Loss of miR-223 and JNK Signaling Contribute to Elevated Statmhm in Malignant Pleural Mesothelioma

Kimberly A. Birnie, Yan Y. Yip, Dominic C.H. Ng, Michaela B. Kirschner, Glen Reid, Cecilia M. Prêle, Arthur W. (Bill) Musk, Y.C. Gary Lee, Philip J. Thompson, Steven E. Mutsaers, and Bahareh Badrian

Malignant pleural mesothelioma (MPM) is often fatal, and studies have revealed that aberrant miRNAs contribute to MPM development and aggressiveness. Here, a screen of miRNAs identified reduced levels of miR-223 in MPM patient specimens. Interestingly, miR-223 targets Statmhm (STMN1), a microtubule regulator that has been associated with MPM. However, whether miR-223 regulates STMN1 in MPM and the functions of miR-223 and STMN1 in this disease are yet to be determined. STMN1 is also regulated by c-Jun N-terminal kinase (JNK) signaling, but whether this occurs in MPM and whether miR-223 plays a role are unknown. The relationship between STMN1, miR-223, and JNK was assessed using MPM cell lines, cells from pleural effusions, and MPM tissue. Evidence indicates that miR-223 is decreased in all MPM tissue compared with normal/healthy tissue. Conversely, STMN1 expression was higher in MPM cell lines when compared with primary mesothelial cell controls. Following overexpression of miR-223 in MPM cell lines, STMN1 levels were reduced, cell motility was inhibited, and tubulin acetylation induced. Knockdown of STMN1 using siRNAs led to inhibition of MPM cell proliferation and motility. Finally, miR-223 levels increased while STMN1 was reduced following the re-expression of the JNK isoforms in JNK-null murine embryonic fibroblasts, and STMN1 was reduced in MPM cell lines following the activation of JNK signaling.

Implications: miR-223 regulates STMN1 in MPM, and both are in turn regulated by the JNK signaling pathway. As such, miR-223 and STMN1 play an important role in regulating MPM cell motility and may be therapeutic targets.

Chromatin, Gene, and RNA Regulation

Mol Cancer Res; 13(7) July 2015

Elevated Stathmin in Malignant Pleural Mesothelioma

Elevated Stathmin in Malignant Pleural Mesothelioma

Malignant pleural mesothelioma (MPM) is often fatal, and studies have revealed that aberrant miRNAs contribute to MPM development and aggressiveness. Here, a screen of miRNAs identified reduced levels of miR-223 in MPM patient specimens. Interestingly, miR-223 targets Statmhm (STMN1), a microtubule regulator that has been associated with MPM. However, whether miR-223 regulates STMN1 in MPM and the functions of miR-223 and STMN1 in this disease are yet to be determined. STMN1 is also regulated by c-Jun N-terminal kinase (JNK) signaling, but whether this occurs in MPM and whether miR-223 plays a role are unknown. The relationship between STMN1, miR-223, and JNK was assessed using MPM cell lines, cells from pleural effusions, and MPM tissue. Evidence indicates that miR-223 is decreased in all MPM tissue compared with normal/healthy tissue. Conversely, STMN1 expression was higher in MPM cell lines when compared with primary mesothelial cell controls. Following overexpression of miR-223 in MPM cell lines, STMN1 levels were reduced, cell motility was inhibited, and tubulin acetylation induced. Knockdown of STMN1 using siRNAs led to inhibition of MPM cell proliferation and motility. Finally, miR-223 levels increased while STMN1 was reduced following the re-expression of the JNK isoforms in JNK-null murine embryonic fibroblasts, and STMN1 was reduced in MPM cell lines following the activation of JNK signaling.

Implications: miR-223 regulates STMN1 in MPM, and both are in turn regulated by the JNK signaling pathway. As such, miR-223 and STMN1 play an important role in regulating MPM cell motility and may be therapeutic targets.

Introduction

Malignant pleural mesothelioma (MPM) arises from the mesothelial cells lining the body’s serosal cavities. MPM has limited therapeutic strategies, and the median survival is 9 to 12 months. The most important risk factor for developing MPM is exposure to asbestos (1). Despite asbestos use and production being banned in many countries, exposure still occurs via nonoccupational exposure and its continued use in developing countries (2).

