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SEMA3A partially reverses VEGF effects through binding to neuropilin-1

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ABSTRACT

Cross-talk between hematopoietic stem cells (HSCs) and bone marrow stromal cells (BMSCs) is essential for HSCs regulation and leukemogenesis. Studying bone marrow of myelodysplasia patients, a pre-leukemic condition, we found mRNA overexpression of vascular endothelial growth factor A (*VEGFA*) in $CD34^+$ HSCs and semaphorin 3A (*SEMA3A*) in BMSCs. To better understand the role of VEGFA and SEMA3A in leukemogenesis, we recruited 30 myelodysplastic syndrome (MDS) patients, 29 acute myeloid leukemia (6 secondary to MDS) patients and 12 controls. We found higher *VEGFA* expression in *de novo* AML patients (without prior MDS) group ($p = 0.0073$) and higher *SEMA3A* expression in all BMSCs patient's samples compared to control group. We then overexpressed *VEGFA* in an acute myelogenous leukemia cell line, KG1 cells, and in normal $CD34^+$ cells. This overexpression increased KG1 ($p = 0.045$) and $CD34^+$ cell ($p = 0.042$) viability and KG1 ($p = 0.042$) and $CD34^+$ cell ($p = 0.047$) proliferation. Moreover, KG1 and $CD34^+$ cells overexpressing *VEGFA* also had increased proliferation when co-cultured with human marrow stromal HS5 cells ($p = 0.045$ and $p = 0.02$, respectively). However, co-culture of these transformed cells with HS5 cells overexpressing *SEMA3A* reduced KG1 ($p = 0.004$) and $CD34^+$ ($p = 0.009$) proliferation. Co-culture of KG1 transformed cells with HS27 cells overexpressing *SEMA3A* reduced KG1 proliferation as well ($p = 0.01$). To investigate whether the dominant *SEMA3A* effect over VEGFA could be due to competition for neuropilin1 receptor (NRP1), we performed immunoprecipitation with anti-NRP1 antibody of cell extracts of co-cultured KG1 and HS5 cells, induced or not by VEGFA and SEMA3A recombinant proteins. Results showed a preferential association of NRP1 with SEMA3A, suggesting that SEMA3A can partially reverse the effects caused by the VEGFA preventing its binding with the NRP1 receptor. Since both hematopoietic cells, leukemic and normal, showed similar behavior, we suppose that the attempt to reversion of VEGF effects by SEMA3A is a homeostatic phenomenon in the hematopoietic niche. Finally, we conclude that VEGFA overexpression confers AML cell advantages and SEMA3A may partially reverse this effect; thus, SEMA3A protein combined with VEGFA inhibitors could be beneficial for AML treatment.

1. Introduction

Hematopoietic stem cells (HSCs) are capable of self-renewal and of differentiating into mature blood cells (Jagannathan-Bogdan & Zon, 2013) and the cross talk between HSCs and Bone marrow stromal cells (BMSCs), in the bone marrow microenvironment, is essential for HSCs regulation (Morrison & Scadden, 2014). This homeostasis may be interrupted by mutations in the HSC, caused by errors induced by aging, environment, hereditary factors among other factors (Wilson et al., 2008). The most frequent hematological disease of aging are the myelodysplastic syndromes (MDS), in which acquired mutations in HSC lead to deregulation of signaling pathways, conferring risk of clonal expansion and progression to acute myeloid leukemia (AML) (Albitar et al., 2002). Nowadays, the differentiation between both MDS and AML is mainly based on the number of immature, supposedly neoplastic, cells in the bone marrow, being 20% the cutoff number (MDS - <20% and AML - 20% or more) Patients with MDS have low or high risk to evolve to AML, depending on genetic, epigenetic and/or immunological factors (Vardiman et al., 2009).

Recent studies have shown that the cross-talk between leukemic stem cells, which are a small fraction of leukemic cells capable of self-

Abbreviations: AML, Acute myeloid leukemia; AML-MRC, Acute myeloid leukemia with myelodysplasia-related changes; BM, Bone marrow; BMSCs, Bone marrow stromal cells; HSCs, Hematopoietic stem cells; MVD, Microvascular density; MDS, Myelodysplastic syndromes; NRP1, Neuropilin1 receptor; SEMA3A, Semaphorin 3A; VEGFA, Vascular endothelial growth factor A; VEGFR1, Vascular endothelial growth receptor 1; VEGFR2, Vascular endothelial growth receptor 2.

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renewal, and BMSCs is essential for leukemogenesis (Lee et al., 2015). Similarly, myelodysplastic cells require a type of cancer stem cell for myelodysplastic syndrome (MDS) development (Woll et al., 2014). For a better knowledge of the interaction between leukemic and stromal cells, our group performed a microarray study using bone marrow cells from MDS patients and found *VEGFA* overexpression in progenitor $CD34^+$ cells and *SEMA3A* overexpression in BMSCs (Baratti et al., 2010).

SEMA3A is a secreted protein characterized by its role in migration and guidance of axons in the developing nervous system. SEMA3A can only bind to neuropilin 1 receptor (NRP1), however neuropilins are unlikely to transduce SEMA3A signals on their own due to their short intracellular domain, thereby, complex formation with Plexin A receptors is necessary for signal transduction (Neufeld et al., 2012).

NRP1 serve as co-receptors for VEGFA; VEGFA is a proangiogenic factor widely known and, due to alternative splicing, the *VEGFA* gene generates multiple isoforms among which $VEGF_{165}$ is the most abundant. VEGFR1 and VEGFR2 are the main receptors for $VEGF_{165}$, however the complex formation with NRP1 co-receptor can enhance their signaling (Neufeld et al., 1999). Studies have described increased *VEGFA* mRNA (Fiedler et al., 1997), plasma levels (Aguayo et al., 2002) and protein expression (Verstovsek et al., 2002) in AML patients and correlation with a worse prognosis (Gerber & Ferrara, 2003), however the role of *VEGFA* in MDS remains controversial (Aguayo et al., 2002; Verstovsek et al., 2002; Bellamy et al., 2001; Wimazal et al., 2006; Gianelli et al., 2013).

An antagonistic role of VEGFA and SEMA3A has been implicated in several solid tumors (Chekhonin et al., 2013; Bender & Mac Gabhann, 2015); while VEGFA increases tumor growth and metastasis (Chekhonin et al., 2013), SEMA3A is associated to lower migration and invasion rates (Pellet-Many et al., 2008), and to a better prognosis (Janssen et al., 2012). Moreover, there is a disagreement as to whether $VEGF_{165}$ and SEMA3A opposite effects could be due to NRP1 binding competition (Pellet-Many et al., 2008). The aim of this study is to better understand the interaction of VEGFA and SEMA3A in the bone marrow of MDS and AML patients.

