INVESTIGATION OF THE ROLE OF FRAGILE X MENTAL RETARDATION PROTEIN IN THE EMBRYONIC NEOCORTEX USING RNA INTERFERENCE.

A Project

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by

Joseph L. Elsbernd

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Abstract

of

INVESTIGATION OF THE ROLE OF FRAGILE X MENTAL RETARDATION PROTEIN IN THE EMBRYONIC NEOCORTEX USING RNA INTERFERENCE.

by

Joseph L. Elsbernd

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a genetic disease characterized by impaired movement coordination, peripheral nervous system damage, limb tremors, cognitive decline, behavioral changes, autonomic nervous system dysfunction, intellectual disability, and Parkinson’s disease like symptoms. FXTAS usually manifests after the age of 50 and affects 1 out of 3000 men and 1 out of 5200 women. FXTAS is theorized to be the result of an expansion of CGG repeats in the 5’ untranslated region (UTR) of the Fragile-X mental retardation 1 (FMR1) gene. FXTAS is one of many disease phenotypes caused by the premutation FMR1 allele. In the United States it is estimated that 1.7 million women and 750,000 men carry the premutation allele and puts them at risk for hypertension, seizures, muscle pain, sensory, and the already mentioned FXTAS. FMR1 encodes for the fragile-X mental retardation protein (FMRP) and it inhibits translation by binding to large number of mRNA transcripts, up to 4% of the total RNA in the nervous system. The specific molecular cause of premutation
phenotypes is not known but is theorized to be caused by FMRP reduction, RNA toxic-gain of function, or some combination of the two. Studies have shown that premutation has an effect on radial glial stem cells and intermediate progenitors during embryonic development. This study validated a new \textit{FMRI} shRNA called GI377 and used RNA interference technique with the shRNA ED03 to experimentally reduce FMRP concentrations in radial glial stem cells and their differentiated daughter cells in embryonic normal type mouse brain. This study determined that, as predicted, the quantity of intermediate progenitors in the ventricular and subventricular zones of the somatosensory cortex decreased when FMRP was knocked down. This evidence is not sufficient to conclude cause nor is it sufficient to rule out other interacting characters. Our work furthers premutation research through the validation of a new shRNA and further adds value to the field through determining that FMRP knockdown alone reduces intermediate progenitor quantity and suggests a differentiation bias towards glial cell fate when FMRP is reduced. FXTAS and other premutation disease have large effects on the lives of those afflicted, their family, and their friends. Discerning the mechanism through which premutation manifests will enable future research to discover cell, gene, and molecular therapies which could reduce or prevent the premutation from manifesting.

\textbf{\_______________, Committee Chair}

Thomas Landerholm

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Date
ACKNOWLEDGEMENTS

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INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a genetic disease characterized by impaired movement coordination, peripheral nervous system damage, limb tremors, cognitive decline, behavioral changes, autonomic nervous system dysfunction, intellectual disability, and Parkinson’s disease like symptoms (Coffey et al. 2008). FXTAS usually occurs after the age of 50 and affects approximately 1 out of 3000 men and 1 out of 5200 women (National Institutes of Health). FXTAS is caused by a premutation expansion of CGG repeats (50-200 CGG repeats) in the 5’ untranslated region (UTR) of the Fragile-X mental retardation 1 (FMR1) gene. The number of repeats found within the 5’ UTR of FMR1 is highly variable from individual to individual and is used to classify four broad allele categories. Each of these categories may have implications ranging from the molecular all the way to organismal level and the categories are called normal, gray-zone, premutation, and full-mutation. These groups are not defined by single-nucleotide polymorphisms (SNP) but they are defined by the quantity of CGG trinucleotide repeats present within the 5’ UTR. In humans the normal phenotype is considered to be any number of CGG repeats less than 45 and normal mice have 9 to 10 repeats, the gray-zone from 46-55, the premutation which is responsible for FXTAS ranges from 55 and 200, and the full-mutation type is any number of repeats over 200 (Nolin et al. 2003). Several disease phenotypes are caused by the FMR1 premutation including FXTAS and FXPOI. In the United States it is estimated that 1.7 million women and 750,000 men carry the premutation allele (Hantash et al. 2011).
The number of CGG repeats found within the 5’ UTR of *FMR1* is highly variable from individual to individual and is used to classify four broad allele categories. Each of these categories may have implications ranging from the molecular to organismal level; the categories are called normal, gray-zone, premutation, and full-mutation. These groups are not defined by single-nucleotide polymorphisms (SNP) but instead are defined by the quantity of CGG trinucleotide repeats present within the 5’ UTR. In humans the normal phenotype is considered to be any number of CGG repeats less than 45 and in mice the normal phenotype has 9 to 10 repeats, the gray-zone from 46-55, the premutation ranges from 55 and 200, and the full-mutation type is any number of repeats over 200 (Coffey et al. 2008).

