pISSN 1976-7447, eISSN 2287-7363
J Biomed Res 2014;15(4):194-199
http://dx.doi.org/10.12729/jbr.2014.15.4.194
Received 21 Nov. 2014, Revised 6 Dec. 2014, Accepted 11 Dec. 2014

Original Article

Na, K-ATPase β2 isoform (*atp1b2*) expressed in the retina of *Xenopus*

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The ubiquitous Na, K-ATPase is a membrane-bound ion pump located in the plasma membrane in all animal cells and plays an essential role in a variety of cellular functions. Studies in several organisms have shown that this protein regulates different aspects of embryonic development and is responsible for the pathogenesis of several human diseases. Na, K-ATPase is an important factor for retinal development, and combinations of the isoforms of each of its subunits are expressed in different cell types and determine its functional properties. In this study, we performed RT-PCR assay to determine temporal expression and *in situ* hybridization to determine spatial expression of Na, K-ATPase β2 isoform (*atp1b2*) in *Xenopus laevis*. Focusing on retinal expression to distinguish the specific expression domain, we used retinal marker genes *sox4, sox11, vsx1*, and *pax6*. *Xenopus atp1b2* was expressed from late gastrulation to the tadpole stage. Using whole mount *in situ* hybridization, we showed that *Xenopus atp1b2* was expressed broadly in the eye, the whole surface ectoderm, and gills. *In situ* hybridization on sections revealed detailed and specific expression in the outer nuclear layer of the retina, which consists of two major classes of photoreceptors, rods and cones, surface ectoderm, pharyngeal epithelium, and gills. These findings indicate that *atp1b2* may play an important role for the development of *Xenopus* retina.

Key words: Na, K-ATPase β2 isoform, outer nuclear layer, retina, pharyngeal epithelium, *Xenopus*

Introduction

The vertebrate retina is a light-sensitive layer of tissue, lining the inner surface of the eye. It's a model for studying mechanism underlying neural cell proliferation, fate choice, signaling and tissue patterning due to its accessibility and simplicity. The retina has six major classes of neurons and a single class of glial cells. The outer nuclear layer consists of cell bodies of rods and cones. The inner nuclear layer contains the Muller cells, horizontal, bipolar and amacrine interneurons. The ganglion cell layer contains nuclei of ganglion cells, the axons of which become the optic nerve fibers for messages and some displaced amacrine cells. The ganglion cells send their axons through the optic nerve to the brain. In amphibians, the site of continuous neurogenesis is in the periphery of the retina and allowing it to grow through the lifespan of the animals [1, 2].

In the retina the Na, K-ATPase restores Na^+ and K^+ gradients used by the photoreceptor dark current, synaptic activity, action potentials, and transmitter uptake. Inner segments of the outer nuclear layer of retina have the high concentrations of Na, K-ATPase [3]. The ubiquitous Na, K-ATPase is a membrane bound ion pump located in the plasma membrane in all animal cells, where it maintains the electrochemical gradients of sodium and potassium ion across the membranes and plays an essential role for variety of cellular functions including osmoregulation, sodium coupled transport of variety of organic molecules, neuronal and muscle cells activity. The Na, K-ATPase is composed of two non-covalently linked subunits: catalytic α subunit as well as β subunit which is required for the structural and functional maturation of α subunit [4-11].

The β subunit is a type II glycosylated membrane protein required for the modulation of sodium and potassium ion of the functional enzyme [12, 13]. In mice, deficiency of β2 (*atp1b2*) exerts motor incoordination, tremors and paralysis of the extremities [14]. The *atp1b2* expression abrogates glioblastoma-derived brain tumor-initiating cells in human [15]. Genetically modified *atp1b2* subunits are associated with apoptosis of photoreceptors in mice [14, 16, 17]. In postnatal mouse, increases in *atp1b2* expression in bipolar cells occur very late, coinciding with synaptogenesis in the inner plexiform layer [18]. Here, we report the temporal and detailed expres-

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sion analysis of *atp1b2* during *Xenopus* embryogenesis.

