MQP-BIO-DSA-6662

MdmX Regulates Mitotic Spindle Polarity of Human Breast Cancer Cells

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biology and Biotechnology

by

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April 25, 2013

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ABSTRACT

Mdm2 and MdmX are oncoproteins that promote cancer by binding and inhibiting the p53 tumor suppressor protein. Amplification and overexpression of the Mdm2 and MdmX genes has been observed in a significant fraction of human cancers, including leukemia, neural tumors, sarcomas, and melanoma. However, MdmX (but not Mdm2) has also been recently shown to inhibit cell proliferation and increase genomic stability in a p53-independent manner in cancer cells and non-transformed cells of murine origin. This project investigates a putative tumor-suppressing role of MdmX in human cells by examining the effects of MdmX on the genomic stability of human breast cancer cells. The results show a correlation between the presence of MdmX, decreased formation of multipolar spindles, and a concomitant increase in chromosome stability during cell mitosis. These findings indicate that MdmX may also have a p53-independent role in suppressing human breast cancer, and could provide a fundamental understanding of the role of genomic stability in neoplasia.

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ACKNOWLEDGEMENTS

I would first like to thank Dr. Stephen Jones for giving me the chance to study in his lab this year. It was a truly great experience that developed me as a person and as a professional. He always made time to accommodate my project and offer me advice. By relating my research to the rest of the scientific world, Steve has taught me much that will relate to my career in the future.

I would also like to thank all the members of the Jones Lab: Chris, Mike, Marilyn, and Kathy, who were always so helpful whenever I had questions or struggled. They provided a welcoming environment that made it exciting to learn and a pleasure to attend every week. In particular, I would like to sincerely thank Zdenka, who instructed and mentored me throughout the length of the project. Despite her busy schedule, she always made time to meet with me when I had questions, edit and advise my paper/presentations, or show me how to do procedures I struggled with. When I made mistakes, she never criticized, but instead took the time to explain what went wrong and how to fix it in the future. Without her guidance, this project would not have happened and I am truly grateful for her help this past year.

Lastly, I would like to thank Dave Adams for helping me find an MQP and always being there to answer my questions. He has given me advice not only with this project, but also regarding job and internship searches, on applying to graduate schools, and on setting personal life goals. Dave has been an amazing advisor and professor that always puts the students first, and I am glad I had the pleasure of working with him.

1.0 BACKGROUND

1.1 The p53 Tumor Suppressor

The p53 transcription factor regulates the expression of multiple genes whose protein products govern cell proliferation, apoptosis, cell metabolism, and the response of cells to DNA damage, hypoxia, and other forms of cell stress. These p53-mediated activities regulate organismal tissue growth and homeostasis, metabolism, ageing, and tumor formation. Given its many important biological functions, it is not surprising that p53 is one of the most studied proteins. This is especially true in the field of Cancer Biology, as mutation of the p53 gene has been found in approximately half of all human cancers (Hollstein et al., 1991).

Under physiological conditions, the p53 protein is present at very low levels within the cell. However, the p53 protein is stabilized and subjected to significant post-translational modification following exposure of cells to DNA damage or other types of stress. This stabilization of p53 leads to a rapid elevation of p53 protein levels, and the subsequent modifications to the p53 protein enhances its activity as a transcription factor (**Fig. 1**). These inputs lead to transcriptional activation of p53 target genes such as Puma or p21. Once expressed, these p53-downstream target proteins can induce apoptosis, cell cycle arrest, senescence, and/or activation of DNA repair. These responses occur in a cell-context-specific manner, and are also partly dependent on the amplitude and nature of the genetic or cellular damage. In addition, p53 can induce expression of its own negative regulators, including Mdm2 and MdmX, thereby limiting the duration of the p53 response within the cell.

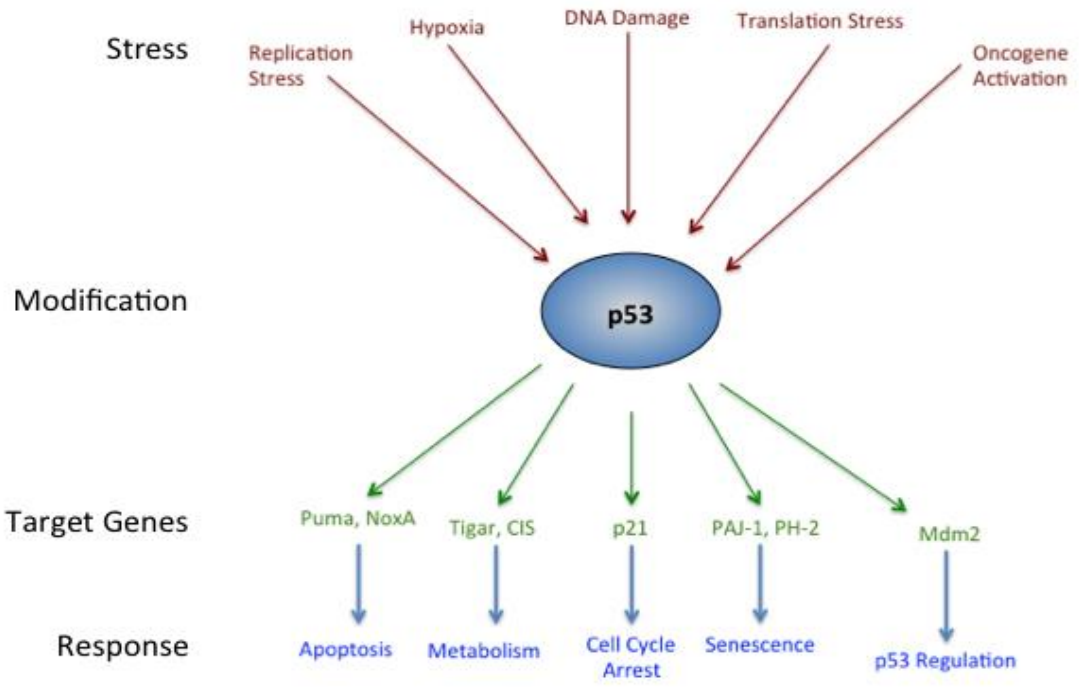

Figure 1: Overview of p53 Activation and Response. The p53 protein can be induced by many different cellular stresses and in turn activates a variety of responses that impact the life cycle of a cell.

1.2 Regulation of p53 by MDM proteins

Though p53 tumor suppressor activity is closely governed within the cell by many different mechanisms, it is clear from genetic experiments that Mdm2 and MdmX, two RING domain proteins, are the major regulators of p53 activities in mammalian cells (Jones et al., 1995; Parant et al., 2001). Mdm2 and MdmX are structural homologues that can bind to each other as well as to the p53 protein. These proteins serve to inhibit p53 functions under normal physiological (unstressed) conditions (Wade et al., 2010), and are encoded by separate genes whose expression is transactivated by p53. Thus, the level of p53 is auto-regulated in cells by the ability of p53 to induce its own negative regulators, Mdm2 and MdmX (Gannon and Jones, 2012).

