Animal Cells and Systems 13: 411-418, 2009

Animal Cells
and
Systems

http://www.AnimalCells.or.kr

Identification and Expression Patterns of *kif3bz* during the Zebrafish Embryonic Development

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Abstract: We are reporting the identification, expression patterns, and possible biological functions of zebrafish *kif3b* (*kif3bz*) encoding 475 amino acids. Kif3Bz contains the kinesin motor domain, catalytic domain, KISc domain, and one single coiled coil domain. Phylogenetic analysis indicates that *kif3bz* is a highly conserved gene among the tested vertebrates. First of all, both maternal and zygotic messages of *kif3bz* were evenly distributed in the blastomeres at 2-cell stage. Its ubiquitous expression throughout the blastomeres continued till 40% epiboly. However, *kif3bz* transcripts became restricted in Kupffer's vesicle at tailbud and 6-somite stages. At 13-somite stage, *kif3bz* expression pattern became specific to the telencephalon, diencephalon, trigeminal placode, and somites. Such expression patterns were further intensified in the telencephalon, diencephalons, hind brain, pronephric ducts, optic vesicles, and spinal cord neurons in the 23-somite stage embryos, and last till 24 hpf. We discussed possible functions of Kif3Bz related to the vertebrate embryonic development.

Key words: motor molecule, kinesin, *kif3bz*, Kupffer's vesicle, cilia, zebrafish embryo

INTRODUCTION

Motor molecules, such as kinesin, myosin, and dynein, are involved in the movement of various molecules and vesicles in cells. These movements are critical to a wide variety of cellular and developmental functions, including organelle movement, localization of developmental determinants, mitosis, meiosis and possibly long range signaling in neurons [Goldstein, 2001a; Guzik and Goldstein, 2004; Hamada, 2007]. The kinesin superfamily consists of a structurally diverse group of microtubule-based motor proteins that produce wide ranges of force-generating movements within cells [Vale and Fletterick, 1997]. Kinesin-like proteins (KLPs), also known as kinesin family proteins (KIFs), share a conserved motor domain of over 340 amino acids, and similarities between this domain have been used to construct molecular phylogenies as grouping the known members of the kinesin superfamily into a number of subfamilies [Moore and Endow, 1996; Kirokawa, 1998]. The members of each subfamily share a common domain organization [Vale and Fletterick, 1997]. Motor domain contains the sequence necessary for ATP hydrolysis and microtubule binding [Kull et al., 1996], driving the movements of membrane-bound organelles and vesicles toward the plus ends of microtubules. Stalk domain consisting of α-helical coiled coil motifs is responsible for mediating the homodimerization of kinesin heavy chains (KHCs) [Diefenback et al., 1998]. Tail domain, the sequences outside of the conserved motor domain, shows few similarities and happens to interact with cargo molecules directly or through adaptor proteins to be responsible for the different cellular roles of the KLPs [Karcher et. al., 2002; Verhey and Rapoport, 2001].

These motor molecules participate in positioning signaling complexes in cells and sending signals to particular directions. For instance, some cases of signal initiation or transmission during development require localization of essential signaling molecules to particular cellular or embryonic regions [Goldstein, 2001a]. In the case of kinesin, it is required for proper posterior localization of *oskar* mRNA and an associated protein, Staufen protein [Brenza et al., 2000], and dynein might be involved in the mechanism of apical localization of *wingless* and pair-rule transcripts in the *Drosophila* blastoderm embryo [Wilkie and Davis, 2001]. In the events of embryonic body planning, KIF3A protein, the subunit of the kinesin motor complex, plays an important role in the earliest cellular determinative events establishing left-right asymmetry in mammalian development [Takeda et al., 1999; Marszalek et al., 1999; Goldstein, 2001b].