The normal type alleles are the only category whose CGG quantity remains stable from one generation to the next. Gray-zone, premutation, and full-mutation alleles generally increase in size from one generation to the next and it appears that as the CGG quantity increases, so does the rate of CGG expansion. Repeat length instability between generations may be related to DNA replication error as a result of slippage as the DNA polymerase moves through the CGG repeat regions. The exact cause of repeat instability is not known but some evidence suggests it may have its roots in meiosis (Nolin et al. 1999). This has important implications for premutation and gray-zone allele carriers who plan on having children as their progeny are at a higher risk of being born with full-mutation sized alleles and thus likely to suffer mental retardation.
Originally the \textit{FMR1} premutation genotype, henceforth referred to as premutation, was believed relevant only in older individuals, through increased chance of having offspring with Fragile-X Syndrome (FXS) and/or development of late onset neurological disease such as FXTAS. A mouse model of \textit{FMR1} premutation was generated by replacing the native 8 CGGs in the mouse with 98 CGGs of human origin (Willemsen 2003). Recent studies found that premutation alleles altered gene expression, cell morphology, cell migration, and neural cell fate as early as embryonic development (6, 7). One study found that in cultured heterozygous premutation neurons the quantity of premutation mRNA was increased 260\% while FMRP was decreased 74\% relative to wild-type (Chen et al. 2010). Previously we determined that FMRP is decreased by 40\% in the embryonic forebrain (Cunningham et al. 2010). There are consequences associated with premutation because FMRP is reportedly involved in the regulation of cell cycle regulation pathways. The decreased FMRP, toxic FMR1 mRNA gain of function, or some other variable or combination could be the cause of premutation phenotypes (Hagerman and Hagerman 2004). Evidence has shown that premutation phenotype may be a result of toxic FMR1 mRNA gain of function (Hashem et al. 2009).

Premutation alleles cause FXTAS, but in addition to FXTAS, premutation can also cause fragile-x premature ovarian insufficiency (FXPOI) in women. FXPOI is a disease where ovary function is disrupted, causing hormone imbalances and decreased fertility. FXPOI affects about 30\% of female premutation carriers and this disease can strike women younger than age 40 (Farzin et al. 2006; Amiri et al. 2012). Homozygous and heterozygous female individuals are at risk for FXPOI and 16\% of those
heterozygous for premutation will suffer from FXPOI (Sherman 2000; Hanson and Madison 2007). Along with FXTAS and FXPOI, the premutation genotype has also been linked to the development of behavioral, mood, and learning disabilities that fall along the autism spectrum (Farzin et al. 2006). Premutation carriers not afflicted with FXTAS have a higher incidence of muscle pain, thyroid problems, seizures, fibromyalgia, tremors, and gait ataxia (Coffey et al. 2008).

The \textit{FMR1} full-mutation genotype (>200 CGG repeats) is responsible for Fragile-X Syndrome. The symptoms of FXS include mental and physical retardation, oversized testes in males at puberty, hyperactivity, impulsive behavior, developmental delay (in crawling, walking, and speech), and learning disabilities (National Institutes of Health). FXS is a dominant trait which affects all male carriers but the symptoms in females vary in severity from minor to the fully affected as a result of female X-inactivation during embryonic development. It is thought that because of the range of symptoms seen in females, that FXS often goes undiagnosed in many women (Nolin et al. 2003). The molecular cause of FXS is the complete silencing of the \textit{FMR1} gene by hypermethylation (Naumann et al. 2009). It is suspected that the CGG repeat expansion causes the cell to lose the ability to differentiate between the \textit{FMR1} gene and the upstream heterochromatin leading to \textit{FMR1} silencing. The loss of FMRP causes altered proliferation, survival rate, and fate of neural progenitor/stem cells in the brains of mice, and likely has the same effect in humans (Luo et al. 2010). The full-mutation is of relevance to premutation carriers as they are more likely to have offspring with full-mutation alleles as a result of the CGG expansion. Furthermore, premutation carriers with CGG expansions
approaching 200 have significantly decreased FMRP levels (Kenneson et al. 2001; Brouwer et al. 2008).