Mdm2 inhibits p53 by binding with p53 and masking the p53 transactivation domain and by altering p53 stability (Kubbutat et al., 1997). Specifically, Mdm2 acts as an E3 ubiquitin ligase, placing ubiquitin moieties onto the p53 protein thereby targeting p53 for proteosomal degradation. Both transactivation domain masking and ubiquitin stimulating Mdm2 activities prevent p53 from functioning as a transcription factor to limit cell growth and promote apoptosis. In contrast, MdmX inhibits p53 by binding to p53 and masking its transactivation domain without destabilizing p53 (Francoz et al., 2006). Together, the two proteins negatively regulate p53 (**Fig. 2**).

Reduced amounts of Mdm2 or MdmX within a cell lead to higher basal levels of p53 activity. Likewise, preventing the ability of these MDM proteins to complex with p53 also promotes p53 stabilization and/or p53 activation, and many of the cell stress signals that promote p53 up-regulation in a cell do so by phosphorylating Mdm2, MdmX, or p53, thereby altering Mdm-p53 signaling (Wade et al., 2010; Gannon and Jones, 2012). Once stress signaling subsides, the MDM proteins are free to complex and inhibit p53 activity, thus returning p53 levels back to unstressed levels within the cell. Conversely, up-regulation or overexpression of Mdm2 or MdmX can result in decreased levels of p53 within the cell, and Mdm2 and MdmX gene amplification and overexpression promotes cancer in mice (Jones et al., 1996; Marine and Jochemsen, 2005) and is frequently associated with human cancers. Thus, extensive efforts have been focused on developing inhibitors of Mdm2-p53 and MdmX-p53 signaling for human cancer therapy.

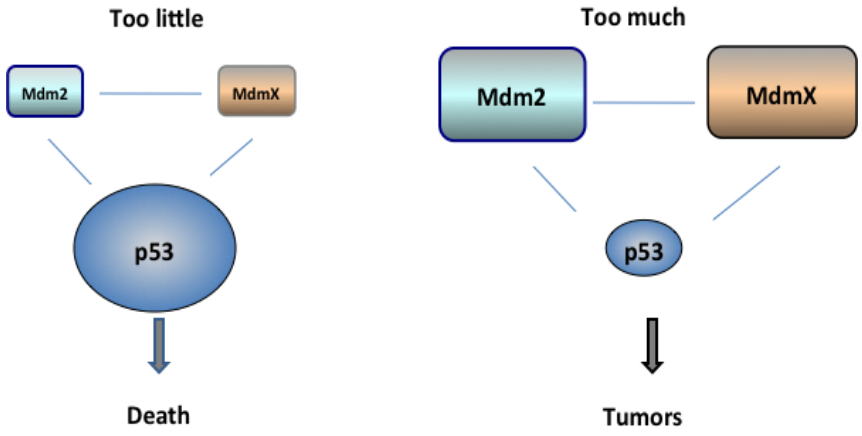

Figure 2: Regulation of p53 by Mdm2 and MdmX. The Mdm proteins work together to negatively regulate the tumor suppressor p53. Amplification or over-expression of Mdm2 and MdmX are seen commonly in human tumors.

1.3 MdmX Suppresses Tumorigenesis in p53-null Mice

The oncogenic potential of Mdm2 and MdmX is well established by numerous studies in the literature (Oliner et al., 1992; Francoz et al., 2006). However, in 2008, MdmX was surprisingly found to also play a role in suppressing tumorigenesis in p53-deficient mice (Matijasevic et al., 2008a). Mice that are deleted for both p53 and MdmX develop tumors at a significantly faster rate than mice lacking only p53. Even mice heterozygous for MdmX display tumorigenesis at a delayed rate compared to p53-null mice homozygous for MdmX. Fifty percent of p53-null mice develop tumors by 25 weeks of age, while fifty percent of double-null mice present with tumors at 20 weeks (Matijasevic et al., 2008a).

At the cellular level, the loss of MdmX increases the growth potential in p53-deficient mouse tumors cells. Tumor cells lacking MdmX divide faster and accumulate to higher densities, suggesting that MdmX is at least partly responsible in controlling proliferation when p53 is absent. A reintroduction of MdmX into double-null mouse cells

reverses the increase in growth potential back to levels equivalent to p53-null cells (Matijasevic et al, 2008a).

Cells harvested from thymic stromal tumors of p53-null mice show that MdmX has a profound impact on chromosome stability and mitotic polarity. FACS analysis and mitotic spreads reveal higher chromosome numbers and DNA content when MdmX is present (Matijasevic et al, 2008a). It is hypothesized that MdmX prevents chromosome loss due to its ability to promote bipolar mitosis when p53 is missing. Bipolar mitosis, or the even segregation of chromosomes in two directions during mitotic division, is seen more frequently in mouse tumor cells containing MdmX than in those p53-deficient cells lacking MdmX. Conversely, there is a greater than 50% decrease in the number of cells with multipolar spindles when MdmX was re-introduced to MdmX/p53 double-null mouse tumor cells (Matijasevic et al, 2008a).

The reintroduction of MdmX into MdmX/p53-null mouse tumor cells decreases proliferation rate, the occurrence of multipolar spindles and stabilizes the number of chromosomes in these cells. These effects may be the reason for the observed suppression of tumorigenesis in p53-deficient mice. Analysis of mouse embryonic fibroblasts showed that MdmX promotes bipolar mitosis and genetic stability in a p53-independent manner (Matijasevic et al., 2008b).

In contrast to what was seen with MdmX, cells deleted for Mdm2 displayed no significant difference on proliferation, chromosome stability or bipolar mitosis in p53-deficient mouse cells (Matijasevic et al., 2008a). Furthermore, exogenous MdmX was capable of suppressing cell proliferation, chromosomal loss, and multipolar mitosis in triple deleted cells (MdmX, Mdm2 and p53-deleted), indicating that Mdm2 is not

required for these p53-independent functions of MdmX (Blodgett, 2012). However, it is unclear if these p53-independent and Mdm2-independent functions of MdmX exist within human cells.

1.4 Cancer and Genomic Stability

Prior to the onset of mitosis in eukaryotes, a cell will have duplicated its centrosome and genomic DNA during S phase. During the early phases of mitosis, the chromosomes condense and align along the metaphase plate. Microtubule spindle fibers attach to the centrosomes and the kinetochore protein structures that form at the centromere of each sister chromatid, linking the chromatids to the two centrosomes (hence "bipolar") located at opposite poles of mitotic spindle and normally creating a highly symmetrical structure (**Fig. 3**). The chromosomes are then diametrically separated during anaphase to yield equal proportions of genetic material to each daughter cell.

