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ORIGINAL ARTICLE

HOXB7 expression by myeloma cells regulates their pro-angiogenic properties in multiple myeloma patients

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The deregulation of the homeobox genes as homeoboxB (HOXB)-7 has been previously associated to tumor progression and angiogenesis; here we investigated the potential role of *HOXB7* in the pro-angiogenic properties of multiple myeloma (MM) cells. We found that HOXB7 was expressed in 10 out of 22 MM patients analyzed at the diagnosis related to high bone marrow angiogenesis and overexpressed in about 40% of myeloma cell lines compared with normal plasma cells. Enforced HOXB7 expression in MM cells by a lentiviral vector significantly modified their transcriptional and angiogenic profile, checked by combined microarray and angiogenesis PCR analyses, upregulating *VEGFA*, *FGF2*, *MMP2*, *WNT5a* and *PDGFA* and downregulating thrombospoindin-2. The pro- and anti-angiogenic HOXB7-related gene signature was also validated in a large independent dataset of MM patients. Accordingly, MM-induced vessel formation was significantly increased by HOXB7 overexpression both *in vitro* angiogenic and chorioallantoic membrane assays, as well as the HOXB7 silencing by small interfering RNA inhibited the production of angiogenic factors, and the pro-angiogenic properties of MM cells. Finally, in SCID-NOD mice we confirmed that HOXB7 overexpression by MM cells stimulated tumor growth, increased MM-associated angiogenesis and the expression of pro-angiogenic genes by microarray analysis supporting the critical role of HOXB7 in the angiogenic switch in MM.
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Introduction

Bone marrow (BM) angiogenesis is typically increased in multiple myeloma (MM) patients in relationship with disease progression being involved in the support of MM cell growth and survival.[1–7] MM-induced angiogenic switch is mainly sustained by the overproduction of pro-angiogenic factors by MM cells or the BM microenvironment[2,8] as the vascular endothelial growth factors (VEGF),[2,8–10] basic fibroblast growth factor (bFGF),[11] interleukin-8 (IL-8),[12] angiopoietin-1,[13] and osteopontin (OPN).[14] Other factors secreted by MM cells may contribute to the angiogenic process including the matrix metalloproteinases (MMPs),[1,15,16] particularly MMP-2, that has been correlated with MM disease progression.[1]

However, the molecular mechanisms involved in the regulation of the angiogenic switch and in the production of the pro-angiogenic molecules by MM cells are not completely known being still under investigation.

The deregulation of the homeoboxB (HOXB) genes has been previously correlated to the angiogenic process, as well as to tumoral-induced neoangiogenesis and tumor progression in solid cancers.[17–22] Particularly, the overexpression of the homeobox HOXB7 has been associated to tumor-related angiogenic switch, cell proliferation and the production of bFGF by breast cancer and melanoma cells.[18–22] In addition, it has been reported that HOXB7 overexpression may also regulate VEGF, IL-8 and Ang-2 by tumor cells.[18] Interestingly, we have recently shown that *HOXB7* is overexpressed in BM microenvironment cells of MM patients as compared with healthy subjects,[23] suggesting its potential role in MM pathophysiology.

Based on all the evidences, to clarify the molecular mechanisms underlying the angiogenic process in MM, in this study we have investigated the expression of *HOXB7* by malignant plasma cells (PCs) and its potential role in the production of pro-angiogenic factors as well as in MM-induced angiogenesis *in vitro* and *in vivo*.

Materials and methods

$CD138^+$ PCs purification from MM patients and controls

$CD138^+$ PC were purified by BM aspirates (5 ml) obtained from iliac crest of 22 consecutive patients (median age 66 years; range 53–73) with newly diagnosed MM (ISS stage I–III) by an immuno-magnetic method using anti-CD138 monoclonal antibody (Ab)-coated microbeads (MACS, Miltenyi Biotec, Bergisch-Gladbach, Germany). Normal PCs were isolated from both reactive tonsils and BM aspirates from healthy subjects undergone to hip surgery after informed consent according to the Declaration of Helsinki. Approval was obtained from the Institutional Reviewer Board of our Hospital. Only samples

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with purity >of 90% checked by flow cytometry (FACScan, Becton Dickinson; San Jose, CA, USA) were tested.

Cell culture conditions

Cell lines. Human myeloma cell lines (HMCLs) were obtained: RPMI-8226 from DSMZ collection (Braunschweig, Germany), JJN3 were kindly provided by Dr Bataille Regis (INSERM, Nantes, France); KMS-27, KMS12 and KMS-20 from Dr Otsuki (Medical School, Okayama, Japan).

HOXB7 overexpression by lentiviral vectors. The amplified HOXB7 complementary DNA sequence was cloned into the pWPI lentiviral vector (kindly provided by Dr Trono, National Center for Competence in Research, Switzerland). Recombinant lentivirus was produced by transient transfection of 293T cells following standard protocol.[24] The transduction efficiency of JJN3 was evaluated by green fluorescence protein expression using flow cytometry whereas the efficacy assessed by the evaluation of HOXB7 messenger RNA (mRNA) and protein expression.

Small interfering RNA (siRNA) transfection. The KMS20 cells were mixed either with 2 nmol of siRNA against *HOXB7* (*si*HOXB7*)* or with a non-specific control siRNA (*si*untarg, Dharmacon Tech, Lafayette, CO, USA) and electroporated using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA) following a single-pulse protocol. 24–48 h (h) after siRNA transfection, cellular pellets were obtained for mRNA and protein extraction and conditioned media stored at −20 °C for further analysis.

Cell proliferation and viability. Measurement of cell viability and DNA synthesis was performed by MTT cell proliferation assay (Cayman Chemical, Ann Arbor, MI, USA) and by tritiated thymidine (Biocompare, South San Francisco, CA, USA) uptake in 96-well microtiter plates, respectively.

Angiogenesis in vitro *and* ex vivo *assays*

In vitro *angiogenesis assay*. Angio-kit was used to test the *in vitro* pro-angiogenic properties of the samples, according to the manufacturer's protocol (TCS Biologicals Buckingham, UK). Briefly, endothelial-like cells were stimulated with VEGF (2 ng/ml, positive control) or suramin (20 μM, negative control), or conditioned medium (CM) of JJN3 cells infected with pWPI empty vector (JJN3-pWPI) or HOXB7 vector (JJN3-HOXB7)/ D-MEM at 10% fetal bovine serum (ratio 1:3), or with CM of KMS20 untarg, or KMS20 siHOXB7/D-MEM at 10% fetal bovine serum (ratio 1:3). At day 11, the cells were fixed and stained using an anti-hCD31 Ab and capillaries formation measured according to the instructions provided (TCS Biologicals).

Chorioallantoic membrane (CAM) assay. Fertilized White Leghorn chicken eggs (20 per group) were treated with: 1 mm^3 sterilized gelatin sponges (Gelfoam Upjohn, Kalamazoo, MI, USA) placed on top of the growing CAM, as previously described[25] and loaded with 1 μl of phosphate-buffered saline (negative control); 1 μl of phosphate-buffered saline with 250 ng VEGF (R&D Systems, Minneapolis, MN, USA) as positive control; 1 μl of CM of JJN3-pWPI 1 μl of CM of JJN3-HOXB7. The supernatants were tested in triplicate.

Mice study

SCID-NOD mice aged 4–6 weeks (Harlan Laboratories, Udine, Italy) were housed under specific pathogen-free conditions. All procedures involving animals were performed in the respect of the National and International current regulations (D.l.vo 116/1992, European Economic Community Council Directive 86/609, OJL 358, 1987). Two groups of 10 animals each were injected sub-cutaneously with 5×10^6 JJN3 cells stably infected with a lentivirus vector carrying HOXB7 (JJN3-HOXB7), or with JJN3 stably infected with an empty vector (JJN3-pWPI). At 23 days after tumor cell inoculation, mice were killed and autopsies were carried out. Tumoral masses were measured as previously described.[26] Maximum length and width of the tumoral masses were measured with a caliper, and tumor volume (mm^3) was calculated according to the following formula: $0.523 \times \text{length} \times \text{width}^2$. Tissue samples and cell extracts of the masses were obtained for immunohistochemical staining and for RNA and protein extraction, respectively.

