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## Induction of angiogenesis by normal and malignant plasma cells

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**Abundant bone marrow angiogenesis is present in almost all myeloma patients requiring therapy and correlated to treatment response and survival. We assessed the expression of 402 angiogenesis-associated genes by Affymetrix DNA microarrays in 466 samples, including CD138-purified myeloma cells (MMCs) from 300 previously untreated patients, in vivo microcirculation by dynamic contrast-enhanced magnetic resonance imaging, and in vitro angiogenesis (AngioKit-assay). Normal bone marrow**

**plasma cells (BMPCs) express a median of 39 proangiogenic (eg, *VEGFA*, *ADM*, *IGF-1*) and 28 antiangiogenic genes (eg, *TIMP1*, *TIMP2*). Supernatants of BMPCs unlike those of memory B cells induce angiogenesis in vitro. MMCs do not show a significantly higher median number of expressed proangiogenic (45) or antiangiogenic (31) genes, but 97% of MMC samples aberrantly express at least one of the angiogenic factors *HGF*, *IL-15*, *ANG*, *APRIL*, *CTGF*, or *TGFA*. Supernatants of MMCs and human myeloma cell lines**

**induce significantly higher in vitro angiogenesis compared with BMPCs. In conclusion, BMPCs express a surplus of proangiogenic over antiangiogenic genes transmitting to the ability to induce in vitro angiogenesis. Aberrant expression of proangiogenic and down-regulation of antiangiogenic genes by MMCs further increases the angiogenic stimulus, together leading to bone marrow angiogenesis at various degrees in all myeloma patients. (Blood. 2009; 114:128-143)**

### Introduction

Multiple myeloma (MM) is an incurable malignant disease of clonal plasma cells that accumulate in the bone marrow (BM), causing clinical signs and symptoms related to the displacement of normal hematopoiesis, formation of osteolytic bone lesions, and production of monoclonal protein.<sup>1</sup>

In the bone marrow microenvironment (BMME) affected by MM, substantial BM neovascularization (“angiogenesis”) is present: compared with healthy persons, a higher microvessel density (MVD),<sup>2</sup> endothelial activation,<sup>3</sup> capillary permeability,<sup>4</sup> and increased perfusion<sup>4</sup> can be detected. BM angiogenesis parallels disease activity, is returned to the normal state after successful treatment,<sup>5,6</sup> and correlates with event-free survival (EFS) and overall survival (OS).<sup>7-10</sup> Several proangiogenic cytokines (eg, *VEGFA*, *FGF2*, and *HGF*) are present in higher concentrations in myelomatous BM and peripheral blood sera<sup>6,11-16</sup> while decreasing after successful treatment.<sup>6,13,14</sup>

In analogy to the “angiogenic switch” model for solid tumors by Folkman et al,<sup>17</sup> the induction of angiogenesis in MM is considered to be related to malignant plasma cells progressively inducing a change in the balance between proangiogenic and antiangiogenic cytokines within the BMME. This change is attributed to malignant plasma cells obtaining the capability of aberrantly producing proangiogenic and concomitantly down-regulating antiangiogenic factors, either directly or by influencing the BMME. Here, it is a matter of debate whether these expression changes in malignant plasma cells take place at the stage of monoclonal gammopathy of

unknown significance (MGUS) or MM. BM angiogenesis has been described to either correlate with the accumulation of MM cells (MMCs; tumor load), or their proliferation. MMCs are thought to benefit in turn from BM angiogenesis by improved oxygen and nutrient supply and likewise antiapoptotic and tumor-promoting effects mediated by endothelial-derived cytokines and myeloma-endothelial adhesion events.<sup>18</sup>

To assess a comprehensive set of “angiogenesis-associated” genes, we combined literature review and association of further related genes by Ingenuity Pathway Analysis (Figure 1). We subsequently assess presence and differential expression of these 402 genes in 466 gene expression profiles, including normal bone marrow plasma cells (BMPCs), primary MMCs, and human myeloma cell lines (HMCLs), BMME from normal healthy donors (NDs) and myeloma patients as well as the association with clinical parameters, genetic abnormalities, and survival.

We report here, for the first time, that already normal BMPCs express several proangiogenic genes, including *VEGFA*, *IGF-1*, and *ADM* and their culture supernatants (n = 11) significantly induce in vitro angiogenesis. Interestingly, this angiogenesis induction cannot be seen as a general property of cells of B-cellular lineage, as memory B-cell (MBC) supernatants do not induce angiogenesis in vitro (n = 6). Expectedly, malignant plasma cells show a various pattern of aberrant expression of several proangiogenic factors, and culture supernatants of primary MMCs (n = 20)

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and HMCLs (n = 10) induce in vitro angiogenesis (tubule formation). None of these factors, however, is expressed in all of the myeloma patient samples, and no significant correlation with in vivo surrogates of perfusion and MVD as determined by dynamic contrast-enhanced magnetic resonance imaging (dce-MRI, n = 64) could be found. Nevertheless, if the 6 most frequently aberrantly expressed factors are considered (*HGF*, *IL-15*, *APRIL* (*TNFSF13*), *ANG*, *TGFA*, *CTGF*), in 2 cohorts of patients 89% (n = 65) and 97% (n = 235) of MMC samples show an aberrant expression of at least one of these factors.

These results shed a different light on our understanding of the mechanism of angiogenesis induction in MM and might change the current paradigm of myeloma pathophysiology in a way that several of the “malignant” properties of MMCs might be attributed to primary plasma cell functions.

## Methods

### Patients and healthy donors

Patients presenting with previously untreated MM (n = 300) or MGUS (n = 23) at the University Hospitals of Heidelberg and Montpellier and 14 healthy ND have been included after written informed consent was obtained in accordance with the Declaration of Helsinki in the study approved by the institutional review boards of the Medical Faculty of the Ruprecht-Karls-University Heidelberg (Heidelberg, Germany), and the Centre Hospitalier Universitaire Montpellier (Montpellier, France), for the respective patients. Patients were diagnosed and staged and their response to treatment was assessed according to standard criteria.<sup>19-22</sup> A total of 207 patients underwent frontline high-dose chemotherapy (HDT) with 200 mg/m<sup>2</sup> melphalan and autologous stem cell transplantation (ASCT) according to or in analogy to the GMMG-HD3 trial.<sup>23</sup> Data were validated by an independent cohort of 345 patients treated within the total therapy 2 protocol.<sup>24</sup> For clinical parameters, see supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

### Samples

For an overview, see Table S2. Bone marrow plasma cells were purified using CD138 microbeads (Miltenyi Biotec), and purity was assessed by flow cytometry (FACS Calibur; BD Biosciences). Aliquots of unpurified (whole) bone marrow (WBM) of patients (n = 57) and healthy donors (n = 7) were obtained after NH<sub>4</sub> lysis as published.<sup>25</sup> BMPCs for supernatant generation were subsequently FACSARIA (BD Biosciences) sorted to purity more than 90% and peripheral CD27<sup>+</sup> MBCs generated as published.<sup>26</sup>

The HMCLs XG-1, XG-2, XG-3, XG-4, XG-5, XG-6, XG-7, XG-10, XG-11, XG-12, XG-13, XG-14, XG-16, XG-19, and XG-20 were generated at Institut National de la Santé et de la Recherche Médicale U847 as published.<sup>27-29</sup> U266, RPMI-8226, LP-1, OPM-2, SKMM-2, AMO-1, JIN-3, NCI-929, KMS-12-BM, KMS-11, KMS-12-PE, KMS-18, MM1S, JIM3, KARPAS 620, L363, and ANBL6 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, and ATCC) were cultured as recommended.