The FMR1 gene is present in many animal species including humans and rodents. The FMR1 gene is located on the X chromosome at q27.3 and is inherited in an X-linked manner. As the FMR1 gene is found only on the X chromosome, men are more susceptible than women to FMR1-related disease. Men are nearly three times as likely to have the premutation condition and twice as likely to suffer from FXTAS. FMR1 encodes the fragile-x mental retardation protein (FMRP).

Previous work has shown that FMRP is a regulatory protein that binds to specific mRNAs to prevent translation (Bardoni and Mandel 2002) and is expressed in many tissues, including neural precursor cells (Cunningham et al. 2010) and neurons (Bardoni and Mandel 2002). FMRP binds 4% of all mRNAs in human embryonic brain (Ashley et al. 1993) and many of these mRNAs are important for cell cycle regulation, differentiation, migration and CNS development (Bardoni and Mandel 2002).

FMRP has an important role in the differentiation of neural precursor cells and immature neurons in the CNS. The role of FMRP is evident in FMR1 knockout mice where there is a 60% reduction in the number of adult neural progenitor/stem cells (aNPC) produce cells in the neural lineage, whereas there is a 75% increase in aNPC’s that produce cells in the astrocytic lineage (Luo et al. 2010). The reduction in neurons and increase in astrocytes in FMR1 knockout leads to changes in the development and
maintenance of the CNS and may manifest as severe mental retardation (Naumann et al. 2009; Luo et al. 2010).

During normal embryonic neocortical development immature excitatory neurons are produced by neural stem and progenitor cells in the dorsal neocortical proliferative zones (ventricular zone and subventricular zones) that line the lateral ventricles of the brain (Noctor et al. 2004). After differentiation, young immature neurons migrate into the subventricular zone (SVZ) and extend a leading process toward and usually migrate towards the lateral ventricle (Noctor et al. 2004). A leading process is an extension of the cell which points in the direction of the cell’s migration (Noctor et al. 2004; Valiente and Martini 2009). After the leading process has been established toward the ventricle, a second process is extended outwards towards the pial surface and the cell subsequently reverses direction and migrates towards the cortical plate. In a mouse model of \textit{FMR1} premutation fewer cells with leading processes were oriented towards the ventricle and fewer cells were located in the subventricular zone, indicating that migration patterns were affected by premutation (Cunningham et al. 2010).

The neocortex has two sources of excitatory pyramidal neurons, radial glial (RG) stem cells and intermediate progenitor (IP) cells (Noctor et al. 2001). RG cells are multipotent stem cells that produce neurons, glial cells, and IP cells, whereas the IP cells primarily produce neurons (Noctor et al. 2004; Miyata et al. 2004; Haubensak et al. 2004; Noctor et al. 2008). RG cells and IP cells are distinguished by unique expression of the transcription factors Pax6 (RG cells) and Tbr2 (IP cells) (Englund et al. 2005). Notably,
we have observed a defect in differentiation of precursor cells in the premutation mice. The proportion of cells that express Pax6 is increased and the proportion of cells that express Tbr2 is reduced in premutation mice relative to wild-type littermate mice (Cunningham et al. 2010). These data suggest a delay in the differentiation of neural precursor cells in premutation mice.

To attempt to model the effects of decreased FMRP that occurs in FMR1 premutation, FMRP knockdown is accomplished in wildtype mice in utero using RNA interference. The innate RNA interference pathway is induced by shRNA coded by and expressed from a plasmid vector. The RNase Dicer recognizes, cleaves, and then unwinds the shRNA (Siomi and Siomi 2009). The ssRNA is recognized and bound by the RISC protein complex, which uses the RNA as a template against the FMR1 mRNA (Siomi and Siomi 2009). The shRNA’s used in this study target a variety of portions along mouse FMR1 mRNA.