2. Materials and Methods

2.1. Patients and controls

The study included patients with a confirmed diagnosis of MDS or AML, untreated at the time of sample collection. Bone Marrow (BM) aspirates from healthy donors, MDS, and AML patients were collected according to institutional guidelines. Assignment to different MDS (high and low risk) and AML (secondary to MDS or Myelodysplasia-Related Changes (MRC) and *de novo*) groups was decided according to the 2008 World Health Organization classification (Vardiman et al., 2009); acute promyelocytic leukemia patients were excluded. For gene expression analysis, $CD34^+$ or BMSCs were isolated from BM aspirates of 12 healthy donors, 30 MDS (22 low risk and 08 high risk) and 29 AML (06 secondary to MDS/MRC and 23 *de novo*) untreated patients who attended the outpatient clinic at the Hematology and Transfusion Center of the University of Campinas (Hemocentro-UNICAMP) from 2006 to 2016 (Table 1). Some patients were donors for both $CD34^+$ cells and BMSCs. For *VEGFA* overexpression assay, normal $CD34^+$ cells were isolated from umbilical cord blood after deliveries and samples were processed within 24 h of collection. This study was approved by the Ethics Committee of the University of Campinas and healthy donors and patients provided informed written consent.

Table 1
Clinical characteristics of the participants in the study.

	$CD34^+$ cells	BMSCs
Normal controls	8	5
Gender (male/female)	3/5	4/1
Age (years), median (range)	40 (29–49)	40 (28–54)
MDS patients	18	25
Gender (male/female)	12/6	18/7
Age (years), median (range)	69 (27–77)	70 (16–90)
Low-risk (RCUD/RCMD/RARS)	11 (0/9/2)	18 (0/16/2)
High-risk (RAEB-1/RAEB-2)	07 (1/6)	07 (2/5)
AML patients	15	17
Gender (male/female)	4/11	7/10
Age (years), median (range)	63 (34–87)	63 (30–86)
Secondary/*de novo*	4/11	5/12

BMSCs, bone marrow stromal cell; MDS, myelodysplastic syndrome; RARS, refractory anemia with ringed sideroblasts; RAEB, refractory anemia with excess of blasts; RCUD, refractory cytopenia with unilineage dysplasia; RCMD, refractory cytopenia with multilineage dysplasia.

2.2. $CD34^+$ cell and BMSCs selection

BM or cord blood mononuclear cells were isolated by Ficoll-Hypaque Plus density-gradient centrifugation (GE Healthcare) and labeled with CD34 MicroBeads (Miltenyi Biotec). $CD34^+$ cells were isolated by MIDI-MACS immunoaffinity columns (Miltenyi Biotec) and purity was determined by flow cytometry (minimum 90%), using anti-CD34 antibody. The remaining mononuclear cells were plated onto Dulbecco's modified Eagle's medium (DMEM; Sigma) supplemented with 10% FBS. Supernatant containing nonadherent cells was removed weekly and replaced with fresh supplemented medium. When the monolayer was established (90% confluence), cells were trypsinized and plated under the same conditions. After three replatings, a homogeneous cell population was obtained and BMSCs were evaluated by flow cytometry for the absence of CD31, CD34, CD45, CD68, and HLA-DR antigens and presence of CD73, CD90, and CD105.

2.3. Cell Lines and Recombinant Proteins

KG1 cell line, which was obtained from a 59-year-old Caucasian male with erythroleukemia that evolved into acute myelogenous leukemia and expresses the CD34 antigen (Morimoto et al., 1994), and the HS5 and HS27 cell lines, two different clones of a long term bone marrow cell obtained from a 30-year-old Caucasian male that were transformed with the amphotropic retrovirus vector LXSN16E6E7 (Roecklein & Torok-Storb, 1995), were obtained from ATCC, Philadelphia, PA. Cells were cultured in RPMI-1640 medium containing 10% FBS, 2 mM glutamine, 100 mg/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B and maintained in a humidified atmosphere at 37 °C, 5% CO_2. For immunoprecipitation assay, co-culture of KG1 and HS5 cells were treated with 100 ng/mL of VEGF 165 (PeproTech) and 250 ng/mL of SEMA3A Fc Chimera (R&D Systems) human recombinant proteins.

2.4. Quantitative polymerase chain reaction

All samples were assayed with cDNA (Revert Aid H Minus First Strand cDNA Synthesis Kit; MBI Fermentas), SYBR Green Master Mix PCR (MBI Fermentas) and specific primers in the MasterCycler ep Realplex (Eppendorf). Relative gene expression was calculated using the equation $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). Control was performed for each primer pair. Amplification specificity was verified using a dissociation curve at the end of each run. Three replicas were run on the same plate for each sample. The following primers were used: *VEGFA*, 5′-ATCGAGACCCTGGTGG ACAT-3′ and 5′-GTGCTGGCCTTGGTGAGG-3′; *SEMA3A*, 5′-TGTATGTTGGAGCAAAGGAT-3′ and 5′-TCTGGTGTAAG ATACTGGCC -3′ (Baratti et al., 2010) and *HPRT*, 5′GAACGTCTTG CTCGAGATGTGA-3′ and 5′-TCCAGCAGGTCAGCAAAGAAT-3′.

2.5. Vector construction and lentivirus transfection

For cell line overexpression, 0.57 kb full-length human VEGF 165 (OriGene) or 2.3 kb full-length human SEMA3A cDNA (OriGene) were amplified by RT-PCR and cloned into pENTR vector (Invitrogen)

according to the manufacturer's instructions. The LR recombination reaction was completed between pENTR-VEGFA 165 or pENTRSEMA3A vectors and pLenti6/V5-Dest vector (Invitrogen) to generate pLenti-VEGF 165 or pLenti-SEMA3A, engineered to express VEGF 165 and SEMA3A respectively. pLenti-LacZ (Invitrogen) was used as control. Lentivirus packaging and tittering were performed by Capital Bioscience Inc. (Gaithersburg, MD). KG1 cells were transduced with lentivirus-mediated pLenti-VEGF 165 or pLenti-LacZ and named VEGFA and Lacz cells, respectively. Briefly, 2×10^5 cells were transduced with lentivirus by spinoculation at multiplicity of infection equal to 5 and selected by 1 μg/mL blasticidin S. HS5 cells were transduced with lentivirus-mediated pLenti-SEMA3A or pLenti-LacZ and named SEMA3A and Lacz cells, respectively. Briefly, HS5 cells were seeded into six-well plates at 2×10^5 cells/well, grown overnight, and transduced with lentiviral vectors at multiplicity of infection equal to 5 and selected by 1 μg/mL blasticidin S. Gene and protein expression of VEGFA and SEMA3A were confirmed by RT-PCR and immunoblotting. Functional assays were performed after 48 h of incubation.