Materials and Methods

Xenopus laevis Husbandry

Xenopus laevis was handled in accordance with animal welfare regulations of Institutional Animal Care and Use Committees (IACUC), Chonbuk National University Laboratory Animal Centre, South Korea. *Xenopus laevis* embryos were maintained according to standard protocols. All efforts were made to minimize the discomfort of animal used.

Reverse Transcriptase Polymerase Chain Reaction

Total RNA was extracted and digested with DNase I and purified with RNeasy cleanup kit (Qiagen). First strand cDNA was synthesized from stage 35 embryos with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas). RT-PCR was performed by using Maxim RT-PCR Premix Kit (iNtRON). The partial sequences of the *X. laevis atp1b2*, *vsx1* and *pax6* ORF were amplified using PCR from stage 30 cDNA using a set of primers designed against two conserved domain regions of each gene in several species. The resulting PCR products of 1,018 bp for *atp1b2*, 1,002 bp for *vsx1* and 1,010 bp for *pax6* were purified, subcloned into a pGEM-T Easy vector (Promega), and sequenced. Primers designed for cloning are shown in Table 1. The PCR conditions were 95°C for 30 seconds, 45°C for 30 seconds, 72°C for two minutes for 35 cycles, and final extension at 72°C for 15 minutes.

In situ hybridizations

Xenopus laevis eggs were collected, fertilized, and embryos were cultured under standard procedure in amphibian [19]. Anti-sense Digoxigenin-labeled probe was transcribed as the standard procedures [20]. The *atp1b2* plasmids were linearized with *Spe* I and transcribed with T7 polymerase. The retinal marker *sox4*, *sox11* plasmids were linearized with *Cla* I and *EcoR* V, respectively, and transcribed with T7 polymerase. The other retinal marker *vsx1* and *pax6* plasmids were linearized with Sac II and transcribed with SP6 polymerase. Whole mount images were taken on an Olympus MV × 10 microscope.

Stage 27, 30, 35, 41 and 47 embryos were dehydrated with a series of ethanol, transferred to xylene, and then embedded in paraffin. Serial sections of 12 μm were cut with microtome (Thermo, MICROM, HM 325). Anti-sense Digoxigenin-labeled probe was transcribed for *in situ* hybridization on sections and counterstained with eosin. The images were acquired digitally using a Leica DM 2500 microscope.

Histological analysis

Stage 47 embryos were dehydrated with a series of ethanol, transferred to Xylene, and then embedded in paraffin. Serial sections of 5 μm were cut with microtome and stained with hematoxylin and eosin.

Results

Temporal expression analysis

The temporal expression patterns of *Xenopus laevis atp1b2* (Fig. 1) were analyzed by RT-PCR using RNA of different developmental stages [21]. The *atp1b2* expression, which was not observed during early cleavage stages (stage 1~9), was observed at the late gastrulation stage (stages 11.5) and continued to be present until stage 40.

Spatial expression analysis

Embryos of stage 15, 20, 30 (tailbud), 40 and stage 45 (tadpole) were used to examine the tissue distribution of *Xenopus atp1b2*. We were interested in establishing the onset of *atp1b2* expression during *Xenopus* retinal development. The results of *in situ* hybridization analysis were shown in Fig. 2. Whole mount *in situ* hybridization showed that in early neurula stage embryos (stage

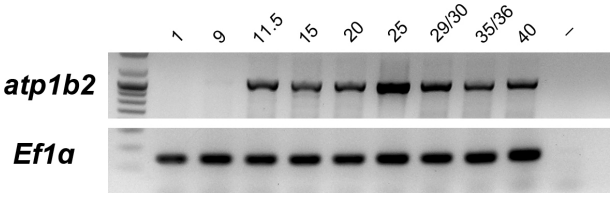

Fig. 1. Temporal expression of *atp1b2* during *Xenopus* development. Expression was analyzed by RT-PCR using RNA isolated from embryos at the indicated developmental stages. Elongation factor 1 alpha (Ef1α) was used as an internal control. Lane (-) shows a negative control in absence of RNA.

