

## Epigenetic Silencing of the Tetraspanin CD9 during Disease Progression in Multiple Myeloma Cells and Correlation with Survival

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**Abstract Purpose:** The purpose of this study was to investigate expression and epigenetic regulation of CD9 in multiple myeloma (MM) cells during disease progression.

**Experimental Design:** CD9 expression was retrospectively analyzed on bone marrow myeloma samples from 81 patients by immunophenotyping. CD9 expression by murine 5TMM cells was detected by flow cytometric staining and quantitative PCR. The methylation status of the CD9 promoter was determined by bisulfite PCR sequencing.

**Results:** Primary plasma cells in the majority of MM patients with nonactive disease ( $n = 28$ ) showed CD9 expression, whereas most cases with active disease ( $n = 53$ ) were CD9 negative. CD9 expression in diagnostic bone marrow samples ( $n = 74$ ) correlated with survival. Moreover, CD9 expression on murine 5T33 and 5T2MM cells was significantly down-regulated during disease development. Treatment of CD9-nonexpressing 5T33MM $vt$  cells with the clinically relevant histone deacetylase inhibitor LBH589 resulted in a significant increase in CD9 expression. In contrast, cells treated with the demethylation agent 5-aza-2'-deoxycytidine barely showed any increase. A combination study with both compounds resulted in a strong synergistic reactivation of CD9. CD9-expressing 5T33MM $vv$  cells and 5T33MM $vt$  cells stably transduced with a mCD9 lentiviral transferplasmid were shown to be more susceptible to natural killer cell – mediated cytotoxicity than CD9-negative 5T33MM $vt$  cells.

**Conclusions:** CD9 expression correlates with disease status and survival of MM patients. In the murine 5T33MM model, we show that histone modifications, and to a lesser extent CpG methylation, are key epigenetic events in CD9 down-regulation. Furthermore, as CD9 expression becomes down-regulated, 5T33MM cells become less susceptible to natural killer cell – mediated cytotoxicity.

Multiple myeloma (MM) is a lethal plasma cell (PC) malignancy accounting for ~1% of all cancer-related deaths in Western countries and is hallmarked by uncontrolled accumulation of monoclonal PCs in the bone marrow (BM);

ref. 1). In the BM, MM cells receive signals to survive and proliferate due to the existence of functional, mutual interactions between the MM cells and the BM microenvironment through growth factors and adhesion molecules (2, 3). Importantly, it has been shown that those interactions also confer resistance to conventional therapies (3).

Multiple genetic aberrations are observed during the pathogenesis of MM (4). However, there is increasing evidence that, in addition to genetic aberrations, epigenetic processes play a major role in the pathogenesis of MM (5). Two major interactive epigenetic modifications that result in a changed transcriptional activity are (a) DNA methylation of cytosine bases within a CpG dinucleotide and (b) posttranscriptional histone modifications. Hypermethylation of CpG islands within gene promoter regions accompanied with deacetylation of histone proteins results in transcriptional silencing of selected (tumor suppressor) genes, a common event in the progression toward malignancy (6, 7). Aberrant methylation of gene promoter regions is one of the most important epigenetic changes found in MM (8). In MM, hypermethylation of genes, including *p15*, *p16*, *DAP-kinase*, *BAD*, *BAK*, *BAX*, *BIK*, *SOCS-1*, and *E-Cadherin*, has been reported (9–14). Moreover, the growth factor interleukin-6 has been implicated in processes regulating epigenetic gene silencing in MM (15). Furthermore,

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histone deacetylase inhibitors (HDACi) have been shown to decrease survival of human MM cells *in vitro* by affecting genes involved in cell cycle and cell death pathways and by potentiating other proapoptotic agents (16–19).

Tetraspanins constitute a family of >30 membrane glycoproteins of which most members are broadly expressed. These glycoproteins are believed to serve as adaptor molecules facilitating the spatial organization and localization of multi-protein complexes in membrane microdomains (20–22). As such, they modulate several biological and pathologic processes, including cell motility and metastasis, adhesion, cell proliferation, differentiation, fusion, cell signaling, cytoskeletal reorganization, and virus infection (22, 23). The tetraspanin CD9 is important in the above-mentioned processes and shows also an inverse correlation between its expression level and tumor progression/metastasis in various solid tumors (24–27). Little is known about the expression and role of CD9 in MM. Recently, Barrena et al. (28) showed significant reduced CD9 surface expression on clonal PCs from MM patients ( $n = 5$ ) compared with PCs from MGUS patients ( $n = 5$ ) and to their normal counterparts, indicating that in MM CD9 expression levels might also inversely correlate with tumor progression. Therefore, reduced CD9 expression suggests a favorable condition toward cancer progression. However, a recent study showed that, in cervical carcinomas, CD9 is indeed globally down-regulated in most invasive cervical carcinomas but reexpressed in cells close to vessels and in the process of transendothelial transition (29). These findings indicate that the role of CD9 in cancer progression is very complex.

In humans, the chromosomal location of the *CD9* gene is 12p13.3. A possible cause of the down-regulation of CD9 might be found within the promoter region of the *CD9* gene. Transcription factors and epigenetic modifications, such as (de)methylation and (de)acetylation, have shown to be major regulators in gene expression. Indeed, screening of human MM cell lines ( $n = 6$ ) for tetraspanin expression (by flow cytometry analysis) revealed the predominant absence of CD9, CD81, and CD82 characterized by reduced steady-state mRNA levels and methylation of the promoter regions. Reexpression of these tetraspanins by demethylation in some of the human cell lines used supported the theory that (hyper)methylation plays an active role in regulating the transcription of CD9 (30).

However, until today, the mechanism in which HDACi alone or in combination with demethylation agents affects CD9 expression remains unclear (31).

