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Original Article

ORIGINAL ARTICLE

Semaphorin-3A inhibits multiple myeloma progression in a mouse model

Noa Lavi[1,2,†], Ofra Kessler[1,†], Keren Ziv[1], Inbal Nir-Zvi[1], Yelena Mumblat[1], Nasrene Eiza[3], Yael Paran[1], Benjamin Brenner[1,2], Zahava Vadasz[3,*] and Gera Neufeld[1]

[1]Cancer Research Center, The Bruce Rappaport Faculty of Medicine, Technion-Israel Institute of Technology, Haifa, Israel, [2]Hematology and Bone Marrow Transplantation Institute, Rambam Health Care Campus, Haifa, Israel and [3]Division of Allergy and Clinical Immunology, Bnai Zion Medical Center, Haifa, Israel

*To whom correspondence should be addressed. Tel: +972-4-8359659; Fax: +972-4-8359961; Email: zahava.vadas@b-zion.org.il
Correspondence may also be addressed to Gera Neufeld. Tel: +972-4-8295430; Fax: +972-4-8523947; Email: gera@tx.technion.ac.il
†These authors contributed equally to this work.

Abstract

Previous studies revealed that progression of multiple myeloma (MM) is associated with downregulation of semaphorin-3A (sema3A) expression in bone marrow endothelial cells. We therefore determined if serum sema3A concentrations are correlated with MM progression and if sema3A can affect MM progression. We find that the concentration of sema3A in sera of MM patients is strongly reduced and that the decrease is correlated with disease progression. A similar depletion is found in patients having acute myeloid leukemia and acute lymphoblastic leukemia but not in cancer forms that do not involve the bone marrow such as in colon cancer. Expression of a modified sema3A [furin-resistant sema3A (FR-sema3A)] stabilized against cleavage by furin-like proprotein convertases in CAG MM cells did not affect their behavior *in-vitro*. CAG cells injected into the tail vein of severe combined immunodeficient (SCID) mice home to the bone marrow and proliferate, mimicking MM disease progression. Disease progression in mice injected with CAG cells expressing FR-sema3A was inhibited, resulting in prolonged survival and a lower incidence of bone lesions. Histological examination and fluorescence-activated cell sorting analysis revealed that FR-sema3A expression reduced the infiltration of the CAG cells into the bone marrow, reduced bone marrow necrosis and reduced angiogenesis induced by the MM cells in the bone marrow. Our results suggest that measurement of sema3A serum concentrations may be of use for the diagnosis and for the monitoring of malignancies of the bone marrow such as MM. Furthermore, our results suggest that FR-sema3A may perhaps find use as an inhibitor of MM disease progression.

Introduction

The semaphorins constitute a large family of secreted and membrane-bound proteins that were initially characterized as repulsive axon guidance factors. Of these, the subfamily of the seven class-3 semaphorins represents the only class of secreted semaphorins. Class-3 semaphorins are characterized, in addition to the hallmark sema domain common to all semaphorins, by a basic domain located at their C-termini that is not present in other semaphorins (1). Class-3 semaphorins, with the exception of semaphorin-3E (sema3E), are distinguished from other semaphorins that bind directly to receptors of the plexin family in that they do not activate plexin-mediated signaling directly. They bind instead to one or to both of the receptors of the neuropilin family that subsequently form complexes with one or two members of the receptors belonging to the plexin family to transduce class-3 semaphorin signals (1–3). The neuropilins also function as co-receptors for several growth factors such as

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Abbreviations

AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
CLL	chronic lymphocytic leukemia
ELISA	enzyme-linked immunosorbent assay
FPPC	furin-like proprotein convertases
FR-sema3A	furin-resistant sema3A
HUVEC	human umbilical vein-derived endothelial cell
MM	multiple myeloma
MGUS	monoclonal gammopathy of undetermined significance
SCID	severe combined immunodeficient
SMM	smoldering multiple myeloma
sema3A	semaphorin-3A
VEGF	vascular endothelial growth factor

vascular endothelial growth factor (VEGF) A (4–6), VEGF-C (7), platelet-derived growth factor (8) and transforming growth factor beta (9) to name but a few. In addition they also form complexes with several tyrosine kinase receptors such as VEGF and platelet-derived growth factor receptors and modulate their function (10). In the case of VEGF this association enhances VEGF-induced signal transduction via the VEGFR-2 tyrosine kinase receptors and thus enhances VEGF-induced angiogenesis (5,11). Semaphorin-3A (sema3A) is possibly the best characterized class-3 semaphorin. It was initially characterized as an axon guidance factor that binds to neuropilin-1 (12–14). It was subsequently found to function as an inhibitor of angiogenesis (15) and as an endogenous inhibitor of the angiogenic switch in the progression of solid tumors (16–19). In addition, sema3A was also found to affect tumor cells directly. For example, sema3A inhibited the migration and spreading of MDA-MB-231 breast cancer cells as well as their ability to form colonies in soft agar, and also inhibited the invasiveness of prostate cancer cells in *in vitro* assays (20–22). Like all of the class-3 semaphorins, sema3A is cleaved by furin-like proprotein convertases (FPPC). Cleavage at the major FPPC site produces an inactive 65 kDa sema3A-derived peptide (23). It is possible that cleavage by FPPC, which are frequently upregulated in malignant cells (24), represents a mechanism by which malignant cells escape from the inhibitory effects of class-3 semaphorins.

Multiple myeloma (MM) is a neoplastic disorder that is characterized by proliferation of malignant plasma cells derived from post-germinal center B cells in the bone marrow microenvironment, monoclonal protein in the blood or urine and associated organ dysfunction. It is also accompanied by increased osteoclast activation along with osteoblast inhibition resulting in bone erosion, pain and fractures (25). MM is thought to evolve from a monoclonal gammopathy of undetermined significance (MGUS). MGUS progresses to smoldering MM (SMM) and, finally, to MM (26). Sema3A has an important role in bone homeostasis and promotes bone formation by competing with receptor activator of nuclear factor kappa-B ligand (RANKL), an inducer of osteoclast differentiation, for binding to the receptor complex consisting of triggering receptor expressed on myeloid cells 2 (TREM2) and adaptor protein DAP12. Furthermore, sema3A also stimulates osteoblast formation by enhancement of Wnt-induced signaling in osteoblasts (27). The expression of sema3A was found previously to be downregulated in endothelial cells isolated from the bone marrow of MM patients, and sema3A inhibited the proliferation of such endothelial cells. Sema3A is a known inhibitor of angiogenesis (15). It was suggested therefore that downregulation of sema3A expression in endothelial cells, which is accompanied by an increase in the production of VEGF-A, promotes angiogenesis and tumor progression in MM (28). We find that the concentration of sema3A in the blood of MM patients is strongly reduced in correlation with the stage of the disease. This decrease is not specific to MM patients but is also observed in patients having other malignancies that develop in the bone marrow such as in various leukemias. Overexpression of a mutated sema3A resistant to inactivation by FPPC inhibited disease progression in a mouse model of MM, suggesting that treatment with sema3A may have therapeutic potential.

