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EphA3 targeting reduces in vitro adhesion and invasion and in vivo growth and angiogenesis of multiple myeloma cells.

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Author contributions

AC designed and performed the in vitro experiments, analysed data and wrote the manuscript; FL, IL, performed experiments in vitro; TA, BN performed confocal experiments; IA performed animal experiments; UF performed immunohistochemistry analysis in patients, ED performed immunohistochemistry in animals, AM, VS, ST performed gene expression profiling experiments; LD performed experiments in vitro, OV, VG, SB, AB, ML, PM, contributed material; ML, AV and GS commented on the manuscript; PM commented on the manuscript and provided financing, FF designed in vivo experiments, provided financing, commented on manuscript and DC designed the study, supervised the experiments, provided financing and wrote the manuscript.

List of abbreviations

BM, Bone marrow; CTCF, Corrected Total Cell Fluorescence; DMEM, Dulbecco's Modified Eagle Medium; ECs, endothelial cells; Efn, ephrin; Eph, Ephrin receptor; FCS, fetal calf serum; FGF-2, fibroblast growth factor-2; FITC, fluorescein isothiocyanate; GPI, glycophosphatidylinositol; H&E, hematoxylin and eosin; HGF, hepatocyte growth factor; MFI, mean fluorescence intensity ; MGUS, monoclonal gammapaties of undetermined significance; MGECs, MGUS endothelial cells; MM, multiple myeloma; MMECs, MM endothelial cells; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; OD, optical density; PE, phycoerythrin, PVDF, polyvinylidene difluoride; RT PCR, real time reverse transcriptase polymerase chain reaction; SFM, serum-free medium; siRNA, small interfering RNA; VEGF, vascular endothelial growth factor;

List of online Supporting information

- Supplementary Materials and Methods
- Supplementary Figure Legends
- Supplementary Figure 1: Antigen phenotype of MMECs
- Supplementary Figure 2: EphA3 expression in MM cell lines
- Supplementary Table 1: Genes up and down regulated in siEphA3 *vs* control siRNA MMECs

ABSTRACT

Background: This study investigates the role of ephrin receptor A3 (EphA3) in the pathogenesis of Multiple Myeloma (MM) and the effects of a selective target of EphA3 by a specific monoclonal antibody on bone marrow endothelial cells (ECs) of MM patients.

Methods: EphA3 mRNA and protein were evaluated in ECs of MM patients (MMECs); in ECs of patients with monoclonal gammapathies of undetermined significance (MGECs) and of those with benign anemia (ECs controls). The effects of EphA3 targeting by siRNA or by the anti EphA3 specific antibody on the angiogenesis was evaluated in vivo and in vitro. The antitumor activity of the antibody targeting EphA3 was measured in vivo in MM xenograft mice.

Results: Compared to the other EC types, MMECs showed a high level of expression of EphA3 both as mRNA transcript and protein. The EphA3 targeting by either siRNA or anti EphA3 antibody impaired the MMECs angiogenesis-related functions both *in vitro* and *in vivo*. Importantly, a clear antitumor activity was demonstrated in NOD SCID mice injected with human MM cell line JJN3 and treated with EphA3 targeting antibody.

Conclusions: EphA3 plays a critical role in MM angiogenesis; the anti EphA3 antibody inhibits this process in vitro and in vivo. EphA3 targeting could represent a novel strategy for the treatment of multiple myeloma patients.

INDRODUCTION

Multiple myeloma (MM) is a clonal disorder originating from post germinal center B cells that have undergone somatic hypermutation and immunoglobulin heavy-chain class switching. These cells typically locate in the bone marrow microenvironment that critically supports MM cell growth and survival.[1] Particularly, angiogenesis plays a critical role in the MM pathophysiology and progression.[2] Despite availability of several new therapeutic agents such as thalidomide, lenalidomide and bortezomib, MM remains incurable.[3] Therefore, there is an urgent need to develop new agents targeting additional pathways relevant for MM cells maintenance to increase the spectrum of available therapies. In this setting, monoclonal antibodies against MM-specific cell surface antigens represent a promising therapeutic approach, which is however hampered by a lack of appropriate membrane target structures expressed across MM cells.

A group of signaling molecules, the ephrins (Efn) and their receptors (Eph), has recently emerged as attractive therapeutic targets, since they control pathways which are critical for the development and maturation of myeloid and lymphoid cells. [4,5]

The Ephs belong to the largest family of receptor tyrosine kinases, divided in two subfamilies: the EphA and EphB according to the preferential ligand. Usually, EphA receptors interact with glycophosphatidylinositol (GPI)-anchored EfnAs, and EphB interact with transmembrane EfnBs, although there are exception to this rule.[6] Eph receptors have vital functions, including cell adhesion, migration and axon guidance, during development and homeostasis of many tissues. [7-10]

Compelling evidence also suggests that Eph-ephrin signaling plays several important roles in cancer development and progression. Expression of ephrin ligands and receptors is often reduced in advanced-stage tumors, and Eph-ephrin signaling regulates tumor growth, metastasis, and angiogenesis by altering cell proliferation, motility, invasion, and migration.[11]

The overexpression of EphA3 has been demonstrated in some cancers, including lung cancers, melanomas, gastric carcinoma and leukemia. [12-15] EphA3 expression is known to be associated with B and T cell malignancies. [16,17] Recently Bryan et al. described EphA3 is highly expressed on the tumor-initiating cell population in glioblastoma multiforme[18] and it is important in angiogenesis and prognosis of gastric and pancreatic carcinoma. [15,19]

No data are available in literature regarding the EphA3 expression in MM patients and its role in favoring MM malignant cell growth and in sustaining angiogenesis in MM microenvironment.

