

ORIGINAL ARTICLE

Role of Growth arrest-specific gene 6-Mer axis in multiple myeloma

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Multiple myeloma is a mostly incurable malignancy characterized by the expansion of a malignant plasma cell (PC) clone in the human bone marrow (BM). Myeloma cells closely interact with the BM stroma, which secretes soluble factors that foster myeloma progression and therapy resistance. Growth arrest-specific gene 6 (Gas6) is produced by BM-derived stroma cells and can promote malignancy. However, the role of Gas6 and its receptors Axl, Tyro3 and Mer (TAM receptors) in myeloma is unknown. We therefore investigated their expression in myeloma cell lines and in the BM of myeloma patients and healthy donors. Gas6 showed increased expression in sorted BMPCs of myeloma patients compared with healthy controls. The fraction of Mer⁺ BMPCs was increased in myeloma patients in comparison with healthy controls whereas Axl and Tyro3 were not expressed by BMPCs in the majority of patients. Downregulation of Gas6 and Mer inhibited the proliferation of different myeloma cell lines, whereas knocking down Axl or Tyro3 had no effect. Inhibition of the Gas6 receptor Mer or therapeutic targeting of Gas6 by warfarin reduced myeloma burden and improved survival in a systemic model of myeloma. Thus, the Gas6-Mer axis represents a novel candidate for therapeutic intervention in this incurable malignancy.

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INTRODUCTION

Multiple myeloma (MM), the second most common hematologic malignancy, is characterized by monoclonal expansion of malignant plasma cells (PC), which acquire numerous genetic abnormalities.^{1,2} The disease is characterized by anemia, immunodeficiency and decreased renal function caused by expansion of malignant PCs, which secrete monoclonal protein.^{1,2} Interaction of myeloma cells with the bone marrow (BM) microenvironment results in a vicious circle leading to osteolyses and disease progression.^{1,2} In recent years, treatment of myeloma has advanced because of stem cell transplantation and availability of novel agents including bortezomib, carfilzomib, thalidomide and lenalidomide, but the disease remains incurable in the majority of patients.^{1,2} Therefore, it is important to develop novel therapies to improve treatment of myeloma patients.

Growth arrest-specific gene 6 (Gas6) was discovered because of its upregulation in fibroblasts under growth-arrest conditions.^{3,4} Gas6 exerts pleiotropic functions in health and disease; it amplifies platelet aggregation in the late phase of thrombus formation^{5,6} enhances erythropoiesis⁷ and increases leukocyte extravasation in inflammatory conditions,⁷ among other functions.^{8,9} Gas6 binds to the TAM family of receptor tyrosine kinases (TAMRs), which consists of Tyro3 (Sky/Rse), Axl (Ufo/Ark) and Mer (Eyk),

though binding affinity of this ligand differs for each receptor (Axl > Tyro3 >> Mer).^{4,10} TAMRs have transforming properties.³ Indeed, expression of Mer in the pro-B BaF3 cell line or of Axl in 32D cells¹¹ leads to growth-factor-independent proliferation.¹²

Gas6 is overexpressed in different human cancers as compared with healthy tissue^{13–17} and promotes proliferation and survival of different cancer cell lines, including prostate and melanoma tumor cells.^{18,19} We recently demonstrated that Gas6 induces cell proliferation and growth of different solid tumors using different preclinical models.²⁰ Furthermore, Gas6 and its receptors also play an important role in hematologic malignancies. Our data indicate that human AML cells express minimal levels of Gas6 but can induce upregulation of Gas6 in the BM stroma to foster their growth and therapy resistance.²¹ In addition, Axl is a negative prognostic factor and therapeutic target in AML.²¹

In normal hematopoiesis, Mer is expressed in tissue macrophages, natural killer cells, dendritic cells and megakaryocytes. Mer is not present at any stage of lymphocytic differentiation. However, transgenic overexpression of Mer in the hematopoietic system of mice induces the development of lymphadenopathy and T-lymphoblastic leukemia.²² Aberrant Mer expression was detected in T-ALL and AML.^{3,23,24} Pre-clinical models indicated that Mer mediates increased cancer cell proliferation, survival and

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therapy resistance.^{25,26} Mer has been shown to induce PI3K/AKT, ERK1/2, NfκB and p38 signaling among other pathways.^{3,24,27} Altogether, these data show an important role of Gas6 and its TAMR signaling in hematologic neoplasia, but they have not yet been studied in myeloma. Therefore, we set out to investigate the expression, biological function and target potential of Gas6 and its receptors in myeloma.

MATERIALS AND METHODS

Detailed methods are provided in the supplement.

Animals

Six to eight-week-old female NSG (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) mice were purchased (Jackson laboratory, Bar Harbor, ME, USA). All animal experiments were carried out according to the institutional guidelines for the welfare of animals in experimental neoplasia and were approved by the local licensing authority (Behörde für Soziales, Gesundheit, Familie, Verbraucherschutz; Amt für Gesundheit und Verbraucherschutz, Hamburg, Germany, project number 53/12).

Patient samples

To analyze Gas6 and Mer expression levels, we used samples from healthy BM donors and BM from patients with primary diagnosis of MM treated at the Medical Center Hamburg-Eppendorf and at the University Hospital Heidelberg. All studies with human samples were carried out in accordance with the declaration of Helsinki with approval of the medical ethics committee of the Hamburg Medical Association (approval numbers PV3400, OB-038/06 and WF-015/10).

Cells and culture conditions

The human multiple myeloma cell lines U266 and RPMI8226 were cultured in RPMI-1640 medium supplemented with 10% of FCS and with 20% of FCS for Molp8 cells, respectively.

Cell-growth and apoptosis assays

After transduction of cell lines with fluorescent protein-encoding lentiviral vectors mediating overexpression or sh-mediated knockdown of Gas6, Mer, Axl or Tyro3, cells were FACS sorted to obtain a purity of >95% (please see below for details of the cloning). Cells were allowed to recover for 3 days after sorting. Apoptosis levels were determined by AnnexinV-APC/propidium iodide assay by flow cytometry (FACS Calibur, Becton Dickinson (BD), Heidelberg, Germany).

Western Blot analysis

Western blot analysis was carried out 3 days after sorting of transgene-expressing cells as previously described.²⁸ The pAkt, tAkt, p-p44/42 MAPK (pErk1/2), p44/42 MAPK (tErk1/2), cleaved caspase-3, Bcl-2 and t-Mer antibodies were purchased from Cell Signaling (distributed through New England Biolabs GmbH, Frankfurt am Main, Germany). β-Actin antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany). pMer antibody was ordered from Abcam (Cambridge, UK). t-Tyro3 antibody was purchased from R&D Systems (Wiesbaden, Germany). t-Axl antibody was a gift from Björn Dahlbäck (Lund, Sweden).

