Zebrafish wnt3 Is Expressed in Developing Neural Tissue

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Wnt signaling regulates embryonic patterning and controls stem cell homeostasis, while aberrant Wnt activity is associated with disease. One Wnt family member, Wnt3, is required in mouse for specification of mesoderm, and later regulates neural patterning, apical ectodermal ridge formation, and hair growth. We have identified and performed preliminary characterization of the zebrafish wnt3 gene. wnt3 is expressed in the developing tailbud and neural tissue including the zona limitans intrathalamica (ZLI), optic tectum, midbrain-hindbrain boundary, and dorsal hindbrain and spinal cord. Expression in these regions suggests that Wnt3 participates in processes such as forebrain compartmentalization and regulation of tectal wiring topography by retinal ganglia axons. Surprisingly, wnt3 expression is not detectable during mesoderm specification, making it unlikely that Wnt3 regulates this process in zebrafish. This lack of early expression should make it possible to study later Wnt3-regulated patterning events, such as neural patterning, by knockdown studies in zebrafish. Developmental Dynamics 238:1788–1795, 2009. © 2009 Wiley-Liss, Inc.

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INTRODUCTION

The Wnt signaling pathway regulates many events in embryonic development and is pivotal in controlling adult stem cell homeostasis. Dysregulation of Wnt signaling has been linked to numerous cancers and other diseases (Logan and Nusse, 2004; Clevers, 2006; Klaus and Birchmeier, 2008). Wnt genes have been highly conserved throughout evolution and mammals have genes for 19 Wnt ligands. A recent census identified putative orthologues for many of these genes in chicken, frog, and fish (Garrick et al., 2007). Study of these genes, because of their pivotal role in development, stem cell biology, and disease, has been intense.

Although Wnt ligands have historically been classified as either “non-canonical” or “canonical” ligands, some recent results have suggested that the distinction between these pathways may be more fluid than previously thought, with particular Wnts able to activate both canonical and non-canonical pathways depending on the particular configuration of receptors present (Tao et al., 2005; Bovolenta et al., 2006; Mikels and Nusse, 2006; Hendrickx and Leyns, 2008; Nusse, 2008). A number of non-canonical pathways are beginning to be defined, but many of the specifics remain to be elucidated (Veeman et al., 2003; Semenov et al., 2007). Canonical signaling proceeds by activation of the transcription of target genes through the stabilization of the multi-functional protein, β-catenin. Under non-signaling conditions, cytosolic and nuclear levels of the signaling pool of β-catenin are kept low by the actions of a constitutively active phosphorylation complex known as the “destruction” complex, which includes the scaffolding protein, Axin, the protein encoded by the adenomatous polyposis coli locus (APC), and Glycogen Synthase Kinase-3 (GSK3), as well as ancillary proteins. Ligand binding to co-receptors of the Frizzled and LDL-receptor related protein (LRP) families leads to disruption of the destruction complex and stabilization of β-catenin, which translocates to the

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nucleus where it can bind DNA-binding proteins of the Tcf/Lef family and activate transcription of Wnt-responsive target genes (Logan and Nusse, 2004).

In anamniotes, the earliest patterning event controlled by the Wnt signaling pathway is specification of the dorsal axis. Stabilization of β-catenin on the future dorsal side of the embryo leads to activation of dorsal organizer genes such as chordin (chd), and eventually formation of head and dorsal structures (Schier and Talbot, 2005). Following the onset of zygotic transcription, Wnt factors such as Wnt8 and Wnt3a then ventralize the developing body plan (Schier and Talbot, 2005).

In mouse, Wnt3 plays one of the earliest roles in embryonic patterning among Wnt ligands. Targeted deletion has revealed that murine Wnt3 is required for primitive streak formation and specification of embryonic mesoderm (Liu et al., 1999; Barrow et al., 2007). Wnt3 transcripts are first detected in the proximal epiblast at embryonic day 6.0 (E6.0) and soon after are found throughout the growing primitive streak (Liu et al., 1999). Wnt3-null animals fail to generate mesoderm or definitive endoderm, although anterior-posterior patterning of visceral endoderm remains intact. Thus, Wnt3 is required for formation of embryonic mesoderm in mice (Liu et al., 1999).