Based on the original anti EphA3 monoclonal antibody IIIA4[12,20] a first-in-class engineered IgG1 antibody targeting the EphA3 (KB004) was developed and it is now under phase I/II clinical trials for the treatment of EphA3 overexpressing hematological myeloid malignancies refractory to conventional treatment.

Here we bring for the first time the proof of concept of the antiangiogenic efficacy of blocking EphA3 with highly-specific human antibody *in vitro* and *in vivo*, showing a potent anti-tumor activity in MM xenograft model in mice, which might have potential therapeutic applications.

MATERIALS AND METHODS

Patients, Endothelial cells (ECs) and Plasma Cells

The study was approved by the Ethics Committee of IRCCS-CROB (Prot 3725; 7-2-2008), all patients provided informed consent. BM primary ECs from control subjects, from MM (MMECs) and from MGUS (MGECs) patients were obtained and cultured as described.[21] The patient information and the MMECs antigen phenotype are described in Supplemental Experimental Procedures and data are showed in Supplementary figure 1.kdskd

The ATCC human myeloma cell lines, MM1S, U266, RPMI8226 and DSMZ JJN3 cells were cultured in RPMI 1640 medium (Gibco) containing 10% fetal calf serum and 1% antibiotics (Gibco) and growed at 37°C under 5%CO_2/95% humidified air.

Absolute real-time PCR (RT-PCR)

RNA from MMECs, MGECs, normal ECs was extracted using Rneasy mini kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized using random hexamers and trascriptor first strand cDNA kit (Roche, Molecular Biochemicals, Mannheim, Germany). Absolute RT-PCR was carried out using Taqman assay (Perkin-Elmer–Applied Biosystems, Massachusetts USA). Cycling conditions are listed in Supplemental Experimental Procedures.

Western blot (WB), Fluorescence-activated cell sorting (FACS) analysis, Immunofluorescence-confocal laser scanning microscopy (IF) and immunohistochemistry(IHC)

For WB, we used anti EphA3 (Abcam, Cambridge, UK) and anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA).

For FACS, a phycoerythrin (PE)-labeled anti-CD105 antibody (Beckman Coulter, Brea, CA, USA), anti-EphA3 (AbCam) and isotype matched control antibodies were used.

For IF, anti-EphA3 antibody (AbCam), then goat anti-mouse Alexa Fluor 488 (Invitrogen) were used.

BM sections of MM patients were stained with anti EphA3-specific monoclonal antibody SL2 (kindly provided by KaloBios Pharmaceuticals, San Francisco, CA, USA). Tissue sections were counterstained with Mayer's hematoxylin solution (H&E). All preparations are indicated in Supplemental Experimental Procedures.

Small interfering RNA (siRNA)

MMECs (4×10^5) were transiently transfected with EphA3–siRNA or control siRNA (Silencer Selecter siRNA Ambion, Lifetechnologies) 5 nM or with the transfection reagent alone (Lipofectamine, RNAiMAX siRNA transfection reagent, Lifetechnologies) for 5 days and submitted to the followed functional studies.

Treatment of MMECs with antibodies

MMECs were treated with anti EphA3 antibody (chIIIA4) or an irrelevant Ab at 7,5 ug/ml or with Dulbecco's Modified Eagle Medium (DMEM; Gibco, Milan, Italy) alone and submitted to the followed functional studies.

Functional studies

Adhesion: One$\times10^4$ siRNA MMECs were plated in DMEM on fibronectin-coated 96-well plates in triplicate for 30 min, fixed with 4% paraformaldehyde and quantified by the crystal violet assay at 595 nm in a Microplate Reader (Molecular Devices Corp., Sunnyvale, CA, USA).

Chemotaxis: Five$\times10^4$ siRNA MMECs as above were tested in Boyden microchamber assay towards 1.5% serum medium with Vascular endothelial growth factor (VEGF 10 ng/ml, Sigma Chemical Co.) and fibroblast growth factor 2 (FGF-2; 10 ng/ml, Peprotech Inc., Rocky Hill, NJ, USA) as chemoattractants. After 8 h at 37°C, the migrated cells were fixed, stained and counted by the EVOS inverted microscope (Euroclone) at X400.

Scratch Wound Healing Assay: MMECs (2 × 104 cells/well) were seeded in 96-well plates, cultured to confluence and then scraped with a pipette tip to generate scratch wounds. Cells were incubated at 37 °C for 24 h with the medium alone or containing anti EphA3 or isotype Ab control. Images were captured using a Nikon Eclipse TE2000-5 microscope. Four selected field of images were captured in each sample, and the wound areas were estimated by Nikon NIS-Elements computer software.

Angiogenesis on Matrigel: MMECs treated as above were plated on Matrigel reduced growth factor (Becton Dickinson Biosciences, Bedford, MA) coated 48-well plates in serum-free medium (SFM) or in presence of anti EphA3 or isotype Ab. After 18h, the skeletonization of the mesh was followed by measurement of mesh areas and vessel length in three randomly-chosen fields with the EVOS microscope at X200.

Gene expression profiling and microarray analysis

Total RNA from both siEphA3 and Control MMECs was extracted using Rneasy mini kit (Qiagen) and processed on Illumina HumanHT-12 v4.0 BeadChips according to the manufacturer's protocol

and subsequently scanned with the Illumina HiScan. Data analyses were performed with GenomeStudio software (Illumina Inc.). Data was normalized with the quantile normalization algorithm, and genes were considered if the detection p-value was lower than 0.05. Statistical significance was calculated with the Illumina DiffScore. Only genes with a DiffScore ≤-30 and ≥30, corresponding to a p-value of 0.001, were considered as statistical significant.

