Published in final edited form as:

Gene Expr Patterns. 2007 January ; 7(3): 233–238.

Expression of the forkhead transcription factor *FoxN4* in progenitor cells in the developing *Xenopus laevis* retina and brain

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Abstract

Forkhead proteins are involved in gene regulation in a large variety of developmental situations. Several forkhead gene products are expressed in the developing eye and brain. Here we characterize the expression of *FoxN4* during *Xenopus* development. We report that *FoxN4* is expressed in the eye from the earliest stages of specification through retinal maturation. *FoxN4* is also expressed in the pallium, optic tectum, isthmus, reticular formation, and in cells lining the ventricle of the tadpole brain.

The forkhead gene products comprise a large family of transcriptional regulators involved in controlling many aspects of embryonic development including cell specification, differentiation, and cell cycle regulation (Carlsson and Mahlapuu, 2002; Hromas and Costa, 1995; Kaufmann and Knochel, 1996; Pohl and Knochel, 2005). Members of this gene family encode a 110-amino acid DNA binding domain, the forkhead box, related to that of the founding member of the family, *Drosophila forkhead* (Weigel and Jackle, 1990; Weigel et al., 1989). Forkhead domains share a high degree of similarity among family members within and across species. Several forkhead genes are expressed in the developing *Xenopus* retina, including *FoxD1* (Mariani and Harland, 1998), *FoxK1* (Pohl and Knochel, 2004), *FoxM1* (Pohl et al., 2005), *FoxO3* (Pohl et al., 2004), and *FoxP1* (Pohl et al., 2005). Another forkhead transcription factor, *FoxN4*, is expressed in progenitor cells of the murine and zebrafish retina (Danilova et al., 2004; Gouge et al., 2001; Li et al., 2004). The *Xenopus* retina has been extensively studied as a model for the development and differentiation of retinal progenitor cells (Perron and Harris, 2000). Additionally, the *Xenopus* tadpole brain is increasingly being used as a model for studies in comparative brain anatomy and development (Bachy et al., 2002; Bachy et al., 2001; Brox et al., 2002; Brox et al., 2004). Recently, *FoxN4* has been identified in *Xenopus*, where it is expressed in the developing retina (Schuff et al., 2006). Here, we report the expression of *FoxN4* in the developing *Xenopus* brain and further characterize its expression in the retina.

1. RESULTS

1.1. Sequence comparisons

We isolated a cDNA encoding *Xenopus laevis* *FoxN4* (Figure 1). The sequence we obtained was essentially identical to a *X. laevis* *FoxN4* sequence independently deposited in GenBank

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(accession number AM114796), differing at only 5 of 506 amino acid positions (99% identity). Overall, *X. laevis* *FoxN4* was similar to previously identified orthologs from other vertebrate species (Fig 1E). Several domains identified in *FoxN4* gene products from other vertebrates were clearly conserved, including the forkhead domain (Fig 1B), a putative activation domain (Fig 1C) and a *FoxN4* domain (Fig 1D). The *FoxN4* domain was identified as a string of amino acids that is conserved among orthologs of *FoxN4* but not the related gene product, *FoxN1* (Danilova et al., 2004).

1.2. Expression in early embryos

We visualized *FoxN4* expression during *Xenopus* development by wholemount in situ hybridization (WISH; Figure 2A-I). The *FoxN4* expression pattern (Fig 2A - D) is similar to that of other eye field transcription factors (Zuber et al., 2003), including *Rx* (Fig 2F - I). Expression is first detected as a single patch at the anterior edge of the neural plate (Fig 2A), corresponding to the developing eye field. As development progresses, this field splits into two distinct regions (Fig 2B). During tailbud stages, the expression of *FoxN4* becomes further restricted to the neural retina and is absent from the lens (Fig 2D). *FoxN4* expression is also observed in several parts of the developing brain (Fig 2B,D). Additionally, *FoxN4* is expressed in the pronephros at tailbud stages (Fig 2E). The expression of *FoxN4* in the developing eye is strikingly similar to that of *Rx* (Fig 2F - I) except for in the brain, where *Rx* is only expressed in the ventral hypothalamus and pineal gland (Casarosa et al., 1997; Mathers et al., 1997). Additionally, the *FoxN4* expression domain does not extend as far medially as the *Rx* expression domain at early tailbud stage (compare Fig 2B and G and see Fig 2J, J'). Expression in the developing brain is first observed during neural tube stages (st 18 - 19, see Fig 2K). At these stages, *FoxN4* is expressed throughout the developing spinal cord, hindbrain, and midbrain. Faint expression is observed in the extreme anterior neural plate, corresponding to the developing forebrain and/or olfactory bulb. This pattern of expression continues into tailbud stages (Fig 2L). Beginning at mid-tailbud stages (st 33/34), forebrain/olfactory bulb expression intensifies (Fig 2M). By late tailbud/early tadpole stage (st 35/36), expression is continuous from the hindbrain through the forebrain (Fig 2N).

