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<Original Article>

Expression analysis of ciliary rootlet coiled coil protein mRNA during *Xenopus* development

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Abstract : Ciliary rootlet coiled coil protein (CROCC), the structural component that originates from the basal body at the proximal end of the ciliary rootlet, plays a crucial role in maintaining the cellular integrity of ciliated cells. In the current study, we cloned *Xenopus* CROCC and performed the expression analysis. The amino acid sequence of *Xenopus laevis* was related to those of *Drosophila*, cow, goat, horse, chicken, mouse and human. Reverse transcription polymerase chain reaction analysis revealed that CROCC mRNA encoding a coiled coil protein was present maternally, as well as throughout early development. *In situ* hybridization indicated that CROCC mRNA occurred in the animal pole of embryo during gastrulation and subsequently in the presumptive neuroectoderm at the end of gastrulation. At tailbud stages, CROCC mRNA expression was localized in the anterior roof plate of the developing brain, pharyngeal epithelium connected to gills, esophagus, olfactory placode, intestine and nephrostomes of the pronephric kidney. Our study suggests that CROCC may be responsible for control of the development of various ciliated organs.

Keywords : ciliary rootlet coiled coil protein, esophagus, nephrostomes, pharyngeal epithelium, *Xenopus*

Introduction

Cilia and flagella are highly ordered organelles in eukaryotes which are involved in generating directional movement. Functional defects of these organelles are linked to several human diseases called ciliopathies [4]. Cilia also play an important role in generating directed fluid flow that is essential for a variety of physiological and developmental processes, including cell migration and left-right asymmetry determination of organ development [2]. Proper understanding of the function of ciliated cells would help to elucidate the molecular basis of animal and human diseases. *Xenopus* embryo is a suitable model for the analysis of ciliated cell fate due to the development of ciliated cells in its surface ectoderm [8, 13].

Rootletin, the ciliary rootlet coiled coil protein (CROCC), is the structural component of ciliary rootlet that originates from the basal body at the proximal end of the cilium found in the cilia of the rootlet. CROCC has two different domains, a globular head domain and a tail domain consisting of extended coiled-coil structures. It is a ubiquitous long-sought component of the motile cilia. Knockdown of rootletin in mice ablates the formation of ciliary rootlets in ciliated cells, which is suggestive of a crucial role for the ciliary rootlet in maintaining the cellular integrity of ciliated cells [13]. In cells with motile cilia such as epithelia lining the airways and brain ventricles, ciliary rootlets appear as a fibrous network [3].

Rootletin is related to C-Nap1, which is a structural component of the ciliary rootlet in murine photoreceptor cells [15]. Subsequently, near complete elimination of rootletin from mice was found to cause photoreceptor degeneration and impaired mucociliary clearance, which is consistent with a key function of this protein in rootlet structures [14].

Though CROCC is a component of the ciliary rootlet and there is some literature available regarding the structure and function of CROCC in other species, the expression pattern during early development remains unclear. We therefore conducted phylogenetic analysis of CROCC and determined the temporal-spatial expression pattern of CROCC mRNA during *Xenopus* early development.

Materials and Methods

Xenopus (*X.*) *laevis* husbandry

X. laevis were handled in accordance with animal welfare regulations of the Institutional Animal Care and Use Committees (IACUC; CBU 2013-0010), Chonbuk National University Laboratory Animal Centre, South Korea. *X. laevis* embryos were maintained according to standard protocols

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[10]. Embryos were staged [6] and raised in 0.1x normal amphibian medium [11]. All efforts were made to minimize the discomfort of animals used.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from stage 1 to 45 embryos [6], digested with DNase I, and purified using an RNeasy cleanup kit (Qiagen, the Netherlands). The gene reference sequence for *Xenopus* CROCC (XM_002942592) was obtained from the NCBI database. *Xenopus* CROCC was amplified by PCR from stage 35/36 cDNA using primers (forward: 5'-ggcaggtcatactggatgct-3'and reverse: 5'-ctgcctctcgttccactctc-3') based on the available gene bank sequence. RT-PCR was performed using a Maxim RT-PCR Premix Kit (iNtRON, Korea). In parallel, human elongation factor 1 alpha (*EF1α*; forward: 5'-accctcctcttggtcgtttt-3' and reverse: 5'-tttggttttcgctgctttct-3') was amplified as an internal control. The PCR conditions were 94°C for 40 sec, 60°C for 40 sec, and 72°C for 1 min for 35 cycles, and a final extension at 72°C for 10 min.

