Generation and Characterization of Fmr1 Knockout Zebrafish

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Abstract

Fragile X syndrome (FXS) is one of the most common known causes of inherited mental retardation. The gene mutated in FXS is named FMR1, and it is well conserved from human to Drosophila. In order to generate a genetic tool to study FMR1 function during vertebrate development, we generated two mutant alleles of the fmr1 gene in zebrafish. Both alleles produce no detectable Fmr protein, and produce viable and fertile progeny with no obvious phenotypic features. This is in sharp contrast to published results based on morpholino mediated knock-down of fmr1, reporting defects in craniofacial development and neuronal branching in embryos. These phenotypes were specifically addressed in our knock-out animals, revealing no significant deviations from wild-type animals, suggesting that the published morpholino based fmr1 phenotypes are potential experimental artifacts. Therefore, their relation to fmr1 biology is questionable and morpholino induced fmr1 phenotypes should be avoided in screens for potential drugs suitable for the treatment of FXS. Importantly, a true genetic zebrafish model is now available which can be used to study FXS and to derive potential drugs for FXS treatment.

Introduction

Fragile X syndrome (FXS) is one of the most common known causes of inherited mental retardation with a frequency of 1:4000 males and 1:6000 females [1]. In almost all cases, FXS is due to the expansion of the unstable CGG trinucleotide repeat sequence in the 5’ untranslated region of the FMR1 gene [2,3]. Once the repeats exceed 200 units (full mutation), the gene is silenced due to the consequent hypermethylation of the CpG island and CGG repeat. Thus, no mRNA is produced, and the lack of the gene product, FMRP, is responsible for the mental retardation in fragile X patients [4]. Other clinical features include macroorchidism, autistic behaviour, epileptic seizures, hyperactivity, attention deficits, and mild craniofacial abnormalities [1].

FMRP is a ubiquitously expressed RNA-binding protein, including two KH domains and an RGG box, with high expression levels in brain and testis [5,6]. The protein can bind to RNAs containing a G-quartet structure and forms together with many other mRNAs and proteins a messenger ribonucleoprotein (mRNP) particle [7,8]. The dynamics and transport of mRNP particles over long distances within the dendrites of neurons is established by movement along microtubules [9].

The development of mouse models of FXS has facilitated cellular studies on the underlying molecular basis of this loss-of-function disorder [10,11]. Fmr1 knock-out mice recapitulate the typical characteristics of FXS, including behavioural abnormalities, learning deficits and audiogenic seizures. Microscopic analysis of brain material from both FXS patients and Fmr1 knockout mice has shown dendritic spine abnormalities [12–17]. The discovery of a spine morphological phenotype indicates a possible defect in synaptic plasticity in FXS. The precise physiological function of FMRP is still not defined; therefore, the role of FMRP at the synapse has become a central research interest. Compelling evidence predicts a model in which FMRP is involved in the regulation (repression) of local protein synthesis at the synapse, which is triggered group 1 mGluR (mGluR1 and mGluR5) activation. Thus, a lack of FMRP may lead to uncontrolled (exaggerated) protein synthesis at the synapse upon group 1 mGluR stimulation and may underlie the enhanced hippocampal and cerebellar LTD found in Fmr1 knock-out mice [16,18,19].

Interestingly, some behavioural abnormalities could be rescued in Fmr1 knock-out mice using mGluR5 antagonists [20,21]. Recently, a rescue of the spine morphological phenotype could be established in cultured Fmr1 knock-out hippocampal neurons using two different mGluR5 antagonists [21].

In 2006, Tucker et al. reported the use of zebrafish embryos to model FXS [22]. Instead of a knock-out approach, a knock-down strategy was applied using microinjection of morpholinos (MOs) into 1–2 cell stage embryos. MOs are antisense oligonucleotides, in which the deoxyribose is substituted with an N-morpholino ring. They can bind to a target mRNA and prevent either translation or normal splicing for up to 4 days. Hence, inhibition of translation is
transient and may not result in a complete loss-of-function. Injection of fmr1 specific MOs resulted in abnormal axonal branching, changes in trigeminal ganglion number and craniofacial abnormalities. Most of these abnormalities in zebrafish embryos could be rescued using MPEP, an mGluR5 antagonist, or by fmr1 overexpression [22].

