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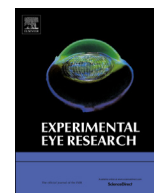

First insights into the expression of VAX2 in humans and its localization in the adult primate retina

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ABSTRACT

VAX2 is a transcription factor specifically expressed in the ventral region of the prospective neural retina in vertebrates and is required for ventral eye specification. Despite its extensive analysis in vertebrates, the biological role of VAX2 in the human is presently unclear. This study was undertaken to investigate VAX2 in humans aiming to gain new knowledge into its involvement in retinal function. Here, we report VAX2 gene expression and protein localization in cultured cells and adult retina. RT-PCR experiments indicated that *VAX2* is enriched in neuronal tissues. Moreover, we identified a novel isoform most abundantly expressed in the retina. We termed the known transcript (NM_012476) isoform-1, and the newly identified transcript as isoform-2. Analysis of protein localization in cultured cells revealed that isoform-1 localizes to the nucleus and isoform-2 is widely expressed within the cell; partial co-localization of isoform-2 and actin filaments was also observed. In nonhuman primate retina VAX2 was seen either in the nuclear or in the cytoplasmic compartment depending on the retinal cell type. In addition, a noteworthy enrichment of the signal was observed in the outer segment of cone photoreceptors.

Overall, this study provides the first insights into the expression of VAX2 in humans and its localization in the adult primate retina. Moreover, preliminary characterization of alternative variants suggests an involvement of VAX2 in multiple cellular pathways. Our findings raise the interesting possibility for further investigation of VAX2 in the retina in health and disease.

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VAX2 belongs to a subfamily of homeobox genes closely related to Drosophila transcription factor gene empty spiracles (*ems*) and to its vertebrate homologues, the *Emx* genes (Simeone et al., 1992). *Vax2* is a transcription factor specifically expressed in the ventral region of the prospective neural retina in vertebrates and is required for ventral eye specification (Barbieri et al., 1999).

The genetic inactivation of *Vax2* in mouse produces, amongst other features, an incomplete closure of the choroidal fissure that results in an incompletely penetrant ocular coloboma (Barbieri et al., 2002; Mui et al., 2002). Considering all the similarities between the phenotype observed in *Vax2* mutant mice and the clinical features of isolated colobomata in human, *VAX2* represents a good candidate gene for this human condition. Thus far, however, no mutations affecting *VAX2* in coloboma patients have been reported. Only negative results for *VAX2* mutational screening in anophthalmia/microphthalmia patients have been published (Slavotinek et al., 2012). A meta-analysis of astigmatism in a Caucasian population identified a locus in the genomic region containing *VAX2*, suggesting a potential involvement of this gene in the pathogenesis of astigmatism (Lopes et al., 2013). Moreover, very recently a homozygous deletion at 2p13.3 encompassing all of *ATP6V1B1* and part of *VAX2* has been reported in a patient affected by Distal Renal Tubular Acidosis. Interestingly, the patient, amongst other features, showed an ocular phenotype diagnosed as bilateral rod/cone photoreceptor dystrophy and mild optic atrophy (Norgett et al., 2015). It has been shown that *Vax2* is also involved in correct intra-retinal retinoic acid distribution, and its action extends to the mature retina, in particular to ensure appropriate gene expression in cone photoreceptor cells (Alfano et al., 2011). *Vax2* inactivation, in mouse, shows alterations in local spectral sensitivity rather than in overall cone function, at least within the range detectable by ERG (Alfano et al., 2011).

Although, the *Vax2* gene has been widely studied in vertebrates its role in humans is presently unclear. This study was undertaken

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with the objective to gain new knowledge into its involvement in human retinal function. Here we report VAX2 gene expression and protein localization in cultured cells and primate retina.

