Zebrafish as a Model of Genetic Disease

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Submitted for partial fulfilment of the requirements for the degree of
Doctor of Philosopy (Ph.D)

Australian Research Council Special Research Center for the Molecular Genetics of Development

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Spring, 2007

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Contents

List of Publications and Contribution

The thesis is based on the following papers

I: Tucker, B., Richards, R. I., and Lardelli, M. (2004). Expression of three zebrafish orthologs of human FMR1-related genes and their phylogenetic relationships. *Dev Genes Evol*. 214(11): 567-74.

Ben Tucker was responsible for performing all work and interpreting all results under the guidance of Michael Lardelli and Robert Richards. Paper written by **Ben Tucker**.

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II: Tucker, B., Richards, R. I., and Lardelli, M. (2006). Contribution of mGluR and Fmr1 Functional Pathways to Neurite Morphogenesis, Craniofacial Development and Fragile X Syndrome. *Hum Mol Genet.* Dec 1; 15(23): 3446-58.

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Ben Tucker

III: Tucker, B., and Lardelli, M. (2007). A Rapid Apoptosis Assay Measuring Relative Acridine Orange Fluorescence in Zebrafish Embryos. *Zebrafish*. 2007, 4(2): 113-116.

Ben Tucker was responsible for performing all work and interpreting all results under the guidance of Michael Lardelli. Paper written by **Ben Tucker**.

Ben Tucker

Acknowledgements

Thanks to Michael Lardelli and Robert Richards
Supervision

Special thanks to the ARC/CMGD and The University of Adelaide
Facilities and funding

Very special thanks to the FRAXA Research Foundation
Mike Tranfaglia and Katie Clapp
For the kind gift of MPEP

And to Jack Da Silva
Statistical advice

Other helpful financial assistance:
-The Network for Genes and Environment in Development (NGED)
-Faculty of Health Sciences

Scientific advice and guidance:
-Past and present members of the Lardelli, Richards, Koblar, Timmis and Kelly Labs.
-Particularly: Clare Van Eyk, John Humphries, Dan Kortschak, Damien Lightfoot, Amanda Lumsden, Rosa McCarty, Catherine McLeod, Simon Wells.

-Mega-thanks to Svanhild Nornes and Helli Meinecke

Thanks also to Family and Friends
George Isadore Ford Hogg (1911-2007)

Introduction

Fragile X is the most common inherited form of human mental retardation. It is is an X-linked disorder most often resulting from expansion of a CGG trinucleotide repeat, leading to a mosaic loss of the Fragile X Mental Retardation Protein (FMRP) (de Graaff et al., 1995; Nolin et al., 1994; Rousseau et al., 1991). Behavioural abnormalities associated with Fragile X Syndrome include developmental delays, hyperactivity, anxiety, autistic behaviours and hypersensitivity to sensory stimuli (Bakker and Oostra, 2003; O'Donnell and Warren, 2002). Gross physical defects observed in patients are craniofacial abnormalities, which include a long thin face with prominent ears, facial asymmetry, a large head circumference and a prominent forehead and jaw, and enlarged testicles (macroorchidisim) (Butler et al., 1993; Lachiewicz and Dawson, 1994; Slegtenhorst-Eegdeman et al., 1998). The primary phenotype at the cellular level is immature/elongated dendritic spines on the dendrites of neurons (Hinton et al., 1991; Irwin et al., 2001; Rudelli et al., 1985). Such morphology has been associated with Down's and Rett syndromes (Kaufmann and Moser, 2000), suggesting underlying similarities in the biological causes of mental retardation in the syndromes.

FMRP is an RNA binding protein that appears to be involved in post translational control. There is extensive evidence to suggest that FMRP protein represses the translation of target mRNAs, and that this function may occur in a localised fashion (Adinolfi et al., 2003; Feng et al., 1997a; Laggerbauer et al., 2001; Li et al., 2001; Mazroui et al., 2003; Mazroui et al., 2002; Zalfa et al., 2003). FMRP controls translation as a messenger ribonucleoprotein particle (mRNP) particle that is shuttled from the nucleus to the cytoplasm. It is hypothesised that the mRNP is the functional unit of FMRP, and that it is transported into growth cones and synapses of neurites via microtubules (Antar et al., 2005; De Diego Otero et al., 2002; Knowles et al., 1996; Kohrmann et al., 1999). The role of FMRP may be spatially dictated, occasionally acting as an enhancer of protein translation (Khandjian et al., 2004; Miyashiro and Eberwine, 2004; Stefani et al., 2004).