Materials and methods

Antibodies

FITC Rat Anti-mouse CD31 (cat. 553372) was from BD Biosciences Pharmingen. The APC/Cy7 anti-mouse CD45 antibody (30-F11) and the Brilliant Violet 510 labeled antihuman CD138 antibody (356518) were from BioLegend. Anti-human neuropilin-1 (7239) was from Santa Cruz Biotechnology. The anti-Sema3A antibody was from R&D (AF1250).

Construction and expression of furin-resistant sema3A

A DNA sequence encoding the FLAG epitope tag was inserted in frame upstream of the stop codon of sema3A. In addition, we point-mutated the major consensus furin cleavage site of sema3A (Figure 2A) using site-directed mutagenesis essentially as described previously for sema3C (29). Briefly, the RTRR furin cleavage consensus site (bp 1671–1683) (Figure 2A) was converted to KTKK using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) as per the vendor instructions, to generate furin-resistant sema3A (FR-sema3A). The complementary DNA encoding FR-sema3A was then subcloned into the NSPI lentiviral expression vector (30) and used to generate third-generation lentiviruses as described previously (31). These viruses were used to infect CAG cells in which we have previously expressed firefly luciferase as described earlier (32) as well as HEK293 cells. The FR-sema3A concentration in conditioned medium derived from these cells after 48 h of incubation at a cell concentration of 5×10^5 cell/ml was 0.45 ng/µl. Pools of control cells containing empty expression vector and pools of cells expressing FR-sema3A were then used in subsequent experiments.

Cell lines

CAG MM cells (33) were kindly provided by Dr Ralph Sanderson, University of Alabama, Tuscaloosa, AL, and cultured in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Human umbilical vein-derived endothelial cells (HUVEC) and HEK293 cells were cultured as described previously (34). All cell lines were recently authenticated at the Genomics Center of the Biomedical Core Facility of the Technion using the Promega GenePrint 24 System to determine short tandem repeat profiles of 23 loci plus amelogenin for gender determination (X or XY). In addition, the male-specific DYS391 locus was included to identify null Y allele results for amelogenin. All of the cell lines were tested free of *Mycoplasma* contamination.

Proliferation assay

Cell proliferation was assayed using a Coulter counter essentially as described previously (35).

Tumor formation

CAG cells expressing firefly luciferase and infected either with empty expression vector or with expression vector encoding FR-sema3A (5×10^6 cells in 0.2 ml phosphate buffered saline) were injected into the tail vein of SCID mice. Following injection, formation of tumors was observed using the IVIS 200 bioluminescent imaging system. Tumors were formed in the bone marrow of the mice and did not form in other organs. Mice were killed when the tumors started to cause clear suffering to the animals,

which in the case of these experiments was manifested by partial paralysis of limbs. Femurs were then taken for histological analysis.

Cell contraction

CAG cells expressing FR-sema3A or CAG cells infected with empty control lentiviruses were grown to high density. The medium was then exchanged to HUVEC growth medium (M199 containing 10% fetal calf serum and antibiotics). The medium was conditioned for 2 days and then added to HUVEC at 37°C in a humidified incubator. Half an hour later the cells were photographed and cell contraction determined as described previously (2)

ELISA assays

Sema3A serum levels were assessed using a commercial ELISA Kit (MyBioSource, San Diego, CA). Serum samples were collected from MM patients at different stages of the disease, from newly diagnosed chronic lymphocytic leukemia (CLL) patients, from acute lymphoblastic leukemia (ALL) patients, from acute myeloid leukemia (AML) patients), from newly diagnosed non-Hodgkin lymphoma patients without bone marrow involvement, from colon cancer patients and from healthy controls. All the samples were stored at –80°C until evaluation. Serum samples were diluted with phosphate-buffered saline and were subjected to enzyme-linked immunosorbent assay (ELISA) evaluation, according the manufacturer's instructions.

Fluorescence-activated cell sorter

Femur bones were collected and bone marrow cells flushed out from each femur with phosphate-buffered saline using a 21 gauge needle. The cells were concentrated using a 70 μm cell strainer, washed and single cell suspensions prepared from the bone marrow of each bone. Aliquots (2 μl) of the cell suspension from each femur were then counted in a Coulter counter. Cells were immunostained using antibodies directed against hCD-138, mouse CD-45$^-$/CD-31$^+$ surface markers. All antibodies were purchased from BD Biosciences or BioLegend (San Diego, CA). Evaluation of the percentage of cells expressing the various antigens was performed using a Cyan-ADP flow cytometer (Beckman Coulter, Nyon, Switzerland) and analyzed with Summit 4.3 software (Beckman Coulter). At least 100 000 events were acquired.

Magnetic resonance imaging

Ex-vivo magnetic resonance imaging (MRI) was performed using a 1 T micro-MRI (Aspect M2, Aspect Imaging, Israel), equipped with a cylindrical radiofrequency volume coil (35 mm inner diameter) for signal excitation and reception. Fixed samples were scanned and T2-weighted images were acquired using fast spin echo sequence with the following acquisition parameters: slice thickness = 0.7 mm, field of view = 6.4 × 6.4 cm, matrix dimension = 200 × 200, spatial resolution = 320 × 320 μm^2, repetition/echo time = 1800/80 ms, number of excitations = 2 and number of averages = 4. On these T2-weighted images, tumors were identified as hyperintense signals in the involved bones.

Histology

Femur bones were collected, fixed and decalcified for 2 weeks in 10% ethylenediaminetetraacetic acid that was exchanged every 2 days. They were then embedded in paraffin, and 6 μm sections were then stained with hematoxylin and eosin.