In this work, we investigated CD9 expression in MM cells during disease progression using human primary MM cells and the 5T33MM and 5T2MM murine models of MM. We showed a correlation between CD9 expression on the one hand and disease status and survival on the other hand. Next, we addressed the involvement of two major interactive epigenetic modifications (i.e., DNA methylation and histone deacetylation) in the CD9 silencing and the possible consequences of this silencing in terms of susceptibility to natural killer (NK) cell-mediated cytotoxicity.

## Materials and Methods

**Patients' samples and clinical characteristics.** Eighty-one patients with MM were retrospectively evaluated in this study. MM patients were staged according to the criteria of Durie and Salmon (32). The patients' characteristics are summarized in Table 1. BM aspirates were collected for routine diagnostic or evaluation purposes after informed consent. The patients were classified as having active ( $n = 53$ ) or nonactive ( $n = 28$ ) disease at the moment of sample collection based on the criteria recently defined by the International Myeloma Working Group (33). Variables recorded at diagnosis were age, gender, BM plasmacytosis, serum  $\beta$ 2-microglobulin ( $\beta$ 2m), serum albumin, hemoglobin (Hb), creatinine, and M-spike. All patients provided informed consent under protocols approved by the Institutional Review Board of the Free University of Brussels.

**Mice.** C57Bl/KaLwRij/Hsd mice were purchased from Harlan CPB. Mice were housed and treated following the conditions approved by the Ethical Committee for Animal Experiments, Vrije Universiteit Brussel (license no. LA1230281).

**Cell lines.** The 5T2MM and 5T33MM $^{uv}$  cells originated from elderly C57Bl/KaLwRij mice that spontaneously developed MM. The cells have since been propagated into syngeneic, young mice by transplantation of diseased BM cells. The clinical and molecular characteristics of these models are very similar to those of the human disease, making them suitable models to study MM progression (34). Tumor-inoculated mice were monitored by weekly quantification of serum paraprotein concentration from the onset of the experiment until the end stage. As reported before, the serum paraprotein concentration is an independent indicator for MM progression and distinctive MM stages in the 5T2MM model (35). For the 5T2MM model, mice were sacrificed during the three different phases in tumor progression, namely, the quiescent stage of slow tumor progression (0–0.2 g/dL), the intermediate stage of moderate tumor growth (0.3–0.57 g/dL), and the end stage of accelerated progression (0.64–1.2 g/dL). For the 5T33MM model, mice were only used in the intermediate (0.2–1.5 g/dL) and end stage (1.6–4 g/dL), as the MM cells are only first detectable by flow cytometry at day 13, a time point at which the intermediate stage is already started. In each of these disease stages, mice were killed and BM cells were flushed out from femora and tibia. Isolation and purification of the MM cells in the BM were done as previously described (35). Cells obtained from end stage mice were 95% pure.

The 5T33MM $^{ut}$  cell line is a clonally identical but *in vitro* stroma-independent growing variant of the 5T33MM $^{uv}$  cell line. Cells were cultured and maintained in RPMI 1640 (BioWhittaker) supplemented with 10% bovine serum (Fetal Clone I; Hyclone), 1% natriumpyruvate, 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin, 2 mmol/L L-glutamine, and 1% MEM (supplements from BioWhittaker).

**Detection of CD9 expression on BM PCs by immunogold-silver staining.** Cytospin preparations of BM mononuclear cells, isolated

**Table 1.** Patient clinical characteristics

| Variable                           | N = 81     |
|------------------------------------|------------|
| Age (y), mean (range)              | 64 (38–91) |
| Male/female                        | 1.1/1      |
| Immunoglobulin type                |            |
| IgG                                | 41         |
| IgA                                | 30         |
| Bence Jones                        | 8          |
| Nonsecretory                       | 2          |
| BM plasmacytosis (%), mean (range) | 25 (2–98)  |
| Durie and Salmon stage             |            |
| Stage I                            | 18         |
| Stage II                           | 24         |
| Stage III                          | 39         |

by Ficoll-Hypaque density centrifugation (Cederlane), were used for immunogold-silver staining as described previously (36). We first added 20  $\mu$ L of 1:50 anti-human CD9 antibody (Dako) for 30 min at room temperature. Next, 50  $\mu$ L of 1:75 goat anti-mouse IgG+IgM-G5 (Amersham) reagent were added for 30 min at room temperature. Silver enhancement was done with the Intense silver enhancement kit (Amersham) for 1 h at 37°C. Next, the preparations were counter-stained with May-Grunwald-Giemsas, air dried, mounted with DPX mounting medium (BDH, VWR International), and examined by bright-field microscopy. A sample was considered positive when >20% of the PCs were CD9 positive.

**Flow cytometry.** CD9 cell membrane expression on the 5TMM cells during disease progression was detected by a double staining procedure. A rat anti-mouse CD9 IgG2a monoclonal antibody (KMC8) was used as a first step followed by FITC-conjugated goat anti-rat IgG monoclonal antibody as a second step. As control, an isotype-matched irrelevant antibody was used. All antibodies were purchased from BD Pharmingen. The proportion of tumor cells was determined by staining the 5T2MM and 5T33MM cells with anti-5T2MM idiotype and anti-5T33MM idiotype antibodies, respectively. Cells were analyzed with a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software or a FACSCanto flow cytometer (Becton Dickinson) using FACSDiva software.

