Multiple myeloma (MM) is an incurable hematologic malignancy characterized by recurrent chromosomal translocations. Patients with t(4;14)(p16;q32) are the worst prognostic subgroup in MM, although the basis for this poor prognosis is unknown. The t(4;14) is unusual in that it involves 2 potential target genes: fibroblast growth factor receptor 3 (FGFR3) and multiple myeloma SET domain (MMSET). MMSET is universally overexpressed in t(4;14) MM, whereas FGFR3 expression is lost in one-third of cases.

Nonetheless, the role of MMSET in t(4;14) MM has remained unclear. Here we demonstrate a role for MMSET in t(4;14) MM cells. Down-regulation of MMSET expression in MM cell lines by RNA interference and by selective disruption of the translocated MMSET allele using gene targeting dramatically reduced colony formation in methylcellulose but had only modest effects in liquid culture. In addition, MMSET knockdown led to cell-cycle arrest of adherent MM cells and reduced the ability of MM cells to adhere to extracellular matrix. Finally, MMSET knockdown and knock-out reduced tumor formation by MM xenografts. These results provide the first direct evidence that MMSET plays a significant role in t(4;14) MM and suggest that therapies targeting this gene could impact this particular subset of poor-prognosis patients.

Methods

Cell culture

KMS-11 (gift of P. Leif Bergsagel, Mayo Clinic, Scottsdale, AZ), U266, NCI-H929, and Raji cells were grown in RPMI-1640 (Invitrogen, Carlsbad, CA).
Figure 1. Stable RNA interference of MMSET I and MMSET II isoforms in t(4;14) + MM cells. (A) Structure of transcripts originating from the MMSET locus. Boxes indicate protein-coding exons, beginning with exon 3 for MMSET I and II. Lines indicate 5’ and 3’ untranslated regions (not shown to scale). MMSET II splices from exon 10 to exons 12-24, whereas MMSET I splices from exon 10 to exon 11, which is shaded gray. The untranslated exons 10 and 12-14 of REIIBP are stippled. REIIBP translation begins in exon 15. Arrows indicate major translation initiation sites. The conserved protein domains PWPP, HMG, PHD, and SET are indicated above the corresponding exons. Thick bars indicate positions of MMSET shRNA 1 (black) and control (gray) short hairpin RNAs. (B) Real-time quantitative RT-PCR analysis of MMSET I, MMSET II, and REIIBP transcripts in KMS-11 cells stably transduced with MMSET shRNA lentivirus or control shRNA lentivirus. Transcript levels are relative to empty vector–transduced cells. Results bars indicate positions of MMSET shRNA 1 (black) and control (gray) short hairpin RNAs. (B) Real-time quantitative RT-PCR analysis of MMSET I, MMSET II, and REIIBP transcripts in KMS-11 cells stably transduced with MMSET shRNA lentivirus or control shRNA lentivirus. Transcript levels are relative to empty vector–transduced cells. Results are means (± SD) of at least 2 independent lentiviral infections (*P < .05, †P = .45 by Student t test). (C) Immunoblotting with antisera recognizing a C-terminal epitope of MMSET demonstrates reduction of full-length MMSET protein by 3 different shRNAs in KMS-11 (lanes 1-5) and NCI-H929 (lanes 6-10). The top arrow indicates full-length MMSET II, translated from the first ATG codon in exon 3. The lower arrows indicate MMSET II isoforms consistent with translation from downstream ATG codons in exons 4 and 6 in NCI-H929 cells, which have a translocation breakpoint between exons 3 and 4.
measured with a BD LSR flow cytometer (BD Biosciences, San Jose, CA), and percentages of G₀/G₁, S, and G₂/M phase cells were determined using Modfit LT software (Verity Software House, Topsham, ME).

Adhesion assay
Cells (10⁶ KMS-11) transduced with either empty vector lentivirus or MMSET shRNA lentivirus were seeded in triplicate in complete RPMI 1640 medium in a 6-well plate coated with growth factor-reduced BD Matrigel (BD Biosciences) according to the manufacturer’s instructions. After allowing cells to adhere 6 hours at 37°C, the medium was removed, and the wells were washed gently with Hanks balanced salt solution (Invitrogen). Washes were pooled with the medium, and the suspended cells were counted using a hemacytometer.