ELISA and RT-PCR

Human Gas6 levels were determined in cell culture supernatants and BM plasma using ELISA kits according to the manufacturers instructions (R&D Systems). Human IgG concentration was assessed in blood plasma from myeloma-bearing mice according to the manufacturers instructions (Human Lambda ELISA KIT; Bethyl Laboratories Inc, Montgomery, TX, USA). Quantitative RT-PCR of Gas6 and Mer was performed using premade GeneExpression Assays from Applied Biosystems (Darmstadt, Germany) and the Eppendorf MasterCycler technology (Eppendorf, Hamburg, Germany). Copy numbers for GAPDH, Mer and Gas6 were calculated using the standard curve method for absolute quantification.

Flow Cytometry

Analysis of apoptosis (Annexin/PI assay) was performed using FACS Calibur with BD CellQuest Pro software (BD). To analyze Mer expression, the PC

population in BM samples was characterized by multicolor flow cytometry using an established panel of antibodies (including CD45, CD38, CD138, CD19, CD56, CD117 all from Beckmann Coulter, Krefeld, Germany).

Multiple myeloma mouse model

U266 cells (2×10^6) were injected into the femur of 6–8-week-old NSG recipients as previously described.²⁹ Animals were considered end-stage and killed when they showed symptoms of hind limb paralysis as published.²⁹ U266 cells were quantified by assessing Venus or GFP fluorescence using FACS Calibur (BD). Myeloma load was monitored by determination of the amount of paraprotein in the blood plasma using ELISA (Human Lambda ELISA KIT; Bethyl Laboratories Inc).

Cloning of LeGO vectors and production of lentiviral particles

Cloning of LeGO vectors for overexpression or silencing of human Gas6, Mer, Axl or Tyro3 was performed as previously described^{30,31} (for detailed protocols and vector maps, refer to <http://www.lentigo-vectors.de>). Sequences of primers used for PCR amplification are provided in Supplementary Table S1. Generation of viral particles and titration were performed as described.^{30,31}

Gene expression arrays

Gene expression profiling of BMMNs and BMPCs was performed as described previously.³² For an in-depth sample overview, refer to Seckinger et al.³³ Absolute gene expression levels are displayed as log2-transformed values.

Statistics

Data represent mean ± s.d., unless otherwise stated. Statistical significance was calculated by Student's *t*-test unless otherwise stated. Gene expression analyses were performed on GC-RMA pre-processed samples as described.³⁴ Because of two different IVT labeling kits used, batch correction was performed using ComBat. (implemented in Bioconductor software, www.bioconductor.org)³⁵ To assess the presence or absence of gene expression, the 'Presence-Absence calls with Negative Probesets' (PANP) algorithm³⁶ was used.

RESULTS

Expression of Gas6 and TAMRs in myeloma patients

In the first step, we studied the expression of Gas6 and its TAM receptors Axl, Tyro3 and Mer in total BM mononucleated cells (BMMNCs) isolated from primarily diagnosed myeloma patients ($n=17$; clinical characteristics are displayed in Supplementary Table S2) in comparison with healthy BM donors ($n=11$) using qRT-PCR. We found that Gas6 and Mer mRNA expression were increased in the BMMNCs of myeloma patients compared with controls, whereas Axl was not and Tyro3 not significantly differentially expressed (Figure 1a and data not shown). The overexpression of Gas6 and Mer in myeloma BMMNCs could be validated using a set of 154 whole BM samples from gene expression profiling of an independent patient cohort³³ (Figures 1b and c). In this larger cohort, Axl and Tyro3 were mostly undetectable in myeloma and control BMMNCs (Figures 1d and e; Supplementary Table S3).

In the next step, we analyzed the mRNA expression level of Gas6 and TAMRs in FACS-sorted CD138⁺ BMPCs from 332 newly diagnosed myeloma patients in comparison with CD138⁺ BMPCs from 11 healthy donors (clinical characteristics are displayed in Supplementary Table S2).³³ We also wished to find out whether the expression levels of Gas6 and its receptors in BMPCs are different between myeloma and monoclonal gammopathy of unknown significance (MGUS), a premalignant stage of myeloma ($n=22$; clinical characteristics are displayed in Supplementary Table S2).

We therefore compared the mRNA expression levels of Gas6, Axl, Tyro3 and Mer as determined by Affymetrix arrays in healthy BMPCs with BMPCs from MGUS and myeloma patients.