Gene expression profiling analysis

The gene expression profile of *HOXB7* was evaluated in the proprietary databases of highly purified $CD138^+$ PC samples from four healthy donors, 11 MGUS, 133 MM, 9 PC leukemia and 23 HMCLs samples profiled on the GeneChip HG-U133A arrays (Affymetrix, Santa Clara, CA, USA) as previously described.[27,28] The CEL files are deposited at the National Center for Biotechnology Information's Gene Expression Omnibus and available through GEO Series accession numbers GSE13591[27] and GSE6205.[28]

The transcriptional profiles of samples from infected HMCLs and tumoral masses in mice were generated on GeneChip HG-U133 Plus 2.0 arrays according to the standard manufacturer's preparation and hybridization protocols. Data were extracted from CEL files and converted to normalized signals using conventional GCOS1.1 scaling. The differential expression of transcripts was tested using Significant Analysis of Microarrays software version 3.0.2 as previously described.[28] NetAffx (https://www.affymetrix.com/analysis/netaffx/), DAVID 2008 (http://david.abcc.ncifcrf.gov) and Gene Set Enrichment Analysis (GSEA, http://www.broad.mit.edu/gsea) tools were used for the functional annotation study of the selected lists.

RNA isolation and reverse transcriptase PCR amplification

For reverse transcriptase PCR analysis, total cellular RNA was extracted from cells using RNeasy mini kit (Qiagen, Milan, Italy). A volume of 1 μg of RNA was reverse-transcribed using the Superscript RnaseH-reverse trascriptase kit according to the manufacturer's protocol (Invitrogen, Milan, Italy).

Complementary DNAs were amplified by PCR with specific primer pairs. PCR reactions were performed in a thermal cycler (MiniCycler MyResearch, Watertown, MA, USA) for 30 cycles. The following specific primer pairs have been used:

- *HOXB7:* F: 5′-AGAGTAACTTCCGGATCTA-3′; R: 5′-TCTGC TTCAGCCCTGTCTT-3′
- *VEGFA*: F: 5′-CGAAGTGGTGAAGTTCATGGATG-3′; R: 5′-T TCTGTTCAGTCTTTCCTGGTGAG-3′
- *FGF2:* F: 5′-GGCTTCTTCCTGCGCATCCAC-3′; R:3′-GGTAA CGGTTAGCACACTCCTTT-3′
- *MMP2:* F: 5′-TGGGCAACAAATATGAGAGAG-3′; R:5′-CGG CATCCAGGTTATCGGGG-3′
- *PDGFA*: F: 5′-CCCCTGCCCATTCGGAGGAAGAGA-3′; R: 5′-TTGGCCACCTTGACGCTGCGGTG-3′

- *TSP2*: F: 5′-GCAACATCAACCGCAAGAC-3′; R: 5′-AAGCA AACCCCTGAAGTGACT-3′
- *WNT5a:* F: 5′-GAGTTCGTGGACGCCCGCGA-3′; R:5′-GGC TCATGGCGTTCACCAC-3′
- *GAPDH*: F: 5′-CAACGGATTTGGTCGTATTG-3′; R:5′-GGA AGATGGTGATGGGATTT-3′

Annealing temperature. *HOXB7*: 58 °C; *VEGFA*: 56 °C; *FGF2*: 57 °C; *MMP2*: 55 °C; *PDGFA*: 56 °C; thrombospoindin-2 (*TSP2*): 59 °C; *WNT5a*: 69 °C; GAPDH: 58 °C.

Product size. *HOXB7*: 274bp; *VEGFA*: 375 bp; *FGF2*: 136 bp; *MMP2:* 750 bp; *PDGFA*: 228bp; *TSP2*: 541 bp; *WNT5a*: bp; *GAPDH:* 209 bp.

Pictures of the electrophoresed complementary DNAs were recorded with a digital DC 120 Kodak camera and quantified.

Real time quantitative PCR and angiogenesis PCR array

Real time PCR was performed by adding 2 out of 20 μl of complementary DNA, obtained from 1 μg of RNA, to an universal master Mix primers and TaqMan probes (Applied Biosystem, Applera, Milan, Italy).

The following TaqMan Gene Expression Assays were used: Hs00270131_m1 (*HOXB7*), Hs00173626_m1 (*VEGFA*), Hs00266645_m1 (*FGF2*), Hs00167093_m1 (OPN), Hs00998537_m1 (*WNT5a*) and Hs01104728_m1 (*ABL*) according to the manufacturer's protocols. For *IL8* and *ANG1* the sequences of the primers and probes used were as follows:

- *IL8*: F: 5′-CTCTTGGCAGCCTTCCTGATT-3′; R: 5′-TATGCAC TGACATCTAAGTTCTTTAGCA-3′; probe: 6-FAM-CTTGGCA AAACTGCACCTTCACACAGA-MGB
- *ANG1*: F: 5′-GCAACTGGAGCTGATGGACACA-3′; R: 5′-CA TCTGCACAGTCTCTAAATGGT-3′; probe: 6-FAM-CAATCTT TGCACTAAAGAAGGTGTTTTACT-MGB

To normalize for differences in RNA quality and reverse transcription efficiency, we applied the comparative *C*t method using the endogenous reference gene *ABL*. The relative HOXB7 mRNA quantification was performed by the Δ*C*t method ($\Delta Ct = \text{mean}C_{T\ HOXB7} - \text{mean}C_{T\ ABL}$). Δ*C*t was evaluated as the difference between the Δ*C*t of a sample and the Δ*C*t of the control. The fold change in HOXB7 mRNA expression (n-fold) was calculated as $2^{-\Delta\Delta Ct}$. The same procedures were used for the other genes analyzed.

The expression level of the pro-angiogenic molecules was evaluated on mRNA extracted from both HMCLs and tumors removed from SCID-NOD mice by Human Angiogenesis RT^2 Profiler PCR Array and RT^2 Real-Timer SyBR Green/ROX PCR Mix (PAHS-024, Superarray, SABiosciences, Frederick, MD) that profiles the expression of 84 key genes involved in modulating the biological processes of angiogenesis.

Western blot analysis and angiogenesis antibody array

Nuclear extracts were prepared using the Nuclear Extraction kit (Active Motive, Vinci Biochem, Italy) according to the manufacturer's protocol. A volume of 40 μg of nuclear extracts and 70 μg of cytosolic extracts were electrotransferred onto PVDF filters (Bio-Rad Laboratories S.r.l. Segrate (MI), Italy) after sodium dodecyl sulphate polyacrylamide gel electrophoresis. After blocking, filters were probed with specific antibodies overnight at 4 °C. The following antibodies were used as primary Abs: polyclonal rabbit anti-HOXB7 Ab. (Zymed Laboratories, Invitrogen; dilution: 1:125), polyclonal goat anti-bFGF Ab. (R&D Systems; dilution: 1:250), anti-VEGF monoclonal Ab (R&D; dilution: 1:250) and anti-HIF-1α (R&D; dilution 1:250). Anti-histone H1 monoclonal Ab (Millipore, Billerica, MA, USA; dilution 1:500) and anti-βactin monoclonalAb (Sigma-Aldrich, Milan, Italy; dilution: 1:5000) were used as internal controls. Appropriate secondary antibodies were used as peroxidase conjugated anti-rabbit (Chemicon, Millipore, Billerica, MA, USA), anti-goat (Rockland, Gilbertsville, PA, USA) or anti-mouse immunoglobulin (BD Pharmingen, Franklin Lakes, NJ, USA). Blots were then developed using the ECLplus (Amersham International plc, Buckinghamshire, UK). Immunoreactive bands were visualized by a 5–15 min exposure (Kodak X-OMAT, Milan, Italy).