Methylcellulose colony formation assays
Methylcellulose media consisted of RPMI 1640 containing 1.1% methylcellulose (Aqua Solutions, Deer Park, TX), 30% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and puromycin at the appropriate concentration for each cell line. Cells were plated at a density of 500 cells/mL in 1 mL volume in humidified 24-well plates. Colonies were counted between 10 and 15 days after plating. For photomicrographs, plates were viewed with an Eclipse TE2000-S microscope using a CFI Plan Fluor DL 4X/0.17 lens (Nikon, Melville, NY). Images were acquired using a SPOT RT KE monochrome cooled CCD camera and SPOT version 4.0.1 software (Diagnostic Instruments, Sterling Heights, MI).

Xenograft studies
Eight- to 10-week-old female athymic nude mice (Harlan, Indianapolis, IN) were injected subcutaneously in the bilateral flanks with 100 µL. Hanks balanced salt solution containing 10⁷ KMS-11 cells stably transduced with empty vector lentivirus or MMSET shRNA lentivirus, or with 10µg parental or gene-targeted KMS-11 cells. Twenty-four-week-old NOD/SCID mice were injected subcutaneously bilaterally with 5×10⁶ (2 mice each) NCI-H929 cells stably transduced with empty vector lentivirus or MMSET shRNA lentivirus. Injection sites were examined for tumors weekly. NCI-H929 tumors grown in NOD/SCID mice were weighed at the end of the observation period (all tumors grew for an identical period of time). The Institute of Laboratory Animals Resources Guide for the Care and Use of Laboratory Animals was followed in all experiments.16

MTT assay
Cells were seeded in 96-well plates at 2500 cells/well in 0.2 mL final volume. Drugs were added at the indicated concentrations. After 44 hours of incubation, MTT (Sigma-Aldrich) was added to a final concentration of 0.5 mg/mL, and cells were incubated for 4 hours. Formazan crystals were solubilized by addition of 10% SDS/0.1 M HCl. Absorbance was read on a plate reader.

Statistical analysis
Student t tests were calculated with Excel version 2003 software (Microsoft, Redmond, WA) using an unpaired 2-sided analysis. Tumor frequencies were compared using a χ² test. A P value less than .05 was considered statistically significant.

Results
Stable knockdown of MMSET expression in MM cell lines
To test the contribution of MMSET disregulation to the pathogenesis of t(4;14) MM, we attempted to deplete MMSET levels with shRNA expressed from a lentiviral vector. Stable pools of virally transduced t(4;14) + KMS-11 and NCI-H929 MM cells were selected with puromycin and analyzed for MMSET isoform expression by quantitative real-time RT-PCR (qPCR) at time points ranging from 4 days to 2 months after infection. As shown in Figure 1B, an shRNA targeting MMSET exon 6 reduced RNA levels of full-length MMSET (MMSET II) and the N-terminal isoform (MMSET I) without significantly affecting the level of the C-terminal isoform (REBH). MMSET I transcript levels were reduced by 85% and MMSET II transcripts by 80% relative to cells transduced with empty lentiviral vector, a variety of shRNA constructs targeting other regions of the MMSET gene, or shRNA against firefly luciferase (Figure 1B and data not shown). To control for a nonspecific induction of interferon response genes, which has been reported with some RNAi studies, we performed qPCR for oligoadenylate synthase 1 and enolase 1.17,18 Neither gene was up-regulated in MMSET shRNA cells versus controls (data not shown).