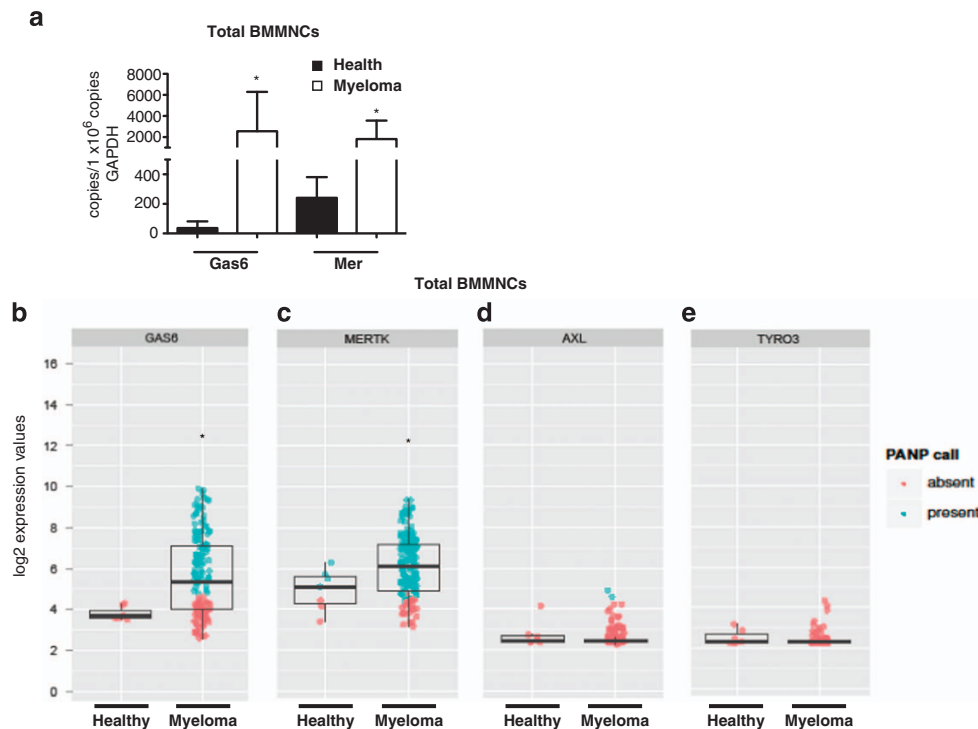


Figure 1. Mer and Gas6 mRNA expression are increased in total BM mononucleated cells (BMNNCs) from myeloma patients. (a) qPCR analysis of Gas6 and Mer mRNAs in BMNNCs from myeloma patients and healthy BM donors normalized to 1×10^6 GAPDH copies. Gas6 and Mer mRNA expression were upregulated in myeloma samples compared with healthy donors ($n = 11/17$, $*P = 0.0355$; $n = 11/15$, $*P = 0.0073$). (b–e) Affymetrix gene expression profiling of Gas6 (b), Mer (c), Axl (d) and Tyro3 (e) showing upregulation of Gas6 ($n = 7/154$, $*P < 0.001$) and Mer ($n = 7/154$, $*P = 0.02$) in myeloma BMNNCs compared with healthy control cells (log2-transformed absolute gene expression levels are displayed).

These analyses indicated that Gas6 was expressed by the majority of BMPCs from myeloma patients, MGUS patients and healthy controls (Supplementary Table S4). Interestingly, Gas6 was upregulated in BMPCs isolated from MGUS ($*P = 0.003$) and myeloma patients ($P < 0.001$) when compared with healthy BMPCs (Figures 2a–d). Subsequently, we analyzed the Gas6 mRNA expression levels in the cytogenetic subgroups del17p, gain1q21, t(4;14), t(11;14) and hyperdiploidy and compared them with cases without the respective aberration. Furthermore, we also investigated the seven subgroups CD1 (CCND1/CCND3 activating translocations), CD2 (CCND1/CCND3 activating translocations, CD-20 and PAX5 overexpression), HY (hyperdiploidy), LB (low bone disease), MF (MAF/MAFB activating translocations), MS (MMSET activating translocations) and PR (proliferation index high) of the gene expression profiling defined molecular classification of myeloma³⁷ in our patient cohort.

These analyses yielded the finding that Gas6 mRNA was significantly lower expressed in t(11;14) myeloma ($*P = 1.8 \times 10^{-6}$) and CD2 cases ($*P = 1.8 \times 10^{-8}$; Supplementary Figures S1a and S2a). It showed significantly higher expression in myeloma with gain1q21 ($*P = 0.004$), LB ($*P = 1.3 \times 10^{-5}$), MF ($*P = 2.4 \times 10^{-3}$) and MS cases ($*P = 9.4 \times 10^{-3}$; Supplementary Figures S1b, S2b, c and d).

To validate our finding that Gas6 is upregulated in myeloma BM at the protein level, we subsequently quantified Gas6 protein expression in the BM plasma (BMP) of newly diagnosed myeloma patients and healthy controls. These analyses revealed a threefold upregulation of Gas6 protein in BMP of myeloma patients at primary diagnosis compared with healthy individuals (Figure 2e).

Also, Mer mRNA was expressed by almost all BMPCs from myeloma patients, MGUS patients and healthy controls at a similar expression level (Supplementary Table S4). Mer mRNA was significantly lower expressed in myeloma with t(4;14) ($*P = 1.6 \times 10^{-6}$), gain1q21 ($*P = 7.7 \times 10^{-5}$) and in CD1 ($*P = 9.2 \times 10^{-6}$) and MS cases

($*P = 5.8 \times 10^{-7}$) (Supplementary Figures S1c–f). The Mer receptor mRNA was significantly higher expressed in HY ($*P = 6.2 \times 10^{-4}$) and MF cases ($*P = 9.1 \times 10^{-4}$; Supplementary Figures S1g and h).

Altogether, BMPC expression of Gas6 and Mer mRNAs showed high variability within the cytogenetic and GEP subgroups and a linear regression of expression levels on the molecular classification entities explained only 17% and 20% of the variability in Gas6 and Mer mRNA expression, respectively (Supplementary Figures S2e and f). In conclusion, most of the variability in gene expression cannot be explained by expression differences between GEP-defined entities.

In summary, in the cytogenetic and GEP subgroups, there is no consistent association of Gas6 or Mer mRNA expression in adverse or good risk groups. Also, we did not find an independent association of Gas6 or Mer mRNA expression levels with overall survival in our patient cohort after correcting for high-risk cytogenetics (data not shown).

We also prospectively determined Mer protein expression in BMPCs of freshly isolated BM of myeloma patients and healthy donors by flow cytometry. Here, we found more than 10-fold higher numbers of Mer⁺ BMPCs in myeloma patients compared with controls (Figures 2f and g).

Axl and Tyro3 were only expressed in a small fraction of 10% and 2%, respectively, of malignant BMPCs isolated from myeloma patients (Figures 2c and d; Supplementary Table S4).

Altogether, our data suggest that malignant PCs express higher level of Gas6 compared with healthy BMPCs and that the fraction of Mer⁺ BMPCs is increased in myeloma patients, whereas the expression level of Mer per PC is not different compared with healthy controls. In contrast, Axl and Tyro3 are not expressed by the majority of myeloma BMPCs.

