A novel link between FMR gene and the JNK pathway provides clues to possible role in malignant pleural mesothelioma

Ajay Srivastava *

Department of Biology and Biotechnology Center, Western Kentucky University, 1906 College Heights Boulevard, TCCW 351, Bowling Green, KY 42101, USA

Abstract

Malignant pleural mesothelioma (MPM) is an aggressive form of thoracic cancer with poor prognosis. While some studies have identified the molecular alterations associated with MPM, little is known about their role in MPM. For example, fragile X mental retardation (FMR) gene is up-regulated in MPM but its role in MPM is unknown. Here, utilizing Drosophila genetics, I investigate the possible role FMR may be playing in MPM. I provide evidence which suggests that FMR may contribute to tumorigenesis by up-regulating a matrix metalloprotease (MMP) and by degrading the basement membrane (BM), both important for tumor metastasis. I also demonstrate a novel link between FMR and the JNK pathway and suggest that the effects of FMR in MPM could in part be mediated by up-regulation of the JNK pathway.

1. Introduction

Malignant pleural mesothelioma (MPM) is an aggressive form of thoracic cancer with no known cure [1,2]. A possible reason could be our lack of understanding of this disease at the molecular level and a concomitant lack of good therapeutic targets [2,3]. While some published reports have focused on the molecular alterations associated with the disease, what role these altered molecules play in the pathogenesis of MPM is unknown [4-7].

Transcriptional profiling studies have reported that the fragile-X mental retardation (FMR1) gene and downstream components of the Jun N-terminal kinase (JNK) pathway, c-Jun and fos-1, are up-regulated in MPM derived tissue samples [8,9]. While no possible role for FMR and the JNK pathway in MPM has been described so far, the role of JNK pathway in tumorigenesis has been documented in several organisms including humans [10-14]. Furthermore, the JNK pathway is involved in regulation of several important biological processes [15] ranging from intercellular adhesion [16], wound healing [17], apoptosis [18], to aging [19].

Down regulation of a single gene FMR1 [20] is responsible for the fragile X-mental retardation in humans, a disease that occurs with a frequency of ~1/5000 males and 1/6000 females [21,22]. This down-regulation of FMR1 can occur due to a variety of reasons ranging from trinucleotide repeat expansion in the UTRs to point mutations in the coding region of the gene to epigenetic mechanisms [21]. Collectively, the phenotypes associated with misregulation of the FMR1 gene are grouped into the fragile X mental retardation syndrome or FMS [21,23]. The FMR1 gene product is responsible for translational regulation of its target genes [24]. It is thought that this is achieved through binding of the mRNA by the FMR protein [23,25]. While the brain and the nervous system have been the focus of studies on FMR function [21,23,25], recent studies have begun to add to the knowledge of FMRs role in other contexts. For example, it was recently demonstrated that the FMR1 gene is up-regulated in cancer cells like the hepatocellular carcinoma where it aids in tumor migration and metastasis [26]. The Drosophila genome consists of a single FMR gene (dFMR) [27] which behaves similarly to its human counterpart [28]. Indeed, human FMR1 gene can rescue Drosophila FMR mutant phenotype [29]. For this reason Drosophila has been used as an attractive genetic model to understand FMR functions during synaptogenesis and neuronal development.
While both the FMR gene and the JNK pathway have been shown to be up-regulated in MPM [8] their roles in MPM and their relationship to each other has not been defined. Herein based on experiments utilizing Drosophila genetics I provide clues to possible role of FMR and JNK pathway up-regulation in MPM. A novel link between the FMR gene and the JNK pathway is also presented.

2. Materials and methods

2.1. Drosophila stocks and culture

All Drosophila stocks and crosses were raised on standard corn meal agar medium at 25 °C in vials and bottles. Both the vials and bottles were also sprinkled with a few pellets of Red Star active dry yeast. Vg-Gal4 (FBti0024054), Ptc-Gal4 (FBti0002124), UAS-dFMR (FBti0026976), UAS-dsRED (FBti0018002), Tubulin-Gal80ts (FBti0027798) are available from the Bloomington Drosophila Stock Center and are described in the indicated Flybase references. PucLacZ is described in Martin-Blanco et al. [30] and Viking-GFP is described in Morin et al. [31] and was obtained from Flytrap (http://flytrap.med.yale.edu).

Genotype used in various figures and in results not presented in figures

The complete genotype used in various figures is given below.