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How the basic body plan of vertebrates is built up is not yet fully understood. In invertebrates such as *Drosophila* and *C. elegans*, their maternally induced processes lay down the information determining the embryonic axes already in the oocyte [Bowerman, 1998; Ray and Schupbach, 1996]. The same case in vertebrates is understood best in *Xenopus laevis*, where dorsal specification is maternally controlled [Wylie et al., 1996] and the cytoplasmic determinants which specify the dorsal fate are present in the eggs just after fertilization [Heasman, 1997]. The yolk, an extra-embryonic structure, is essential for the induction of both dorsal and marginal cell fates. The embryos that are removed the vegetal yolk mass during the 1-cell stage present strong ventralized phenotypes as missing of axial structures [Mizuno et al., 1999]. These results suggest that dorsal determinants are located in the vegetal pole of yolk cell after fertilization and then transported to the future dorsal side of the embryo. The results of embryological manipulation data using cold treatment, UV-irradiation, or treatment of nocodazole which causes depolymerization of microtubules show cortical microtubule arrays are required for the transport of determinants from the vegetal pole into the future dorsal side of the embryo and proper translocation of β-catenin into nuclei [Jesuthasan and Strahle, 1996]. The molecular nature of the dorsal determinant is still unknown, but the stabilization and nuclear translocation of the β-catenin, a component of the Wnt signaling pathway that contains dorsalizing activity, in both *Xenopus* and zebrafish embryos at the early blastula stage seems to be involved in dorsal determinants [de Robertis et al., 2000; Schneider et al., 1996; Larabell et al., 1997; Fuentealba et al., 2007]. The more detail studies in *Xenopus* demonstrated that β-catenin colocalizes with subcortical microtubules at the dorsal side of the egg and the microtubules that extend from the sperm entry point to the dorsal side of the embryo mediate the transport of key molecules toward their dorsal blastomeres during cleavage stages [Rowning et al., 1997; Weaver et al., 2003]. We also currently reported that a novel kinesin-like protein, Surhe is associated with dorsalization in the zebrafish embryonic development [Kim et al., 2008].

In order to investigate biological functions of Kif3Bz, we identified a zebrafish homologue of *kif3b* and found that it is maternally and zygotically expressed. *kif3bz* transcripts are present throughout the early embryos. In particular, *kif3bz* is expressed in Kupffer's vesicle. We discussed possible roles of Kif3Bz in light of left-right asymmetry establishement.

MATERIALS AND METHODS

Zebrafish maintenance and embryo culture

Zebrafish and embryos were maintained at 28.5°C on a 14 h/10 h light/dark cycle. Embryos were collected from natural mating, cultured at 28.5°C in embryo water and staged by developmental time and morphological criteria (Westerfield, 1995).

cDNA cloning and DNA sequencing

Total RNA was isolated from the shield stage of embryos using TRI REAGENT (Molecular Research Center) and 3 μg of total RNA from zebrafish embryos at 24 hpf was used for reverse transcription (RT) reaction. The first strand cDNA was synthesized by using Superscript III Reverse Transcriptase (Invitrogen). The full length cDNA of *kif3bz* was amplified with forward (5'-GATCCTCGAGATGTCC ATGAAATCAAAGACGG-3') and reverse (5'-GATCG AATTCTCAGACATACCTTAAATTTTGGCT-3') primers containing Eco RI and Xho I sites by using pfu DNA polymerase. The RT-PCR products were digested with Eco RI and Xho I restriction enzymes, cloned in the Eco RI and Xho I sites of pcDNA3 vector and then verified by sequencing analysis.

RT-PCR

Total RNA was isolated from different embryo stages using RNAzol B (TEL-TEST, Inc.), and 3 μg total RNA was used for RT-PCR. Using *kif3bz*-specific primers, a 400 bp fragment was obtained by RT-PCR amplification (pre-denature 94°C, 120 sec, denaturation 94°C, 30 sec, annealing 55°C, 30 sec, elongation 72°C, 30 sec, post-elongation 72 °C, 120 sec, 30 cycles). For the loading control, zebrafish β-*actin*-specific primers were used under the same condition. Two sets of primers were used to amplify *kif3bz*- and β-*actin*-specific products: *kif3bz,* -GATCCTCGAGATGTCC ATGAAATCAAAGACGG-3' (forward) and 5'-GATCGA ATTCTCAGACATACCTTAAATTTTGGCT-3' (reverse); β-*actin*, 5'-GAGGAGCACCCCGTCCTGC-3' (forward) and 5'-GATGGCTGGAACAGGGCC-3' (reverse).