Mice studies

Four-week-old NOD/SCID mice (Harlan Laboratories, Udine, Italy) were housed under specific pathogen-free conditions. All procedures were performed in the respect of the National and International current regulations (D.l.vo 27/01/1992, n.116, European Economic Community Council Directive 86/609, OJL 358, Dec. 1, 1987). Three groups (A-C) of five animals were injected sub-cutaneously with $10x10^6$ of human JJN3 MM cell line. Starting from three days after tumor cell inoculation, group A received subcutaneously (s.c.) 75µg of anti EphA3 (chIIIA4) three times a week, group B received control isotype Abs and group C received PBS. Fourteen days after JJN3 inoculation, mice were sacrificed and autopsies were carried out.

Tissiue Studies

For immunohistochemistry tumor sections were immunostained with rat anti-mouse CD31 (clone SZ31; Dianova, Hamburg, Germany), mouse anti-ki-67 (Dako, Glostrup, Denmark), rat anti-mouse F4/80 (Caltag, Burlingame,CA, USA) Abs. Immunocomplexes were detected using the Bond™ Polymer Refine Detection Kit according to the manufacturer's protocol (Leica, Wetzlar, D), then sections were counterstained with H&E. Apoptosis was detected by TUNEL staining with the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, Billerica, MA) according to the manufacturer’s protocol.

Results

EphA3 is upregulated in MMECs *vs* MGECs and normal ECs

EphA3 mRNA levels, measured by absolute RT-PCR, increases from ECs to MGECs, reaching the highest values in MMECs (Figure 1A). Accordingly, western blot and immunofluorescence showed a progressive increase of EphA3 protein from EC to MGEC to MMEC (Figure 1B-C). EphA3 protein expression in MMECs was confirmed using flow cytometry; representative expression profiles are shown in Figure 1D. EphA3 stained intensely and diffusely the MM microvessels and the plasma cells in MM BM biopsies (Figure 1E).