**Methylation pattern of the CD9 gene promoter region.** 5T33MMvt cells were treated, respectively, with 80 nmol/L 5-aza-2'-deoxycytidine (5'aza-dc; Sigma-Aldrich), 4 nmol/L LBH589 (Novartis), and a combination of both inhibitors. Cells were replenished with fresh medium containing the compound(s) every 24 h. Genomic DNA from treated 5T33MMvt cells as well as nontreated 5T33MMvt and 5T33MMvv cells was isolated after 48 h (QIAamp DNA Mini kit, Qiagen). Bisulfite treatment was done on genomic DNA using the EpiTect Bisulfite kit (Qiagen). The bisulfite-treated gDNA was purified using an EpiTect spin column (Qiagen). We analyzed the 3-kb region upstream the transcription start of CD9 with Methyl Primer Express version 1.0 (Applied Biosystems). For the region containing the highest concentration of CpG sites, we designed bisulfite-specific primers: forward primer, 5'-AGAGTTTACGATGAATTAGGATG-3'; reverse primer, 5'-CCTAAACTCCACTTTTAAAAA-3'. A PCR was carried out amplifying 50 ng of bisulfite-treated gDNA in 40 cycles: 30 s at 95°C, 30 s at 54°C, and 30 s at 72°C. The amplicon (338 bp) was gel purified and TA cloned into pTZ57R (Fermentas). The resulting plasmids were sequenced by DNASion SA.

**Real-time quantitative PCR analysis.** Cells were lysed in 250  $\mu$ L Trizol (Invitrogen) and maintained at -80°C. Total RNA was extracted using the RNeasy Mini kit (Qiagen). The concentration and purity of RNA were determined by spectrophotometric measurement (Gene Quant II, Pharmacia Biotech). Total RNA (2  $\mu$ g) was converted into cDNA by the SuperScript First-Strand Synthesis System (Invitrogen) using random hexamers as primers. Quantitative real-time PCR was done using the ABI PRISM 7700 Sequence Detector. For the detection of mouse CD9 mRNA, an Assay-on-Demand (Applied Biosystems) was used as described by the manufacturer. To standardize the amount of sample RNA, we amplified the endogenous reference gene *GUS* also using an Assay-on-Demand. Each sample was amplified in triplicate. Relative standard curves were prepared for CD9 and *GUS* using cDNA from 5TMM-diseased BM.

**Lentiviral construction and production.** A lentiviral transferplasmid encoding mouse CD9 was constructed. The *mCD9* gene was obtained by *EcoRI* digestion of pBlueScript II-mCD9 (a kind gift from A. Kudo, Tokyo Institute of Technology, Yokohama, Japan; ref. 37) and inserted into the transferplasmid pHR'ripCMV-IRES-tNGFR-SIN (38) to yield pHR'ripCMV-mCD9-SIN. Mouse CD9-encoding lentiviral vector particles were produced in 293T cells by transient cotransfection of the transfer (pHR'ripCMV-mCD9-SIN), envelope (pMD.G), and packaging plasmid (pCMV $\Delta$ R8.9) as previously described (39). The vector stock was collected 48 and 72 h after transfection and concentrated by ultracentrifugation as described (39). The viral titer was determined by

infection of 293T cells with serial dilutions of the vector stock. Seventy-two hours after infection, the number of mCD9-positive cells was determined by fluorescence-activated cell sorting (FACS) analysis.

**Generation of a mCD9-positive 5T33MMvt cell line.** Fresh 5T33MMvt cells were transduced at a multiplicity of infection of 20. Cells were frequently replenished with fresh medium. Two weeks after transduction, cells were surface stained for CD9 and subcloned by FACS sorting single cells into a 96-well plate (Becton Dickinson FACSVantage using CellQuest software). Proliferating clones were FACS analyzed for CD9 expression.

**Isolation of NK cells.** Polyinosinic acid:poly-CMP (Amersham) was injected i.p. (200  $\mu$ g/mouse) into naive mice 24 h before using their spleen as a source of NK cells. Spleen cells were isolated by crushing the organ. Mononuclear cells were separated by Ficoll centrifugation and incubated on nylon wool columns to remove monocytes and B lymphocytes.

**Cytotoxicity assay.** NK cells were used as effectors in a <sup>51</sup>Cr release assay as previously described (40). Briefly, aliquots of the appropriate number of effectors were added in a total volume of 100  $\mu$ L per well of 96-well U-bottomed tissue culture plates. The E:T ratios used were 100:1, 50:1, 25:1, and 12.5:1, all in triplicate. Aliquots of <sup>51</sup>Cr-labeled targets (5  $\times$  10<sup>3</sup> cells) in a total volume of 100  $\mu$ L medium were added to each well. Subsequently, plates were incubated at 37°C for 4 h. After incubation, plates were centrifuged at 400 rpm, aliquots of 50  $\mu$ L were removed from each well and <sup>51</sup>Cr release was detected with a 1450 Microbeta liquid scintillation counter. As controls, targets were incubated with 100  $\mu$ L of 10% SDS detergent (maximal release) or 100  $\mu$ L of medium alone (spontaneous release). MM cell lines used as targets included 5T33MMvt, 5T33MMvv, and 5T33MMvt mCD9. The NK cell-sensitive T-cell line Yac-1 was used as positive control. The percent-specific release was calculated for each E:T ratio by the following formula:

$$\% \text{ specific release} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})} \times 100$$

**Statistics.** The Mann-Whitney *U* test was used for statistical significance of the *in vitro* data. Correlations between CD9 expression and tumor characteristics or clinical data were studied by the use of contingency tables. Statistical significance was evaluated by the Fisher's exact test. Survival curves were plotted using the Kaplan-Meier method. The statistical significance of differences in overall survival between groups of patients was estimated by the log-rank test. Overall survival was defined as the time from diagnosis until death from any cause, with those still alive censored at the time of the last follow-up. The clinical follow-up ended on August 31, 2007 with a median follow-up of survivors of 48 mo (range, 4-117 mo). The following baseline variables were considered for their prognostic value: age at presentation, gender, serum albumin, serum creatinine, Hb, BM plasmacytosis,  $\beta$ 2m, M-spike, and CD9 expression. Univariate analyses were done to screen for prognostic variables by using Cox proportional hazards regression. The Cox model was also used for multivariate analysis to identify the most significant variables related to outcome. A *P* value of <0.05 was considered significant in all statistical analyses. Statistical analysis of the study data was done using the MedCalc statistical analysis software (version 7.5.0.0) for Windows.