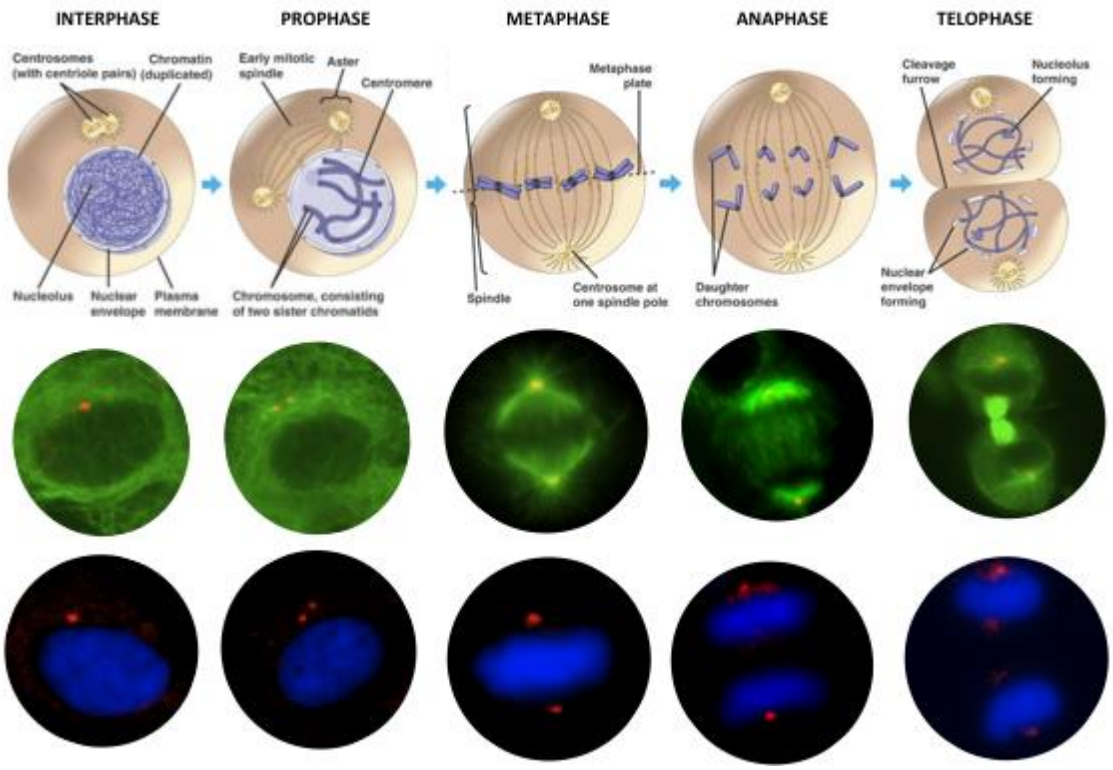

Figure 3: Stages of Normal Mitosis Resulting in Bipolar Chromosome Segregation. Fluorescent images are of MB-157 cells from this MQP experiment, while textbook images are from mrsnatzkesbiology.blogspot.com.

In many cancer cells, the number of centrosomes at cell division exceeds two. These "supernumerary centrosomes" lead to the formation of more that two spindle poles ("multipolar") and promotes asymmetric segregation of the chromosomes (Fukasawa, 2005). Thus, the resulting daughter cells inherit abnormal numbers of chromosomes. Once the progenitor cells replicate their DNA, they will have aberrant chromosome numbers (ploidy). This lack of duplicated chromosome number (diploidy) results in aneuploidy, with the daughter cell displaying either hyperploidy (more than normal) or hypoploidy (less than normal). Interestingly, aneuploidy is a very common feature of most cancer cells (Saunders, 2005), and the genetic instability of cancer cells may facilitate subsequent tumor progression or metastasis.

Although the precise mechanisms leading to aneuploidy in cancer cells remain to be established, a potential role for unequal inheritance of chromosomes in cancer development was hypothesized by Theodor Boveri almost a century ago. In his 1902 seminal work, Boveri discovered that abnormal chromatin composition in the daughter cells was likely due to abnormal spindle pole formation and not due to inadequate separation of cytoplasm (Boveri, 1902). The multipolar segregation of chromosomes to daughter cells leads to insufficient genetic material that is missing regulatory elements for cell growth, and leads to a cancerous cell. With this information, it is easy to see that when more multipolar divisions occur, there is a greater probability of a chromosome combination that causes malignant tumors (Boveri, 1902).

In tumor cell lines, supernumerary centrosomes correlate with the reduction or loss of p53 activity (Fukasawa et al., 1996; Fukasawa, 2005). After cytokinesis, each daughter cell receives one centrosome that must be duplicated once before mitosis. The initiation of centrosome duplication during S phases of the cell cycle is dependent on the cell passing the restriction checkpoint at the G1-S boundary. This checkpoint is governed by cyclin-dependent kinase 2 (CDK2) and cyclinE (Dulic et al., 1992; Koff et al., 1992), which phosphorylate the Rb protein and facilitate entry of the cell into S phase. The CDK2/cyclinE complex is regulated by the cyclin dependent kinase inhibitor p21. As noted previously, the gene encoding p21 (CDKN1A) is an important target of p53 transactivation (Harper, 1997). By continuously inhibiting CDK2/CyclinE, p21 (when upregulated by p53) blocks S phase and the initiation of centrosome duplication. In cells lacking p53, premature activation of CDK2/CyclinE triggers multiple centrosome duplications (Tarapore et al., 2001; Nayak and Das, 2002), leading to supernumerary

centrosomes. However, while centrosome amplification appears necessary for multipolar spindle formation during M phase, it is unclear if additional changes within the cell must also occur.

As most tumor cells possess multiple centrosomes, it has been proposed that the tumor cells retain some semblance of normal cell division by clustering their supernumerary centrosomes into two poles, allowing the cell to undergo a pseudo-bipolar mitosis. This would prevent the wholesale loss of massive numbers of chromosomes during a cell division, permitting survival of the tumor cell, albeit with some potential loss or gain of ploidy. Thus, pseudo-bipolar mitosis in cells with amplified centrosomes may facilitate cancer progression. Alternatively, this mechanism for preventing multi-polar mitosis may also enhance the genomic stability of the cell, reducing the likelihood of loss of other tumor suppressors or other further neoplastic changes within the cancer cell. Both possibilities have been proposed, but neither has been examined directly in an experimental setting (Fukasawa, 2005).

Based upon the Boveri hypothesis and previous studies of the ability of MdmX to both promote pseudo-bipolar mitosis and suppress spontaneous tumorigenesis in p53-deficient mice, the Jones lab has proposed that the genomic stability promoted by MdmX in p53-deficient mouse cells is linked with its tumor suppressing functions in mice, and that MdmX plays a similar role in human cancer. My research within this lab has examined the effects of MdmX on chromosome number and multipolarity in two human breast cancer cell lines. I hypothesize that the introduction of MdmX into human cells will increase chromosome stability and decrease the frequency of multipolar spindles in cells with compromised p53 function.

2.0 PROJECT PURPOSE

Previous research established that MdmX plays an Mdm2-independent role in establishing genomic stability in p53-deficient mouse cells. The purpose of this project is to determine the effect of MdmX on chromosome number and spindle polarity in human cancer cells. A better understanding of the mechanisms of MdmX on chromosome maintenance and spindle polarity will give insight on how MdmX suppresses tumorigenesis. MdmX has been proposed to be an important target for clinical intervention against cancer, and several labs are presently investigating small molecule interruption of MdmX function with the goal of up-regulating p53 tumor suppressing activity in cancer cells. Thus, it is crucial that we achieve a finer understanding of the tumorigenic effects of MdmX in human cancers that contain or lack functional p53. Two different human mammary tumor lines will be studied: MB-231 (containing high levels of mutated p53, low p53 activity and high levels of MdmX) and MB-157 (containing no detectable p53 protein and very low levels od MdmX). Cells transfected with a plasmid encoding human MdmX will be assayed for cell proliferation, chromosome number, and spindle polarity.