The small but powerful regulators of gene expression miRNAs are potentially novel therapeutic targets for the treatment of cancers. MiRNAs are regulators of developmental and cellular pathways and most commonly silence genes by binding to the 3’- untranslated region (UTR) of target mRNA. This binding induces either mRNA degradation or inhibition of translation (3). More than 50% of miRNA genes are found in cancer-associated genomic regions, and their altered expression in numerous cancers supports the notion that these small RNAs serve as a novel class of oncogenes or tumor suppressors (4).

More recently, the aberrant expression of miRNAs has also been demonstrated in MPM (5). The identified miRNAs are predicted to target some of the more frequently affected genes in MPM, and many are located in chromosomal areas known to be deleted or gained in this disease (6). Specific miRNAs such as miR-31 (7), miR-34b/c (8), miR-29c’ (9), miR-16 (10), and miR-205 (11) have been shown to influence functional characteristics of MPM cells. Others, including the miR-200c family (12), miR-126 (13), miR-103 (14), and miR-625-3p (15), have been investigated for their diagnostic potential.

The focus of this study was miR-223. Recent evidence suggests that miR-223 may behave as an oncogene or tumor suppressor in a range of hematopoietic and solid tumors (16–21) and can regulate functions, such as proliferation, migration (16, 22), and
chemoresistance (23) in these malignancies. The role of miR-223 in MPM is unknown. MiR-223 is predicted to target many genes including the microtubule regulator Stathmin (STMN1; refs. 17, 21) that was previously linked to MPM (24).

STMN1 is a ubiquitously expressed cytosolic phosphoprotein in humans (25) and modulates microtubule dynamics, therefore playing an essential role in mitosis, intracellular transport, motility, and maintenance of cell shape (26). STMN1 is highly expressed in a number of cancers, and this expression is believed to promote carcinogenesis (27). STMN1 was previously shown to be overexpressed in MPM (24); however, the functional role of STMN1 in this cancer has not been determined.

The mechanisms regulating STMN1 overexpression during malignancy are complex. They not only include posttranscriptional regulation by miRNAs like miR-223 (17, 21) but also the loss of tumor suppressor p53 (28, 29) and constitutive activity of several oncogenic transcription factors (30–32). In a previous study, we demonstrated negative regulation of STMN1 expression by c-Jun N-terminal kinase (JNK) signaling through c-Jun in embryonic fibroblasts (33). The extent to which JNK signaling is involved in regulating STMN1 in MPM is unclear.

In this study, we examined expression levels of miR-223 in MPM. Because STMN1 may be highly expressed in MPM and STMN1 is regulated by miR-223, we determined whether miR-223 regulates STMN1 in MPM and explored the regulatory mechanisms involved. Finally, we investigated the functional relevance of altered miR-223 and STMN1 levels in the pathogenesis of MPM.

Materials and Methods

Cell lines

Eight MPM cell lines (five human and three mouse), primary human and mouse mesothelial cells, and the immortalized mesothelial cell line Met-Sa were included in the study. The human (JU77, LO68, N036) and mouse MPM cell lines (AB1, AB2, AB22) and Met-Sa were kindly provided by Prof Bruce Robinson (Department of Medicine and Pharmacology, University of Western Australia, Australia). The CRL2081 and CRL5820 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were grown in DMEM (Life Technologies) supplemented with L-glutamine (Life Technologies) and 10% FCS (SerumCo). Mesothelial cells were grown under the same conditions but with 15% FCS. Mouse mesothelial cells were isolated from the omentum, and fat pads of BALB/C mice and human mesothelial cells were isolated from pericardial fluid as previously described (34). JNK-null murine embryonic fibroblasts (MEF; iJNK1, iJNK2, and iJNK1/2) were used for the controlled re-expression of JNK isoforms as described previously (33).

Patient samples

The 17 MPM tumor samples used in this study are part of a previously described series of archived formalin-fixed paraffin-embedded (FFPE) specimens collected from patients undergoing extrapleural pneumonectomy at Strathfield Private or Royal Prince Alfred Hospitals (RPAH) in Sydney, Australia, between 1994 and 2009 (35). The six formalin-fixed normal pleural tissues were obtained from patients without cancer undergoing cardiac or aortic surgery at RPAH. Informed written consent was obtained from all patients prior to surgery.