Phylogenetic tree with protein homology search

The amino acid sequence homology was searched using the BLAST program. The multiple alignment of the amino acid sequences of Kif3Bz and other members of Kinesin II family were made by the ClustralW program. Based on the alignment, a phylogenetic tree using the maximum likelihood method was generated.

Whole-mount in situ hybridization

The C-terminal *kif3bz* was PCR amplified then subcloned into the multiple cloning site of pcDNA3 (Invitrogen) vector. Antisense digoxigenin-labeled riboprobes were generated from the linearized pcDNA3 *kif3bz*, according to the instructions provided from the DIG labeling kit (Roche). To prevent pigmentation, embryos were raised in 1-phenyl-2-thiourea solution at starting through somitogenesis before harvested. Whole-mount in situ hybridiation

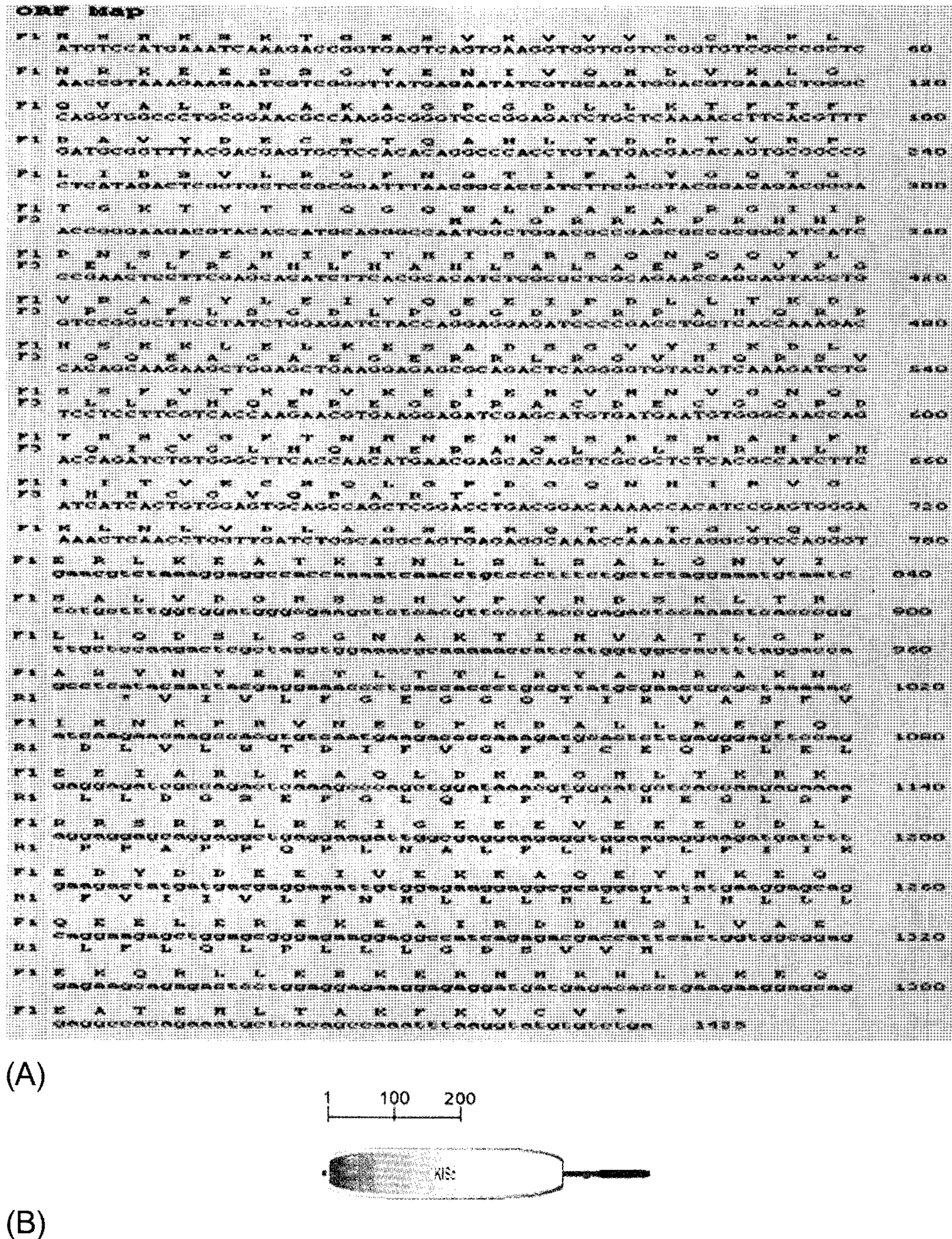

Fig. 1. Amino acid sequences deduced from the DNA sequences of *kif3bz* and structural domains. **A**. *ki3fbz* ORF ranges between postion 1 and 1424, which encodes 475 amino acids. **B**. Analysis of the amino acids sequences of Kif3Bz using SMART program. KISc domain at the N-terminus contains the kinesin motor domain, catalytic domain domain, ATPase domains. There is a coiled coil domains (green bar) at the C-terminus.

analysis followed the protocol of Westerfield (1995) with minor modification. Proteinase K treatment (10 μg/mL) was performed for 3 to 20 min depending upon the stages of embryos. The hybridized probes were detected using pre-absorbed anti-digoxigenin-AP Fab fragments (Roche) diluted (1:2000) in blocking solution (PBS, 0.1% Tween-20, 5% sheep serum). After 4-10 staining, embryos were mounted in a 2:1 mixture of benzylbenzoate:benzylalcohol, then examined by microscopy.

RESULTS

Structural organization of *kif3bz*