### iFISH

Interphase fluorescence in situ hybridization (iFISH) analysis was performed on CD138-purified plasma cells as described<sup>30,31</sup> using probes for chromosomes 1q21, 4p16, 6q21, 8p21, 9q34, 11q13, 11q23, 13q14.3, 15q22, 17p13, 19q13, 22q11, and translocations t(4;14)(p16.3;q32.3), t(11;14)(q13;q32.3) (Poseidon Probes, Kreatech Diagnostics). Ploidy status and clonal/subclonal aberrations (ie, present in ≥ 60% vs 20%-59% of assessed

MMCs) were defined as published.<sup>30</sup> A modified copy number score<sup>30</sup> (excluding gains of 1q21) was used to assess ploidy state.

### Gene expression analysis

Gene expression profiling (GEP) was performed as previously published.<sup>31</sup> In brief, after RNA extraction, labeled cRNA was generated using the small sample labeling protocol vII (Affymetrix, Santa Clara, CA) and hybridized to U133 A + B GeneChip microarray (Affymetrix) for the training group (TG) and U133 2.0 plus arrays for the validation group (VG), according to the manufacturer's instructions. When different probe sets were available for the same gene, we chose the most specific probe set showing the maximal variance and the highest signal. Expression data for MMC samples are deposited in ArrayExpress under the accession numbers E-MTAB-81 and E-GEOD-2658.

To validate the Affymetrix gene expression data, expression of *VEGFA* (Hs00173626\_m1), *TGFA* (Hs00608187\_m1), *CTGF* (Hs00170014\_m1), and *ADM* (Hs00181605\_m1; all Applied Biosystems) was assessed by quantitative real-time polymerase chain reaction (RT-PCR) using the ABI Prism 7700 Sequence Detection System and the  $\Delta\Delta C_t$  method.<sup>32</sup>

### Intracellular staining for VEGF

Intracellular vascular endothelial growth factor (VEGF) expression (clone 23410; R&D Systems) of 10 HMCLs, primary samples of 3 MM and one MGUS patient was measured by flow cytometry using a fixation and permeabilization kit (eBioscience). Overlays were established using the Infinicyt Software (Cytognos).

### Protein detection by ELISA

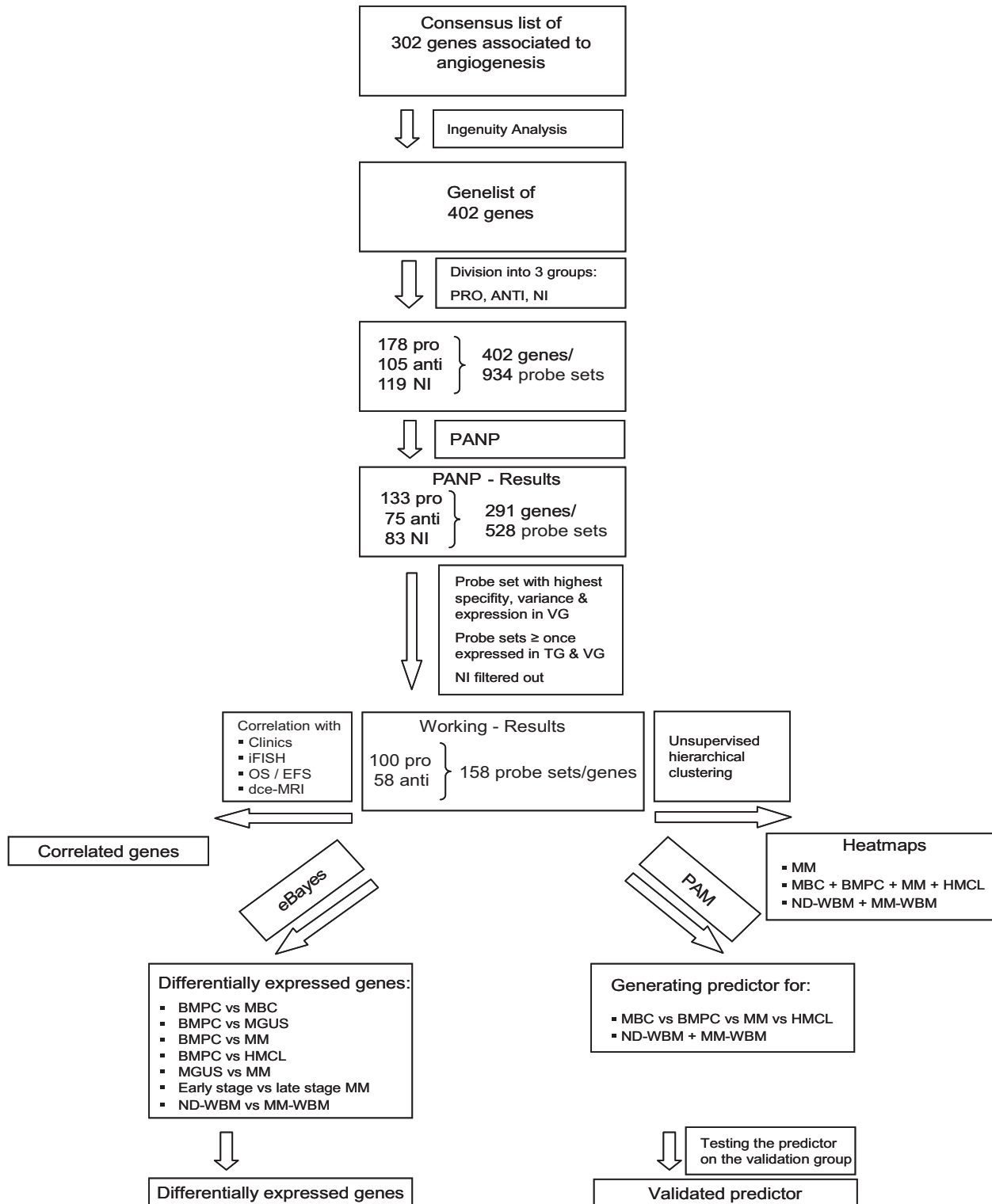
Levels of VEGF, HGF, interleukin-15 (IL-15), TGFA, and IGF-1 were measured in culture supernatants of HMCLs (n = 10), primary MMCs (n = 2), and BM sera of myeloma patients (n = 10) and NDs (n = 3) according to the manufacturer's instructions (RayBio for VEGF, HGF and IL-15; R&D Systems for TGFA and IGF-1). Culture supernatants were obtained by growing 10<sup>6</sup> cells per mL for 24 hours in serum-free RPMI 1640 without addition of IL-6 (R&D Systems).