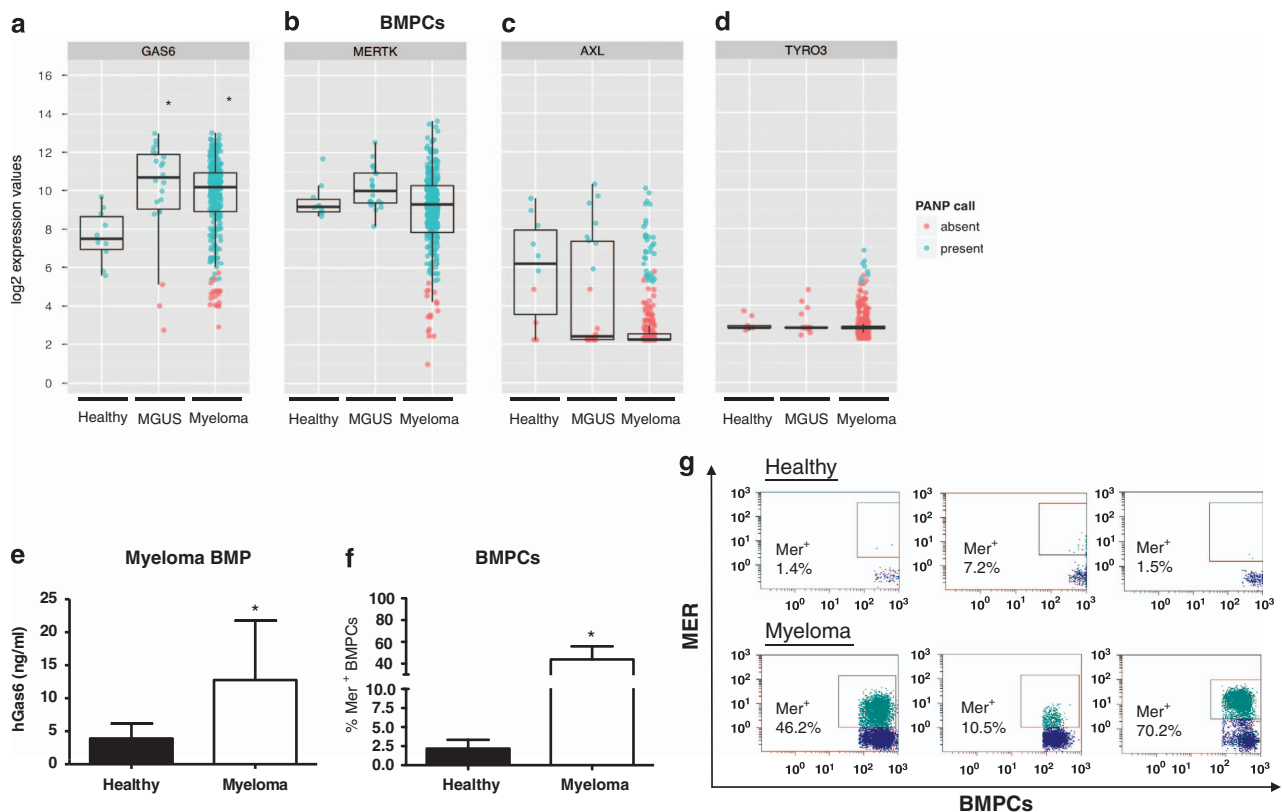


Figure 2. Gas6 expression is increased in malignant PCs and BMP from myeloma patients compared with healthy controls. (a–d) Gene expression profiling of Gas6 (a), MERTK (b), Axl (c) and Tyro3 (d) showing upregulation of Gas6 ($n = 11/331$) in MGUS ($*P = 0.003$) and myeloma BMPCs ($*P < 0.001$) compared with healthy BMPCs (log2-transformed absolute gene expression levels are displayed). (e) hGas6 ELISA indicating upregulation of Gas6 expression in the BMP of myeloma patients compared with healthy controls ($n = 15/6$, $*P = 0.0016$). (f) Quantification of Mer⁺CD45⁺CD19⁺CD38⁺ BMPCs cells by flow cytometry indicates a higher percentage of Mer⁺ BMPCs in myeloma patients compared with healthy controls ($n = 8/7$, $*P = 0.0359$). (g) Representative FACS plots from healthy BM donors and myeloma patients. The quantification of Mer⁺ BMPCs cells by flow cytometry indicates a higher percentage of Mer⁺ BMPCs in myeloma patients compared with healthy controls ($n = 8/7$, $*P = 0.04$).

Function of Gas6 and Mer in myeloma cells

Because of their expression in > 90% of myeloma patients, we first chose to investigate the functional implications of Gas6 and Mer in myeloma biology. To identify suitable cell line models for *in vitro* assays, we quantified expression of Gas6 and Mer mRNA in five different human myeloma cell lines (RPMI8226, U266, JLN3, Molp-8 and Lp-1). Consistent with the patient data, all cell lines expressed Mer, whereas Axl was mostly not or expressed at very low levels. Tyro3 was expressed by RPMI8226, U266 and JLN3 cells. RPMI8226, U266 and JLN3 cells co-expressed Gas6, whereas Molp-8 and Lp-1 cells were negative for Gas6 (Supplementary Table S5). Because in myeloma patients Gas6 and Mer were both expressed by malignant PCs, we decided to mainly focus on RPMI8226 and U266 cells. PCR data were validated by western blot and ELISA, revealing expression of Mer and Gas6 protein by both cell lines, albeit at different levels (Figures 3a and b). We could detect Mer phosphorylation in U266 and RPMI8226 cells indicating active receptor signaling in baseline conditions (Figures 3a, d and e). Interestingly, serum starvation augmented Mer phosphorylation in RPMI8226 and U266 cells, revealing increased Mer signalling in challenging conditions (Figure 3c). In contrast, Gas6 mRNA levels remained constant upon serum starvation (data not shown).

Subsequently, we induced the downregulation of Mer by means of shRNA in RPMI8226 and U266 cells (Supplementary Figure S3 and Figures 3d and e). Proliferation assays demonstrated that knockdown of Mer expression inhibited growth of both cell lines in comparison with control-transduced cells (Figures 4a and b).

In addition, starvation-induced apoptosis was increased in U266 cells and RPMI cells with knockdown of Mer compared with control-transduced cells (Figure 4c and data not shown). In a next step, we investigated the impact of Mer knockdown on phosphorylation of MAPK and Akt, important signal transduction intermediates fostering myeloma proliferation and survival. In these experiments, we found that the inhibition of Mer led to a decrease of phosphorylated MAPK and Akt in U266 cells (Figure 4d). Furthermore, the anti-apoptotic protein Bcl-2 was downregulated after blockade of Mer and cleaved caspase 3 levels were increased showing that Mer signalling activates anti-apoptotic pathways in myeloma cells (Figure 4d). Similar data were obtained in RPMI8226 cells (data not shown).

To study the role of Axl, we utilized a genetic knockdown approach with shAxl.²¹ We found that Axl expression was quite low in U266 and RPMI8226 cell lines and higher in Lp-1 cells (Supplementary Figure S4a). Proliferation assays indicated that in U266 cells shAxl did not inhibit proliferation (Supplementary Figure S4b). In addition, we overexpressed Axl in RPMI8226 and U266 cells (Supplementary Figure S4c). Consistent with the previous results, Axl overexpression did not promote myeloma cell proliferation (Supplementary Figures S4d and e).