Evaluation of muscle weakness

Mice were observed and scored every other day. For clinical examination, mice were evaluated for myasthenic weakness and assigned clinical scores as described previously (36). Briefly, mice were observed on a flat platform for a total of 2 min. They were then exercised by gently dragging them suspended by the base of the tail across a cage top grid repeatedly (20–30 times) as they attempted to grip the grid. They were then placed on a flat platform for 2 min and again observed for signs of muscle weakness. Clinical muscle weakness was graded as follows: grade 0, mouse with normal posture, muscle strength, and mobility at baseline and after exercise; grade 1, normal at rest but with muscle weakness characteristically shown by a hunchback posture, restricted mobility and difficulty in raising the head after exercise; grade 2, grade 1 symptoms without exercise during observation period; grade 3, dehydrated and moribund with grade 2 weakness; and grade 4, dead.

Statistical analysis

Statistical significance of ELISA assays for the determination of sema3A levels in serum was performed using one-way analysis of variance (Kruskal–Wallis test) followed by Dunn's multiple comparison test. The Mann–Whitney nonparametric test was used to compare the effects of FR-sema3A with control groups in the rest of the experiments.

Ethics statement

The animal studies were all conducted according to the National Institutes of Health guidelines and were approved by the institutional review board of the Technion. The study parts involving patients were approved by the Helsinki Committee of the Rambam Medical Center, Haifa, Israel (approval number 0125-13-RMB). Patients signed on informed consent before recruitment.

Results

The concentration of sema3A is reduced in sera of leukemia and MM patients

In a previous study, it was observed that endothelial cells isolated from the bone marrow of MM patients express reduced amounts of sema3A (28). We reasoned that it may be possible to screen for MM patients and to follow MM disease progression by monitoring sema3A concentrations in body fluids. Indeed, we find that sera derived from MM patients contain significantly reduced concentrations of sema3A as determined using an ELISA assay, and that the reduction seems to be correlated, at least roughly, with disease progression (Figure 1). In patients with advanced MM following relapse, the serum concentration of sema3A was on average 3-fold lower than in the sera of healthy controls (Figure 1). The reduction in the sema3A concentration was not caused by treatments as it was observed in newly diagnosed MM patients and was also observed in sera derived from patients responsive to treatment with bortezomib (Figure 1). In contrast, the sema3A concentrations in sera derived from patients diagnosed in the earliest stages of MM in which there were still no clinical manifestations (MGUS and SMM) were not different from those observed in healthy controls (Figure 1).

To determine if the depletion of sema3A is specific to MM, we also measured the concentration of sema3A in sera derived from patients with AML, ALL or CLL. All the samples were taken from newly diagnosed patients before they received any treatment. Interestingly, the average concentration of sema3A in sera derive from ALL and AML patients was even lower than that in sera derived from MM patients (14% and 19%, respectively, compared with healthy controls) (Figure 1). The average concentration of sema3A in sera obtained from newly diagnosed CLL patients was also half the concentration of sema3A in healthy controls, similar to the average concentration in MM patients, but failed to reach statistical significance, possibly because of the relatively small number of patients we tested (Figure 1). A 30% decrease in serum sema3A concentration was also observed in patients with non-Hodgkin follicular lymphomas without bone marrow involvement. However, in this case the decrease was not statistically significant despite the higher number of patients tested. There was also no decrease in the serum sema3A concentration of patients with colon cancer (Supplementary Figure 1, available at *Carcinogenesis* Online). Taken together, these findings suggest that reduced sema3A serum concentrations are

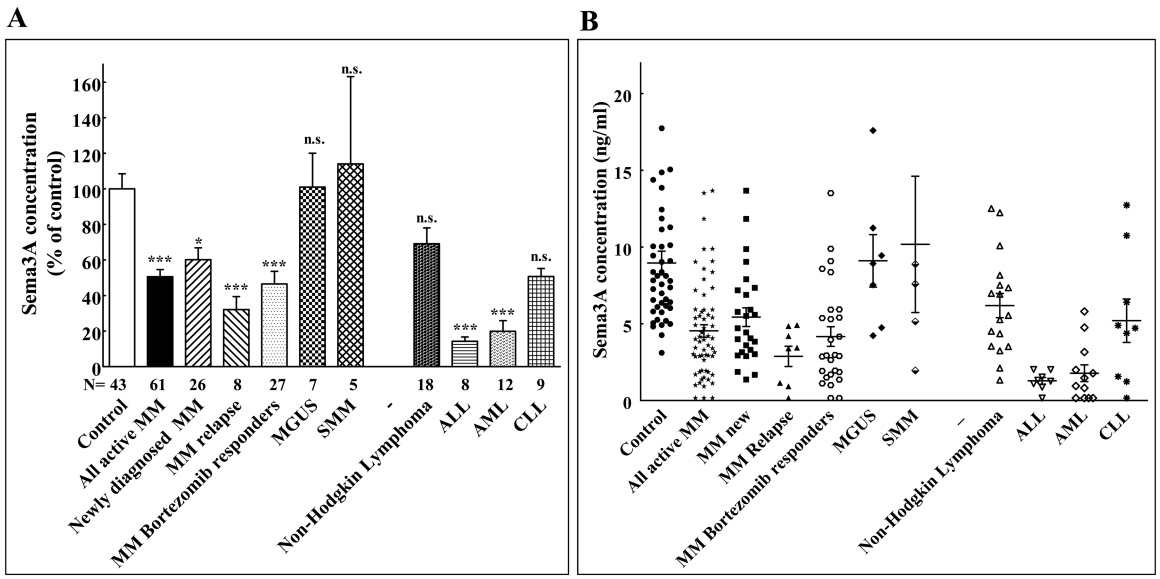

Figure 1. Serum sema3A concentrations of cancer patients. (**A**) Sema3A concentration in sera taken from MM patients at different stages of the disease was measured as described in Materials and methods. Sera from newly diagnosed MM patients were taken before starting therapy. Sera from bortezomib responders were obtained while on treatment. Sera from patients who relapsed were taken after relapse. These patients were not treated at the time that the serum was taken. The sera from all patients diagnosed with ALL, AML, CLL or from non-Hodgkin lymphoma patients without bone marrow involvement were obtained from newly diagnosed patients before treatment. Statistical significance was determined using one-way analysis of variance as described. *N* designates the number of patients assayed in each category. $^{*}P < 0.05$, $^{***}P < 0.001$, n.s.: nonspecific. (**B**) The same data as in panel A are presented as a scatter plot and the actual serum concentrations in nanogram per milliliter as determined by ELISA are given.

associated with malignancies in which tumor cells proliferate and replace normal cells in the bone marrow.