The objective of this project is to validate the effectiveness of three novel shRNA’s targeting FMR1 mRNA and to determine if knockdown of FMRP through RNA interference alters neuronal migration and the differentiation of neural precursor cells during embryonic cortical neurogenesis. We hypothesized that reducing the expression of FMRP in RG stem cells will cause wild-type mice to exhibit defects consistent with the premutation condition, including defects in neuronal migration, a reduction of Pax6-expressing cells (RG), and an increase in Tbr2-expressing cells (IP cells). Our work allowed us to test the hypothesis that the 40% reduction in the expression of FMRP in the
embryonic brain of premutation mice is responsible for the defects in neuronal migration and precursor cell differentiation that we have characterized (Cunningham et al. 2010). This work provides a number of benefits. First it has helped identify new targets for therapeutic intervention. Next it provided a new tool for examining mechanisms of brain development. Lastly we helped bring better insight into the mechanisms of \textit{FMR1} premutation by investigating the effect of FMRP knockdown on neocortical development.
METHODS

Animal handling and surgery

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of California at Davis and performed within the guidelines set by the National Institute's of Health Guide for Care and Use of Laboratory Animals.

Wild-type pregnant mice (strain C57/BL6 or Swiss Webster) were anesthetized at embryonic (E) day 14 with 5% isoflurane and surgically opened using a laparotomy. The uterus containing embryos was removed from the peritoneal cavity and lavaged with warm artificial cerebrospinal fluid (aCSF; 125mM NaCl, 2.5mM KCl, 1mM MgCl₂, 2mM CaCl, 1.25mM NaH₂PO₄, 25mM NaHCO₃). The uterus was returned to the peritoneal cavity and was rinsed with 30-35 °C aCSF. After closing the incision, the pregnant mice were removed from anesthesia and allowed to recover under observation. Shortly after removal from anesthesia, the animals were administered buprenophine (0.5 mg/kg) for analgesia. Animals were monitored twice daily and given additional analgesia as needed. After three days the pregnant mice were anesthetized, a laparotomy performed and the embryos were removed from the uterus. Embryos were collected, anesthetized with ice, and then transcardially perfused with 0.1 M phosphate buffered saline followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed in 4% PFA overnight, followed by cryoprotection in 30% sucrose.
In utero intracerebral electroporation

A shRNA cloning vector (Origene Rockville, MD) containing a 29mer \textit{FMR1} shRNA, the previously validated ED03 \textit{FMR1} shRNA were used (generous gift from Kim McAllister and Morgan Sheng) or a non-effective 29-mer scrambled shRNA (Origene) as negative control (Edbauer et al. 2010). Wild-type pregnant mice (strain C57/BL6 or Swiss Webster) were anesthetized at embryonic (E) day 14 with 5% isoflurane and surgically opened using a laparotomy as above. The uterus was removed and approximately 1 µg/µL of plasmid solution and dye was injected into the lateral ventricle of each embryo for a total of 1 µL total volume using a thinly pulled glass micropipette made from a microcapillary tube (Martínez-Cerdeño et al. 2012).

After all embryos were injected, unidirectional electrodes with a disc size of 3 mm were placed flanking the cerebral hemispheres of the embryos. The electrodes were aligned such that the positive electrode was directly superficial to the developing dorsal somatosensory cortex. Five square-wave electronic pulses at 30 V for 50 ms with 950 ms interpulse intervals were used to induce the uptake of the plasmid into the cells lining the ventricle. The uterus was returned to the peritoneal cavity and was rinsed with 30-35°C aCSF. After closing the incision, the pregnant mice were removed from anesthesia and allowed to recover and observed. Post-electroporation the embryos were allowed to continue normal development for three days, after which they were transcardially perfused and cryoprotected as above.
Tissue Processing

Embryonic brains were sectioned at 300 µm by vibratome (Ted Pella Vibratome Series 1000). Sections were analyzed using a Fluoview FV1000 confocal microscope (Olympus). Only sections with a regular evenly distributed electroporated field as determined by microscopy in the dorsal somatosensory cortex were analyzed. Successfully transfected sections were cryoprotected in 30% sucrose, embedded in OCT, sectioned at 30 µm using a cryostat (Microm HM 550), and mounted on slides with high tissue binding affinity (Superfrost Plus ® by Fisher Scientific).