Finally, we performed the knockdown of Axl in Lp-1 cells (Supplementary Figure S4f). Also, in this cell line with higher Axl levels, we did not detect inhibition of proliferation upon blockade of Axl (Supplementary Figure S4g).

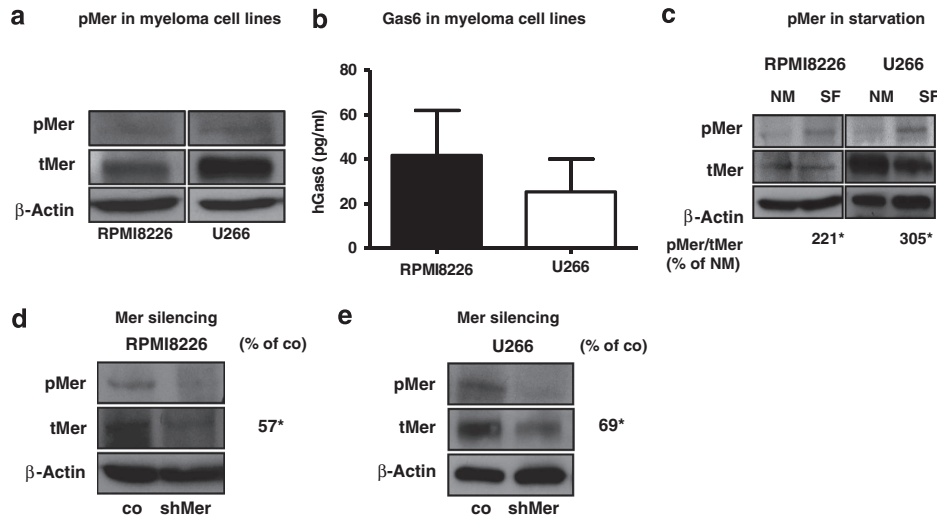


Figure 3. Expression of Gas6 and Mer by different myeloma cell lines. **(a)** Immunoblot showing protein levels of phosphorylated Mer (pMer) and total Mer (tMer) in RPMI8226 and U266 cells. **(b)** Gas6 secretion by RPMI8226 and U266 cell lines as determined by ELISA. **(c)** Immunoblot and densitometric quantification indicating the upregulation of pMer after 3 h in serum-free (SF) versus normal medium (NM) in RPMI8226 and U266 cells ($n = 3$; $*P < 0.05$). **(d, e)** Immunoblot of RPMI8226 **(d)** and U266 **(e)** cells showing lower levels of Mer (tMer) and phospho Mer (pMer) after lentiviral silencing of Mer by shRNA. Densitometric quantification of (pMer/β-actin)/(tMer/β-actin) normalized to control-transduced cells ($n = 3$; $*P < 0.05$).

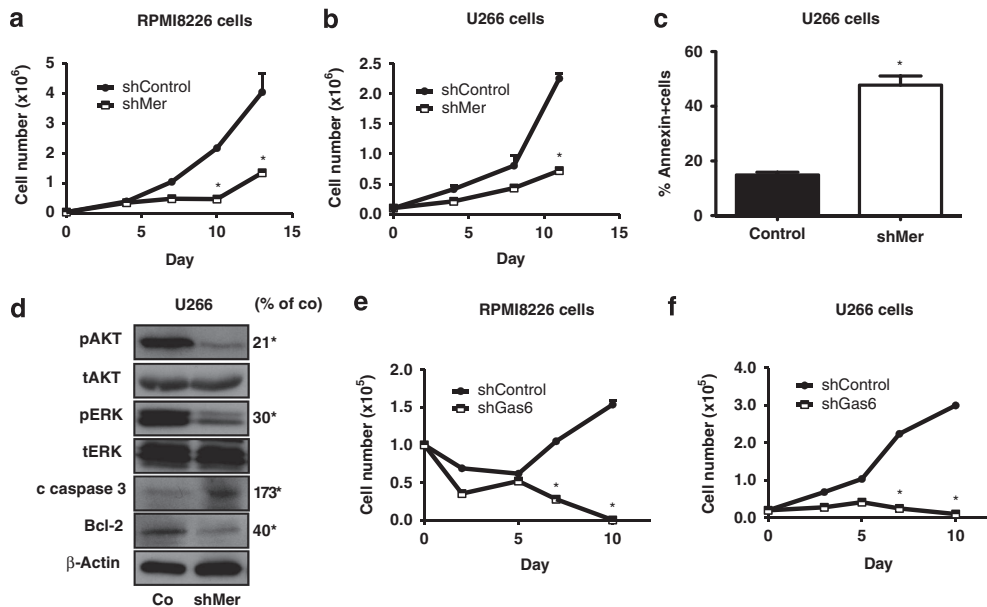


Figure 4. Mer silencing inhibits proliferation and myeloma-promoting signal transduction pathways. **(a, b)** Lentivirally mediated silencing of Mer with shRNA in RPMI8226 **(a)** and U266 **(b)** cells inhibits proliferation in comparison with control-transduced cells ($n = 3$; $*P < 0.05$). **(c)** Mer knockdown leads to increased numbers of Annexin⁺ U266 cells in starvation conditions ($n = 3$; $*P = 0.01$). **(d)** Immunoblots from U266 protein extracts indicate inhibition of Akt, Erk and Mer phosphorylation and reduction in Bcl-2 expression as well as induction of cleavage of caspase 3 after knockdown of Mer. Densitometric quantification of (phosphorylated protein/β-actin)/(total protein expression/β-actin), (Bcl-2/β-actin) and (cleaved caspase 3/β-actin) normalized to control-transduced cells ($n = 3$; $*P < 0.05$). **(e, f)** shGas6-infected RPMI8226 **(e)** and U266 **(f)** cells show reduced proliferation compared with control-transduced cells ($n = 3$; $*P < 0.05$).

In addition, we down-modulated Tyro3 by means of shRNA in U266 and RPMI8226 cells. We found that the proliferation of these cell lines was unchanged in comparison with control-transduced cells (Supplementary Figures S5a–d).

In conclusion, these results suggest that in myeloma Axl and Tyro3 seem to play a negligible role whereas Mer influences the proliferation of myeloma cells.