3.0 METHODS

3.1 Cells

All experiments were performed using human tumor cells cultured from the mammary gland. Cell lines MDA-MB-231 and MDA-MB-157 were obtained from American Type Culture Collection (ATCC). p53 in MB-231 cells has a substitution mutation in codon 8 and p53 in MB-157 cells has a truncating mutation. Western blot analyses show that MB-231 has high levels of a mutated p53 protein and high levels of MdmX, while MB-157 has no detectable p53 protein and very low levels of MdmX (Lam et al, 2010).

MB-231 and MB-157 cells have very different growth characteristics and morphology. MB-157 cells grew at a much slower rate than MB-231 cells, and are more rounded and loosely attached to the surface (**Fig. 4**). MB-231 cells were grown in DMEM media supplemented with 10% serum, penicillin and streptomycin. To aid growth, MB-157 cells were grown on gelatinized plates in DMEM media supplemented with nonessential amino acids, 15% serum, penicillin and streptomycin.

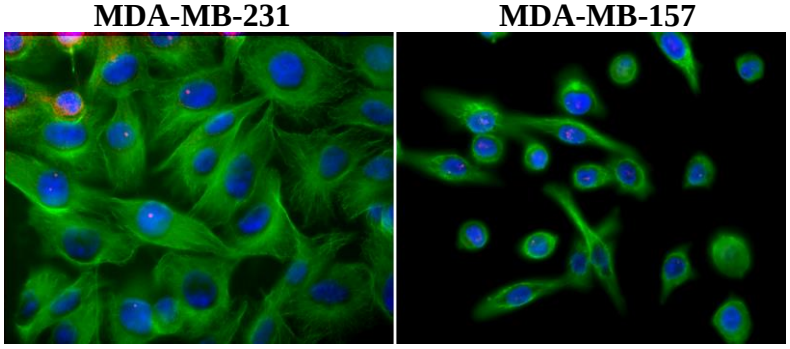

Figure 4: Morphology Differs Between MB-231 and MB-157 Cell Lines. MB-231 cells (low p53 activity, high MdmX) are more flattened and attached to the plate while MB-157 cells (no p53 activity, low MdmX) are rounded and loosely attached.

3.2 Plasmid Purification, Digestion and Linearization

Plasmid DNA encoding mouse MdmX was isolated from *E. coli* using the *Qiagen Maxiprep* kit. Plasmid identity was confirmed by a double digest using restriction enzymes Xho1 and EcoRV, which cut near the beginning and end of the MdmX gene (**Fig. 5A**). The plasmid map indicated a band of 1.6kb for MdmX and roughly 5kb for the remaining plasmid vector. Gel electrophoresis showed bands at each of these locations, verifying that the MdmX gene was present (**Fig. 5B**). The plasmid was linearized for transfection with Pvu1. Gel electrophoresis was used to confirm the Pvu1 digestion.

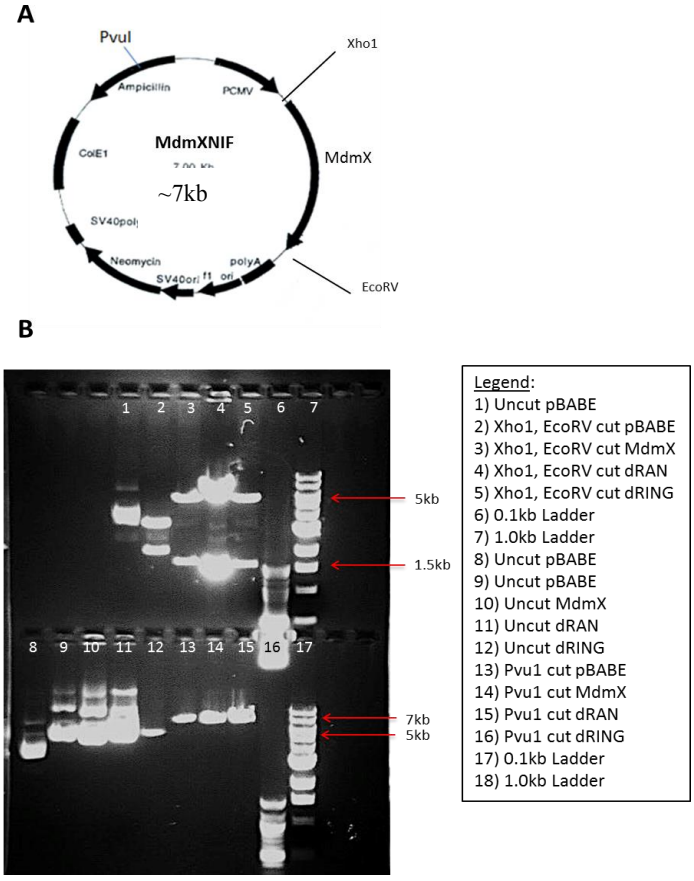

Figure 5: Digestion of MdmX with Xho1, EcoRV and Pvu1. Plasmid DNA was double digested using Xho1 and EcoRV to confirm plasmid length (A). Undigested plasmid was linearized with Pvu1 to increase transfection efficiency. Gel electrophoresis was done to confirm digestions (B). dRAN and dRING are MdmX plasmids with deleted Ran and RING domains used in parallel studies.

3.3 Transfection

Cells were plated on a 6-well plate and grown in Dulbecco's Modified Eagles media (DMEM) with no antibiotics until they were 70-80% confluent. A co-transfection of MdmX and pBABE plasmid containing puromycin resistance gene for selection on puromycin was performed for both cell lines. *Invitrogen*'s transfection reagent (TR) *Lipofectamine 2000* (Invitrogen) was used for transfection of MB-231 cells. The transfection of MB-157 cells with Lipofectamine 2000 was unsuccessful, so two different transfection reagents from *Roche XtremeGene-9* (X9) and *XtremeGene-HP* (HP) were used for MB-157 cells under a variety of conditions to provide a greater chance of a successful transfection. Transfection reagents work by surrounding the plasmid DNA with a positive coating that has a similar biochemistry to the cell membrane. This allows for the passage of the plasmid into the cell, where it can integrate into the genome of the transfected cell (**Fig. 6**). There are many conditions that can impact the success rate of this integration, such as the charge or polarity of the transfection reagent, which is why different cells require different transfection reagents.

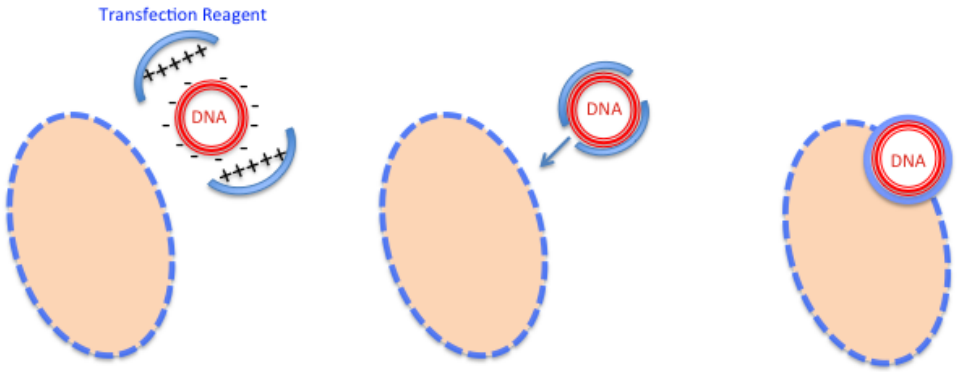

Figure 6: Transfection Reagent Mechanism. Transfection reagents surround negatively charged DNA to allow passage into positively charged cell membrane.