The pleural effusions (26 MPM and 10 benign pleural diseases) of patients from Sir Charles Gairdner Hospital in Perth Western Australia (approximately 300 mL) were centrifuged for 10 to 15 minutes to separate the supernatant from cells. In hemorrhagic samples, the cells were incubated in 1× red blood cell lysis buffer (BioLegend) before the cells were washed with 1× PBS (Sigma-Aldrich), and resuspended in QAozol (Qiagen).

Collection and use of human and mouse mesothelial cells and human pleural effusions were approved by the Sir Charles Gairdner Hospital Human Research Ethics Committee and the University of Western Australia Animal Ethics Committee, respectively. Consent for the use of archival tissue blocks was waived by the Sydney Local Health District Human Research Ethics Committee (Concord), consistent with the Human Tissue Act (1983) and the NHMRC National Statement on Ethical Conduct in Human Research (Commonwealth of Australia, 2007).

RNA isolation

To enrich for tumor content, tumor specimens were laser-capture micro-dissected using the PALM System (Zeiss) as described previously (15) and deparaffinized in xylene. RNA was isolated from deparaffinized tumor samples and formalin-fixed normal pleural tissue using the RNeasy FFPE Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA or enriched miRNA was extracted from cells using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol.

Reverse transcription and real-time qPCR

Total RNA from tumor samples (50 ng) and cells (10 ng) was reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Life Technologies) together with an equimolar mix of primers specific for miR-223 and endogenous controls (RNU16B for tumor and RNU48, RNU44, or SNOR202 for cells). The resulting cDNA (5 μL) was diluted by addition of 28.9 μL ultrapure water, and 2.25 μL per well was used as template in real-time qPCR. qPCR was performed in duplicates using the appropriate TaqMan primer/probes together with the TaqMan Universal Master Mix no AmpErase UNG (Life Technologies) in a total reaction volume of 10 μL. To analyze STMN1 expression, 1 μg of RNA was reverse transcribed using the Omniscript Reverse Transcription Kit (Qiagen) and random hexamer primers (Life Technologies). The qPCR was performed in duplicates with the STMN1 Taqman primer probe and Taqman Gene Expression Master Mix (Life Technologies). STMN1 expression relative to 18S ribosomal RNA was calculated. qPCR was run on the StepOne Plus Real-Time PCR system (Life Technologies) or the Stratagene Mx3000q qPCR Instrument (Stratagene/Agilent Technologies).

RT2 miRNA PCR array and Taqman OpenArray profiling

Enriched miRNA (100 ng) was reversed transcribed using the RT2 miRNA First Strand Kit (Qiagen), diluted 1/10, mixed with RT2 qPCR master mix, and loaded onto the RT2 miRNA PCR Array for analysis. For Taqman OpenArray profiling (Life Technologies), total RNA (100 ng) was reverse transcribed using the Taqman MicroRNA Reverse Transcription Kit with pool A and B RT Megaplex primers, preamplified with the Taqman PreAmp...
Mol Cancer Res; 13(7) July 2015

Birnie et al.

Master Mix and Megaplex PreAmp pool A and B primers, diluted 1 in 20, mixed with Taqman OpenArray Real-Time PCR Master Mix, loaded onto the Taqman OpenArray miRNA panel, and run on the OpenArray cycler. Results were analyzed in the DataAssist 3.0 (Life Technologies) and Graphpad PRISM 4 programs.

Western blot

Protein (20 μg) was separated on a 12% acrylamide/bisacrylamide (BIO-RAD) gel with sodium dodecyl sulphate electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane (Merck Millipore). After blocking with 5% skim milk for an hour, the membrane was incubated overnight with primary antibodies at 4°C. The membrane was washed and incubated with rabbit or mouse Horseradish peroxidase (HRP)–conjugated antibodies (Agilent Technologies). The membrane was washed again and bands detected using Immobilon Western HRP Substrate (Merck Millipore) and an AGFA CP1000 X-ray Processor (AGFA Healthcare). To reprobe with antibodies to determine total protein loading, membranes were incubated with glycine stripping buffer (50 mmol/L glycine, 35 mmol/L SDS, 1% Tween-20, pH 2.5), blocked in 5% skim milk, and incubated with α-tubulin primary antibody overnight before the detection of α-tubulin the following day.