A number of later developmental processes are also regulated by Wnt3, such as hair growth (Millar et al., 1999; Kishimoto et al., 2000), apical ectodermal ridge (AER) formation (Barrow et al., 2003), and neural patterning (Krylova et al., 2002; Braun et al., 2003; Lie et al., 2005; Schmitt et al., 2006; Lewis et al., 2008). Wnt3 regulates retinal ganglion axon guidance and the topography of tectal wiring through a repulsive interaction with the Ryk receptor, which has recently been shown to have high affinity for Wnts (Schmitt et al., 2006). Finally, Wnt3 likely regulates synapse formation between motoneurons and sensory neurons in the spinal cord (Krylova et al., 2002). Unfortunately, loss of function studies in mouse to elucidate the neural patterning functions of Wnt3 have been impeded by the fact that Wnt3 is required at earlier stages for primitive streak formation and specification of mesoderm (Liu et al., 1999; Lewis et al., 2008), although conditional deletion strategies have been helpful in revealing some later functions such as regulation of AER formation (Barrow et al., 2003).

Despite the importance of Wnt3 in vertebrate body plan formation, cloning and characterization of a zebrafish Wnt3 orthologue has not yet been reported, although a putative wnt3 locus in the genome has been identified (Garriock et al., 2007). Here we report the cloning and preliminary characterization of zebrafish wnt3.

RESULTS

Discovery of the Zebrafish wnt3 Gene

To determine if there is a zebrafish gene homologous to mouse Wnt3, we searched for genes that had been annotated as wnt3 in the zebrafish genomic database at Ensembl.org (http://www.ensembl.org), and found EntrezGene LOC569420, which corresponded to the predicted reference sequence XM_692803. This locus was also described in a recent census of vertebrate Wnt genes, but the message was not identified (Garriock et al., 2007). We designed PCR primers to amplify the entire putative coding sequence from cDNA, but we were unable to amplify a product. One possible explanation for our inability to amplify the putative sequence was that the 5′-most coding exon given in Ensembl could be incorrectly predicted, thus there would be no transcripts containing the 5′-primer sequence. We compared the mouse transcript to the putative zebrafish transcript and noticed that the homology deteriorated upstream of the most 5′ splice junction in the open reading frame, also suggesting that the 5′-most exon was incorrectly annotated. We, therefore, queried the translated peptide corresponding to the mouse 5′-most coding exon against the zebrafish Ensembl genome by TBLASTN, which yielded a single hit, directly upstream of the putative wnt3 coding exons. These observations suggested that the predicted transcript is incorrect and that the true transcript contains sequence from a different 5′-exon. In support of this hypothesis, two ESTs were found that contained the putative upstream exonic sequence. A nucleotide sequence closely conforming to our new prediction was recently deposited in GenBank under accession number EU203154.

