Gene Expression Profiling of Bone Marrow Endothelial Cells in Patients with Multiple Myeloma

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Abstract

Purpose: To determine a "gene/molecular fingerprint" of multiple myeloma endothelial cells and identify vascular mechanisms governing the malignant progression from quiescent monoclonal gammopathy of undetermined significance.

Experimental Design: Comparative gene expression profiling of multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells with the Affymetrix U133A Arrays was carried out in patients at diagnosis; expression and function of selective vascular markers was validated by real-time reverse transcriptase-PCR, Western blot, and small interfering RNA analyses.

Results: Twenty-two genes were found differentially expressed (14 down-regulated and eight up-regulated) at relatively high stringency in multiple myeloma endothelial cells compared with monoclonal gammopathy of undetermined significance endothelial cells. Functional annotation revealed a role of these genes in the regulation of extracellular matrix formation and bone remodeling, cell adhesion, chemotaxis, angiogenesis, resistance to apoptosis, and cell-cycle regulation. Validation was focused on six genes (*DIRAS3, SERPINF1, SRPX, BNIP3, IER3*, and *SEPW1*) not previously found to be functionally correlated to the overangiogenic phenotype of multiple myeloma endothelial cells in active disease. The small interfering RNA knockdown of *BNIP3, IER3*, and *SEPW1* genes affected critical multiple myeloma endothelial cell functions correlated with the overangiogenic phenotype.

Conclusions: The distinct endothelial cell gene expression profiles and vascular phenotypes detected in this study may influence remodeling of the bone marrow microenvironment in patients with active multiple myeloma. A better understanding of the linkage between plasma cells and endothelial cells in multiple myeloma could contribute to the molecular classification of the disease and thus pinpoint selective gene targets for more effective antiangiogenic treatments. (Clin Cancer Res 2009;15(17):5369–78)

The unique markers expressed by tumor vasculature distinguish it from normal endothelium. These abnormalities reflect the pathologic nature of its induction and attempts to discover tumor endothelial cell markers have always been hampered by technical difficulties in isolating functionally intact and phenotypically stable endothelial cells from patient samples. St. Croix et al. (1) were the first to show that colorectal cancer endothelial cells overexpress specific transcripts as a result of qualitative

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Translational Relevance

Bone marrow angiogenesis is a constant hallmark of patients with multiple myeloma. Here, we identified genes differentially expressed in multiple myeloma endothelial cells with respect to monoclonal gammopathy of undetermined significance endothelial cells, providing for the first time a "gene/molecular fingerprint" of multiple myeloma endothelial cells. Deregulated genes are mostly involved in extracellular matrix formation and bone remodeling, cell-adhesion, chemotaxis, angiogenesis, resistance to apoptosis, and cell-cycle regulation. Validation was focused on *DIRAS3, SERPINF1, SRPX, BNIP3, IER3*, and *SEPW1* genes, which were not previously found to be functionally correlated to the overangiogenic phenotype of multiple myeloma endothelial cells. Small interfering RNA for three up-regulated genes (*BNIP3, IER3*, and *SEPW1*) affected critical multiple myeloma endothelial cell functions mediating the cell overangiogenic phenotype, that is, proliferation, apoptosis, adhesion, and capillary tube formation. Our data may help the molecular classification of the disease and identify new therapeutic targets, such as *BNIP3, IER3*, and *SEPW1* genes, for its antiangiogenic management.

differences in gene profiling compared with endothelial cells of the normal colorectal mucosa. Of 79 transcripts differentially expressed, 46 were at least 10-fold more elevated compared with normal endothelial cells, whereas 33 were expressed at lower levels.

Further studies on the genomic features of freshly isolated tumor endothelial cells have been circumstantial. Endothelial cells associated with gliomas (2) and invasive breast carcinomas (3) have distinct gene expression patterns related to extracellular matrix and surface proteins characteristic of proliferating and migrating endothelial cells, and point to specific roles for genes in driving tumor angiogenesis and progression (growth, invasion, and metastasis) of tumor cells. van Beijnum et al. (4) compared transcriptional profiles of angiogenic endothelial cells isolated from colorectal cancer and normal mucosa, as well as from placenta, and identified 17 genes that were overexpressed in tumor endothelial cells but not in angiogenic endothelial cells of the normal tissues. Antibodies targeting four tumor cytoplasmic/cell-surface or secreted molecules (vimentin, CD59, High Mobility Group Box 1, and IGFBP7) inhibited angiogenesis *in vitro* and *in vivo*.

Bone marrow angiogenesis is a constant hallmark of multiple myeloma progression. It accompanies the transition from monoclonal gammopathy of undetermined significance to multiple myeloma or from remission multiple myeloma to relapse and the leukemic phase (5). Induction of the "vascular phase" is partly sustained by angiogenic cytokines, such as vascular endothelial growth factor (VEGF), fibroblast growth factor-2, and matrix metalloproteinases, secreted by the bone marrow plasma cells. Monoclonal gammopathy of undetermined significance and multiple myeloma plasma cells can be distinguished from normal ones, whereas their own differentiation is difficult. This finding suggests that disease-controlled remodeling of the bone marrow microenvironment rather than a cell-intrinsic genetic change may account for the malignant changeover in keeping with the crucial role of the tumor microenvironment in inducing the angiogenic switch (6).

Gene expression profiling is a powerful mean of dissecting the biology of multiple myeloma. Here, it has been used for the first time to characterize and compare multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells and thus obtain a better understanding of the genes governing interactions between plasma cells and endothelial cells, the role of multiple myeloma endothelial cells in the progression of multiple myeloma, and new potential therapeutic targets.

Patients, Materials, and Methods

Patients and endothelial cell cultures

Ten consecutive patients fulfilling the International Myeloma Working Group diagnostic criteria (7) for multiple myeloma ($n = 5$) and monoclonal gammopathy of undetermined significance ($n = 5$) were studied at diagnosis with gene expression profiling. Multiple myeloma patients (3 M, 2 F) were with age of 54 to 81 y (median, 70.5 y) and staged as IIIA (8); the M component was immunoglobulin G ($n = 4$) or immunoglobulin A ($n = 1$). Monoclonal gammopathy of undetermined significance patients (3 M, 2 F) were with the age of 52 to 79 y (median, 69.6 y) and were immunoglobulin G ($n = 3$) or immunoglobulin A ($n = 2$). Gene expression validation by real-time reverse transcriptase-PCR (RT-PCR) and Western blot analysis was done on additional 55 consecutive patients at diagnosis: 32 multiple myeloma (18 immunoglobulin G, 9 immunoglobulin A, 5 κ or λ; age, 48-86 y; median, 72.4 y; 18/8 IIIA/B, 5/1 IIA/B) and 23 monoclonal gammopathy of undetermined significance (14 immunoglobulin G, 6 immunoglobulin A, 3 immunoglobulin M; age, 52-82 y; median, 70.7 y). The study was approved by the local Ethics Committee at the University of Bari Medical School, and all patients gave their informed consent in accordance with the Declaration of Helsinki.

Multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells were obtained as described (9). Briefly, centrifugation on Ficoll gradient of heparinized bone marrow aspirates was followed by polystyrene flask adherence to isolate stromal cells that were immunodepleted of macrophages and plasma cells with CD14 and CD38 monoclonal antibody–coated flasks (Immunotech) and incubated with magnetic microbeads coated with Ulex europaeus-1 lectin (its receptor is highly and specifically expressed by endothelial cells). Beads with bound endothelial cells were cultured in complete RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and 1% glutamine to allow cell detachment, spreading, and growth.

The purity and viability of endothelial cells grown at least one passage (>97% viable cells) were assessed by flow cytometry (FACScalibur, Becton Dickinson) for positivity of endothelial cell markers: factor VIII–related antigen and CD105, and negativity of CD14 and CD38. *FVIII-RA, CD38, CD105*, and *IgH VDJ* gene expression was investigated by RT-PCR; endothelial cell viability was assessed by trypan blue viable staining.

Human umbilical vascular endothelial cells (HUVEC) were purchased from Clonetics Biowhittaker and cultured in EGM-2MV media (Clonetics Biowhittaker).

Gene expression profiling analysis

Total RNA was extracted from frozen endothelial cells with TRIzol reagent (Invitrogen), purified with the Rnasy total RNA Isolation Kit (Qiagen), and verified for integrity with an Agilent Bioanalyzer (Agilent Technologies). Samples for which at least 5 μg of total RNA were

available were analyzed according to Affymetrix protocols (Affymetrix, Inc.) on GeneChip Human Genome U133A Arrays, as previously described by us (10). The images were acquired with MicroArray Suite 5.0 software (Affymetrix), and the probe level data converted to expression values with the Bioconductor function for the Robust Multi-Array average procedure (11).

Unsupervised analyses were applied to a subset of genes whose average change in expression levels varied at least 2-fold from the mean across the whole panel (2-Fold Change of Average Expression). For hierarchical agglomerative clustering, Pearson's *r* and average linkage (11) were respectively used as distance and linkage methods in DNA-Chip Analyzer software (10, 12, 13). Supervised gene expression analysis was done with the Gene@ Work software platform (10), which is a gene expression analysis tool based on the pattern discovery algorithm Structural Pattern Localization Analysis by Sequential Histograms (14). Gene@Work is able to discover global gene expression "signatures" that are common to an entire set of at least *n* experiments (the support set), where *n* is a user-selectable parameter called the "minimum support." Briefly, differentially expressed genes are identified by comparing an expected gene expression probability density $p(e)$, empirically computed from the experimental set with a predefined threshold (the parameter δ). Patterns of differentially expressed genes are then ranked according to their statistical significance (the pattern score or *z* score). Here, the value of δ was set to 0.03, and the support value was chosen as $n = n_0 - 1$, being $n_0 = 5$ the number of samples in our phenotype set. For each gene, the statistical significance of the differential expression across the phenotype and control sets (z_g) was computed using the formula $z_g = (\mu_p - \mu_c) / (\sigma_p + \sigma_c)$, wherein μ_p and σ_p are, respectively, the mean and SD computed from the gene expression values for that gene in the phenotype group, and μ_c and σ_c are their corresponding values computed from the control group. Supervised multiclass analysis was done using the Significant Analysis of Microarrays software version 3.00[8] (15).

The functional study on the selected lists was done by means of NetAffx[9] and of the Database for Annotation, Visualization and Integrated Discovery Tool 2008 (U.S. NIH).[10] The gene expression data have been deposited at the National Centre for Biotechnology Information's Gene Expression Omnibus.[11]

Real-Time RT-PCR

Total RNA (1 μg) was reverse transcribed into total cDNA with the "iScript cDNA Synthesis Kit" (Bio-Rad; ref. 16). Primers (TaqMan Gene Expression Assays, Applied Biosystems) were (forward/reverse) as follows: BCL2/adenovirus E1B 19-kDa interacting protein (BNIP3), 5′-CCACCTCGCTCGCAGACACCAC-3′/5′-GAGAGCAGCAGAGATGGAAGGAAAAC-3′; DIRAS3, 5′-TCTCTCCGAGCAGCGCA-3′/5′-ATCTTCCTGTGGGGCTTGAAGG-3′; selenoprotein W1 (SEPW1), 5′-GGCGCTTGAGGCTACAAGTCC-3′/5′-TATTGAATCGTGAACGGTTGC-3′; serpin peptidase inhibitor, clade F member 1 (SERPINF1), 5′-TATGACTTGATCAGCAG-3′/5′-AGCTTCATCTCCTGCAGGGA-3′; immediate early response 3 (IER3), 5′-GCCACCCGACATGACATCC-3′/5′-CTGGTGCGCGAGCGTATCC-3′; sushi repeat–containing protein, X-linked (SRPX), 5′-CAGTCTGGTCGTGGTATCTTTG-3′/5′-CTCCAATAAGACACCGACGC-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-CCCTCCAAAATCAAGTGGGG-3′/5′-CGCCACAGTTTCCCGGAGGG-3′, together with a fluorochrome FAM- or VIC-labeled TaqMan probe premixed at the optimal concentration for amplification. Reaction mixture and amplification conditions were done according to the manufacturer's instructions (Applied Biosystems). Each RNA was tested in triplicate and the threshold cycles values were averaged ± 1 SD. The expression of each gene was normalized on glyceraldehyde-3-phosphate dehydrogenase. The relative amounts of the genes studied and comparison of their expression in multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells were calculated with the $\Delta\Delta$Ct method.

Western blot analysis

Total proteins (50 μg) were subjected to immunoblot analysis with the following antibodies: monoclonal antibodies to BNIP3 (GeneTex, Inc.), DIRAS3 (ARHI, Abcam), and SERPINF1 (PEDF, Upstate), and antisera to IERF3 (IEX-1, Abcam), SRPX, SEPW1 (Santa Cruz Biotechnology, Inc.), and β-actin (Sigma-Aldrich Co.). Immunoreactive bands were detected by SuperSignal West Pico Chemiluminescent Substrate (ThermoScientific) and signals visualized with the Gel Logic 1500 Imaging System (Eastman Kodak Co.).

Functional studies

RNA interference. Small interfering RNA duplexes for BNIP3 (BNIP3–small interfering RNA) were synthesized using the Silencer small interfering RNA construction kit (Ambion, Inc.). The sequences were as follows (17): sense, 5′-CACGAGCGUCAUGAAGAAAUU-3′ starting at nucleotide 439 from AUG start codon of human BNIP3 coding sequence (Gen-Bank Accession Number MM 004052); and antisense, 5′-UUUCUUCAUGACGCUCGUGUU-3′, applied as previously described (16). For IER3 knockdown, multiple myeloma endothelial cells were transfected with 2 μg/mL of either Stealth negative control–small interfering RNA or Stealth IER3–small interfering RNA (Invitrogen), according to manufacturer's instructions. For SEPW1 knockdown, multiple myeloma endothelial cells were transfected with 2 μg/mL of control (sc-36869) or SEPW1–small interfering RNA (sc-40932, Santa Cruz Biotechnology, Inc.), according to manufacturer's instructions.

