Neurobiology of Disease 32 (2008) 510–520

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

Genes and pathways differentially expressed in the brains of *Fxr2* knockout mice

Sebastiano Cavallaro [a,*], Sabrina Paratore [a], Francesco Fradale [a], Femke M.S. de Vrij [b], Rob Willemsen [b], Ben A. Oostra [b]

[a] *Functional Genomics Center, Institute of Neurological Sciences, Italian National Research Council, Catania, Italy*
[b] *Department of Clinical Genetics, Erasmus MC, Rotterdam, The Netherlands*

ARTICLE INFO

Article history:
Received 14 December 2007
Revised 26 July 2008
Accepted 9 September 2008
Available online 30 September 2008

Keywords:
Fragile X syndrome
FXR1
FXR2
Genomics
Knockout
Mental retardation
Microarray

ABSTRACT

Fragile X syndrome is a common inherited form of mental retardation and originates from the absence of expression of the *FMR1* gene. This gene and its two homologues, *FXR1* and *FXR2*, encode for a family of fragile X related (FXR) proteins with similar tissue distribution, together with sequence and functional homology. Based on these characteristics, it has been suggested that these proteins might partly complement one another. To unravel the function of *Fxr2* protein, the expression pattern of 12,588 genes was studied in the brains of wild-type and *Fxr2* knockout mice, an animal model which shows behavioral abnormalities partly similar to those observed in *Fmr1*-knockout mice. By genome expression profiling and stringent significance tests we identify genes and gene groups de-regulated in the brains of *Fxr2* knockout mice. Differential expression of candidate genes was validated by real-time PCR, *in situ* hybridization, immunohistochemistry and western blot analysis. A number of differentially expressed genes associated with the *Fxr2* phenotype have been previously involved in other memory or cognitive disorders.

Introduction

Fragile X syndrome is the most common inherited form of mental retardation and originates from the loss of *FMR1* expression due to trinucleotide repeat expansion (Fu et al., 1991; Verkerk et al., 1991). In addition to global cognitive deficits, the disorder can also be manifest as specific impairments in visual–spatial learning as well as auditory and visual short-term memory (Freund and Reiss, 1991; Fisch et al., 1996; Fisch et al., 1999). The function of the *FMR1* gene product, FMRP is partly known, with the presence of three RNA binding regions (two KH domains and an RGB box) suggesting that FMRP is an RNA-binding protein (Ashley et al., 1993; Siomi et al., 1993). Indeed, *in vitro*-translated FMRP has been demonstrated to preferentially bind certain RNA homopolymers and to selectively bind a subset of brain transcripts including its own message (Siomi et al., 1993).

The observation that FMRP is an RNA binding protein and may be implicated in RNA metabolism suggests that other genes, whose products may vary in the absence of FMRP, could play a significant role in the cognitive deficits associated with fragile X syndrome.

In view of the broad variety of genes and the cross talk of genetic pathway, gene expression profile by microarray technology today offer a new dynamic and functional dimension to the exploration of mental retardation and provide the unprecedented opportunity to survey the molecular basis underling fragile X syndrome. In a previous study, we have performed genome-wide expression analysis in *Fmr1* knockout (KO) mice, an animal model showing a phenotype that mimic the human syndrome, such as macro-orchidism and behavioral abnormalities (The Dutch-Belgian Fragile X Consortium, 1994). Among the gene expression changes that result from a deficiency of FMRP, our analysis involved a number of genes previously involved in other memory or cognitive disorders (D'Agata et al., 2002).

Two homologues of *FMR1*, *FXR1* and *FXR2*, have been identified (Siomi et al., 1995; Zhang et al., 1995; Coy et al., 1995). Together, these genes encode for a family of fragile X related (FXR) proteins with sequence and structural homology, similar expression patterns and cellular function (Tamanini et al., 1996; Bakker et al., 2000). Based on these characteristics, it has been suggested that FXR proteins might partly complement one another. Indeed, *Fmr1* KO mice shows behavioral abnormalities partly similar to those observed in *Fxr2* KO mice (Bontekoe et al., 2002), whereas *Fmr1*/*Fxr2* double-KO mice have exaggerated impairments in open-field activity, prepulse inhibition of acoustic startle response and contextual fear conditioning relative to the single gene knockouts (Spencer et al., 2006). *Fmr1* and *Fxr2* genes, therefore, appear to contribute in a cooperative manner to pathways controlling locomotor activity, sensorimotor gating and cognitive processes.