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2.2. Beta Galactosidase assay for larval wing disc

Beta galactosidase assay was performed as described in Srivastava et al. [34]. Briefly, wandering third instar larvae of the correct genotype (as described in 2.2 Fig. 1D and E) were obtained from the cross, assessed for the presence of GFP expression under a

![Fig. 1](image_url)
stereo-fluorescence microscope, dissected in cold 1X PBS and then fixed for 10 min in 1% Glutaraldehyde. The dissected and fixed larvae were washed three times in 50 mM phosphate buffer. After the final wash the larvae were transferred into 1 ml of staining solution with fresh X-Gal. The mixture was incubated for six hours and then washed in 50 mM phosphate buffer. The discs were stored in this buffer at 4°C or wing discs were dissected out from the carcass and mounted in 70% glycerol and imaged on a Leica Stereomicroscope.

2.3. Immunostaining

Antibody staining was performed as described in Srivastava et al. [34]. The discs were dissected in cold 1X PBS and then transferred to a paraformaldehyde based fixative solution for 10 min. Following fixation the discs were washed 2X in 1XPBTA for 20 min each. Subsequent to this the discs were placed in a blocking solution made up of 760ul of 1XPBTA and 40ul of normal goat serum. The discs were blocked for 30 min at room temperature and then incubated with the primary MMP1 antibody [35] obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa, IA. The secondary antibody used was a Cy3 conjugated anti mouse IgG obtained from Jackson Immunoresearch and used at a dilution of 1:400.

2.4. Basement membrane degradation assay

Basement membrane degradation assay [34] was performed by dissecting wing discs from five third instar larvae of the correct genotype (w; PtcGal4, Viking-GFP, UAS-dsRED/+; UAS-dFMR1/Tubulin-Gal80ts), raised at 18°C and then shifted to 25°C for 48–72 h. The dissected discs were fixed, mounted in Vectashield mounting media (Vector Laboratories) and then imaged on a Zeiss LSM510 Confocal Microscope using the green and the red channels in a sequential setting. The Z-stack obtained was further processed to generate orthogonal XZ sections and assess basement membrane degradation. The observed BM degradation was most seen in the hinge region of the wing disc and was incompletely penetrant.

3. Results

3.1. Overexpression of dFMR results in cell death and up-regulation of the JNK pathway

To better understand the role of FMR in MPM and to mimic the FMR up-regulation observed in MPM I chose to over-express the dFMR gene in the developing Drosophila wing disc using the UAS-Gal4 system [36]. Overexpression of dFMR in different regions
of the wing resulted in phenotypes indicative of cell death (Fig. 1A–C). For example, overexpression of \textit{dFMR} along the anterior posterior compartment border (using \textit{Ptc-Gal4} driver) resulted in less tissue in the region of overexpression between wing vein 3 and wing vein 4 (bracketed region in Fig. 1B compared to 1A, \( n = >50 \) flies with complete penetrance). Similarly, overexpression of \textit{dFMR} in the wing margin (using \textit{Vg-Gal4} driver) resulted in wing notches (Fig. 1C). These phenotypes confirm previously reported phenotypes where the \textit{dFMR} overexpressing cells were shown to be apoptotic as judged by their incorporation of Acridine Orange [27].

Because cell death can be caused by up-regulation of the JNK pathway [37], I reasoned that the \textit{dFMR} overexpressing cells in the wing imaginal disc could be up-regulating the JNK pathway. The up-regulation of \textit{puckered} (\textit{puc}) gene has been exploited as a reliable readout of the status of JNK pathway activity in \textit{Drosophila} [30,38]. This is because activation of the JNK pathway is associated with an increased expression of the dual specificity phosphatase encoded by the \textit{puc} gene. The \textit{puc} assay has been facilitated by the existence of an enhancer trap insertion in the \textit{puc} gene (\textit{Puc} \textit{LacZ}) with \( \beta \)-galactosidase as the reporter [30,38]. In control wing discs (Fig. 1D, arrowheads) the \( \beta \)-galactosidase reporter from the \textit{Puc} \textit{LacZ} is localized to a region of the wing disc called the peripodial stalk (arrowheads in Fig. 1D). Overexpression of \textit{dFMR} gene along the anterior posterior compartment border [39] of the wing disc (using the \textit{Ptc-Gal4} driver) and in a genetic background that also contained \textit{Puc} \textit{lacZ} resulted in an increased expression of the JNK pathway (arrows in Fig. 1E compared to arrowheads in 1D).