The isolated cDNA encoding *kif3bz* was subjected to DNA sequence analysis. It contained an open reading frame between base pair position at 1 and 1424, encoding a protein of 475 amino acids. Amino acid sequence analysis using SMART program found that it includes kinesin motor domain and catalytic domain as well as one coiled coil

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Mus musculus (mouse)               MPINKSEKP---ESCDNVKVVVRCRPLNEREKSMCYRQAVSVDEMRGTIT  47
Rattus norvegicus (brown rat)      MPINKSEKP---ESCDNVKVVVRCRPLNEREKSMCYRQAVSVDEMRGTIT  47
Homo sapiens (man)                 MPINKSEKP---ESCDNVKVVVRCRPLNEREKSMCYKQAVSVDEMRGTIT  47
Gallus gallus (chick)              MPINKPEKPDRPESCDNVKVVVRCRPLNEREKATGYKMAVNVDEMRGTIT  50
Xenopus laevis (clawed frog)       MPINRADKP---ESCDNVKVVVRCRPLNERERAMSSKMAVGVDEIRGTIS  47
Danio rerio (Zebrafish)            MPSNKLDKPEKLEVSDNVKVVVRCRPLNEKEKIMGHKQSVTVDEIRGTIT  50

Mus musculus (mouse)               VHKTDSSNEPPKTFTFDTVFGPESKQLDVYNLTARPIIDSVLEGYNGTIF  97
Rattus norvegicus (brown rat)      VHKTDSSNEPPKTFTFDTVFGPESKQLDVYNLTARPIIDSVLEGYNGTIF  97
Homo sapiens (man)                 VHKTDSSNEPPKTFTFDTVFGPESKQLDVYNLTARPIIDSVLEGYNGTIF  97
Gallus gallus (chick)              VHKTDSSNEPPKTFTFDTVFGPESKQLDVYNLTARPIIDSVLEGYNGTIF  100
Xenopus laevis (clawed frog)       VHKVDSMNEPPKTFTFDTVFGPDSNQLDVYNLTARPIIDSVLEGYNGTIF  97
Danio rerio (Zebrafish)            VNKLDISSEPPKTFTFDTVFGPDSKQLDVYNLTARPIIDSVLEGYNGTIF  100

Mus musculus (mouse)               AYGQTGTGKTFTMEGVRAVPGLRGVIPNSFAHIFGHIAKAEGDTRFLVRV  147
Rattus norvegicus (brown rat)      AYGQTGTGKTFTMEGVRAVPGLRGVIPNSFAHIFGHIAKAEGDTRFLVRV  147
Homo sapiens (man)                 AYGQTGTGKTFTMEGVRAIPELRGIIPNSFAHIFGHIAKAEGDTRFLVRV  147
Gallus gallus (chick)              AYGQTGTGKTFTMEGVRAVPELRGIIPNSFAHIFGHIAKAEGDTRFLVRV  150
Xenopus laevis (clawed frog)       AYGQTGTGKTFTMEGVRTVPELRGIIPNSFAHVFGHIAKAEGDTRFLVRV  147
Danio rerio (Zebrafish)            AYGQTGTGKTFTMEGVRAVPELRGIIPNSFAHVFGHIAKAEGDTRFLVRV  150