Next, we wished to determine influence of loss- and gain-of-function of Gas6 on myeloma cells. shRNA-mediated knockdown of Gas6 strongly inhibited the proliferation of RPMI8226 and U266 cells and resulted in cell death shortly after sorting of shGas6-transduced cells, whereas control-transduced cells were proliferating (Figures 4e and f). Because of rapid cell death in Gas6-silenced cells, we were unable to obtain sufficient cell

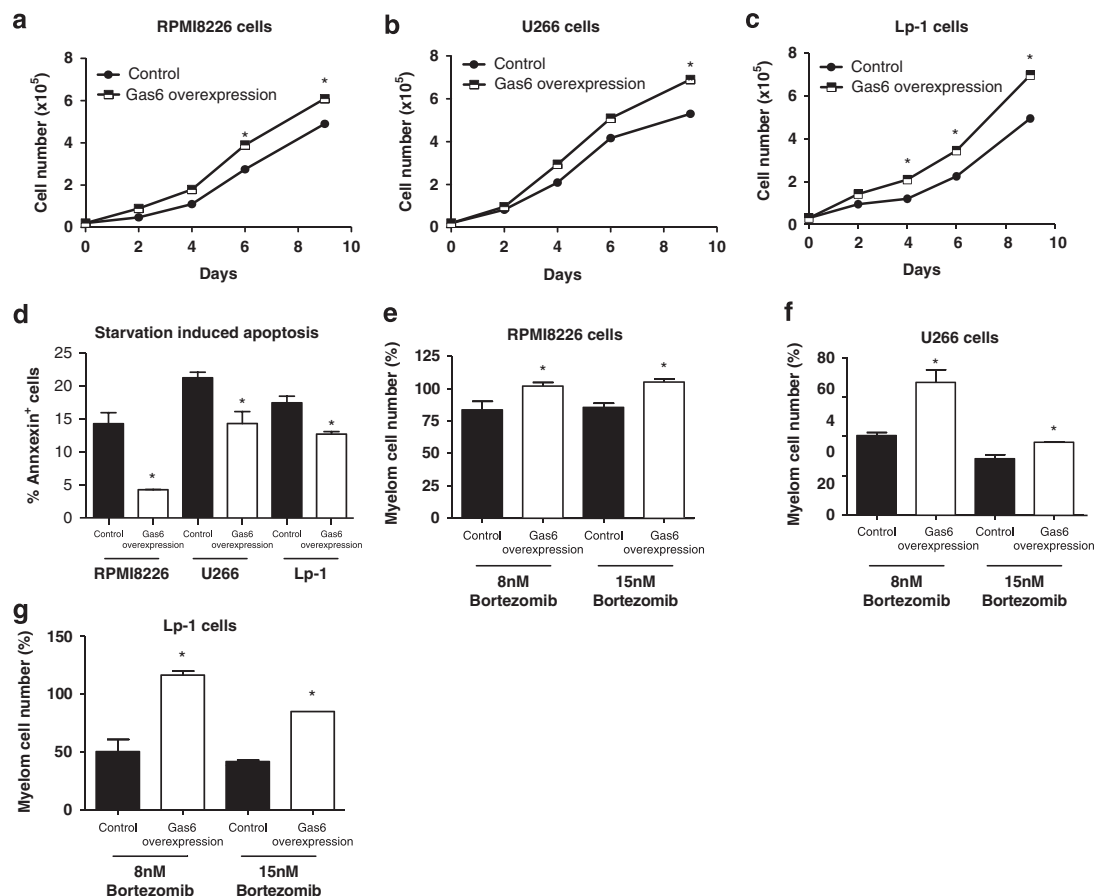


Figure 5. Gas6 promotes proliferation and survival of myeloma cells and protects them against bortezomib treatment. (a–c) Gas6 overexpression in Gas6⁺ RPMI8226 (a), Gas6⁺ U266 (b) and Gas6⁺ Lp-1 (c) cells fosters their proliferation ($n=3$; $*P<0.05$). (d) Gas6 overexpression leads to decreased numbers of Annexin⁺ cells in starvation conditions in RPMI8226 and Lp-1 cells ($n=3$; $*P<0.05$). (e–g) Gas6 overexpression in RPMI8226 (e), U266 (f) and Lp-1 (g) cells induces reduced sensitivity toward bortezomib treatment compared with control-transduced cells ($n=3$; $*P<0.001$).

numbers for Gas6 quantification. In contrast, Gas6 overexpression led to the increased proliferation rate of Gas6⁺ U266, Gas6⁺ RPMI8226 and Gas6⁺ Lp-1 cells (Figures 5a–c; Supplementary Figures S6a–c). Overexpression of Gas6 also protected these myeloma cells from starvation-induced apoptosis (Figures 5d and c). In addition, Gas6-overexpressing U266, RPMI8226 and Lp-1 cells were more resistant toward bortezomib, representing an established anti-myeloma agent (Figures 5e–g).³⁸ Consequently, Gas6 can render myeloma cells more resistant to therapy *in vitro*.

Thus, our functional data indicate that autocrine Gas6 and its receptor Mer mediate proliferation and survival of myeloma cells.

Blockade of Mer prolongs survival of mice in a systemic myeloma model

Encouraged by the *in vitro* data suggesting inhibition of myeloma cell proliferation by downregulation of Mer, we investigated the therapeutic potential of Mer blockade on myeloma progression *in vivo*. For this purpose, we utilized the well-documented systemic orthotopic U266 myeloma model allowing monitoring of disease progression by FACS.²⁹ Diseased mice reach end-stage characterized by limb paralyses and severe spinal deformity after 7–8 weeks.²⁹

We injected Venus-labeled Mer-silenced and control-transduced U266 cells into NSG mice. At end-stage, we found a reduction of myeloma infiltration both in the initially injected femur and in the contralateral femur (Figure 6a). In addition, Igλ light chain quantification by ELISA revealed reduced paraprotein in the peripheral blood of U266-bearing animals after 3 weeks and at

end-stage of the disease (Figure 6b).²⁹ Moreover, we found a prolongation of survival by 41 days in mice ($n=4/5$, $*P=0.004$) injected with Mer-silenced U266 cells in comparison with mice injected with control-transduced cells (Figure 6c). Thus, inhibition of Mer reduces myeloma burden and prolongs survival in a clinically relevant myeloma model.