FMRP contains three RNA-binding motifs that provide a level of specificity. The domain structure of FMRP includes two ribonucleoprotein K homology domains (KH domains), and a cluster of arginine and glycine residues (RGG box). The RGG box recognises a three-

dimensional RNA structure called a G-quartet. A large number of putative mRNAs have been isolated, and despite the specificity imbued by the binding domains, different interactors are identified by different studies. A number of genes important for microtubule and spine assembly or maintenance have been identified as part of the mRNP. These include microtubule-associated protein 1B (MAP1B), (Brown et al., 2001; Zalfa et al., 2003; Zhang et al., 2001) Rac1 (rho family, small GTP binding protein (Lee et al., 2003)), and calcium/calmodulin-dependent protein kinase IIα (Zalfa et al., 2003). Synaptic plasticity, long-term potentiation (LTP) and long-term depression (LTD), require local protein synthesis (Klann et al., 2004). Synaptic stimulation induces local translation (Steward and Schuman, 2003) and induces relocation of FMRP into the spines (Ostroff et al., 2002). In this way there is a clear link between FMRP repression of mRNAs and synaptic maturation / spine-pruning.

Synaptic stimulation induces local translation via the metabotropic glutamate receptors (mGluRs) which include *FMR1* (Todd et al., 2003; Weiler et al., 1997). In mice lacking functional FMRP mGluR induced LTD is increased (Bear et al., 2004; Huber et al., 2002; Koekkoek et al., 2005). The connection is supported as mGluR antagonists rescue defects in mice and *Drosophila* strains that lack the *FMR1* (McBride et al., 2005; Yan et al., 2005).

The mechanism of FMRP regulation is unknown. Phosphorylation of FMRP suppresses translation in polyribosomes complexed with FMRP (Ceman et al., 2003) and stimulation of mGluR5 is known to decrease the activity of the phosphatase (Pp2A; Protein Phosphatase 2A (Mao et al., 2005)). Pp2Ac has also been shown to be linked to microtubule dynamics (Gong et al., 2000). It may be speculated that FMRP is part of a regulatory loop where FMRP controls its own activity by controlling the level of pp2a, which activates FMRP function.

The primary cellular phenotype of defects in spine morphology is entirely consistent with the expression of FMRP. FMRP has been detected along dendrites and at synapses where it is thought to regulate synaptic protein synthesis locally (Antar et al., 2004; Feng et al., 1997b; Zalfa et al., 2003). This suggests that alleviation of such repression leads to Fragile X syndrome. A number of target mRNAs of FMRP are involved in neuronal development and plasticity/maturation of synapses (Aschrafi et al., 2005; Brown et al., 2001; Chen et al., 2003; Darnell et al., 2001), including MAP1B (microtubule-associated protein 1B). Translation of

mRNAs at neurites provides growth cones and synapses the capacity to regulate their structure and function (Churchill et al., 2002). Given that spine elongation is a translation-dependent mechanism (Vanderklish and Edelman, 2002; Vanderklish and Edelman, 2005), this may represent cohesive model of the connection between the biological pathway and the cognitive outcomes.

Neurite Morphology

The abnormal shape of Fragile X Syndrome dendritic spines, as well as the behavioural abnormalities, has lead to the theory that FMRP may act as part of LTD machinery in spine morphology. Given that the machinery for protein synthesis is found in the dendrites near synapses (Steward and Schuman, 2001; Steward and Schuman, 2003), and that FMRP is a translational repressor it appears that the FMRP may inhibit translation of specific mRNAs that are required for LTD. It is known that group 1 mGluRs are a stimulus for local protein synthesis, and there is strong evidence for a connection between mGluRs and FMRP. This is known as the 'mGluR theory of fragile X Syndrome' (Bear, 2005; Weiler et al., 1997).