For this study the following methodologies were used. *VAX2* gene expression was tested by RT-PCR using primers located within exon 1 and exon 3 (Fw – GGTCAGCATGGGCGATGG; Rv-GTGGGAGTCTTAAGTGTTAGC) allowing the amplification of the full-length coding cDNA (NM_012476). Analysis was performed using human tissues (commercially available RNA, Clontech), as well as Y79 and hTERTRPE-1 (RPE-1) cell lines (RNA prepared using Trizol, Invitrogen). cDNA was prepared using both the QuantiTect® Reverse Transcription kit (Qiagen) and Superscript II (Invitrogen) with oligo dT primers according to the manufacturer's instructions. RT-PCR was performed using GoTaq® Colorless Master Mix 2X (Promega). The *HPRT* gene (Fw- GGGACATAAAAGTAATTGGTG; Rv-GCGACCTTGACCATCTTTGG) was used as an internal control. Retina cDNA PCR products were sub-cloned in pcRII TOPO Vector (Invitrogen) and clones with different inserts, discriminated by size, were analyzed by direct sequencing.

Real-time quantitative PCR (qPCR) was carried out with the StepOnePlus System (Applied Biosystem) and performed as described by Alfano and co-workers (Alfano et al., 2005). Differences between the mean Ct values of tested genes and those of reference genes were calculated as $\Delta Ct = CT_{VAX2} - CT_{HPRT}$ and represented as $2^{-\Delta Ct}$. *VAX2* full-length cDNAs were cloned at the *Xho*I–*Kpn*I sites (Fw-ccctcgagGTCAGCATGGGCGATGG; Rv-ggggtaccGTGGGAGTCTTAAGTGTTAGC) in pEGFP-C3 (Clontech, 6082-1) and at *Hin*dIII-*Kpn*I sites (Fw-ccaagcttGGTCAGCATGGGCGATGG; Rv-ggggtaccGTGGGAGTCTTAAGTGTTAGC) in p3XFLAG-*myc*-CMV™-26 (Sigma, E6401). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. RPE-1, SK-N-SH and HeLa cells were grown at 37 °C and 5% CO_2 in DMEMF12 or DMEM, respectively, supplemented with 10% FCS, penicillin (100 U/ml) and streptomycin (50 μg/ml). Y79 cells were cultured in RPMI 1640 supplemented with 20% FCS, penicillin (100 U/ml) and streptomycin (50 μg/ml). RPE-1 cell line was purchased from Clontech, whilst the other cell lines were purchased from ATCC. Experiments were performed with cells of early passages.

For immunocytochemistry cells were fixed in 4% PFA, blocked 2 h with blocking reagent (6% BSA/0.3% Tween20) and hybridized (1–12 h) with the relevant primary antibodies. An anti-Flag antibody (F3165) was used diluted 1:10 000. Endogenous expression of VAX2 protein was analyzed using a commercially available antibody (S-17, sc-79339) diluted 1:10. *Ex vivo* analysis was performed on adult monkey retina (*Macaca fascicularis*, 2 and 16.5 years old) and mouse eyes (C57/BL6, 2 month old). Monkey eyes were obtained from animals sacrificed in accordance with local and national ethical rules for purposes not related to this project. Eyes of 2 month old C57/BL6 mice were obtained from the UCL Institute of Ophthalmology (IOO) Biological Resources Unit (BRU); the work was approved by the IOO Institutional Animal Care and User Committee (IACUC #70/2710).

Tissues were fixed in 4% PFA, treated with a sucrose gradient (10–30%) and embedded in O.C.T. compound (VWR, UK). Cryosections (5–10 μm) were treated at high temperature in 0.01 M Citrate buffer for antigen retrieval followed by permeabilization with 0.3% Triton-X in PBS (5 min). Sections were blocked (2 h at room temperature) and hybridized (18 h at room temperature) with PBS containing 5% donkey serum, 6% BSA and 0.3% Tween20. A goat VAX2 antibody (S-17, sc-79339) diluted 1:10 and an anti M/L-Opsin antibody (gift from Prof. Jeffery) diluted 1:500 were used. Peanut agglutinin (PNA) staining was performed using a Rhodamine conjugated PNA (Vector Lab) diluted 1:100 (1 h at room temperature). Alexa Fluor®488 (Molecular Probe, 1:400) and Cy3 conjugated AffiniPure (Jackson ImmunoResearch lab, 1:400) were used as secondary antibodies. Negative controls (−ve control) were undertaken by omitting the primary antibody. Nuclei were stained (10 min at room temperature) with 4′, 6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, 1:5000). Slides were mounted using Dako fluorescent medium (DAKO) and viewed on a LSM700 confocal microscope. Z-stack confocal images were analyzed using the Zen lite (black edition) Digital imaging free software (Zeiss); the final pictures of both cells and tissues were the results of maximum projections.