Transfection conditions applied in this study are summarized in **Table 1**. When cell survival following transfection was satisfactory (+++), cells were split 24 hours after transfection and selection for puromycin resistance started 24 hours later. Transfected cells with lower survival rates were propagated for several days or weeks before beginning selection.

	MB-231	MB-157	
	Lipofectamine	**XtremeGene-9**	**XtremeGene-HP**
pBABE : MdmX	**1 : 6**	**1 : 6**	**1 : 6**
TR : DNA	**2.5 : 1**	**6 : 1**	**1 : 1**
Survival	**+++**	**++**	**+**

Table 1: Transfection and Cell Survival

3.4 qRT-PCR

Cells were harvested by scraping in Trizol and stored at -80°C before RNA extraction. RNA was isolated using *Zymo* and *Qiagen* RNA extraction kits. 2µg of RNA was used for first-strand cDNA synthesis by combining with 2µL of random hexamers, 1 µL of 10mM dNTP mix, and DEPC water up to 10µL. Samples were then incubated for 5 minutes at 65°C and cooled for one minute. A mixture of 2µL of 10X RT buffer, 4µL of 25mM $MgCl_2$, 2µL of 0.1M DTT, and 1µL of RNaseOUT Recombinant Ribonuclease inhibitor was added to each sample. SuperScript II RT was added to each sample and then incubated for 10 minutes at 25°C, 50 minutes at 42°C, 15 minutes at 70°C, and then cooled. 1µL of RNase H was added to each sample and incubated at 37°C for 20 minutes. All RT-PCR reagents used were from *Invitrogen*.

2µL of each cDNA sample was then placed in PCR tubes containing a master mix of 12.1µL GoTag Master Mix, 10.5µL H_20, 0.2µL forward, and 0.2µL reverse primer.

All samples were tested in replicates for mouse MdmX, for human actin and for human MdmX. Quantitative, real-time polymerase chain reaction, or qRT-PCR, was performed for all samples in replicates for mouse MdmX, human housekeeping gene (β-actin), and for endogenous, human MdmX. The results were normalized for equal levels of β-actin in each sample.

3.6 Functional Analyses

Transfected cells were analyzed for cell proliferation, chromosome number, and spindle polarity.

Proliferation Assay. MB-231 cells were plated at a density of $0.02x10^6$ cells per well in a 6-well plate, with media being changed every three days. Each day, two wells per genotype were harvested and counted. MB-157 cells were not used in this experiment.

Metaphase Spreads. Proliferating cells were incubated in pre-warmed media containing colcemid (0.02μg/mL) for 90 minutes to inhibit progression of mitotic cells to anaphase, harvested and then incubated in hypotonic solution for 18 minutes at 37°C. Cells were fixed using a few drops of 3:1 methanol to acetic acid and were placed on ice for an hour. The fixed, swollen cells were dropped on microscope slides, dried and then DAPI (4'-6'-diamidino-2-phenylindole) stained for counting using fluorescent microscopy.

Immunofluorescence Staining. Cells were grown on coverslips, fixed in methanol and stored at -20°C. Primary antibodies used were mouse anti-α-tubulin (Sigma) for

microtubules and rabbit anti-γ-tubulin (Sigma) for centrosomes. Secondary antibodies were anti-mouse Alexa 488 and anti-rabbit Alexa 594 (Invitrogen) (**Fig. 7**). DAPI was used to counter-stain chromosomes blue. Cells were then analyzed using a fluorescence microscope and images were captured using *Metamorph* imaging software.

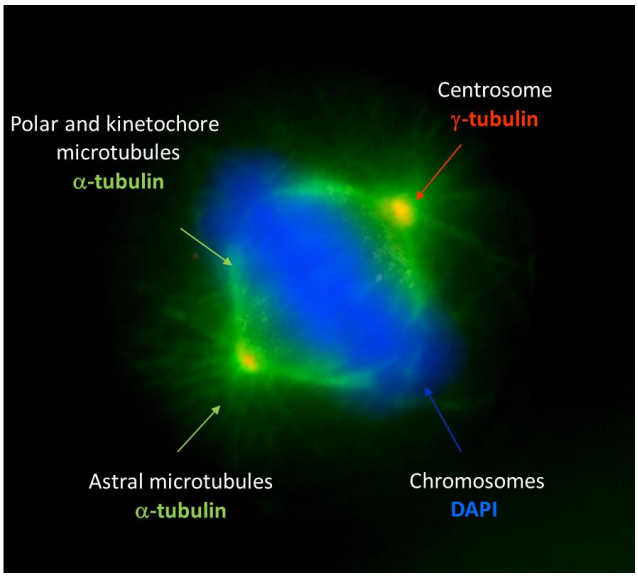

Figure 7: Immunofluorescence Staining. For immunofluorescence staining, anti-α-tubulin was used for microtubules (green), anti-γ-tubulin for centrosomes (red), and DAPI for DNA (blue).

4.0 RESULTS

The purpose of this project is to determine the effect of MdmX on chromosome stability and spindle polarity in human breast cancer cells. Human breast cancer cells lacking functional p53 were co-transfected with plasmid containing a full-length mouse MdmX cDNA placed under transcriptional control of a viral long terminal repeat (MLV-LTR) promoter and a second plasmid bearing puromycin drug resistance gene placed under transcriptional control of a cytomegalovirus (CMV) promoter. Transfected cells were selected for resistance to puromycin, and screened by quantitative polymerase chain reaction for the presence of MdmX. Functional assays were performed using the MdmX transfected cells to determine the effect of MdmX on cell proliferation, chromosome number and spindle polarity. All functional assays were done in parallel with cells transfected with only the puromycin resistance plasmid as a control.

4.1 qRT-PCR

Transfected cells were screened for the levels of exogenous mouse MdmX mRNA by performing multiple quantitative RT-PCR reactions (**Fig. 8**). All results were normalized to the housekeeping gene, human actin, and expressed as a fold increase over the mock control. The levels of mouse MdmX mRNA are four to forty times above the levels detected in mock control, indicating that transfection was successful. MB-157 transfectants generated by HP transfection reagent show lower levels of mouse MdmX compared to the clones generated by X9 reagent. The levels of MB-157 HP transfectants are still roughly four times higher than in control cells. Basal levels of endogenous

human MdmX (hMDMX in green) were higher in MB-231 cells than MB-157 cells, as expected from the literature (Parant et al., 2001).