An anticonvulsant drug, mGluR5-specific antagonist 2-methyl-6-phenylethynyl-pyridine (MPEP), has been used to demonstrate this biochemical connection (Anwyl, 1999; Gasparini et al., 1999; Renner et al., 2005; Schoepp et al., 1999). In particular, *Drosophila* and mouse fmr1 knockout models have been used to variously demonstrate rescue of behaviour, courtship defects and mushroom body defects using MPEP (McBride et al., 2005; Yan et al., 2005).

We have described the usefulness of the embryos of the zebrafish, *Danio rerio*, as a model of Fragile X mental syndrome (Tucker et al., 2004; Tucker et al., 2006). Using morpholino knock-down of FMRP we can recapitulate the symptoms of Fragile X Syndrome seen in other animal models of the disease, and of the human condition itself. In brief, advantages of using zebrafish embryos as a model organism include optical transparency, availability of large numbers of embryos for statistical analysis, external development and simple drug delivery.

Using this model, we have demonstrated a rescue of neurite morphology using MPEP, suggesting that the interaction between the mGluR and FMRP has broader morphological implications than regulation of translation at the synapse. We suggest a model connecting neural morphology, mGluR signalling and FMRP via calcium dynamics (Tucker et al., 2006).

A number of studies have been published subsequent to (Tucker et al., 2006). These are generally consistent with our model, but hint further at the relationship between FMRP, spine dysmorphogenesis (LTP/LTD), neurite dysmorphogenesis, and calcium signalling. Our findings may be circumstantially related to those of Castren et al. (2005), who have described an increase in intense oscillatory Ca2+ responses to neurotransmitters in differentiated cells lacking FMRP.

Our model of neurite morphogenesis suggests a regulatory relationship between FMRP and CaMKIIα. CaMKIIα is a Ca^{2+}–calmodulin-dependent protein kinase that has been found to localise to growth cones as part of the FMRP granule (Kanai et al., 2004; Zalfa et al., 2003). A recent study has demonstrated further that CaMKIIα is dysregulated in response to mGluR activation in Fmr1 knock-out mice (Muddashetty et al., 2007). This finding is consistent with a model in which glutamate receptor signalling interacts with FMRP repression of mRNA.

Examining dendritic spine dysmorphogenesis, Pfeiffer and Huber (2007) have demonstrated that FMRP rescues the number of dendritic spines, but not the structure or maturity of those spines in an FMR1-KO background. This indicates that FMRP is involved in removing spines. This may be relevant given that the LTP process appears to be associated with increase in dendritic spine number and LTD appears to be associated with synaptic strength (Lynch, 2004). Our model of neurite morphogenesis may fit this data if both LTP and LTD are due to a similar calcium influx, (depending on the timing and frequency of the influx; (Dudek and Bear, 1993)) . It is postulated that low calcium influx leads to LTD, and influx above a threshold leads to LTP (The Bienenstock, Cooper and Munro model; BCM model – 1982 (Bienenstock et al., 1982)). The model we describe might be intrinsically related to these calcium dynamics.

LTP has also been implicated in aspects of Fragile X Syndrome. (Li et al., 2002; Wilson and Cox, 2007; Zhao et al., 2005). Interestingly Meredith et al. (2007) report that FMR1-KO mice

lack L-type calcium channels in spines. This is contradictory to our model of upregulated calcium, but may also be related to a loss of granule transport of the channel subunits by FMRP. However, this study also found that LTP activity can be restored in FMR1-KO mice by improving reliability and amplitude of calcium signalling. They found that raising mice in enriched environments leads to such a stabilising increase in neuronal activity, restoring LTP to WT levels. They concluded that synaptic plasticity is functional in FMR1-KO mice, and can be stimulated by strong neuronal activity. This may also be reflected in the highly transient, but activity dependent/ inducible nature of FMRP expression (Gabel et al., 2004).