Immunohistochemistry

Slides containing 30 µm thick sections were washed three times in 0.1 M phosphate buffered saline (PBS), and antigen retrieval was performed by boiling samples in a microwave for 15 min in 10mM citrate buffer (pH 6) containing 10 mM Sodium Citrate and (v/v) 0.1% Tween-20. Slides were allowed to cool to room temperature and then rinsed three times in 0.1 M PBS. Slides were blocked for 1.5 hours at room temperature in blocking buffer consisting of (v/v) 10% donkey serum, 0.1% Triton X-100 and (v/v) 0.2% gelatin. After blocking, slides were incubated overnight (>16 hrs) at ambient temperature with (v/v) 2% donkey serum, 0.02% Triton X-100, (w/v) 0.04% gelatin, and primary antibodies. The primary antibodies used included mouse anti-tGFP (Origene), chicken anti-FMRP (generous gift of Paul Hagerman), rabbit anti-Tbr2 (Abcam), and rabbit anti-Pax6 (Covance). Tissue samples were rinsed with PBS and
then incubated for 1 to 2 hours at ambient temperature in (v/v) 2% Donkey serum, 0.02% Triton X-100, 0.2% DAPI (Roche), and (w/v) 0.04% gelatin. The secondary antibodies included donkey anti-mouse (Cy2), donkey anti-rabbit (Cy5), and donkey anti-chicken (Cy2), all from Jackson Immunoresearch. Tissue samples were rinsed three times with PBS for 5 minutes each before the slides were coverslipped with Mowiol.

Tissue samples were viewed and imaged using a Fluoview FV1000 Confocal Microscope by Olympus. Single optical planes were processed and fluorescence was quantified using Olympus Fluoview software and ImageJ (Version 1.44p). ImageJ was used to analyze and adjust the images for contrast, size, and color application to view expression of markers.

Analysis of FMRP knockdown effectiveness

Tissue samples electroporated at E14 and perfused at E17 were used to determine the effectiveness of shRNA knockdown. The Origene shRNAs tested were GI374 (TTGCTGAAGATGTCATACAGGTTCCACGA), GI375 (GTGATGAAGTTGAGGTTTATTCCAGAGCA), and GI377 (AAGGAAGTAGACCAGTTGCGTTTGGAGAG), all of which were generated to target FMR1 mRNA. A noneffective scrambled vector T13 (Origene) was used for control experiments. Nuclear staining with DAPI was used to evaluate the total number of cells. The FMRP intensity value for transfected cells (tGFP+, FMRP+, and DAPI+) and the non-transfected control cells group (GFP-, FMRP+, and DAPI+) for each image were
determined by manually drawing a region of interest (ROI) around each cell body and recording the reported pixel intensity value. Each treatment contained sections from three embryos except for GI375 which had only two. A total of 1,673 cells were quantified and an analysis of variance (ANOVA) test was conducted on the treatment groups to determine significance.

Analysis of Pax6 and Tbr2 expression

To determine if FMRP knockdown recreated the premutation phenotype in the neural stem cells of wild-type embryonic somatosensory cortex, the expression of Pax6 and Tbr2 in transfected cells with the FMRP knockdown were analyzed to indicate the effects of FMRP knockdown on neural stem (Pax6+) and progenitor (Tbr2+) populations. The vectors used in this comparison were ED03 shRNA (GTGATGAAGTTGAGGTTTA) and empty control vector (pSUPER). The ED03 FMR1 shRNA vector was reported to decrease FMRP levels by 40% and thus was used for further analysis (Edbauer et al. 2010). ED03 was co-transfected along with pEGFP-C1 to act as a marker. A minimum of three animals were used for each condition. Electroporated sections from dorsal somatosensory cortex were analyzed from each animal from each condition. Samples used for Pax6 and Tbr2 expression analysis were immunostained with DAPI, anti-GFP, and either anti-Pax6 or anti-Tbr2 and imaged on a confocal microscope. The total quantity of GFP+ cells in the dorsal cortex was determined for each image by manual count. The total number and location of cells
coexpressing tGFP and either Pax6 or Tbr2 were determined for each image by manual count.
RESULTS