2.6. Vector construction and Nucleofection®

For $CD34^+$ overexpression, a 0.57 kb full-length human VEGF 165 (OriGene) were amplified by RT-PCR and cloned into pMig vector. The empty vector pMig was used as control. For Nucleofection®, 5×10^4 $CD34^+$ cells were suspended in 20 μl of Nucleofection® buffer solution for human primary cells (P3 Primary Cell 4DNucleofector™ X Kit, Lonza) containing pMig-VEGFA or pMig plasmid DNA using the EO-100 program of the Amaxa 4D-Nucleofector. Immediately after Nucleofection®, cells were transferred into 500 μl of pre-warmed DMEM medium supplemented with 20% FBS, 50 ng/mL SCF and 10 ng/mL IL-3. Gene expression was confirmed by RTPCR.

2.7. Co-culture of HS5 or HS27 and KG1 or $CD34^+$ cells

Stromal feeder layers from HS5 or HS27 cells in the nontransduced, Lacz/SEMA3A transduced conditions were seeded in 6-well culture plates (2×10^5 cells/well for co-culture with KG-1 cells) or 24-well culture plates (2×10^4 cells/well for co-culture with $CD34^+$ cells) in RPMI-1640 medium containing 10% FBS and incubated for 12 h at 37 °C. After this period, nonadherent cells were seeded onto stromal cell monolayers (1×10^6 KG1 or 1×10^5 $CD34^+$ cells/well) and incubated for 72 h at 37 °C. Nonadherent cells were removed and functional assays were performed.

2.8. Cell viability assay

KG1 cell viability was determined by MTT assay as previously described (Mosmann, 1983). Briefly, 2.5×10^4 cells/well was seeded in 100 μl RPMI-1640 10% FBS medium. Next, 10 μl of a 5 mg/mL solution of MTT were added to the cells followed by incubation at 37 °C for 4 h. The reaction was stopped using 100 μl of 0.1 N HCl in anhydrous isopropanol. Cell viability was evaluated by measuring the absorbance at 570 nm, using an automated plate reader. Conditions were tested in six replicates.

$CD34^+$ cell viability was determined by Cell-titer 96 aqueous solution cell proliferation assay kit (Promega) according to the manufacturer's instructions. Briefly, 1×10^4 cells/well were seeded in DMEM 20% FBS, 50 ng/mL SCF and 10 ng/mL IL-3 supplemented medium. Next, 20 μl of cell-titer 96 aqueous solution were added to the cells followed by incubation at 37 °C for 2 h. Absorbance was read at 490 nm, using an automated plate reader. Conditions were tested in two replicates.

2.9. Cell proliferation assay (Ki-67 staining)

5×10^5 cells were fixed with 70% ethanol and stored at −20 °C. Ki-67 staining was performed according to the manufacturer's instructions (BD Bioscience). Mean of fluorescence intensity (M.F·I) was obtained by flow cytometry using a FACS Calibur (Becton Dickinson). Blank cells (without KI-67 staining) were used as negative control for each condition. Acquired data were analyzed using FlowJo software.

2.10. Colony formation assay

Colony formation was carried out in semisolid methylcellulose medium (2×10^3 cell/mL; MethoCult 4230 for KG1 cells or 4435 for $CD34^+$ cells; StemCell Technologies Inc.). KG1 colonies were detected after 7 days of culture by adding 1 mg/mL of MTT reagent and scored by Image J quantification software (U.S. National Institutes of Health). $CD34^+$ colonies were characterized and scored according to their morphology with an inverted microscope at 40× magnification in a culture dish marked with a scoring grid. The colonies were classified into CFU-GM (Colony-forming unitgranulocyte/macrophage) and BFU-E (Burst-forming unit-erythroid). Conditions were replicated twice.

2.11. Detection of apoptosis by Annexin V and PI staining

1.25×10^5 cells were fixed with 70% ethanol and stored at −20 °C. Cells were washed twice with PBS and resuspended in binding buffer containing 1 μg/mL PI and 1 μg/mL APC labeled Annexin-V (BD Bioscience). All specimens were analyzed by flow cytometry (FACS Calibur) after incubation for 15 min at room temperature in a light protected area. Acquired data were analyzed using FlowJo software. Conditions were replicated twice.

2.12. Immunoprecipitation and Western blot

Equal amounts of protein were used for total extracts or for immunoprecipitation with specific antibodies, followed by SDS-PAGE and Western blot analysis with the indicated antibodies (carried out using the ECL™ Western Blotting Analysis System; Amersham Pharmacia Biotech Ltd., Buckinghamshire, UK). Antibodies against Plexin-A4 (Santa Cruz, CA), VEGF Receptor 2 (Cell Signaling Technology, MA), Neuropilin (NRP-1) (Santa Cruz, CA). For VEGFA and SEMA3A protein expression, the antibodies used were Anti-VEGF 165A (ABCAM, Cambridge, AM), anti-SEMA3A (Santa Cruz, CA) and normalized by anti-Actin (Santa Cruz, CA).

2.13. Statistical analysis

Statistical analyses were performed using GraphPad Instat 5 (GraphPad Software, Inc., San Diego, CA, USA) and SAS System for Windows (Statistical Analysis System, 9.4. SAS Institute Inc., 2002–2012, Cary, NC, USA.). For patient's comparisons, *Mann–Whitney* test was used. For functional assay comparisons, *Student t*-test and Anova test were used, as appropriate. A P value < 0.05 was considered as statistically significant.

3. Results

3.1. Increased expression of VEGFA and SEMA3A in patient bone marrow cells

The first step of our study comprised evaluation of mRNA expression of *VEGFA* in $CD34^+$ cells and *SEMA3A* in BMSCs from MDS, AML and healthy donor BM aspirates. *VEGFA* mRNA expression was significantly increased in CD34+ cells from *de novo* AML patients compared with healthy donors (5.06 [0.42–57.52] vs 1.00 [0.8–2.19], $P = 0.0073$) (Fig. 1 A). *SEMA3A* mRNA expression was significantly increased in