In the present study, we first sought to investigate *VAX2* gene expression. By performing 40 cycles PCR assays we observed that *VAX2* is enriched in the neuronal tissues (Fig. 1A, top panel); *HPRT* amplification was used as control (Fig. 1A, bottom panel). Expression was also detected in the cell lines tested (data not shown). Amongst the neuronal tissues, not including the retina, an enrichment of the signal was seen in the cerebellum. Previous studies have described *Vax2* localizing to the adult cerebellum in *Xenopus* (Liu et al., 2008). Therefore, our results suggest an involvement of VAX2 in the homeostasis of this brain compartment. Interestingly, by RT-PCR we constantly obtained two amplicons: a lower band of the expected size and an additional upper band (Fig. 1B, indicated by the red asterisk). Sub-cloning and sequencing of PCR products obtained, both from retina and RPE-1 cell line cDNAs, showed that both bands correspond to specific products. The longer amplicon is due to the amplification of a novel *VAX2* splice-variant, consisting of four exons, three of which are shared with the canonical NM_012476 transcript, plus an additional exon of 63 bp (AACCAGCCCCAGTCCTGAAACTACCTAGA-GATCCACCCTGAATCACCTCATTAGCATACAACA) located between the second and third exon of the NM_012476 transcript. The newly identified transcript partially overlaps with the EST H92142 of retinal origin reported in the UCSC database (http://genome.ucsc.edu/); this EST contains the 63 bp exon. We termed the canonical NM_012476 transcript isoform-1, and the newly identified transcript as isoform-2. Isoform-1 is predicted to encode a 290 amino acid (aa) protein, whereas isoform-2, a shorter protein of 150 aa. The extra-exon introduces an earlier stop codon resulting in a truncated protein which still retains, however, part of the HOX domain sequence, spanning from aa 102 to 164, according to SMART online prediction tool (http://smart.embl-heidelberg.de/). The 63 bp nucleotide sequence is conserved in primates and absent in other species as revealed by Blast alignment (http://blast.ncbi.nlm.nih.gov/Blast.cgi) against the genome of different organisms (fish, amphibian, avian, mammals). However, experimental validations are required to assess whether or not this is a coding sequence in nonhuman primates. Suitable material for further investigation was not available in this study.

Moreover, RT-PCR suggests that the two isoforms are differentially expressed. Interestingly, isoform-2 appears highly detectable in retina compared with other tissues (Fig. 1B). Real Time qPCR experiments, using primers located within the second and the third exon of isoform-2 (Fw-GCCTGGAGATGGAGTTCC; Rv-, GTATGCTAATGAGGTGATTCAG) and therefore specific for this transcript, confirmed that isoform-2 is most abundantly expressed in the retina (Fig. 1C) suggesting a potentially important role in retinal cells.

With regards to investigating the biological function of *VAX2* isoforms, we next analyzed their *in vitro* sub-cellular localization. Transient transfection assays, performed with both constructs pEGFP-VAX2 and p3XFLAG-VAX2, showed that isoform-1 localizes to the nucleus (Fig. 1D and data not shown), whilst isoform-2 displays a widespread localization within the nucleus and the cytoplasm (Fig. 1E and data not shown). Both isoforms show a consistent localization pattern even when they are co-expressed in

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