## Results

**CD9 expression by primary MM cells and correlation with disease activity.** The CD9 expression of primary MM cells from BM of 81 different patients was found to be heterogeneous and present in 43.2% of all MM patients tested. The expression of CD9 inversely correlated with disease activity: the percentage of MM patients being CD9 positive was higher in the population with nonactive disease than among patients with active

**Table 2.** Relationship between CD9 expression and clinical variables

|                    | CD9              |                  | P*          |
|--------------------|------------------|------------------|-------------|
|                    | CD9 <sup>+</sup> | CD9 <sup>-</sup> |             |
| Age (y)            |                  |                  |             |
| <65                | 20               | 25               | 0.82        |
| ≥65                | 15               | 21               |             |
| Gender             |                  |                  |             |
| Male               | 15               | 28               | 0.12        |
| Female             | 20               | 18               |             |
| Hb (g/dL)          |                  |                  |             |
| >10                | 25               | 21               | <b>0.02</b> |
| ≤10                | 10               | 25               |             |
| BM PC (%)          |                  |                  |             |
| <30                | 20               | 29               | 0.65        |
| ≥30                | 15               | 17               |             |
| β2m (mg/dL)        |                  |                  |             |
| <3.5               | 22               | 18               | <b>0.04</b> |
| ≥3.5               | 13               | 28               |             |
| Albumin (g/dL)     |                  |                  |             |
| ≥3.5               | 18               | 20               | 0.5         |
| <3.5               | 17               | 26               |             |
| Creatinine (mg/dL) |                  |                  |             |
| <2                 | 31               | 40               | 1           |
| ≥2                 | 4                | 6                |             |
| M-spike (g/dL)     |                  |                  |             |
| <3                 | 16               | 21               | 1           |
| ≥3                 | 19               | 25               |             |

NOTE: Significant ( $P < 0.05$ ) correlations are in bold.

\*By Fisher's exact test.

disease (60.7% versus 33.9%, respectively;  $P = 0.033$ , Fisher's exact test).

**Correlation of CD9 expression with clinical variables.** Several clinical variables were compared in patients with and without CD9 expression (Table 2). A significant correlation between CD9 expression and serum β2m, as well as Hb level, was observed. Higher levels of serum β2m and lower levels of Hb were observed in MM patients lacking CD9 expression ( $P = 0.04$  and  $0.02$ , respectively). CD9 expression did not correlate with age, gender, BM plasmacytosis, serum creatinine, albumin, or M-spike.

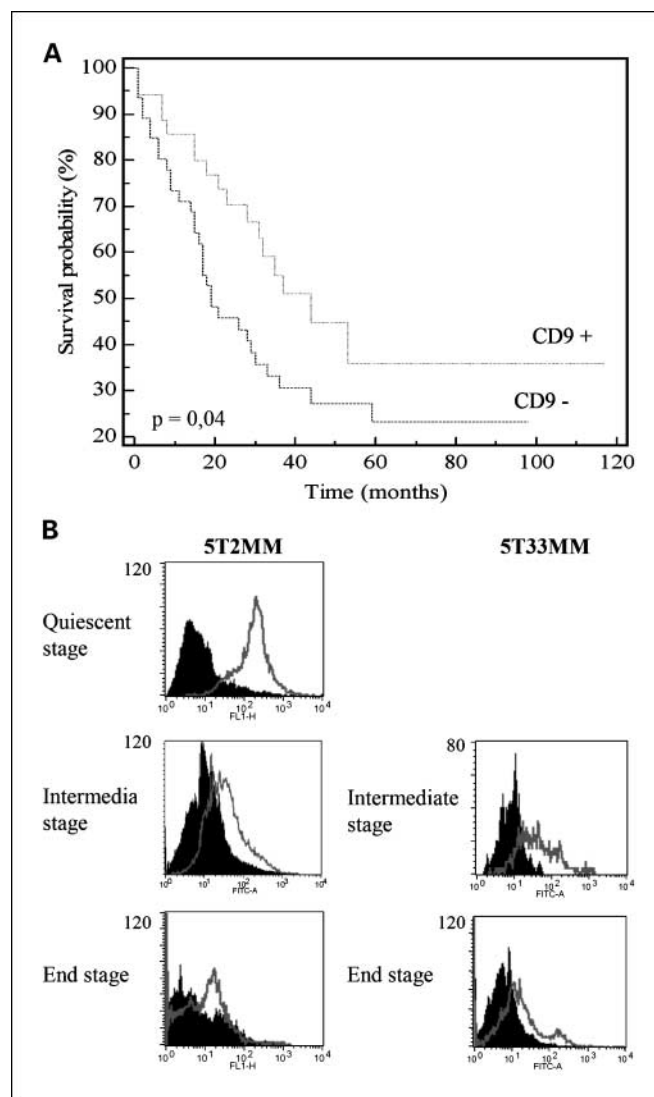
**CD9 expression at diagnosis correlates with survival.** We next investigated whether CD9 expression of 74 MM patients from whom BM samples were taken at diagnosis was predictive of MM outcome using the Kaplan-Meier method. These patients represent a subset of our study population. Survival probabilities of subgroups with or without CD9 expression were estimated and then compared by the log-rank test. There was a significant survival difference between the two groups ( $P = 0.04$ ), with a median survival of 43 and 24 months for the CD9-expressing and the CD9-nonexpressing group, respectively (Fig. 1A).

Using univariate Cox regression survival analysis, CD9 expression as well as various clinical variables, including β2m, albumin, and Hb, were identified as variables associated with disease outcome (Table 3A).

Subsequently, CD9 expression as well as these clinical variables, which were significant in univariate analysis, were included in a multivariate Cox stepwise regression model as covariates. Multivariable analysis showed that

only β2m was retained as independent predictor of survival (Table 3B).