Transfection studies and immunoprecipitation

All transfection studies were carried out using the Lipofectamine 2000 reagent (Life Technologies). For miR-223 overexpression studies, cells were transfected with 10 nmol/L of the Ambion Pre-miR miRNA-223 mimic or Ambion Pre-miR miRNA scrambled control (Life Technologies). For STMN1 inhibition studies, cells were transfected with 10 nmol/L of the STMN1 siRNA or control (Santa Cruz Biotechnology). For JNK knockdown, cells were transfected with ON-TARGETplus siRNA (20 nmol/L; Dharmacon), targeting JNK1 or JNK2 isoforms individually or in combination, and in order to activate JNK signaling, cells were transfected with constitutively active MKK7 and MEKK1 constructs. Immunoprecipitation (IP) of the AGO2 protein was carried out as previously described (36).

Cell proliferation, clonogenicity, and migration assays

Cell proliferation was analyzed using the Methylene Blue and WST-1 assays (Roche). Cells were seeded in 96-well plates, transiently transfected, and serum-starved for 24 hours before grown in 10% FCS medium. Proliferation was ceased 0, 24, and 48 hours after scratching, and the area of the scratch that was covered by cells (percentage) was determined using Image J software (National Institute of Health). To confirm the role of STMN1 in MPM cell migration, a transwell membrane with 0.8 μm pores (BD Biosciences) was used. Transiently transfected cells were seeded on top of the transwell membrane (5.0 × 104 cells/well) in serum-free DMEM with serum-free or 10% FCS growth media (500 μL) added to the bottom. After 24 hours, cells were fixed using 4% PFA. Cells that had not migrated through the membrane were scraped off, and the remaining cells were stained using 0.05% crystal violet for 30 minutes. Cells in 4 random fields for each membrane were counted, and percentage migration was calculated.

Immunofluorescence staining of microtubules

JU77 cells were chosen to investigate the impact of miR-223 on tubulin acetylation given the high expression of STMN1 observed in this cell line and the demonstrated reduction of STMN1 expression in these cells following miR-223 overexpression. The cells cultured on uncoated glass cover slips were transfected with miRNA precursor 223 or scrambled control and cultured for 48 hours for fixation with 4% (w/v) PFA (20 minutes, room temperature). Cells were permeabilized [0.2% (v/v) Triton X-100 in PBS; Sigma-Aldrich] and pre-blocked [10% (v/v) FCS in PBS] before incubation with anti-tubulin or anti-acetylated tubulin primary antibodies followed by Alexa555-conjugated secondary antibodies (Life Technologies) diluted in 1% (w/v) BSA (Sigma-Aldrich) in PBS. The cells were then mounted (GelMount, Biomeda Corp), and images were captured on a Leica TCS SP5 confocal microscope using a 100× 1.35 NA objective.

Statistical analysis

Data are expressed as mean ± SEM of three experiments unless otherwise specified. Statistical significance was determined using Graphpad PRISM 4 with a two-tailed unpaired test or a Mann–Whitney test for data that were not normally distributed. A P value less than 0.05 was considered statistically significant.

Results

miR-223 expression is low in MPM

To identify miRNAs with an altered expression in MPM, we performed RT2 miRNA PCR Array analysis of 88 miRNAs in human mesothelial cell controls and three MPM cell lines (NO36, JU77, LO68). When we compared miRNA expression between the mesothelial and MPM cells, we identified a number of miRNAs that were either up- or downregulated in the MPM cell lines. We selected miR-223 for further study, as this was the only miRNA consistently downregulated in the MPM cell lines (Fig. 1A). In order to confirm this finding, we then measured the expression of miR-223 in a range of MPM samples.

The levels of miR-223 were validated in human and mouse mesothelial cells and five human and three mouse MPM cell lines by qPCR. Compared with the mesothelial cell controls, miR-223 was expressed significantly lower in both human (P < 0.01,
miR-223 and JNK Signaling Target STMN1 in MPM

