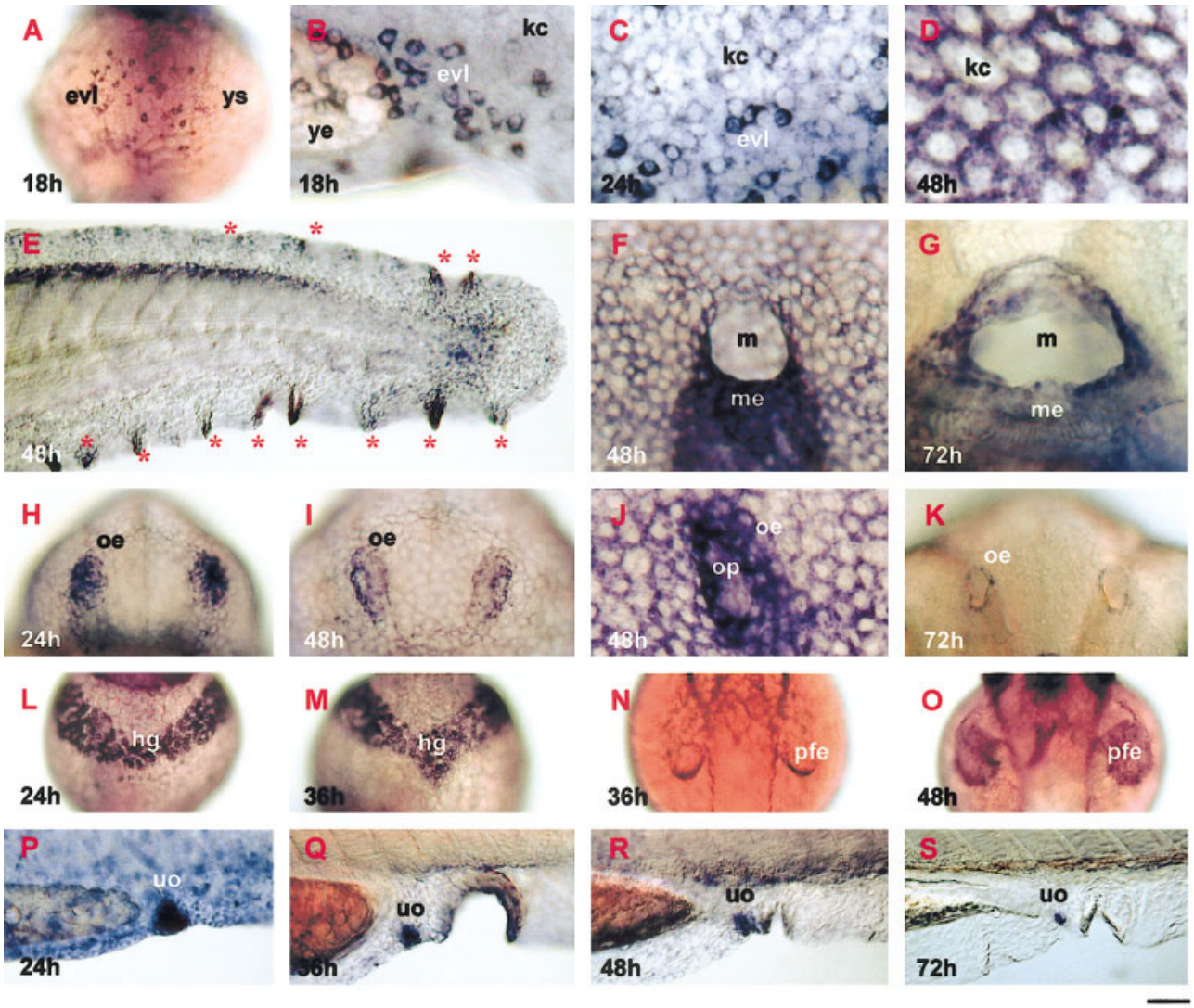
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Fig. 2.