The formation of bone marrow tumors from CAG multiple myeloma cells injected into the circulation is inhibited following expression of a recombinant mutated sema3A resistant to furin cleavage (FR-Sema3A)

We reasoned that it may be possible that a reduction in the production of sema3A in the bone marrow of MM patients contributes to disease progression, and that forced reexpression of sema3A in the bone marrow of MM patients may perhaps have a beneficiary effect. To examine this hypothesis, we expressed in human MM-derived CAG cells the complementary DNA encoding firefly luciferase as described previously (32). We subsequently infected the cells with a lentiviral expression vector containing a complementary DNA that encodes a point-mutated sema3A rendered resistant to cleavage by FPPC (FR-sema3A), to which we have also fused in frame a FLAG epitope tag upstream of the stop codon (Figure 2A) (37). Control cells were infected with the empty lentiviral expression vector. FPPC are frequently expressed in tumor cells (24) and cleavage of sema3A by these enzymes inhibits sema3A activity (23), which is why we chose to use the mutated sema3A. Conditioned medium derived from these cells, but not from control cells infected with the empty lentiviruses, induced contraction of HUVEC indicating that FR-sema3A is active (Figure 2C). CAG cells home to the bone marrow of mice following their injection into the circulation and form tumors (38). The recombinant FR-sema3A expressed by the CAG cells was intact, but the expression level was significantly lower than the expression levels obtained using HEK293 cells (Figure 2B). Neither the proliferation (Supplementary Figure 2A, available at *Carcinogenesis* Online) nor the transwell migration rate of the CAG cells (Supplementary Figure 2B, available at *Carcinogenesis* Online) were inhibited following the expression of recombinant FR-sema3A in the cells.

To determine if sema3A can inhibit the progression of MM, we injected equal numbers of control cells or FR-sema3A expressing cells into the tail vein of age-matched SCID mice. The injected cells formed tumors in the bones of the animals, and the development of these tumors over time was followed by live imaging using an IVIS 200 live imaging system. Postmortem analysis did not reveal tumors in any organs except in bones although a small number of CAG cells could also be detected in lungs (data not shown). We found that tumor development from FR-sema3A expressing cells in the bone marrow of the animals was retarded compared with that from control cells (Figure 3A). All the mice that were injected with control cells had to be killed between days 36 and 38 after injection due to severe clinical problems (partial or complete paralysis, for example) whereas most of the mice that were injected with FR-sema3A expressing cells survived longer and three were still alive and in reasonable condition even 76 days postinoculation (Figure 3A, B and E). From around day 35 postinoculation, mice inoculated with control cells looked unhealthy with fluffy fur compared with the slick fur of mice inoculated with FR-sema3A expressing cells. The control mice also lost weight compared with mice inoculated with FR-sema3A expressing cells (Figure 3C). MM patients may have muscle weakness resulting from tumor-induced compression of the spinal cord (39). We, therefore, evaluated muscle weakness in mice inoculated with control cells and those inoculated with FR-sema3A expressing cells using a previously published semi-quantitative assay (36). Indeed, we found that mice inoculated with control cells had significantly more muscle weakness than those inoculated with FR-sema3A expressing cells (Figure 3D).

To better characterize the effects of FR-sema3A on the development of bone marrow tumors from injected CAG cells, we repeated the experiment, but this time we sacrificed age-matched mice on day 20 postinoculation regardless of their clinical condition. In this experiment too expression of FR-sema3A retarded disease progression that was manifested by lower