### In vivo assessment of angiogenesis by dynamic contrast-enhanced magnetic resonance imaging

The entire spine of MM (n = 57) and MGUS patients (n = 7) was examined on a 1.5-Tesla-Tomograph (Symphony; Siemens) from the 1st cervical vertebra to the sacrum with a sagittal STIR and a sagittal T1-weighted SE as published.<sup>33</sup> Two model variables are used to describe the tissue-specific information of the signal intensity-time curves: amplitude *A* (arbitrary units) is proportional to the relative signal enhancement as a surrogate for MVD and perfusion, the exchange rate constant *kep* (minutes) reflects the contrast agent transit between the extravascular and intravascular compartment.

### In vitro assessment of angiogenesis

The angiogenic potential of 20 primary MMCs, 11 BMPCs, 6 MBC samples, and 10 HMCLs was investigated in the AngioKit assay (TCS Cellworks) according to the manufacturer's instructions. Culture supernatants were obtained as described for the enzyme-linked immunosorbent assays (ELISAs). Equal volumes of cell culture supernatants were added to the supplied growth medium. RPMI 1640, VEGF (2 ng/mL), and suramin (20  $\mu$ M) served as medium, positive, and negative controls, respectively. All experiments were performed in triplicate, except for BMPCs and MBCs, because of limitations in achievable sample size (“Results”). After 11 days, cells were analyzed using a combined CD31 ELISA/CD31 tubule staining kit (TCS Cellworks). Tubular density was monitored using an Olympus IX-70 microscope (Olympus) at 40 $\times$  magnification.



**Figure 1. Genes and probe sets included in the respective parts of the analysis.** Shown is our strategy for selecting angiogenesis-related genes. On the initial set of 402 genes after review of Medline and the Cytokines & Cells Online Pathfinder Encyclopaedia as well as Ingenuity Pathway Analysis, PANP-derived judgment of expression ("presence" vs "absence") was assessed, leading to 291 genes being present at least once. Of these, 83 genes with no exploratively attributable information (NI) on proangiogenic or antiangiogenic activity were excluded. For further analyses, the 100 proangiogenic and 58 antiangiogenic genes present at least once in the training (TG) and validation group (VG) were retained.

### Consensus list of proangiogenic and antiangiogenic genes

A consensus list of 302 genes associated with angiogenesis has been obtained by review of Medline and the Cytokines & Cells Online Pathfinder Encyclopaedia ([www.copewithcytokines.de](http://www.copewithcytokines.de)). Subsequently, genes were analyzed using Ingenuity Pathway Analysis (Ingenuity Systems) and

100 genes added. These 402 genes were divided into 3 groups: proangiogenic, antiangiogenic, and "no information," although some limitations apply to a gene expression-based analysis, as especially angio-inhibitory molecules are generated in vivo by cleavage of proteins by various proteases.

## Statistical analysis

Gene expression data were normalized to GC-robust multi-array average (GC-RMA).<sup>34</sup> To assess presence or absence of gene expression independently of Affymetrix-mismatch probe sets, the “Presence-Absence calls with Negative Probe sets (PANP)” algorithm<sup>35</sup> was used. “Aberrant expression” of a gene within the MMC samples compared with BMPCs is defined as “presence” within the MMC samples, but not at least once in BMPCs within TG and VG. Differential gene expression was assessed using empirical Bayes statistics in linear models for microarray data.<sup>36</sup> *P* values were adjusted for multiple testing controlling the false discovery rate as defined by Benjamini and Hochberg at a level of 5%.<sup>37</sup> Expression profiles of 466 samples (13 MBCs, 14 BMPCs, 23 MGUS, 300 MM, 52 HMCLs [the same 20 HMCLs on different microarrays in TG and VG as well as AMO-1, JIN-3, NCI-929, KMS-12-BM, KMS-11, KMS-12-PE, KMS-18, MM1S, JIM3, KARPAS 620, L363 and ANBL6 in VG only], and 64 WBM) divided in TG (*n* = 113, MM *n* = 65) and VG (*n* = 353, MM *n* = 235) were analyzed. To assess the association of expressed angiogenic genes (signature) with EFS<sup>23</sup> and OS<sup>23</sup> for patients undergoing HDT and ASCT (Heidelberg/Montpellier group: 48 TG, 159 VG; Arkansas group: 345), Goeman global test<sup>38</sup> was applied. Findings were validated using an independent set of 345 patients from the Arkansas group. Association of chromosomal aberrations and clinical parameters with gene expression was calculated using the 2-sample *t*-statistic. Differences in clinical parameters between defined groups were investigated by analysis of variance. Correlation was measured using the Spearman correlation coefficient ( $r_s$ ). Correlation with categorical variables was measured using the Kendall tau coefficient ( $\tau$ ). For assessing the relationship between categorical variables, Fisher exact test was used. The gene expression-based proliferation index is calculated as previously published.<sup>31</sup>

In all statistical tests, an effect was considered statistically significant if the *P* value of its corresponding statistical test was not greater than 5%. All statistical computations were performed using R,<sup>39</sup> version 2.8.1; Bioconductor,<sup>40</sup> version 2.3; and the Affymetrix Annotation Release 27. Results of the TG are shown in the supplemental data.

## Results

### Expression of angiogenesis-related genes

Gene expression of angiogenesis-related genes was evaluated using U133 A + B and U133 2.0 plus Affymetrix microarrays. Of the 402 genes initially included (Figure 1, selection strategy), 283 genes could be exploratively attributed using Medline review to be either proangiogenic (178 genes) or antiangiogenic (105 genes). Of these, 158 genes were expressed at least once in TG and VG, that is, 100 proangiogenic and 58 antiangiogenic genes, shown in Table 1. Genes not fulfilling these criteria are depicted in supplemental Table 3.

Using PANP-derived judgment of expression (“presence” vs “absence”), we found BMPCs to express 49 proangiogenic and 32 antiangiogenic genes with a median of 39 proangiogenic and 28 antiangiogenic genes in the VG, respectively (Table 1). MBCs express 47 proangiogenic and 30 antiangiogenic genes with a median of 32 proangiogenic and 19 antiangiogenic genes in the VG, respectively (Table 1). Of the proangiogenic BMPC genes, 21 genes are expressed significantly lower in MBCs, including major angiogenic factors, such as *VEGFA*, *IGF-1*, and *ANG*. Twelve genes show a significantly higher expression in MBCs, eg, *HDGF* and *PGF*; 4 antiangiogenic genes are up-regulated, 15 genes are significantly down-regulated in MBCs versus BMPCs (eg, *BMP6*, *TIMP1*, *TIMP2*; Figure 2; Table 2).