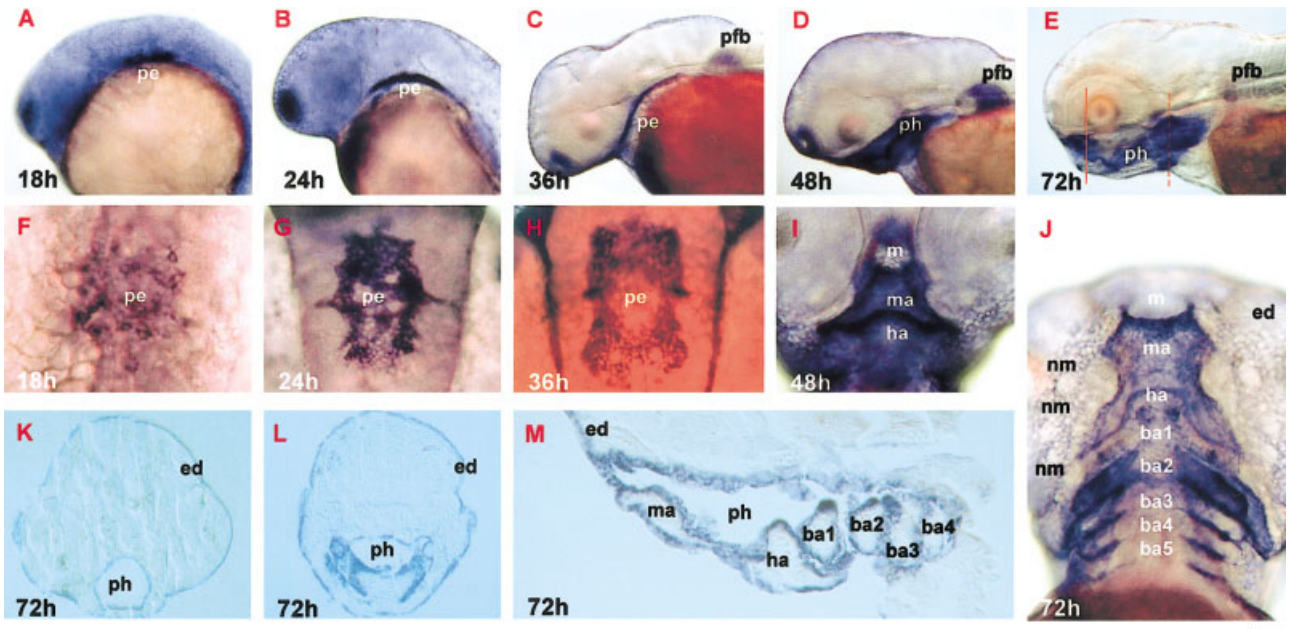
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Fig. 3.

transcripts were restricted to the apical margin of the pectoral fin buds epidermis (Fig. 2N). From 36 hpf onward, the *ictacalcin*-positive epidermal cells extended from the apical margins to cover the entire pectoral fin buds (Fig. 2O), and a strong expression level was maintained until 72 hpf, the latest stage we examined. Other organs showing *ictacalcin* staining include the urogenital opening. Compared with *evx1* (Thaeron et al., 2000), which labels the putative urogenital opening as early as the 11-somite stage, we found that the *ictacalcin* staining in the urogenital opening was not visible until 24 hpf (Fig. 2P). From 24 hpf onward, the *ictacalcin* staining in the urogenital opening became gradually restricted (Fig. 2P–S); therefore, only a spot-like appearance of *ictacalcin* staining was detected in 72 hpf-old embryos (Fig. 2S).