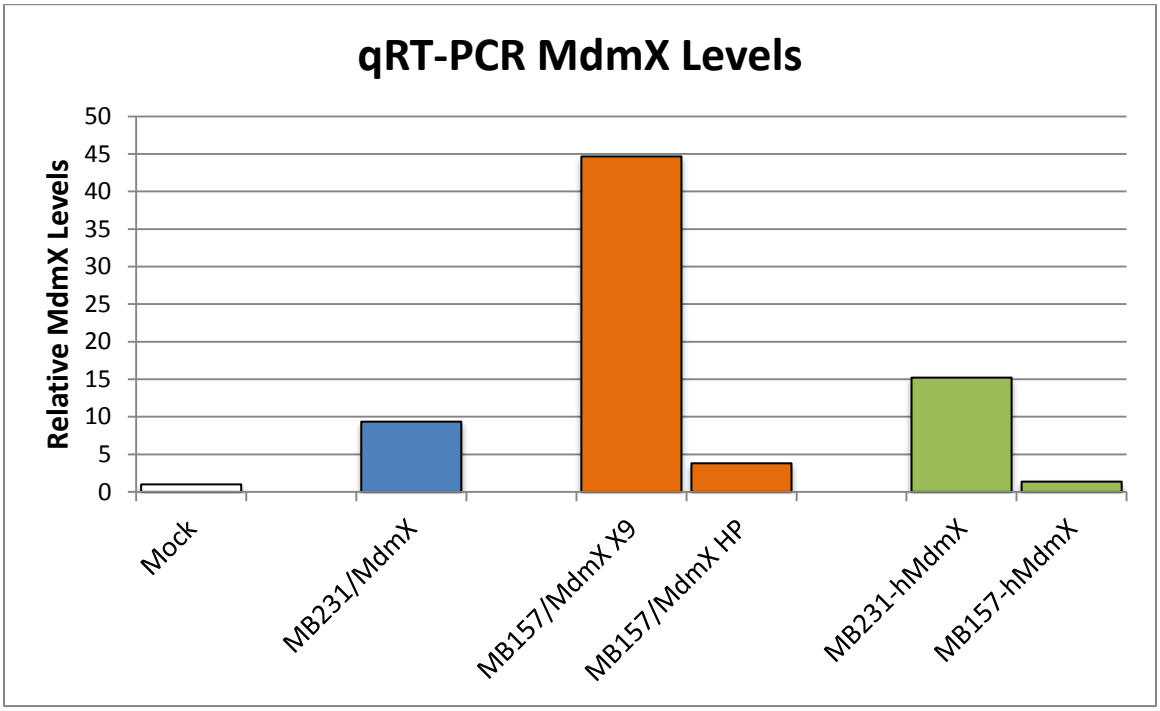

Figure 8: Ectopic MdmX Expression in Transfected Cells. qRT-PCR results for mouse MdmX and for the endogenous hMdmX were normalized to human actin. The results for MB-231 cell line represent the average from four separate clones and human MdmX levels are the average from both genotypes for each cell line. (X9 and HP stand for the transfection agents used).

4.2 Exogenous MdmX does not Affect Proliferation Rate of MB-231 Cells

A cell proliferation assay showed that MB-231 cells transfected with mouse MdmX proliferate at a similar rate to mock cells (**Fig. 9**). There was no significant difference in growth seen between MdmX transfectants and mock cells after 12 days of counting.

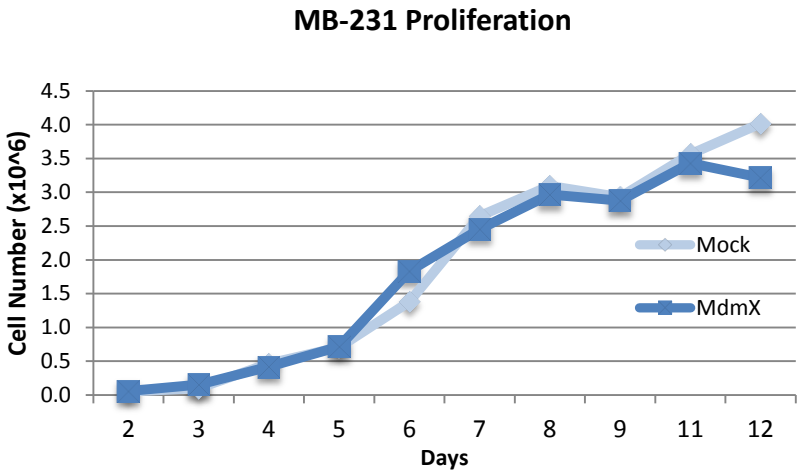

Figure 9: Exogenous MdmX does not Effect Proliferation Rate in MB-231 Cells. $0.05x10^6$ cells were plated to each well in 6 well plates and two separate samples were counted daily for each genotype with 3 measurements for each sample.

4.3 Effect of MdmX on Chromosome Maintenance

Metaphase spreads were prepared from MdmX transfectants and control cells, and then DAPI stained for chromosome counting. Exogenous MdmX does not seem to affect chromosome number in the population of MB-231 cells (low p53, high endogenous MdmX) (**Fig. 10**), while MB-231, MB-157 cells (no p53, low endogenous MdmX) transfected with MdmX show drastic increase in chromosome number compared to the mock control cells. On average, there were nearly twice as many chromosomes in MdmX transfected cells. The results for MB-157 in Figure 10 represent mean value (plus/minus standard deviation) of chromosomes in both HP and X9 clones. The increase in chromosome number is similar for the two clones and does not reflect the difference in the levels of MdmX expression between HP and X9.

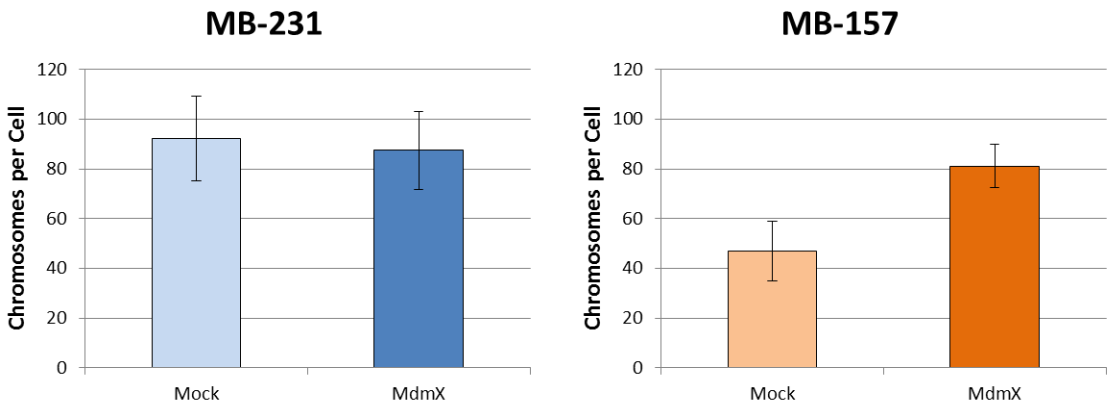

Figure 10: Effect of MdmX on Chromosome Number. Metaphase spreads of cells were stained with DAPI and at least 30 spreads for each genotype were counted using fluorescence microscopy. The data shown are the averages from two clones. For MB-157 p-value < 0.0001; for MB-231, p-value= 0.1794).