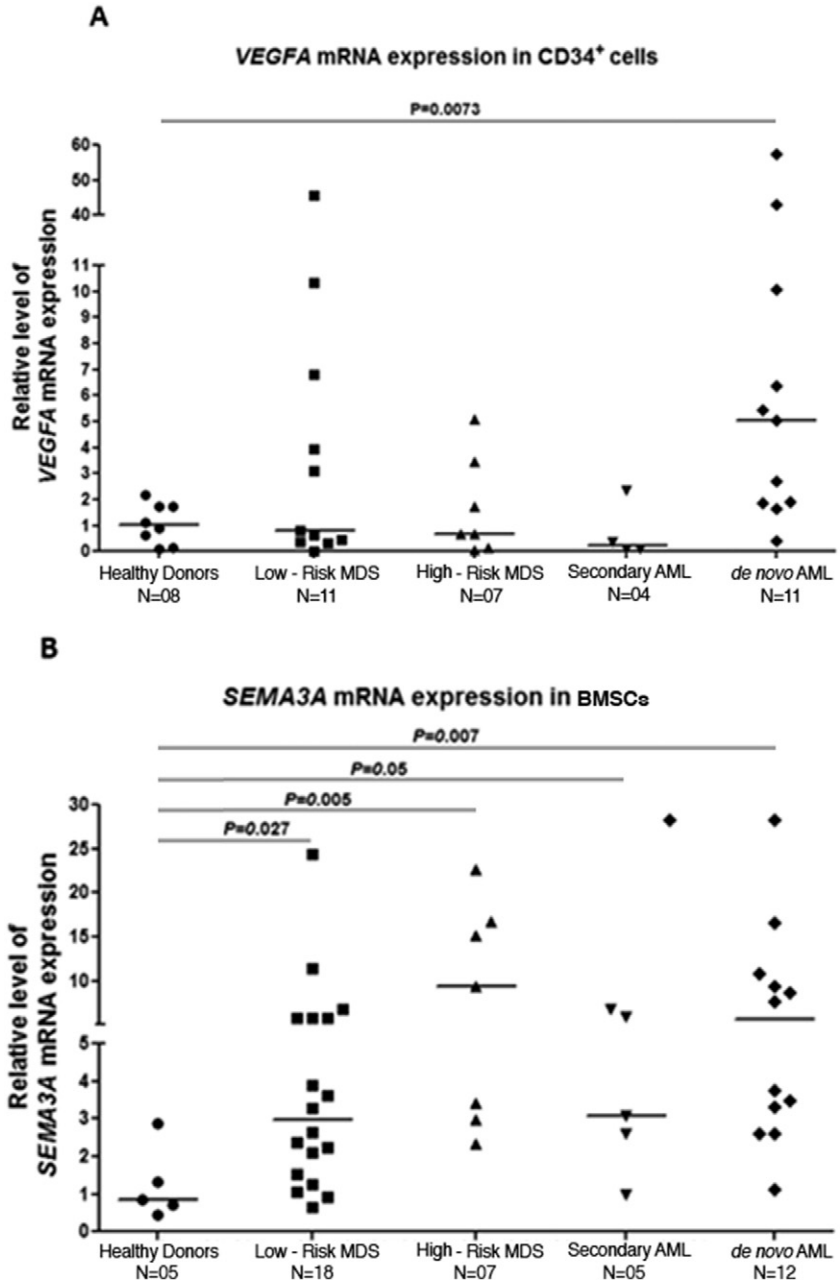

Fig. 1. VEGFA expression is increased in CD34$^+$ cells of *de novo* AML patients and SEMA3A is increased in BMSCs of MDS and AML patients compared to control cells. VEGFA and SEMA3A mRNA expression in healthy donors and MDS and AML patients. RTPCR was performed on cDNA from BM CD34$^+$ or BMSCs. Each dot indicates the relative *VEGFA* or *SEMA3A* expression for each patient. Horizontal lines represent medians. mRNA expression levels were normalized by HPRT endogenous control. (A) *VEGFA* mRNA expression was significantly higher in CD34$^+$ cells from *de novo* AML patients compared with healthy donors (P = 0.0073). (B) *SEMA3A* mRNA expression was significantly higher in BMSCs from MDS low-Risk (P = 0.027), MDS High-Risk (P = 0.005), secondary AML (P = 0.05) and *de novo* AML (P = 0.007) patients compared with healthy donors; *Mann-Whitney test.*

BMSCs increased derived from MDS and AML patients compared with BMSCs from healthy donors: MDS low-risk (2.97 [0.64–24.39] vs 0.83 [0.43–2.86], P = 0.028), MDS high-risk (9.50 [2.34–22.60] vs 0.83 [0.43–2.86], P = 0.005), Secondary AML (3.07 [0.97–6.97] vs 0.83 [0.43–2.86], P = 0.05) and *de novo* AML (5.73 [1.10–28.21] vs 0.83 [0.43–2.86], P = 0.007) (Fig. 1 B).

3.2. VEGFA overexpression increased proliferation of KG1 and CD34 + cells

We further evaluated the role of *VEGFA* overexpression. We chose the leukemic cell line KG1 because it expressed a low level of VEGF and then could be used for VEGF overexpression (Supplementary data). However, normal CD34$^+$ cells exhibited an even lower level of *VEGF* mRNA (Supplementary data). The KG1 cell line was stably transduced with lentivirus-mediated targeting *VEGFA* or *Lacz* (Control). After antibiotic selection, the efficiency of *VEGFA* overexpression was verified by qPCR and Western blotting (Supplementary data). CD34$^+$ cells were nucleofected® with Mig-VEGFA or Mig vectors and the efficiency of *VEGFA* overexpression was verified by qPCR (Supplementary data). We next evaluated the effects of *VEGFA* overexpression on cell viability, proliferation, colony formation and apoptosis.

VEGFA ovexpression significantly increased the viability of KG1 (124.4 ± 23.03% vs 100 ± 0.62%; P = 0.003) (Fig. 2 A1) and CD34$^+$ cells compared to control (159.6 ± 41.2% vs 102.5 ± 16.93%; P =

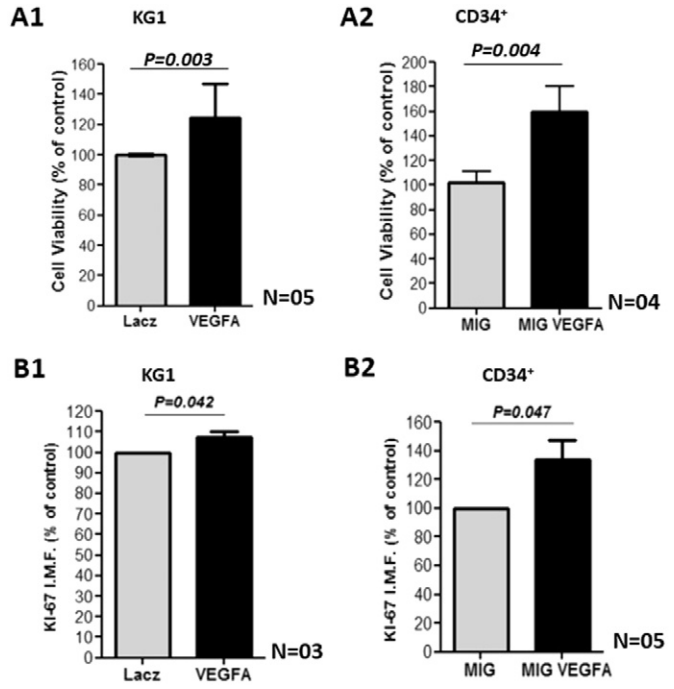

Fig. 2. VEGFA overexpression increased cell proliferation. (A1) KG1 viability was determined by MTT assay after 48 h of incubation. (A2) CD34$^+$ viability was determined by Cell-titer 96 aqueous solution cell proliferation assay kit after 96 h of incubation. Results are shown as mean ± SD, the numbers of independent experiments are indicated; *Anova test*. (B1) KG1 and (B2) CD34$^+$ cell proliferation was determined by Ki-67 M.F.I. after incubation for 48 h. Results are shown as mean ± SD, the numbers of independent experiments are indicated; *Student t-test*.