STMN1 is overexpressed in MPM cell lines
STMN1 was first confirmed as a target of miR-223 by Wong and colleagues in 2008 (21). These authors also demonstrated that within both hepatocellular carcinoma (HCC; ref. 21) and gastric cancer (17), a downregulation of miR-223 contributed to high levels of STMN1. We found low levels of miR-223 in MPM and as STMN1 was previously shown to be expressed at high levels in MPM cell lines and tumors (24), we hypothesized that a loss of miR-223 may contribute to the overexpression of STMN1 in MPM. We measured STMN1 mRNA by qPCR and found that STMN1 levels were significantly higher in human MPM cell lines compared with mesothelial cell controls (P < 0.05, Fig. 2A). STMN1 mRNA levels were also significantly higher in two of the three mouse MPM cell lines examined (P < 0.05, Fig. 2B). Levels of STMN1 protein were assessed by Western blot and quantified using densitometry. In the human MPM cell lines, STMN1 protein was significantly increased compared with mesothelial cell controls (P < 0.01, Fig. 2C). In the mouse MPM cell lines,

Fig. 1B) and mouse (P < 0.01, Fig. 1C) MPM cell lines, validating our preliminary observation. To confirm that miR-223 is reduced in MPM patients, we also examined miR-223 levels in tumor tissue and pleural effusion cells. MiR-223 was analyzed in 17 human FFPE MPM and six control pericardial mesothelium samples by qPCR. MiR-223 was expressed significantly lower in the MPM tissues (P < 0.001, Fig. 1D). An miRNA profiling study in cells isolated from pleural effusions of MPM and benign pleural disease (Benign) patients showed miR-223 is significantly lower in MPM. F, qPCR validation confirmed this result.

Figure 1. MiR-223 expression is low in MPM.
A, MiR-223 expression is lower in three MPM cell lines compared with human mesothelial cells (control) as determined by superarray analysis (n = 1). MiR-223 expression in (B) human and (C) mouse MPM cell lines is significantly lower compared with mesothelial cells (control) as determined by qPCR (n = 3). D, MiR-223 expression is significantly lower in human MPM tissues (MPM) compared with normal mesothelium from the pericardium (NP). E, Taqman OpenArray analysis of miRNAs in cells isolated from pleural effusions of MPM and benign pleural disease (Benign) patients showed miR-223 is significantly lower in MPM. F, qPCR validation confirmed this result. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
STMN1 protein was also significantly higher in two of the three MPM cell lines, consistent with the mRNA data ($P < 0.05$, Fig. 2D).

**MiR-223 regulates STMN1 in MPM**

To determine if miR-223 targets STMN1 in MPM, the miR-223 mimic or a control construct was transfected into JU77 and CRL2081 cells. Of the five cell lines used in this study, JU77 had one of the lowest STMN1 levels, whereas CRL2081 had one of the highest. The day following transfection, cells were serum starved, and 24 and 48 hours later, the cells were harvested. To ensure that the transfection was successful and that miR-223 expression had increased, miR-223 levels were measured by qPCR. Levels of miR-223 were significantly higher at 24 and 48 hours compared with controls in both cell lines ($P < 0.01$, Fig. 3A). STMN1 mRNA and protein were then measured by qPCR and Western blot, respectively. Both STMN1 mRNA ($P < 0.05$, Fig. 3B) and protein ($P < 0.05$, Fig. 3C) were reduced at both time points in both cell lines. To validate these findings, we used IP to isolate the AGO2 protein (Fig. 3D) following transfection of JU77 cells with the miR-223 mimic. AGO2 is the protein found within the RNA-induced silencing complex (RISC) to which miRNAs and their targets bind. Therefore, if STMN1 is a target of miR-223, in miR-223–transfected cells, there should be higher levels of endogenous STMN1 bound to miR-223 and AGO2 compared with cells transfected with a scrambled control. Our results show that following AGO2-IP, more STMN1 was bound to miR-223 and AGO2 in miR-223–transfected cells versus cells transfected with control constructs (Fig. 3D). These results confirm that miR-223 can bind to and regulate STMN1 in MPM cells.