The expression profile of angiogenesis-related proteins was analyzed by both human angiogenesis array kit (ARY007, R&D Systems) and Quantibody angiogenesis array (RayBiotech Inc. Norcross GA, USA) according to the manufacturer's protocols. The intensity of each band was quantified by ID Image Analysis Software (Kodak).

Enzyme-linked immunosorbent assay assays

Aliquots of 48-h cell CM were tested for soluble VEGF, PDGFA, MMP-2 and TSP2 (R&D Systems) protein levels according to the manufacturer's protocols. Cytokine levels in the CM were normalized to the total protein levels at the end of culture period.

BM angiogenesis and immunohistochemical staining of VEGF, bFGF, CD31 and Ki-67

BM angiogenesis was evaluated on bone biopsies of the 22 MM patients analyzed as previously described.[13,14] Angiogenesis was measured as density of microvessels by staining endothelial cells using anti-CD34 monoclonal Ab (working dilution 1:50, clone QBEnd/10 NeoMarkers, Fremont, CA, USA). The mean of vascular density and the mean number of microvessels in each biopsy were expressed on the basis of total number of microvessel transversal section (capillaries and/or venules) per the total cellular area (in mm2) and per the total number of fields (× 400 magnification), respectively.[13,14]

Tissue samples obtained from tumors removed from SCID-NOD mice colonized by JJN3-pWPI and JJN3-HOXB7 (10 mouse for each group) were fixed in 10% neutral-buffered formalin, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin. In addition, fixed sections were immuno-stained with mouse anti-human VEGF (R&D; dilution 1:20) or biotinylated goat anti-human bFGF (R&D; dilution 1:20) or with 1:100 diluted mouse anti-Ki67 primary antibody (Clone MIB-1, Dako, Carpinteria, CA, USA) for 30 min. VEGF staining was revealed using the UltraVision LP Large Volume Detection System horseradish peroxidase polymer (Thermo Scientific) whereas bFGF staining by horseradish peroxidase-labeled streptavidin (Kit LSAB2, DakoCytomation Denmark, Glostrup, Denmark) and quantified according to semiquantitative immunohistochemical score.[13] Detection of Ki-67 has been carried out using high-sensitive detection system (Advance-horseradish peroxidase, Dako) and 3,3-diaminobenzidine has been employed as chromogen substrate. Angiogenesis was evaluated on frozen tissues samples obtained from SCID-NOD mice fixed in acetone and treated with rabbit anti-mouse CD31/PECAM-1 (Millipore; 1:1000). After washing sections were incubated with a secondary antibody (Rat anti-IgG horseradish peroxidase; Millipore; 1:250) and reaction revealed with a solution of 3-3′-diaminobenzidine tetrahydrochloride (liquid DAB substrate chromogen system, DAKO). Slides were then incubated.

Results

HOXB7 expression characterizes a fraction of MM primary tumors: relationship with BM angiogenesis

HOXB7 mRNA expression in a proprietary microarray database of highly purified PC from MM primary tumors was first analyzed (Figure 1a). To investigate whether *HOXB7* differential expression could be associated with distinct transcriptional profiles, the MM cases, based on *HOXB7* expression, were ordered and divided into four quartiles. The supervised analysis led to the identification of 168 genes (at a *q*-value = 0), 91 of which upregulated in the fourth quartile (Supplementary Figure 1). Interestingly, among them, we identified both the pro-angiogenic genes *MMP2* and *PDGFA*.

HOXB7 mRNA expression was then evaluated in freshly isolated CD138$^+$ MM cells obtained from 22 MM patients at the diagnosis. The presence of *HOXB7* mRNA transcript was observed in 10 out of 22 CD138$^+$ MM PC analyzed but not in the PC from tonsils or BM of healthy individuals (Table 1). Interestingly, we found that BM angiogenesis was significant higher in patients positive for HOXB7 mRNA as compared with those negative for HOXB7 mRNA (number of microvessels per field: mean ± s.e.: 8.54 ± 0.31 vs 5.78 ± 0.2, median: 8.05 vs 5.2; $P = 0.03$ and MVD: mean ± s.e.: 37.6 ± 1.83 vs 16.05 ± 0.6, median: 34.6 vs 14.5; $P = 0.005$). HOXB7 protein expression was also evaluated and detected at nuclear level in 5 out of 16 MM patients available but not in CD138$^+$ PC from healthy donor (data not shown).