Generally, LTP has been overlooked in favour of LTD as a mechanism of FMRP function. Fascinatingly, an important study by (Desai et al., 2006) suggests that LTP is affected in Fragile X Syndrome, whilst LTD remains normal. These researchers indicate that *Fmr1* is highly selective in its effects on plasticity, but suggest that this finding is not completely contradictory to the mGluR theory of Fragile X Syndrome.

Given that increased stimulation of neurons enhances calcium signaling, these observations would appear to be consistent with our model.

Conclusion

MPEP treatment restored neurite morphology in which morphants to normal. Given the success of the analysis, our zebrafish Fragile X Syndrome model enables an assessment of the ability of small molecules, proteins and RNAs to modify these symptoms and provide leads as therapeutic agents with which to prevent / treat these symptoms in humans.

A weakness in the zebrafish model of the Fragile X Syndrome is a difficulty in studying elements of the mutation mechanism. Furthermore, examination of testicular-related abnormalities is difficult in a morpholino knockdown model of Fragile X Syndrome as the active period of the morpholino does not overlap with differentiation of testicular cells in zebrafish development. To study these aspects adequately, a mutant model will be required.

TILLING projects or improvements in zebrafish homologous recombination technology may provide resources that increase the validity of the zebrafish model in examination of these aspects of the syndrome, and the range of questions the model can address.

Despite limitations, morpholinos are an extremely useful as a tool for disease modelling in examining genetic interactions that affect penetrance or severity of the disease, and further elucidating the genetic pathways through which the genes involved operate. Morpholinos (and other microinjected substances used for overexpression or for knockdown) will certainly remain a useful tool in future research.

Summary of Papers I-III and Continuity

The zebrafish is rapidly becoming a vital tool in studies of genetic disease. Use of the zebrafish embryo as an experimental model combines the efficiency of techniques specific to invertebrates with the human applicability of vertebrate studies, along with a number of other advantages such as optical transparency and high spawn number. Sequencing maps and mutant screen data are available, and gene ontology annotation is progressing. Furthermore, a number of highly important projects are underway to expand the utility of the zebrafish still further (eg. Mutant screens and TILLING projects; see (Lieschke and Currie, 2007) for review). As such the zebrafish has become a vital model organism for study of a variety of genetic defects, toxicology and pharmacological screens etc.

These papers trace the development of zebrafish embryos as a model organism for both genetic disease and, as part of this, the development of a relatively high throughput approach to analysing relative levels of apoptosis.

The first paper describes the *fmr1* gene family in zebrafish (*fmr1*, and its orthologs *fxr1* and *fxr2*). This paper includes a phylogenetic analysis of the gene family that demonstrates the high conservation between human and zebrafish, in the context of *Drosophila*. We then describe expression of the genes in the embryo (using *in situ* hybridisation) and adult (using real time pcr). The conclusions are that the zebrafish is an appropriate model in which to study Fragile X Mental Retardation genetic disease.

The second paper builds upon this conclusion and further establishes the appropriateness of the model by recapitulating elements of the disease that had already been modelled in other model organisms. The research is validated using a number of controls. We describe a number of original findings that extended the body of knowledge regarding pharmacological rescue of the FMRP loss phenotypes. A craniofacial phenotype is identified, the first such discovery in a model of Fragile X syndrome. These findings are a vital step toward understanding the pathway from gene, to molecular phenotype, to cellular morphology, to gross morphology. As part of these studies, we found it necessary to analyse apoptosis. The technique developed to facilitate this analysis is described in our third paper.

Given the highly stochastic nature of the apoptotic patterns we developed a method to take full advantage of the characteristics of zebrafish embryos, primarily their transparency and availability in large numbers. As the zebrafish becomes more widely accepted as a model for a diverse range of scientific questions, the development of such a technique is doubly important given the necessity of a cheap, reliable and simple generalizable method of analysing processes affecting cell viability in fish. This has clear importance for pharmacological studies, but is also a long overdue addition to the battery of controls available for highly invasive techniques such as microinjection, in which apoptosis is regularly found among its non specific effects.