**Expression of CD9 by 5T2MM and 5T33MM cells during tumor progression.** To further confirm the down-regulation of CD9 expression during disease progression in MM, we used the representative mouse models 5T2MM and 5T33MM. By a double-flow cytometric staining procedure with CD9 and tumor-specific anti-idiotypic monoclonal antibodies, we showed CD9 expression on the majority of the MM cells of the quiescent stage (82%; range, 80-85%) for the 5T2MM



**Fig. 1.** CD9 expression in MM and association with disease activity and survival. **A**, survival probability of MM patients according to the expression of the tetraspanin CD9. The Kaplan-Meier curve shows the probability of survival for MM patients with CD9 expression (gray dot line) compared with MM patients without CD9 expression (black dot line). The log-rank test was used to compare survival probabilities of the two groups. There was a statistically significant difference in survival between the two groups ( $P = 0.04$ ), with a median survival of 43 and 24 mo for the CD9-expressing and the CD9-nonexpressing group, respectively. **B**, down-regulation of the CD9 expression on the murine 5T2MM and 5T33MM cells during MM progression. Left, flow cytometric analysis of the 5T2MM cells during the different disease stages (quiescent, intermediate, and end stage); right, flow cytometric analysis of the 5T33MM cells during the intermediate and end stages. Black filled histograms, isotype control; gray line, CD9 staining. CD9 staining is shown on gated anti-5T33MM and anti-5T2MM idiotype-positive cells. Results from one representative experiment of three are shown.



**Table 3.**

| Variable   | RR (95% CI)      | P*            |
|--|------------------|---------------|
| A. Univariate analysis of the correlation of CD9 expression and clinical variables with survival |                  |               |
| Age  | 1.5 (0.87-2.67)  | 0.14          |
| Albumin  | 2.1 (1.19-3.81)  | <b>0.01</b>   |
| $\beta$ 2m   | 3.3 (1.83-6.09)  | <b>0.0001</b> |
| BM PC%   | 1.5 (0.87-2.66)  | 0.14          |
| CD9  | 2.5 (0.99-3.22)  | <b>0.04</b>   |
| Creatinine   | 1.8 (0.85-4.22)  | 0.12          |
| Gender   | 0.8 (0.93-2.9)   | 0.08          |
| Hb   | 1.9 (1.1-3.38)   | <b>0.02</b>   |
| M-spike  | 1.7 (0.93-2.9)   | 0.08          |
| B. Multivariate analysis: results of Cox stepwise regression analysis                            |                  |               |
| Albumin  | 1.6 (0.82-2.98)  | 0.18          |
| $\beta$ 2m   | 2.7 (1.38-5.33)  | <b>0.004</b>  |
| CD9  | 1.6 (0.85-2.83)  | 0.16          |
| Hb   | 0.99 (0.51-1.93) | 0.99          |

NOTE: Significant correlations are in bold ( $P < 0.05$ ).

Abbreviations: 95% CI, 95% confidence interval; RR, risk ratio.

\* $\chi^2$  test.

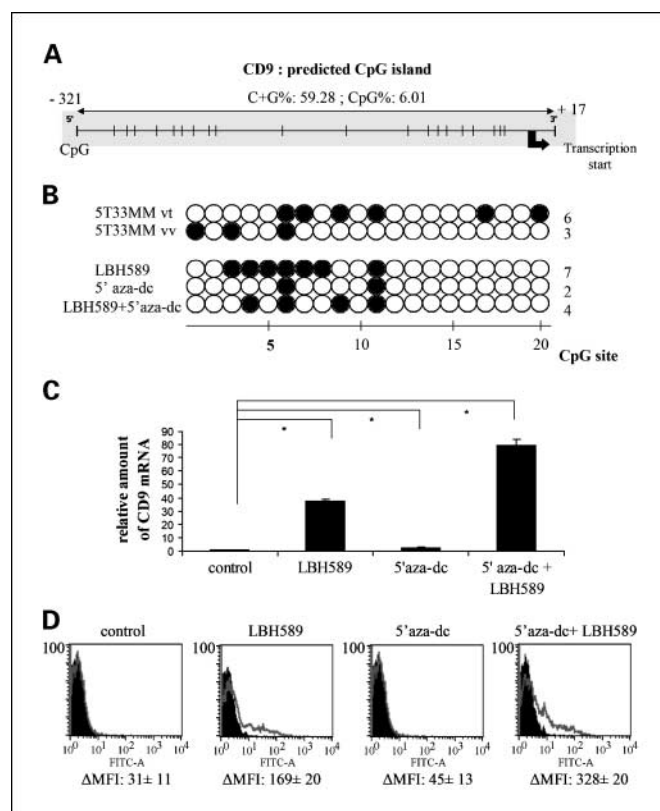
model (Fig. 1B). This percentage was already declining in the intermediate stage (50%; range, 45-55%), whereas in the end stage CD9 expression is almost completely lost (3%; range, 0-5%). This was also confirmed in the 5T33MM model (Fig. 1B): in the intermediate stage, ~70% (range, 65-75%) of the MM cells expressed CD9 compared with only 20% in the end stage (range, 18-32%). These data are clearly in line with the results observed in MM patients.

**CD9 promoter status.** We next sought to define the mechanisms involved in the down-regulation of CD9 in MM cells. As previously shown, 5T33MM $vv$  cells of terminally diseased mice express CD9 at low levels, whereas the stroma-independent variant 5T33MM $vt$  does not (41). To establish if the expressing (5T33MM $vv$ ) and nonexpressing cell lines (5T33MM $vt$ ) harbor different methylation patterns, we amplified the CD9 promoter region containing 20 CpG sites from bisulfite-modified gDNA (shown in Fig. 2A). Bisulfite converts all unmethylated cytosines to uracils, leaving the methylated cytosines unconverted. The specificity of the primers was analyzed by their failure to amplify non-bisulfite-treated gDNA. The amplified product of three independent PCRs was cloned to ensure that methylation patterns are fully representative for the original sample (42). For each sample, 10 different clones were sequenced and their methylation pattern was determined. As shown in Fig. 2B, the CD9 promoter region of 5T33MM $vt$  cells contains twice as much methylated CpG sites compared with the 5T33MM $vv$  cells. The distribution of methylated CpG sites is also different among samples.