Mus musculus (mouse)               SYLEIYNEEVRDLLGKDQTQRLEVKERPDVGVYIKDLSAYVVNNADDMDR  197
Rattus norvegicus (brown rat)      SYLEIYNEEVRDLLGKDQTQRLEVKERPDVGVYIKDLSAYVVNNADDMDR  197
Homo sapiens (man)                 SYLEIYNEEVRDLLGKDQTQRLEVKERPDVGVYIKDLSAYVVNNADDMDR  197
Gallus gallus (chick)              SYLEIYNEEVRDLLGKDQSQRLEVKERPDVGVYIKDLSAYVVNNADDMDR  200
Xenopus laevis (clawed frog)       SYLEIYNEEVRDLLGKDQNQRLEVKERPDVGVYIKDLSGYVVNNADDMDR  197
Danio rerio (Zebrafish)            SYLEIYNEEVRDLLGKDQMQRLEVKERPDVGVYIKDLSGYVVNNADDMDR  200

Mus musculus (mouse)               IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGVDGNMHVRMGKLH  247
Rattus norvegicus (brown rat)      IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGVDGNMHVRMGKLH  247
Homo sapiens (man)                 IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGIDGNMHVRMGKLH  247
Gallus gallus (chick)              IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGVDGNMHVRMGKLH  250
Xenopus laevis (clawed frog)       IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGADGNIHVRMGKLH  247
Danio rerio (Zebrafish)            IMTLGHKNRSVGATNMNEHSSRSHAIFTITIECSEKGVDGNQHVRMGKLH  250

Mus musculus (mouse)               LVDLAGSERQAKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVPY  297
Rattus norvegicus (brown rat)      LVDLAGSERQAKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVPY  297
Homo sapiens (man)                 LVDLAGSERQAKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVPY  297
Gallus gallus (chick)              LVDLAGSERQAKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVSY  300
Xenopus laevis (clawed frog)       LVDLAGSERQAKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVPY  297
Danio rerio (Zebrafish)            LVDLAGSERQGKTGATGQRLKEATKINLSLSTLGNVISALVDGKSTHVPY  300

Mus musculus (mouse)               RNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  347
Rattus norvegicus (brown rat)      RNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  347
Homo sapiens (man)                 RNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  347
Gallus gallus (chick)              RNSKLTRLLGDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  350
Xenopus laevis (clawed frog)       RNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  347
Danio rerio (Zebrafish)            RNSKLTRLLQDSLGGNSKTMMCANIGPADYNYDETISTLRYANRAKNIKN  350
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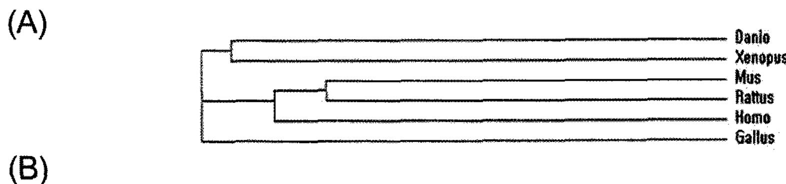
(A)



(B)

Fig. 2. Amino acids sequence homology analysis and phylogenetic analysis of Kif3Bz in conjunction with mouse, brown rat, human, chick, and clawed frog. **A.** Kif3Bz shares 82%, 74%, 73%, 72% with Xenopus, Gallus, human, and mouse, respectively. **B.** Phylogenetic analysis using ClustalW program (http://www.ebi.ac.uk/clustalw/). Kif3Bz is most closely related to Xenopus.

domain. The motor domain at the N-terminus of Kif3Bz includes the sequence necessary for ATP hydrolysis and microtubule binding, driving the movements of membrane-bound organelles and vesicles toward the plus ends of microtubules. Stalk domain consists of á-helical coiled coil motif responsible for dimerization of kinesin heavy chains. It also contains a P-loop sequence characteristic for ATP- or GTP-binding proteins and the consensus sequence of the nucleotide binding motif for kinesins. Tail domain, the sequences outside of the conserved motor domain, shows few similarities and happens to interact with cargo molecules directly or through adaptor. Taken together, Kif3Bz belongs to the kinesin family member 3, as evidenced by the presence of the kinesin features in the amino-terminal motor domain.