Immunoblotting with antisera generated against C-terminal epitope common to full-length MMSET and REBH demonstrated knockdown of MMSET II at the protein level.9 Depletion of MMSET II protein correlated with depletion of mRNA and was observed with 3 different MMSET shRNAs (Figure 1C). Unlike KMS-11 cells, NCI-H929 cells have a translocation breakpoint 3’ of the major translation initiation site of MMSET.7,8 It has been demonstrated by transient expression of MMSET-GFP fusion cDNAs that MMSET proteins can be translated in vivo from ATG codons in MMSET exons 4 and 6.5,10 We demonstrate that NCI-H929 cells do indeed express truncated MMSET II proteins consistent with translation from exons 4 and 6. The smallest isoform, consistent with usage of the exon 6 ATG, is the most abundant and is expressed in excess over the full-length protein, which is expressed from the nontranslocated allele (Figure 1C). These results confirm that the translocation (4;14) results in selective overexpression of the translocated MMSET allele, most likely from the influence of the strong IgH enhancer.

Selective disruption of the translocated MMSET allele by somatic cell gene targeting
Off-target and other nonspecific effects have been described in RNA interference studies.19-21 In addition, RNA interference does not always elucidate the same phenotypes that are seen with more traditional methods of ablating gene function, such as gene knockout by homologous recombination.22 To independently validate our RNA-interference studies and with the goal of creating an MMSET-null phenotype in human myeloma cells, we attempted to disrupt the MMSET gene in KMS-11 cells by homologous recombination. A targeting construct was designed to delete MMSET exon 7, replacing it with a neomycin resistance cassette flanked by loxP sites (Figure 2A). On subsequent Cre-loxP recombination, the neomycin resistance cassette is removed, leaving an intronic loxP site in place of exon 7. Deletion of exon 7 is predicted to shift the reading frame of the 3’ transcript, leading to a premature stop codon just after exon 6. The predicted truncated protein would lack all conserved domains except for the N-terminal PWWP domain. Successful homologous recombination events were identified by PCR screening (Figure S1). Three clones with heterozygous deletion of exon 7 were expanded, along with 2 random integrant clones, where the neomycin resistance cassette had integrated nonhomologously elsewhere in the genome. To determine whether homologous recombination in the targeted clones had occurred on the translocated allele or the intact chromosome 4 allele of MMSET, we performed a series of experiments. First, RT-PCR was used to detect and verify IgH-MMSET fusion transcripts from the translocated allele as described.23 As expected, fusion transcripts

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were detected in all clones (Figure 2B). Next, RT-PCR using the IgH JH6 forward primer and a neomycin resistance gene reverse primer was performed to determine whether any of the clones that had undergone homologous recombination had disrupted the translocated allele. As shown in Figure 2B, a product of the correct predicted size is seen in one targeted clone (hereafter referred to as T-KO) indicating disruption of the translocated \textit{MMSET} allele. Finally, using RT-PCR primers that span the deleted exon 7, the targeted T-KO clone shows a smaller fusion transcript compared with randomly integrated clones and clones that targeted the nontranslocated \textit{MMSET} allele (hereafter referred to as non-T-KO). This analysis was performed on clones after Cre-loxP deletion of the neomycin resistance cassette, using the JH6 forward primer and a reverse primer on \textit{MMSET} exon 12. As shown in Figure S2, the T-KO clone displays a transcript size consistent with splicing from exon 6 to exon 8.

\textit{MMSET} transcript levels were measured by qPCR using an exon 7-specific forward primer to detect intact \textit{MMSET} I and \textit{MMSET} II transcripts. Intact \textit{MMSET} I levels were reduced by 90% and \textit{MMSET} II levels by 85% in T-KO cells, compared with levels in non-T-KO cells and 2 random integrants (Figure 2C and data not shown). The more than 50% reduction is consistent with increased transcription from the translocated allele, as would be predicted from the influence of the strong IgH intronic enhancer. Immunoblotting confirmed significant reduction in full-length intact \textit{MMSET} II protein levels in T-KO cells. As predicted, REIIBP levels do not differ significantly between targeted and control clones (Figure 2D).

In preliminary attempts to disrupt the second \textit{MMSET} allele in T-KO cells, we were only able to isolate clones that had retargeted the translocated allele (data not shown). This may reflect lethality of complete disruption of \textit{MMSET} or insufficient transcription from the remaining allele to generate adequate levels of neomycin resistance because the neomycin resistance gene is expressed from the endogenous promoter of the targeted locus. Nonetheless, T-KO cells are genotypically near-knockouts by virtue of the differential allelic expression of \textit{MMSET} and recapitulate expression levels found in stable shRNA knockdown clones.