Sequence comparison and phylogenetic analysis

The amino acid identity was searched with BLAST services available at the National Center for Biotechnology Information (USA) website. Amino acid sequences of *Drosophila* (NP_651216.2), cow (XP_005203428.1) goat (XP_005 677122.1), horse (XP_005607667.1), chicken (XP_425750.4), mouse (XP_006538865.1), and human (AA126912.1) were obtained from the GenBank database and aligned with the MegAlign program (DNASTAR) using Clustal W [12]. The aligned amino acid sequences were used to construct a phylogenetic tree with the neighbor-joining method [9].

***In situ* hybridization**

Anti-sense digoxigenin (DIG)-labeled probe was transcribed according to standard procedures [5]**.** CROCC was linearized with Nco1 (Promega, USA) and transcribed with SP6 (Roche, Germany). *In vitro* fertilized *X. laevis* embryos were fixed with MEMFA (MOPS, EGTA, $MgSO_4$, and formaldehyde) at required stages for whole mount *in situ* hybridization, as previously described [5]. Embryos were stored in methanol at −20°C overnight, rehydrated through a graded series of methanol and washed in phosphate-bufferd saline (PBS). Embryos were treated with proteinase K for 10 min at room temperature before re-fixation and pre-hybridization over night at 60°C. On addition of the probe, the embryos were incubated at 60°C overnight. Embryos were then subjected to a series of saline-sodium citrate buffer washes at 60°C. Blocking was done prior to overnight incubation with anti-DIG antibody (1 : 2000) at 4°C. Embryos were washed 4 times for 1 h in PBT (PBS-tween 20) followed by 5 min washes in alkaline phosphatase buffer. Color reactions were developed with BM-purple substrate (Roche, Germany). Reactions were stopped by washing in PBS, followed by re-fixation. Wild type embryos were bleached in methanol (2) : 30% hydrogen peroxide (1) to fully reveal expression patterns. Whole mount pictures were taken on an Olympus MVX10 microscope (Olympus Corporation, Japan).

For conducting *in situ* hybridization on sections, stage 29/30, 35/36, 40 and 45 embryos were selected and dehydrated with a series of ethanol, transferred to xylene and then embedded in paraffin. Serial 12-μm sections were cut with a rotary microtome (Microm HM 325 Rotary Microtome; Thermo Scientific, USA) and hybridized with the appropriate probes. Sections were then briefly counterstained with eosin. Images were acquired digitally using a Leica DM2500 microscope (Leica Microsystems, Germany).

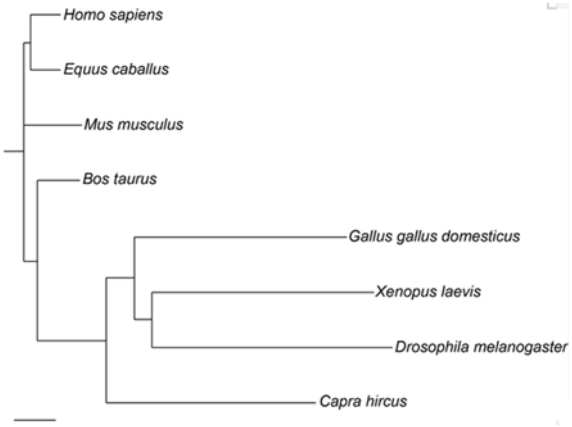

Fig. 1. Phylogenetic tree based on amino acid alignment showing the relationships among species specific CROCC proteins. The tree is based on an amino acid sequence alignment performed with the Clustal W method. The scale bar measures the distances between sequences. The GenBank accession numbers are as follows: *Drosophila melanogaster*, NP_651216.2; *Bos Taurus* (cow), XP_005203428.1; *Capra hircus* (goat), XP_005677122.1; *Equus caballus* (horse), XP_005607667.1; *Gallus gallus domesticus* (chicken), XP_425750.4; *Mus musculus* (mouse), XP_006538865.1; *Homo spiens* (human), AA126912.1.

Results

***X. laevis* CROCC**

The partial *Xenopus* CROCC cDNA sequences (301 amino acids) encoded proteins that show varying degrees of sequence conservation with other species. In particular, the partial sequence of *Xenopus* CROCC amino acids shared 29%, 33%, 35%, 34%, 35%, 34% and 33% identities with *Drosophila melanogaster, Bos taurus*, *Capra hircus*, *Equus caballus, Gallus gallus, Mus musculus* and *Homo sapiens*, respectively. Figure 1 shows a phylogenetic tree of CROCC protein including the above species. In addition, we performed phylogenetic analysis based on sequence alignment in order to further investigate the relationship among these species. The phylogenetic tree indicated that CROCC proteins in different species segregate and are distinct from one another (Fig. 1).