Phylogenetic analysis of *kif3bz*

In order to investigate relatedness of Kif3Bz to the homologues of other species, we analyzed the overall homology of the amino acids among various vertebrates using the BLAST program. Kif3Bz shares 82%, and 74% homology with Xenopus and Gallus, respectively whereas it does 73, 72, and 72% homology with human, rattus, and mouse, respectively (Fig. 1A). Both amino acid sequence homology studies and phylogenetic analysis with ClustralW program found that Kif3Bz is most related to Xenopus (Fig. 1B).

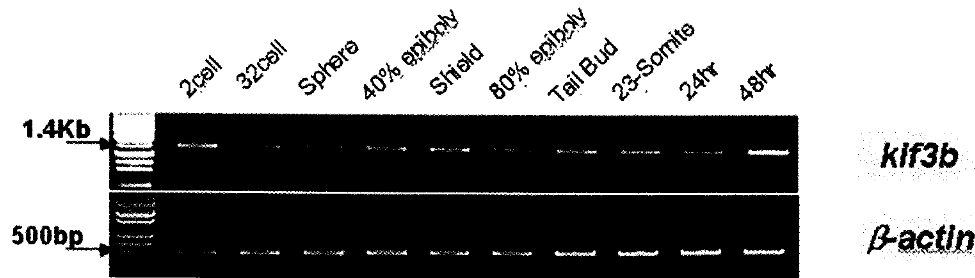


Fig. 3. Temporal expression patterns of *kif3bz* in the various zebrafish developmental stages. The transcripts level of β-*actin* was measured as the internal control.

Spatio-temporal expression pattern of *kif3bz* in the zebrafish embryos

In order to circumvent biological roles of Kif3Bz, we initially conducted RT-PCR to analyze its temporal expression patterns at the various zebrafish embryonic stages. *kif3bz* transcripts were found at high level in the 2 cell, 32 cell, and sphere stages, suggesting that it is maternally expressed. They were zygotically expressed in the later stages with gradual reduction. To visualize spatial distribution of *kif3bz* mRNAs along the zebrafish embryonic development, digoxygenin-labeled antisense RNAs were generated and its expression patterns were analyzed by *in situ* hybridization on whole mount embryos. *Kif3bz* transcripts were already detected at 2-cell stage and were evenly distributed to all blastomeres (Fig. 4A). The messages were ubiquitously distributed throughout the blastomeres through 40% epiboly (Fig. 4B, C). The detection of *kif3bz* mRNAs prior to mid-blastula transition suggests that it is maternally inherited. At bud stage, its transcripts were present in the distal forerunner cells (DFCs) (Fig. 4D, G), the precursor cells for Kupffer's vesicle (KV). KV is a transient embryonic 'organ of asymmetry' that regulates the earliest known step in left-right (LR) axis specification [Essner et al., 2005]. *kif3bz* transcripts were clearly found in KV on the embryos at 6-somite stage (Fig. 4E, H). Because KV does an essential function in generating Nodal flow that is critical to left-right asymmetry establishment [Okabe et al, 2008], it is noteworthy that *kif3bz* is expressed in the DFCs and KV. In the embryos at 13-somite stage, *kif3bz* transcripts were detected in the telencephalon, diencephalon, hindbrain, trigeminal placode, and somites (Fig. 4F, I). Such expression patterns were intensified in the telencephalon, diencephalons, hind brain, and further specified in the pronephric ducts, optic vesicles, and spinal cord neurons in the 23-somite stage embryos (Fig. 4J, K) and last till 24 hpf (Fig. 4L, M).