**JNK signaling regulates miR-223 and STMN1**

Previously, we demonstrated that in the absence of JNK signaling, STMN1 was substantially overexpressed in murine fibroblasts and that the re-expression of JNK decreased STMN1 to normal wild-type levels (33). In the current study, we found that miR-223 levels were significantly elevated and inversely
Figure 3. MiR-223 regulates STMN1 in MPM cells. A, MiR-223 was significantly overexpressed in the JU77 and CRL2081 cell lines following transfection of the miR-223 mimic and as determined by qPCR. The increase in miR-223 reduced STMN1 (B) mRNA and (C) protein in both cell lines as shown by qPCR and Western blot analysis (*C3, P < 0.05; **C3, P < 0.01). D, the specificity and efficiency of the anti-AGO2 antibody were tested on whole-cell lysate, AGO2-IP, and AGO2-IP flow through (FT) from cells transfected with either miR-223 or control. Levels of STMN1 were measured following AGO2-IP using qPCR and were higher in cells transfected with the miR-223 mimic (*n = 2).
correlated with reduction in STMN1 mRNA following 4-hydroxytamoxifen (4-HT)–induced re-expression of JNK isoforms in a null (jnk1−/-, jnk2−/-) background (P < 0.05, Fig. 4A). These results suggest that firstly JNK signaling is upstream of miR-223 as the activation of JNK is itself sufficient to induce miR-223 expression, and secondly, that miR-223 may have a role in the posttranscriptional attenuation of STMN1 expression by JNK. In support of this occurring in MPM, expression, and phosphorylation of c-Jun, the downstream archetypal substrate of JNK is reduced while STMN1 expression is elevated in human MPM cell lines when compared with control primary mesothelial cells (Fig. 4B). This also occurs in mouse MPM cell lines (data not shown). In addition, JNK activation in response to hyperosmolarity or ectopic expression of constitutively active upstream activators (MKK7/MEKK1) triggered decreased STMN1 levels in JU77 MPM cells (Fig. 4C and D). These results indicate that JNK signaling regulates STMN1 and miR-223 expression, and this may occur in MPM cells.

**MiR-223 regulates MPM cell motility**

Emerging evidence suggests that miR-223 has a role in regulating cell functions, such as proliferation and migration (16, 17, 22, 37). We examined the effect of increased levels of miR-223 on MPM cell proliferation using WST-1 and methylene blue assays. Overexpression of miR-223 did not alter cell proliferation in either JU77 or CRL2081 cells over 48 hours (Fig. 5A). The effect of increased miR-223 levels on cell clonogenicity over 7 to 21 days was also assessed, and the results were also negative (Fig. 5B). However, overexpressing miR-223 significantly inhibited MPM cell motility at 24 hours when this was assessed using an in vitro...
miR-223 and JNK Signaling Target STMN1 in MPM

Figure 5.

MiR-223 inhibits wound closure. A, MiR-223 overexpression did not alter cell proliferation as determined by the WST-1 and methylene blue assays. B, clonogenicity was also not altered following miR-223 overexpression, but MiR-223 overexpression significantly reduced (C) wound closure for 24 hours in both the JU77 and CRL2081 cell lines as determined by a wound-healing assay. Magnification, x10. D, overexpression of miR-223 induced tubulin acetylation in MPM cells (n = 3). *, P < 0.05.
wound/scratch assay ($P < 0.05$, Fig. 5C). Our findings indicate that low levels of miR-223 may enhance cell motility in MPM. We also observed an increase in acetylated microtubules following miR-223 overexpression while the total microtubule network remained relatively unchanged (Fig. 5D). This suggests increased microtubule stabilization following miR-223 overexpression and is consistent with downregulated STMN1 expression as a consequence.

Figure 6.
Reducing STMN1 using siRNA inhibits MPM cell proliferation. STMN1 (A) mRNA and (B) protein were significantly reduced in the JU77 and CRL2081 cell lines following transfection of STMN1 siRNA and as determined by qPCR and Western blot analysis. C, the reduction of STMN1 using siRNA significantly inhibited proliferation for 24 hours in both cell lines ($n = 3$). *$P < 0.05$. 

Mol Cancer Res; 13(7) July 2015

Molecular Cancer Research
STMN1 regulates MPM cell motility

It has been proposed that STMN1 plays an oncogenic role in MPM (24). To determine if STMN1 is important in MPM tumorigenesis, STMN1 levels were reduced in MPM cells using specific siRNAs and cell proliferation, clonogenicity, and motility assessed. Following transient transfection of the STMN1 siRNA or control construct, STMN1 mRNA and protein were measured. STMN1 mRNA ($P < 0.05$, Fig. 6A) and protein ($P < 0.05$, Fig. 6B) were significantly reduced at both 24 and 48 hours in JU77 and CRL2081 cells. Reducing STMN1 also significantly inhibited cell proliferation at 24 hours in both cell lines ($P < 0.05$, Fig. 6C), although this inhibition was not apparent at 48 hours. Reducing STMN1 did not affect MPM cell clonogenicity (Fig. 7A) but significantly reduced cell motility at 24 hours when assessed using a wound/scratch assay ($P < 0.05$, Fig. 7B). However, the closing of the wound/scratch can involve both cell proliferation and migration, and as already mentioned, we observed an inhibition of