Compared with normal BMPCs, MMCs maintain expression of proangiogenic BMPC genes but show an aberrant expression of 51 proangiogenic and 26 antiangiogenic genes (Table 1; Figure 2A,B). The most frequently aberrantly expressed genes comprise

*HGF* in 74.7%, HGF-receptor *MET* in 70%, *IL-15* in 65.3%, *TGFA* in 46%, *ANG* in 30.3%, and *CTGF* in 28.3% of MMC samples (Figure 2C; Table 1). Of the proangiogenic BMPC genes, 7 show a significantly higher expression in MMCs, eg, *HGF* and *ADM*; 13 proangiogenic genes, however, are expressed significantly lower in MMCs (Figure 2; Table 3). Five antiangiogenic genes are significantly down-regulated in MMCs versus BMPCs (*PF4*, *AKAP12*, *TIMP2*, *LAMA5*, and *SERPINF1*), and 3 are up-regulated (Table 3).

Comparing MMCs of patients with early (MGUS and MMI) versus advanced-stage plasma cell dyscrasia (MMII and MMIII), we found 4 proangiogenic genes (including *IL-6*) to be significantly up-regulated and 8 down-regulated in the advanced stage. For the antiangiogenic genes, 2 genes (*IFII6* and *ERAPI1*) were significantly up-regulated and 3 (including *PF4*) down-regulated (Table 3). Comparing samples obtained from MGUS patients with MM samples, 9 genes are differentially expressed (Table 3); if this analysis is restricted to MGUS patients showing any clonal aberrations by iFISH (*n* = 5), no gene remains significant.

HMCLs maintain expression of aberrantly expressed MMC genes (Figure 2; Table 1) and show an additional aberrant expression of 3 proangiogenic and 3 antiangiogenic genes. No proangiogenic gene is aberrantly expressed or any antiangiogenic gene is lost in all HMCLs.

The unsupervised hierarchical clustering based on the proangiogenic and antiangiogenic genes shows BMPCs clustering together in a sub-branch within the MMCs of the VG. The 20 HMCLs cluster together with the MBCs, both appearing in a separate sub-branch (Figure 2D). A comparable picture was obtained with MMCs of the TG (supplemental Figure 1D).

A PAM-based predictor for MBCs, BMPCs, MMCs, and HMCLs of 133 genes calculated on the proangiogenic and antiangiogenic genes in the consensus list predicts group attribution with an estimated error rate of 3% (TG) and 3% (VG), respectively (supplemental Table 5A).

In the BMME of normal donors (ND-WBM) and myeloma patients (MM-WBM), 63 and 90 (of 100) proangiogenic as well as 34 and 53 (of 58) antiangiogenic genes are expressed. Twelve genes are differentially expressed between ND-WBM and MM-WBM (Table 4). In the unsupervised hierarchical clustering of the WBM samples, MM-WBM and ND-WBM separate (supplemental Figure 2).

A PAM-based predictor for ND-WBM and MM-WBM calculated on the 158 expressed proangiogenic and antiangiogenic genes and comprising 49 genes allows predicting the group attribution with an estimated error rate of 9% (supplemental Table 5B).

### Validation of gene expression data

To validate gene expression data, quantitative real-time PCR, flow cytometry, and ELISAs were performed. Gene expression measured by quantitative RT-PCR verifies expression of *VEGFA* ( $r_s = -0.45$ ,  $P = .2$ ), *ADM* ( $r_s = -0.84$ ,  $P = .004$ ), *CTGF* ( $r_s = -0.9$ ,  $P < .001$ ), and *TGFA* ( $r_s = -0.42$ ,  $P = .2$ ) in 10 HMCLs as detected by Affymetrix gene-chip (Figure 3A, supplemental Figure 3). An additional validation is given by the flow cytometric measurement of intracellular VEGF. VEGF expression can be detected in 10 of 10 HMCLs, 3 of 3 primary MMCs, and 1 of 1 MGUS cell samples. An exemplary primary MMC and MGUS sample as well as 2 HMCL samples are shown in Figure 3B.

Secretion of VEGF, IGF-1, HGF, IL-15, and TGFA was measured by ELISA (Table 5). Of the proangiogenic factors already expressed by BMPCs, VEGF levels above the detection threshold of 20 pg/mL can be detected in all MMC and HMCL supernatants as well as all BM sera of myeloma patients and NDs.