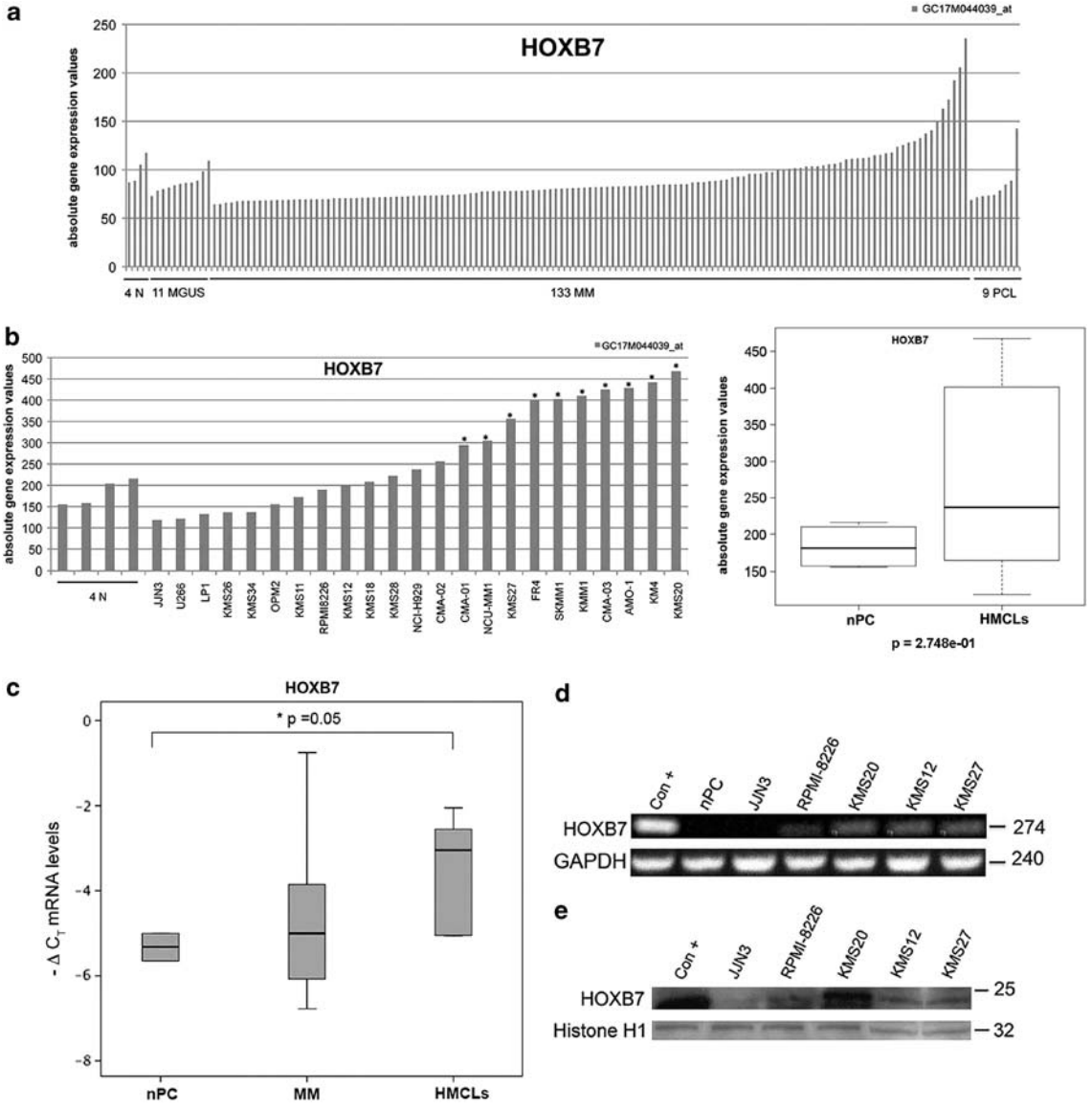

Figure 1 HOXB7 expression by MM cells. Distribution of *HOXB7* expression level profiled on HG-U133A arrays in 133 MM samples, 4 normal (N) plasma cells (PC), 11 MGUS and 9 plasma cell leukemia PCL (**a**) and in human myeloma cell lines (HMCLs) (**b**). Graphs represent the absolute gene expression level of the specific probset GC17M044039_at. *Indicates HMCLs with *HOXB7* expression level more than three standard deviations as compared with BM PCs from healthy subjects. Box plot of HOXB7 mRNA gene expression levels in 4 normal plasmacells (nPC) and 23 HMCLs (**b** on the left). Box blots of *HOXB7* mRNA level in 4 nPC, 20 MM and 5 HMCLs quantified by real time PCR (**c**). *HOXB7* mRNA expression by HMCLs (JJN3, RPMI-8226, KMS12, KMS20 and KMS27) and normal plasma cells (nPC) was evaluated using qualitative PCR (**d**). HOXB7 protein expression was detected in HMCLs at nuclear level by westernblot analysis. (Con+, positive control—HOXB7-transduced cells) (**e**).

Table 1 HOXB7 mRNA expression and BM angiogenesis in MM patients

Samples	*ISS*	*Type*	*HOXB7 mRNA*	*BM MVD*	*BM no. of vessels*
Healthy tonsil CD138+	/	/	–	/	/
Healthy tonsil CD138+	/	/	–	/	/
Healthy tonsil CD138+	/	/	–	/	/
Healthy tonsil CD138+	/	/	–	/	/
Healthy BM CD138+	/	/	–	/	/
MM1	I	κ	+	25.75	8.08
MM2	III	κ	+	16.52	5.78
MM3	I	κ	–	17.93	10.5
MM4	III	λ	–	41.73	9.56
MM5	III	κ	–	29.92	6.06
MM6	III	λ	+	21.18	5.57
MM7	III	κ	+	63.01	14.61
MM8	III	κ	+	34.66	8.05
MM9	III	κ	+	35.74	7.96
MM10	III	κ	–	17.4	3.0
MM11	III	λ	–	49.17	8.06
MM12	II	κ	–	12.8	5.40
MM13	I	λ	–	30.89	7.13
MM14	I	λ	–	14.9	3.49
MM15	II	κ	–	14.2	2.94
MM16	I	κ	+	63.54	7.69
MM17	I	κ	–	9.58	3.59
MM18	I	λ	–	16.38	5.15
MM19	II	λ	+	50.99	12.47
MM20	II	κ	–	11.67	4.53
MM21	I	λ	+	34.69	6.11
MM22	II	κ	+	30.99	9.08

MVD, microvascular density; no. of vessels, total number of vessels for field.

HOXB7 *is overexpressed by HMCLs*

Thereafter, *HOXB7* mRNA expression was analyzed in a proprietary microarray database including 23 HMCLs finding that 10 (43%) of them overexpressed *HOXB7* in comparison with $CD138^+$ samples from healthy donors (Figure 1b). Based on microarray dataset, *HOXB7* mRNA expression was analyzed in a subgroup of HMCLs by both quantitative and qualitative PCR. We confirmed that *HOXB7* mRNA level was significantly higher in HMCLs as compared with normal $CD138^+$ PC whereas the difference between HMCLs and $CD138^+$ MM cells did not reach a statistical significance as well as between normal PC and $CD138^+$ MM cells (Figure 1c). Moreover, among the HMCLs analyzed, we found that RPMI-8266, KMS12, KMS20 and KMS27 expressed *HOXB7* mRNA transcript, whereas JJN3 was negative as well as normal $CD138^+$ PC (Figure 1d). Data were then confirmed at protein level finding that RPMI-8226, KMS12, KMS20 and KMS27 show a constitutive HOXB7 expression at nuclear level, whereas JJN3 did not express HOXB7 (Figure 1e).

Effect of HOXB7 *overexpression on whole transcriptional and angiogenic profiles of MM cells: upregulation of pro-angiogenic molecules*

In order to investigate the potential role of HOXB7 on the whole transcriptional and specifically pro-angiogenic profile of MM cells, first we enforced HOXB7 expression by a lentiviral vector in JJN3 cells (JJN3-HOXB7) that do not constitutively express HOXB7. The efficiency of HOXB7 transduction was more that 95% as assessed by green fluorescence protein evaluation by flow cytometry (Figure 2a). Consequently, the efficacy of lentiviral transduction was demonstrated by the significant increase of *HOXB7* mRNA level (median fold change: 450) in JJN3-HOXB7 as compared with JJN3 transduced with the empty vector (JJN3-pWPI) and confirmed by qualitative PCR and at protein level by western blot (Figure 2a, left and middle panel). A slight stimulatory effect on cell proliferation was observed in JJN3-HOXB7 in comparison with JJN3-pWPI (data not shown).

First, the effect of HOXB7 overexpression on the main pro-angiogenic molecule production by MM cells was evaluated. By real time PCR, using the TaqMan Gene Expression Assays, we found that *VEGFA* and *FGF2* were significantly upregulated ($P=0.001$ and $P=0.01$, respectively) but not *OPN*, *IL8* and *ANG1* (Figure 2b). Second, by means of a gene-expression profiling study on JJN3 triplicates we found that 756 genes were significantly modulated by HOXB7 overexpression including chemokine and chemokine receptor genes as *CCL3*, *CCL5*, *CXCL1* and *CCR1*; genes belonging to cell adhesion molecule family as *SDC1* (syndecan1), *CDH1* (E-Cadherin), *CDH2* (N-Cadherin), *CDH11*, *NCAM1* and *CD28* and genes belonging to Janus Kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) and p53 signaling pathways (Supplementary Table 1). Particularly, among the pro-angiogenic genes we found the significant upregulation of *VEGFA*, *MMP2*, *WNT5A*, *PDGFA* and the specific downregulation of the MMP inhibitor *TIMP2* (Table 2 (i) and Supplementary Table 1). These data were in accordance with those observed in primary MM tumors, being the large part of these genes identified at a q-value <0.03 in the previously described supervised analysis. These data were then validated and integrated by PCR (Figures 2c and d, respectively) for *VEGFA*, *FGF2*, *MMP2* and *PDGFA*. Interestingly, using angiogenesis PCR arrays we also found that the angiogenic inhibitor *TSP2* was significantly inhibited. Data were confirmed at protein level by western blot for bFGF (Figure 2e) and enzyme-linked immunosorbent assay for VEGF, MMP-2, PDGF and TSP2 (Figure 2f).