We successfully amplified a zebrafish Wnt3 homologue by designing a new 5′ primer corresponding to a portion of the 5′ untranslated region (5′-UTR) using the alternative upstream exonic sequence. The amplified cDNA contained 1,131 base pairs (bp) including 1,068 bp of putative open reading frame (ORF), with an in-frame stop codon upstream of the putative initiation codon in the 5′-UTR. The protein encoded by this ORF is 90% identical and 97% similar to the mouse and human Wnt3 proteins (Fig. 1A). By comparison, the known zebrafish Wnt3l protein (also known as Wnt3a) is 82% identical and 90% similar to the mouse and human Wnt3 proteins. Phylogenetic analysis shows that our novel protein sequence segregates most closely with human and mouse Wnt3, while the known Wnt3l segregates with mouse Wnt3a (Fig. 1B). A comparison of the loci of Wnt3 in human and mouse to that of our newly discovered gene on chromosome 12 shows that synteny is conserved, with the identified zebrafish gene having the same 3′-proximal
Zebrafish wnt3 is the orthologue of other vertebrate Wnt3 genes. A: Sequence conservation of the deduced amino acid sequence for zebrafish Wnt3, comparing mouse Wnt3 (mWnt3, top), human WNT3 (hWNT3, second from top), zebrafish Wnt3l (zWnt3l, second from bottom), and mouse Wnt3a (mWnt3a) bottom. B: Phylogenetic analysis of zWnt3 compared to similar genes in zebrafish mouse and human. zWnt3 clusters with known human and mouse Wnt3 genes, whereas zwnt3l clusters with mWnt3a. mWnt8a was used as the outgroup. Numbers indicate bootstrap confidences at branch points. C: Conservation of synteny between Wnt3 genes in human, mouse, fugu, and zebrafish. The Nsf gene is the nearest 3'-neighbor in all four species.
neighbor, nsf, as in mouse (Nsf) and human (NSF; Fig. 1C; Garriock et al., 2007). Synteny with the 5′-neighbor is not conserved in either of the two fish genomes examined. Based on the high identity between mouse and human Wnt3 and our novel gene, as well as conservation of synteny, we propose that our novel zebrafish gene be named wnt3. Zebrafish wnt3 has been deposited in GenBank under the accession number FJ915144.

Zebrafish wnt3 Is Expressed in the Developing Nervous System

Because Wnt3 has been shown to have important roles during embryonic development in other organisms, we wanted to determine where and when zebrafish wnt3 is expressed.

We first examined the course of temporal expression in whole embryos by reverse transcriptase-polymerase chain reaction (RT-PCR; Fig. 2A). RNA was isolated from embryos at different time points during development, and the presence of wnt3 transcripts was assayed. We found that wnt3 is first expressed at tailbud stage (10 hr post-fertilization; hpf) and levels increased through 50 hpf, the latest time point examined. Notably, wnt3 transcripts are not detected at shield or earlier (Fig. 2A). By whole mount in situ hybridization (WISH), expression continues through at least 6 days post-fertilization (dpf, Fig. 2B). These results indicate that, unlike in mouse, zebrafish wnt3 is not expressed prior to the specification of the germ layers and is thus unlikely to play a role in primary axis specification or mesoderm induction. We can-
Fig. 2. Temporal and spatial expression of wnt3 during zebrafish embryonic development. A: RT-PCR analysis of wnt3 (top row) in total cDNA from whole embryos at the indicated stages of development. ef-1α was used as a positive control. B: WISH analysis of wnt3 expression. Embryos at various ages processed for expression of the mRNAs are shown. In all cases, anterior is to the left. a–c: Flat-mounted head views at 3-s show that wnt3 displays stronger expression in the presumptive ZLI (black arrows, pZLI), whereas wnt3l displays stronger expression in the prospective midbrain just posterior. d–f: Flat-mounted tailbud views at 3-s. Note wnt3 shows stronger expression in the lateral and posterior border of the tailbud. g–i: Lateral views of the tailbud at 18-s. wnt3l shows stronger and deeper expression in the tailbud. Comparative flatmount views of wnt3 and fgf24 at 22-s (j) and magnified images at the level of the finbud progenitors (k, l). wnt3 is expressed bilaterally (red arrowheads) in tissue just medial to the finbud progenitor fields marked by fgf24 (black arrowheads, fb). m–o: Dorsal flatmount views at 18-s. Both wnt3 and wnt3l are expressed in the developing diencephalon and mesencephalon, but wnt3 is expressed farther posterior into the presumptive cerebellum. p,q: Lateral views of the brainstem with eyes removed at 18-s confirm wnt3 expression in the presumptive cerebellum and demonstrate that wnt3 extends much farther ventrally at both the presumptive ZLI and the midbrain hindbrain boundary, reaching into the presumptive basal plate. r,s: At 24 and 30 hpf (lateral brain stem views, eyes removed), this trend continues. Double WISH with shha confirms the basal plate expression. At 4 dpf (t) and 6 dpf (u), wnt3 continues to be expressed in the optic tectum, and is visible in the otic vesicles (dorsal head views). bp, basal plate; cer, cerebellum; fb, finbud progenitors; MHB, midbrain-hindbrain boundary; pZLI, presumptive zona limitans intrathalamica; ZLI, zona limitans intrathalamica; *, optic tectum.
and known