**Table 1. Expression of proangiogenic and antiangiogenic genes as judged by PANP**

**A**

Gene Symbol	Probeset	MBC present [%]	BMPC present [%]	MGUS present [%]	MM present [%]	HMCL present [%]	ND-WBM present [%]	MM-WBM present [%]
		(n=13)	(n=14)	(n=23)	(n=300)	(n=52)	(n=7)	(n=57)
ELK3	221773_at	76.9	100.0	100.0	98.7	92.3	100.0	100.0
ETS1*	224833_at	100.0	100.0	100.0	78.3	90.6	100.0	100.0
F11R*	223000_s_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
GRN	216041_x_at	100.0	100.0	95.7	100.0	98.1	100.0	100.0
HDGF	200896_x_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
HIF1A	200989_at	100.0	100.0	87.0	77.7	100.0	100.0	100.0
HSP90AA1	210211_s_at	100.0	100.0	100.0	99.7	100.0	100.0	100.0
IL6ST	212195_at	92.3	100.0	100.0	100.0	100.0	100.0	100.0
JUN	201466_s_at	100.0	100.0	100.0	100.0	75.0	100.0	100.0
MYC	202431_s_at	100.0	100.0	91.3	92.0	98.1	100.0	100.0
NCL	200610_s_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
ODC1	200790_at	100.0	100.0	100.0	99.7	100.0	100.0	100.0
PGF	215179_x_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
YARS	212048_s_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
ADM	202912_at	46.2	92.9	100.0	96.7	82.7	100.0	100.0
CXCL12	209687_at	0.0	92.9	60.9	41.3	3.8	42.9	50.9
FOS	209189_at	92.3	92.9	100.0	99.3	15.4	100.0	100.0
VEGFA	210512_s_at	46.2	92.9	95.7	96.7	100.0	100.0	100.0
IL6R	205945_at	15.4	85.7	91.3	92.7	94.2	100.0	100.0
IGF1	209541_at	0.0	78.6	91.3	97.3	57.7	0.0	64.9
IL8	202859_x_at	46.2	78.6	43.5	34.7	1.9	100.0	100.0
NFKB1	209239_at	100.0	78.6	65.2	85.3	86.5	100.0	100.0
PPBP	214146_s_at	69.2	78.6	60.9	39.3	0.0	100.0	100.0
CITED2	207980_s_at	38.5	71.4	91.3	98.3	28.8	100.0	100.0
RNASE4	213397_x_at	7.7	71.4	87.0	83.0	44.2	0.0	38.6
CD40	215346_at	100.0	64.3	73.9	81.0	7.7	57.1	91.2
SIRT1	218878_s_at	100.0	64.3	73.9	86.7	90.4	71.4	100.0
SOD2	215223_s_at	100.0	64.3	69.6	80.0	98.1	100.0	100.0
VEZF1	202173_s_at	92.3	64.3	65.2	74.7	90.4	100.0	100.0
AAAMP	201511_at	15.4	57.1	73.9	79.7	98.1	85.7	94.7
AGGF1*	222661_at	100.0	57.1	75.0	92.8	96.9	100.0	100.0
GPI	208308_s_at	100.0	57.1	95.7	97.7	100.0	100.0	100.0
IGF2R	201393_s_at	30.8	57.1	91.3	87.0	96.2	100.0	100.0
EPAS1	200878_at	0.0	50.0	60.9	58.3	11.5	14.3	57.9
MYH9	211926_s_at	92.3	50.0	30.4	44.7	88.5	100.0	100.0
PLAUR	210845_s_at	46.2	50.0	30.4	12.7	7.7	100.0	100.0
SEMA4D	203528_at	30.8	50.0	65.2	82.0	84.6	100.0	100.0
MDK	209035_at	0.0	42.9	60.9	39.0	13.5	0.0	36.8
CCL2	216598_s_at	0.0	28.6	0.0	1.0	1.9	0.0	1.8
CTNNA1	201533_at	92.3	28.6	65.2	71.7	75.0	100.0	100.0
CXCL16*	223454_at	66.7	28.6	31.3	17.0	18.8	100.0	96.5
IGFBP7	201163_s_at	0.0	28.6	8.7	26.3	28.8	100.0	100.0
RUNX2*	232231_at	33.3	28.6	12.5	29.8	90.6	100.0	100.0
CXCL2	209774_x_at	0.0	21.4	0.0	3.7	0.0	85.7	77.2
ENPP2	209392_at	0.0	21.4	4.3	11.3	21.2	14.3	10.5
IL1B	39402_at	0.0	21.4	8.7	7.3	0.0	100.0	98.2
PTPRJ	227396_at	46.2	21.4	52.2	42.3	36.5	100.0	100.0
NRP1	212298_at	0.0	14.3	17.4	2.7	0.0	0.0	1.8
TF	203400_s_at	0.0	14.3	4.3	4.7	0.0	0.0	7.0

Percentage of MBCs, normal BMPCs, and malignant plasma cells (MGUS, MM) as well as HMCLs; ND-WBM and MM-WBM expressing (A) proangiogenic and (B) antiangiogenic genes as judged by PANP. Depicted are only genes found to be expressed at least once in the 113 samples of the training and the 353 samples of the validation group. Proangiogenic genes expressed already at BMPC stage are depicted in gray, aberrantly expressed genes in MMC in light gray, and those significantly overexpressed in MMC with white letters on a dark gray background. Results are listed according to the percentage of BMPC samples, and those aberrantly expressed according to the percentage of MMC samples expressing the respective gene.

\*As PANP can only be assessed for U133A and U133 2.0 plus arrays, for probe sets located on the U133B chip presented data are based on the validation group only.

**Table 1. Expression of proangiogenic and antiangiogenic genes as judged by PANP (continued)**

**A (continued)**

Gene Symbol	Probeset	MBC present [%]	BMPC present [%]	MGUS present [%]	MM present [%]	HMCL present [%]	ND-WBM present [%]	MM-WBM present [%]
		(n=13)	(n=14)	(n=23)	(n=300)	(n=52)	(n=7)	(n=57)
HGF	210997_at	0.0	0.0	56.5	74.7	26.9	42.9	52.6
SRC	213324_at	23.1	7.1	73.9	73.3	30.8	0.0	1.8
MET	203510_at	0.0	7.1	65.2	70.0	76.9	0.0	33.3
IL15	205992_s_at	7.7	14.3	43.5	65.3	48.1	0.0	70.2
SMARCC1	201075_s_at	84.6	0.0	30.4	54.0	94.2	71.4	70.2
TGFA	205016_at	0.0	7.1	52.2	46.0	23.1	100.0	86.0
TNFSF13	210314_x_at	23.1	14.3	69.6	41.7	11.5	100.0	100.0
ANG	205141_at	0.0	14.3	52.2	30.3	19.2	0.0	14.0
CTGF	209101_at	0.0	0.0	13.0	28.3	1.9	0.0	36.8
HPSE	219403_s_at	0.0	0.0	26.1	25.3	44.2	100.0	96.5
VEGFC	209946_at	0.0	0.0	39.1	25.3	3.8	0.0	7.0
APOLD1	221031_s_at	0.0	0.0	30.4	21.3	57.7	0.0	8.8
PDGFD	219304_s_at	0.0	0.0	26.1	20.7	15.4	57.1	68.4
TYMP	204858_s_at	0.0	0.0	17.4	20.7	21.2	100.0	96.5
IGF1R*	225330_at	16.7	0.0	0.0	19.6	81.3	100.0	89.5
TGFB2	209909_s_at	0.0	0.0	4.3	16.7	19.2	0.0	8.8
BIRC5	202095_s_at	0.0	0.0	13.0	16.3	100.0	100.0	96.5
MMP9	203936_s_at	0.0	7.1	17.4	12.7	0.0	100.0	100.0
FGFR3	204379_s_at	0.0	0.0	0.0	12.3	17.3	0.0	8.8
VEGFB	203683_s_at	0.0	0.0	8.7	11.0	50.0	0.0	0.0
S1PR1	204642_at	38.5	0.0	0.0	10.3	5.8	100.0	91.2
FGF2	204421_s_at	0.0	0.0	8.7	9.7	7.7	0.0	3.5
KLF5	209212_s_at	0.0	0.0	0.0	9.0	3.8	100.0	93.0
EDIL3*	225275_at	0.0	0.0	0.0	8.5	12.5	0.0	5.3
PDGFB	216061_x_at	0.0	7.1	4.3	7.7	3.8	0.0	0.0
AMOTL1*	225450_at	0.0	0.0	18.8	6.4	37.5	0.0	5.3
EDN1	218995_s_at	0.0	0.0	0.0	6.3	0.0	0.0	8.8
TEK	217711_at	0.0	7.1	0.0	6.0	1.9	0.0	3.5
CAMP	210244_at	0.0	0.0	8.7	5.7	1.9	100.0	100.0
CALCRL*	234996_at	0.0	0.0	0.0	5.5	6.3	0.0	1.8
SPP1	209875_s_at	0.0	0.0	8.7	5.0	13.5	14.3	15.8
F13A1	203305_at	7.7	0.0	13.0	4.7	0.0	100.0	96.5
GNLY	37145_at	0.0	0.0	4.3	4.7	0.0	100.0	100.0
IL6	205207_at	0.0	0.0	0.0	4.7	13.5	0.0	5.3
FGF9	206404_at	38.5	0.0	0.0	4.0	38.5	0.0	15.8
SLIT2	209897_s_at	0.0	0.0	0.0	3.7	0.0	0.0	3.5
HBEGF	203821_at	0.0	0.0	4.3	3.0	30.8	100.0	73.7
ID1	208937_s_at	7.7	0.0	0.0	2.3	9.6	0.0	7.0
TNFAIP2	202510_s_at	0.0	0.0	4.3	2.3	3.8	100.0	100.0
ANGPTL6*	223967_at	0.0	0.0	18.8	1.7	0.0	0.0	0.0
PROK2*	232629_at	0.0	0.0	12.5	1.7	21.9	100.0	100.0
TNF	207113_s_at	0.0	0.0	0.0	1.3	0.0	14.3	29.8
FN1	211719_x_at	0.0	0.0	0.0	1.0	23.1	0.0	0.0
SEMA3C	203789_s_at	0.0	0.0	4.3	1.0	28.8	0.0	1.8
UNC5B*	226899_at	0.0	0.0	0.0	0.4	0.0	0.0	1.8
EGFL7	218825_at	0.0	0.0	0.0	0.3	5.8	0.0	0.0
FGFR2	203638_s_at	0.0	0.0	0.0	0.3	5.8	0.0	0.0
TERT	207199_at	0.0	0.0	0.0	0.3	30.8	0.0	0.0
MMP13	205959_at	0.0	0.0	0.0	0.0	3.8	0.0	0.0
NRP2*	225666_at	0.0	0.0	0.0	0.0	9.4	0.0	0.0
SMOC2*	223235_s_at	0.0	0.0	0.0	0.0	6.3	0.0	0.0