shRNA knockdown results

In order to determine the effectiveness of purchased FMRP knockdown vectors, embryonic mice were transfected with one of three shRNA vectors or a scrambled control. FMRP intensity readings from each neuron were normalized for each image to correct for differences in image quality. A one-way analysis of variance (ANOVA) test using Tukey-Kramer HSD p=0.05 was used to determine if TR13, GI374, GI375, or GI377 significantly differed from each other. Neurons located in the cortical plate (CP) and subplate (SP) were used in the analysis because these cells are the post-mitotic daughter cells of RG stem cells, see Figure 1 for zone locations. GI374 and GI375 were not significantly different from the scrambled control whereas GI377 was significantly different from all other treatments and the control, see Table 1. Relative to the control, GI377 reduced FMRP intensity by 6.0% (p=0.001).
Figure 1. Composite coronal image of the E16 mouse brain including the somatosensory cortex. Red color = FMRP. Blue color = DAPI nuclear stain. (A) (1) Pial surface of the brain. (2) Ventricular surface of the brain. (3) Lateral ventricle. (B) Zoomed in portion of the somatosensory cortex showing the zones. (CP) Cortical plate. (SP) Sub-plate. (IZ) Intermediate zone. (SVZ) Sub-ventricular zone. (VZ) Ventricular zone. (V) Ventricle. FMRP is expressed throughout the brain and there are specific regions (VZ, SP, and CP) which have higher levels of FMRP immunoreactivity. FMRP is expressed by RG stem cells in the VZ and IP cells in the SVZ (Cunningham et al. 2010).
Table 1. Results of one-way ANOVA comparing GI374, GI375, GI377, and TR13. One-way ANOVA performed using Tukey-Kramer HSD p=0.05 on shRNA knockdown vectors. GI377 was significantly different (p < 0.05) from scrambled control (TR13), GI374, and GI375.

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RG cells in the ventricular zone and subventricular zone

Pax6 is a transcription factor expressed by pluripotent RG stem cells which are located in the VZ, see Figure 1 for location and Figure 3(A) for banding pattern. We examined Pax6 expression in cells transfected with Fmr1 knockdown or control vectors as evidenced by GFP expression. The specific expression pattern of Pax6+ cells can be seen in Figure 3(A). A two-tailed student’s t-test was used to determine significance (p=0.05). In this experiment it was determined that the number of Pax6+/GFP+ cells in the VZ and SVZ were not significantly different in the ED03 shRNA group when compared to the control, VZ p=0.5902 and SVZ p=0.7911, see Figure 2.
Figure 2. Relative quantities of radial glial and intermediate progenitor cells in the ventricular (VZ) or subventricular zone (SVZ) after FMRP knockdown. FMRP knockdown was performed by in utero electroporation at E14, analysis at E17. Radial glia cells were identified by expression of Pax6 and intermediate progenitors were identified by Tbr2 expression. The relative quantities of cells in each cortex zone were determined by dividing the number of Pax6+/GFP+ or Tbr2+/GFP+ cells by the total number of GFP+ cells in the electroporation field. The quantity of intermediate progenitors was significantly lower than that of the control in both the ventricular and subventricular zones. The control was empty vector and the shRNA was ED03. The population size was 3 for all conditions except radial glial shRNA which had a size of 2. A two-tailed student’s t-test was used to determine significance (p=0.05). * indicates significance of p < 0.05. Mean error bars are shown in blue. Standard deviation bars are shown in red.
Figure 3. Expression of Pax6 and Tbr2 at E16 in dorsal somatosensory cortex. Blue = DAPI. Red = Pax6 (A) or Tbr2 (B). The transcription factor Pax6 is a marker of radial glial (RG) stem cells and the transcription factor Tbr2 is marker for intermediate progenitor (IP) cells. The RG cells are primarily found in the ventricular zone. IP cells are primarily located in the SVZ.
IP cells in the ventricular zone and subventricular zone

Tbr2 is a transcription factor expressed by IP cells, which are primarily located in the SVZ during embryonic development, see Figure 1 for location and Figure 3(B) for banding patterns. We analyzed the proportion of GFP+ electroporated cells that expressed Tbr2. A two-tailed student’s t-test was used to determine significance (p=0.05). The number of Tbr2+/GFP+ cells present in the VZ and SVZ in the ED03 shRNA group were significantly different from that of the control, VZ p=0.0109 and SVZ p=0.0283 (see Figure 2).