transcription of these canonical Wnt target genes. As expected, 50% of injected embryos assayed for gfp did not carry the transgene (since they were the progeny of a heterozygous outcross) and, therefore, did not show any gfp transcript, serving as an internal control (data not shown). These results indicate that Wnt3 is capable of activating the canonical Wnt pathway.

DISCUSSION

We have identified a previously uncharacterized wnt3-like gene in the zebrafish genome. Based on the fact that the deduced Wnt3 protein segregates most closely with human and mouse Wnt3 proteins, and based on conservation of synteny between zebrafish Wnt3 and known Wnt3 genes in mouse, human, and fugu, we propose that we have identified the zebrafish orthologue. Surprisingly, zebrafish Wnt3 does not appear to be expressed prior to the beginning of somitogenesis, making it highly unlikely that Wnt3 participates in specification of mesoderm, as has been shown for mouse Wnt3 (Liu et al., 1999). This difference should permit the study of later roles for Wnt3 in embryological processes such as neural patterning, which have been refractory to study by targeted deletion in mouse.

Zebrafish wnt3 is expressed in embryos beginning at the tailbud stage in the developing neural plate and the tailbud. wnt3 expression in bilateral stripes near the finbud progenitors marked by gfp24 during somitogenesis is intriguing. The expression appears to be medial to the developing finbud fields and is transient. Future studies should address whether Wnt3 helps in establishing the finbud field. We were not able to detect wnt3 in the finbuds at later time points, making it unlikely that zebrafish Wnt3 regulates AER outgrowth as observed in mouse (Barrow et al., 2003). One possibility is that wnt3l, which is expressed later in the finbuds proper (Norton et al., 2005), substitutes for wnt3 in zebrafish AER regulation.

Neural expression of wnt3 closely parallels that seen in other vertebrates (Roelink et al., 1990; Roelink and Nusse, 1991; Salinas and Nusse, 1992; Bulfone et al., 1993; Parr et al., 1993; Braun et al., 2003; Robertson et al., 2004), with notable expression in the diencephalon, dorsal mesencephalon, MHB, and dorsal hindbrain and spinal cord. In other vertebrates, Wnt3 is thought to be responsible for posteriorizing the forebrain, opposing the actions of transcription factors such as Six3 and Wnt antagonists such as Dkk1, and playing a role in the formation of the ZLI (Braun et al., 2003; Wilson and Houart, 2004; Lewis et al., 2008). Wnt3 has also been suggested to be responsible for helping instruct the topography of retinal axon wiring to the optic tectum (Schmitt et al., 2006). The pattern of zebrafish wnt3 expression makes it likely that these functions are conserved in fish. Knockdown of function using antisense morpholino oligonucleotides should help to elucidate how Wnt3 functions in neural patterning across phyla. Interestingly, zebrafish wnt3 alone among the known putatively canonical zebrafish Wnt genes is expressed in the basal plate. This expression may be indicative of some yet unknown role for Wnt signaling in patterning the tegmentum and/or ventral diencephalon.

EXPERIMENTAL PROCEDURES

Fish Maintenance and Microinjection

Zebrafish stocks (AB*, WIK, and Tg(TOP:GFP)μ251+) were cared for and embryos were obtained through natural spawning and staged according to established procedures (Westfield, 2000). One-cell-stage embryos were microinjected with the indicated masses of RNA in a volume of 1 nl (calibrated by injection of test drops into halocarbon oil series 27 [Sigma Aldrich, St. Louis, MO] on a stage micrometer), using a femtojet microinjector (Eppendorf, Westbury, NY).