Percentage of MBCs, normal BMPCs, and malignant plasma cells (MGUS, MM) as well as HMCLs; ND-WBM and MM-WBM expressing (A) proangiogenic and (B) antiangiogenic genes as judged by PANP. Depicted are only genes found to be expressed at least once in the 113 samples of the training and the 353 samples of the validation group. Proangiogenic genes expressed already at BMPC stage are depicted in gray, aberrantly expressed genes in MMC in light gray, and those significantly overexpressed in MMC with white letters on a dark gray background. Results are listed according to the percentage of BMPC samples, and those aberrantly expressed according to the percentage of MMC samples expressing the respective gene.

\*As PANP can only be assessed for U133A and U133 2.0 plus arrays, for probe sets located on the U133B chip presented data are based on the validation group only.

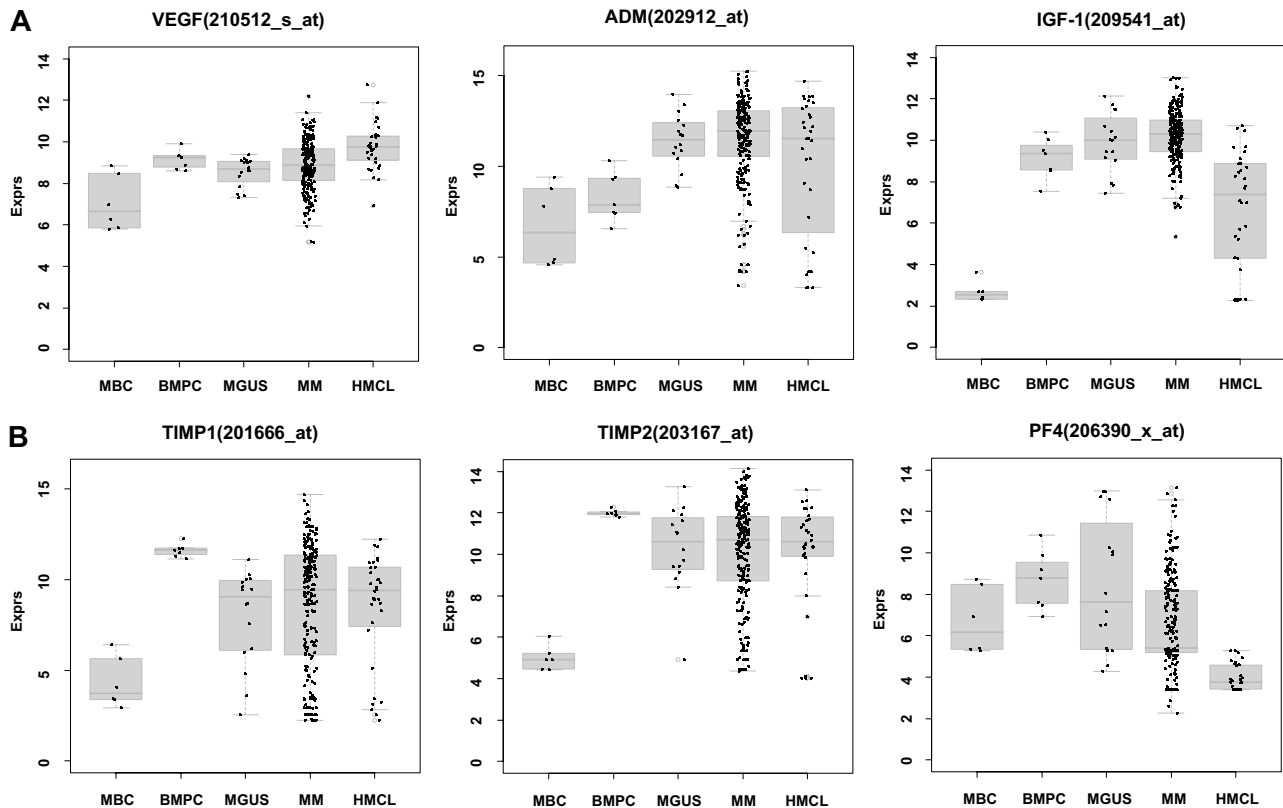
Table 1. Expression of proangiogenic and antiangiogenic genes as judged by PANP (continued)