Paper I:

Tucker, B., Richards, R. I., and Lardelli, M. (2004). Expression of three zebrafish orthologs of human FMR1-related genes and their phylogenetic relationships. *Dev Genes Evol* 214(11): 567-74.

-PMID: 15378363
-Impact factor 2005: 2.549
-http://www.springerlink.com.proxy.library.adelaide.edu.au/content/4h521bbnf4lnmvgk/?p=159598c92a424ee99bd9f94771d27702&pi=1
-doi: 10.1007/s00427-004-0438-9

Tucker, B., Richards, R. and Lardelli, M. (2004) Expression of three zebrafish orthologs of human FMR1-related genes and their phylogenetic relationships. *Development Genes and Evolution, v.214 (11) pp. 567-574, November 2004*

Paper II:

Tucker, B., Richards, R. I., and Lardelli, M. Contribution of mGluR and Fmr1 Functional Pathways to Neurite Morphogenesis, Craniofacial Development and Fragile X Syndrome. *Hum Mol Genet.* 2006 Dec 1;15(23):3446-58.

-PMID: 17065172
-Impact factor 2005: 7.764
-http://hmg.oxfordjournals.org/cgi/reprint/ddl422v1
-doi: 10.1093/hmg/ddl422

Tucker, B., Richards, R. and Lardelli, M. (2006) Contribution of mGluR and Fmr1 functional pathways to neurite morphogenesis, craniofacial development and fragile X syndrome.
Human Molecular Genetics, v. 15 (23) pp. 3446-3458, December 2006

Paper III:

Tucker, B., and Lardelli, M. (2007). A Rapid Apoptosis Assay Measuring Relative Acridine Orange Fluorescence in Zebrafish Embryos. *Zebrafish*. 2007, 4(2): 113-116.

-ZFIN ID: ZDB-PUB-070907-2
-http://www.liebertonline.com/doi/abs/10.1089/zeb.2007.0508
-doi: 10.1089/zeb.2007.0508.

Tucker, B. and Lardelli, M. (2007) A Rapid apoptosis assay measuring relative acridine orange fluorescence in zebrafish embryos.
Zebrafish, v. 4 (2) pp. 113-1176, August 2007

Future Work

Our model of Fragile X Syndrome has led to development of a hypothesis of FMRP functional pathways. We have hypothesised that intracellular Ca++ mediates FMRP function in concert with mGluR signalling. Future work includes exploration of the use of various molecules to test this hypothesis further. These will be directed toward altering intracellular Ca++ levels or modulation of mGluR signalling. The primary results are to be assessed on the basis of recapitulation or modulation of fragile-X syndrome like symptoms in zebrafish.

Obtaining and using 'next generation' mGluR5 antagonists (Renner et al., 2005) to rescue FMRP morphant neural phenotypes will define with greater precision the interaction of the mGluR signalling with FMRP machinery. The eventual aim of this research will be to increase the efficacy of mGluR antagonism. Other, previously characterised mGluR antagonists such as (E)-2-methyl-6-(2-phenylethenyl)-pyridine (SIB-1893; (Varney et al., 1999)) and 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP; (Cosford et al., 2003)), which has greater mGluR5 selectivity than MPEP (Lea et al., 2005), and agonists such as (RS)-2-chloro-5-hydroxyphenylglycine (CHPG; (Doherty et al., 1997)), will be assessed in terms of FMRP morphant neural phenotypes.

A second future direction to pursue is to identify wether subcellular location of the FMRP protein contributes to its role in interacting with the mGluR pathway. mGluR signalling requires FMRP to regulate appropriate localisation of postsynaptic mRNA. This is possible using fish transgenic for GFP-tagged FMRP. FMRP is thought to be localised in response to mGluR signalling (De Diego Otero et al., 2002; Li et al., 2003). Use of transgenic fish in this manner will allow live imaging of localisation. The Tg(sensory:GFP) transgenic line will be used to image live developing trigeminal ganglion neurons (Sagasti et al., 2005). Live imaging of the trigeminal neurites will allow real time analysis of neurite development, which in turn allows a better understanding of the activity of the underlying processes giving rise to neurite morphology.