In the present study, we generated two independent fmr1 knock-out alleles using TILLING (targeted induced local lesions in genomes). TILLING combines random induced mutations by ENU treatment and subsequent screening for null mutations [23]. We provide a characterization of both homozygous and transheterozygous mutants with special emphasis on the phenotypic features reported earlier in the fmr1 knock-down study [22].

Results
Isolation of Two Fmr1 Mutant Alleles

In order to develop a genetic model in which the effects of FMRP on brain development can be easily studied during development we screened for knock-out alleles in the zebrafish system. From a randomly mutagenized library we isolated two independent mutant alleles: hu2787 defines a C to T change in the coding region of fmr1 (ENSDARG00000037433), leading to the introduction of a premature stop at codon position 113 (Figure 1A). The mRNA derived from this allele is less stable than that derived from the wild-type fmr1 locus. This is illustrated in Figure 1B using whole mount in situ hybridisation with an fmr1 specific probe, on a batch of embryos obtained from a cross between heterozygous parents. Presumably this is the result from a well-known phenomenon named nonsense-mediated-decay (NMD). Furthermore, using a C-terminal antibody we are unable to detect expression of Fmr in neurons using immunocytochemistry on paraffin sections, whereas a high expression could be detected in neurons from wild type zebrafish. Figure 1C illustrates high Fmr expression in Purkinje cells in the cerebellum and neurons in the telencephalon. In addition, we determined Fmr expression in total brain homogenates by Western blot analysis. Consistent with the immuno-stainings, no Fmr was detectable (Figure 1D). The second allele we isolated, hu2898, has a mutated splice acceptor site at the end of the 7th intron. Sequencing of splicing products from this allele shows that hu2898 leads to the use of an alternative splice acceptor site 2 bases downstream of the original site. This induces a frameshift with regard to the original reading frame and an opal stop codon 27 nucleotides downstream (Figure 1A). Animals carrying any combination of the two mutant alleles show loss of Fmr in both immunocytochemistry (Figure 1C) and Western blot analysis (Figure 1D).

Fmr1 Mutant Zebrafish Are Viable

Animals lacking zygotic Fmr are found at Mendelian frequencies in crosses between heterozygous parents. They display wild-type development, and develop into fertile adults with no gross abnormalities. Progeny from homozygous mutant parents were also analyzed to check the potential effect of maternally provided protein and/or mRNA on development. Also these maternal-zygotic (MZ) mutant animals develop normally, and display no obvious defects in behaviour or fertility. Importantly, we did not observe selective pressure against homozygous mutant combinations in any of the crosses we performed (not shown), strongly suggesting that potentially lethal phenotypes are not repressed by the presence of genetic modifiers in our genetic backgrounds.

Lack of Fmr1 Does Not Induce Craniofacial Defects

The results described above contrasts with morpholino induced fmr1 knock-down studies that have been published before [22]. More specifically, it was demonstrated that these morphants display aberrant expression of three markers: axial, dlx-2a and islet-1. We therefore analyzed the expression of these genes by in situ hybridisation in fmr1 MZ null embryos. The results of these
experiments are depicted in Figure 2A. We observed no significant differences between wild-type and \textit{fmr1} null embryos in any of the analyses.

In addition, we measured the width of Meckel's cartilage and the angle it makes to the anterior-posterior axis, to address whether these mutant animals develop abnormalities that may be related to the craniofacial defects seen in fragile X patients, as described in the morpholino knockdown morphants [22]. In both the MZ homozygous stop mutants and embryos derived from homozygous stop mutant mothers and homozygous splice mutant fathers (not shown) the width as well as the angle of this structure is indistinguishable from that in wild-type animals (Figure 2B).

**Figure 2. Phenotypic assays on wild-type and \textit{fmr1} mutant embryos.** A) Wild type and mutant embryos were analyzed using whole mount \textit{in situ} hybridisation using probes against \textit{dlx-2a}, \textit{axial} and \textit{islet-1}. B) The width of Meckel's cartilage was measured in wild type (\(n = 9\)) and MZ \textit{fmr1} mutant (\(n = 11\)) embryos. The angle of this structure with regard to the anterior-posterior axis was also measured in wild-type (\(n = 6\)) and \textit{fmr1} mutant (\(n = 9\)) embryos. Indicated errors represent SD. C) Neurite branching was measured on Rohon-Beard neurites using the monoclonal antibody zn-12. Plotted is the branching frequency per 1000 \(\mu\)m in both wild-type and MZ stop mutant embryos. In total \(n = 25\) neurites (wild-type) and \(n = 28\) neurites (MZ\textit{fmr1}) were traced in a total of 8 embryos of each genotype. Error bars represent SD.