Figure 1

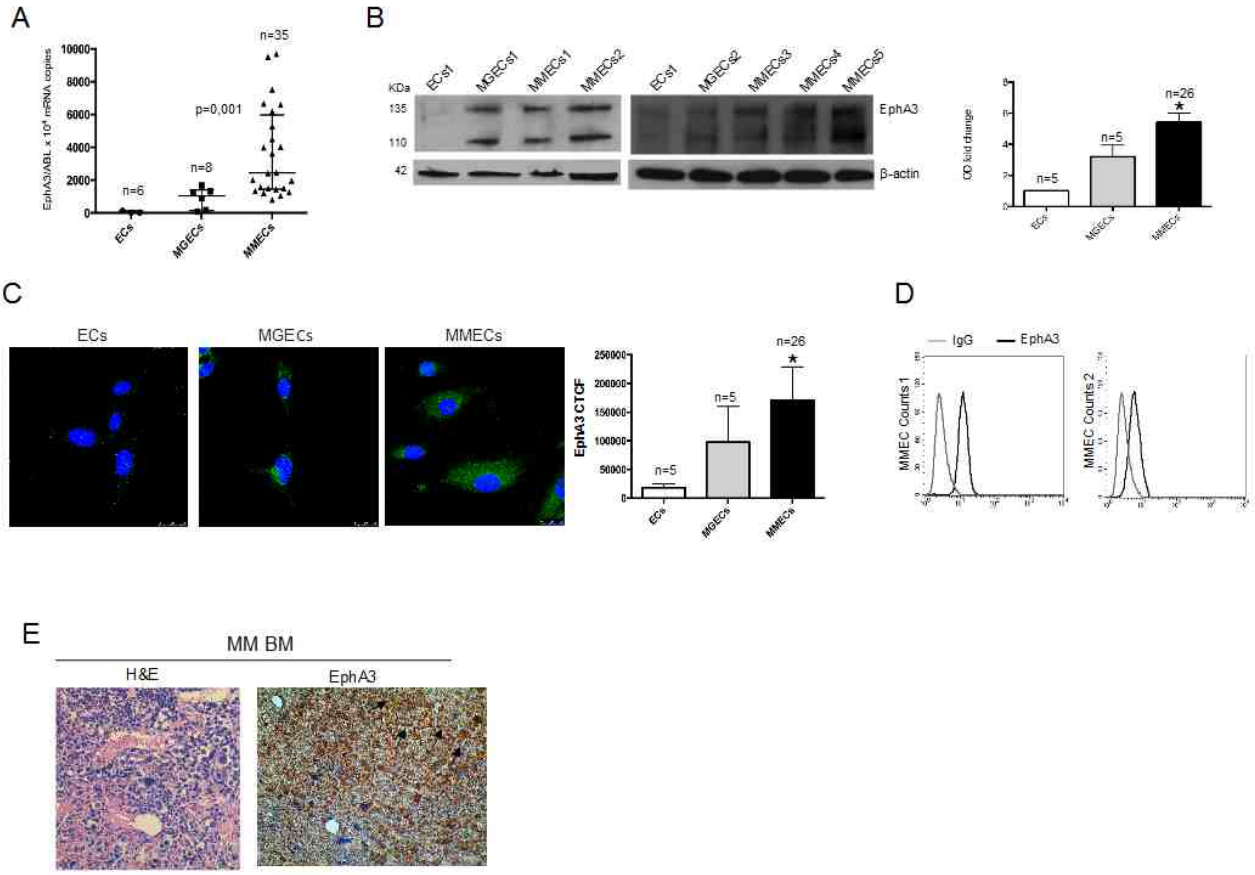

Figure 1. Analysis of EphA3 expression in normal ECs vs MGECs vs MMECs. A. Absolute Real Time -PCR of EphA3 mRNA copies /10*4 *ABL* copies as median + DS of 6 normal (□) and 8 MGUS (□) and 35 MM subjects (□) respectively. p= 0,001 by One Way ANOVA and Kruskal-Wallis test. **B.** Western blot of representative 2 normal, 2 MGUS and 5 MM subjects (β-actin=loading control). EphA3 fold change of Optical Density (OD) as means +DS of 5 normal, 5 MGUS and 26 MM subjects. **C.** Confocal immunofluorescence of EphA3 in MMECs *vs*. MGECs *vs.* ECs. Corrected Total Cell Fluorescence (CTCF) as mean + DS of 26 MM and 5 MGUS and 5 normal subjects. Pictures by confocal laser scanning microscope with 40X objective lenses. *=*P*<0.03 or better by Wilcoxon signed-rank. D) FACS analysis of EphA3 protein expression in MMECs from 2 representative patients. **E.** EphA3 immunohistochemical staining of BM biopsies from representative MM patients. EphA3 stains both neovessels (arrows) and plasma cells. Hematossilin/eosin staining (H&E) of BM biopsies is showed. Pictures by an Olympus photomicroscope (Olympus, Milan, Italy) with a CCD camera (Princeton Scientific Instr., Princeton, NJ, USA);

Loss of EphA3 inhibits tubular morphogenesis *in vitro*

In order to demonstrate the role of EphA3 in MMEC angiogenesis, we knocked down its gene by silencing RNA (siRNA). In EphA3-siRNA MMECs the protein was reduced by over 80% *vs* untreated or non-targeted siRNA cells (Figure 2A). EphA3-siRNA did not affect cell viability nor induce apoptosis (data not shown), but impacted cell adhesion (-35%) and chemotaxis (-40%) (Figure 2B). The EphA3-siRNA MMECs seeded onto the Matrigel surface (which mimics the subendothelial basement membrane) gave no angiogenesis, i.e. it lost the arborized constitutive assembly with significant reduction in the vessel areas and length (Figure 2C).