RNA Isolation, cDNA Preparation, and RT-PCR

RNA was isolated from groups of 20 whole embryos at stages noted in the text using TRIzol (Invitrogen, Philadelphia, PA) according to the manufacturer’s instructions. cDNA was generated from total RNA using the Superscript III RT-PCR Kit (Invitrogen). PCR analysis of stage-specific cDNA was performed with Taq polymerase (Invitrogen) using 35-cycles. PCR products were analyzed on an agarose gel and were digitally recorded using a UVP BioDoc-It gel visualization system (UVP, Upland, CA). RT-PCR primers were previously described for ef-1α (Chen and Kimelman, 2000). New primers for wnt3 RT-PCR were

Fig. 3. Zebrafish Wnt3 can activate the canonical Wnt pathway. Zebrafish heterozygous for a gfp transgene under the control of a Wnt/β-catenin-dependent promoter (Tg(TOP:GFP)μ251+) were outcrossed, and the resulting embryos were allowed to develop un.injected (left column), or injected with either 30 pg (middle column) or 70 pg (right column) of wnt3 mRNA. At shield stage, embryos were fixed and processed by WISH for expression of either gfp (top row) or chd (bottom row) as indicated. Injected embryos showed a dose-dependent increase in canonical Wnt signaling-driven transcript abundance. One hundred percent of embryos displayed the phenotypes depicted for each condition. Non-transgenic siblings provided an internal control for gfp in situ (not pictured).
zWnt3-RT-F (5′-TACGCTTCTCTTCAAGCATCC-3′) and zWnt3-RT-R (5′-CTCTTTTCGAATTCTCTGACC-3′).

**Constructs and mRNA Synthesis**

A putative wnt3 homologue was identified as described in the text and amplified using 40 cycles of PCR with Taq (Invitrogen) from 24 hpf AB* embryo cDNA with the primers 5′-Wnt3-UTR-F (5′-CCTCTCTTCTGGAATCCTGTTAGT-3′) and zWnt3-R (5′-cttataTTATTTACATGTATGTCGTGCTGACAC-3′), which introduces a 3′ Xba1 site to facilitate subcloning. The 1,137-bp product was cloned to the pCRII vector (Invitrogen) from 24 hpf AB* embryo mRNA as described in the text and amplified amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994). Aligned sequences were prepared for publication using the BOX- SHADE server (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic comparisons were performed using the PHYLIP software (http://evolution.genetics.washington.edu/phylip.html) by the neighbor-joining distance method with bootstrap analysis (100 replications). Sequences used for comparison were mWnt3 (NP_033545), hWnt3 (NP_110308), zWnt3l (AAT38336), mWnt3a (NP_033545), and mWnt8a (NP_033316). Conservation of synteny was established by examination of neighboring genes using the Ensembl genome browser (http://www.ensembl.org).

**In Situ Hybridization**

Digoxigenin-labeled probe synthesis was as previously described (Clements and Kimelman, 2005). pCRII wnt3 was linearized with SpeI (NEB) and transcribed with T7 polymerase (NEB). Digoxigenin gfp probe was produced from pcS2-1794 CLEMENTS ET AL.

**References**


crocon YM-100 centrifugal filter (Millipore, Billerica, MA).

**Genomic and Phylogenetic Analysis**

Identification of a putative wnt3 homologue was as described in the text. Deduced amino acid sequences were aligned using the ClustalW program (Thompson et al., 1994). Aligned sequences were prepared for publication using the BOX-SHADE server (http://www.ch.embnet.org/software/BOX_form.html). Phylogenetic comparisons were performed using the PHYLIP software (http://evolution.genetics.washington.edu/phylip.html) by the neighbor-joining distance method with bootstrap analysis (100 replications). Sequences used for comparison were mWnt3 (NP_033547), hWnt3 (NP_110308), zWnt3l (AAT38336), mWnt3a (NP_033545), and mWnt8a (NP_033316). Conservation of synteny was established by examination of neighboring genes using the Ensembl genome browser (http://www.ensembl.org).

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