**Reactivating CD9 expression.** To confirm the central role of (hyper)methylation and/or deacetylation in the down-regulation of CD9 during disease progression, 5T33MM $vt$  cells were treated with the demethylation agent 5'aza-dc, the clinically relevant HDACi (LBH589), and a combination of both. Treatment with 80 nmol/L 5'aza-dc up to 4 days resulted only in a modest 2- to 4-fold increase in CD9 mRNA levels as determined by real-time quantitative PCR (Fig. 2C). Increasing the concentration of 5'aza-dc resulted in massive cell death from day 2 onwards (data not shown). In contrast, treatment

with 4 nmol/L LBH589 up to 2 days induced a 40-fold increase in CD9 mRNA levels, indicating that promoter demethylation might not be required for CD9 induction. Nevertheless, cotreatment of cells for 48 h resulted in a strong synergistic retranscription of CD9, confirming that epigenetic regulation plays an important role in CD9 silencing in MM. Using flow cytometric analysis, consistent with the changes observed in CD9 mRNA levels, a synergistic effect on CD9 protein levels was observed after cotreatment (Fig. 2D).

Next, we analyzed methylation patterns of 5T33MM $vt$  cells treated with 5'aza-dc, LBH589, and a combination of both by bisulfite sequencing, as described before. Figure 2B shows methylation distribution over 20 analyzed CpG sites within the CD9 promoter. As expected, the net amount of methylated cytosines in samples treated with 5'aza-dc was found to be much lower than in untreated 5T33MM $vt$  control cells. In



**Fig. 2.** The epigenetic regulation of the CD9 expression in the 5T33MM model. **A**, schematic representation of the amplified CD9 promoter region containing 20 CpG sites from bisulfite-modified genomic DNA. **B**, genomic bisulfite sequencing of the selected CD9 promoter region of 5T33MM $vt$ , 5T33MM $vv$ , and 5T33MM $vt$  cells treated for 48 h with LBH589 (HDACi); 5T33MM $vt$  cells treated for 48 h with 5'aza-dc (demethylation agent); and 5T33MM $vt$  cells treated for 48 h with both agents. Each circle represents an individual CpG site. Closed circles, methylated CpG sites; open circles, unmethylated sites. **C**, strong synergistic reexpression of CD9 mRNA in the 5T33MM $vt$  cells after combined treatment with LBH589 and 5'aza-dc. Real-time quantitative PCR analysis showing relative fold induction of CD9 mRNA in the 5T33MM $vt$  cells after treatment with LBH589, 5'aza-dc, and the combination compared with control. Columns, mean of three independent experiments; bars, SD. \*,  $P < 0.05$ . **D**, synergistic membranous reexpression of CD9 on the 5T33MM $vt$  MM cells after treatment with LBH589 and 5'aza-dc. Flow cytometric analysis showing induced CD9 expression on the 5T33MM $vt$  cells after treatment with LBH589, 5'aza-dc, and the combination compared with control. Black filled histograms, isotype control; gray line, CD9 staining. Results from one representative experiment of four are shown.  $\Delta$ MFI, increase in mean fluorescence intensity compared with control staining. Points, mean of four mice; bars, SD.  $P < 0.05$ .

contrast, treatment with LBH589 resulted in a slight increase and shift in methylated CpG sites compared with untreated cells. Cotreatment with both compounds reduced the amount of methylated CpG sites compared with cells treated with LBH589 alone but not to the same extent as cells treated with 5'aza-dc alone. The above data thus suggest that deacetylation is the key epigenetic mechanism resulting in a higher CD9 reactivation in the 5T33MM model.

**The CD9-transduced 5T33MMvt cells are more sensitive to cell-mediated lysis than control transduced or nontransduced parental cells.** In the past, Shallal and Kornbluth (40) showed that CD9 expression enhances the susceptibility of MM cell lines to cell-mediated cytotoxicity. We thus speculate that, as CD9 expression becomes down-regulated during disease progression, MM cells probably become less susceptible to cell-mediated cytotoxicity and thus can escape the immune system. To confirm this, we did NK cell cytotoxicity assays using the CD9-expressing 5T33MMvt and CD9-nonexpressing 5T33MMvt cell lines. As expected, the CD9-expressing MM cells were clearly more susceptible to NK cell-mediated cytotoxicity than the 5T33MMvt cell line lacking CD9 (Fig. 3B). To further examine the involvement of CD9 in NK cell-mediated cytotoxicity, the 5T33MMvt cell line was stably transfected with the lentiviral transfer plasmid pHR'ripCMV-SIN or pHR'ripCMV-mCD9-SIN. Two weeks after transduction, cells were subcloned by FACS sorting and proliferating clones were screened by flow cytometric analysis. As shown in Fig. 3A, a 99% pure clone with strong CD9 expression was obtained. Next, NK cell assays were done with control and CD9-transduced cells as targets. Again, the CD9-expressing MM cells were more sensitive to NK cell-

mediated lysis than nontransduced parental cells (Fig. 3B). No difference was observed in CD9 expression and susceptibility to NK mediated lysis between nontransduced parental cells and control transduced cells (data not shown).