0.045) (Fig. 2 A2). Ki-67 analysis revealed that VEGFA overexpression increased proliferation of KG1 (107.7 ± 2.82%; P = 0.042) (Fig. 2 B1) and CD34$^+$ cells (134.0 ± 7.68%; P = 0.047) (Fig. 2 B2). Regarding colony formation and apoptosis, VEGFA ovexpression had no effect in KG1 or CD34$^+$ cells (data not shown).

3.3. SEMA3A effects are dominant over VEGFA in co-culture assays

To determine whether VEGFA and SEMA3A had opposite effects in leukemia cells, we performed co-culture assays using HS5 or HS27 stromal and KG1 leukemic cell lines. It is important to point out that stromal cell line HS5 as well as normal BMSCs extracted from cord blood showed low levels of *SEMA3A* mRNA (Supplementary data). HS5 and HS27 cell lines were stably transduced with lentivirus-mediated targeting *SEMA3A* or *Lacz* (Control). After antibiotic selection, the efficiency of *SEMA3A* overexpression was verified by qPCR and Western blotting (Supplementary data). KG1 and HS5 cells were co-cultured for 72 h under three conditions: KG1 VEGFA or KG1 Lacz and HS5 non-transduced cells; HS5 SEMA3A or HS5 Lacz and KG1 nontransduced cells; and KG1 VEGFA or KG1 Lacz and HS5 SEMA3A and HS5 Lacz cells. KG1 and HS27 cells were co-cultured for 72 h under one condition: KG1 VEGFA or KG1 Lacz and HS27 SEMA3A and HS27 Lacz cells. KG1 non-adherent cells were then harvested, and proliferation, apoptosis and colony formation assays were performed.

In HS5 and KG1 co-cultures, Ki-67 analysis revealed an increased proliferation of KG1 cells overexpressing VEGFA when co-cultured with HS5 cells (176.9 ± 46.34%; P = 0.045) (Fig. 3A). However, co-culture with HS5 SEMA3A cells decreased the proliferation of KG1 cells compared with HS5 Lacz co-culture (93.15 ± 0.79%; P = 0.004) (Fig. 3B). Finally, SEMA3A effects were dominant over VEGFA effects as co-culture of KG1 VEGFA and HS5 SEMA3A showed reduced proliferation compared to KG1 Lacz plus HS5 Lacz (67.15 ± 22.09%; P < 0.001) or KG1 VEGFA plus HS5 Lacz coculture (118.8 ± 13.67% vs 91.97 ± 5.26%; P < 0.001) (Fig. 3C). In HS27 and KG1 cocultures, Ki-67 analysis also revealed that SEMA3A effects were dominant over VEGFA effects as co-culture of KG1 VEGFA and HS27 SEMA3A showed reduced proliferation compared to KG1 VEGFA plus HS27 Lacz (137.3 ± 10.83% vs 159.4 ± 15.12%; P = 0.016) or KG1 Lacz plus HS27 SEMA3A co-culture (137.3 ± 10.83% vs 149.6 ± 16.34%; P = 0.016) (Fig. 3D).

Co-culture with HS5 SEMA3A cells did not alter colony formation of KG1 cells. However, co-culture of KG1 VEGFA and HS5 Lacz cells increased the number of KG1 colonies compared to KG1 Lacz and HS5 Lacz co-culture (163.9 ± 37.81%; P = 0.004). The number of KG1 colonies decreased in KG1 VEGFA and HS5 Lacz co-culture compared to KG1 Lacz and HS5 SEMA3A (163.9 ± 37.81% vs 101.0 ± 31.27%; P = 0.008); and in the co-culture of KG1 VEGFA and HS5 Lacz cells compared to KG1 VEGFA and HS5 SEMA3A cells (163.9 ± 37.81% vs 106.9 ± 35.42%; P = 0.011) (Fig. 3E).

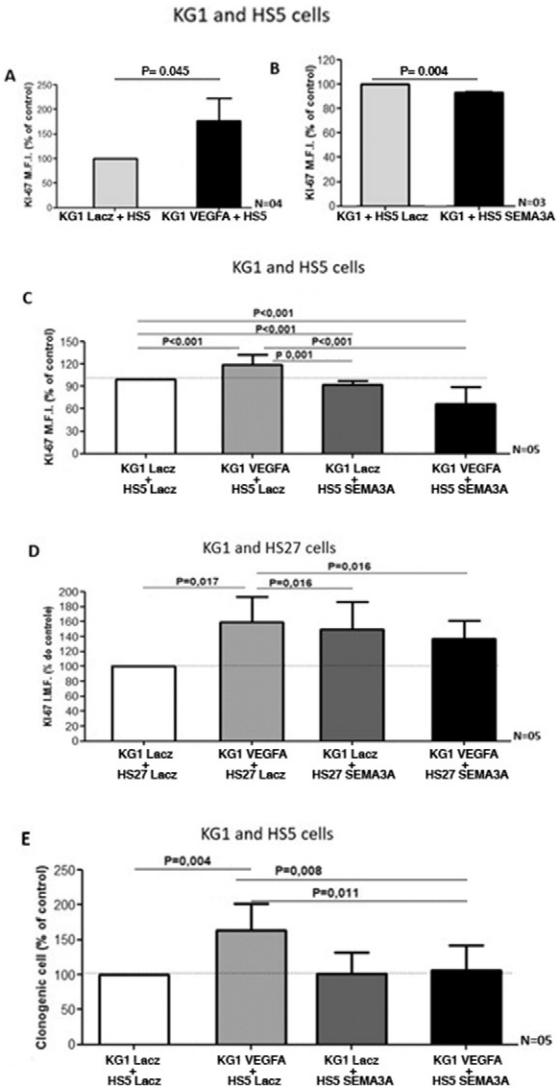

Fig. 3. SEMA3A effects are dominant over VEGFA in co-culture assays using KG1 and stromal cell lines (HS5 and HS27). KG1 proliferation was determined by KI-67 M.F.I. flow cytometry after co-culture of: (A) KG1 VEGFA or KG1 Lacz and HS5 non-transduced cells, (B) HS5 SEMA3A or HS5 Lacz and KG1 non-transduced cells, (C) KG1 VEGFA or KG1 Lacz and HS5 SEMA3A or HS5 Lacz. (D) KG1 VEGFA or KG1 Lacz and HS27 SEMA3A or HS27 Lacz. Results are shown as mean ± SD, the numbers of independent experiments are indicated; *Student t-test*, and *Anova test*. (E) Colonies containing viable KG1 cells were detected by MTT in cells co-cultured for 72 h with HS5 cells, followed by 07 days in culture with methylcellulose. Results are shown as mean ± SD, the number of independent experiments are indicated; *Anova test*.