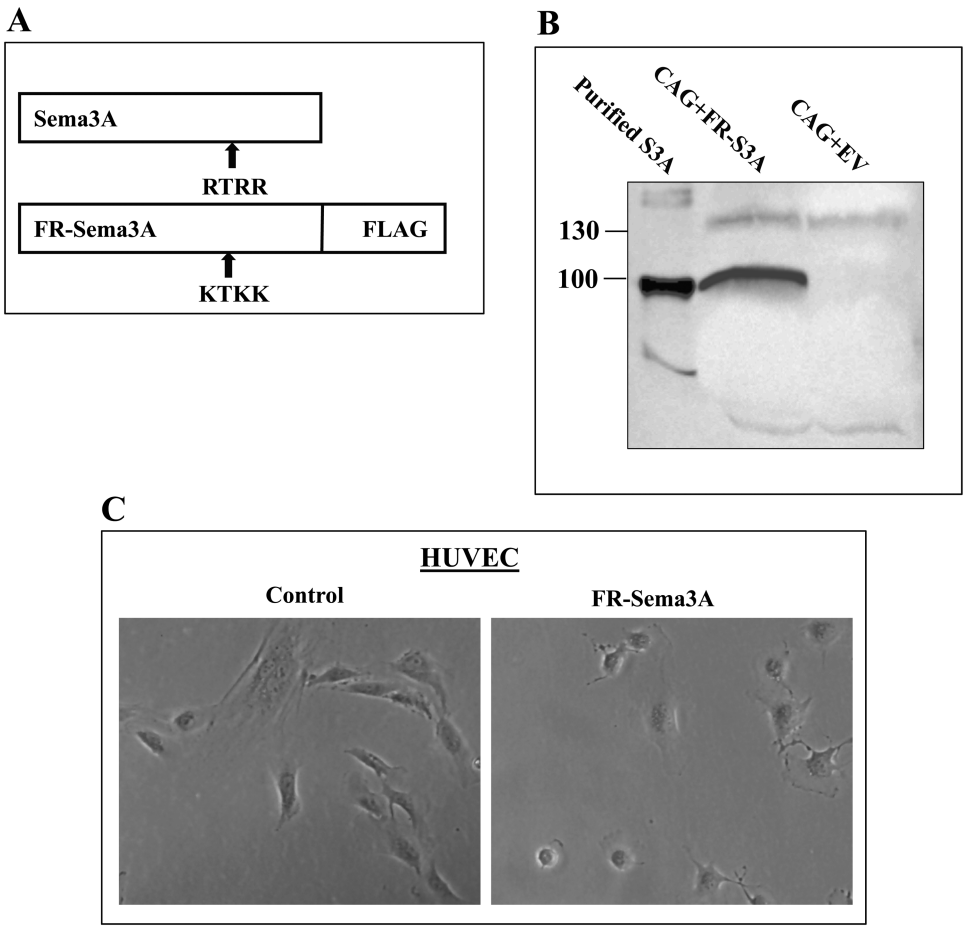

Figure 2. The effects of FR-sema3A expression on the behavior of CAG cells. (**A**) To create FR-sema3A, the wild-type sema3A complementary DNA was point-mutated at the site encoding the major FPPC consensus site as shown, and a FLAG epitope tag was inserted in-frame upstream of the stop codon. (**B**) Equal volumes of conditioned medium were collected from CAG cells (5×10^5 cells/ml) infected with empty expression vector (CAG+EV) or with expression vector directing expression of FR-sema3A (CAG+FR-S3A) after a 48 h incubation. Aliquots (50 µl) from these conditioned mediums as well as 50 ng of recombinant FR-sema3A purified from HEK293 cells (purified S3A) were separated on an sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel, blotted onto nitrocellulose and analyzed by western blot analysis using an anti-sema3A antibody. (**C**) HUVEC (15×10^3) were seeded on a gelatin coated 12-well dish, control conditioned medium from empty vector-infected CAG cells (EV) or conditioned medium from CAG cells expressing FR-sema3A was added to the dishes. Cells were photographed after 30 min at 37°C.

tumor burden in bones of mice inoculated with FR-sema3A expressing CAG cells (Figure 4A and B). Histological sections of femurs derived from mice inoculated with control cells that were stained with hematoxylin and eosin revealed large necrotic areas (Figure 4C, black arrows), whereas in the bone marrow of mice inoculated with FR-sema3A expressing cells there were no necrotic areas at this stage (Figure 4C). When sections of bone marrow derived from areas containing live cells derived from mice inoculated with control cells or cells expressing FR-sema3A (Figure 4C, red arrows) were examined at a higher magnification, we found that the bone marrow of mice inoculated with control CAG cells contained a high concentration of tumor cells (Figure 4D, upper panel), whereas that of mice inoculated with FR-sema3A expressing cells appeared more normal and only contained sparsely distributed tumor cells (Figure 4D, lower panel, arrows).

The bone marrow of mice inoculated with FR-sema3A expressing cells contained a significantly higher concentration of cells compared with bone marrow derived from the femur bones of mice inoculated with control cells (Figure 5A), indicating independently that the bone marrow of these mice is significantly less necrotic. The bone marrow of mice inoculated with FR-sema3A expressing CAG MM cells also contained significantly lower concentrations of human CD138-positive CAG cells (Figure 5B and Supplementary Figure 3A, available at *Carcinogenesis* Online). These FR-sema3A-induced changes were accompanied by a significant, 6.5-fold reduction in the concentration of $CD45^-/CD31^+$ endothelial cells in the bone marrow of mice that received FR-sema3A expressing cells compared with the concentration of endothelial cells in the bone marrow of mice inoculated with control cells, suggesting that FR-sema3A inhibits CAG cell-induced angiogenesis (Figure 5C and Supplementary Figure 3B, available at *Carcinogenesis* Online). Examination of mice injected with control cells using MRI at day 20 after inoculation revealed a significant presence of CAG MM tumor cells in the spinal vertebrae of these mice demonstrated as a hyperintense signal enclosed within the vertebral bones on T2-weighted images. In contrast, cells expressing FR-sema3A infiltrated the spinal vertebrae much less at this stage (Figure 5D, yellow arrows). The spinal cord of animals inoculated with control CAG cells looked compressed whereas that of animals inoculated with FR-sema3A expressing CAG cells seemed much more similar to the spinal cord of healthy mice (Figure 5D, red arrows). These observations suggest that spinal cord compression may contribute to the prominent muscle weakness observed in mice inoculated with control CAG cells.