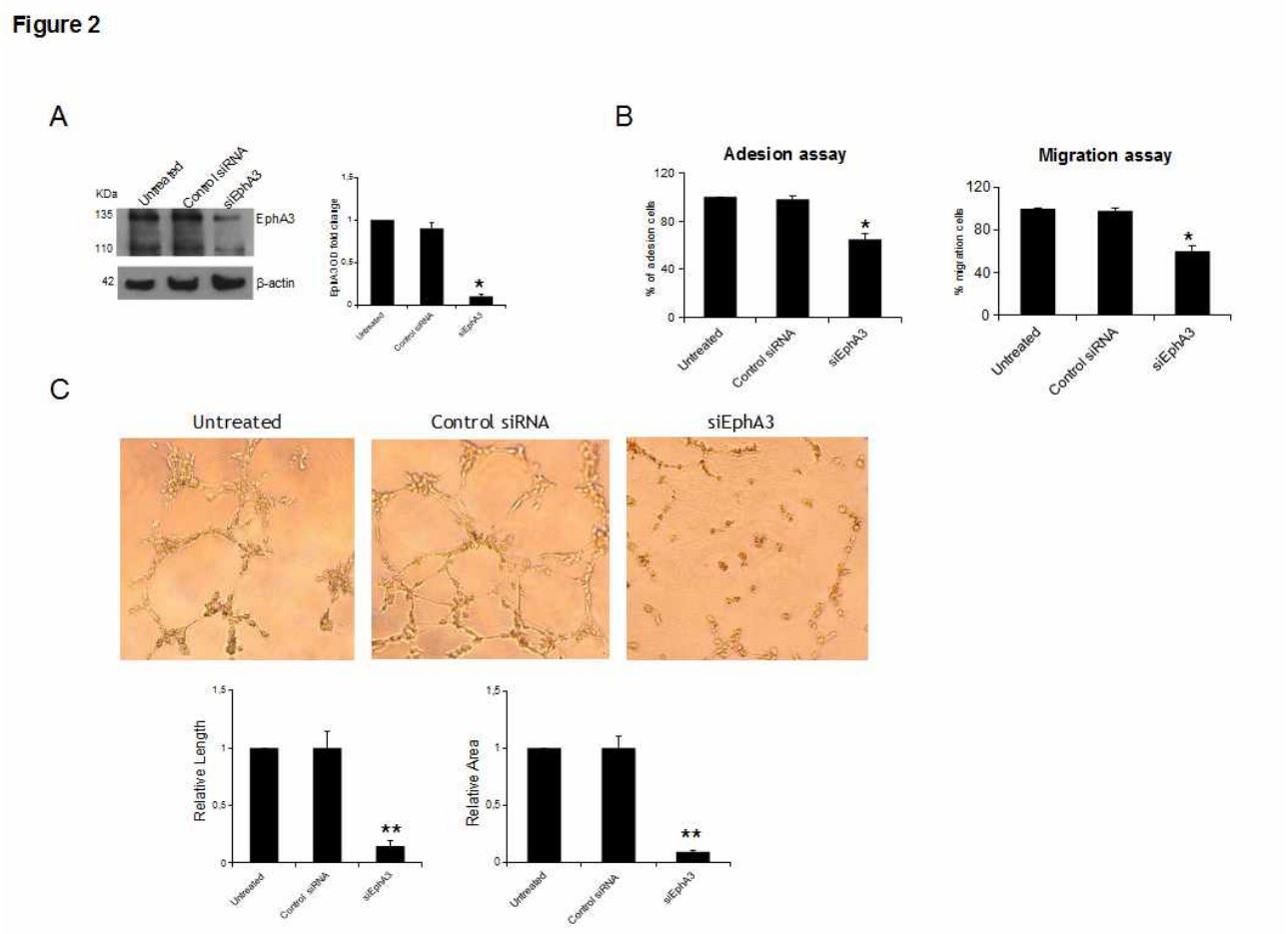

Figure 2. Effects on EC functions and angiogenesis in MMECs transfected with EphA3 siRNA (siEphA3). The cells were also transfected with non-targeting siRNA (Control siRNA) or lipofectamine only (Untreated) and analyzed after transfection**. A.** Western blot for knockdown analysis of EphA3 (β-actin=loading control). **B.** siRNA-transfected cells were tested for adhesion to fibronectin, chemotaxis and **(C)** angiogenesis on Matrigel (quantified by vessel length and areas in the bottom panels) and compared with control siRNA and untreated cells by the EVOS image software. Matrigel magnification=200X. Data are means +SD of 18 MM patients. **P*<0.03 or better and ***P*<0.01 or better by Wilcoxon signed-rank test.

The transcriptional profiles of EphA3-siRNA MMECs were compared with those of non-targeted siRNA cells. Among the significantly modulated genes (190 genes (Supplementary Table 1), we

found downregulation of the adhesion, migration and angiogenesis molecules *RYK, JAM2, VEGFA , FLNA, CD148* in EphA3-siRNA MMECs. (Table 1)

Table 1. Down regulated genes in siEphA3 *vs* control siRNA MMECs

ENTREZ GENE SYMBOL ID	NAME	FUNCTION	FOLD CHANGE
57124	**CD248**	Angiogenesis[22]	0,8
2316	**FLNA**	Adhesion, migration[21]	0,8
7422	**VEGFA**	Angiogenesis[23]	0,7
58494	**JAM2**	Cell-cell adhesion[24]	0,7
6259	**RYK**	Focal adhesion[25]	0,3

EphA3-specific antibody inhibits MMECs *in vitro* migration and tube formation

To examine whether anti-EphA3 Ab (chIIIA4) affected the angiogenic functions of MMECs, we performed wound healing and tube formation assays. First, MMECs treated with anti EphA3 were not affected for viability nor apoptosis (data not shown). MMECs in wound healing assay, decreasing the percentage of migrated cells from ≈ 90% (control) to 15% in the presence of Ab anti-EphA3(chIIIA4). As expected, MMECs migration was not affected by an irrelevant Ab (Figure 3A). MMECs treatment with Ab anti-EphA3(chIIIA4) significantly inhibited the formation of tube-like structures (Figure 3B). Overall, these results provided strong evidence of the antiangiogenic activity of anti EphA3, *in vitro*, affecting endothelial cell migration and tubulogenesis.