RNA secondary structure predictions with CyloFold

Secondary structure predictions of the shRNA vectors after GI374, GI375, GI377, and ED03 were generated using CyloFold (Bindewald et al. 2010) and are shown in Figure 4(B). GI374 and ED03 had no predicted structure. GI375 and GI377 were predicted to have short-hairpin loops.
Figure 4. shRNA target locations and RNA secondary structure predictions. (A) Predicted target locations for GI374, GI375, GI377, and ED03 against *Mus musculus* *FMR1* mRNA (NM_008031, 4,412 bp). (B) shRNA secondary structure predictions were made using CyloFold (Bindewald et al. 2010).
DISCUSSION

The success of the shRNA experiment in recreating some of the premutation phenotype through the use of shRNA adds evidence to the hypothesis that the reduction of FMRP expression contributes to the premutation phenotype. This evidence is not sufficient to conclude cause nor is it sufficient to rule out other interacting characters. This work furthers premutation research by validating a new \textit{FMR1} shRNA tool and demonstrating the effect of FMRP reduction on IP cells. More research is needed to fully ascertain if FMRP knockdown affects RG stem cells or not. Cell signaling is complex and extremely interconnected and so broad expression studies are needed to truly determine if mRNA, protein, unknown characters, or unknown combinations induce premutation. We must keep in mind the importance of determining the premutation mechanism of action because there are many affected individuals who need more assistance than the current strategy of symptom mitigation provides.

Determination of the mechanism of action for premutation is of tremendous importance because this knowledge will greatly enhance our ability to discovery therapeutic interventions for the many thousands suffering from it. Towards this end we conducted an experiment to validate the efficacy of \textit{FMR1} shRNA for use as tools in premutation research and attempted to experimentally replicate the premutation phenotype in unaffected mice as a way to narrow down the potential causes of premutation.
We characterized the novel shRNA GI377 as having the ability to reduce FMRP levels by 6% (p=0.001). This vector will be a valuable tool for studying the interactions of FMRP in the cell for diseases like FXS, FXTAS, and FXPOI. The vector will allow investigators to reduce FMRP in specific cells using an RNA interference technique. The slight reduction is a benefit because it will inform as to which pathways downstream of FMRP are the most sensitive to its reduction and how this affects the individual’s phenotype.

Of the two shRNA vectors that failed to affect FMRP levels, GI375 is of note for its similarity to the established and previously characterized \textit{FMR1} shRNA ED03 (Edbauer et al. 2010). ED03 is a 19-mer and GI375 a 29-mer and the first 19 bases of GI375 are identical to ED03. With their large similarity in sequence but differing effectiveness in mind, a RNA secondary structure prediction using CyloFold was performed (Bindewald et al. 2010). The prediction indicated that GI375 formed a short hairpin loop, see Figure 4(B). The RNA interference pathway of mammalian cells recognizes, cleaves, and unwinds double stranded RNA (dsRNA) using the DICER protein (Siomi and Siomi 2009). The single stranded RNA (ssRNA) fragments generated by DICER are recruited by the RISC protein complex which uses the ssRNA as a template to guide the complex and template to the target RNA (Siomi and Siomi 2009). Once the recruited ssRNA template forms dsRNA with its mRNA target, the RISC protein complex will cleave the dsRNA and form smaller fragments which destroys the function of that particular mRNA (Siomi and Siomi 2009). It is conceivable that after the protein DICER cleaves the original dsRNA that the fragments then may fold again into a
new dsRNA which could hinder its use by the RISC protein complex or even make it a target for further DICER cleavage, leading to a loss of specificity. The sequences of the other non-functional vector GI374 and the functional vector GI377 vector were subjected to predictions with CyloFold (Bindewald et al. 2010). The functional GI377 was predicted to have secondary structure similar to GI375 whereas the non-functional GI374 had no secondary structure predictions. Of the vectors used in this experiment, GI377’s target sequence lays the farthest from the 5’ UTR and inside exon 13, see Figure 4(A). GI377’s effectiveness could be a factor of its distance from the 5’ UTR as this could indicate it does not have to compete with translation machinery or regulatory proteins.