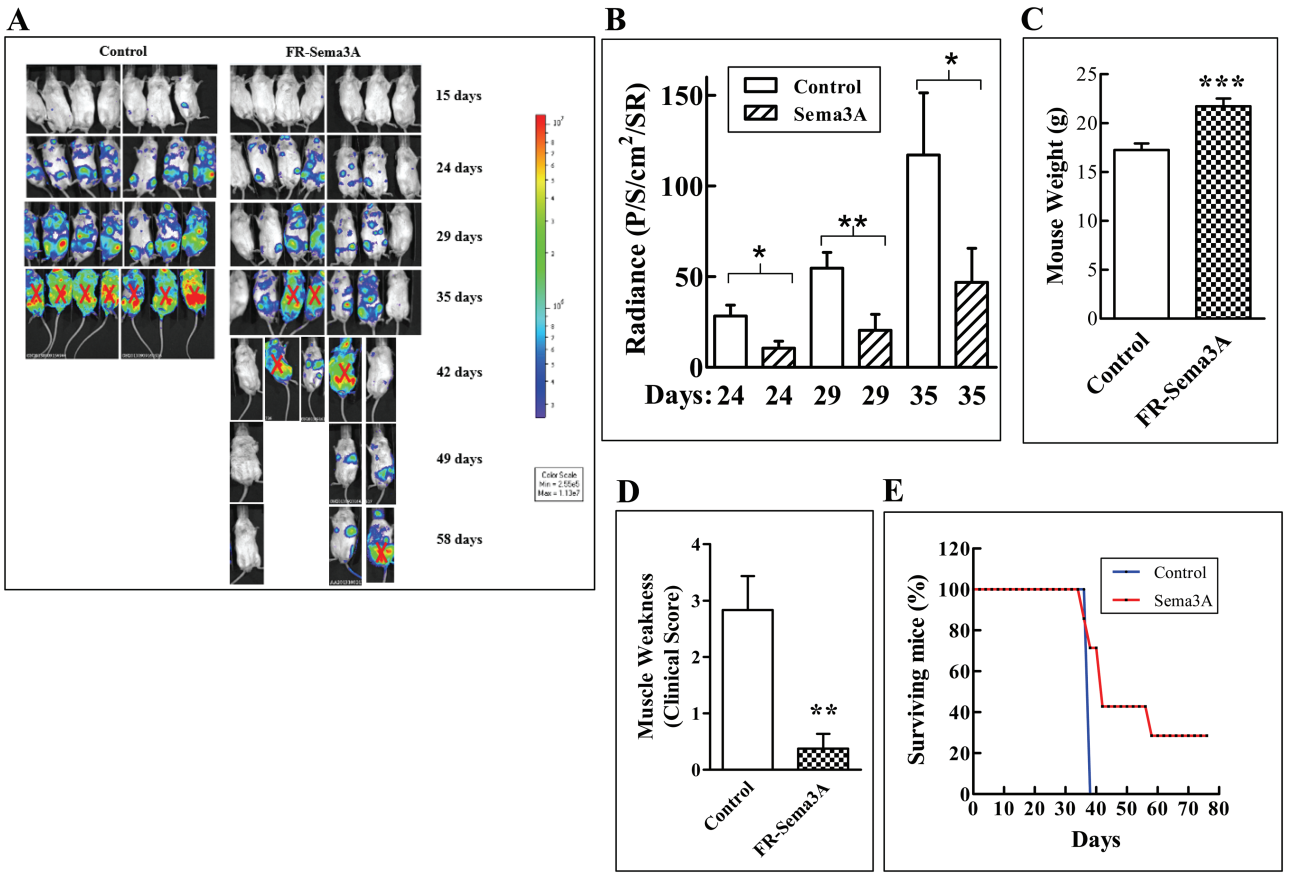

Figure 3. The effect of FR-sema3A on tumor progression. (**A**) CAG cells (control) or CAG cells expressing FR-sema3A (FR-sema3A) (5×10^6 cells), which also expressed firefly luciferase, were injected into the tail vein of SCID mice. The development of tumors was monitored using bioluminescence imaging as described in Materials and methods. Mice were killed when they began to suffer excessive muscle weakness. (**B**) Quantification of tumor load as derived from bioluminescence imaging. Shown is the average bioluminescence signal per mouse. Error bars represent the standard error of the mean. *$P < 0.05$, **$P < 0.01$. (**C**) Mice inoculated with control CAG cells (control) or FR-sema3A expressing CAG cells were weighted on day 35 postinoculation. ***$P < 0.001$ (**D**) Mice inoculated with control CAG cells (control) or with CAG cells expressing FR-sema3A (FR-sema3A) were tested 35 days after inoculation for muscle weakness as described in Materials and methods. **$P < 0.01$. (**E**) Survival curve of mice inoculated with control CAG cells or CAG cells expressing FR-sema3A.

We also tried to purify FR-sema3A from conditioned medium of HEK293 cells to inject purified FR-sema3A to mice inoculated with CAG cells. However, we encountered difficulties as the purified FR-sema3A lost activity following freezing and thawing. Because large amounts of FR-sema3A are required for such an experiment, we were unable to conduct this experiment.

Discussion

We have found that sema3A is present in nanogram per milliliter amounts in sera, and that the serum concentration drops significantly in patients who have hematologic malignancies characterized by extensive replacement of bone marrow cells by malignant cells such as in MM or various forms of acute leukemia, but not in patients with non-Hodgkin lymphoma without involvement of the bone marrow or in patients with colon cancer in which there is also no involvement of the bone marrow. In MM patients, we could not detect a decrease in the concentration of sema3A in sera from MM patients with the earliest stages of the disease in which there are no clinical manifestations as yet. In newly diagnosed patients with active disease the concentration of sema3A in the serum was about 60% of the concentration in healthy controls, and in patients with advanced disease that have relapsed after bortezomib treatment the average sema3A concentration dropped further to about 30% of the level found in healthy controls. In some patients with advanced disease, the serum sema3A concentration was 20-fold lower than that in healthy controls. It is, therefore, possible that the monitoring of sema3A levels may turn out to be an additional independent prognostic marker for MM progression. We also hypothesize that it may be useful for the identification of patients progressing from asymptomatic SMM to active disease. This will need to be further tested in the future as we have been unable to obtain access to a sufficient number of SMM patients. An even sharper drop in serum concentration of sema3A was observed in acute leukemias. In newly diagnosed ALL patients, the average sema3A serum concentration was only 14% of the concentration found in healthy controls and in AML patients it was 19% of the concentration found in healthy controls. In newly diagnosed CLL patients, we also observed a ~50% reduction in the sema3A concentration, but it did not reach statistical significance. However, the number of CLL patients that we sampled was relatively low and it is likely that the reduction is real although it seems less pronounced than in ALL and AML patients. Early in the development of CLL, there may not be a lot of CLL cells in bone marrow, whereas in advanced stages of CLL, the bone marrow is usually full with malignant cells. In contrast, in malignant diseases that do not involve the bone marrow such as in colon cancer or in lymphomas, we could not detect significant changes in the concentration of serum sema3A compared with healthy controls.