Expression of *ictacalcin* in Pharynx

In vertebrates, the reiterated outgrowth of the endodermal wall of the pharynx is the first morphologic sign of segmentation in the pharyngeal region (Goodrich, 1930). We also detected abundant *ictacalcin* transcripts in the developing pharynx from 18 hpf onward (Fig. 3A,F). By 24 hpf, the *ictacalcin* staining in the pharyngeal region became sharper and more condensed (Fig. 3B,G). From 36 hpf onward, *ictacalcin*-expressing cells extended anteriorly, up to the protrusion of the mouth (Fig. 3C,H). By 48 hpf, the epithelium in the pharyngeal region folded to form a hollow pharynx, and the *ictacalcin* transcripts are detected in abundance in the mucosal and gill epithelia, which covered the entire pharyngeal arches (including the mandibular arch, hyoid arch, and the five branchial arches; Fig. 3D,E,I,J). Tissue sections revealed that both the endoderm-derived epithelial cells lining the pharynx and the ectoderm-derived striated epithelium covering the pharyngeal arches are *ictacalcin*-positive, whereas the mesoderm-derived muscles as well as the neural crest-derived chondrocytes in the pharyngeal arches are all *ictacalcin*-negative (Fig. 3K–M). Therefore, unlike the epidermis-specific E-type *keratin* gene, both the epithelial cells covering the epidermis (ectoderm-derived) and lining the pharynx (endoderm-derived) are positive for *ictacalcin* staining.

Concluding Remarks

ictacalcin was found to be the most abundant transcript of the skin transcriptome in adult catfish (Porta et al., 1996; Karsi et al., 2002). In this report, we cloned the zebrafish *ictacalcin* ortholog and assayed its spatiotemporal expression during embryogenesis. Phylogenetic analyses strongly support *ictacalcin* as being a new member of the vertebrate *S100* gene family. The *ictacalcin* transcripts in keratinocytes are anchored to the peripheral domain and show distinct subcellular localization from the E-type *keratin* gene. Similar to its catfish ortholog, zebrafish *ictacalcin* gene expression was also preferentially detected in the epidermal cells covering the entire embryo. The noticeably high expression of *ictacalcin* in the epithelial lining cells of the mouth and pharynx allows us to follow the pharynx development in zebrafish.

EXPERIMENTAL PROCEDURES

The zebrafish EST database maintained at NCBI (http://ncbi.nlm.nih.org) was searched for sequence annotations indicative of possible homology to *ictacalcin* gene by either BLAST or keyword search. The full-length cDNA of zebrafish *ictacalcin* gene was obtained by performing 5′ and 3′ rapid amplification of cDNA ends, following the manufacturer's instruction (Clontech). To

Fig. 2. Expression of zebrafish *ictacalcin* gene in striated epithelium covering the entire body. Lateral views (B–E,P–S), dorsal views (N,O), ventral views (A,F–I,K–M), and flatted mount (J) of zebrafish embryos followed in situ hybridization for *ictacalcin* riboprobe. Anterior is to the left in B, C, E, P–S. Anterior is to the top in A, D, F–O. A–D: Expression of *ictacalcin* in the envelope cells and keratinocytes. **A,B:** At 18 hours postfertilization (hpf), *ictacalcin*-positive envelope cells scattered in the yolk sac, yolk extension, and epidermis. **C:** At 24 hpf, *ictacalcin* transcripts are detected in both envelope cells and keratinocytes. **D:** At 48 hpf, high-power magnification showing the enriched expression of *ictacalcin* in the peripheral margin of keratinocytes. **E,F:** At 48 hpf, *ictacalcin* is strongly expressed in the tail fin folds (indicated by asterisks) and mucosal epithelium. **G:** At 72 hpf, strong expression of *ictacalcin* in the mucosal epithelium. **H–K:** Expression of *ictacalcin* in the olfactory epithelium. Olfactory epithelium in olfactory placodes (H, 24 hpf) and olfactory pits (I and J, 48 hpf) are heavily stained with *ictacalcin*. K: 72 hpf, only the peripheral margin of the nasal cavities is positive for *ictacalcin* staining. **L,M:** Expression of *ictacalcin* in the hatching gland at 24 (L) and 36 hpf (M). **N,O:** Expression of *ictacalcin* in the pectoral fin buds epidermis at 36 (N) and 48 hpf (O). **P–S:** Expression of *ictacalcin* in the developing urogenital opening. evl, envelop cells; hg, hatching gland; kc, keratinocytes; m, mouth; me, mucosal epithelium; oe, olfactory epithelium; op, olfactory pits; pfe, pectoral fin buds epidermis; uo, urogenital opening; ye, yolk extension; ys, yolk sac. Scale bar = 100 μm in A,L–O, 50 μm in E,H,I,K,P–S, 25 μm in B,C,F,G,J, 12.5 μm in D.