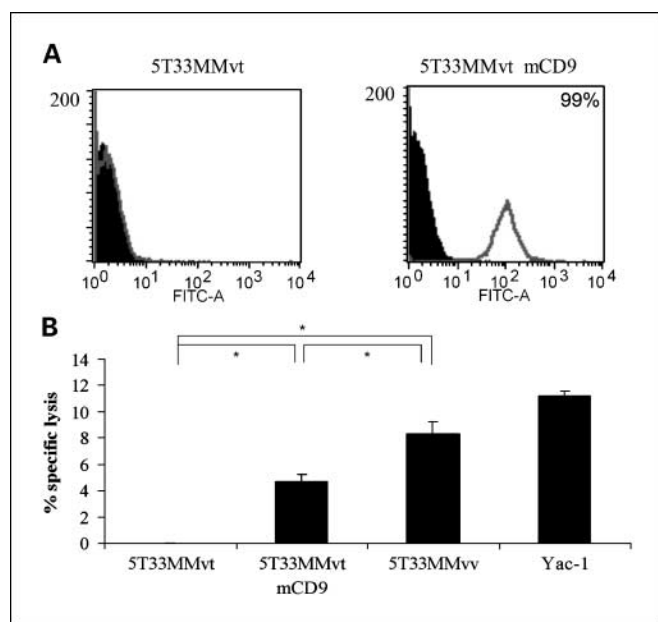
## Discussion

MM remains a lethal malignancy despite the intensive research in the last decades. Crucial for their survival, the MM cells are dependent of functional interactions with the BM microenvironment through membrane-embedded molecules (3). The tetraspanin CD9 showed a lot of potential as a possible target due to an inverse correlation between protein expression and tumor progression/metastasis in various solid tumors (24–27). To determine CD9 expression throughout MM disease, we examined protein expression on PCs in a large cohort of MM patients ( $n = 81$ ). We found that CD9 expression by primary MM cells was heterogeneous among the MM patients tested. In addition, patients with active disease expressed significantly less frequent CD9 compared with MM patients with nonactive disease, indicating that CD9 expression levels inversely correlate with MM progression. This suggests that CD9 expression can be altered when disease progresses. These data were further confirmed in murine 5T2MM and 5T33MM cells at the different stages of disease progression. It was found that CD9 expression was also clearly down-regulated on MM cells during disease progression in both the 5T2MM and 5T33MM *in vivo* mouse models ( $P = 0.045$ ).

Interestingly, CD9 expression also correlated with clinical variables such as serum  $\beta 2m$  and Hb levels. Using the Kaplan-Meier method, the prognostic value of CD9 expression in MM patients at the time of diagnosis was investigated. A significant survival difference was found between CD9-expressing and CD9-nonexpressing groups, with a survival advantage for the CD9-expressing group. Higher levels of serum  $\beta 2m$  and lower levels of Hb, both indicators of poor prognosis, therefore correlate well with the negative prognostic profile of the MM patient subgroup lacking CD9 expression. Previous studies have shown in various cancer types, including breast, esophageal, and lung cancer, that loss of CD9 expression is associated with worse prognosis (43, 44). This indicates that evaluation of CD9 expression in individual patients could identify subgroups who may have a high risk of rapid disease progression.

Univariate Cox regression survival analysis showed that serum  $\beta 2m$  and albumin levels predicted disease outcome in our study population. These findings allow us to conclude that this study population is representative for evaluating the clinical significance of CD9 expression in MM.

We, however, failed to show in multivariate analysis that CD9 represented an independent factor influencing survival. Nevertheless, the association between CD9 expression and  $\beta 2m$  levels clearly reflects a feature of worse outcome. Analysis of CD9 expression in a larger number of patients is necessary to make the statistical conclusions more robust and to further evaluate its value as biomarker in MM. In the presence of statistical significance, CD9 expression may add independent information to standard prognostic factors and may therefore, together with established prognostic markers such as  $\beta 2m$  and albumin, act as a marker to predict prognosis in individual MM patients. Although there is not enough evidence to use CD9



**Fig. 3.** The sensitivity of CD9-transduced 5T33MMvt cells to NK cell-mediated cytotoxicity is significantly higher than the control transduced and parental cell lines. **A**, comparison of CD9 expression on the surface of the CD9-transduced and parental 5T33MMvt cell lines. Left, parental cell line; right, CD9 transduced cell line. Black filled histograms, isotype control; gray line, CD9 staining. Results from one representative experiment of three are shown. **B**, 5T33MMvv, 5T33MMvt, and CD9-transduced 5T33MMvt cells were used as targets in 4-h  $^{51}Cr$  release assays. NK cells were used as effectors. E:T ratios shown are 100:1. Columns, mean of three independent experiments; bars, SD. \*,  $P < 0.05$ . Yac-1 cells were used as positive control for the NK cell assay.

expression in clinical practice, the present findings should, however, stimulate more work on CD9 in MM.

DNA methylation and modification of histones have both proven to be important regulators of gene expression. Therefore, we examined if either or both phenomena play a role in the down-regulation of CD9 during MM disease progression. Treatment of 5T33MM*vt* cells with a methylation inhibitor (5'aza-dc) resulted in a modest but significant elevation of CD9 expression. A similar small (14%) but significant increase in membranous expression of CD9 in RPMI 8226 human MM cells was seen after demethylation (30). However, Drucker et al. showed methylation of specific CpG sites within the CD9 promoter but failed to analyze a complete CpG island inside the promoter, making it difficult to determine whether the promoter is in fact hypermethylated or not. Therefore, we confirmed our findings by bisulfite PCR sequencing amplifying a complete CpG island inside the promoter and showed a reduced amount of methylated CpG sites in samples treated with the methylation inhibitor. CD9 is found to be rarely methylated in the non-small cell lung cancer cell lines and in primary lung tumor tissue, but we showed methylation at low frequencies in both 5T33MM*vt* and 5T33MM*vt* cells. Moreover, in the article written by Drucker et al. (30), the U266MM cell line did not show any (re)activation of CD9 after treatment with 5'aza-dc despite the presence of methylation as observed by methylation-specific PCR. Therefore, we were convinced that methylation could not be the only epigenetic event regulating CD9 expression.