Table 1. Primer sequences for cloning and RT-PCR

Gene Name		Primer Sequence		Gene ID
		Forward	Reverse	
atp1b2	RT-PCR	TCGCATCAACAAGTGAAAGC	TCGCATCAACAAGTGAAAGC	NM_001086893
	Cloning	CCGTCATCTTCCTCATTGGT	CCGTCATCTTCCTCATTGGT	
vsx1	Cloning	AGCAAAATCAAAGGGCAAGA	AGCAAAATCAAAGGGCAAGA	NM_001096722
pax6	Cloning	GCCACATTCCCATTAGCAGT	GCCACATTCCCATTAGCAGT	NM_001085944

15) *Xenopus atp1b2* was expressed in the whole neuroectoderm (Fig. 2A) and at late neurula stage (stage 20) it was expressed in the whole surface ectoderm (Fig. 2B). At tailbud stage (stage 30) *atp1b2* was expressed in the eye and the developing brain with entire epidermal cells (Fig. 2C). *Xenopus atp1b2* expression was observed in the developing gills in tadpoles (stage 40 and 45) along with other expression domain (Figs. 2D and 2E). Gene expression persisted at a high level at least until stage 45, the last stage examined in the present study.

Whole mount *in situ* hybridization revealed that *Xenopus atp1b2* was expressed in the eye at stages 30 to 45. To determine the specific layer in which *atp1b2* was expressed, we performed *in situ* hybridization on the serial sections of stage 30, 35, 41 and 47 embryos. At tailbud stage (stage 30) *Xenopus atp1b2* was expressed in the retina and distinguishingly in the epidermal cells (Fig. 2F). At stage 35 we found an exclusive expression domain that was pharyngeal epithelium (Figs. 2G and 2H) and its expression persisting as the same pattern at stage 41 including the lateral prominence for gills (Figs. 2I and 2J). In tadpole (stage 47), the expression of almost the same domains including neural tube and the future brain also persisted, but at this stage the retinal expression was more specific in the outer nuclear layer of retina (Fig. 2K).

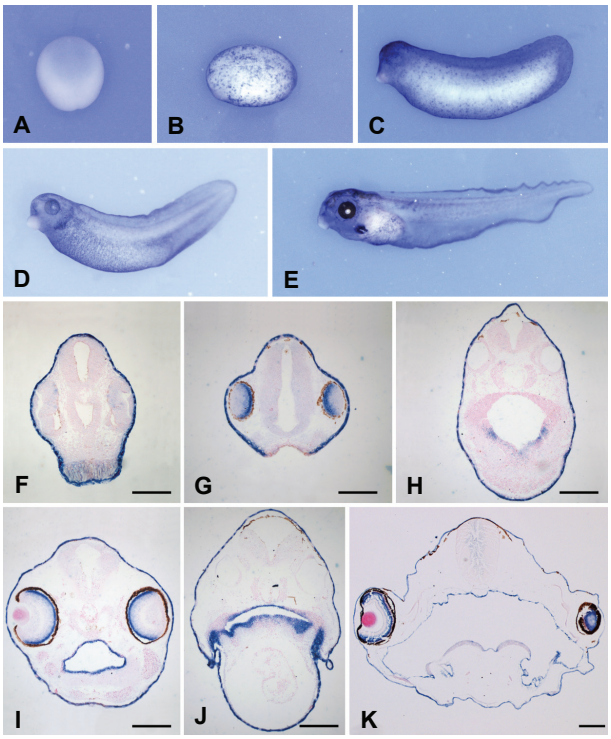

Fig. 2. Developmental expression of *atp1b2* by *in-situ* hybridization. Whole mount in situ hybridization results (A-E). *Xenopus atp1b2* was expressed in the neuroectoderm at stage 15 (A), in the entire epidermal cell layer at stage 20 (B), in the surface ectoderm and in the eye and brain at stage 30 (C), and in the brain, eye, developing gills along with surface ectoderm at stage 40 and 45 respectively (D, E). F, G, H, I, J and, K are the results of in situ hybridization on serial sections at different stages. The *atp1b2* was expressed in the epidermal cell layer and retina at stage 30 (F) and 35 (G), respectively. It was expressed in the pharyngeal epithelium at stage 35 (H) and in the epidermal cell layer, retina, pharyngeal epithelium along with a projections for developing gills at stage 41 (I, J). The expression of *atp1b2* persisted in the brain and specifically in the outer nuclear layer of retina at stage 47 (K). Scale bar=200 μm.

