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The transcription factor Foxk1 is expressed in developing and adult mouse neuroretina

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ABSTRACT

The forkhead transcription factor Foxk1 is an important regulator of myogenic progenitor cells. Since our previous data from mouse retina revealed that Foxk1 is upregulated in Ptf1a-deficient mice we investigated the spatial and temporal expression of Foxk1 during development of mouse retina. Expression of Foxk1 was analyzed on both mRNA and protein level. To identify Foxk1 transcripts, retina and cerebrum (positive control) of adult animals (postnatal day 90 (P90)) was subjected to reverse transcription polymerase chain reaction (RT-PCR) and sequencing of the amplified cDNA. The Foxk1 protein was analyzed in adult retina by Western blotting and in developing eyes at embryonic day (E) 13, 15, E17, P0, P4, P7, P10 and P90 by immunohistochemistry. Localization of Foxk1 expression was determined using cell-specific markers by double labelling. Foxk1 transcripts were detected in adult retina by RT-PCR and confirmed by sequencing. Western blot analysis confirmed the expression of Foxk1 protein in the adult retina. Immunohistochemical examination of developing eyes localized the protein to bipolar, amacrine and ganglion cells with an onset of Foxk1 expression from E15 onwards. The expression pattern during development suggests that Foxk1 may have an important role in retinal cells.

Adult mammalian retina consists of six principal types of neuronal cells (cone photoreceptors, rod photoreceptors, horizontal cells, bipolar cells, amacrine cells and ganglion cells) and one type of glia cell (Mueller cell). The stratified structure of the retina is attributed to the highly organized cells in three cellular (nuclear) layers separated by two synaptic (plexiform) layers. The outer nuclear layer (ONL) accommodates the cell nuclei of cone and rod photoreceptors. In the inner nuclear layer the nuclei of horizontal, bipolar, amacrine and Mueller cells are localized. The ganglion cell layer (GCL), which borders on the vitreous body, is comprised of both nuclei of displaced amacrine cells and ganglion cells. The outer plexiform layer (OPL) and inner plexiform layer (IPL) contain the dendrites and axons of these neuroretinal cells (Nakhai et al., 2007).

During vertebrate retinogenesis the generation of different retinal cell types from a pool of multipotent retinal progenitor cells (RPCs) proceeds in relatively ordered chronological sequence. Ganglion cells are generated first, followed in overlapping phases by horizontal cells, cone photoreceptors, amacrine cells, rod photoreceptors, bipolar cells and Mueller glia cells (Marquardt and Gruss, 2002).

The forkhead box (Fox) gene family encodes regulatory proteins that are involved in a wide spectrum of important biological processes from embryonic development to metabolic homeostasis. It has been shown that FOX genes play a crucial role in human diseases (Hannenhalli and Kaestner, 2009; Jackson et al., 2010). Fox transcription factors are characterized by an evolutionary highly conserved DNA-binding domain (DBD), known as the winged helix-turn-helix domain (WHD) or forkhead (FH) domain. The mouse Foxk1 (also called myocyte nuclear factor or MNF) gene is located on the chromosome 5 and consists of 9 exons. Foxk1 was first described 1994 as a sequence-specific DNA-binding protein to the myoglobin CCAC box motif (Bassel-Duby et al., 1994). At the amino acid (aa) level, the human FOXK1 (733 aa) and mouse Foxk1 (719 aa) gene show 88.7% identity (Katoh, 2004). In a yeast two hybrid analysis the LIM-only protein, Fhl2, has been found to interact

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with Foxk1 and corepresses Foxo4 activity in myogenic progenitor cells (Shi et al., 2010). In addition, Foxk1 interacts with SWI-independent-3 (Sin3) protein through Sin3 interacting domain (SID) and regulates myogenic progenitors (Shi and Garry, 2012). As upstream genes, Sox15 and Fhl3 transcriptionally coactivate Foxk1 gene expression (Meeson et al., 2007). In addition, Foxk1 is a direct upstream regulator of the cyclin dependent kinase inhibitor, p21(CIP), and regulates the cell cycle progression (Hawke et al., 2003). Besides these interactions that have been reported for mouse myogenic progenitor cells, very little data are available on the function and involvement of Foxk1 in molecular pathways. In a human rhabdomyosarcoma cell line it has been shown that FOXK1 interacts with the MADS-box transcription factor SRF and coregulates the expression of SM alpha-actin and PPGB (Freddie et al., 2007). Furthermore, analysis of cell cycle regulating proteins showed that FOXK1 binds to the promoter and regulates the DHFR, TYMS, GSDMD and TFDP1 genes (Grant et al., 2012).

We and others have shown that the pancreas-specific transcription factor 1a (Ptf1a) is essential for the differentiation of GABAergic and glycinergic amacrine cells and horizontal cells in the mouse retina (Fujitani et al., 2006; Dullin et al., 2007; Nakhai et al., 2007). Since our microarray analysis revealed that Foxk1 is upregulated in Ptf1a-deficient retinae and that there are yet no data available about the expression of Foxk1 in the retina, we analyzed the expression pattern in the developing and adult murine neuroretina.

1. Materials and methods

1.1. Tissue preparation

All experiments were performed with C57Bl/6N mice in accordance with the ARVO (Association for Research in Vision and Ophthalmology) statement for the use of animals in ophthalmic and vision research and the study was approved by the Institutional Animal Care and Use Committee of the University Halle-Wittenberg and University Erlangen. Eyes and cerebrum were explanted from sacrificed embryonic and adult animals. For developmental staging of the animals, midday of the day of vaginal plug formation was defined as embryonic day 0.5 (E0.5), and day of birth was considered as postnatal day 0 (P0). Eyes or retinal tissue was investigated at embryonic day (E) 13, E15, E17, P0, P4, P7, P10 and P90 (adult).