In this study we performed genome-wide expression analysis in the brains of *Fxr2* KO mice to identify mRNAs which may be transcriptionally misregulated in the brain when the *Fxr2* protein is absent.

* Corresponding author. Istituto di Scienze Neurologiche, CNR, Viale Regina Margherita 6, 95123 Catania, Italy. Fax: +39 095 7122426.
E-mail address: s.cavallaro@isn.cnr.it (S. Cavallaro).
Available online on ScienceDirect (www.sciencedirect.com).

0969-9961/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.nbd.2008.09.008

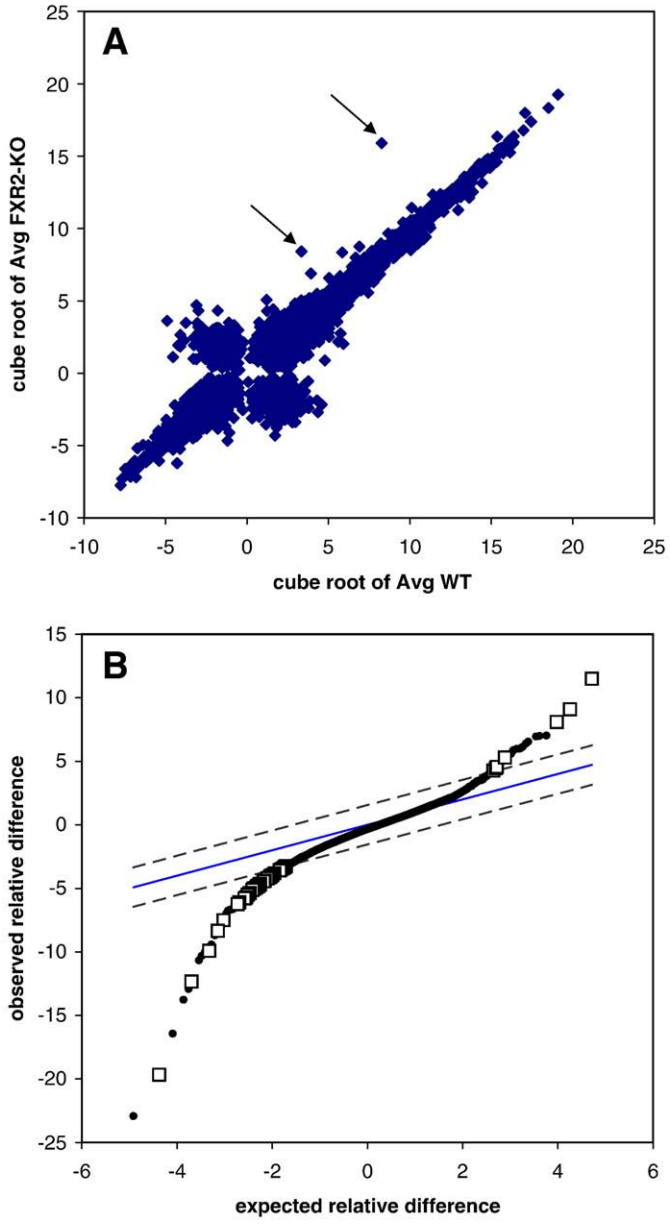

Fig. 1. Gene expression measured by microarrays. (A) Cube root scatter plot of gene expression among wild type (WT) and *Fxr2* knockout (*Fxr2 KO*) mice. The average raw expression values of each experimental condition are plotted on a cube root scale. Two of the genes whose expression is altered are indicated by arrows. (B) Identification of genes with statistical significant changes in expression. Using SAM, the observed test statistic is plotted against the statistic expected by chance for 12,588 genes. Genes that were expressed higher or lower in *Fxr2* KO mice appear on the positive or negative side of the *x*-axis, respectively. By setting a threshold level (Δ)=1.5 (dotted lines) and two fold changes, SAM predicted 50 genes (indicated by the squares) as being differentially expressed with a % FDR of 0.