**MMSET knockdown severely reduces clonogenic growth of MM cells**

Both KMS-11 and NCI-H929 cells with MMSET knockdown grew more slowly than their control counterparts, although they could be cultured for several months under drug selection (Figure 3A). Although the differences in growth kinetics in liquid culture were subtle, such culture conditions do not adequately assess all relevant aspects of growth. To assay their clonogenic capacity, we cultured control and knockdown cells in methylcellulose. MMSET knockdown KMS-11 and NCI-H929 cells exhibited a striking loss of colony formation relative to controls (Figure 3B,C). T-KO cells were also severely impaired in their ability to grow in methylcellulose, as indicated by reduced colony number and size. In contrast, non-T-KO cells were indistinguishable from random integrant control clones (Figure 3D). MMSET knockdown was repeated in the non-t(4;14) MM cell line U266 and the Burkitt lymphoma cell line Raji. In both cell lines, MMSET knockdown severely reduces clonogenic growth of MM cells.
MMSET knockdown preferentially affects adherent growth of KMS-11 cells

KMS-11 cells grow as a mixture of adherent and nonadherent cells in standard tissue culture flasks. MMSET-knockdown KMS-11 cells exhibited a striking shift to nonadherent growth. Although total cell numbers expanded at a rate that was only slightly slower in the knockdown cells, control cells grew preferentially adherent, whereas more than 90% of the MMSET knockdown cells were nonadherent. Adherent MMSET knockdown cells proliferated more slowly than control cells (Figure 4A). Similar effects were seen when T-KO cells were cultured (Figure 4B). Cell-cycle analysis by flow cytometry showed that adherent knockdown cells shifted from S-phase to G0/G1 relative to controls. In contrast, there was little difference between the nonadherent population of the control and knockdown cells (Table 1).

Reduced adhesion of MMSET-knockdown KMS-11 cells

Loss of adhesion was also observed when MMSET knockdown cells were plated on Matrigel, a more physiologic adhesion substrate consisting of extracellular matrix. Because of the...
short-term nature of this experiment, differences in cell proliferation do not affect the interpretation of cell numbers adhering to this substrate. Under these conditions, MMSET knockdown cells demonstrated a markedly reduced ability to adhere relative to control cells (Figure 5). To discover why the MMSET knockdown cells had such a reduction in substrate adhesion, we screened a panel of adhesion molecules reported to be expressed in MM cells by qPCR. For most of the genes analyzed, transcript levels were not reduced by MMSET knockdown (Table S2). Reductions greater than 2-fold were observed for integrin α5 (ITGA5), N-cadherin (CDH2), and CD56 (Figure 6A). The CD56 antigen (also known as neural cell adhesion molecule) was reduced approximately 10-fold in the MMSET knockdown cells.

We also analyzed the expression of several genes whose expression has been reported to correlate with the t(4;14) in microarray gene expression profiling of primary MM samples, including activated leukocyte cell adhesion molecule (ALCAM), amphiregulin (AREG), nerve growth factor receptor associated protein 1 (NGFRAP1), CDH2, centaurin γ2 (CENTG2), microphthalmia transcription factor (MITF), Kruppel-like factor 4 (KLF4), and Cystatin C (CST3). The initiator of DNA binding (ID1) gene, which has been reported as a potential MMSET target gene based on its up-regulation after transient transfection of MMSET I into K562 leukemia cells, was also analyzed. KMS-11 cells showed reductions in ALCAM, AREG, NGFRAP1, and CDH2 after MMSET knockdown (Figure 6A). In contrast, we did not find any differential expression of ID1 or several other potential target genes. Comparison of our T-KO cells with control clones also showed similar down-regulation of these same genes, consistent with them being either direct or indirect targets of MMSET (Figure 6B). Non-T-KO cells did not show reduced expression of these transcripts (data not shown).