Thirdly, an increasing body of research connects FMRP to activity of the GABA receptor (El Idrissi et al., 2005; Gantois et al., 2006). Therefore it is intended to explore this interaction in

terms of modulation of demonstrated fragile X-like phenotypes (Tucker et al., 2006), and elucidate any role of GABA receptors in the FMRP pathway. The initial approach to this will be using the $GABA_A$ receptor antagonists pentylenetetrazole (PTZ) or picrotoxin or agonists such as muscimol, THIP, or isoguvacine.

A critical line of inquiry will be to explore the connection between craniofacial defects and neural defects (Tucker et al., 2006). It will be important to understand shared pathways of these apparently disparate syndromic aspects if an effective treatment is to alleviate all aspects of the syndrome. Preliminary research (Ben Tucker, *unpublished observations*) suggests that the craniofacial abnormalities are not rescued by MPEP treatment, if not unaffected by such treatment. These defects are expected to be related to migratory or differentiation defects in neural crest. It will be important to explore the connection, if any, between neural crest mechanics and calcium dynamics. Other modifiers of craniofacial defects will be investigated in order to identify the pathogenic pathway(s) responsible for the observed phenotype. It seems likely that a combination of treatments will be necessary for comprehensive treatment of Fragile X Syndrome.

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---------Comments regarding Paper 1 made by the external referee---------

- I feel the author did not fully elaborate on the principal aim underpinning the portfolio of publications, i.e. why should the fmr1 gene of the zebrafish be targeted to achieve a transient knockdown of gene expression. [....] I would have appreciated reading the author's thoughts on why the zebrafish should be used for modelling a human neurological disease.

The zebrafish is a useful model organism for the study of human neurological disease for a number of reasons. For this series of studies the primary advantages of the zebrafish are its transparency (allowing easy fluorescent gene and protein visualisation), its high number of embryos (for statistical analysis) and its status as a vertebrate (increasing its relevance to human disease). Furthermore, zebrafish have a neural system very similar to that of humans, whilst simultaneously being much easier to access or visualise. The zebrafish has fewer, larger neurons in the trigeminal ganglion than found in humans, but its function is largely reflective of human function and vertebrates in general. While the experiments described in this thesis were initially based on findings from Drosophila (in terms of branching of dendritic arborising neurons; Lee et al., 2003), the important results found by Lee et al. are limited in scope by virtue of the simplicity of the organism, and its genetic distance from humans (the Drosophila has one fragile x related gene, as opposed to the full family of three genes). It is difficult to make assertions about the human disease based on such findings. Experiments in mouse and human, however, have many problems with access and number of subjects or samples. Again, implications regarding neuron morphology had been made prior to the work described in this thesis (Comery, et al. 1985; Rudelli, et al. 1997), but the system in which these findings were made allowed for little or no manipulation and for poor techniques of visualisation, as well as being restricted to observations of individuals rather than a statistical analysis.

- Zebrafish carries apparent orthologues of the FMR1 gene, together with the autosomal paralogues, *FXR1* and *FXR2*. I thought the author could have undertaken a reciprocal BLAST of predicted zebrafish protein sequences against the human genome to identify appropriate 'hits', and also splice site analysis of the zebrafish sequences against the Zv2 assembly.

We have done a reciprocal blast and a splice site analysis as part of the preliminary work for the paper, however we did not include the analyses for brevity. The human FMR1 family genes were the highest similarity genes in the reciprocal blast, and the splice site analysis is mostly reflective of the organisation in humans.

- I'm surprised that the apparent orthologues of other species were not included as well; the author mentions mouse, chicken, Drosophila and Xenopus (strange that nothing has been done in pufferfish).

Again, we have done these phylogenetic analyses as part of the preliminary work for the paper, however we did not include the analyses for simplicity and brevity. Fugu *was not included as the fmr1 gene family doesn't appear to have been verified in this species.*

- What evidence is there that the zebrafish elongation factor 1-alpha gene expresses a transcript that could be used as a normaliser for gene expression studies?

ef-1 alpha is routinely used as a normaliser in adult and embryonic zebrafish tissues. While its suitability for time course experiments may be questionable, it is used here only in adult zebrafish tissues of the same age, where it has been demonstrated to be consistent (Bauer, 2001; Frost & Nilsen 2003; Goutel et al., 2000; Herzog et al., 2003; Kramer et al., 2002; Nordnes et al., 1994; Olsvik et al., 2005).