Gene Symbol	Probeset	MBC present [%]	BMPC present [%]	MGUS present [%]	MM present [%]	HMCL present [%]	ND-WBM present [%]	MM-WBM present [%]
		(n=13)	(n=14)	(n=23)	(n=300)	(n=52)	(n=7)	(n=57)
ACVR2A	205327_s_at	100.0	100.0	100.0	100.0	96.2	0.0	64.9
JUND	203752_s_at	100.0	100.0	100.0	100.0	98.1	100.0	100.0
PTEN*	225363_at	100.0	100.0	100.0	100.0	100.0	100.0	100.0
WARS	200629_at	100.0	100.0	100.0	100.0	98.1	100.0	100.0
ZFP36	201531_at	100.0	100.0	100.0	100.0	98.1	100.0	100.0
IFI16	208966_x_at	100.0	100.0	100.0	99.7	92.5	100.0	100.0
CD55	201926_s_at	100.0	100.0	100.0	99.0	92.5	100.0	100.0
SP100	202863_at	100.0	100.0	91.3	99.0	88.7	100.0	100.0
HSD17B11	217989_at	100.0	100.0	95.7	98.7	94.3	100.0	100.0
CALR	214315_x_at	61.5	100.0	95.7	96.3	96.2	85.7	98.2
FOXO3	204132_s_at	15.4	100.0	95.7	94.0	90.6	100.0	100.0
ERAP1	210385_s_at	53.8	92.9	95.7	100.0	96.2	100.0	100.0
BMP6	206176_at	0.0	92.9	100.0	99.0	86.8	0.0	84.2
NDRG1	200632_s_at	100.0	92.9	91.3	99.0	96.2	100.0	100.0
FOXO1	202724_s_at	92.3	92.9	91.3	93.3	90.6	100.0	100.0
ZFP36L1	211962_s_at	100.0	92.9	91.3	88.7	69.8	100.0	100.0
TIMP2	203167_at	7.7	92.9	87.0	82.7	84.9	100.0	100.0
COL4A3	222073_at	100.0	92.9	34.8	43.3	66.0	0.0	3.5
EGR1	201693_s_at	0.0	78.6	56.5	64.7	3.8	71.4	70.2
PF4	206390_x_at	53.8	78.6	65.2	38.7	0.0	100.0	100.0
DAPK1	203139_at	0.0	71.4	82.6	82.3	73.6	100.0	96.5
TIMP1	201666_at	0.0	71.4	73.9	69.0	67.9	100.0	100.0
JAG1	216268_s_at	0.0	64.3	69.6	82.7	66.0	100.0	96.5
LAMA5	210150_s_at	53.8	64.3	69.6	75.3	54.7	14.3	78.9
BMPR2*	225144_at	100.0	57.1	93.8	94.0	100.0	71.4	94.7
SCYE1	202541_at	100.0	57.1	82.6	87.7	92.5	100.0	100.0
HEY1	44783_s_at	7.7	57.1	60.9	82.3	69.8	85.7	84.2
SEMA3F	209730_at	38.5	57.1	82.6	63.3	45.3	14.3	42.1
ACVR1	203935_at	0.0	50.0	69.6	86.7	66.0	0.0	71.9
BMPR1A	213578_at	38.5	50.0	65.2	61.3	77.4	0.0	21.1
APP	200602_at	92.3	50.0	47.8	52.7	66.0	100.0	100.0
SPARC	200665_s_at	30.8	21.4	60.9	50.7	49.1	100.0	100.0
HTATIP2	210253_at	84.6	0.0	47.8	46.3	81.1	100.0	98.2
TP53	201746_at	7.7	0.0	43.5	37.3	52.8	0.0	8.8
WARS2*	222734_at	0.0	0.0	50.0	35.7	46.9	14.3	38.6
ACVR2B	220028_at	0.0	0.0	30.4	28.3	50.9	0.0	3.5
ING4	218234_at	15.4	0.0	47.8	25.7	49.1	0.0	8.8
SPRY1	212558_at	0.0	7.1	17.4	20.0	39.6	0.0	35.1
PTHLH	211756_at	0.0	0.0	8.7	15.0	3.8	0.0	8.8
VASH2*	235343_at	0.0	0.0	6.3	14.9	31.3	0.0	1.8
AKAP12	210517_s_at	0.0	14.3	17.4	14.3	5.7	14.3	28.1
BAI3	205638_at	0.0	0.0	0.0	10.7	1.9	0.0	5.3
BMPR1B*	229975_at	0.0	0.0	18.8	8.5	4.3	0.0	7.0
MMP19	204574_s_at	0.0	0.0	0.0	5.3	1.9	0.0	5.3
NRG2	206879_s_at	0.0	0.0	0.0	4.7	3.8	0.0	1.8
BPI	205557_at	7.7	0.0	8.7	3.7	5.7	100.0	100.0
CXCL10	204533_at	0.0	0.0	17.4	3.7	9.4	0.0	14.0
PTN	211737_x_at	0.0	0.0	0.0	3.7	26.4	0.0	0.0
IL12A	207160_at	0.0	0.0	0.0	3.0	5.7	0.0	7.0
ADAMTS1	222162_s_at	0.0	0.0	0.0	2.7	15.1	0.0	1.8
SEMA3A	206805_at	0.0	0.0	0.0	1.0	15.1	0.0	0.0
TP73*	232546_at	0.0	0.0	0.0	0.9	0.0	0.0	0.0
SERPINF1	202283_at	0.0	7.1	0.0	0.7	13.2	42.9	19.3
IFNG	210354_at	0.0	0.0	0.0	0.3	7.5	28.6	36.8
TIMP3	201150_s_at	0.0	0.0	0.0	0.3	3.8	0.0	1.8
THBS1	201110_s_at	0.0	7.1	0.0	0.0	5.7	57.1	66.7
AGT	202834_at	0.0	0.0	0.0	0.0	20.8	0.0	0.0
CXCL14*	222484_s_at	0.0	0.0	0.0	0.0	3.1	0.0	0.0

Percentage of MBCs, normal BMPCs, and malignant plasma cells (MGUS, MM) as well as HMCLs; ND-WBM and MM-WBM expressing (A) proangiogenic and (B) antiangiogenic genes as judged by PANP. Depicted are only genes found to be expressed at least once in the 113 samples of the training and the 353 samples of the validation group. Proangiogenic genes expressed already at BMPC stage are depicted in gray, aberrantly expressed genes in MMC in light gray, and those significantly overexpressed in MMC with white letters on a dark gray background. Results are listed according to the percentage of BMPC samples, and those aberrantly expressed according to the percentage of MMC samples expressing the respective gene.

\*As PANP can only be assessed for U133A and U133 2.0 plus arrays, for probe sets located on the U133B chip presented data are based on the validation group only.





**Figure 2. Expression of proangiogenic and antiangiogenic genes.** Expression of (A) the proangiogenic genes *VEGFA*, *ADM*, and *IGF-1*, (B) the antiangiogenic genes *TIMP1*, *TIMP2*, and *PF4*, and (C) the aberrantly expressed genes *HGF*, *CTGF*, and *TGFA* as well as *MET*, *IL-15*, and *ANG* within the validation group. Supplemental Figure 1 contains information on the training group. (D) The unsupervised hierarchical clustering shows BMPCs (depicted in blue) clustering together in a sub-branch within the MMCs (depicted in white). All HMCLs (depicted in orange) clustering together with the MBCs (depicted in light blue) in a separate branch each. Supplemental Figure 1D contains information on the training group.