The FMR1 shRNAs GI377 and ED03 were used in this project. GI377 was previously uncharacterized and is now known to knockdown FMRP in vivo. ED03 was characterized by Edbauer and colleagues previously in vitro against HEK293 cells. ED03 was used for the RG and IP cell experiment because the characterization of GI377 was not complete when the RG and IP cell experiment began. Both vectors target FMR1 mRNA but differ in length and target site, see Figure 4(A). Access to both of these shRNA is important because each reduces FMRP to different extents which are useful in determining FMRP dosage related effects, if any, that cause premutation. GI377 and ED03 may also be used in conjunction with each other to more fully knockdown FMRP. With two functional shRNA a new fusion vector could be made with the goal of more complete knockdown or avoidance of off-target effects by administering a smaller dose of each shRNA but maintaining the same efficacy.
Wild-type mouse brains were electroporated with ED03 to knockdown FMRP and transcription factor markers examined in an attempt to determine if affected cells would manifest the premutation phenotype. The transcription factors Pax6 and Tbr2 mark RG cells and IP cells respectively (Englund et al. 2005). Previous work indicates that in premutation mice the quantity of Pax6+ RG cells is increased whereas the quantity of Tbr2+ IP cells is decreased (Cunningham et al. 2010). Since Pax6 cells undergo divisions that generate Tbr2 cells, the results suggest either reduced production of Tbr2 cells or a delay in differentiation. In this study I determined that FMRP knockdown in wild-type mice altered proportions of Tbr2+ cells but not Pax6+ cells.

The transcription factors Pax6 and Tbr2 mark RG stem cells and IP’s respectively (Englund et al. 2005). In premutation mice the quantity of Pax6+ RG stem cells is increased whereas the quantity of Tbr2+ IP cells is decreased. In this study it was determined that FMRP knockdown in wild-type mice by the ED03 shRNA vector was consistent with the premutation phenotype in regards to Tbr2+ cells in the VZ and SVZ but not with Pax6+ cells (Figure 2).

The success in recreating some features of the premutation phenotype in IP cells but failing to do so in RG cells could be a result of sample size or unknown molecular mechanisms. A sample size of only two was used in the analysis of Pax6+ cells, as this isn’t a large enough n value for statistical significance it is difficult to know what the effects of the vector are on the stem cells. The range of values for the two data points for the Pax6+ shRNA group completely encompassed the range of values for the control, see
Figure 2. A possibility for RG cell numbers not reflecting premutation phenotype could be that FMRP reduction is not solely responsible for the phenotype. It has been suggested by others that \textit{FMR1} mRNA gain of function toxicity, where the increased mRNA concentration is believed to be toxic for the cells, could be partially or fully responsible for the premutation phenotype (Hagerman and Hagerman 2004). It is also a possibility that both elevated mRNA and protein reduction leads to alterations in stem cell differentiation.

The diseases FXTAS, FXS, and FXPOI have large effects on the lives of those afflicted, the family and friends of the afflicted, and those yet to be born. Projects like this one are important for determining the specific molecular basis for the premutation phenotype and its related diseases FXTAS and FXPOI. Discerning the mechanism through which premutation phenotype manifests will enable future research to discover cell, gene, and molecular therapies which can reduce or prevent the manifestations of the premutation genotype. The recreation of the premutation phenotype in IP cells of the VZ and SVZ using \textit{FMR1} shRNA provides evidence for but does not prove that FMRP reduction causes premutation. More work is needed to determine if FMRP reduction creates premutation phenotype throughout the brain and not just the IP of the somatosensory cortex. This research is important because, aside from symptom management, there are no treatments for FXTAS and FXPOI syndromes.
LITERATURE CITED


Willemsen, R. (2003). The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. Human Molecular Genetics, 12, 949-959.