MMSET deregulation affects cell cycle progression and adhesion regulons in t(4;14) myeloma plasma cells

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Design and Methods

Comparison of expression arrays samples

MMSET knockdown samples were compared to control samples using dCHIP, for each cell line. In addition, patient samples (n=231) were used to compare those with and without a t(4;14) translocation. Patients with a t(4;14) translocation were identified by both FISH and using the expression-based TC classification system (Bergsagel and Khuel *et al.* 2005). FISH probes and patient samples have been described previously.[13, 14] Of the 231 samples, 37 were assigned to the t(4;14) group, 40 to t(11;14), 8 to t(14;16), 6 to t(14;20), 73 to D1 group, 37 to D2 group, 5 to D3 group, 20 to D1+D2 group and 5 remained unassigned. Data from expression arrays were normalised using dCHIP default invariant set normalisation method and the model-based expression summarisation. Control samples (Negative MMSET siRNA treated cells or patients without a t(4;14) translocation) were used as the baseline (B) and compared to samples treated with MMSET siRNA or patients with a t(4;14) translocation (E). Comparison criteria used were lower bound-fold change [E/B>1.2] or [B/E>1.2], mean difference [E-B>50] or [B-E>50], *t*-test *p*-value <0.05. The generated probeset lists were compared using a Venn diagram to identify probesets whose expression is effected by MMSET in cell lines and patient samples.

Multiple myeloma cell lines and cell culture

All cell lines were acquired from either ATCC or DSZM, with the exception of KMS-11, which was kindly provided by Dr. Otsuki (Kawasaki Medical School, Japan). All cells were grown in the absence of antibiotics and mycoplasma contamination was excluded. All myeloma cell lines were cultured in RPMI-1640 medium (Invitrogen Life Technologies, UK) supplemented with 10% fetal calf serum (Invitrogen Life Technologies, UK). Cultures were maintained in exponential growth phase at 37°C in a humidified atmosphere of 95% air/5% carbon dioxide.

Nuclear-cytosol extraction and Western blotting analysis

The cytosolic and nuclear fractions of MM cell lines were isolated and Western blotting analysis performed. MM cell lines growing exponentially were washed with PBS and lysed in ice cold Buffer A (10 mM HEPES pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF; 10% (v/v) Nonidet NP-40). The cytosol fraction was collected by centrifugation. The nuclei were lysed in ice cold Buffer B (20mM HEPES pH 7.9; 0.4 M NaCl; 1mM EDTA; 1mM EGTA; 1mM DTT; 1mM PMSF). The detection of MMSET COOH-terminal was determined using the 5306 antibody raised *in house*. Alpha-tubulin (Sigma-Aldrich, UK) and PARP-1 (Santa Cruz Biotechnology) was used to determine the purification of the cytosol and nuclear fractions, respectively.

Western blot analysis on whole cell lysates was performed by washing 1×10^7 MM cell lines growing exponentially in cold PBS followed by lysis in cell lysis buffer (50mM Tris –HCl, pH 7.5; 150mM NaCl; 1% (v/v) Triton X-100 + 0.5% (w/v) Na-Deoxycholate; 1mM EDTA; 1mM PMSF) containing a cocktail of protease inhibitors (Roche Applied Science, UK). Protein concentration was estimated by using the BCA™ Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, US). Twenty micrograms of total proteins were fractionated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with primary and secondary antibodies followed by chemiluminescence detection using ECL Plus™ (GE Healthcare, UK). Blots were stripped and immunoblotted with anti-α-tubulin or GAPDH antibody to control for protein loading. Immunoblots were scanned and digitised images were quantitatively analysed by densitometry using AIDA 2.0 densitometry software (Raytek, Sheffield, UK).

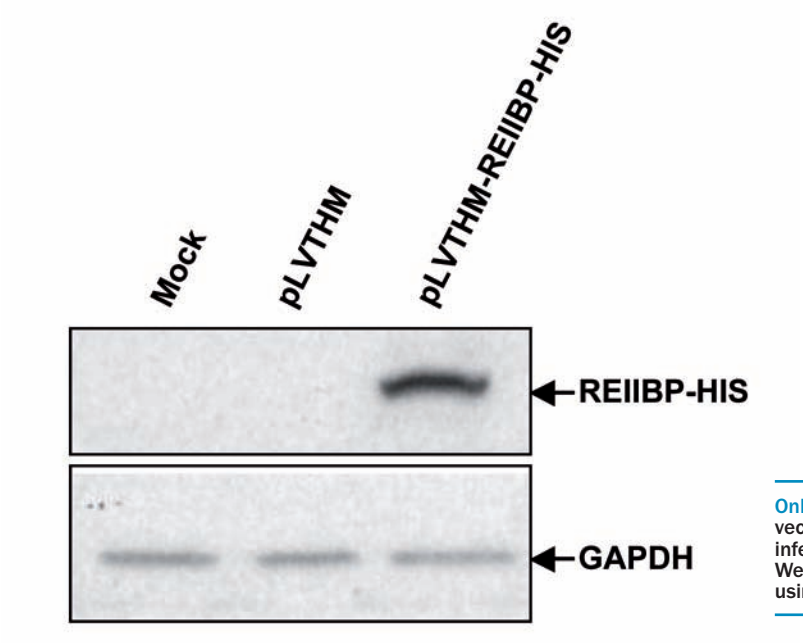
MMSET knockdown and REIIBP cloning

MM cell lines were permeabilised to siRNAs using streptolysin-O (SLO) as described previously.[17-19] Briefly, 1×10^6 cells were incubated with previously optimised concentrations of SLO (8 Units for H929 and RPMI8226 and 6 Units for JIM-3), followed by exposure to 2.5-5μM of single siRNA. siRNAs were synthesized by Invitrogen or Qiagen targeting human MMSET (273 siRNA: 5'- CCAAAGACCUCGACGACCCUUUCAU-3'; 490 siRNA: 5'- GGGAAUGUCCUUGGCAUCAUUGUGA -3'. A negative control siRNA was also used (5'-UUCUCCGAACGUGUCACGU-3'). MMSET knockdown was assessed 48 hours post-permeabilisation by Western blotting analysis. The transfection efficiency of JIM-3, H929 and RPMI8226 was kept at ≥83% in all experiments as monitored by FACS using Alexa 488 fluorescently labelled negative control siRNA.

REIIBP was cloned and ligated into the pLVTHM lentivector. RPMI8226 was infected with lentiviral supernatant and REIIBP infected cells (GFP+) were sorted.

Cell proliferation assay

Cell proliferation was assessed using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, UK). Cells were seeded in 96-well plates at density of 30,000 cells/well in RPMI-1640 supplemented with 10% (v/v) FCS. Plates were incubated in a humidified incubator with 5% CO_2 at 37°C and cell proliferation was assessed at 0, 24, 48 and 72 hours according to the manufacturer's instructions (Promega, UK) on a Dynatech Laboratories MRX plate reader. Each experimental condition was performed in triplicate.



Online Supplementary Figure S1. The REIIBP was cloned into the pLVTHM vector and lentiviral supernatant was produced. RPMI8226 cell line was infected with pLVTHM-REIIBP. Expression of the REIIBP was verified by Western blotting analysis as described in the Design and Methods section using the 5306 antibody.