Na, K ATPase β2 subunit expression in the retina of eye

We also performed *in situ* hybridization on sections with different proneural and panneural retinal cell markers. We used *sox4* and *sox11* to visualize ganglion cell layer, *pax6* to visualize amacrine and ganglion cells, *vsx1* to detect bipolar cells and compare the expression with *atp1b2*. After conducting *in situ* hybridization on sections, it was observed that *atp1b2* was expressed in the retina at stage 27, in contrast to *atp1b2*, and all other marker genes were expressed in the retina at this stage (Figs. 3A-E). *Xenopus atp1b2*, *vsx1*, and *pax6* were weakly expressed in the retina at stage 30 whereas *sox4* and *sox11* were strongly expressed (Figs. 3F-J). Since *Xenopus atp1b2* was expressed in the retina we have sectioned later stage to see the specific layer of expression. At stage 35, *atp1b2* was expressed in outer part of the retina and among the retinal marker genes *sox4* and *sox11* were expressed strongly in the ciliary marginal zone (CMZ) whereas *pax6* was not expressed in the CMZ (Figs. 3K-O).

The specific retinal layer was not clearly detectable at

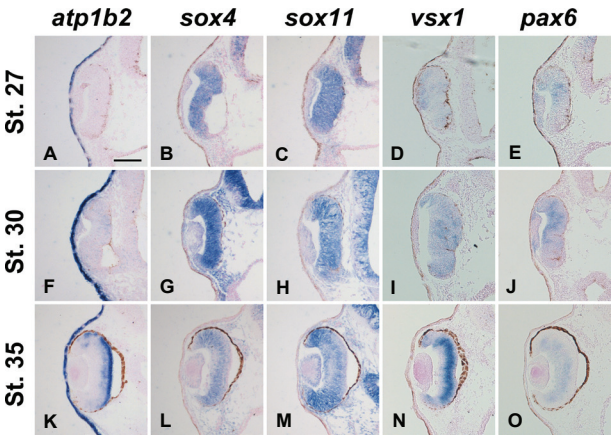

Fig. 3. Expression analysis of *atp1b2* in the retinal development before cellular differentiation. Transverse sections were performed at three different stages along the anteroposterior axis. Each row shows sections of different stages embryos and each column shows expression of different marker genes for retinal development. Expression of *atp1b2, sox4, sox11, vsx1* and *pax6* at stage 27 (A-E), at stage 30 (F-J), at stage 35 (K-O) respectively. Scale bar=100 μm.

stage 41 but we found the different domain of expressions (Figs. 4A-E). At stage 47, *Xenopus atp1b2* was specifically expressed in the outer nuclear layer of retina whereas *sox4* and *sox11* in the inner nuclear layer and ganglion cell layer, *vsx1* in the inner nuclear layer, and *pax6* were expressed both in the inner nuclear layer and ganglion cell layer (Figs. 4F-J).

Discussion

The *atp1b2* was first cloned as an adhesion protein and there is some evidence for its mediation of cell–cell interactions [14]. The question arises whether it could play a role in cell adhesion and histogenesis. Its only early expression in the mouse retina, however, was in photoreceptors that have already taken up position scleral to the still proliferating progenitor cells [22, 23]. Its expression in bipolar cells occurred long after histogenesis and appeared to reflect the increased need for ion transport consequent to synaptogenesis [16]. Investigation of the retina thus supports a role for *atp1b2* only in Na, K-ATPase activity.

In this study, we focused that *atp1b2* is expressed in the outer nuclear layer of retina in developing *Xenopus* embryo. We here provide a detailed description of the tissue specific expression of *atp1b2* isoform during *Xenopus* embryogenesis and thereby extend earlier finding by others.