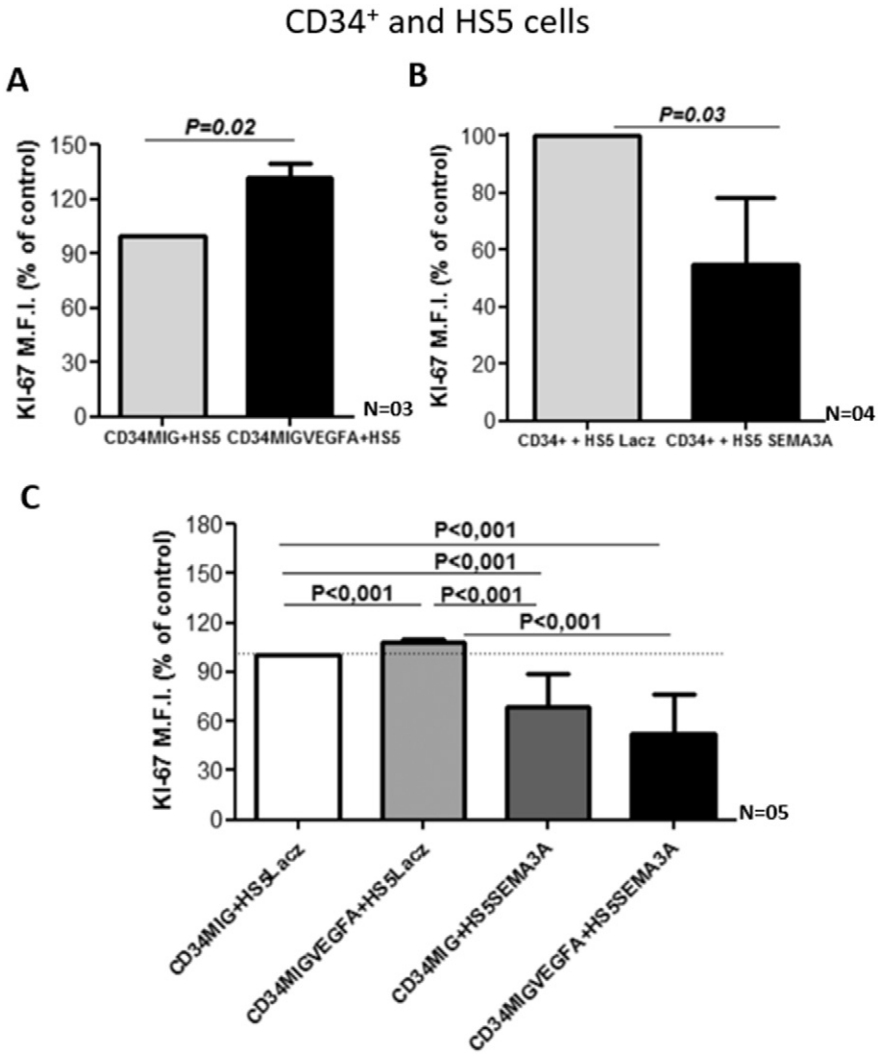

Fig. 4. SEMA3A effects are dominant over VEGFA in co-culture assays using $CD34^+$ cells from cord blood and HS5 cell line. $CD34^+$ proliferation was determined by KI-67 M.F.I. flow cytometry after co-culture of: (A) $CD34^+$ MIG VEGFA or $CD34^+$ MIG and HS5 nontransduced cells, (B) HS5 SEMA3A or HS5 Lacz and $CD34^+$ non-transduced cells, (C) $CD34^+$ MIG VEGFA or $CD34^+$ MIG and HS5 SEMA3A or HS5 Lacz. Results are shown as mean ± SD, the numbers of independent experiments are indicated, *Student t-test* and *Anova test.*

VEGFA or *SEMA3A* ovexpression did not alter KG1 apoptosis in any of the coculture conditions (data not shown). Thus, these results indicated that in the absence of SEMA3A, VEGFA overexpression increased proliferation of KG1 cells co-cultured with HS5 cells. However, in the absence of *VEGFA*, *SEMA3A* overexpression decreased proliferation of KG1 cells. When we combined KG1 VEGFA e HS5 SEMA3A cells, the SEMA3A effects were dominant over VEGFA.

To determine whether VEGFA and SEMA3A had opposite effects in $CD34^+$ normal cells, we performed co-culture assays using HS5 stromal cell line and $CD34^+$ cells from umbilical cord blood. $CD34^+$ and HS5

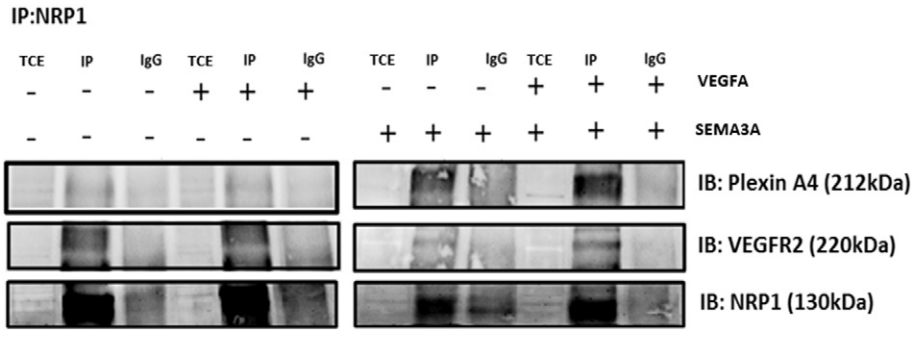

Fig. 5. Preferential association of NRP1 receptor with SEMA3A over VEGFA. Immunoprecipitation (IP) assay with NRP1 antibody and immunoblotting (IB) with Plexin-A4, VEGFR2 and NRP1 antibody were performed using total extracts from KG1 and HS5 cocultured cells under four conditions: Control cells (Without recombinant protein treatment), VEGFA cells (treated with recombinant VEGFA protein (100 ng/μL)), SEMA3A cells (treated with recombinant SEMA3A protein (250 ng/μL)) and VEGFA + SEMA3A cells (Treated with recombinant VEGFA protein (100 ng/μL) and with recombinant SEMA3A protein (250 ng/μL)). Isotype IgG antibody was used as a negative control of the immunoprecipitation; total cell extracts (TCE) were also analyzed.

cells were co-cultured for 72 h under three conditions: $CD34^+$ MIG VEGFA or $CD34^+$ MIG and HS5 non-transduced cells; HS5 SEMA3A or HS5 Lacz and $CD34^+$ non-transduced cells; and $CD34^+$ MIG VEGFA or $CD34^+$ MIG and HS5 SEMA3A or HS5 Lacz cells. $CD34^+$ non-adherent cells were then harvested and proliferation, apoptosis and colony formation assays were performed.