To better illustrate the difference in chromosome number between mock and MdmX transfectants in MB-157 cells, values were plotted as a range of chromosomes versus cell number (**Fig. 11**). The data in Figure 11 demonstrates a drastic shift in the number of cells with increased chromosome number upon MdmX introduction. Most cells transfected with the mock plasmid have between 36 and 55 chromosomes, while a majority of MdmX transfected cells have between 73 and 84 chromosomes.

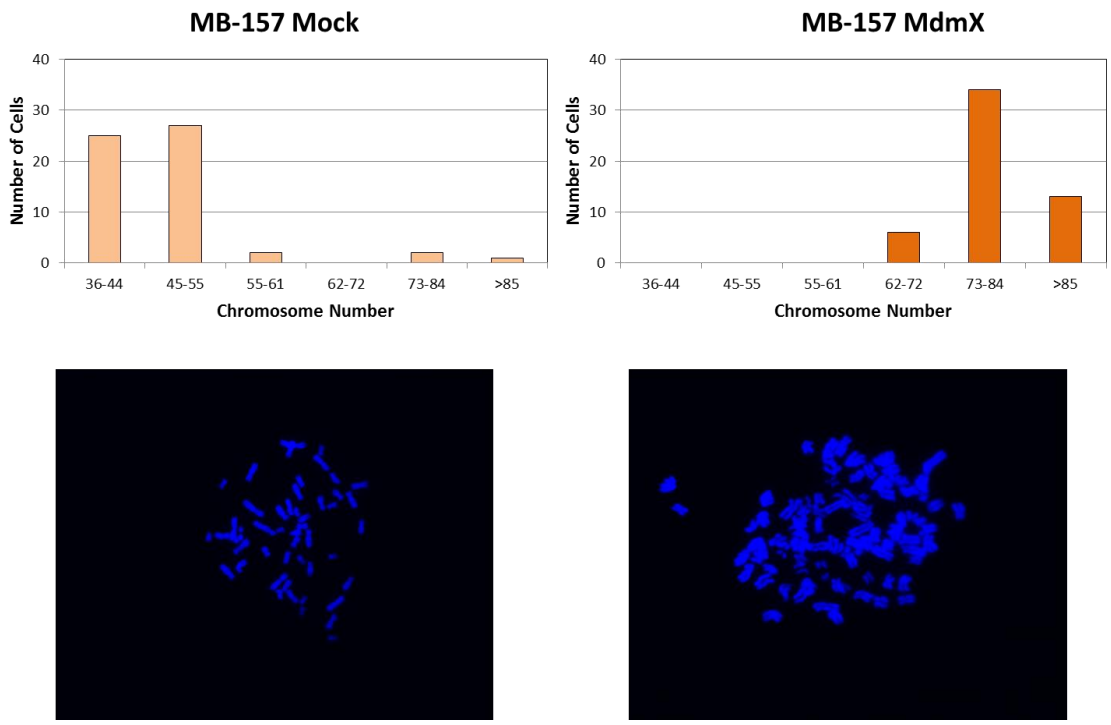

Figure 11: MdmX Increases Chromosome Number in MB-157 Cells. Chromosome spreads were prepared for mock and MdmX transfected MB-157 cells. Chromosomes were stained with DAPI and counted in two separate experiments. Data is based on at least 50 counts for both mock and MdmX transfectants.

4.4 Effect of MdmX on Spindle Polarity

MdmX was previously found to have an Mdm2-independent function in promoting bipolar mitosis in mouse tumor cells. To examine the effect of MdmX on spindle polarity, immunofluorescence staining was done on both human breast cancer cell lines. Both MB-231 and MB-157 cells show a significant decrease in the percent of cells containing multipolar spindles in MdmX transfectants (**Fig. 12**). When MdmX is transfected, the occurrence of multipolar cells drops to roughly half of those seen in mock transfectants. Additionally, MB-231 cells as a whole display only one third the number of multipolar cells as compared to MB-157 cells.

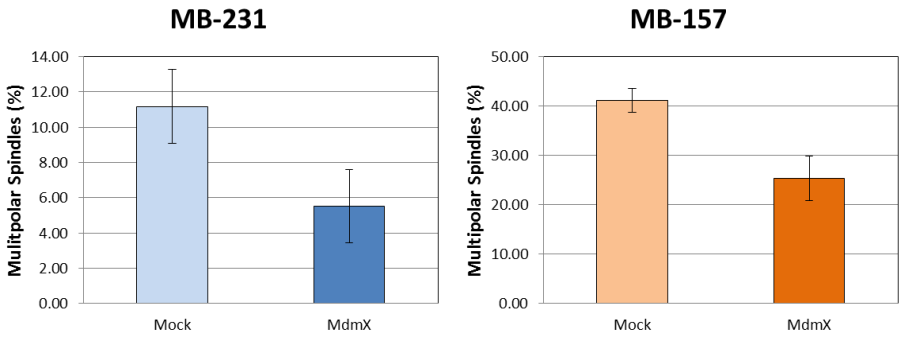

Figure 12: MdmX Decreases Number of Multipolar Spindles in Human Breast Cancer Cells. Cells were stained for immunofluorescence and observed using fluorescence microscopy. Mitotic cells were counted to determine the incidence of multipolar spindles. MB-231 data is based on over 2000 mitotic cells scored and for MB-157 data over 1000 mitotic cells scored for each genotype. The p-value between mock and MdmX transfectants is $< 0.0001$for both cell lines.

The high initial number of multipolar mitosis in the MB-157 cell line allowed for more detailed analysis of the transition between multipolarity and bipolarity. These examinations suggest that the reduction in multipolar spindles is linked to centrosome clustering. Closer inspection of bipolar mitotic cells shows that nearly 20% of the bipolar spindles in MdmX transfectants can be classified as pseudo-bipolar (**Fig. 13A**). Pseudo-bipolar mitotic cells contain supernumerary centrosomes, yet still segregate chromosomes in a bipolar fashion. MB-157 cells with MdmX tend to cluster centrosomes together at opposite poles, preventing multipolar segregation of chromosomes (**Fig. 13B**). Panels B1 in Figure 13 display an image of a normal, bipolar mitotic spindle with two centrosomes. B2 shows several examples of pseudo-bipolar spindles with supernumerary centrosomes clustered together, symmetrically or asymmetrically, toward two poles. Images on the left-most and right-most sides in panel B3 illustrate relatively well organized tetra- and tri-polar spindles, respectively, that may or may not result in 4 or 3 viable progeny. The results suggest that the role of MdmX in promoting bipolar mitosis derives from its

function in centrosome clustering. In this process, mitotic cells with three centrosomes can generate the asymmetric bipolar spindle with one centrosome on one pole and two centrosomes on the opposite pole, resulting in an equal, bidirectional segregation of chromosomes. The far right column of Figure 13B shows how three centrosomes can act to pull the chromosomes in three different directions, or that two of the centrosomes can cluster together and segregate the chromosomes in two opposite directions. In a similar fashion, the left-hand column in Figure 13B shows how a mitotic cell with four centrosomes can act in the same way.