Table 1. Partial cell-cycle arrest of adherent MMSET knockdown cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>G0/G1, %</th>
<th>S, %</th>
<th>G2/M, %</th>
</tr>
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<tbody>
<tr>
<td>Control suspension</td>
<td>47.74 ± 0.95</td>
<td>43.99 ± 1.10</td>
<td>8.28 ± 2.05</td>
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<tr>
<td>Knockdown</td>
<td>46.44 ± 5.62</td>
<td>41.60 ± 3.81</td>
<td>11.97 ± 1.81</td>
</tr>
<tr>
<td>Control adherent</td>
<td>32.69 ± 3.04</td>
<td>57.17 ± 0.05</td>
<td>10.15 ± 2.99</td>
</tr>
<tr>
<td>Knockdown adherent</td>
<td>43.52 ± 8.46</td>
<td>40.88 ± 4.44*</td>
<td>15.60 ± 3.99</td>
</tr>
</tbody>
</table>

Cell cycle profiling by flow cytometric analysis of DNA content was performed on KMS-11 cells transduced with empty vector lentivirus or MMSET shRNA lentivirus growing in suspension or adherent. Percentages of G0/G1, S, and G2/M phase cells were calculated with ModFit LT software. Results are the average of 2 experiments derived from independent lentiviral infections. Values are mean (± SD).

*P = .03 versus control adherent.
**MMSET knockdown does not alter drug sensitivity of t(4;14) MM cells**

Because patients with the t(4;14) have a short disease-free and overall survival, we examined the effect of MMSET knockdown on the sensitivity of KMS-11 and NCI-H929 cells to a variety of drugs, including dexamethasone, doxorubicin, and lenalidomide, using a MTT viability assay. KMS-11 and NCI-H929 cells were resistant to many of these drugs, but MMSET knockdown did not enhance sensitivity (Figure 7). Interestingly, KMS-11 cells remained quite sensitive to the histone deacetylase (HDAC) inhibitor trichostatin A, despite being resistant to other drugs. Because MMSET has been reported to interact with HDACs, one might have predicted synergy between MMSET knockdown and HDAC inhibition. However, we did not observe any enhanced sensitivity to trichostatin A.

**MMSET expression is required for MM tumor growth in vivo**

To test the contribution of MMSET expression to the tumorigenicity of KMS-11 cells, stable pools of control and MMSET shRNA-bearing KMS-11 cells were injected subcutaneously in the flanks of athymic nude mice. Control KMS-11 cells formed tumors within 1 week in all cases, whereas MMSET-knockdown cells failed to form tumors in the majority of cases even after 5 weeks after inoculation (Table 2). Gene–targeted KMS-11 clones differed in their tumorigenicity according to which allele was targeted. The non-T-KO clone formed tumors with similar frequency to parental KMS-11 cells, whereas the T-KO clone failed to form tumors (Table 2). We were unable to establish NCI-H929 xenografts using a nude mouse model. However, in preliminary experiments with xenografting NCI-H929 cells into NOD/SCID mice, MMSET knockdown cells formed tumors that were more than 4-fold smaller than control tumors, although the sample size used in these studies was not large enough to statistically assess an effect on tumor frequency. In total, these in vivo studies strongly suggest that MMSET has an important role in the tumorigenicity of multiple myeloma cells.