For retina isolation adult eyes were enucleated, hemisected at the pars plana of the eye globe, the lens removed and the retina peeled away from the retinal pigment epithelium (RPE). For RNA extraction retina and cerebrum were submerged with the RNAlater RNA stabilization reagent (Qiagen, Hilden, Germany) and stored at −20 °C.

For protein extraction retinal and cerebral tissues from adult mice were snap-frozen in liquid nitrogen and kept at −80 °C until use.

For immunohistochemical analysis, the entire eyes were submerged in 4% formaldehyde fixation buffer (A + E. Fischer, Wiesbaden Germany) and stored in this fixative. Only sections through the central retina were analysed.

1.2. RT-PCR and sequencing

Total RNA from retina and cerebrum was extracted with RNeasy Micro kit (Qiagen, Hilden, Germany). Genomic DNA was digested with 1 U DNaseI per µg total RNA (Fermentas, St. Leon-Rot, Germany) at 37 °C for 30 min. Subsequently, the reaction was inactivated in 2.5 mM EDTA (Fermentas) for 10 min at 65 °C. The first strand cDNA synthesis, 1 µg RNA was incubated at 65 °C for 5 min with 10 nmol of each dNTP, 50 pmol oligo(dT)20-primer (both from Invitrogen, Karlsruhe, Germany) in a total volume of 13 µl and chilled on ice for 1 min. The first strand buffer (1 µmol Tris–HCl (pH 8.3), 1.5 µmol KCl, 60 nmol $MgCl_2$, 0.1 µmol DTT, 200 U of the SuperScript III RT (all reagents from Invitrogen) and 40 U RiboLock RNase Inhibitor (Fermentas) were given to the mixture and further incubated for 60 min at 50 °C in a total volume of 20 µl. The reaction was stopped by heating 15 min at 70 °C. In negative controls, the addition of the enzyme SuperScript III RT was omitted.

For PCR, 1 µl cDNA (0.1 µg/µl) from each sample was used in a final volume of 20 µL PCR reaction mix (1×PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 250 mM of each dNTP, 5 U Taq polymerase (Invitrogen) 1 µM primer listed in Table 1).

The PCR was carried out for 45 cycles, each cycle consisting of 94 °C for 45 s, 53 °C for 45 s, 72 °C for 45 s and a final extension at 72 °C for 5 min. The ß-actin (actin) mRNA was amplified as positive control (Table 1). In negative controls, cDNA was replaced by ddH_20 (double distilled water) or the cDNA synthesis mix without transcriptase. Results from RT-PCR were verified by sequencing (Eurofins MWG Operon, Ebersberg, Germany) with the dideoxy chain-termination sequencing technique (Sanger et al., 1977) using the sense primers (Table 1) and the internet-based BLASTn program for sequence alignment (Altschul et al., 1997).

1.3. Western blot analysis

Retinal and cerebral tissues were homogenized (Ultra Turrax T25 basic, IKA Werke, Staufen, Germany) in 200 µl RIPA lysis buffer (Cao et al., 2005) with 218 mM PMSF and protease inhibitor cocktail (Complete, Roche, Mannheim, Germany). Of each tissue protein extract, 40 µg were resolved in 10% SDS gel. Proteins and molecular weight marker (PageRuler™ Prestained Protein Ladder, Fermentas, Germany) were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and blocked in 5% filtered non-fat dry milk (Roth, Karlsruhe, Germany) in PBST (137 mM NaCl, 1.47 mM KH_2PO_4, 7.81 mM Na_2HPO_4, 2.68 mM KCl, 0.1% Tween 20) at room temperature (RT) for 30 min under agitation. The blot was incubated with rabbit anti-mouse Foxk1 antibody (1:100, Table 2) in 0.5% filtered non-fat dry milk in PBST overnight at 4 °C and the membrane was incubated with HRP-conjugated secondary goat anti-rabbit IgG antibody (Table 3) in PBST for 1 h at RT. Immunoreactivity was detected using chemiluminescence (ECL plus®, Amersham-Pharmacia, Uppsala, Sweden) in a LAS 3000

Table 1
Sequences of the intron spanning primers used for the detection of Foxk1 in RT-PCR and sequencing analysis.

Primer	Sense primer, 5′→3′	Antisense primer, 5′→3′	bp	Annealing temperature (°C)
Mouse Foxk1	CTA CCC CAA CAA TGC ACC TT	GAG GAC TTG CTG GAC AGG AG	226	60
Gene Bank ID: NM_199068 (for PCR)	Exon 1 (ENSMUSE00000590046) Exon 2 (ENSMUSE00001081865)	Exon 2 (ENSMUSE00001077487)		
Mouse Foxk1 (for sequencing)	AAA GCG GAG ACA GAG AGG TG Exon 5 (ENSMUSE00001014867)	CCA GAG TAC CGC TAT TCC CA Exon 6 (ENSMUSE00001024108)	116	60
Mouse β-Actin Gene Bank ID: NM_007393 (control)	ATA TCG CTG CGC TGG TCG TC Exon 2 (ENSMUSE00001112660)	AGG ATG GCG TGA GGG AGA GC Exon 4 (ENSMUSE00000517504)	300	57