- Did the author analyse the PCR efficiencies of the primers he used to show that they were equally efficient, and therefore satisfied the appropriate threshold for use in quantitative real-time RT-PCR (qRT-PCR) experiments?

Levels of gene expression were not compared across genes, only across tissues. As the external referee suggests, comparisons between the primer pairs would be meaningless given that the efficiency of the primers has not been assessed.

- Did the author show that his amplicons were 'pure' by performing dissociation profiling or undertake sequencing?

Disassociation curves suggest that the primer pairs were acceptable.

- Was the RNA isolated from the zebrafish assessed to determine its quality prior to reverse transcription as quality has a direct bearing on the validity of qRT-PCR data?

The RNA used was run over a nanosep column to remove small molecules, and was assessed for quality using imaging of ethidium bromide staining, and was assessed in terms of contamination using spectrophotometry.

- I liked the analysis of the long and short transcript forms expressed by the zebrafish *fxrl* gene, but did the author show these forms existed prior to undertaking qRT-PCR? I understand that the zebrafish EST database contained the variants, but I would have appreciated a comment that the author had verified it experimentally although the qRT-PCR suggests that they exist.

We identified the splice variants in Genbank and the Zv2 assembly, and as noted by the external referee, the results of the Real Time PCR are also strongly supportive of the existence of the splice variants.

- How many zebrafish were used for the tissue analysis of transcript levels? Was only one fish used or more, and were tissues pooled?

Tissue samples were taken from five fish and the RNA extracted was pooled.

---------Comments regarding Paper 2 made by the external referee---------

- Were the levels of Fmrp also examined at 5dpf? In this context, the author rightly states ignorance of the long-term (>2 days) efficacy of the injected morpholinos. The transient nature of the knock-down approach places some limitations on the usefulness of modelling Fragile X syndrome in the zebrafish and it is possible that *fmr1* gene expression recovers by 5dpf, or earlier. This does not discount the "genotype"-phenotype correlations that the author makes, but warrants some discussion.

No. given the uncertainty of the morpholinos effect at this late stage we felt that this would not be an informative experiment given the proposed hypotheses. We accept that the observed craniofacial defects are a downstream, possibly mechanically related phenotype rather than a direct consequence of Fmrp knockdown. The presentation of the human craniofacial phenotype may be similarly downstream or mechanically related, however.

- Injected embryos were immediately placed in E3 medium containing MPEP, but no reasoning was presented to help the reader understand why 250ng/ml MPEP was used. In addition, I wonder why the effect of varying the time at which the addition of MPEP can bring about phenotypic rescue was not examined.

250ng/m1 MPEP was used as the results of a titration suggested this was the optimal

concentration (minimising toxic reactions and death). Varying the time of application was trialed, but appears to have little effect on the resulting phenotype (if applied before brain development). Given the a) maternal expression of fmr1 and b) injection of morpholinos at 1 cell stage we decided to immediately apply MPEP to maintain consistency in our treatments.

- MPEP also showed craniofacial anomalies (Figure 6 legend), but in combination with the *fmr1MO*, the twin insults appeared to cancel each other out. The former effect using MPEP alone did not appear to be discussed.

This effect cannot be reliably discussed until a longer lasting/verifiable/consistent knockdown is achieved in 5 dpf embryos.

- The imperative, however, is to take this to the next level in achieving stable/regulated knockdown of targeted zebrafish genes. The author appears not to have speculated on how this might be achieved.