HOXB7 silencing inhibited the production of pro-angiogenic molecules by MM cells

To further evaluate the potential role of HOXB7 in the production of angiogenic molecules by MM cells we performed HOXB7 silencing by siRNA in the KMS20 HMCL that

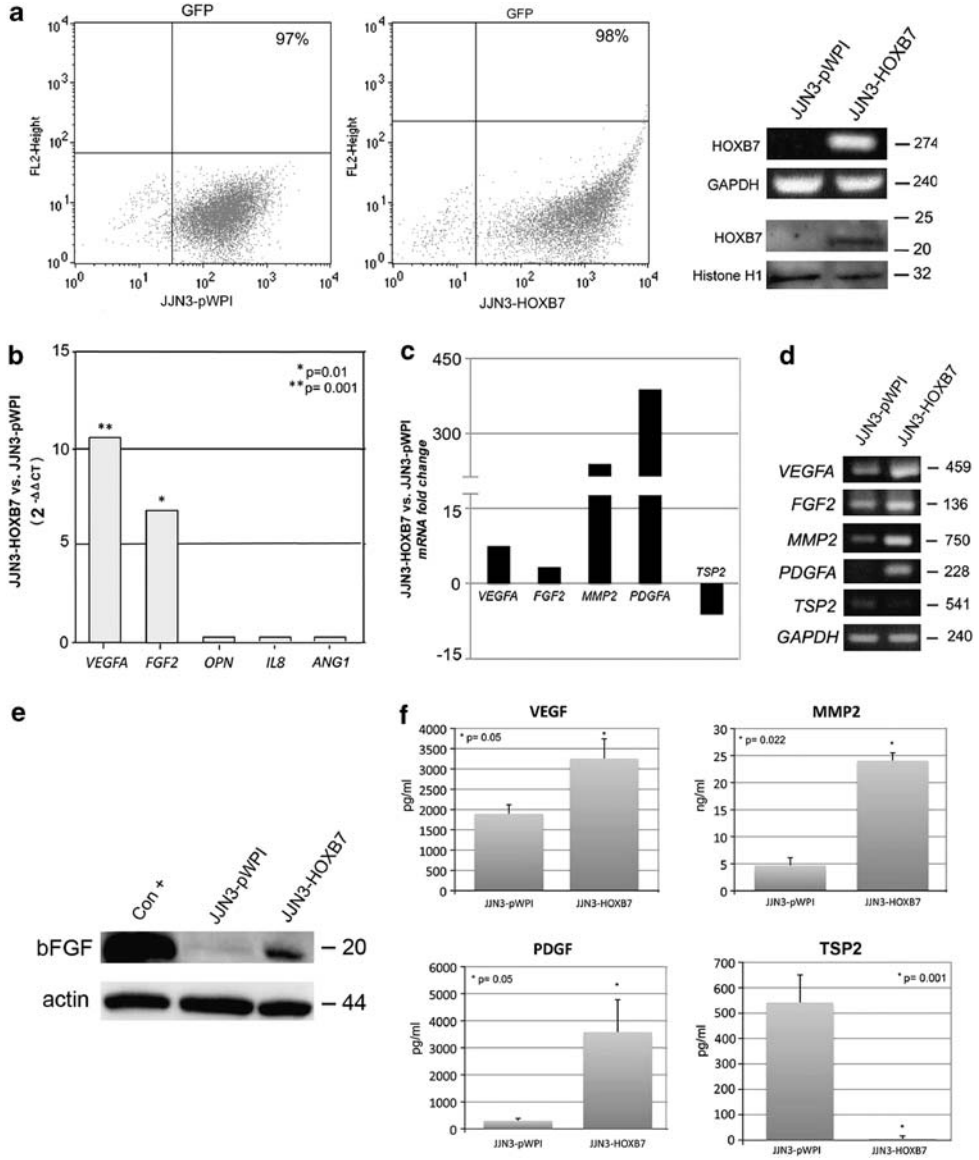

Figure 2 Effect of HOXB7 overexpression in MM cells: upregulation of the pro-angiogenic molecules. JJN3 was infected either with *HOXB7* lentivirus vector (JJN3-HOXB7) or the empty vector (JJN3-pWPI). The efficiency of the transduction was evaluated by GFP evaluation using flow cytometry, whereas the efficacy was assessed evaluating HOXB7 expression by qualitative PCR and western blot (**a**). The expression of *VEGFA*, *FGF2*, *OPN*, *IL-8* and *ANG1* was evaluated at mRNA level by real time PCR by TaqMan Gene Expression Assays in transduced cells. Graphs represent the median mRNA fold expression value ($2^{-(\Delta Ct\ JJN3\text{-}HOXB7\ -\ \Delta Ct\ JJN3)}$) in JJN3-HOXB7 vs JJN3-pWPI assessed in triplicate (**b**). By angiogenesis PCR array using the Real-Timer SyBR Green/ROX PCR Mix, the pro-angiogenic and anti-angiogenic genes were assessed in JJN3-HOXB7 as compared with JJN3-pWPI. Graphs represent the median mRNA fold change of the genes significantly modulated according to the manufacturer's protocol (**c**). The expression of *VEGFA*, *FGF2*, *MMP2*, *PDGFA* and *TSP2* mRNA was evaluated by qualitative PCR in JJN3-HOXB7 and JJN3-pWPI (**d**). bFGF protein expression was evaluated by western blot analysis in JJN3-HOXB7 and JJN3-pWPI. rhbFGF has been used as positive control (Con +) (**e**). Aliquots of CM of JJN3-HOXB7 and JJN3-pWPI were tested for VEGF, MMP-2, PDGF, TSP-2 protein levels by ELISA assay. Graphs and bars represent the mean protein values ± s.d. of three independent experiments performed twice (**f**).

constitutively expresses HOXB7. A significant reduction of HOXB7 transcript level was obtained by siRNA transfection after 24 h (fold change of reduction: KMS20 *si*HOXB7 vs KMS20 *si*untarg: 0.13), confirmed by qualitative PCR (Figure 3a), as well as a dowregulation of its nuclear protein level after 48 h (Figure 3a). A slight reduction on cell viability and proliferation was observed in KMS20 transfected with *si*HOXB7 as compared with *si*untarg (data not shown). HOXB7 silencing significantly reduced *VEGF*, *FGF2* and *TGFB1* mRNA level (Figure 3b), whereas any significant effect was not observed on the other angiogenic molecules (data not shown). Data were then validated by qualitative PCR (Figure 3c) as well as by angiogenesis antibody array showing a significant reduction of VEGF, bFGF and TGFβ protein levels in KMS20 *si*HOXB7 as compared with KMS20 *si*untarg (Figure 3d).

HOXB7 involvement in the pro-angiogenic effect of MM cells

Given that HOXB7 regulates the expression of the pro-angiogenic molecules in HMCLs, the role of HOXB7 modulation on MM-induced endothelial-cell proliferation and survival was

Table 2 Angiogenesis-related genes significantly modulated by HOXB7 overexpression in JJN3 (i) and in JJN3-HOXB7 tumoral mass in mice (ii)

Gene name	*Gene symbol*	*Probe set*	*Fold change*
(i)			
Homeobox B7	*HOXB7*	204778_x_at	7.95
Matrix metallopeptidase 2	*MMP2*	201069_at	15.85
Chemokine (C-C motif) ligand 5	*CCL5*	1405_i_at	15.39
Wingless-type MMTV integration site family, member 5A	*WNT5A*	213425_at	13.73
Platelet-derived growth factor alpha polypeptide	*PDGFA*	205463_s_at	13.38
Vascular endothelial growth factor A	*VEGFA*	212171_x_at	5.88
TIMP metallopeptidase inhibitor 2	*TIMP2*	231579_s_at	−2.20
(ii)			
Homeobox B7	*HOXB7*	204778_x_at	4.27
Fibroblast growth factor receptor 3	*FGFR3*	204379_s_at	31.38
Chemokine (C-C motif) ligand 5	*CCL5*	1405_i_at	6.71
Platelet-derived growth factor alpha polypeptide	*PDGFA*	205463_s_at	4.44
Wingless-type MMTV integration site family, member 5A	*WNT5A*	213425_at	3.86
Transforming growth factor, beta-induced 68 kDa	*TGFB1*	201506_at	2.29
Placental growth factor-2	*PGF*	215179_x_at	2.08
Vascular endothelial growth factor A	*VEGFA*	212171_x_at	2.0
TIMP metallopeptidase inhibitor 2	*TIMP2*	231579_s_at	−2.0