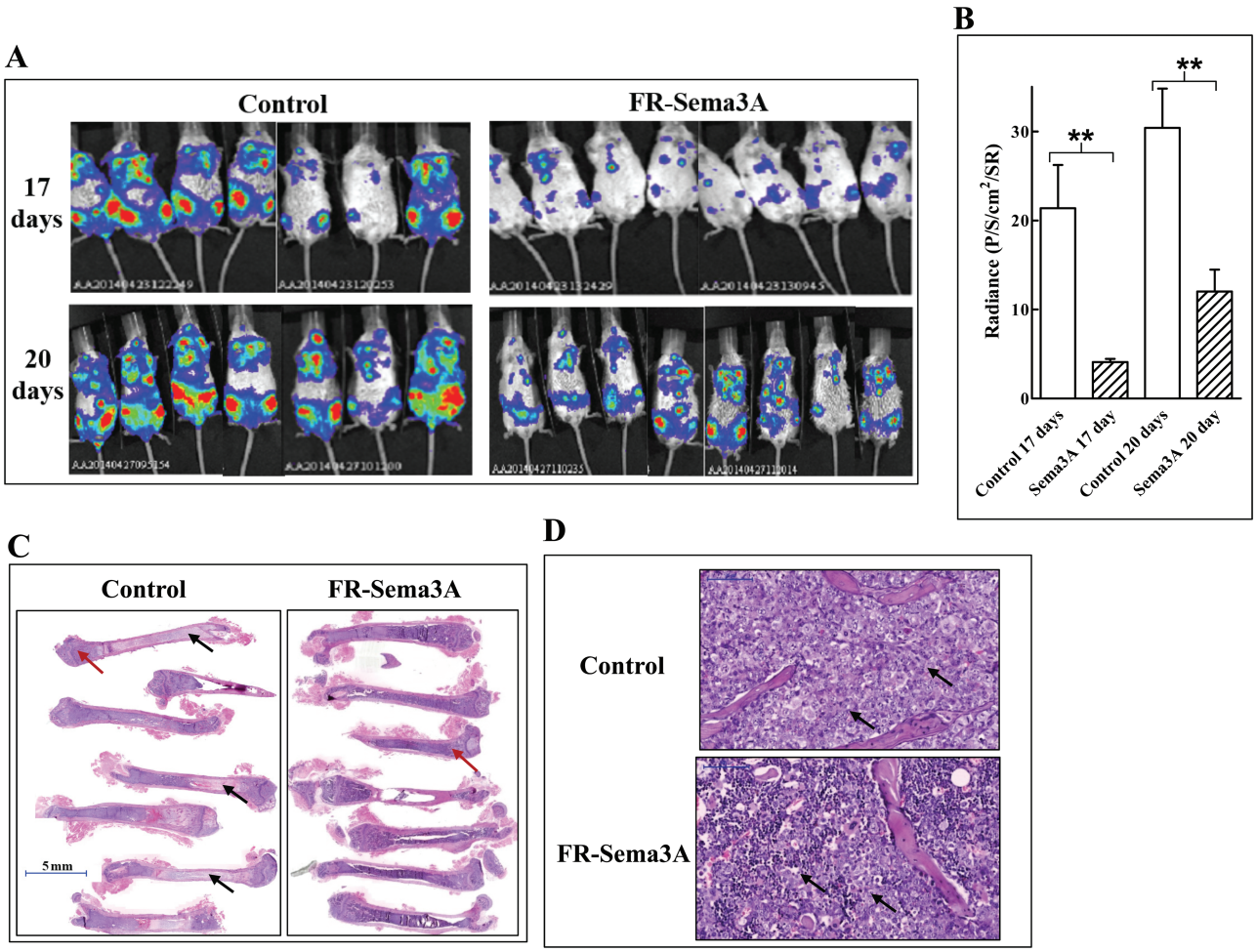

Figure 4. Comparison of the bone marrows of mice 20 days after inoculation with myeloma cells. (**A**) Age-matched SCID mice were inoculated with CAG cells expressing luciferase and either empty expression vector (control) or FR-sema3A and tumor progression was monitored by bioluminescence photography as described in Figure 3. Shown are bioluminescence pictures of the mice 17 and 20 days after inoculation. The mice were then killed on day 20 regardless of the stage of tumor development. (**B**) Quantification of tumor load as derived from bioluminescence imaging. Shown is the average bioluminescence signal per mouse. Error bars represent the standard error of the mean. $^{**}P < 0.01$. (**C**) Femur bones were collected from mice inoculated with control cells or cells expressing FR-sema3A on day 20 postinoculation. The bones were decalcified, fixed, embedded, sectioned and stained with hematoxylin/eosin as described. Black arrows point at necrotic regions. The blue and red arrows point at areas containing live cells shown at a higher magnification in panel C. (**D**) The areas designated by the red arrows in panel C are shown at a higher magnification. The bone marrow of mice that received control cells contains a high proportion of tumor cells (arrows) whereas that of mice inoculated with FR-sema3A expressing cells contains at this time a lower concentration of tumor cells (arrows) and seems much more normal.

Taken together, our observations suggest that the normal bone marrow contains a population of cells that secrete sema3A into the circulation. Our observations suggest that replacement of normal bone marrow cells by malignant cells depletes the sema3A producing cells, resulting in reduced serum sema3A concentrations. MGUS and SMM represent two earlier stages in MM development, which are not accompanied by clinical manifestations (26). It is thus likely that in MGUS and SMM there is as yet little depletion of normal bone marrow cells, and that as a result patients diagnosed with MGUS and SMM do not as yet display a significant reduction in their serum sema3A concentration. It is possible that the reduction observed in the serum concentration of sema3A in CLL patients was not as pronounced as in MM, AML and ALL patients because of the heterogeneity of the CLL patients we sampled and simply reflects the average degree of bone marrow replacement by the malignant CLL cells, which may depend on the stage of the disease at diagnosis.

The physiological function of the circulating sema3A is as yet unknown although it may function as a regulator of blood vessel permeability (15). A previous report demonstrated that normal bone marrow–derived endothelial cells express sema3A, and that the production of sema3A is reduced significantly in endothelial cells isolated from the bone marrow of MM patients. This reduction was accompanied by a concurrent increase in VEGF expression in these cells. It was suggested that this drop in sema3A production, which was associated with an increase in $VEGF_{165}$ expression, serves as the trigger that enables the angiogenic switch that enables the transition from MGUS to MM (28). More work will be required to determine if bone marrow endothelial cells do indeed represent the major source of the sema3A found in the circulation, and if the changes in the serum sema3A concentrations observed during MM progression are indeed correlated to changes in sema3A expression in bone marrow endothelial cells. It is possible that additional types of bone marrow cells also produce sema3A. Indeed, it was reported that stem cells in the bone marrow also produce sema3A (40). However, we could not detect an additional sema3A expressing bone marrow cell type in which sema3A expression is inhibited