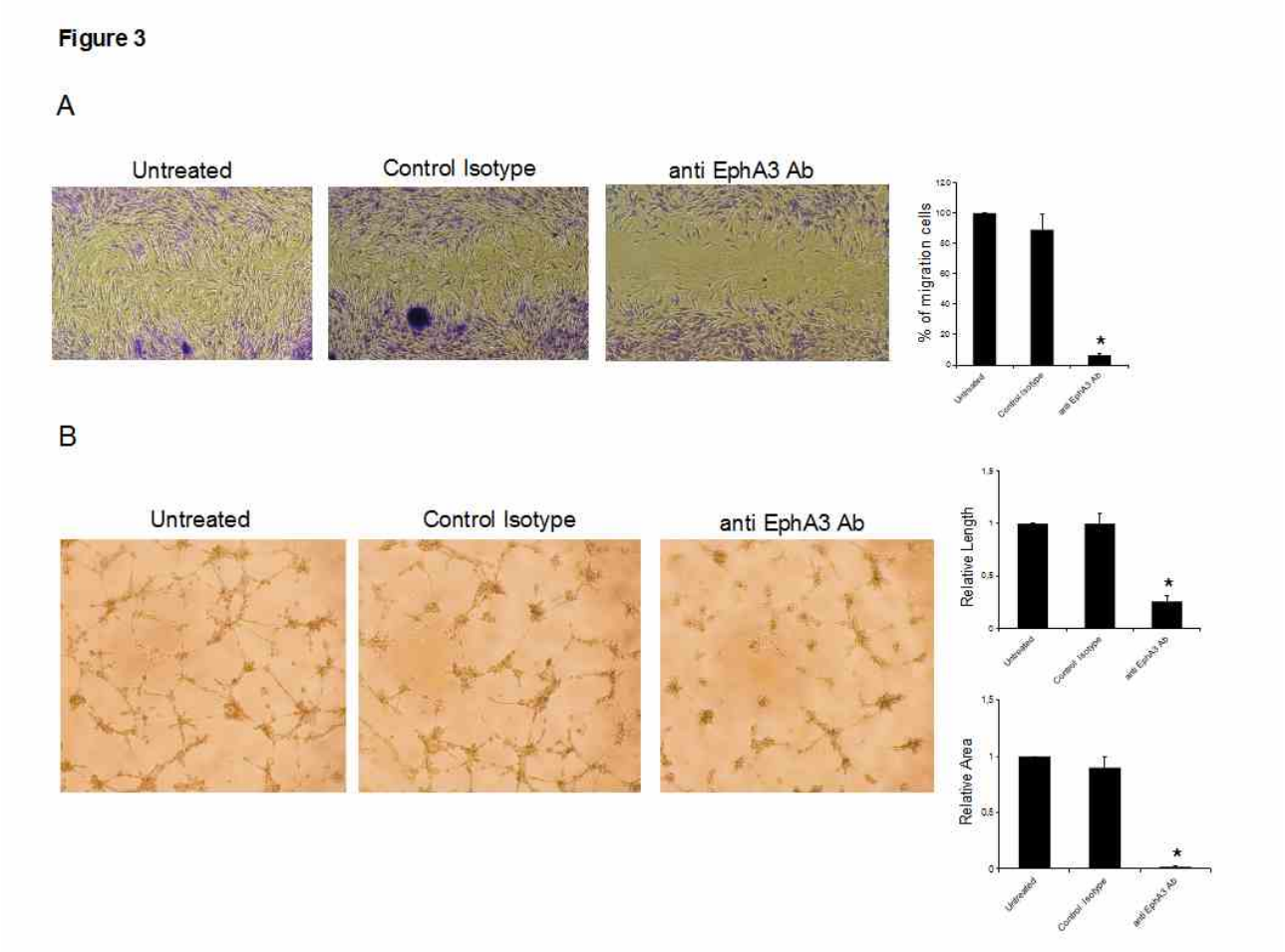

Figure 3. Characterization of anti angiogenic capability of anti EphA3-specific antibody . A. Analysis of migration of MMECs by wound healing assays *in vitro*. Representative photographs (4X magnification) taken 24h after scratching are shown. Cell migration was monitored over 24 h with 7,5 µg/ml of anti EphA3-Ab or an irrelevant Ab (Control) or in medium (Untreated) as indicated. **B.** MMECs were cultured on standard Matrigel in the absence (Untreated) or presence of anti-EphA3 or an irrelevant Ab (Control). Representative microphotographs of tube formation after 18 hours of culture (4X magnification) are shown. Quantification of lateral migration and tube formation (vessel length and areas) is shown at right panels. Anti EphA3 was assayed at least three times and the corresponding values (means ± SD) were represented. * P<0.01 versus control.

Anti-EphA3 inhibits tumor xenograft growth

Having demonstrated effects of anti EphA3 on MMECs *in vitro*, we next sought to assess the *in vivo* efficacy of anti EphA3 using a mouse model of human MM.

First, we checked EphA3 expression in the MM cell lines such as MM1S, U266, RPMI8226 and JJN3. Our results indicated that JJN3 cells expressed the highest levels of EphA3, followed by MM1S, RPMI8226 and U266 cells with a moderate expression of EphA3 (Supplementary Figure 1A). Furthermore, to study whether the treatment with anti-EphA3 antibody could directly affect to

the tumor cell proliferation and survival, the MM cell lines were cultured in the presence of anti EphA3 antibody. No direct effect on tumor cell growth was observed *in vitro* in any of the MM cell lines (data not shown).

We studied the effect of anti EphA3 on tumor growth by using JJN3 cells in NOD/SCID mice. As shown in Figure 4A, anti EphA3 treatment significantly inhibited tumor growth. Tumor developed was formed by round to oval cells showing a morphological range from mononuclear to occasionally multinucleated cells with prominent nucleoli and frequent mitotic figures (Figure 4B(a). The tumor was supplied by a fairly developed microvascular network, as assessed by CD31immunostaining (b), and showed high proliferative activity, as revealed by Ki-67 immunostaining (c) and low apoptotic index, as assessed by the TUNEL assay (d). Similar histologic (e) and immunohistochemical features (f, g, h) were observed in tumors developed in mice treated with control isotype Abs. By contrast, tumor masses developed in mice treated with anti-EphA3 Abs (i) were smaller in size (figure 4A) and showed foci of ischemic-hemorrhagic necrosis (N), in association with a significant ($P < 0.05$) reduction in the number of intact tumor microvessels (j). The proliferative activity (k) was not significantly different from that observed in tumors from untreated or control isotype treated mice, while the apoptotic index was significantly ($P < 0.05$) increased (l) in comparison with tumors from both groups of mice (d and h). F4/80 stained monocytes and macrophages were frequently found, particularly at the tumor edge, in close contact with apoptotic tumor cells evidences by aspects of nuclear fragmentation (inset in l). Taken together, these results demonstrate that anti EphA3 triggers *in vivo* inhibition of tumor growth, decreased angiogenesis, and increased MM-cell apoptosis.