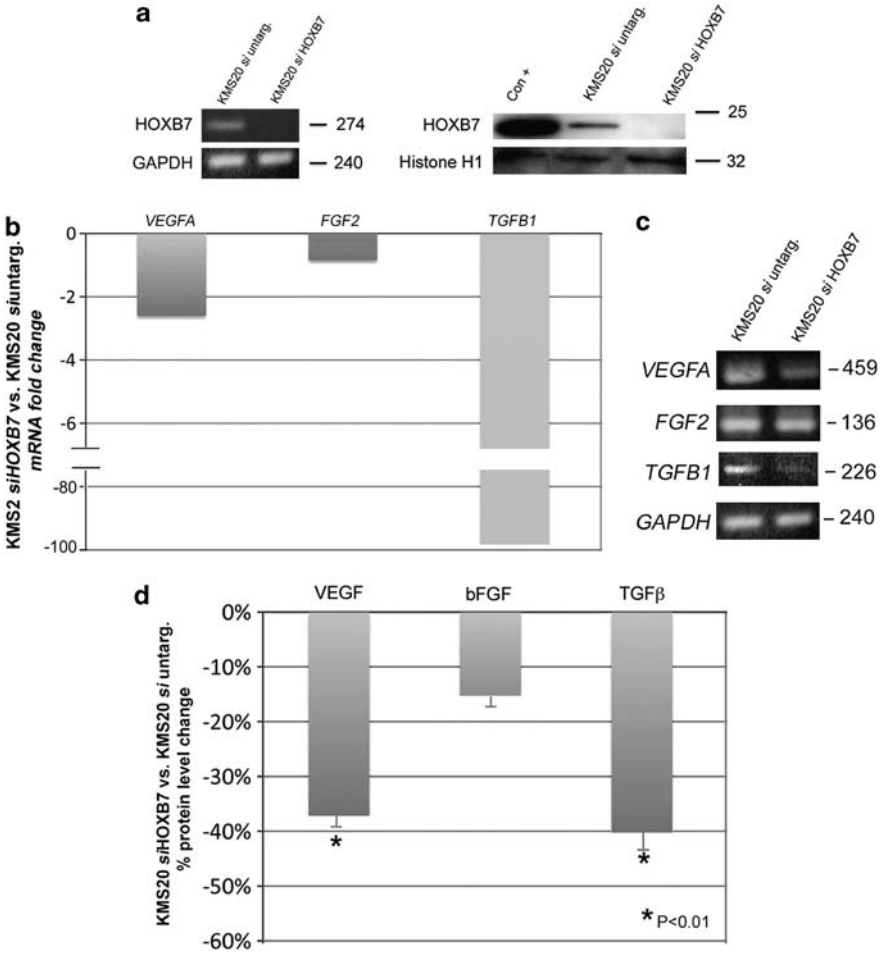

Figure 3 Effect of HOXB7 silencing in MM cells: downregulation of pro-angiogenic molecules. KMS20 were transfected by electroporation with siRNA against HOXB7 (KMS20 *si*HOXB7) or with a non-specific control siRNA (KMS20 *si*untarg). HOXB7 mRNA and protein expression was evaluated by RT–PCR and by westernblot analysis on nuclear extracts (Con+, positive control—JJN3-HOXB7-transduced cells), respectively (**a**). Angiogenic genes significantly modulated were assessed in KMS20 *si*HOXB7 as compared with KMS20 *si*untarg by angiogenesis PCR array. Graphs represent the median mRNA fold change of *VEGFA, FGF2* and *TGFβ1* (**b**). mRNA expression of these molecules was evaluated by qualitative PCR in KMS20 *si*HOXB7 and KMS20 *si*untarg (**c**). Graphs and bars represent the mean % ± s.d. of protein level change of VEGF, FGF2 and TGFβ in *si*HOXB7 as compared with KMS20 *si*untarg cells checked by angiogenesis antibody array (**d**).

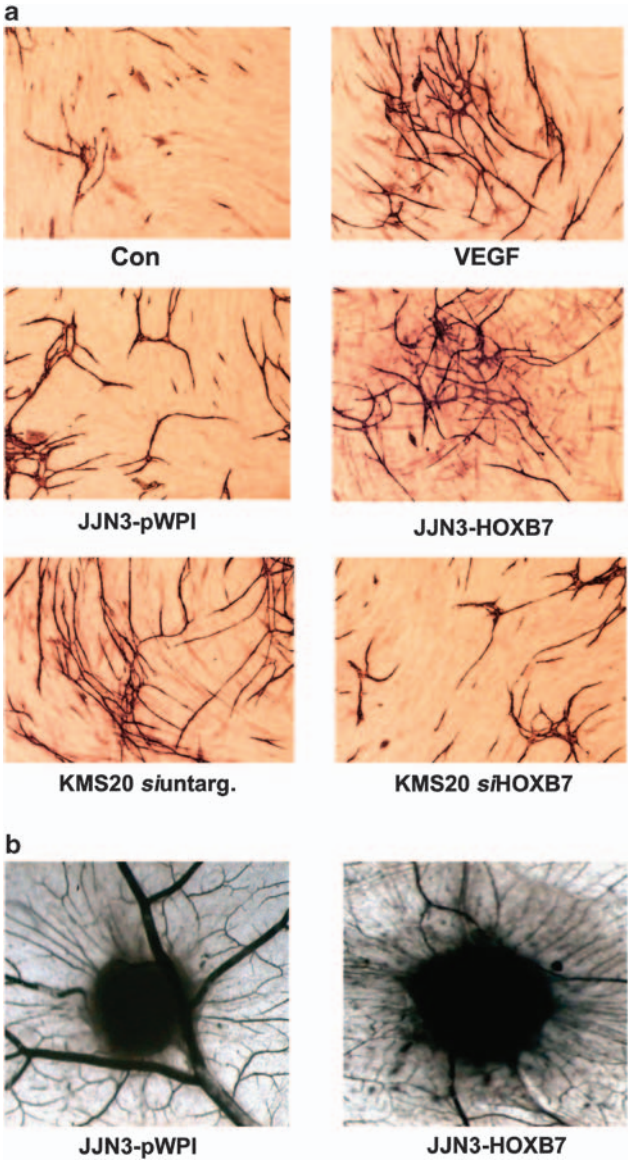

Figure 4 HOXB7 overexpression by MM cells increases their pro-angiogenic properties. Endothelial-like cells were incubated with DMEM medium at 10% FBS (Con) or with DMEM medium at 10% FBS plus VEGF or with CM of JJN3-pWPI or JJN3-HOXB7/D-MEM at 10% FBS or KMS20 *si*untarg or KMS20 *si*HOXB7 (dilution 1:3). (Original magnification $\times 10$) (**a**). Fertilized white leghorn chicken eggs were treated with 1 mm^3 sterilized gelatin sponges placed on top of the growing CAMs and loaded with 1 μl of CM of JJN3-pWPI or 1 μl of CM of JJN3-HOXB7. CAMs were photographed with a camera and image analyzer system (Olympus Italia, Italy) (**b**).

then tested using two different angiogenesis assays as Angiokit and CAM assays. First, we found that CM of JJN3-HOXB7 significantly stimulated vessel formation as compared with JJN3-pWPI (JJN3-HOXB7 vs JJN3-pWPI: number of capillar junctions: mean ± s.d.: 18.5 ± 1.9 vs 10 ± 1, $P=0.01$; number of tubules: 76.5 ± 4 vs 55 ± 3.9, $P<0.01$; tubule length: 2542 ± 108 mm vs 1836 ± 150 mm, $P<0.01$) (Figure 4a). VEGF treatment was used as positive control of the system. (VEGF vs vehicle (Con): number of capillary junctions: 22 ± 0.8 vs 6 ± 1.12, $P=0.001$; number of tubules: 90.2 ± 1.17 vs 40 ± 3.9, $P=0.001$; tubule length: 3000 ± 78 mm vs 1036 ± 150 mm, $P=0.001$).