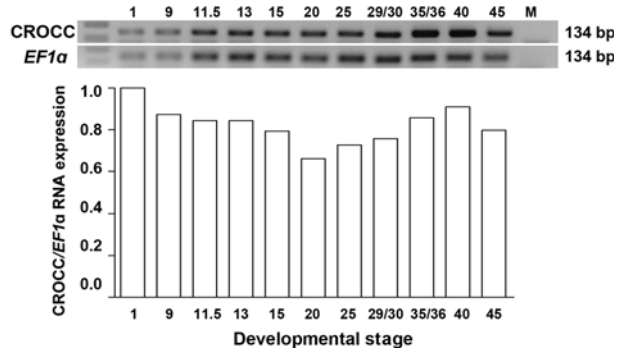

Fig. 2. Temporal expression profile of CROCC during Xenopus early development. Expression was analyzed by RT-PCR of RNA extracted from embryos at the indicated developmental stages. *EF1α* was used as a loading control. Lane M shows a negative control (absence of RNA). In *Xenopus laevis*, CROCC expression was initiated from stage 1 (maternal) onward (zygotic).

Temporal expression of CROCC mRNA

The temporal expression patterns of *X. laevis* CROCC mRNA (Fig. 2) were analyzed by RT-PCR using total RNA from different developmental stages including the early cleavage stages [6]. CROCC RNA expression was observed in all analyzed stages, including the early cleavage stages. The CROCC transcript was maternally synthesized and persisted until the stage 45, examined.

Localization of CROCC mRNA

The spatial expression patterns were analyzed using whole mount *in situ* hybridization and *in situ* hybridization on sections of different stages of embryos. *Xenopus* CROCC mRNA was strongly enriched in the animal hemisphere at the early blastula stage (stage 15) (Fig. 3A). *In situ* hybridization on sections indicated specific expression in the surface ectoderm. Initially, the CROCC mRNA was expressed diffusely in the surface ectoderm at both blastula and neurula stages (Fig. 3B and C).

Whole mount *in situ* hybridization showed that in the tailbud stage (stage 29/30), CROCC mRNA expression was unambiguously detected in the anterior part of the developing brain (Fig. 4A); however, at the late tailbud stage (stage 35/36), it was expressed in the anterior part of the brain and very weakly expressed in the esophagus and nephrostomes (Fig. 4B). *In situ* hybridization on sections revealed more detailed expression. At stage 29/30, expression was localized to the roof plate of the anterior part of the neural tube (Fig. 4C). CROCC mRNA expression was detected in the olfactory region at stage 35/36 and expression in the anterior part of the developing brain also persisted at this stage (Fig. 4D and E). Interestingly, on this stage, the expression was observed in the nephrostomes of the pronephric kidney (Fig. 4F). At stage 40, along with the nephrostomes, CROCC mRNA expression was detected very weakly in the esophagus (Fig. 4G). At the tadpole stage (stage 45), CROCC mRNA was first detected in the pharyngeal epithelium that may be connected to the gills (Fig. 4H) and subsequently the expression was confined to a ring-like structure of the esophagus (Fig. 4I) that was finally connected to the intestine (Fig. 4J).

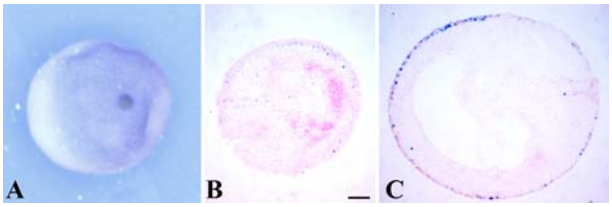

Fig. 3. Developmental expression of CROCC in *Xenopus* by *in situ* hybridization. (A) CROCC mRNA was expressed in the animal hemisphere at stage 15. (B) *In situ* hybridization on sections obtained at stage 15 and (C) at stage 20, CROCC was expressed in the surface ectoderm. Scale bar = 100 μm.