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Fmr1 Mutants Do Not Show Rohon-Beard Neurite Branching Defects

Finally, Tucker et al. [22] described a defect in neurite branching in Rohon-Beard neurons in fmr1 morphant embryos. We therefore analyzed the branching frequency of the Rohon-Beard neurons, similar to what was reported by Tucker et al. [22]. In Figure 2C we show that also in this analysis we find no significant difference between wild-type and MZ fmr1 stop mutant animals.

Discussion

We describe the generation of two fmr1 knockout alleles in zebrafish, and as such provide a new genetic model system to study FXS, a highly prevalent form of inherited mental retardation. FXS is caused by the loss of the gene product of fmr1, Fmr. FXS models have been described in multiple systems and from these models it has become clear that FMRP is acting at the synapse to regulate the translation of target mRNAs upon group 1 mGluR stimulation and whose protein products mediate synaptic strength [16,18]. The translation of target mRNAs at the synapse. Potentially, such a process can be well affected by mGluR antagonists that would ameliorate the phenotypes no numbers are given related to the penetrance of the defect; injection of antibodies against alpha-acetylated tubulin resulted in a dramatic axon defect only in 3/30 axon defasciculation in 13/30 fmr1 morphants. Finally, the craniofacial dysmorphology could only be observed in 9/15 fmr1 morphants.

In summary, we find the loss of fmr1 in zebrafish at most induces very subtle phenotypes that are not readily detectable using light-microscopy and techniques like immunocytochemistry and in situ hybridisation, at least in the strains used in our laboratory. It remains well possible that subtle defects are induced by lesions in fmr1, and that these may be used to develop sensitive and robust essays to probe fmr1 function, which may in turn be used for screening of small molecules libraries in order to find drugs suitable for treatment of FXS. At present, however, we have to conclude that the phenotypes as described by Tucker et al. [22] may be based on morpholino induced artefacts, and as such not useful to study fmr1 function in the zebrafish.

Materials and Methods

Zebrafish Strains and Screening F1 ENU- Mutation Library

Adult zebrafish were bred and maintained under standard conditions. Staging of embryos was according to Kimmel et al. [26]. Embryos at different developmental stages were fixed with 4% PFA/PBS overnight.

ENU induced mutation library was screened for a mutation in the fmr1 gene. Amplicons were designed for exon 5–6 and exon 7–9 and screened for mutation as described [23]. Fish with mutant alleles (fmr1fmr1hu2898 (splice); fmr1fmr1hu2770 (stop)); fmr1hu2770 (splice)) were outcrossed against TL and crossed to obtain homozygous or transheterozygous embryos.

Immunocytochemistry Adult Brain

Adult zebrafish were sacrificed by euthanasia using high dose of MS222, brains were dissected immediately and fixed overnight in 3% paraformaldehyde. The brains were embedded in paraffin according to standard protocols. Sections (7 µm) were deparaffined, followed by antigen retrieval using microwave treatment in 0.01 M sodium citrate solution. Endogenous peroxidase activity blocking and immunoincubation was performed as described before using polyclonal rabbit 730 antibodies against zebrafish Fmrp [27].

Western Blotting

Half brains (saggital) from adult zebrafish were homogenised in 500 µl HEPES-buffer (10 mM HEPES, 300 mM KCl, 3 mM

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MgCl₂, 100 µM CaCl₂, 0.45% Triton X-100 and 0.05% Tween-20, pH 7.6, with Complete protease inhibitor cocktail (Roche Diagnostics), while kept on ice. After incubating the homogenates on ice for 30 minutes, they were sonicated twice for 20 seconds. Cell debris was spun down and the supernatant was collected. Loaded albumin to 100 µg protein, heated at 95°C for 5 minutes and loaded onto a 10% SDS-PAGE gel. After electroblotting the gel onto a nitrocellulose membrane, the membrane was incubated overnight at 4°C with the rabbit polyclonal 758 antibody specific for zebrafish FMRP [27], in PBS-T with 5% milk powder. The next day the membrane was incubated with a hors eradish peroxidase conjugated secondary antibody rabbit-α-mouse (DAKO), allowing chemiluminescence detection with an ECL KIT (Amersham).