Fig. 3. Expression of zebrafish *ictacalcin* gene in the developing pharynx. Anterior is to the left in (A–E,M). Anterior is to the top in (F–J). Lateral views (A–E), dorsal views (F–H), ventral views (I,J), transverse sections (K,L), and longitudinal section (M) of whole-mount embryos followed in situ hybridization for *ictacalcin* riboprobe. **A,F:** At 18 hours postfertilizationh (hpf), onset of *ictacalcin* transcription in pharyngeal region. **B,G:** At 24 hpf, *ictacalcin* is up-regulated in the shaping pharyngeal region. **C,H:** At 36 hpf, the *ictacalcin* staining in the developing pharynx is extending more anterior to the mouth position. **D,I:** At 48 hpf, when the mouth is protruding, the mucosal epithelium covering the developing mandibular and hyoid arches shows condensed *ictacalcin* staining. **E,J:** At 72 hpf, the mucosal and gill epithelia covering the entire pharyngeal arches (one mandibular arch, one hyoid arch, and five branchial arches) shows heavy *ictacalcin* staining. Note that the neuromasts in the cephalic lateral line are devoid of *ictacalcin* staining. **K–M:** Transverse (K,L) and longitudinal (M) sections at the level of the head show the superficial distribution of *ictacalcin* transcripts in the epidermis and lining of pharynx. The solid and dotted lines in E indicate the section levels in K and L, respectively. ba1–5, branchial arches 1–5; ed, epidermis; ma, mandibular arch; m, mouth; me, mucosal epithelium; ha, hyoid arch; nm, neuromast; pe, pharyngeal endoderm; pfb, pectoral fin bud; ph, pharynx. Scale bar = 100 μm in A–E, 50 μm in F–J, 25 μm in K,L, 12.5 μm in M.

identify potential *ictacalcin* genes in other vertebrates, we performed BLAST searches in all vertebrate EST databases using the zebrafish *ictacalcin* sequence. The protein sequences were translated from nucleotide sequences using the sequence utilities available through the BCM Search Launcher interface (http://search-launcher.bcm.tmc.edu). Protein sequences of *ictacalcins* were aligned by using CLUSTAL W (Thompson et al., 1994), and phylogenetic trees were constructed by the neighbor-joining method (Pearson et al., 1999) through the DDBJ interface (http://www.ddbj.nig.ac.jp/E-mail/clustalw-e.html). Confidence in the branch nodes was assessed with 1,000 bootstrap replicates. RT-PCR and WISH were performed by using the procedures described by Hsiao et al. (2003). The PCR primers used for detecting zebrafish *ictacalcin* gene transcripts were as follows: *ictacalcin*-F (5′-GCCTACTGGACTCGCTTGCT-3′) and *ictacalcin*-R (5′-TCATTTTTTCCTTTACAGACAAATCTATTC-3′). Digoxigenin-labeled, full-length, antisense (for experiment) and sense (for negative control) riboprobes for zebrafish *ictacalcin* gene were used for WISH. For tissue sectioning, stained embryos were embedded in paraffin and sectioned at 5 μm thickness. For chromosomal mapping, PCR was performed on the LN54 radiation hybrid panel and the data were analyzed with RHMAPPER program (Hukriede et al., 2001).

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