DISCUSSION

Kinesin is the most abundant motor in many cell types and is responsible for the movement of many different cargoes [Goldstein, 2001; Vale and Fletterick, 1997; Moore and Endow, 1996; Hirokawa, 1998]. In the case of Kinesin-2 family, The two motor domains of Kif3A/B coordinate for motility and move at different speeds [Zhang and Hancock, 2004] whereas stability and specificity of heterodimer formation for the coiled coil neck regions of the motor proteins Kif3A and Kif3B is driven by non-sepcific attraction of the oppositely unstructured charged regions without affecting stability of the heterodimeric coled-coil structure [Chana et al., 2005]. In fact, they play critical roles in mitosis [Haraguchi et al., 2006], photoreceptor cell death [Jimeno et al., 2006], and endocytic protein recycling [Schonteich et al., 2008]. Kif3A by itself is involved in various essential signaling process and organogenesis, such as Hedgehog signaling topography [Koyama et al., 2007], development and patterning of the vertebrate skeleton by Hedgehog- and Gli3-dependent mechanisms [Kopakova-Hart et al., 2007], Hedgehog pathways-dependent tumorigenesis [Wong et al., 2009], Wnt signaling pathways [Gerdes and Katsanis, 2008], beta-catenin-dependent Wnt signaling through dual ciliary and non-ciliary mechanisms [Corbit et al., 2008], and N-cadherin and organizes the developing neuroepithelium [Teng et al., 2005]. It also associates with protein phosphatase Dusp26 to promote N-cadherin-mediated cell and cell adhesion [Tanuma et al., 2009]. Kif3B alone also interacts with CLC-5 to facilitate CLC-5 plasma membrane expression, endocytosis and microtubular transport [Reed et al., 2009].

We showed that Kif3Bz is expressed in Kupffer's vesicle (Fig. 4E, H) in which cilia conduct important roles in generating Nodal flow [Okabe et al., 2008]. LR asymmetry is initiated by Nodal flow direction in KV [Hirokawa et al., 2006]. Three developmental events are essential for maintenance of Nodal flow direction from right to left; normal development of DFCs, ciliary motor activity in KV [Essner et al., 2005], and midline barrier [Bisgrove et al., 1999]. However, mechanisms how Nodal signaling pathway is involved in initiation of DFCs development and cilia motility along the zebrafish embryonic development are unknown. KV, a derivative of the DFCs [Cooper and D'Amico, 1996], was reduced or absent in *sqt* $^{-/-}$;*cyc*$^{+/+}$