Proliferation and apoptosis assays. Multiple myeloma endothelial cells (10^4 per well) were incubated with 1 μCi of ^{3}[H]-thymidine 8 h before DNA synthesis measuring by using a filter scintillation counter (1430 MicroBeta, Wallac). The IC_{50} inhibitory drug concentration indicates a 50% decrease in proliferation compared with control. For survival analysis, cells were washed with ice-cold PBS, fixed in 70% cold ethanol, treated with DNase-free RNase (Boehringer Mannheim), and stained with 10 μg/mL propidium iodide (Sigma-Aldrich Co.). Cell cycle analysis was assessed on the FACScalibur and Cell-quest software. The ranges for G_0/G_1, S, G_2/M, and sub-G_1 phase cells were established on the basis of the corresponding DNA content of histograms. At least 10,000 cells per sample were considered in the gate regions used for calculations.

Adhesion and Matrigel assays. Multiple myeloma endothelial cell adhesion to fibronectin (Sigma Chemical Co.) and angiogenesis on Matrigel (Becton Dickinson) were done as previously described (9).

Results

Gene Expression Profiling. To determine whether bone marrow endothelial cells from monoclonal gammopathy of undetermined significance and multiple myeloma patients could be distinguished from HUVECs according to the natural grouping of their gene expression profiles, we did an unsupervised analysis using the hierarchical clustering algorithm on the two AVEFC probes in 15 endothelial cell samples data set. The 663 probe sets found to be highly variable along the entire data set generated a dendrogram (Fig. 1A) with two major branches: one containing multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells, and the other grouping the HUVECs. Neither multiple myeloma endothelial cells nor monoclonal gammopathy of undetermined significance endothelial cells could be identified as a distinct cluster of the dendrogram. The most significant modulated functions recognized for the 663 probe sets were associated to cell motility, blood vessel development, and morphogenesis, together with cell death and

[8] Excel front-end publicly available at http://www-stat.stanford.edu/~tibs/SAM/index.html.
[9] https://www.affymetrix.com/analysis/netaffx/
[10] http://david.abcc.ncifcrf.gov/
[11] http://www.ncbi.nlm.nih.gov/geo; accession number GSE14230.

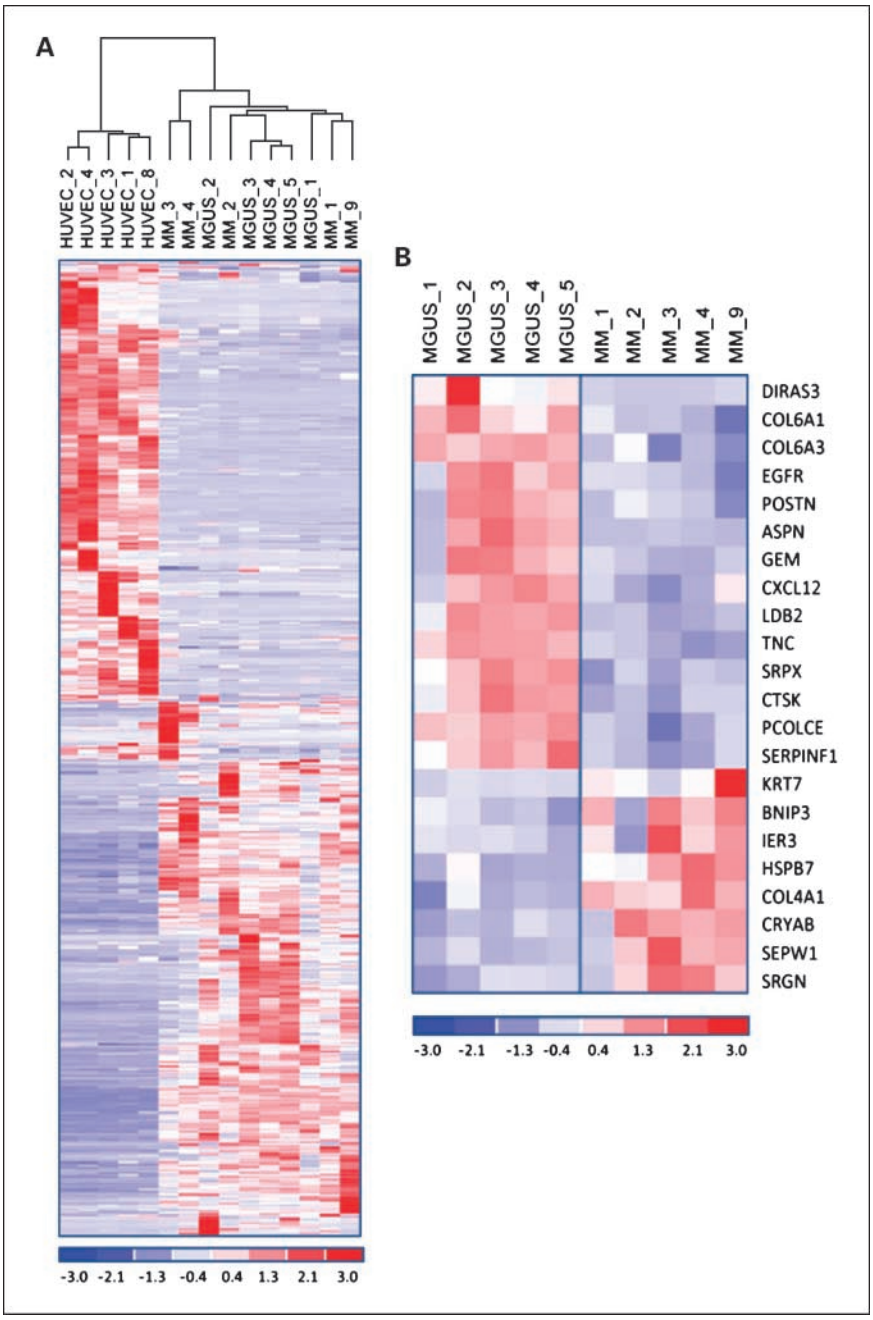

Fig. 1. Unsupervised (*A*) and supervised (*B*) analysis of gene expression profiles from a data set composed of five monoclonal gammopathy of undetermined significance endothelial cell, five multiple myeloma endothelial cell, and five HUVEC samples. *A,* the dendrogram was generated with a hierarchical clustering algorithm based on the average-linkage method. In the matrix, each column is a sample and each row a gene. The 15 samples are grouped according to their expression levels of the 663 most variable probe sets. *B,* identification of the 22 genes differentially expressed in five multiple myeloma endothelial cells versus five monoclonal gammopathy of undetermined significance endothelial cells. The δ value was set at 0.03 and the support value at $n = 4$. Color scale bar, the relative gene-expression changes normalized by the SD; the color changes in each row is gene expression relative to the mean across the samples (with gene symbols).

growth processes. Furthermore, signal transduction, ion transport and homeostasis, and development and differentiation of different tissues were found among the most important modulated processes between HUVECs, monoclonal gammopathy of undetermined significance endothelial cells, and multiple myeloma endothelial cells. Supplementary Table S1 summarizes all the significant functional categories identified by the Database for Annotation, Visualization and Integrated Discovery Tool 2008.