Treatment with a clinically relevant acetylation inhibitor (HDACi) LBH589 showed, despite a shift in methylated sites and slight increase in methylation in the bisulfite-treated LBH589 samples, a much stronger reactivation of CD9 expression. Our results are in analogy with Zhong et al. (45), who showed that CD9 expression is dominantly regulated by histone acetylation in lung cancer. These results indicate that LBH589 is capable of reactivating CD9 in MM cells at a much higher rate than 5'aza-dc can. The presence of some specific methylated CpG sites might be beneficial and might function as a docking site for HDAC and DNA methyltransferase-related molecules, which results in a changed chromatin structure leading to a changed CD9 expression (31).

The molecular mechanisms by which LBH589 and trichostatin A reactivate silenced estrogen receptor gene expression in the breast cancer cell line MDA-MB-231 were very recently shown (46). By chromatin immunoprecipitation analysis, trichostatin A and LBH589 treatment was found to release DNA methyltransferase 1, HDAC1, and the H3 Lys<sup>9</sup> (H3-K9) methyltransferase SUV39H1 from the promoter (the latter only with LBH589). These changes resulted in accumulation of acetylated histones H3 and H4 (histone modifications associated with active chromatin), a decrease in methylated H3-K9 (repressive histone modification), and impaired binding of the heterochromatin protein 1 $\alpha$ , leading to an open chromatin structure. Furthermore, LBH589 treatment was not accompanied by demethylation of the CpG island. For CD9, Zhong et al. (45) showed accumulation of acetylated histone H4, increase in histone H3 Lys<sup>4</sup> methylation, and a decrease in methylated H3-K9 in lung cancer cells after treatment with trichostatin A. It is thus plausible to suggest that LBH589 restores expression of CD9 in MM by reorganization of the heterochromatin-associated proteins and opening of the chromatin structure.

To test our hypothesis, we did a combination study with 5'aza-dc and LBH589. The results show a strong synergistic effect, although the methylation status determined by bisulfite sequencing shows a decrease in the number of methylated CpG sites compared with LBH589 treatment alone. In accordance with our data, a synergistic effect of 5'aza-dc and LBH589 was also shown for the estrogen receptor gene expression (46). Our findings show that, even in the presence of some specific methylated CpG sites, HDACi are able to open the chromatin structure and induce increased CD9 expression. Our data indicate that, when the amount of methylated CpG sites is extreme (too high or too low), too many or too few possible docking stations are available for HDAC and DNA methyltransferase-related molecules, which results in none or a modest activation of CD9. This is shown in samples treated with LBH589, 5'aza-dc, and the combination, where an intermediate amount of methylated CpG sites results in the highest expression of CD9. Not only the amount but also the localization of the methylated CpG sites seems to be crucial for expression. For instance, 5T33MM*vt* samples with no detectable CD9 expression show methylated CpG sites 17 and 20 compared with all other samples that do express CD9. In contrast, CpG site 6 is methylated in all samples. Some specific CpG sites are protected from methylation; Höller et al. (47) described protection from methylation by the SP1 transcription factor. CpG sites 12 and 14 are part of an SP1 transcription factor and are in none of the samples methylated. In short, our findings support the hypothesis that specific methylated CpG sites can serve as adapter sites for epigenetic modifying proteins (HDAC, DNA methyltransferase-related proteins, etc.), which change chromatin structure.

We and others recently showed that although CD9 expression is globally down-regulated during tumor progression to promote expansion of the malignant cells, CD9 is locally reexpressed to adjust microenvironmental requirements (29, 41). Indeed, we showed that CD9 expression by the MM cells is locally up-regulated *in vivo* by close interaction of the cells with BM endothelial cells and that CD9 is involved in trans-endothelial invasion and thus metastasis of the MM cells to anatomically distant BM sites or extramedullary sites at the end stage of the disease (41). It has become clear that DNA methylation is not a primary cause of inactivation of transcription but rather maintains long-term silencing of genes that had already been switched off by histone modifications, such as histone deacetylation and H3-K9 methylation (31). These modifications are more dynamic modifications and are believed to occur earlier in epigenetic silencing (31, 48). Thus, down-regulation by promoter histone modifications is a reversible and thus more "immature" state of silencing, which can be modulated or selected for in relation to the microenvironment according to the needs (48, 49). This further confirms our observation that histone modifications, rather than DNA methylation, are key epigenetic modifications in CD9 down-regulation during MM progression.

It has now become widely accepted that epigenetic changes cooperate with genetic changes to cause cancer development and progression. Indeed, epigenetic mechanisms are essential for development and progression of cancers by complementing, amplifying, and diversifying genetic alterations (49, 50). Very recently, the epigenetic progenitor model has suggested that cancer cells might arise from stem cells by polyclonal epigenetic

silencing of epigenetic gatekeepers (such as p16 or APC) locking them in a stem cell-like state, thus fostering clonal expansion and providing a substrate for risk of subsequent genetic and epigenetic alterations that further drive tumor progression (50). Thus, genetics and epigenetics cooperate at all stages of cancer development. Together with the fact that (1) the observed epigenetic regulation of CD9 was proven to be functional in MM in terms of immune escape and that (2) immune escape mechanisms are early events in carcinogenesis (51), it is very likely that the epigenetic regulation of CD9 is rather a cause than a consequence of MM progression. However, it cannot be ignored that not all epigenetically silenced genes play a direct role in MM progression because it has become clear that a substantial number of silenced genes may represent by-products of an abnormal epigenetic program (50).

In summary, a correlation between CD9 expression on the one hand and disease status and survival on the other hand was

shown. We clearly showed that epigenetics (especially histone modifications and to a lesser extent DNA methylation) play an important role in the down-regulation of CD9. Furthermore, we provide evidence that, as CD9 expression becomes down-regulated during disease progression, MM cells become less susceptible to NK cell-mediated cytotoxicity and thus might escape the immune system.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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