Ki-67 analysis revealed an increased proliferation of $CD34^+$ cells overexpressing *VEGFA* when co-cultured with HS5 cells (131.8 ± 7.9%; $P = 0.02$) (Fig. 4A). However, co-culture with HS5 SEMA3A cells decreased the proliferation of CD34+ cells compared with HS5 Lacz co-culture (55.05 ± 23.29%; $P = 0.03$) (Fig. 4B). Finally, SEMA3A effects were dominant over VEGFA effects since the co-culture of $CD34^+$ MIG VEGFA and HS5 SEMA3A showed reduced proliferation compared to $CD34^+$ MIG plus HS5 Lacz (52.24 ± 23.77%; $P < 0.001$) or $CD34^+$ MIG VEGFA plus HS5 Lacz co-culture (107.7 ± 2.76% vs 52.54 ± 23.77%; $P < 0.001$) (Fig. 4C).

VEGFA or *SEMA3A* ovexpression did not alter $CD34^+$ colony formation or apoptosis in any of the co-culture conditions (data not shown). These results indicated that in the absence of *SEMA3A*, *VEGFA* overexpression increased proliferation of $CD34^+$ cells co-cultured with HS5 cells. However, in the absence of *VEGFA*, *SEMA3A* overexpression decreased proliferation of $CD34^+$ cells. When we combined *VEGFA* and *SEMA3A* overexpression, the SEMA3A effects were dominant over VEGFA.

3.4. Preferential association of NRP1 receptor with SEMA3A over VEGFA

To investigate whether the dominant SEMA3A effect over VEGFA could be due to competition for the NRP1 receptor, we performed immunoprecipitation assay using antibody anti-NRP1 receptor followed by immunoblotting of anti-VEGFR-2 (VEGFA receptor) and anti-Plexin-A4 (SEMA3A coreceptor). The protein was collected from KG1 and HS5 cells after co-culture and treated under these conditions: Control (No recombinant protein), VEGFA Recombinant (100 ng/μL), SEMA3A Recombinant (250 ng/μL) and both recombinant proteins. Immunoblotting revealed that, in the combined presence of VEGFA and SEMA3A recombinant proteins, there was a greater association of NRP1 with Plexin-A4 (SEMA3A coreceptor) than with VEGFR-2 (VEGFA receptor), probably favoring SEMA3A signaling (Fig. 5). These results suggest that the dominant effect of SEMA3A could be over preferential association with NRP1 receptor.

4. Discussion

The importance of bone marrow microenvironment in leukemogenesis becomes more evident each day. Kim and colleagues described the ability of leukemic stem cells to reset the communication between hematopoietic stem cells and the bone marrow microenvironment, inducing leukemogenesis (Kim et al., 2015). With the aim of better understanding this interaction, we investigated the relationship between VEGFA and SEMA3A in AML and MDS bone marrows. We found a significant increase of *VEGFA* in $CD34^+$ cells of *de novo* AML bone marrows. Moreover, *SEMA3A* was significantly increased in BMSC of AML and MDS samples, compared to healthy controls. *VEGFA* increase has been described in several types of cancer and correlates with worse prognosis in solid tumors (Ferrara, 2004).

The increase of *VEGF* mRNA, plasma levels and protein expression in hematopoietic diseases has been reported and may be correlated with increased angiogenesis and microvascular density (MVD) in bone marrows of AML patients (Pruneri et al., 1999; Aguayo et al., 2000; Hussong et al., 2000; de Bont et al., 2001; Song et al., 2015). Increased VEGFA plasma levels (Aguayo et al., 2002; Aguayo et al., 2000; Lee et al., 2007; Brunner et al., 2002) and mRNA in mononuclear cells from AML bone marrows has also been described (Fiedler et al., 1997; Hussong et al., 2000; de Bont et al., 2001; Mourah et al., 2009); however, to our knowledge, no data has been reported regarding *VEGFA* in $CD34^+$ cells. In our study, we found no difference between *VEGFA* mRNA expression in $CD34^+$ cells from MDS patients and healthy donors. The disclosed data regarding *VEGFA* and MDS patients is controversial. Some authors described a gradual increase of VEGFA plasma levels and MVD according to MDS progression (Pruneri et al., 1999; Aguayo et al., 2000), however these results have not yet been confirmed (Zorat et al., 2001; Madry et al., 2007).

To investigate how VEGF increase influences leukemic cells, we overexpressed *VEGFA* in KG1 leukemic cell line, which is $CD34^+$ positive (Morimoto et al., 1994), and in $CD34^+$ cells isolated from umbilical cord blood and performed proliferation, viability, apoptosis and colony formation assays. *VEGFA* overexpression increased KG1 and $CD34^+$ cells viability and proliferation. Our data are in accordance with the results of List and colleagues that described increased proliferation of KG1 cells after treatment with VEGF recombinant protein (List et al., 2004). They also reported an unexplained increase in apoptosis rates of KG1 cells treated with VEGFA. However, our study did not find differences in the apoptosis rates of KG1 VEGFA and KG1 Lacz cells, confirming that the increased viability herein described may be related to increased proliferation. Further corroborating our data, Xu and colleagues described increased viability in leukemic cell line HL-60 after *VEGFA* overexpression, despite having also reported decreased apoptosis and increased colony formation (Xu et al., 2003). Thus, we suggest that increased VEGFA expression confers advantages for leukemic cells that become more capable of proliferating over normal hematopoietic cells.

Increased *VEGFA* expression was found only in $CD34^+$ cells from *de novo* AML patients; this result is in agreement with the "paracrine-autocrine loop" theory suggested by some authors (Dias et al., 2000; Casella et al., 2003; Kampen et al., 2013). Leukemic cells are believed to produce and secrete VEGFA and in a paracrine manner, stimulate BMSCs from bone marrow microenvironment to produce more VEGFA, other cytokines, and growth factors, therefore benefiting leukemic cells in an autocrine manner (Dias et al., 2000; Casella et al., 2003; Kampen et al., 2013). Our group investigated *VEGFA* gene expression in BMSCs from low and high risk MDS, secondary and *de novo* AML bone marrows and found increased *VEGFA* expression in BMSCs from *de novo* AML patients compared to the control group (unpublished observations) corroborating the "paracrine-autocrine loop" theory.