Figure 4

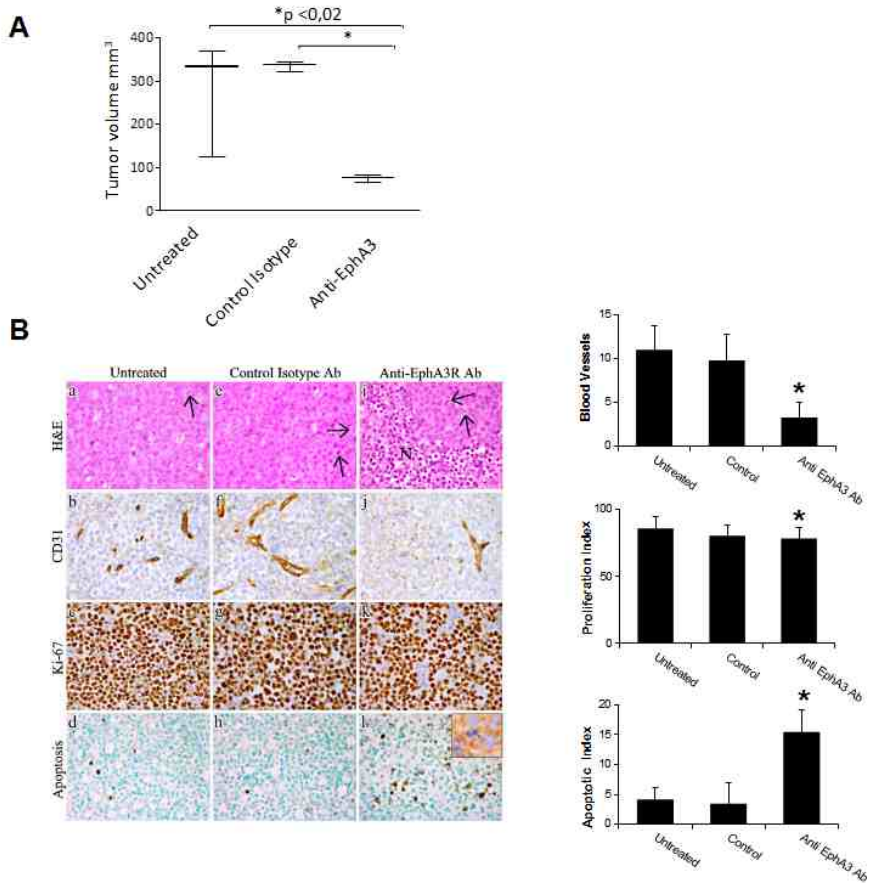

Figure 4. *In vivo* antitumor activity of the anti EphA3-specific antibody on MM xenograft mice. The SCID/NOD animals were injected subcutaneously with $10*10^6$ of JJN3. Three days after cell inoculation, mice were divided in three groups of five mice and treated with control isotype or anti EphA3 antibody o PBS (Untreated). After fourteen days mice were killed, tumors removed and measured as described in the methods. **A.** Box plots represent the median volume of the masses removed from all the mice of each group . **B.** Morphological aspects of tumors developed in a representative untreated (a), control isotype Ab (e) or anti-EphA3 Abs treated mice (ì). Round to oval neoplastic plasma cells with frequent mitotic figures (arrows) are showed. Microvascularization was fairly developed in tumors from both groups of mice (b, f), tumor cell proliferation was fast (c, g) and apoptosis was scanty (d, h). By contrast, tumor masses developed in mice treated with anti-EphA3 Abs (i) showed foci of ischemic-hemorrhagic necrosis (N), in association with a defective microvascular supply (j). Tumor cell proliferation (k) was similar to that observed in control tumors (c, g), while apoptotic figures, frequently wrapped by F4/80 stained monocyte/macrophages (inset), were clearly increased (l) in comparison with tumors from both untreated (d) and control isotype Ab treated (h) mice. (a-l: X400; inset in l: X1000). Left panel:

Results are expressed as mean ± SD of CD31 positive microvessels per field; or Ki-67 or TUNEL positive cells/number of total cells (Proliferation Index and Apoptotic Index respectively) evaluated on formalin-fixed paraffin embedded tissue sections by immunohistochemistry. * $p < 0.001$ One-way ANOVA for comparisons between 3 groups; $p < 0.05$ Tukey-test compared with tumors from untreated mice; $p < 0.05$ Tukey-test compared with tumors from control isotype Ab treated mice.

DISCUSSION

MM cells strictly depend on the BM microenvironment for survival and disease progression. The activation/generation of endothelial cells and the consequent angiogenesis seems to be crucial in this process. The microenvironment consists of a 'niche' of BM stromal cells (BMSCs), including fibroblasts, osteoclasts (OCs), osteoblasts, vascular endothelial cells, lymphocytes and extracellular matrix (ECM). The crosstalk between MM and the BM niche, mediated by cytokines and adhesion molecules is critical for the homing of malignant cells to the BM, production of tumor survival factors and inhibition of osteoblastogenesis. A number of novel agents including thalidomide, lenalidomide and bortezomib have revolutionized the treatment of MM resulting in a significant improvement of the overall survival; their activity is through an anti-angiogenetic effect. Complete remission can be achieved but recurrence of the disease remains the main obstacle to cure. Considering the critical role of angiogenesis in sustaining the survival and proliferation of MM cells, there is a need of more effective drugs targeting the microenvironment and particularly the angiogenesis to improve the clinical outcome for MM patients.

EphA3 receptor plays critical role in many solid tumors as well as hematological malignancies.

Eph receptors and their membrane-bound ephrin ligands are involved in many biological processes including adhesion, cell migration and angiogenesis. Ephrin-B2 and the Eph receptors EphB3 and EphB4 are also present in vascular endothelium. During embryonic development, ephrin-B2 and EphB4 are expressed in arteries and veins, respectively, and are required for vascular remodeling during blood vessel maturation.[26] However, the ephrin-A1 ligand and its EphA2 receptor are expressed in tumor angiogenesis.[27] Antibody inhibition experiments show that Eph/ephrin signaling is required for angiogenesis to proceed.[28] Further studies have shown that soluble EphA2-Fc and EphA3-Fc constructs inhibit tumor angiogenesis and growth in vivo,[29] providing the first functional evidence for EphA receptor in the regulation of tumor angiogenesis.

While their expression and function in normal adult tissues is limited, Eph and ephrin overexpression in human cancers often correlates with aggressive, invasive, and metastatic phenotypes. Eph/ephrin signaling activation can lead to different nearly opposite biological effects

such as cell adhesion or cell repulsion. Data from the literature show that these opposite outcomes mediated by Eph/ephrin signaling are dependent on the Eph receptor signaling strength, cell type presenting the receptor and density of the receptor. Several variables direct the type of cellular responses.