A supervised analysis was done to find which genes specifically differentiated multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells. Twenty-two differentiating genes were detected based on our experimental and gene expression profiling analysis approaches; 14 were down-regulated and eight up-regulated in multiple myeloma endothelial cells (Fig. 1B; Table 1). Interestingly, among the differentially expressed genes, nine encode for proteins located in the extracellular matrix: periostin (POSTN), tenascin-C (TNC), CXCL12, cathepsin K (CTSK), SERPINF1, collagen type VI alpha1 and alpha3 (COL6A1 and COL6A3), and procollagen C–endopeptidase enhancer (PCOLCE), all of which negatively modulated in multiple myeloma endothelial cells; and the collagen type IV alpha1 (COL4A1) was found to be positively modulated. Furthermore, five genes have been described to be involved in the control of apoptosis: BNIP3, IER3, and two members of the heat shock protein family, crystallin alpha B (CRYAB), and heat shock 27-kDa protein family, member 7 (HSPB7), all of them up-regulated in multiple

Table 1. Functional annotations of the 22 differentially expressed genes found in the multiple myeloma endothelial cells versus monoclonal gammopathy of undetermined significance endothelial cells comparison

Probe set ID	Gene symbol	z_g Score	Gene title	GO biological process/ GO molecular function	Ref.
218934_s_at	*HSPB7*	2.63	Heat shock 27-kDa protein family, member 7 (cardiovascular)	Response to stress/apoptosis	28
211981_at	*COL4A1*	2.24	Collagen, type IV, α 1	Phosphate transport	33
201194_at	*SEPW1*	1.90	Selenoprotein W, 1	Cell redox homeostasis	52
209283_at	*CRYAB*	1.90	Crystallin, α B	Antiapoptosis	44
209016_s_at	*KRT7*	1.69	Keratin 7	Cytoskeleton organization and biogenesis	34
201858_s_at	*SRGN*	1.35	Serglycin	Ossification	50
201849_at	*BNIP3*	1.24	BCL2/adenovirus E1B 19-kDa interacting protein 3	Response to hypoxia/apoptosis	18
201631_s_at	*IER3*	1.18	Immediate early response 3	Antiapoptosis	24
210809_s_at	*POSTN*	-1.25	Periostin, osteoblast-specific factor	Skeletal development/cell adhesion	35
201983_s_at	*EGFR*	-1.60	Epidermal growth factor receptor [erythroblastic leukemia viral (*v-erb-b*) oncogene homolog, avian]	Positive regulation of cell proliferation/cell-cell adhesion	50
204472_at	*GEM*	-1.86	GTP-binding protein overexpressed in skeletal muscle	Immune response/signal transduction	54
212940_at	*COL6A1*	-1.97	Collagen, type VI, α 1	Cell adhesion	33
204955_at	*SRPX*	-2.05	Sushi repeat–containing protein, X-linked	Cell adhesion/apoptosis	29
209687_at	*CXCL12*	-2.14	Chemokine (C-X-C motif) ligand 12 (stromal cell–derived factor 1)	Chemotaxis/cell adhesion	36
206481_s_at	*LDB2*	-2.19	LIM domain binding 2	Multicellular organismal development	51
201438_at	*COL6A3*	-2.30	Collagen, type VI, α 3	Cell adhesion	33
202450_s_at	*CTSK*	-2.50	Cathepsin K	Proteolysis	42
215506_s_at	*DIRAS3*	-2.56	DIRAS family, GTP-binding RAS-like 3	Regulation of cyclin-dependent protein kinase activity	53
202283_at	*SERPINF1*	-2.65	Serpin peptidase inhibitor, clade F (α-2 antiplasmin, pigment epithelium derived factor), member 1	Negative regulation of angiogenesis	45
219087_at	*ASPN*	-2.81	Asporin	Protein binding	41
202465_at	*PCOLCE*	-3.58	Procollagen C–endopeptidase enhancer	Multicellular organismal development	39
201645_at	*TNC*	-3.85	Tenascin C	Cell adhesion	37

NOTE: Genes are ranked according to their z_g score values expressed using the Monoclonal Gammopathy Endothelial Cells group as baseline.
Abbreviation: GO, gene ontology.

myeloma endothelial cells, and SRPX, was found to be down-regulated. Finally, three genes, *EGFR*, *GEM* (a GTP-binding protein), *DIRAS3* (a GTP-binding RAS-like protein), involved in cell proliferation and signal transduction have been found to be downmodulated in multiple myeloma endothelial cells.

Next, we did a supervised multiclass analysis applying the Significant Analysis of Microarrays algorithm (15) and found that all of the 22 genes were significantly modulated in HUVECs with respect to monoclonal gammopathy of undetermined significance endothelial cells and multiple myeloma endothelial cells (Supplementary Table S2 and Supplementary Fig. S1 for details).

Real-Time RT-PCR and Western blot validation. The gene expression profiling data were validated on 32 multiple myeloma endothelial cell and 23 monoclonal gammopathy of undetermined significance endothelial cell samples by testing mRNA and protein levels of six genes more closely related to angiogenesis: *DIRAS3*, *SERPINF1*, *SRPX*, *BNIP3*, *IER3*, and *SEPW1*. Specifically, DIRAS3 was 4.3 times less expressed (Fig. 2A); SERPINF1, 2.5 times less (Fig. 2B); and SRPX, five times less (Fig. 2C). In contrast, BNIP3 was five times more expressed (Fig. 2D); IER3, 2.5 times more (Fig. 2E); and SEPW1, only 1.2 times more (Fig. 2F). Immunoblotting analysis disclosed similar differences in the protein expression levels (Fig. 3). Although DIRAS3, SERPINF1, and SRPX proteins were significantly inhibited in multiple myeloma endothelial cells, BNIP3 and IER3 were significantly more expressed, but SEPW1 only slightly increased.