Materials and methods

Animals

Animals, housed 1/cage, were given access to food and water, and maintained on a 12:12 light/dark cycle in a constant temperature (23 °C). The animals were sacrificed by cervical dislocation, and the brains were rapidly removed, dissected, snap-frozen in liquid nitrogen and stored in an ultra-low-temperature freezer at −80 °C.

The number of animals used in this study was 15 wild type and 15 *Fxr2 KO* two month old mice. The *Fxr2* KO mice were generated by crossing a heterozygous *Fxr2* KO female with a heterozygous *Fxr2* KO male, both derived from a cross between a wild type C57BL/6J male and a female heterozygous for the *Fxr2* KO allele (N9 congenic on C57BL/6J). Animals were genotyped by PCR using DNA obtained from tail clippings (Bontekoe et al., 2002).

Microarray analysis

Six individual animals (n=3/genotype) were used for microarray analysis. Brain total RNA (n=3 per group) was extracted, reverse transcribed, biotinylated and hybridized to a single Affymetrix GeneChip Murine Genome Array U74A array as previously described (Cavallaro et al., 2002) with the protocol outlined in the GeneChip Expression Analysis Technical Manual (Affymetrix, CA, USA). The arrays were washed and stained by using a fluidics system with streptavidin-phycoerythrin (Molecular Probes, Eugene, OR, USA), amplified with biotinylated anti-streptavidin antibody (Vector Laboratories, Burlingans, CA, USA) and then scanned with a GeneArray Scanner (Affymetrix, CA, USA). To determine the quality of labeled targets prior to analysis on GeneChip Murine Genome Array U74A arrays, each sample was hybridized to one GeneChip Test3 array. The image data were analyzed by MicroArray Suite 4.0 Gene Expression analysis program (Affymetrix, CA, USA). Detailed protocols for data analysis of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described (Lipshutz et al., 1999). Average difference values of less than zero represent probe sets where the intensity of the mismatched probe is on average greater than the perfect matched probe and, thus the probe set is performing poorly. For this reason, values below 0 were set to 0.01. To identify changes with statistical confidence we used Significance Analysis of Microarrays (SAM) version 3.00, a method that assigns a score to each gene on the basis of change in gene expression relative to the standard deviation of repeated measurements (Tusher et al., 2001). For genes with scores greater than an adjustable threshold, SAM uses permutations of the repeated measurements to estimate the percentage of genes identified by chance, the false discovery rate (FDR) (Tusher et al., 2001). Fig. 1 shows the variability between the raw gene expression of wild type and three *Fxr2 KO* mice. By setting a threshold level (Δ)=1.05 and two fold changes, SAM predicted a FDR of 5% for 52 altered genes (Table 1).

Table 1
Estimated number of significant genes estimated by SAM

SAM delta	Fold	No. of genes falsely called (median)	No. of genes called significant	% FDR (median)
0.2		3861.0	6326.0	61.0
0.4		1065.6	3193.0	33.4
0.6		322.7	1342.0	24.0
0.8		66.6	450.0	14.8
1.0		25.6	207.0	12.4
1.2		9.4	119.0	7.9
1.4		6.8	86.0	7.9
1.6		4.3	51.0	8.4
1.8		1.7	32.0	5.3
2.0		0.9	26.0	3.3
2.3		0.4	25.0	1.7
0.2	2	561.4	822.0	68.3
0.4	2	170.8	481.0	35.5
0.6	2	47.8	249.0	19.2
0.8	2	10.2	108.0	9.5
1.0	2	4.3	64.0	6.7
1.2	2	3.0	45.0	6.6
1.4	2	1.7	37.0	4.6
1.6	2	0.0	22.0	0.0
1.8	2	0.0	14.0	0.0
2.0	2	0.0	11.0	0.0
2.3	2	0.0	11.0	0.0

The number of genes predicted by SAM to be significantly altered was calculated with different parameters (delta value and fold change). The False Discovery Rate (FDR) was the percentage of falsely significant genes compared to the genes called significant.