The role of EphA3 in MM has not been previously investigated.

In this study we first showed that EphA3 is highly overexpressed in MM bone marrow derived ECs. The over expression was detected with different approaches at mRNA and protein level. Immunohistochemistry reaction in bone marrow biopsies from MM patients allows to identify the EphA3 in ECs and in hematopoietic cells. Interestingly, we could detect EphA3 expression also in ECs from MGUS, although at lower levels, but not in normal ECs. MMECs are analyzed ~30 days after harvesting from the BM, we suggest, tentatively, that changes in EphA3 gene and protein are stably acquired by these cells, with transition from MGUS (avascular phase) to MM (vascular phase). Perhaps these gene/protein changes are a consequence of genomic or epigenetic changes. Therefore, epigenetic regulation, such as changes in gene methylation, may play a dominant role in upregulating the expression of EphA3 in neoplastic cells and may be the result of already abnormal cell biology, supported by the fact that EphA3 expression is not regulated by methylation of the promoter in normal tissue.[16] However, literature data show that little characterization of MM at the epigenetic level (for example gene methylation and acetylation) has been performed.[30]

Angiogenesis is complex and tightly orchestrated multistep processes that involve several biological systems such as cell proliferation, migration and tube formation. Suppression at any step of these processes will inhibit the formation of new vessels. We have demonstrated antiangiogenic effects of EphA3 knockdown (KD) *in vitro* at three levels: inhibition of adhesion, migration and tubular structure formation. This is consistent with previously reported data that showed EphA3 silencing prevents spreading of LK63 cells on fibronectin surface.[31] Gene expression profiling showed that EphA3 KD inhibited some adhesion and pro angiogenic factor genes such as RYK, VEGFA, FLNA. The capacity of EphA3 to regulate these molecules by MMECs supports EphA3 as a regulator of angiogenesis. [22-25,32]

Interestingly, we obtained the same effects, a decreased migration and impaired capacity to form vessels *in vitro* by treating MMECs with an antibody targeting EphA3 (chIIIA4 mAb), *in vitro*.[20] chIIIA4 mAb targets a site closely adjacent to the heterotetramerization site on the N-terminal of EphA3's extracellular domain adjacent to the ligand-binding site and has a high affinity for EphA3.[33] The mechanism through which EphA3-specific antibody blocks MMECs adhesion and

motility has not been investigated. Hence, it is possible that the anti-EphA3 antibody themselves may modulate EphA3 signaling by forcing it into a conformation that interfere with the signal transduction cascade such as the antibody binding could modulate ligand oligomerization and clustering or impair reverse signaling.[34] Additional work will be needed to conclusively address the precise mechanisms of these function-modulating antibody.

Our *in vitro* data are clearly supported by the *in vivo* data.

First, we demonstrated that all MM cell lines studied expressed high EphA3 levels. Interestingly the EphA3 mRNA copies are higher than those measured in MMECs. Therefore EphA3 also becomes potential target in cancer cells.

Treatment of NOD/SCID mice bearing the JJN3 MM cell line with chIIIA4 mAb resulted in an effective inhibition of tumor xenograft growth. Immunohistochemistry analysis performed on tumor cells after treatment with chIIIA4 mAb clearly show that apoptosis was significantly increased in tumors treated with chIIIA4 mAb, whereas cell proliferation was not affected; the increased apoptosis over similar proliferation rates may account for the observed reduction in tumor growth in treated mice. Tumor xenografts showed a dramatic reduction in the number of blood vessels indicating that anti-EphA3 antibody mediated angiogenic suppression. Interestingly, in the tumor after anti EphA3 treatment we observed a macrophage infiltration (insert in figure 4B) that could be responsible for an antibody-dependent cellular phagocytosis (ADCP).[35] In fact, anti-EphA3 antibody failed to inhibit the growth of cultured MM cell lines and primary MMECs (data not shown) *in vitro*, consistent with the hypothesis that anti-EphA3 affect tumor growth through a killing cell mediated mechanism rather than through inhibiting tumor cell proliferation *per se*. Therefore, the observed induction of apoptosis would be consequence of eliminating blood vessels.

The effect of IIIA4 *in vivo* against EphA3 using mouse xenograft models of LK63 pre-B ALL, HEK293 cell line and SK-MEL28 melanoma cell lines was assessed. Rapid internalization of the monoclonal antibody is observed on administration of IIIA4 in mice tumor xenograft models and the mouse leukemia model.[20]

Clinical and pre-clinical studies suggest that some therapeutic monoclonal antibodies, such as trastuzumab and rituximab may also enlist immune effector cells to attack and kill tumor cells by cytotoxicity (ADCC) and phagocytosis (ADCP). [36,37] Actually, IIIA4 has been modified using antibody Humaneering™ technology to produce KB004, which has now in multi-center open-label Phase 1 trial, (http://clinicaltrials.gov/ct2/show/NCT01211691) in patients with EphA3-positive hematologic malignancies including chronic myeloid leukemia (CML), acute myeloid leukemia

(AML), acute lymphoblastic leukemia (ALL), myelodysplastic syndromes (MDS) who are refractory to, have failed, or have not received standard-of-care treatment. KB004 induces apoptosis of malignant cells through different mechanisms, mainly a immune effect induced by ADCC.[38]

In summary, we have identified EphA3 as potential target in MM and described the initial characterization of anti-EphA3 that represents a new approach to target tumor and angiogenesis for MM treatment and, possibly, other angiogenesis-dependent pathologies. In the future, it will also be of interest to investigate whether anti EphA3 antibody can complement or synergize with other established anti angiogenic agents.

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