Functional Validation of BNIP3, IER3, and SEPW1 in Multiple Myeloma Endothelial Cells by RNA Interference. The biological relevance of the three up-regulated genes, that is, *BNIP3*, *IER3*, and *SEPW1*, was validated as for their potential implication in proliferation, resistance to apoptosis, adhesion to fibronectin, and overangiogenic activity of multiple myeloma endothelial cells. Although a small interfering RNA control oligo (Fig. 4A, *lane b*) had no effect on protein levels, we observed a dose-dependent down-regulation by using 50 to 150 nmol/L of small interfering RNA for BNIP3 and 0.1 to 2 μg/mL of small interfering RNA for IER3 and SEPW1 (Fig. 4A, *lanes c-e*), with a maximum effect at 150 nmol/L for BNIP3 and 2 μg/mL for IER3 and SEPW1. In Fig. 4B, a small interfering RNA–silenced expression of BNIP3 and IER3 but not of SEPW1 reduced multiple myeloma endothelial cell proliferation compared with control small interfering RNA or untreated cells, as also confirmed by cell-cycle profiling (Fig. 4C). Interestingly, increasing sub-G_1 peak of the cell cycle (Fig. 4C) indicated that down-regulation of IER3, particularly of BNIP3, correlated

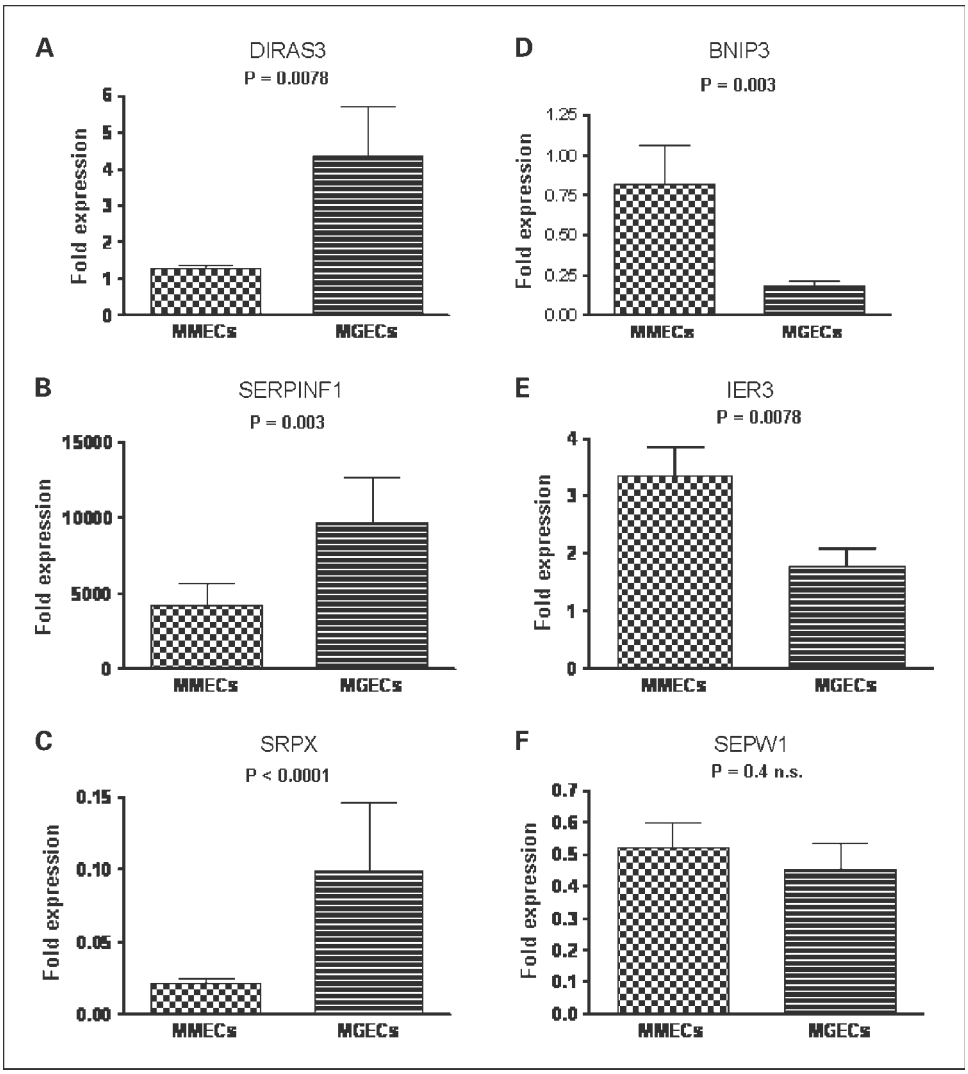

Fig. 2. Measurement of gene expression by real-time RT-PCR. Significant decrease of DIRAS3 (*A*), SERPINF1 (*B*), and SRPX (*C*) in multiple myeloma endothelial cells, as opposed to a significant increase of BNIP3 (*D*) and IER3 (*E*), and only a trend to increase for SEPW1 (*F*). Values are expressed as mean ± 1SD for 32 multiple myeloma and 23 monoclonal gammopathy of undetermined significance patients. Significance assessed by the Wilcoxon signed-rank test.

with a decreased multiple myeloma endothelial cell viability (54.2% for BNIP3 and 22.2% for IER3 compared with 1.2% of control small interfering RNA–treated multiple myeloma endothelial cells), whereas silencing of SEPW1 seemed dispensable (1.8%). These data were further corroborated by specific apoptosis tests (Fig. 4D). Conversely, we observed a dose-dependent inhibition of multiple myeloma endothelial cell adhesion to fibronectin (Fig. 5A), as well as of angiogenic activity on Matrigel (Fig. 5B) of multiple myeloma endothelial cells treated with small interfering RNA for BNIP3, IER3, and SEPW1.

Discussion

Previous gene expression profiling studies designed to identify genes perhaps involved in the initiation and progression of multiple myeloma (10, 18) revealed that monoclonal gammopathy of undetermined significance and multiple myeloma plasma cells can be distinguished from normal plasma cells, whereas their own differentiation is problematical. These findings suggest that modulation of the bone marrow microenvironment rather than genetic alterations of the tumor cells may partly account for the malignant conversion of monoclonal gammopathy of undetermined significance. Because multiple myeloma mainly progresses in the bone marrow, signals from this microenvironment are thought to play a critical role in maintaining plasma cell growth, migration, and survival. Reciprocal positive and negative interactions between plasma cells and bone marrow stromal cells, namely endothelial cells, endothelial cell progenitor cells, hematopoietic stem cells, osteoblasts/osteoclasts, chondroclasts, fibroblasts, macrophages, and mast cells, are mediated by an array of cytokines, receptors, and adhesion molecules (6). In the present study, the analysis and comparison of multiple myeloma endothelial cell and monoclonal gammopathy of undetermined significance endothelial cell gene expression profiling identified 22 genes differentially expressed that may play an important role in multiple myeloma progression. Specific pathway analysis indicated their involvement in the control of apoptosis, extracellular matrix formation and bone remodeling, cell adhesion, angiogenesis, and cell proliferation.