The key way to approach stable/regulated knockdown of zebrafish genes would be to use a TILLING (Targeting Induced Local Lesions in Genomes) approach. This method involves mutagenesis followed by DNA screening for single base mutations in the target gene (McCallum et al., 2000). This method requires significant resources however, as a high level of throughput as well as a high level of sensitivity is required (but might be outsourced to the ACVMD; Australian Centre for Vertebrate Mutation Detection).
An attractive in-house approach would be to make a stable transgenic expressing (possibly in a tissue specific manner) siRNAs against fmr1. However this technology is less frequently used than morpholinos, and tends to have poor specificity in zebrafish. Validating a number of siRNAs and producing transgenics appears to be a more time intensive approach than use of the morpholinos. Given the success of our early work, however, this transgenic approach may be justifiable.
Overall, targeted knockout in zebrafish is of great importance to the use of the organism as a model in the study of human diseases, and this importance will become critical as the technologies become more reliable or widely accepted.

---------Comments regarding Paper 3 made by the external referee---------
The author could have discussed:

- The lack of specificity of acridine orange in detecting apoptosis.

Acridine orange has been reported as being specific for apoptotic forms of cell death, not significantly labelling necrotic cells (Abrams et al., 1993).

- An analysis of reducing the number of biological replicates or the group size of 10. Why did the author choose ten groups of ten embryos?

Given the method of analysis, each sample must be the same size. 10 embryos is a convenient number for the equipment used (range of the microscope using a x2.5 objective lens). 10 groups were used for statistical validity.

- Other means of inducing apoptosis. The author relied on a simple positive control morpholino (*MOfmr1*) at a single dose and a single concentration. The possibility of using a defined dose of UV irradiation for varying lengths of time would have provided a greater range of "insult" and hence apoptotic outcome. This type of study might have provided a more comprehensive correlation between the two methods he describes.

Agreed, but this is outside of the scope of the paper.

- The well known work of Coles and Ross and their study of apoptosis in the zebrafish. The analysis of regions of the zebrafish other than the tail could have been included.

Counting cells would be complicated in areas of tissue thicker than the tail, as apoptotic cells may be out of focus. This is one of the advantages of using a densitometric analysis.

- A comparison of acridine orange staining and TUNEL in terms of specificity in detecting apoptosis.

*At least one study has made this comparison (*Martins et al., 2007). *As 'tunel' stains cells in the early stages of apoptosis, it tends to estimate a higher number of apoptotic cells. However, a key advantage of our technique is that very little treatment of the embryos is required in preparation for analysis, whereas 'tunel' staining requires both fixation and peeling of the embryos. As the levels of fluorescence staining in Acridine orange staining and 'tunel' staining will be different (due to different treatment protocols), it would be difficult to compare specificity of each treatment using this method.*

---------Other comments made by the external referee---------

The author could have discussed:

- The possibility of using tools other than morpholinos for down-regulation of the *fmr1* gene was not discussed. The author appears to be limiting future zebrafish studies to a morpholino-restricted window of a few days post fertilisation.

This topic was not discussed to maintain focus of the thesis. There is a great potential for knockdown tools other than morpholinos, such as siRNAs (small interfering RNA), m/PNA (modified/peptide nucleic acid), S-DNAs (phosphorothioate-linked DNA), and LNAs (locked nucleic acid). Furthermore, knockout approaches in the zebrafish are gradually becoming a viable option.

It would be interesting to pursue lines of study involving siRNAs, as they can be used to create transgenic embryos. This would allow longer term analysis, as well as the potential for neural crest or neuron specific knockdown. However this technology is less frequently used than morpholinos, accounted for by a still poor specificity in zebrafish. PNAs have a number of advantages, primarily being more effective, but less targeted, than morpholinos. It would be interesting to use PNAs (or the more reliable mPNAs) as an alternative approach to morpholino use. Again, however, this technology is less broadly accepted and in an earlier stage of development than morpholinos.

- The author makes an interesting observation regarding the lack of rescue of craniofacial abnormalities using MPEP (if not unaffected by such treatment?). I wonder if having a population of zebrafish exhibiting equally compromised fmr1 gene expression might offer a better platform for future studies, and the assessment of combinatorial treatments, which I think is a good point made by the author.

If we can establish with certainty a standard level of knockdown or establish a knockout at 5 dpf then the effects of MPEP on craniofacial development would be a critical line of research.

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