MPM cell proliferation following the knockdown of STMN1. Therefore, we cannot conclude whether the reduction seen in wound/scratch closure following STMN1 reduction was due to cell migration or proliferation or a combination of these. In order to confirm that STMN1 does have a role in regulating MPM motility, we then assessed MPM cell migration using a transwell migration assay. Migration of both JU77 and CRL2081 cells was significantly reduced through the transwell membrane following the knockdown of STMN1 expression ($P < 0.05$, Fig. 7C). These results suggest that STMN1 is potentially involved in the regulation of MPM proliferation and motility.

Discussion

MPM is a fatal cancer as current treatments rarely extend patient survival beyond 12 months (1). In order to develop novel treatments for MPM, a better understanding of the molecular
Here, we show for the first time that loss of miR-223 and JNK-signaling through the c-jun transcription factor contribute to the overexpression of STMN1 in MPM.

Aberrant miR-223 expression has been shown in a number of cancers, but its role in carcinogenesis remains to be clarified, as opposing results have been reported. MiR-223 is highly expressed and thought to act as an oncogene in bladder cancer (39), recurrent ovarian cancer (20), and gastric cancer (40). In contrast, miR-223 is downregulated in osteosarcoma (41), esophageal carcinoma (16), and HCC (21). Consistent with these latter studies, we found low levels of miR-223 in a range of MPM samples, including cell lines, cells isolated from MPM patient pleural effusions and tissue.

One target of miR-223 is STMN1, a microtubule regulator protein that has been associated with tumorigenesis in various cancers. STMN1 modulates cell mitosis, movement, and shape through its regulation of microtubule dynamics (26) and is highly expressed in leukemia, lymphoma, neuroblastoma, and prostate, breast and ovarian cancer, and many others (42). This high expression often correlates with a poorer prognosis and the development of local and distant metastasis (27). We found STMN1 to be highly expressed in both human and mouse MPM cell lines compared with mesothelial controls. This observation supports the results of earlier research in which high levels of STMN1 were observed in MPM cell lines and tumors (24) and suggests STMN1 may have an oncogenic role in MPM.

STMN1 was first identified as a target of miR-223 when a putative miR-223 binding site in the 3’UTR of the STMN1 gene encompassing 12 perfectly matched nucleotides was found (21). Within this study, a strong inverse correlation between STMN1 and miR-223 levels in HCC cell lines was demonstrated. After transfection with the miR-223 mimic, a consistent reduction of the STMN1 protein was observed (21). Similarly, in gastric cancer cell lines, luciferase reporter assays were used to demonstrate the specific interaction between miR-223 and the 3’UTR of STMN1. Transfecting miR-223 into these cells reduced the STMN1 protein and addition of an miR-223 blocker rescued STMN1 expression, suggesting that the suppressive effect was specifically caused by miR-223 (17). More recently, the interaction between miR-223 and STMN1 in p53 mutant breast and colon cancer cells was demonstrated (23). Mutant p53 was shown to upregulate STMN1 via miR-223 downregulation and as a consequence enhances the chemoresistance of these cells. In the current study, STMN1 mRNA and protein were consistently reduced following overexpression of miR-223, and higher levels of endogenous STMN1 were observed bound to miR-223 and AGO2 in cells transfected with the miR-223 mimic compared with cells transfected with a control. These results strongly suggest that STMN1 is regulated by miR-223 in MPM cells and that this regulation also occurs in MPM.

To further investigate the regulatory mechanisms underlying low miR-223 expression in MPM and subsequent upregulation of STMN1, we analyzed whether the JNK signaling pathway was involved. We chose JNK signaling because we previously identified this pathway as a regulator of STMN1 expression (33). Following the re-expression of the JNK isoforms in JNK knock-out MEFs, we observed a correlation between JNK-mediated miR-223 upregulation and STMN1 downregulation. We also observed downregulation of STMN1 in MPM cell lines following activation of JNK signaling. These results imply that within MPM, the low expression of miR-223 and the high levels of STMN1 may be caused by aberrant JNK signaling and suggest a potential tumor-suppressive role for the JNK-miR-223–STMN1 axis. JNK signaling has been shown to play a role in MPM cell death (43, 44); however, other studies have shown that JNK can have various functions in other malignancies (45). Our findings are novel in this regard, revealing a potential new role for JNK in MPM biology.