In Situ Hybridisation
ISH experiment were performed as described in Thess et al. [28]. The RNA-probes were made according standard protocols. Probes against fmr1 (EST clone f56d6b.1, IRBOp99110101D from RZPD, Berlin, Germany) from which the cDNA fragment was cloned into pCS2plusclx2a-2a [29]; axial [30] and idet-1 [31] were used in the described experiments. ISH to show NMD on the hu2787 allele was done in one batch, so that wild-type, heterozygous and homozygous mutant embryos received identical treatments. Embryos were genotyped afterwards, revealing a consistent loss of fmr1 mRNA in homozygous hu2787 mutants.

Cartilage Staining
fmr1 hu2787 in cross embryos (5 dpf) were Alcian blue stained according to Neuhaus et al. [32]. The width and the angle of Meckel’s cartilage were measured and embryos were genotyped.

References
1. Hagerman RJ (2002) The physical and behavioural phenotype. In: Hagerman RJ, Hagerman P, eds. Fragile-X syndrome: diagnosis, treatment and research. Baltimore: The Johns Hopkins University Press. pp 3–109.
2. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, et al. (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65: 905–914.
3. Oberli I, Rousseau F, Heitz D, Kretz C, Devy D, et al. (1991) Instability of a 500-base pair DNA segment and abnormal methylation in fragile X syndrome. Science 252: 1097–1102.
4. Verheij C, Bakker CE, de Graaff E, Keulemann J, Willemsen R, et al. (1993) Characterization and localization of the FMR-1 gene product associated with fragile X syndrome. Nature 363: 722–724.
5. Devy D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL. (1993) The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. Nat Genet 4: 335–340.
6. Tamanini F, Willemsen R, van Uilen L, Bontekoe C, Galjaard H, et al. (1997) Differential expression of FMR-1, FXR1 and FXR2 proteins in human brain and testis. Hum Mol Genet 6: 1315–1322.
7. Schaeffer C, Bardoni B, Mandel JL, Ehresmann B, Ehresmann C, et al. (2001) The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. Embo J 20: 4803–4813.
8. Darnell J, Jensen KB, Jin P, Brown Y, Warren ST, et al. (2001) Fragile X Mental Retardation Protein Targets G Quartet mRNAs Important for Neuronal Function. Cell 107: 489–99.
9. De Diego Otero Y, Severijnen LA, Van Cappellen G, Schrier M, Oostra B, et al. (2006) The generation of a conditional Fmr1 knock out mouse model to study Fmr1 knockout mice: A model to study fragile X mental retardation. Cell 78: 71–33.
10. Verheij C, Bakker CE, Verheij C, Willemsen R, Vanderheiden R, Oerlemans F, et al. (1994) Fmr1 knockout mice: A model to study fragile X mental retardation. Cell 76: 39–53.
11. Menterie J, Nieuwenhuizen I, Kirkpatrick I, Zu T, Hoogenveen-Westerveld M, et al. (2006) The generation of a conditional Fmr1 knockout mouse model to study Fmrp function in vivo. Neurobiol Dis 21: 549–555.
12. Hinton VJ, Brown WT, Wismierski K, Rudelli RD (1991) Analysis of neocortex in three males with the fragile-X syndrome. Am J Med Genet 41: 289–294.
13. Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, et al. (1997) Abnormal dendritic spines in fragile X knockout mice: Maturation and pruning defects. Proc Natl Acad Sci U S A 94: 5401–5408.
14. Nimmicki NS, Oberlander AM, Svbodha K (2001) Abnormal development of dendritic spines in fmr1 knock out mice. J Neurosci 21: 5339–46.
15. Galvez R, Gopal AR, Greenough WT (2003) Somatosensory cortical barrel dendritic abnormalities in a mouse model of the fragile X mental retardation syndrome. Brain Res 971: 85–98.
16. Kooiwek SK, Yamasuchi K, Mišojković BA, Dorland BR, Ruijgrok T, et al. (2005) Deletion of Fmr1 in Purkinje Cells Enhances Parallel Fiber LTD, Enlarges Spines, and Attenuates Cerebellar Eyelid Conditioning in Fragile X Syndrome. Neuron 47: 339–351.
17. Grouman AW, Allridge GM, Weller JL, Greenough WT (2006) Local protein synthesis and spine morphogenesis: Fragile X syndrome and beyond. J Neurosci 26: 7151–5.
18. Huber KM, Gallagher SM, Warren ST, Bear MF (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proc Natl Acad Sci U S A 99: 7746–50.
19. Bear MF, Dolan G, Osterweil E, Nagarajan N (2007) Fragile X: Translation in Action. Neuropsychopharmacology 33: 84–87.
20. Van QJ, Rammal M, Tranfaglia M, Bauchwitz RP (2005) Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. Neuropharmacology 49: 1053–1066.
21. de Vrij FMS, Levenge J, Van der Linde HC, Koeckkoek K, De Zeewe CI, et al. (2008) Rescue of behavioral phenotype and neuronal protrusion morphology in Fmr1 KO mice. Neurobiol Dis 31: 127–132.
22. Tucker B, Richards RI, Lardelli M (2006) Contribution of mGluR and Fmr1 Functional Pathways to Neurite Morphogenesis, Craniofacial Development and Fragile X Syndrome. Hum Mol Genet 15: 3446–3450.
23. Winneholt E, Plasterk RH (2004) Target-selected gene inactivation in zebrafish. Methods Cell Biol 77: 69–90.
24. Bear MF (2005) Therapeutic implications of the mGluR theory of fragile X mental retardation. Genes Brain Behav 4: 393–5.
25. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, et al. (2007) p53 Activation by Knockdown Technologies. PLoS Genet 3: e76.
26. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF (1995) Stages of embryonic development of the zebrafish. Dev Dyn 203: 253–310.
27. van ‘t Padje S, Engels B, Blonden L, Severijnen LA, Verheijen F, et al. (2005) Characterisation of Fmrp in zebrafish: evolutionary dynamics of the fmr1 gene. Dev Genes Evol 215: 189–206.
28. Thiése C, Thiése B, Schilling TF, Posledovat JH (1993) Structure of the zebrafish snail1 gene and its expression in wildtype, spadetail and no tail mutant embryos. Development 119: 1203–15.