The vertebrate retina consists of three nuclear layers of retina, the outer nuclear layer, the inner nuclear layer, and ganglion cell layer. Among them the outer nuclear layer consists of two major classes of photoreceptors, rods and cones [24]. Our data showed the expression of *atp1b2* in the outer nuclear layer. To detect the specific layer of expression we also observed the expression of other retinal marker genes, where *sox4* and *sox11* were expressed in the inner nuclear layer and ganglion cell layer [25], *pax6* was expressed in the inner nuclear layer and ganglion cell layer [26], and *vsx1* was expressed in the inner nuclear layer of retina [27]. The *pax6* expression was also devoid of the mature cells of the outer half of the retina and CMZ [28]. Tissue specific expression was summarized in Table 2. It is notable that in some cell types, elevated levels of particular Na, K-ATPase subunit isoforms (*atp1a3* with *atp1b2* in photoreceptors) preceded significantly other phenotypic differentiation. In most other cases, however, detectable expression coincided with the adoption of differentiated characteristics and upregulation corresponded with increases in retinal function.

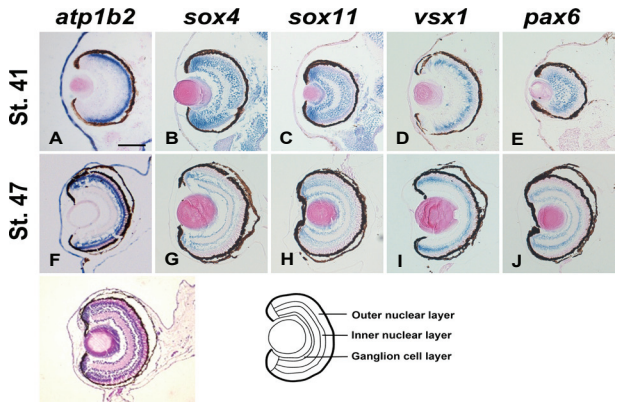

Fig. 4. Expression analysis of *atp1b2* in the retinal development after cellular differentiation. Expression of *Xenopus atp1b2* in the retina at stage 41 (A-E) and at stag 47 (F-J), hematoxylin and eosin stained embryos (stage 47) showing different layer of retina (K), and diagram of eye showing different layer of retina (L). Scale bar=100 μm.

Numerous studies have now confirmed that Na, K-ATPase subunit isoform composition has effects on the affinities of the enzyme for Na+, K+, and ouabain [3, 29]. Substitution of *atp1b2* or *atp1b3* for *atp1b1* (as occurs in photoreceptors) increases affinity for Na+ in Sf-9 cells [3, 30] but may have different effects in other cells with other isoforms [31, 32].

We also observed the expression of *atp1b2* in the pharyngeal epithelium. The homologues of *atp1b2* have also been confirmed in some other vertebrates [33, 34]. Along with these expression *atp1b2* was also expressed in the developing brain, developing gills, and whole epidermal cells.

The overall conclusion of the present study is that the *atp1b2* may have an important role for the development of outer nuclear layer of retina in developing *Xenopus*. However, further studies will be necessary to demonstrate a direct role of *atp1b2* in the retinal development along with its other expression domain.

Acknowledgements

This study was financially supported by grants from the Korea Research Foundation Grant (2011-0014454). We are grateful to Dr. Susanne Kuhl, Institute of Biochemistry and Molecular Biology, Ulm University, Ulm, Germany for giving *sox4* and *sox11* plasmids.

Table 2. Tissue-specific expression of *atp1b2* along with other marker in the retina during *Xenopus* development

Tissue Specified	*atp1b2*	*sox4*	*sox11*	*vsx1*	*pax6*
Ciliary Marginal Zone (CMZ)	+	−	−	−	−
Retinal pigment epithelium	−	−	−	−	−
Outer nuclear layer	+	−	−	−	−
Inner nuclear layer	−	+	+	+	+
Ganglion cell layer	−	+	−	−	+

"+", gene strongly expressed in the corresponding tissue; "−", undetected.

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