Zebrafish as a Model of Genetic Disease

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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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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.**

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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.