1.3. Expression in retinal progenitor cells

The expression pattern of *FoxN4* during retinal development was further analyzed by in situ hybridization using sectioned material (Fig 2O - S). At st 36, *FoxN4* expression is strongest in the developing ciliary marginal zone (CMZ; Fig 2O), the portion of the retina adjacent to the lens containing retinal progenitor cells (Perron and Harris, 2000). *FoxN4* expression is mostly absent from the rest of the retina, where cell differentiation is beginning to take place. We found that *FoxN4* expression becomes restricted to the CMZ of the neural retina as retinal maturation proceeds (Fig 2P - S). *FoxN4* is expressed in all but the tip region of the CMZ, where most of the stem cells are found. In contrast, *Rx* is expressed throughout the CMZ at this stage (Figs 2T) (Mathers et al., 1997; Van Raay et al., 2005).

1.4. Expression in the tadpole brain

To further characterize the expression of *FoxN4* in the developing brain, WISH was performed using isolated tadpole brains (Fig 3). We identified brain regions expressing *FoxN4* by comparison to the expression patterns of *Lhx2*, *Lhx5*, *Lhx9* (Moreno et al., 2004; Moreno et al., 2005), and *Dlx2* (*xDll-4*) (not shown) (Brox et al., 2004). We found that *FoxN4* is expressed along the entire rostral/caudal length of the dorsal brain. It is expressed in the dorsal pallium, in the region of prosomeres 1 - 3 of the forebrain, optic tectum and isthmus (compare Figs 3A, B with D - J). Analysis of sections of stained brains suggests that *FoxN4* is expressed in prosomere 3 (Fig 3L) and the lining of the ventricle/neural tube in the region of prosomeres 1 and 2 (Fig 3K). Direct comparison of *FoxN4* and *Lhx9* expression by double in situ

hybridization demonstrates at best minimal overlap of expression in prosomere 2 (Fig 3J). Analysis of sections of a brain subjected to double in situ hybridization for *FoxN4* and *Lhx9* demonstrate that *FoxN4* is primarily expressed in the lining of the ventricle and not in the region of prosomere 2, expressing *Lhx9* (Fig 3J'). The expression of *FoxN4* in this region is similar to that of *Sox2* (Fig 3N), primarily expressed in progenitor cells (Wegner and Stolt, 2005). Expression appears to be largely absent from prosomere 4. In the hindbrain, *FoxN4* expression is evident in the dorsal ridge of the reticular formation (compare Figs 3A and H). Ventrally, *FoxN4* is expressed in the subpallium, medial olfactory bulbs and the preoptic area (Fig 3C). Thus, both in the developing brain and the retina, *FoxN4* is primarily expressed in regions containing progenitor cells.

2. MATERIALS AND METHODS

2.1 Isolation of *Xenopus laevis* FoxN4

We identified a *Xenopus laevis* EST with significant similarity to mouse *FoxN4* in a BLAST search. We obtained and sequenced a *FoxN4* EST from NIBB (Japan; URL: <http://xenopus.nibb.ac.jp/>; accession number BJ061286; NIBB Mochii normalized *Xenopus* tailbud library *Xenopus laevis* cDNA clone XL072i03). We amplified DNA flanking the 5'-end of the *FoxN4* sequence included in the EST by one-armed (OA) PCR (Macrae and Brenner, 1994; Sparrow et al., 1998) from a St 17 neurula library (Sparrow et al., 1998) using the following primers: 5'-GSP-1: 5' -CAAGCACCTTTGCGTGAAGATCCAC - 3'; 5'-GSP-2: 5' - GTTCTTCCAGCCGTCTGGAGCTATC - 3'; and vector-specific primers as described previously (El-Hodiri et al., 2003). We then similarly performed 3'- OAPCR using the gene-specific primers 3'-GSP-1: 5' - CTGCTCACTCACTGCAGCTTC - 3' and 3'-GSP-2: 5' - CACTAATCATACCAGAGATC - 3'. The *FoxN4* coding region was then amplified using 3'-GSP-2 and CDS R: 5' - GTCCCGCTATTAAAGCAAAGC - 3'. The amplified products were cloned in the TOPO TA PCR II plasmid (Invitrogen) according to manufacturer's instructions. The *FoxN4* cDNA was then liberated from the TOPO TA plasmid using EcoRI and subcloned into the EcoRI site of pCS2+ (Turner and Weintraub, 1994).