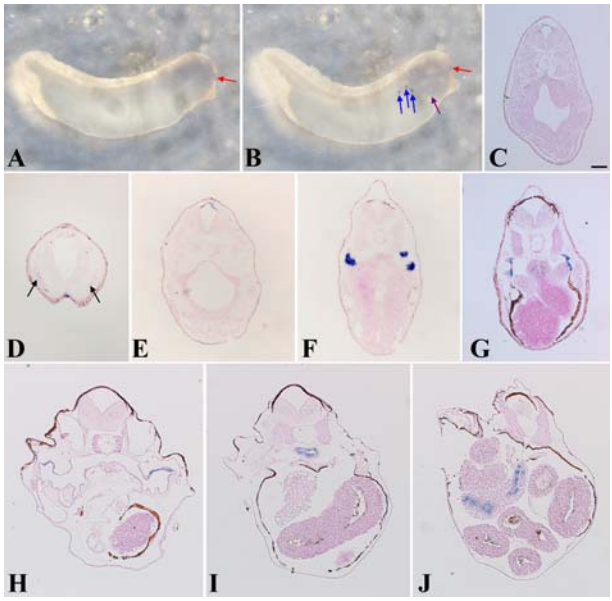

Fig. 4. Expression analysis of CROCC gene at different stages of development in *Xenopus*. (A) Whole mount *in situ* hybridization showed the expression of CROCC mRNA in the anterior brain at stage 29/30. (B) CROCC was expressed in the anterior part of the brain, surface ectoderm at neck region and nephrostomes at stage 35/36. (C) In the sections, CROCC was expressed in the roof plate of the neural tube at stage 29/30. (D) CROCC was expressed in the olfactory placode at stage 35/36. (E) CROCC expression persisted in the roof plate of the neural tube at stage 35/36. (F) Expression was strongly detected in the nephrostomes. (G) CROCC was expressed in the nephrostomes at stage 40. (H) CROCC also expressed in pharyngeal epithelia at stage 45 that are connected to the gills. (I) Expression was confined to esophagus (stage 45) and (J) Esophageal expression continued to the intestine (stage 45). Red arrow, roof plate of the neural tube; Blue arrow, nephrostomes; Purple arrow, surface ectoderm at the pharyngeal region; Black arrow, olfactory placode. Scale bar = 100 μm.

Discussion

We reported the CROCC mRNA expression pattern in developing *X. laevis* embryos. The primary function of the ciliary rootlet is to provide structural support for the cilium [14]. The ciliary rootlet is composed of polymerized rootletin bundle fibers into thick filaments [15]. In the present study, the phylogenetic relationship between the *Xenopus* CROCC and its known homologues from different species including *drosophila*, rat, chicken, goat, cow, horse and human was analyzed using neighbor joining methods. As a results, CROCC proteins were conserved among all of the species studied. According to RT-PCR results, CROCC is a maternal gene which expression begins in the early cleavage stage and continues onward (zygotic) where expression was persisted up to stage 45, the last stage examined in this study.

Based on *in situ* hybridization, *Xenopus* CROCC mRNA was expressed in the ciliated epidermis at the early cleavage stage and in the surface ectoderm at the early neurula stage. In the tailbud stage, we found that CROCC mRNA was also expressed in the roof plate of the anterior neural tube and the olfactory organ. Mouse CROCC was localized in discrete structures consistent with dendritic knobs of the olfactory sensory neurons [7]. These findings suggest that CROCC may have a role in the development of neural tissue and sensory organs. In *Xenopus,* CROCC may also participate in the development of the pharynx and esophagus as indicated by its expression in the pharyngeal epithelium and esophagus.

Whole mount *in situ* hybridization revealed surface ectodermal expression of CROCC in the pharyngeal region that is connected to the gills. The gills are the respiratory organs of *Xenopus* comprising comb-like filaments, the gill lamellae, which increase the surface area for oxygen exchange. Along with other cilia-related genes, mouse CROCC mRNA is also expressed in the respiratory epithelium [7]. Thus, CROCC may be responsible or play a role in the development of the respiratory organs in both vertebrates and mammals.

In the present study, CROCC mRNA was exclusively expressed in nephrostomes of *Xenopus* that are specific to the amphibian pronephric kidney. Nephrostomes are funnel-shaped ciliated openings of the excretory tubules into the coelom that propel water, metabolic waste, unnecessary hormones and other substances into the metanephridium [1]. In *Xenopus*, CROCC may thus have a vital role in regulation of the disposal function of the pronephric kidney by activating the cilium of the nephrostomes.

The overall conclusion of the present study is that cilia-related *Xenopus* CROCC is a maternally enriched gene that is expressed in the roof plate of the anterior part of the developing brain, the pharyngeal epithelium connected to the gills, and is exclusively expressed in another ciliated parts including the nephrostomes, through which *Xenopus* disposes of waste materials. Functional analysis will, however, be necessary to reveal the specific location-dependent functions of CROCC.

Acknowledgments

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