**Discussion**

MMSET is implicated in MM pathogenesis by its constant overexpression in t(4;14) MM and its persistent association with a poor prognosis irrespective of FGFR3 expression. However, to date, there has not been any direct evidence that MMSET is a critical gene for either the pathogenesis or maintenance of MM. In the present study, we have demonstrated that t(4;14)–positive MM cells depend on continued MMSET overexpression for their clonogenic growth in semisolid medium, the type of growth most closely linked to the transformed phenotype. In addition, our data suggest that MMSET overexpression resulting from chromosomal translocation dramatically affects the tumorigenicity of MM cells in a xenograft model. These results support a contributing role for MMSET in the translocation (4;14). Interestingly, cells without the t(4;14), including the non-MM cell line Raji, also showed reduced clonogenic growth with MMSET knockdown. This suggests that the MMSET gene may have a more global role in cell growth, adhesion, and tumorigenicity. The fact that knockdown and knockout of MMSET I and II cause a severe growth phenotype suggests that this translocation results in a gain-of-function of one or both of these isoforms. Consistent with this hypothesis, targeted disruption of the translocated MMSET allele reduced MMSET I and II
expression by 80% to 90% and generated loss-of-function phenotypes that were not seen with targeting of the nontranslocated allele. Our shRNA constructs do not reduce expression of the C-terminal isoform, REIIBP, and we have thus far been unable to specifically target REIIBP by RNAi. Therefore, our findings do not exclude a possible role for REIIBP in t(4;14) MM. We are currently generating isoform-specific knockouts of MMSET to delineate the contributions of the individual isoforms to MM pathogenesis. Furthermore, our results do not exclude a role for FGFR3 overexpression or activating mutations in FGFR3 in t(4;14) MM.

Reduction of MMSET expression led to a dramatic decrease in the ability of KMS-11 MM cells to adhere to substrate and also led to relative G0/G1 cell-cycle arrest in the adherent cells. Whether this effect on adhesion is related to the loss of clonogenicity and tumor formation is unclear at present. There is a great deal of experimental evidence linking anchorage-independent growth in vitro with tumorigenicity in vivo, although most of the data apply to epithelial or mesenchymal tumors. Nontransformed fibroblasts and epithelial cells undergo cell-cycle arrest and apoptosis on loss of attachment to substrate, and they are incapable of growth in semisolid media. In contrast, MMSET knockdown in KMS-11 cells causes cell-cycle arrest preferentially under adherent growth conditions. In MM, abnormal adhesion to bone marrow stromal cells may play an important role in the proliferation and survival of the malignant clone.28 Indeed, the transcription factor c-maf, which is overexpressed as a result of the t(14;16) in MM, has been linked to myeloma pathogenesis by up-regulating integrin B7 and increasing adhesion to bone marrow stroma.29,30 It is possible that aberrant up-regulation of adhesion proteins may be a pathogenetic mechanism common to recurrent MM chromosomal translocations. In this light, it is intriguing that several adhesion molecules, including α5 integrin, ALCAM, CDH2/N-cadherin, and CD56/neural cell adhesion molecule, are down-regulated when MMSET expression is reduced. We are currently performing functional studies to determine the role of adhesion proteins in the growth and adhesion phenotypes we have observed in MMSET knockdown cells.

Putative MMSET target genes have been previously identified by transiently transfecting MMSET cDNA into nonmyeloma cell lines and by comparing microarray gene expression profiles between t(4;14)+ and t(4;14)– myeloma cases.24,27 The latter approach is likely to miss genes such as CD56, which are commonly expressed in both t(4;14)+ and t(4;14)– cases, whereas the former approach does not recapitulate the context and temporal regulation of MMSET in MM cells. We have shown that several genes whose expression correlates with the presence of the t(4;14) in primary myeloma cells, including AREG, ALCAM, CDH2, and NGFRAP1, are down-regulated by MMSET knockdown and by disruption of the translocated, overexpressed MMSET allele, suggesting that they may be bona fide MMSET target genes. Other genes whose expression correlates with the t(4;14), such as KLF4 and MITF, are unaffected. Some of these genes may be targets of FGFR3 overexpression, as is likely for KLF4.31 MM cell lines with differential allelic knockout of MMSET will provide a useful system to determine the target genes of MMSET overexpression in a biologically relevant manner. Identification of MMSET target genes will guide our understanding of how dysregulation of this gene contributes to this aggressive form of MM and may lead to novel therapeutic approaches for patients with this poor-prognosis translocation.

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Authorship

Contribution: J.L. and B.H.P. conceptualized work, analyzed data, and wrote the manuscript; J.L., A.M.A., H.K., J.P. Garay, J.P. Gustin, and Q.W. performed essential laboratory research; and R.J.A. and W.M. provided critical evaluation of the work.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References


The multiple myeloma–associated \textit{MMSET} gene contributes to cellular adhesion, clonogenic growth, and tumorigenicity

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