Three apoptosis-related genes were up-regulated in multiple myeloma endothelial cells. (*a*) BNIP3 belongs to the Bcl-2 family and is induced by hypoxia-inducible factor–1α (19). Notably,

hypoxia-inducible factor–1α is up-regulated in the overangiogenic endothelial cells isolated from patients with active multiple myeloma at diagnosis, relapse, or leukemic phase (9), thus promoting the expression of VEGF (19) and the vascular phase of the active disease (20). Similarly, BNIP3 is up-regulated in breast cancer (21) and in non–small cell lung cancer (22), and its expression correlates with a poor prognosis. Because BNIP3 is proapoptotic (Table 1), its overexpression in tumors with poor prognosis may represent a paradox. However, high levels of Bcl-2 and epidermal growth factor or a different location from mitochondria (wherein the protein is normally present) are all possible mechanisms inhibiting the BNIP3 proapoptotic activity (23). Of note is that BNIP3 behaves as an antiapoptotic gene in multiple myeloma endothelial cells because BNIP3–small interfering RNA cells studied here increase apoptosis and decrease growth. It may be well that one or more of the above mechanisms inhibit BNIP3 in multiple myeloma endothelial cells and render it supportive for the cell overangiogenic phenotype. (*b*) IER3 is a member of the "immediate early response gene" family induced by the antiapoptotic factor nuclear factor κB in response to the tumor necrosis factor-α and ligand-mediated FAS (24). It is an antiapoptotic (Table 1) and a stress-inducible gene and plays a pivotal role in cell survival under stress conditions (24). In addition, its role in reducing intracellular reactive oxygen species for cell homeostasis is correlated to its antiapoptotic ability (25). Its up-regulation in multiple myeloma endothelial cells from patients at diagnosis may account for the overangiogenic activation of these endothelial cells (9), hence for multiple myeloma progression. Here, we show the antiapoptotic role of IER3 in multiple myeloma endothelial cells because their small interfering RNA–silenced IER3 expression reduced cell proliferation and induced apoptosis. Notably, IER3 has been reported to be overexpressed in multiple myeloma plasma cells too (26). (*c*) HSPB7 is an IER3-related gene. It is overexpressed in skeletal muscle, constituting an essential cellular response to fiber aging (27). It is overexpressed in prostate cancer in association with a poor prognosis and alters the balance between proliferation and apoptosis pending toward proliferation (28). Its overexpression in multiple myeloma endothelial cells may further explain the overangiogenic phenotype of these endothelial cells. On the other hand, SRPX, a tumor suppressor gene with proapoptotic function (29), is down-regulated in multiple myeloma endothelial cells, as also observed in human tumor cell lines and prostate and lung cancer (30, 31). Its proapoptotic ability has been also shown with gene knockout in mouse (32). Its down-regulation may contribute to the overangiogenic phenotype of multiple myeloma endothelial cells in active multiple myeloma patients. Overall, our data strongly suggest that coordinated antiapoptotic mechanisms are activated in multiple myeloma endothelial cells and contribute to their overangiogenic phenotype.

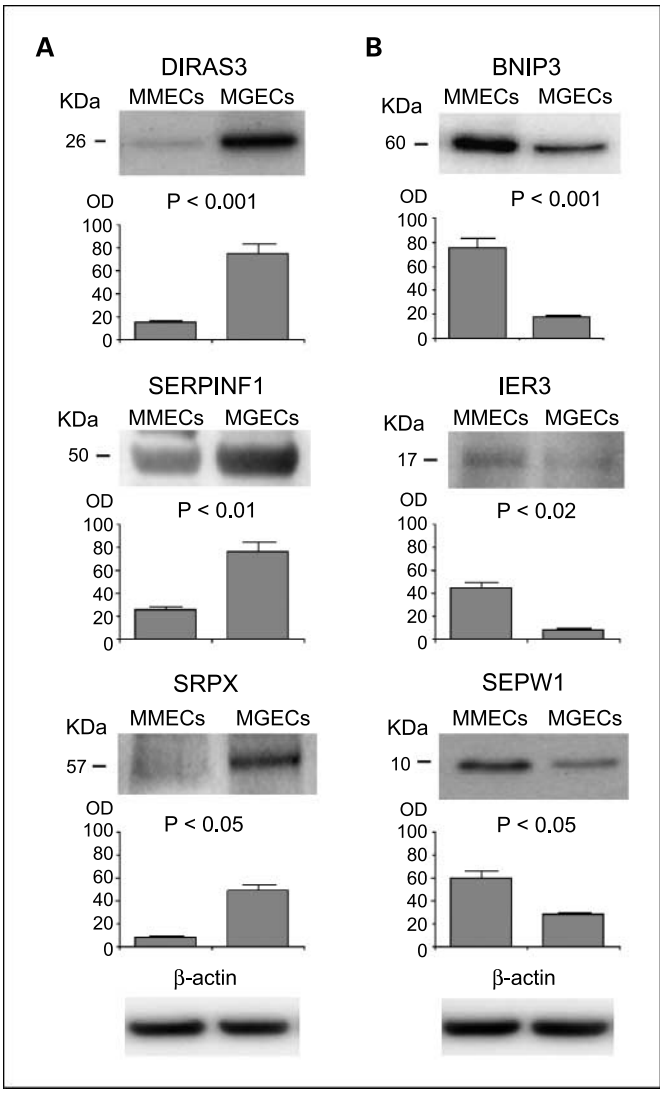

Fig. 3. Protein expression evaluated by Western blot. DIRAS3, SERPINF1, and SRPX (*A*) proteins significantly decrease in multiple myeloma endothelial cells, whereas BNIP3, IER3, and SEPW1 (*B*) proteins significantly increase. The band intensity is evaluated as optical density units by a Kodak software in 32 multiple myeloma and 23 monoclonal gammopathy of undetermined significance patients, and given as mean ± 1 SD. Gels refer to representative patients. Significance assessed by ANOVA by the Fisher and Kruskal-Wallis test followed by the Duncan (*t*), Bonferroni (*t*), and Wilcoxon paired tests.

In the extracellular matrix proteins and cell microfilaments, COL6A1 and COL6A3, governing cell anchorage to the extracellular matrix (33), were down-regulated in multiple myeloma endothelial cells, which may account for the increased migration of multiple myeloma endothelial cells and fast sprouting of neovessels in active multiple myeloma patients (9). In contrast, COL4A1, a major component of the vascular basement membrane, was up-regulated and probably provides a track support for the assembly and maturation of newly formed multiple myeloma microvessels. COL4A1 is closely involved in angiogenesis, one reason being that it is a substrate of metalloproteinase-2 and matrix metalloproteinase-9 secreted by multiple myeloma plasma cells and multiple myeloma endothelial cells (9).