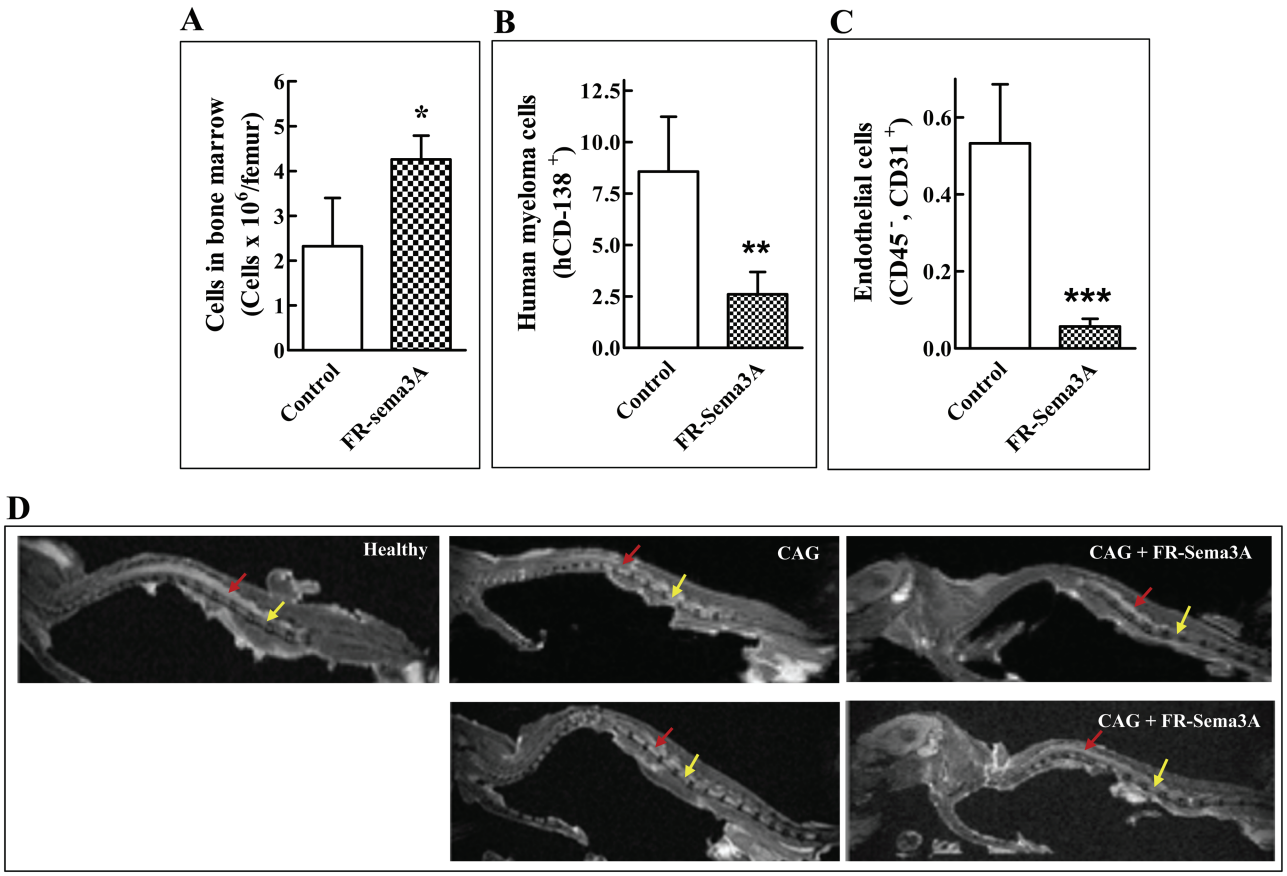

Figure 5. The effect of FR-sema3A expression on bone marrow cell populations and on spinal vertebrae. (**A**) Bone marrow cells were collected from the femurs of the mice as described in Materials and methods. The average number of cells recovered from the bone marrow of femurs of mice inoculated with control cells (control) or FR-sema3A expressing cells (FR-sema3A) was determined as described in Materials and methods. *$P < 0.05$. (**B**). The percentage of human CD-138-positive CAG myeloma cells in the total population of bone marrow cells recovered from femur bones of mice inoculated with control cells or cells expressing FR-sema3A was determined using fluorescence-activated cell sorting analysis as described in Materials and methods. **$P < 0.01$. (**C**). The percentage of $CD\text{-}45^{-}/CD\text{-}31^{+}$ endothelial cells was determined in cells isolated from each of the femur bone marrows of mice inoculated with control cells (control) or cells expressing FR-sema3A (FR-sema3A) using fluorescence-activated cell sorting analysis as described in Materials and methods. ***$P < 0.001$. (**D**). Healthy mice (healthy), mice inoculated with control cells (CAG) and mice inoculated with cells expressing FR-sema3A (CAG+FR-sema3A) were imaged using MRI as described in Materials and methods. Yellow arrows point at spinal vertebrae and red arrows point at the spinal cord.

in MM or in other malignancies involving the bone marrow (data not shown).

Sema3A functions as a potent inhibitor of angiogenesis (15) and inhibits the progression of solid tumors (16–19,22). The aforementioned observations suggested that reexpression of sema3A in the bone marrow of MM patients may perhaps inhibit the progression of MM. Cleavage of sema3A at its major conserved FPPC site produces products with strongly reduce activity (23). Because FPPC expression is known to be strongly upregulated in malignant cells, and because cleavage by FPPC may represent an additional mechanism by which tumors escape the antiangiogenic effects of sema3A, we introduced point mutations into the major FPPC cleavage site of sema3A to generate FR-sema3A, a sema3A variant that is fully biologically active yet resists FPPC-mediated cleavage. CAG MM cells expressing FR-sema3A still colonized specifically the bone marrow of SCID mice following their injection into the circulation as do wild-type CAG. The progression of MM in this MM model was inhibited significantly as a result of the expression of FR-sema3A as assessed by several parameters that include prolonged survival, reduced colonization of spinal vertebrae and consequently significantly less incidences of muscle weakness, reduced bone marrow necrosis and reduced concentrations of endothelial cells in the bone marrow indicating reduced angiogenesis. It is also possible that parts of the beneficial effects of FR-sema3A are due to direct inhibition of the bone erosion that is associated with MM progression as sema3A also promotes bone formation by inhibition of osteoclast precursor differentiation and through enhancement of osteoblast differentiation (27,41).

Our results indicate that monitoring sema3A concentrations in serum could perhaps be used to monitor disease progression in MM patients and possibly also in various additional hematological malignancies. Our results also suggest that FR-sema3A could perhaps have potential as a therapeutic agent although much more work will be required to better characterize its mode of action and biological effects. We have not yet been able to synthesize, purify and concentrate sufficient amounts of FR-sema3A to determine whether it can be used as a systemic drug. Such experiments will need to be performed in the future.

Supplementary material

Supplementary data are available at *Carcinogenesis* online.

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