### 3.2. Overexpression of \textit{dFMR} results in up-regulation of MMP

Having established that the \textit{dFMR} gene can up-regulate the JNK pathway I hypothesized that \textit{dFMR} expression could also result in the up-regulation of MMP. This was based on the observation that JNK signaling regulates the expression of MMPs [12,34]. To test my hypothesis I overexpressed \textit{dFMR} in wing discs using the \textit{Ptc-Gal4} driver and simultaneously immunostained these discs with an antibody directed towards the \textit{Drosophila} MMP1 (see materials and methods). The domain of \textit{Ptc-Gal4} expression in these discs was marked with Green Fluorescent Protein (GFP) expression from a UAS-GFP transgene. Control wing discs (Fig. 2A) expressing GFP and stained for MMP protein expression resulted in no MMP expression in the domain of \textit{dFMR} expression (Fig. 2A–A’’). The domain of expression of GFP in these cells was tightly localized to the region of \textit{Ptc-Gal4} expression with sharp boundaries (arrows in Fig. 2A, A’’). However, overexpression of \textit{dFMR} in the wing disc using the \textit{Ptc-Gal4} driver resulted in robust MMP expression (arrows in Fig. 2B’, B’’ compared to Fig. 2A’, A’’) in a cell autonomous and non-cell autonomous manner. Furthermore, the cells overexpressing \textit{dFMR} and consequently MMP were not restricted to the tight domain of GFP expression observed in control discs (arrows in Fig. 2A, A’’). Instead, the \textit{dFMR} overexpressing cells appear to leave the domain of \textit{Ptc-Gal4} expression (as judged by GFP labeled cells in Fig. 2B pointed with arrows) and migrate into the opposite posterior compartment (arrows in Fig. 2B–B’’). This phenotype has been previously observed in cells that display...
migratory behavior in the *Drosophila* wing disc [40]. Based on the previous observations it is inferred that cells that overexpress dFMR up-regulate the JNK pathway and an MMP and in the process acquire migratory properties that allow them to move away from their primary site. The acquisition of migratory properties and the degradation of BM is an important turning point in tumor cells becoming metastatic [41]. I next asked if dFMR overexpression can lead to the degradation of BM.

3.3. Overexpression of dFMR results in degradation of the BM

To test the idea that dFMR overexpression can lead to BM degradation I overexpressed dFMR using the Ptc-Gal4 driver in a genetic background that also harbored a GFP protein trap [31] transgene in the *viking* gene (see materials and methods) [34]. One of the major constituents of BM is the Collagen IV protein [42] which is encoded by the *viking* gene in *Drosophila*. The protein trap in the *viking* gene also labels the BM with GFP. Overexpression of dFMR in wing discs using the Ptc-Gal4 driver resulted in modest degradation of the BM (arrows in Fig. 3). This degradation of the BM was incompletely penetrant and primarily observed in the hinge region of the wing disc. A reason for the incomplete penetrance could be the variable expression of the transgenes with maximal expression of dFMR by Ptc-Gal4 occurring in the hinge region.

3.4. Overexpression of dFMR does not cooperate with activated RasV12

It has been shown in *Drosophila* that JNK pathway cooperates with an activated oncogene, RasV12, during tumor growth and metastasis [10]. Furthermore, the JNK pathway is also misregulated in human tumors [11,13]. Because FMR up-regulation leads to an up-regulation of the JNK pathway, it is possible that the FMR up-regulation in MPM collaborates with other oncogenes or mutations in tumor suppressor genes to bring about the metastatic effect associated with MPM. One possibility could be cooperation between FMR overexpression and the overexpression of RasV12 (Ras is frequently affected in human tumors). To test this possibility I utilized the MARCM system [43] in *Drosophila* to overexpress dFMR in clones of GFP labeled cells also simultaneously overexpressing RasV12. No over proliferation or migration of tumor tissue to contiguous organs was observed in these larvae when compared to the control RasV12 only expressing larvae. These results suggest a lack of cooperation between overexpression of dFMR and RasV12 (data not shown).