Cytokeratin 7 (KRT7) is up-regulated in multiple myeloma endothelial cells, as in invasive renal and ovarian carcinomas (34). This finding is consistent with the fast invasive spreading and tube formation by multiple myeloma endothelial cells observed on an artificial subendothelial basement membrane (Matrigel; ref. 9). *POSTN* gene, involved in cell adhesion and modulated in breast and ovarian carcinomas (35), is down-regulated in multiple myeloma endothelial cells, in keeping with the fast vessel sprouting in active multiple myeloma. CXCL12 is up-regulated in multiple myeloma endothelial cells; noteworthy is that the CXCL12/CXCR4 is a critical

regulator for homing of tumor cells and multiple myeloma plasma cells (36).

Another five genes with similar functional annotations were down-regulated in multiple myeloma endothelial cells: TNC, a basement membrane component and inducer of matrix metalloproteinase expression (37) that regulates organ morphogenesis (38); PCOLCE, a product of the maturation of type I and III collagen (39) that is functional in angiogenesis through interaction with some integrins (40); asporin (ASPN), an extracellular matrix protein that participates in several bone diseases (41); and CTSK, a lysosomal cysteine proteinase that favors cancer invasion and metastasis (42). Down-regulation of these genes could be responsible for modification of the extracellular matrix that helps the invasive capacity of multiple myeloma endothelial cells.

In the angiogenesis-related genes, *CRYAB*, which belongs to the heat shock proteins and participates in tube morphogenesis, transformation of immortalized human mammary epithelial cells (43), and survival of tumor endothelial cells (44), was up-regulated, whereas SERPINF1, a serine protease inhibitor of angiogenesis (45) through Fas/Fas ligand mediated apoptosis (46), was accordingly down-regulated in multiple myeloma endothelial cells. Of note, CRYAB is antiapoptotic by inhibiting TRAIL-induced apoptosis through suppression of caspase-3 activation (47). It is up-regulated in gliomas and breast, prostate, and renal cell carcinomas (48). It has been proposed as one of the strongest candidates for resistance against DNA-damaging drugs by means of genomewide analysis on malignant melanoma cell lines (49). Its up-regulation in multiple myeloma endothelial cells shown here, along with that of BNIP3, IER3, and HSPB7, further implies that antiapoptotic pathways can be operative in multiple myeloma endothelial cells and help the cell overangiogenic phenotype.

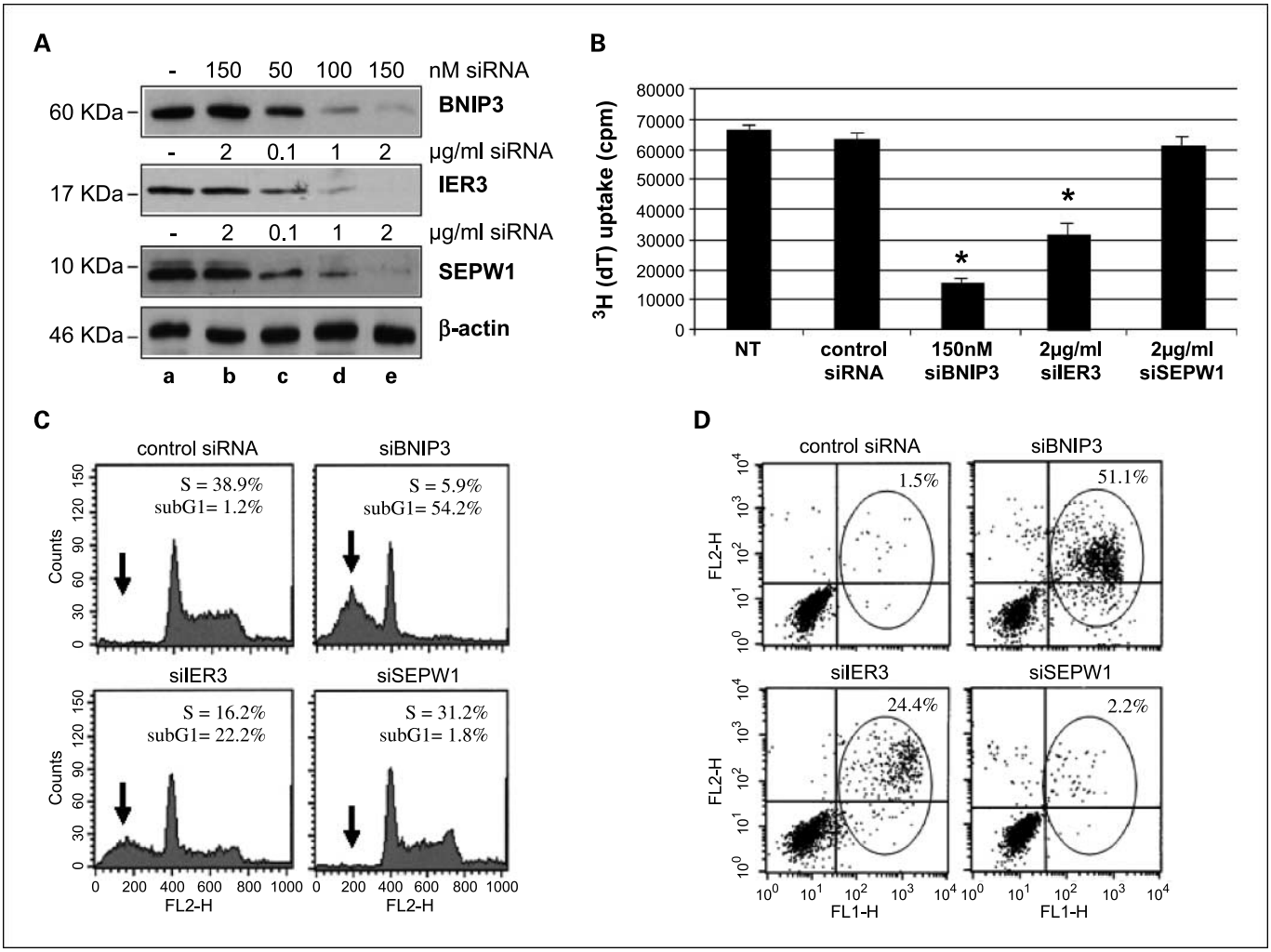

Fig. 4. Silencing of BNIP3, IER3, and SEPW1 protein expression in multiple myeloma endothelial cells. *A,* multiple myeloma endothelial cells were transfected with carrier alone (*lane a*), scrambled control small interfering RNA (*lane b*), or small interfering RNA for BNIP3, IER3, or SEPW1 (*lanes c-e*) at the indicated doses. After 48 h, total lysates were analyzed for BNIP3, IER3, or SEPW1 protein levels, and β-actin as loading control. *B,* proliferation of multiple myeloma endothelial cells transfected with either a control small interfering RNA or small interfering RNA specific for BNIP3, IER3, or SEPW1, or left untreated for 48 h. Values are presented as mean ± 1 SD of three independent experiments. *, $P < 0.05$ compared with control small interfering RNA by Wilcoxon signed-rank test. Cell-cycle distribution (*C*) and apoptosis rate (*D*) of small interfering RNA–transfected multiple myeloma endothelial cells (150 nmol/L small interfering RNA for BNIP3, 2 µg/mL small interfering RNA for IER3 and SEPW1) at 48 h were assessed by propidium iodide and Annexin V stainings, respectively, and fluorescence-activated cell sorting analysis. The percentage of cells in S/sub-G1 phase (*arrows*) or undergoing apoptosis (*circles*).