Role of Gas6 in myeloma progression *in vivo*

Subsequently, we wished to determine the influence of Gas6 overexpression by U266 on myeloma progression *in vivo*. Therefore, we injected fluorescently labelled control and Gas6-overexpressing U266 cells into NSG mice as described above. These experiments revealed that mice bearing Gas6-overexpressing U266 cells survived shorter than mice injected with control-transduced U266 cells (Figure 7a). To study a potentially clinically applicable pharmacologic approach to inhibit Gas6, we utilized warfarin, which blocks γ -carboxylation, a posttranslational modification necessary for the biologic activity of Gas6. Treatment started 3 days after intra-femoral application of U266 cells at a dose level of 1 mg/l in drinking water. This dose level was chosen because previous publications indicated inhibition of Gas6-mediated biologic effects *in vivo*.³⁹ This experiment indicated reduction of myeloma burden and prolongation of survival of U266 myeloma-bearing mice upon treatment with warfarin (Figures 7b and c).

Altogether, our data show that blockade of the Gas6-Mer axis has therapeutic potential in a systemic preclinical myeloma model.

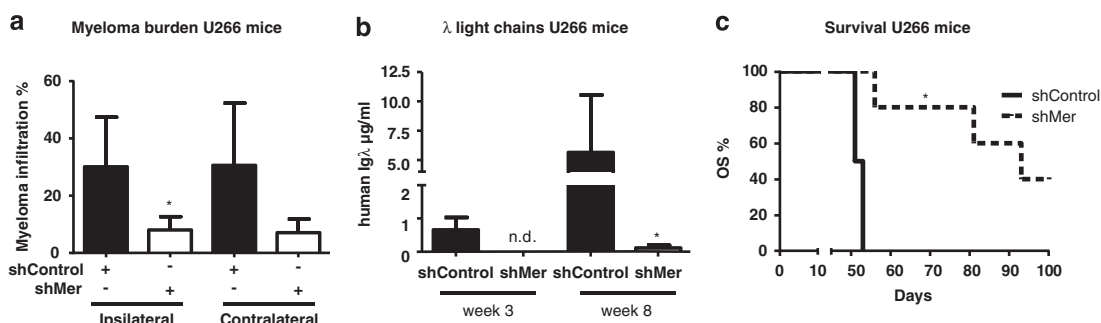


Figure 6. Mer blockade reduces myeloma burden and prolongs survival in the U266 systemic myeloma mouse model. (a) Silencing of Mer in U266 cells causes reduced myeloma burden in the ipsilateral and contralateral femur compared with control-transduced cells ($n=4/4$, $*P=0.0489$; $P=0.0789$). (b) Silencing of Mer in U266 cells reduces human Ig λ light chain paraprotein in the peripheral blood of mice compared with control-transduced U266 cells ($n=4/5$, $P=0.2143$). (c) Prolongation of overall survival in mice that were injected with Mer-silenced U266 cells in comparison with control transduced cells ($n=4/5$, $*P=0.0039$).

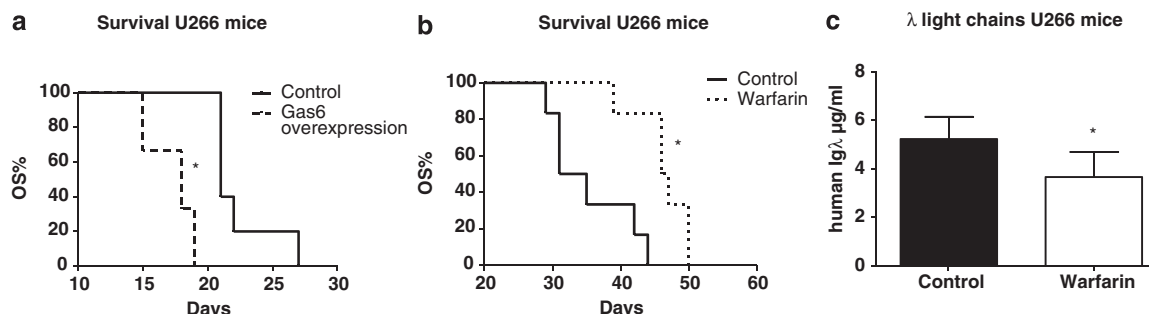


Figure 7. Therapeutic targeting of Gas6 promotes survival in the U266 myeloma mouse model. (a) Overexpression of Gas6 in U266 cells reduces overall survival in mice in a MM model ($n=5/6$, $*P=0.0018$). (b, c) Administration of warfarin at 1 mg/ml drinking water reduces overall survival (b) and decreases human Ig λ light chain paraprotein in the peripheral blood of mice compared with non-treated mice (c) ((b) $n=6/6$, $*P=0.005$; (c) $*P=0.022$).

DISCUSSION

By analyzing myeloma patient samples as well as *in vitro* and *in vivo* models, this study yielded the following major findings: (i) Gas6 is upregulated by malignant CD138⁺ BMPCs in comparison with healthy PCs; (ii) the Gas6 receptor Mer is expressed by malignant BMPCs in the majority of patients and the fraction of Mer⁺ PCs is increased in initially diagnosed myeloma patients when compared with healthy controls; (iii) Axl and Tyro3 are not expressed or expressed by a low proportion of myeloma BMPCs only; (iv) downregulation of Mer or Gas6 inhibits myeloma proliferation and apoptosis resistance *in vitro*; and (v) downregulation of Mer or therapeutic blockade of Gas6 exert a therapeutic anti-myeloma effects on relevant clinical endpoints in a systemic myeloma model.

Until now, no evidence exists about increased expression levels of Gas6 in myeloma patients. Our functional studies show that Gas6 contributes to myeloma pathobiology. Indeed, Gas6 knock-down in myeloma cell lines induces rapid cell death compared with control-transduced cells. Vice versa, overexpression of Gas6 leads to enhanced proliferation of myeloma cells. Furthermore, in starvation conditions, myeloma cells showed increased survival upon overexpression of Gas6. In addition, increased autocrine Gas6 production augments the resistance of myeloma cells to bortezomib. These findings were obtained upon overexpression of Gas6 in Gas6⁺ myeloma cells and upon expression of Gas6 in Gas6⁻ myeloma cells. These data suggest that both overexpression and *de novo* expression of Gas6 by myeloma cells can increase their proliferation, survival and therapy resistance. However, data concerning the impact of Gas6 expression on therapy resistance were generated *in vitro*. Hence, validation of the implications of

Gas6 expression in the response of myeloma cells to bortezomib (and other anti-myeloma agents) in preclinical models is still warranted.