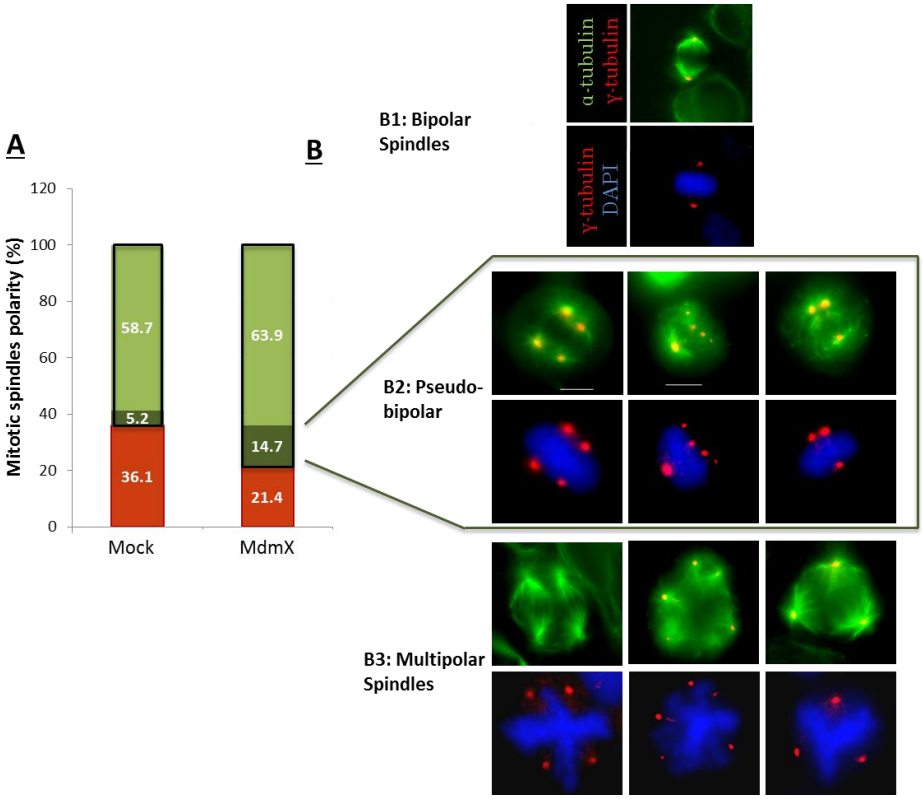

Figure 13: MdmX Increases Number of Pseudo-Bipolar Spindles and Decreases Number of Multipolar Spindles. Immunofluorescence staining of MB-157 cells shows high levels of multipolar cells and increased levels of pseudo-bipolar spindles in MdmX transfectants (A). Shown here are examples of bipolar, pseudo-bipolar and multipolar spindles (B). Scale bars = 10 µm.

5.0 DISCUSSION

5.1 Conclusions

MdmX has been recently found to suppress immortalization and inhibit tumorigenesis in p53-deficient cells and mice, respectively (Matijasaevic et al., 2008a). Recent work in the Jones lab indicates that MdmX increases the chromosomal stability of p53-null mouse tumor cells by suppressing multipolar spindle formation during cell mitosis (Matijasaevic et al., 2008a). In this MQP, we sought to determine whether MdmX could likewise promote genomic stability in p53-deficient human breast cancer cells. Our results demonstrate that MdmX expression correlates with the decreased formation of multipolar mitotic spindles and with an increase in chromosome number in p53-deficient, human breast cancer cells. These findings suggest that MdmX may also have a p53-independent role in suppressing human breast cancer.

Two different breast cancer cell lines, MB-231 and MB-157, were used in this study. MB-231 cells contain high levels of mutated p53 protein and high levels of endogenous MdmX protein. In contrast, the p53 protein is undetectable in MB-157 cells due to a truncating mutation within the p53 gene, and endogenous MdmX is present at very low levels in these cells. Both cell lines display multipolar mitosis, with MB-157 cells (low MdmX) having approximately four times the amount of multipolar mitosis (40%) as MB-231 cells (10%) (high MdmX). Introduction of exogenous MdmX by transfection reduces the incidence of multipolar mitosis by half, and increases the incidence of pseudo-bipolar mitosis in both cell lines. Interestingly, increased chromosomal stability was detected in MB-157 cells, but not in MB-231 cells, suggesting that either the MdmX-induced threshold for altering ploidy was different in the two cell

lines, or that the already large amounts of hyperploidy in the MB-231 cells made it difficult to assess increased chromosomal stability. Alternatively, differences in chromosomal stabilization between these two MdmX-transfected cell lines may reflect the differences in their p53 status. If MdmX-mediated centrosome clustering promotes transition from multipolar-to-pseudo-bipolar mitosis and consequently prevents chromosome loss, then the non-functional p53 protein present in MB-231 cells may interfere with the process downstream from centrosome clustering.

A proliferation assay was performed with MB-231 cells. The results indicate that MdmX transfection does not alter the rate of human breast cancer cell proliferation. This contrasts with previous findings made in the Jones Lab, in which exogenous MdmX expression was found to suppress the proliferation of mouse MdmX-null, p53-null tumor cells (Matijasevic et al., unpublished). The differing effects of MdmX on the proliferation of mouse and human tumor cells could be due to the presence of mutated p53 protein and already high levels of endogenous MdmX in the human MB-231 cells. Alternatively, the results may suggest that the effects of MdmX on spindle polarity are unlinked to cell proliferation. This latter possibility would underscore our hypothesis that MdmX does not suppress tumorigenesis by altering cell proliferation, but rather by increasing pseudo-bipolar mitosis and genomic stability.

5.2 Experimental Setbacks

Due to the absence of p53 protein and low levels of MdmX protein, the MB-157 cells are ideal for the study of p53-independent, MdmX-mediated cellular processes in human cells. However, these cells grow slowly and are extremely difficult to transfect.

Therefore, transfections had to be repeated several times using different transfection reagents and different combinations of conditions for transfection and selection. These "trouble-shooting" types of experiments, while common in science, precluded complete functional analyses of the MdmX-transfected cells within the timeframe of this project.

5.3 Future Experiments

Once *in vitro* analyses are completed, the next step will be to perform *in vivo* experiments with nude mice to compare the tumorigenic potential of MdmX-transfected and control human breast tumor cells by transplanting them into the mouse mammary fat pad and monitoring tumor formation. Those experiments would directly assess the effects of pseudo-bipolar mitosis with tumorigenesis *in vivo*, and address *in vivo* the validity of the Boveri hypothesis that abnormal spindle formation and unequal chromosome numbers lead to cancer.

Additionally, in unpublished studies by the Jones lab, the effects of MdmX on proliferation and genomic stability have been linked to a specific domain on the MdmX gene. This MdmX domain is a Zn-Finger like structure that is analogous to other cellular proteins that bind Ran, a GTPase protein that regulates nuclear compartmentalization and genome segregation. Future experiments will include structure-function comparisons between full-length MdmX and mutant MdmX lacking the Ran domain in control of centrosome clustering and genome stability. Eventually, complex formation between Ran and MdmX via this Zn-finger domain will also be examined. Understanding the link between domain and function will develop a better understanding of the MdmX gene and its role in tumor suppression.

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