4. Discussion

Malignant Pleural Mesothelioma (MPM) is an aggressive cancer where molecular alterations and their role in MPM pathogenesis are not well understood [3–5,8]. It has been known that *FMR1* and the JNK pathway are up-regulated in MPM derived tissue samples [8] however their role in MPM and their relationship to each other was unknown (Fig. 4A, question marks). In this study, using *Drosophila* genetics, a set of data are presented that provide a novel link between FMR and the JNK pathway and offer clues to a possible role of these two molecules in MPM. First, it is demonstrated that overexpression of dFMR can up-regulate the JNK pathway (Fig. 1D–E) as assessed by the expression of the dual specificity phosphatase enhancer trap, *puc*odc. Second, the FMR overexpressing cells acquire migratory properties as they overcome the restrictive barriers imposed by the organ compartment boundary and move to the posterior compartment of the wing disc (Fig. 2). This acquisition of migratory properties by tumor cells is an important event in tumor metastasis and could be playing a similar role in MPM. Third, dFMR up-regulation results in up-regulation of an MMP (Fig. 2) and degradation of the BM (Fig. 3). Because a link between JNK pathway up-regulation and tumorigenesis has been well established in *Drosophila* [10,12,34] and in hepatocellular carcinoma and skin cancer in mice and humans [11,14] and because up-regulation of MMP and the degradation of BM are important events during tumor metastasis [41], it is an attractive possibility that through its up-regulation of the JNK pathway, FMR possibly contributes to tumorigenesis in MPM (Fig. 4). However, this connection is speculative and remains to be experimentally verified. Whether the up-regulation of JNK by FMR is direct or indirect remains to be seen and the possibility of FMR acting through non-JNK mediated mechanisms in MPM cannot be ruled out either (Fig. 4B, question marks).

Because JNK pathway and RasV12 collaborate during tumorigenesis in *Drosophila* [10], it was an interesting possibility that FMR overexpression and RasV12 may collaborate as well by way of JNK pathway up-regulation in response to FMR overexpression. My results however, failed to demonstrate a collaboration between these two genes. A possible reason could be that Ras is very rarely misregulated in MPM patients [5] but instead most of the MPM patients have a defect in *neurofibromatosis 2* gene (*NF2*) which is a tumor suppressor gene and works through the recently identified Hippo signaling pathway [44]. It is possible that FMR cooperates with *NF2* mutations to bring about the tumorigenic effects seen in MPM. Future efforts could focus on this hypothesis and utilize mutations in the *Drosophila* *NF2* gene *merlin* to test the cooperation between FMR overexpression and *NF2* mutations.

The fact that Fragile-X Mental Retardation Syndrome (FMS) patients are less likely to develop tumors is intriguing [45]. A possible explanation comes from studies where FMS patients display a mild reduction in the ligand for the β-catenin pathway (an important oncogenic pathway) [46]. Furthermore, in hepatocellular carcinoma [47] and breast cancer tissues [26] the *FMR1* gene has been found to be highly up-regulated. It has been proposed that the *FMR1* encoded FMR protein regulates cell adhesion, epithelial mesenchymal transition in these breast cancer patients, both of which are important events in tumor metastasis [26]. A decrease of *FMR1* in FMS patients would result in down-regulation of the

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**Fig. 4.** A model proposing how FMR may contribute to tumorigenesis in MPM. (A) It is known that genetic alterations in MPM result in FMR and the JNK pathway up-regulation. The relationship between FMR and the JNK pathway and the role of FMR in tumorigenesis was unknown (depicted by question marks). (B) A proposed model based on preliminary findings in this study illustrates how FMR could contribute to tumorigenesis in MPM. Briefly, overexpression of FMR results in up-regulation of the JNK pathway which in turn up-regulates MMP leading to degradation of the BM and thereby possibly contributing to the MPM phenotype. The possibility of JNK and FMR acting through other mechanisms to effect tumorigenesis cannot be ruled out and is indicated with arrows and boxed question marks. Boxed question marks also signify proposed, speculative connections for which experimental verification is required.
above mentioned tumor causing effects and thereby make tumor formation and metastasis difficult. Future studies could focus on exploring these links as causative reasons for preventing tumorgenesis in FMS patients.

The data presented by me in this study offer another possible explanation for why FMS patients are less likely to develop tumors. In FMS patients the FMR1 gene is either silenced or mutated. The findings in this study of a “FMR-JNK” connection and the knowledge of FMR [26] and JNK pathway’s involvement in hepatocellular carcinoma [11] suggest that the “FMR-JNK” connection could also be active in hepatocellular carcinoma, supporting its progression. Future studies could test this idea by knocking down FMR1 and observing the resultant suppression of hepatocellular carcinoma.

My findings, taken together, provide a unique link between FMR and JNK pathway activation might play during MPM. It also offers a hint to the possibility of using FMR1 stocks and antibodies used in this study. Sean Schaffer, the Bloomington Drosophila Stock Center, and the Developmental Studies Hybridoma Bank are acknowledged for maintaining FMR1 and JNK pathway reagents in this study. Schaffer at the Vanderbilt University Core Imaging Facility is acknowledged for coordinating my usage of the Confocal Microscope.

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Conflict of interest

The author declares that there is no conflict of interest.

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