2.2 In situ hybridization

All in situ hybridization analysis was performed using digoxigenin or fluorescein-labelled antisense riboprobes. Probes were prepared by in vitro transcription of linearized templates. *FoxN4* probe was prepared from the EST plasmid linearized with NotI and transcribed with T7 RNA polymerase. Probes for *Dlx2* (Papalopulu and Kintner, 1993; Seufert et al., 2005), *Lhx2* (Bachy et al., 2001; Viczian et al., 2006), *Lhx5* (Bachy et al., 2001), *Lhx9* (Bachy et al., 2001), *Rx2A* (Mathers et al., 1997) and *Sox2* (Mizuseki et al., 1998) were prepared as described previously. Wholemount in situ hybridization was performed using fixed *Xenopus* embryos as described previously (Sive et al., 2000). Embryos were staged using accepted standard staging criteria (Nieuwkoop and Faber, 1994). In situ hybridization was also performed using 8 µM sections of fixed, paraffin-embedded st 36, 38, 40, 41, or 45 tadpoles (Viczian et al., 2003). Expression in tadpole brains was visualized by wholemount in situ hybridization of brains isolated from fixed st 46 embryos using digoxigenin-labelled riboprobes as described previously (Colombo et al., 2004). Double in situ hybridization was performed using the same protocol modified as described previously (Seufert et al., 2005). Some brains subjected to wholemount in situ hybridization were further analyzed in section. To prepare these sections, samples were incubated overnight at 4°C in 25% (w/v) sucrose and cryopreserved in Tissue-Tek OCT Compound (Sakura). Sections (14 µm thickness) were prepared, dehydrated, and mounted using Permount (Fisher Scientific).

3. ACKNOWLEDGEMENTS

The authors are indebted to Jean Ille for help in interpretation of brain in situ hybridization patterns. We thank Rob Grainger, Milan Jamrich, Sylvie Retaux and Michael Zuber for plasmid DNAs and Monica Vetter and members of the El-Hodiri lab for helpful and critical discussions. We also thank Holly Moose for helping with the sequence comparisons and for critical reading of the manuscript. This work was supported by NIH/NEI grant EY015480 to HME.

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FIGURE 1.

Xenopus laevis FoxN4 is closely related to other vertebrate FoxN4 gene products. A. Predicted amino acid sequence of the *X. laevis* FoxN4 gene product. The forkhead domain is underlined. The previously denoted FoxN4-specific sequence and a putative activation domain are marked by a dashed line and a double underline, respectively. B - D. Alignment of forkhead (B), FoxN4-specific (C), and activation domains (D) from *X. laevis* and other vertebrate FoxN4 gene products sequences. Sequences were aligned using ClustalW 1.8 at the EMBL-EBI webpage (URL: <http://www.ebi.ac.uk/clustalw/index.html>) and then formatted using BOXSHADE at the EMBnet website (URL: http://www.ch.embnet.org/software/BOX_form.html). E. Phylogenetic analysis performed using the MegAlign program (part of the DNASTar/Lasergene

suite of programs) from a ClustalW 1.8 alignment of the sequences shown. Accession numbers for sequences used are as follows: mouse Foxn1 - NP_032264; mouse Foxn4 - AAL06288; chicken FoxN4 - XP_415189; zebrafish FoxN4 - AAG27086.

FIGURE 2.

FoxN4 is expressed in developing eye fields, brain, and retinal progenitor cells. A – J: Detection of *FoxN4* (A – E) and *Rx* (F – I) expression in neural plate (st 16 – A, F), neural tube (st 19 – B, G) and tailbud (st 25 – C, H; st 31 – D, E, I) embryos by wholemount in situ hybridization (WISH) using antisense riboprobes. Arrow in I denotes pineal gland. E: high magnification view of pronephric area from an embryo similar to that shown in D (box) but subjected to reduced proteinase K treatment and prolonged color reaction. J: Comparison of *FoxN4* and *Rx* expression in a st 19 embryo visualized by double in situ hybridization using antisense probes for *FoxN4* (turquoise color) and *Rx* (magenta color). Arrowhead denotes region of *Rx* single staining. J': digital enlargement of left eye region in J to more clearly show *Rx* expression (arrowhead). K – N: analysis of *FoxN4* expression in the developing brain of whole *Xenopus* embryos at neural tube and tailbud, at indicated stages. O – T: analysis of *FoxN4* (O – S) and *Rx* (T) expression in sections obtained from paraffin-embedded fixed embryos at early tadpole stages. Arrowheads in R, T indicate the tips of the CMZ. Abbreviations: C: cement gland; CMZ (bracket): ciliary marginal zone; E: eye; FB: forebrain; HB: hindbrain; L: lens; MB: midbrain; O: olfactory bulb/placode/organ; R: retina.

FIGURE 3.

Expression of *X. laevis* *FoxN4* in the tadpole brain. Visualization of *FoxN4* expression in isolated tadpole brains by WISH. Brains were isolated from fixed st 45 tadpoles and subjected to WISH using antisense riboprobes for *FoxN4* (A-C), *Lhx2* (D, E), *Lhx5* (F, G), or *Lhx9* (H, I) as indicated. Brain regions are labeled (abbreviations are explained in box). A, D, F, H: dorsal views; B, E, G, I: lateral views; C: ventral views. J: Simultaneous visualization of *FoxN4* (turquoise) and *Lhx9* (magenta) expression in the region of prosomere 2 by double in situ hybridization performed using an isolated st 45 tadpole brain. J'- Section of brain shown in J. K - M: Sections of isolated tadpole brains subjected to WISH using *FoxN4* (K, L) or *Lhx5* (M) antisense riboprobes. N: In situ hybridization performed on a sectioned st 41 tadpole

probed with an antisense *Sox2* riboprobe. Red lines in B, G, and J indicate planes of section shown in panels J', K, L, and M.