To explore miR-223’s role in MPM, the effect of overexpressing miR-223 on MPM cell function was assessed. Increasing the levels of miR-223 had no effect on cell proliferation or clonogenicity but significantly reduced cell motility. This is consistent with previously reported findings for esophageal carcinoma, whereby the overexpression of miR-223 inhibited cell migration across a wound/scratch (46). In support of the observed reduction in cell motility, overexpressing miR-223 induced the acetylation of tubulin within MPM cells. The acetylation of tubulin is consistent with microtubule stabilization and a reduction in cell motility (47).

The role of STMN1 in MPM cell function was assessed by inhibiting STMN1 using siRNA. The inhibition of STMN1 reduced cell proliferation over the first 24 hours but not by 48 hours, and reducing STMN1 had no effect on MPM cell clonogenicity. However, inhibiting STMN1 reduced MPM cell motility, supporting earlier studies in gastric (17) and colon cancers (48).

In conclusion, we have shown that miR-223 targets STMN1 in MPM, and miR-223 and STMN1 are potential regulators of MPM cell motility, and both STMN1 and miR-223 are regulated by JNK signaling. It remains feasible that miR-223 may behave as a tumor suppressor in MPM, whereas STMN1 has potential oncogenic characteristics. MiR-223 and STMN1 are attractive targets for future MPM therapeutic studies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: K.A. Birnie, C.M. Prêle, A.W. (Bill) Musk, P.J. Thompson, S.E. Mutsaers, B. Badrian
Development of methodology: K.A. Birnie, A.W. (Bill) Musk, P.J. Thompson, S.E. Mutsaers, B. Badrian
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.A. Birnie, Y.Y. Yip, D.C.H. Ng, M.B. Kirschner, G. Reid, A.W. (Bill) Musk, S.E. Mutsaers, B. Badrian
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K.A. Birnie, Y.Y. Yip, D.C.H. Ng, G. Reid, C.M. Prêle, P.J. Thompson, S.E. Mutsaers, B. Badrian
Writing, review, and/or revision of the manuscript: K.A. Birnie, D.C.H. Ng, M.B. Kirschner, G. Reid, C.M. Prêle, A.W. (Bill) Musk, Y.C.G. Lee, P.J. Thompson, S.E. Mutsaers, B. Badrian
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Birnie, Y.C.G. Lee, S.E. Mutsaers
Study supervision: A.W. (Bill) Musk, P.J. Thompson, S.E. Mutsaers, B. Badrian

Acknowledgments
The authors thank Richard Alcock (LottCWste State Biomedical Facility Genomics, Perth, Western Australia) for providing access to Taqman OpenArray miRNA platform. They also thank Brian McCaughan, James Edelman, and...
Michael Vallely for providing clinical samples, Sonja Kløve for marking tumor area on FFPE blocks, Yunn Yee Cheng for laser-capture micro-dissection, and Yvonne Yeap for the MIKE7 and MEK1 transfection experiments.

Grant Support

This work was supported by grants from Dust Diseases Board N.S.W., Maurice Blackburn, and Sir Charles Gairdner Hospital Research Committee.

References


Mol Cancer Res; 13(7) July 2015 1117

miR-223 and JNK Signaling Target STMN1 in MPM

www.aacrjournals.org

This article was published OnlineFirst on March 30, 2015; DOI: 10.1158/1541-7786.MCR-14-0442

Published OnlineFirst on June 15, 2017. © 2015 American Association for Cancer Research.


Molecular Cancer Research

Loss of miR-223 and JNK Signaling Contribute to Elevated Stathmin in Malignant Pleural Mesothelioma

Kimberly A. Birnie, Yan Y. Yip, Dominic C.H. Ng, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-14-0442

Cited articles
This article cites 48 articles, 14 of which you can access for free at:
http://mcr.aacrjournals.org/content/13/7/1106.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/13/7/1106.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.