This observation was confirmed in the CAM assay finding that the angiogenic response induced by the JJN3-HOXB7 was significantly higher as compared with JJN3-pWPI (mean number of vessels = 20 ± 4 vs 2 ± 2, $P<0.001$; VEGF = 24 ± 4; Con = 7 ± 2) (Figure 4b).

Second, to demonstrate unambiguously that HOXB7 is a key molecule in the regulation of the angiogenic properties of MM cells, we tested the effect of HOXB7 silencing on the angiogenic response of MM cells by the Angiokit. We found that CM of KMS20 *si*HOXB7 induced a significantly lower pro-angiogenic effect as compared with KMS20 *si*untarg. (capillary junctions: 11 ± 0.96 vs 21 ± 0.8; tubules: 50.75 ± 1.23 vs 85.5 ± 4.6; tubule length: 1862 ± 57.78 mm vs 2833 ± 122 mm, $P<0.01$) (Figure 4a).

HOXB7 overexpression stimulated MM-induced angiogenesis in vivo *in SCID-NOD mice*

Next, the effect of HOXB7 overexpression by MM cells was investigated *in vivo* in SCID-NOD mice. Two groups of ten animals were injected sub-cutaneously with JJN3-HOXB7 or JJN3-pWPI and killed after 23 days. By the end of the follow-up period, all mice developed tumors that grew in the site of injection, in the absence of metastases at distant sites. Mice injected with the JJN3-HOXB7 cells developed tumors significantly bigger than mice inoculated with the JJN3-pWPI (JJN3-HOXB7 tumors, mean volume 214 mm^3; range 142–2063 mm^3; JJN3-pWPI tumors, mean volume 134 mm^3; range 3–718 mm^3, $P=0.0039$) (Figure 5a). However, we failed to find a significant difference in Ki-67 immunohistochemical staining in the two groups of tumors analyzed (data not shown). Histological samples were then both frozen and paraffin-embedded to stain CD31 and VEGF. We found that the number of vessels for field (400 ×) positive for CD31 was significantly higher in JJN3-HOXB7 tumoral masses as compared with JJN3-pWPI (median value: 4.50 vs 3.20; $P=0.05$) (Figure 5b panel *i*). Consistently we found that VEGF immunostaining was at higher intensity in JJN3-HOXB7 tumoral masses (immunohistochemical score: ++/+++) vs JJN3-pWPI (−/+) (Figure 5b panel *ii*).

The overexpression of HOXB7 in JJN3-HOXB7 tumoral masses as compared with JJN3-pWPI was checked by real time PCR (HOXB7 vs pWPI $2^{-\Delta\Delta CT}$: 500) and by western blot (data not shown). Following, in order to investigate whether *HOXB7* overxpression may also affect *in vivo* the transcriptional (and specifically the pro-angiogenic) profile of MM, we performed microarray analysis on cellular pellets from both JJN3-HOXB7 and JJN3-pWPI tumoral masses. The supervised analysis revealed 631 differentially expressed genes including genes belonging to cell adhesion molecules and focal adhesion factors as *CDH2*, *NCAM*, *CD28*, *CTNNB1* (beta-catenin) and *CCL3* (Supplementary Table 2) or to angiogenesis as *VEGFA*, *PGF*, *PDGFA*, *WNT5A*, *TGFB1*, *CCL5*, *TIMP2* and *FGFR3* (Table 2 (ii) and Supplementary Table 2). Data obtained by microarrays were then validated by real time PCR on selected genes (HOXB7 vs pWPI $2^{-\Delta\Delta CT}$ for *VEGFA*: 8.2; *FGF2*: 3.2; *WNT5a*: 5.0) and integrated by angiogenesis PCR array showing the upregulation of *VEGFA*, *VEGFC*, *PGF*, *FGF2*, *FGFR3*, *MMP2*, *PDGFA* and *CD31* mRNA expression levels and the decrease of *TSP2* (Figure 5c) in JJN3-HOXB7 tumoral masses as compared with JJN3-pWPI. Finally, by angiogenesis antibody array a significant upregulation of VEGF, bFGF and PDGFA at protein levels in JJN3-HOXB7 as compared with JJN3-pWPI (Figure 5d) was found.

Validation on MM primary tumor dataset of HOXB7-related angiogenic molecules

Finally, we attempted to validate the pro-angiogenic and anti-angiogenic genes (*VEGFA*, *WNT5A*, *MMP2*, *PDGFA*, *TGFB1*,

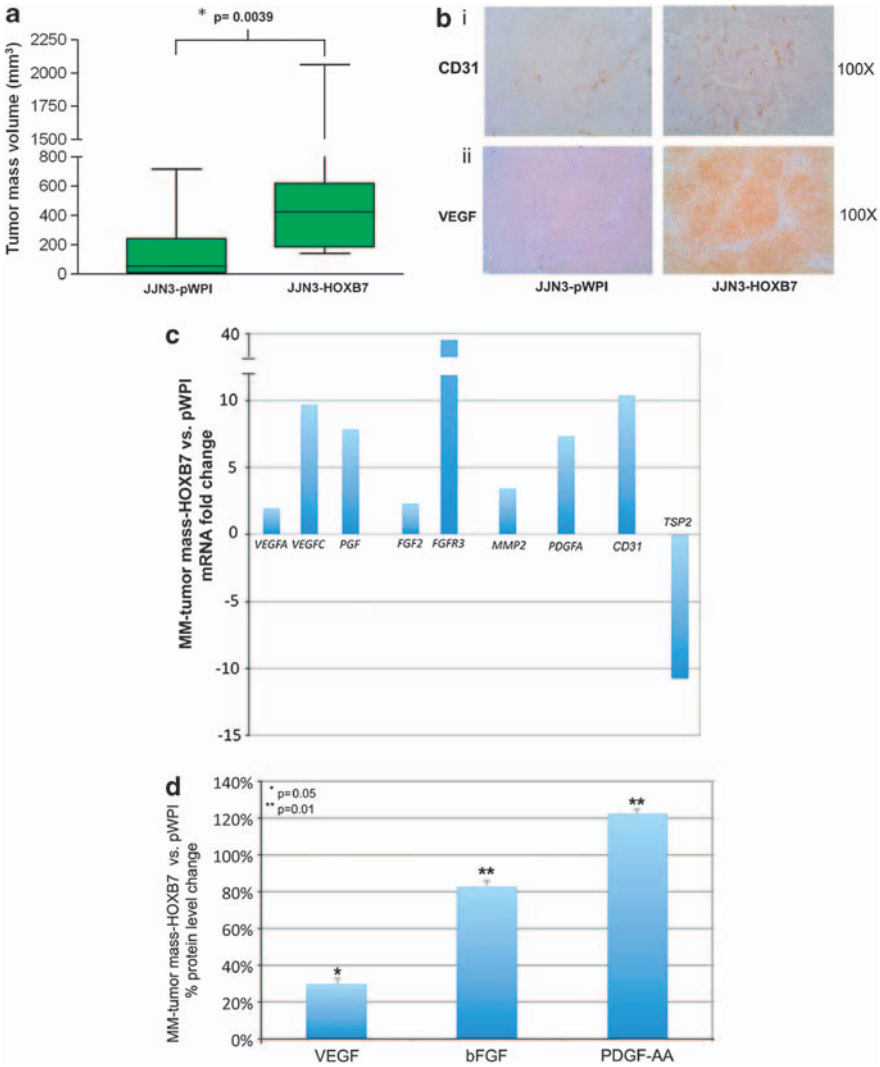

Figure 5 Effect of HOXB7 overexpression by MM on pro-angiogenic factors *in vivo* in SCID-NOD mice. Two groups of *SCID-NOD* mice each were injected sub-cutaneously with 5×10^6 JJN3-HOXB7 or with JJN3 JJN3-pWPI. Box blots represent the median tumoral mass volume of JJN3-HOXB7 and JJN3-pWPI colonized mice (**a**). CD31 and VEGF immunostaining was performed on frozen tissues (**b** panel i) and on formalin-fixed paraffin embedded sections (**b** panel ii), respectively, (original magnification $\times 100$). Graphs represent the median mRNA fold change of the angiogenic genes significantly modulated in JJN3-HOXB7 tumoral masses as compared with JJN3-pWPI performed in triplicate by angiogenesis PCR array (**c**). VEGF, bFGF and PDGFA protein expression was assessed in cell lysates obtained from tumoral masses by angiogenesis antibody array. Graphs represent the mean % ± s.d. of protein level change JJN3-HOXB7 tumoral masses as compared with JJN3-pWPI performed twice (**d**).