Antibody Staining Embryos
For whole-mount immunohistochemistry, embryos were fixed in 4% paraformaldehyde for four hours at RT, washed with PBT and incubated overnight at 65°C in FST solution (50% formamide, 2x SSC, 0.1% Tween-20). Next day, washed in PBT and blocked in ABS (PBT, 2% DMSO, 0.1% IGEPAL, 2% lamb serum, 2% BSA) and incubated overnight with the monoclonal antibody zn-12 (1:200, Developmental Studies Hybridoma Bank). Primary antibody was washed off by ABS buffer, and embryos were incubated overnight with secondary antibody goat anti-mouse conjugated with Alexa-488 (1:250, Molecular Probe) [Tucker et al, 2006]. Embryos were imaged with a Leica DM6000 microscope, Leica camera DFC 360 FX and Leica LAS AF Software. Images were analysed using the NeuronJ plugin [33].

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Author Contributions
Conceived and designed the experiments: BAO RW RK. Performed the experiments: MJdB HvdL JRB. Analyzed the data: MJdB BAO RW RK. Wrote the paper: RW RK.
29. Akimenko MA, Ecker M, Wegner J, Lin W, Westerfield M (1994) Combinatorial expression of three zebrafish genes related to distal-less: part of a homeobox gene code for the head. J Neurosci 14: 3475–86.

30. Strahle U, Blader P, Henrique D, Ingham PW (1993) Axial, a zebrafish gene expressed along the developing body axis, shows altered expression in cyclops mutant embryos. Genes Dev 7: 1436–46.

31. Appel B, Korzh V, Glasgow E, Thor S, Edlund T, et al. (1995) Motoneuron fate specification revealed by patterned LIM homeobox gene expression in embryonic zebrafish. Development 121: 4117–25.

32. Neuhauss SC, Solnica-Krezel L, Schier AF, Zwartkruis F, Stemple DL, et al. (1996) Mutations affecting craniodfacial development in zebrafish. Development 123: 357–67.

33. Meijering E, Jacob M, Sarria JCF, Steiner P, Hirling H, Unser M (2004) Design and Validation of a Tool for Neurite Tracing and Analysis in Fluorescence Microscopy Images. Cytometry 58A: 167–176.