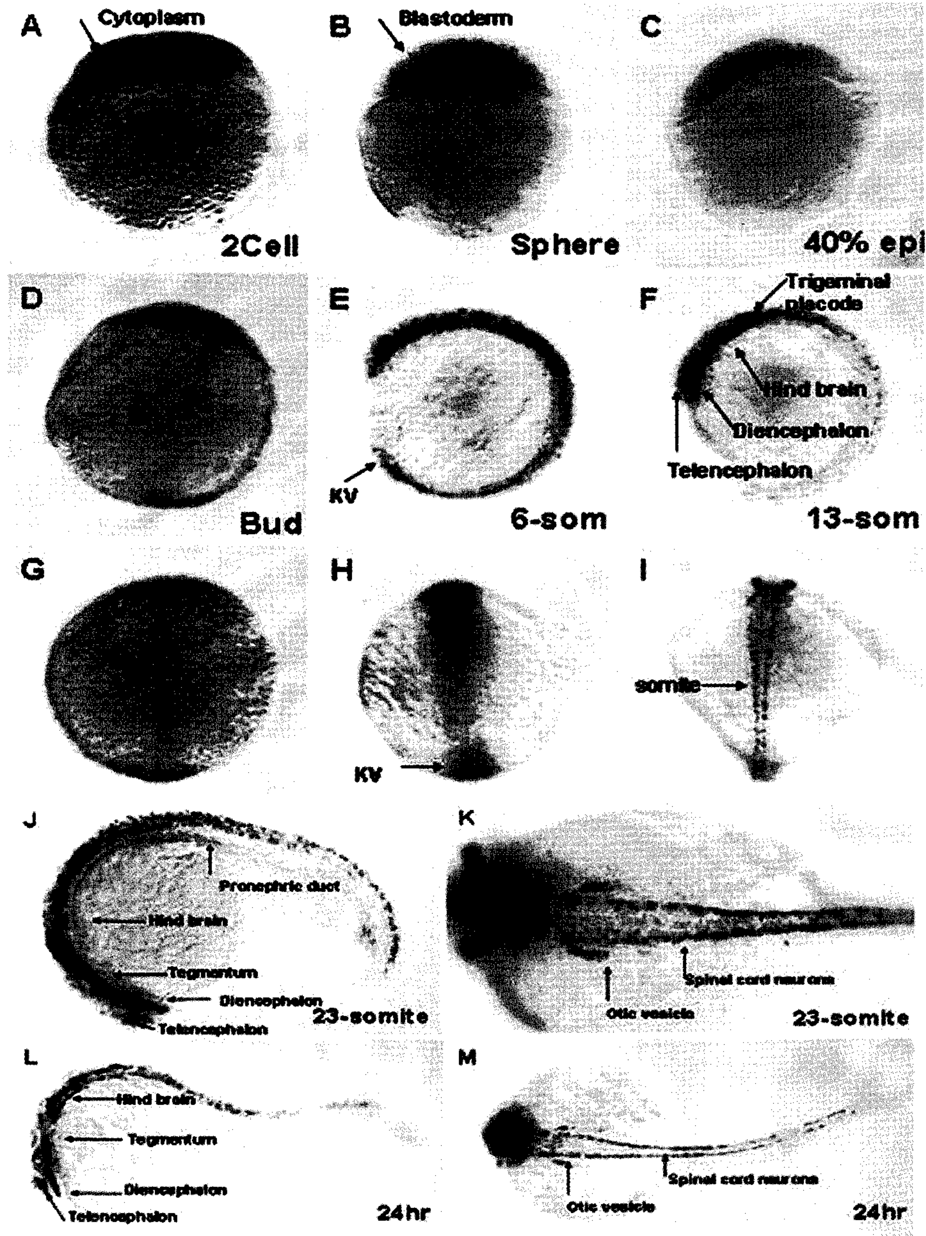


Fig. 4. Spatiotemporal expression patterns of zebrafish *kif3a* in the various developmental stages (A) 2 cell, (B) sphere, (C) 40% epiboly, (D, G) bud, (E, H) 6-somite (F, I) 13-somite (J, K) 23-somite, (L, M) 24 hpf, (A~F, J, L) lateral view, (G~I, K, M) dorsal view.

mutants at 6 somite stage [Dougan et al., 2003]. DFCs were also reduced or absent in *sqt* $^{-/-}$; *cyc*$^{+/+}$ mutants [Dougan et al., 2003], as revealed by expression of *sox17* [Alexander and Stainier, 1999]. Zygotic *sqt* expression begins in the YSL at midblastula stage [Chen and Kimelman, 2000]. DFCs were detected at 30~40% epiboly by SYTO-11 staining [Cooper and D'Amico, 1996]. *sqt* appears thereby to be a critical morphogen for KV at around 30% epiboly stage. Cas, a endoderm inducer modulates *sqt* [Kikuchi et al., 2001] and *ntl* expression in DFCs [Amack and Yost, 2004] to maintain DFCs development and *lrdr1* expression [Essner et al., 2005]. In addition, *ntl* and *cas* mutants all lead to the same endpoint of defective KV morphogenesis and affect downstream LR gene expression in dramatically different manners [Essner et al., 2005]. These observations suggest that 1) DFCs and *lrdr1* expression are activated by Sqt protein activity 2) DFCs development and *lrdr1* expression are maintained by auto-activation loops of Sqt-mediated Nodal/Cas signaling cascade in DFCs. Taken al together, concerted modulation of the genes have KV generated to develop ciliary structure. The ciliary structure obviously plays critical roles in left-right asymmetry establishment. It is of great interest if Kif3Bz is an element defining the DFC as well as KV in zebrafish embryos.

Immunohistochemical localization of Kif3Bz, and gene knock-down experiment using *kif3bz*-specific morpholino shall find clue to the questions.

ACKNOWLEDGEMENT

This research was supported by National Research Foundation of Korea Grant funded by the Korean Government (2009-0074496); the Korean Ministry of Environment as "The Eco-technopia 21 Project" (Grant number; 091-081-077). We thank the Korea Zebrafish Organogenesis Mutant Bank (ZOMB) for maintenance and providing zebrafish lines.

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[Received November 13, 2009; accepted December 17, 2009]