TSP2, *TIMP2* and *FGF2*) found to be significantly associated to HOXB7 modulation in HMCLs both *in vitro* and *in vivo*. Specifically, using a classification approach on the large GSE2658-independent datasets[28] of primary MM tumors, we evaluated whether the combination, in whole or in part, of such genes could distinguish the samples showing lower and higher HOXB7 expression. The most powerful combination was represented by *VEGFA*, *WNT5A*, *TGFB1*, *TIMP2* and *FGF2*, which correctly classified 62% of the 280 samples, included in the first and fourth HOXB7 expression-based quartiles (Supplementary Table 3).

Discussion

HOXB7 is a homeobox-containing transcription factor mediating several developmental process including hemopoietic differentiation and lymphoid development as well as leukemogenesis.[29,30] HOXB7 overexpression has been previously reported in solid tumors as breast,[20,21] melanoma[22,31] and ovarian cancers[32] in relationship with tumor cell-invasion ability, metastasis and angiogenesis suggesting the potential role of this gene in tumoral progression.[18,21,22,32] Among hematological malignancy *HOXB7* overexpression has been recently reported in chronic lymphocytic leukemia samples as compared with B and T lymphocytes.[33] In the present study, analyzing *HOXB7* gene profile in primary MM cells, we show that *HOXB7* is overexpressed by more than 40% of HMCLs and a fraction of MM patients at the diagnosis related to BM angiogenesis. Recently, in-line with our observation an overexpression of HOXB7 has been reported in extramedullary MM as compared with intramedullary cases.[34]

Then we focalized our attention on the potential involvement of HOXB7 in the production of pro-angiogenic molecules by MM cells and their pro-angiogenic properties in order to clarify the molecular mechanisms underling MM-induced angiogenic

switch. Two different approaches were used to investigate the pro-angiogenic role of HOXB7, either by enforcing HOXB7 expression by lentivirus vectors in JJN3 or silencing its constitutive expression in KMS20 by appropriate siRNA. Thus, we evaluated the potential effect on the transcriptional and angiogenic profile of MM cells using combined gene-expression profiling analysis and PCR angiogenesis arrays demonstrating that HOXB7 overexpression increased *VEGFA*, *FGF2*, *MMP2* and *PDGFA* gene expression and the corresponding proteins. Among these factors, VEGF, bFGF and MMP-2 are well known pro-angiogenic factors produced by MM cells[1,9–11,15,16] and critically involved in the angiogenic process, whereas the role of PDGFA in MM cells is not known even if its pro-angiogenic activity in cancer cells is well established.[35] In-line with our results it has been previously reported that HOXB7 overexpression regulates bFGF in breast and melanoma cancer cells[19,22] as well as VEGF.[18] On the contrary we failed to observe a modulation of Ang-2 and IL-8 by HOXB7 overexpression as reported by others.[18] Interestingly, other than the induction of pro-angiogenic molecules we found that HOXB7 overexpression inhibited the production of anti-angiogenic factors, particularly thrombospondin-2, which is known to act as a potent inhibitor of tumoral angiogenesis[36] and is deregulated in MM patients.[37] The capacity of HOXB7 to modulate the production of the pro-angiogenic factors by MM cells was confirmed by silencing HOXB7 in KMS20, that significantly inhibited the expression of VEGF and bFGF. TGFβ was also inhibited by HOXB7 down-regulation in KMS20, as well as several genes belonging to TGFβ signal pathway, including *BMP2* and *BMP7* were regulated by HOXB7 overexpression in JJN3. Furthermore, the correlation among *HOXB7* overexpression and the modulation of pro-angiogenic factors was observed in large datasets of proprietary and publicly available primary tumors, with the additional validation given by the good prediction capacity of five genes (*VEGFA*, *WNT5A*, *TGFB1*, *TIMP2* and *FGF2*) to classify a consistent number of independent samples from GSE2658 series. This suggests that the association between *HOXB7* upregulation and higher expression of pro-angiogenic factors is a conserved characteristic of MM disease, and is not affected by lab- or cohort-specific biases.

Consistently with the role of HOXB7 in the production of pro-angiogenic molecules by MM cells, we found that their angiogenic properties were significantly regulated by HOXB7 modulation in two different widely used and validated *in vitro* and *ex vivo* angiogenic models.[1,13,14,25] Finally, to confirm the pathophysiological role of HOXB7 in MM-induced angiogenesis, we tested the effect of its overexpression in MM cells using a SCID-NOD mice model previously tested.[38] Interestingly, together with higher MM-associated angiogenesis and VEGF expression we found that the tumor burden was significantly higher in JJN3-HOXB7 injected mice as compared with JJN3-pWPI suggesting that the increased vessel formation might stimulate MM growth. However, we can also suppose that HOXB7 directly affects MM cell growth given that its overexpression stimulated JJN3 proliferation rate as well as HOXB7 silencing inhibited cell proliferation and survival in KMS20. Accordingly, it has been previously reported that HOXB7 expression regulates cell proliferation in breast cancer[21] and myeloid progenitor cells.[30] The role of HOXB7 in the regulation of the pro-angiogenic profile of MM cells was then further confirmed by combined microarray analysis and angiogenesis PCR arrays in tumoral masses obtained from mice. Interestingly, among the genes significantly modulated by HOXB7 overexpression, we found *WNT5a* upregulation. WNT5a is an activator of non-Wnt canonical pathway transduced through FZD and Ror2 co-receptors to several cascades, either disheveled pathways involving Rho family small GTPase and JNK or Ca^{++} dependent pathways.[39] Consistently to our data, it has been previously shown that *HOXB7* overexpression by breast-cancer cells induced Rho and Ras GTP-ase activation and consequently cell migration and invasion.[20] Recent evidences also indicate that WNT5a signaling stimulates endothelial cell proliferation and survival showing a pro-angiogenic effect.[40] In addition, it has been demonstrated that WNT5a expression could be associated to tumor proliferation and VEGF expression,[41] as well as metastasis.[42] Thus, based on these evidences we can suppose that the pro-angiogenic effect of HOXB7 in MM cells could also involve WNT5a overexpression together with VEGF and other pro-angiogenic factor overproduction. Previous evidence suggested that the use of antagonists or inhibitors of a single pro-angiogenic factor as VEGF inhibitors had a minimal activity in MM[43] probably because of the redundant production of angiogenic molecules by tumor cells. On the other hand multitarget drugs as thalidomide have shown anti-angiogenic and anti-MM activity in MM patients. Interestingly, it has been recently reported that thalidomide inhibits HOXB7-induced gene expression in glioblastoma cell line.[44] This evidence, together with the capacity of HOXB7 to regulate cell proliferation and the production of several pro-angiogenic factors, suggests that it might be an attractive therapeutic target for a broad inhibition of angiogenic molecules and consequently of the MM-induced angiogenesis.

Conflict of interest

The authors declare no conflict of interests.

Acknowledgements

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)