Herein, we observed increased *SEMA3A* gene expression in BMSCs of all patients compared to the control group. In the literature, we found two studies of *SEMA3A* in AML: The first reported that epigenetic changes increases *SEMA3A* expression and contributes to EZH2 silencing in patients with secondary AML (Yang et al., 2015). The second described lower expression of *SEMA3A* in nonadherent cells from bone marrow stroma of AML patients compared to the control group (Wang et al., 2005). Thus, not much is known of the role of SEMA3A in the bone marrow microenvironment and more studies are needed to affirm SEMA3A involvement in the pathogenesis of MDS and AML.

In order to investigate VEGFA and SEMA3A effects in leukemic cells, we proceeded to co-culture of HS5 stroma cells overexpressing *SEMA3A* and KG1 leukemic cells or $CD34^+$ cells overexpressing *VEGFA*. The *VEGFA* overexpression increased proliferation and clonogenicity of KG1 cells and increased $CD34^+$ cell proliferation; however, had no influence in apoptosis. On the other hand, co-culture of KG1 or $CD34^+$ cells with HS5 overexpressing *SEMA3A* decreased KG1 proliferation and clonogenicity and decreased $CD34^+$ cell proliferation. These data are consistent with the anti-angiogenic effects of SEMA3A described in solid tumors (Tamagnone, 2012; Vacca et al., 2006). When we combined KG1 cells overexpressing *VEGFA* and HS5 cells overexpressing *SEMA3A*, the effect of SEMA3A was dominant over VEGFA, causing decreased proliferation and colony formation (Tamagnone, 2012; Vacca et al., 2006). In co-culture of KG1 cells overexpressing VEGFA and HS27 cells overexpressing *SEMA3A*, the effect of SEMA3A was also dominant over VEGFA, causing decreased KG1 proliferation. In co-culture assays using normal $CD34^+$ cells, the SEMA3A effect was also dominant and caused decreased $CD34^+$ cell proliferation.

In contrast to KG1 cells, *VEGFA* overexpression did not increase the colony formation of $CD34^+$ cells isolated from umbilical cord blood. However, increased clonogenicity of bone marrow mononuclear cells from high-risk MDS patients, after treatment with recombinant VEGF, has been previously described (Bellamy et al., 2001). These discrepant results may be related to different signaling pathways activated in normal and leukemic cells. List and colleagues reported increased colony formation of KG1 cells dependent on the PI3K/Akt pathway and independent of ERK (List et al., 2004). Diversely, Koistinen et al. described increased clonogenicity of the leukemic line OCI/AML-2-dependent on nitric oxide (Koistinen et al., 2001). Leukemic cells have frequent mutations that may contribute to dysregulation of signaling pathways (van Gisbergen et al., 2015), and are not present in $CD34^+$ normal cells, therefore, we hypothesized that leukemic cells are more committed and increased *VEGFA* may be enough to stimulate clonal growth. On the other hand, normal $CD34^+$ cells may be more effective in controlling mechanisms of clonogenicity induced by *VEGFA* overexpression.

Thus, our results suggest that the model proposed for solid tumors, wherein SEMA3A inhibits the effects caused by VEGFA (Chekhonin et al., 2013; Bender & Mac Gabhann, 2015) also applies to AML. The overexpression of *VEGFA* confers advantages to leukemic cells, by increasing their proliferation capacity, and SEMA3A inhibits these effects, preventing the uncontrolled proliferation of leukemic cells. The mechanism mediating this interaction has not yet been elucidated, however there is speculation that this could be through the competition between VEGFA and SEMA3A for NRP1 receptor binding (Pellet-Many et al., 2008). To investigate this hypothesis, we co-cultivated KG1 and HS5 cells treated or not with VEGFA and SEMA3A recombinant proteins and obtained protein extracts. We then immunoprecipitated anti-NRP1 receptor and performed immunoblotting with anti-VEGFR2 and anti-Plexin A4 receptor antibodies. Our results indicated that in the presence of the two recombinant proteins, the NRP1 receptor preferentially forms complexes with Plexin A4 rather than VEGFR2. The formation of NRP1 SEMA3Aplexin A4-complex is essential for SEMA3A signaling (Goshima et al., 2012). These data suggest that the dominant effect of SEMA3A may be related to the competition for NRP1 binding.

Herein, we describe increased *VEGFA* and *SEMA3A* expression in bone marrow cells from *de novo* AML patients and the possible dominant effect of SEMA3A, suggesting that SEMA3A could control the effects caused by VEGFA. However, some questions remain: Why is *SEMA3A* expression increased in BMSCs from MDS and secondary AML patients? If SEMA3A inhibits the effects caused by VEGFA, why do patients still exhibit uncontrolled proliferation of leukemic blasts?

Little is known regarding the effect of SEMA3A in bone marrow microenvironment. Due to their tissue-specific effect, SEMA3A could delay effects caused by other growth factors or could act upon the pathogenesis of myeloid diseases. Considering only VEGFA and SEMA3A interaction in *de novo* AML patient bone marrow, our data suggest that SEMA3A may have a therapeutic role through inhibition of the effects caused by VEGFA. Under physiological conditions, $VEGF_{165}$ and SEMA3A have been suggested to be involved in a negative feedback. In endothelial cells, increased $VEGF_{165}$ expression induces increased *SEMA3A* expression as a part of an angiogenic balance; and this feedback is dysregulated in malignant processes (Vacca et al., 2006; Catalano et al., 2004). Despite these results, we are aware that SEMA3A protein alone is not capable of reversing the clinical condition of patients, as many factors contribute to AML pathogenesis, acting in synergy with VEGFA (Kornblau et al., 2010). VEGFA has been a target of cancer treatment, including AML and many inhibitors of pathways triggered by VEGFA have been produced and are under test in clinical protocols (Garcia-Manero et al., 2015; Gupta et al., 2013). The use of SEMA3A may add new possibilities to improve this treatment. However, although our study provides insights regarding the clinical relevance of SEMA3A and VEGFA in MDS and AML, taking into consideration the heterogeneity of these diseases, any application as a possible treatment would no doubt require additional studies in large and unselected AML patient cohorts, necessary to clarify whether there are exceptional subsets of patients where this effect is weaker or cannot be seen.

5. Conclusion

Our results suggest that *VEGFA* overexpression confers advantages to leukemic cells, by increasing their proliferation, and SEMA3A protein may partially reverse the effects caused by VEGFA. Thus, the combined administration of VEGFA inhibitors and SEMA3A protein may be beneficial for treatment of AML patients.

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Conflict of interest

The authors have no potential conflicts (financial, professional, or personal) that are relevant to the manuscript to disclose.

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