Our results are in concordance with published work indicating that Gas6 can promote proliferation, survival and therapy resistance of solid and hematologic cancer cells.^{3,18–21} Consistently, we found that Gas6 overexpression led to shortened overall survival *in vivo* in the U266 orthotopic myeloma model whereas pharmacologic blockade of Gas6 induced survival prolongation in this model. Our data indicate that autocrine Gas6 plays a specific role in myeloma, because in AML, Gas6 expression by leukemia is negligible and Gas6 is mainly produced by BM stroma cells.²¹ Our results also show that the amount of Gas6 as secreted by myeloma cell lines is much lower compared with the Gas6 levels present in the BMP of myeloma patients. Obviously, it is impossible to directly compare the supernatant of *in vitro* cultured cells with BMP, but the finding might point to potential other sources of Gas6 within the BM besides the myeloma cells. Future studies need to investigate whether Gas6 is also produced by BM stromal cells in myeloma and how stroma-derived Gas6 contributes to the biology of this malignancy. In addition, further work is necessary to unravel the functions of autocrine versus paracrine Gas6 in different hematologic malignancies.

Which Gas6 receptor is relevant in myeloma? Our data show that Mer is expressed by malignant PCs and that the fraction of Mer⁺ BMPCs is increased in myeloma patients compared with healthy individuals. In contrast, Axl and Tyro3 were not expressed by the majority of patients included in this study. Thus, it is highly likely that Mer represents the most important Gas6 receptor expressed by malignant PCs in newly diagnosed myeloma. Further

work is necessary to determine the role of Gas6 and TAM receptors in relapsed myeloma. The expression pattern in primarily diagnosed myeloma differs from other hematologic malignancies. In AML, Axl and Mer are expressed and were identified as novel therapeutic targets.^{21,24} In chronic lymphocytic leukemia, Axl represents a potential therapeutic target, whereas expression of Mer has not yet been investigated.³⁷ Thus, expression and biological relevance of different TAMRs vary in a cell-type and context-specific way in hematologic cancer. We found increased Mer expression in myeloma BMMNCs compared with BMMNCs isolated from healthy donors. Importantly, Mer expression per BMPC was unchanged in myeloma patients compared with healthy BMPCs but numbers of Mer⁺ BMPCs were increased in myeloma patients. Thus, the increased numbers of Mer⁺ BMPCs in the BM of myeloma patients are most likely responsible for the increased Mer expression detected within the total population of myeloma BMMNCs. However, there is considerable heterogeneity in the numbers of Mer-expressing BMPCs between individual patients. It is possible that the amount of Mer⁺ BMPCs influences the prognosis of myeloma patients and/or the response to (anti-Mer) therapies. The observation that only a fraction of BMPCs express Mer could also mean that only a part of the malignant PC clone would be accessible for Mer-targeted therapies.

Our analysis of association of BMPC Gas6 and Mer expression with cytogenetic risk groups yielded the finding that Gas6 is higher expressed in one out of three subgroups with a bad prognostic implication, whereas its expression is lower in a subgroup with standard risk. Mer is lower expressed in two out of three cytogenetic subgroups associated with bad prognosis. Thus, Gas6 expression or Mer expression levels are not consistently associated with adverse or good cytogenetic risk factors and also show considerable heterogeneity within the different subgroups. Similar findings were obtained when analyzing the association of expression of these genes with GEP subgroups.⁴⁰

In any case, future work in larger cohorts is necessary to assess the potential prognostic and predictive value of Mer expression in myeloma. Altogether, our data indicate that Mer represents a druggable target in this disease. Therefore, we subsequently investigated the therapeutic potential of Mer blockade *in vitro* and *in vivo*.

We found that downregulation of Mer by shRNA inhibited the proliferation and survival of different myeloma cell lines *in vitro*, whereas expression levels of Axl or Tyro3 did not influence the proliferation of myeloma cells. Thus, when looking at these findings and those derived from analyzing the clinical samples (please see above), Mer seems to be the most relevant TAMR in myeloma. However, we cannot exclude that Axl or Tyro3 play a role in myeloma biology in other contexts such as communication with the microenvironment.

Upon investigation of important signal transduction pathways promoting myeloma growth and survival,⁴¹ we found pronounced reduction of phosphorylated MAPK and Akt. In addition, we detected reduced Bcl-2 levels and increased cleaved caspase 3 levels. Altogether, these data indicate that blockade of Mer potentially reduces the proliferation and survival of myeloma cells. These results are in line with findings in AML indicating that Mer promotes the proliferation and survival of AML cells.²⁴ Future studies are needed to unravel which additional signal transduction pathways might be activated by Mer in myeloma cells.

Why do myeloma cells overexpress Gas6? On the basis of our data, increased Gas6 expression by myeloma cells does increase their proliferation, but importantly also their survival in challenging growth factor-deprived conditions and possibly upon treatment. Thus, Gas6 overexpression confers a growth advantage to malignant PCs. In addition, we found that the fraction of Mer-expressing BMPCs is increased in myeloma cells compared with healthy BMPCs which points to the fact that Mer⁺ BMPCs can

expand in myeloma. Thus, one can speculate that these Mer⁺ BMPCs might express increased Gas6 levels thereby further increasing the pro-proliferative and pro-survival signalling via the Gas6-Mer axis. Future work is necessary to dissect the quantitative impact of Gas6 and Mer levels on myeloma proliferation, survival, therapy resistance and other mechanisms involved in myeloma pathobiology.

Our data indicate that inhibition of Mer or Gas6 reduces myeloma burden and increases survival of mice bearing an orthotopic myeloma model. Further work is necessary to determine whether inhibition of Gas6 and/or Mer can sensitize myeloma cells for established anti-myeloma agents *in vivo*. Altogether, our work shows that Gas6 and Mer might represent novel treatment targets in myeloma. Agents such as warfarin that are able to inhibit gamma-carboxylation and thus activation of Gas6 are already available. However, as they also inhibit the plasmatic coagulation, their use might be difficult in myeloma patients with disease- or therapy-induced thrombocytopenia. Thus, development of anti-Gas6 monoclonal antibodies might be warranted to study therapeutic effects of Gas6 inhibition in myeloma patients. Development of clinically applicable small molecule Mer inhibitors is underway, thus Mer blockade might be able to enter clinical trials in the future.^{42,43}

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

SL initiated, conceived of, designed and supervised research, wrote the manuscript and analyzed data. JSW and IBB designed experiments, wrote the manuscript, performed experiments and analyzed data. NW, TM, MW, KR, HT, SS, VG, MCC and MJ performed experiments and data analysis. MB, DA, DS, WF, EH, NK, BF, DH, BK, MSR, KP and CB contributed vital new reagents and commented on the research direction and edited the manuscript. All authors discussed and commented on the manuscript.

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