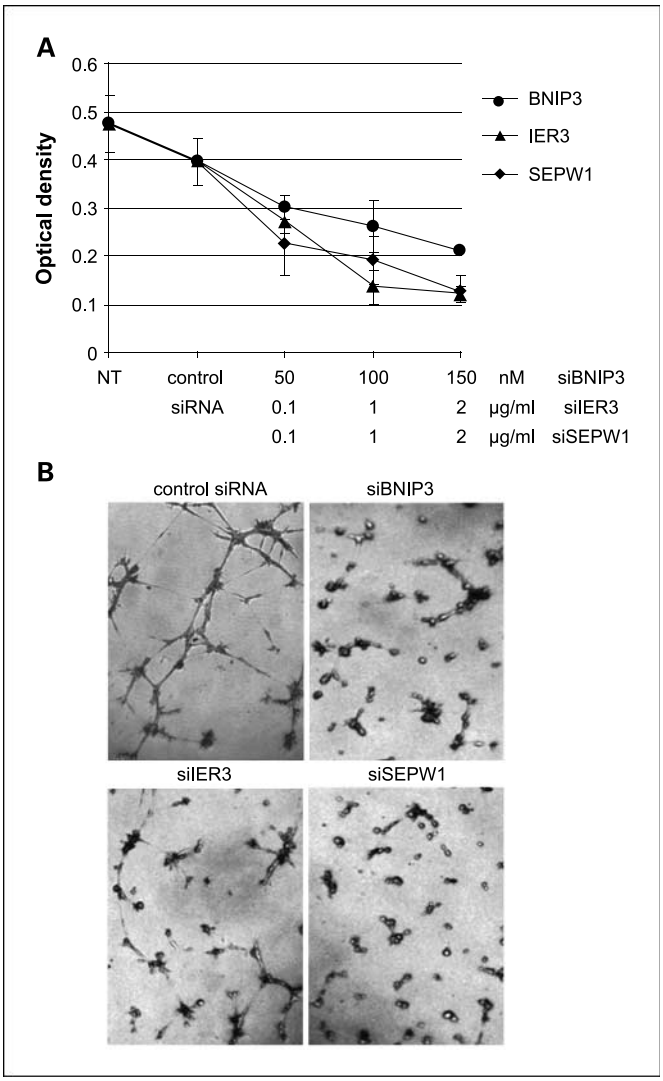

Fig. 5. Effect of BNIP3, IER3, and SEPW1 down-regulation on fibronectin-mediated adhesion and angiogenesis on Matrigel of multiple myeloma endothelial cells. *A,* multiple myeloma endothelial cells were transfected with the indicated doses of small interfering RNA for BNIP3, IER3, and SEPW1, or a control small interfering RNA. Upon 24 h, the attachment to and spreading on fibronectin of small interfering RNA–transfected multiple myeloma endothelial cells was analyzed at 90 min and compared with untreated cells; no changes were observed at 30 min. *B,* capillary formation was observed under a phase contrast microscope and pictures taken at ×10 after an 18-h incubation.

In the cell proliferation and homeostasis genes, serglycin (SRGN) and epidermal growth factor receptor (EGFR), both involved in cell proliferation (50), were respectively up- and down-regulated in multiple myeloma endothelial cells. The LIM domain binding 2 (LDB2), involved in the maintenance of cell homeostasis (51), and *SEPW1*, a gene with antioxidant function (52) that could play a role in protection from oxidative stress, are respectively down- and up-regulated in multiple myeloma endothelial cells. SEPW1-mediated homeostasis seems to be crucial for angiogenesis in multiple myeloma because small interfering RNA–silenced expression of the gene inhibited multiple myeloma endothelial cell adhesion and angiogenic activity.

In the series of signal transduction, cell-cycle regulation, and bone remodeling genes, two genes are down-regulated in multiple myeloma endothelial cells: (*a*) DIRAS3, which negatively regulates cell growth and is associated with disease progression in breast and ovary carcinomas (53), and (*b*) GEM, a protein with GTPase activity that plays a role in intracellular signal transduction and regulation of the cell cycle (54).

A comparison of the 367 well-characterized genes resulting from the HUVECs versus multiple myeloma endothelial cells supervised gene expression profiling analysis done here (data not shown) with the genes previously reported as differentially expressed between these two endothelial cell types by means of a 96-gene cDNA array (9) indicates that, among 36 genes previously described, eight genes are differentially expressed at high stringency: the isoform 7 of the fibroblast growth factor, the VEGF isoforms VEGF-A and VEGF-C, fibronectin 1, and thombospondin 2 were up-regulated in multiple myeloma endothelial cells, whereas the endothelial differentiating factor 1, CD105, and CD31 were down-regulated, further implying that marked differences do exist between multiple myeloma endothelial cells and normal endothelial cells.

Overall, these findings imply that multiple myeloma endothelial cells (*a*) are functionally different from monoclonal gammopathy of undetermined significance endothelial cells, (*b*) are characterized by an overangiogenic phenotype, and (*c*) resemble transformed cells because they down- or up-regulate some genes like tumor cells. Whether these changes are influenced by the multiple myeloma microenvironment and/or plasma cells or are associated with genomic alterations in multiple myeloma endothelial cells is now being investigated in our laboratory. It is conceivable that microenvironmental factors (such as hypoxia, inflammation, expression of multiple cytokines, and growth factors, etc.) regulating tumor-associated blood vessels (such as other tumor bone marrow–stromal elements) may display unstable, heterogeneous, and progressive characteristics to an extent comparable with (and causally linked to) the instability of the cancer cell genome. In addition, those factors may have genetic causes and consequences (i.e., increased expression of oncogenes, loss of tumor suppressor genes). This reciprocal interrelationship and heterogeneity may translate into site- and stage-specific changes in the regulation of bone marrow–microvessel density and angiogenesis dependence, and ultimately to changes in the proliferation and antiapoptotic potential of multiple myeloma tumor cells, even in the same patient.

In conclusion, we have identified 22 genes differentially expressed in multiple myeloma endothelial cells and monoclonal gammopathy of undetermined significance endothelial cells. They may represent new molecular markers for prognostic stratification of multiple myeloma patients and prediction of the response to antiangiogenic drugs. Furthermore, the gene expression profiling of multiple myeloma endothelial cells may lead to the identification of a number of new therapeutic targets, including *BNIP3*, *IER3*, and *SEPW1* genes, for the antiangiogenic management of multiple myeloma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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