Measured values correlate well with *VEGFA* expression assessed by GEP for HMCLs ( $r_s = 0.74$ ,  $P < .01$ ). *IGF-1* levels above the mean detection threshold of 26 pg/mL (range given by the manufacturer, 7-56 pg/mL) can be found in 1 of 2 MMC and 4 of 10 HMCL supernatants as well as all BM sera. The values for BM sera are by several orders of magnitude higher compared with MMC or HMCL supernatants. Measured values correlate with *IGF-1* expression assessed by GEP for HMCLs ( $r_s = 0.64$ ,  $P = .05$ ). Of the aberrantly expressed factors, measured *HGF* levels are by orders of magnitude above the detection threshold within the BM sera of all samples. For HMCL supernatants, *HGF* secretion above the detection level of 8 pg/mL can be detected in all samples; however, 2 HMCL supernatants show a level around the detection threshold (8.1 and 8.4 pg/mL). Measured values correlate well with *HGF* expression assessed by GEP for HMCLs ( $r_s = 0.89$ ,  $P < .001$ ). *IL-15* levels above the median detection threshold of 3 pg/mL cannot be found in MMC or HMCL supernatants, whereas they are detectable in all BM sera, including normal BM. *TGFA* secretion above the median detection threshold of 2.27 pg/mL (range given by the manufacturer, 0.55-7 pg/mL) can be detected in 8 of 10 HMCL, 1 of 2 MMC supernatants, and all BM sera.

#### **In vitro tubule formation by supernatants of memory B cells, normal and malignant plasma cells, and myeloma cell lines**

The angiogenic potential of supernatants of 6 MBC (but for 2 in a single measurement), 11 BMPC (but for 2 in a single measurement), 20 primary MMC (in triplicates), and 10 HMCL samples (in triplicates twice) was investigated in the AngioKit model. After 11 days, in vitro tubule formation was quantified using a

CD31 ELISA and tubules were visualized by staining with an anti-CD31 (PECAM-1) antibody. Unlike those of MBCs, supernatants of BMPCs, MMCs, and HMCLs show a significant induction of tubule formation compared with medium control ( $P = .04$ ,  $P < .001$ , and  $P < .001$ , respectively; Figure 4A). Three exemplary MBC, BMPC, and MMC samples as well as HMCLs, respectively, are shown in Figure 4B.

#### **Correlation of angiogenic gene expression with biologic and clinical parameters**

When considering only genes correlated significantly in TG and VG with a coefficient more than 0.4, the only chromosomal aberration correlating with one of the angiogenic genes is t(4;14) with *FGFR3* expression (TG  $\tau = 0.47$ ,  $P = .002$ ; VG  $\tau = 0.73$ ,  $P < .001$ ). Only *BIRC5* (survivin), a gene also associated with proliferation, correlates significantly with the plasma cell labeling index ( $n = 67$ ,  $r_s = 0.54$ ,  $P = .001$ ). By correlating expression of angiogenic genes with our gene expression-based proliferation index, of the genes not part of this index, one gene shows a significant positive correlation coefficient more than 0.4 (*GPI*, TG  $r_s = 0.46$ ,  $P = .001$ ; VG  $r_s = 0.51$ ,  $P < .001$ ), 3 a negative correlation (*TERT*, TG  $r_s = -0.51$ ,  $P < .001$ ; VG  $r_s = -0.43$ ,  $P < .001$ ; *TEK*, TG  $r_s = -0.53$ ,  $P < .001$ ; VG  $r_s = -0.56$ ,  $P < .001$ ; *PDGFB*, TG  $r_s = -0.63$ ,  $P < .001$ ; VG  $r_s = -0.47$ ,  $P < .001$ ), all being proangiogenic. Thus, no obvious connection could be found between MMC proliferation and angiogenic gene expression.

The dce-MRI surrogates for perfusion (*A*) and the exchange rate constant (*kep*) do not correlate significantly with any of the

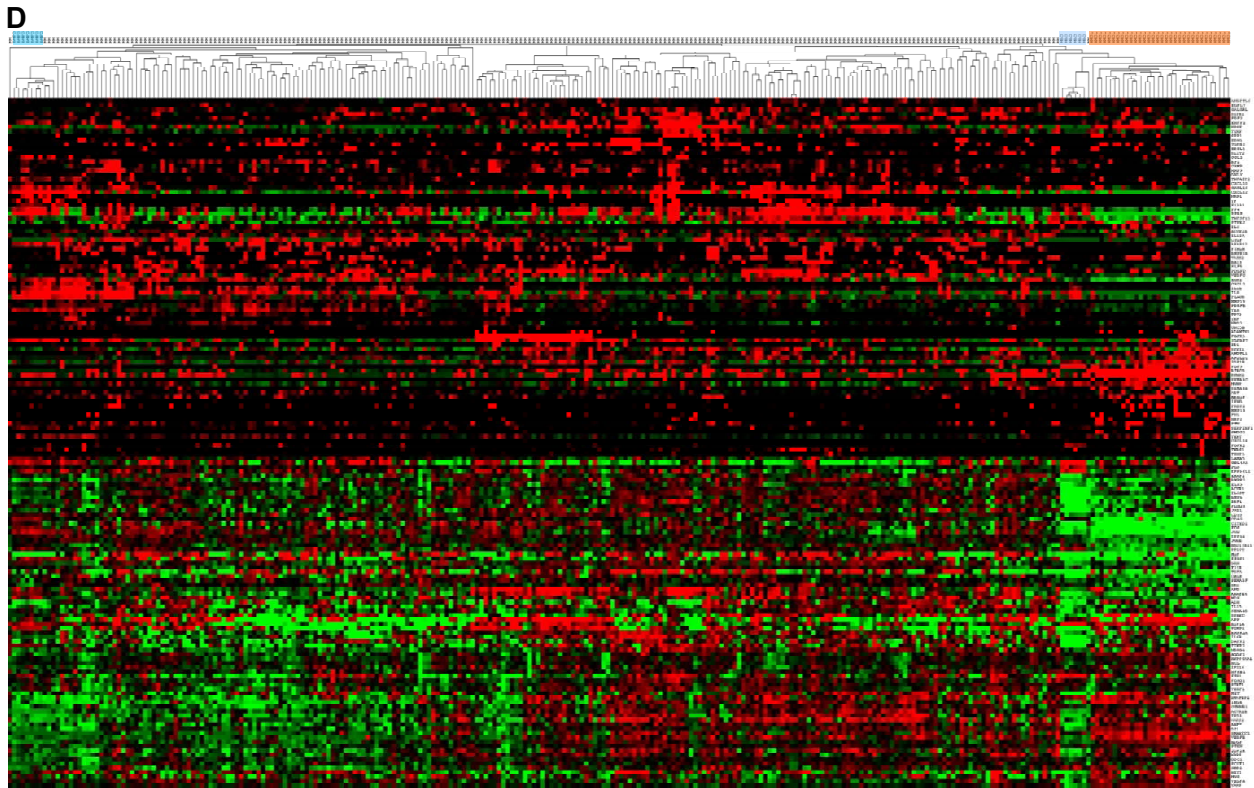
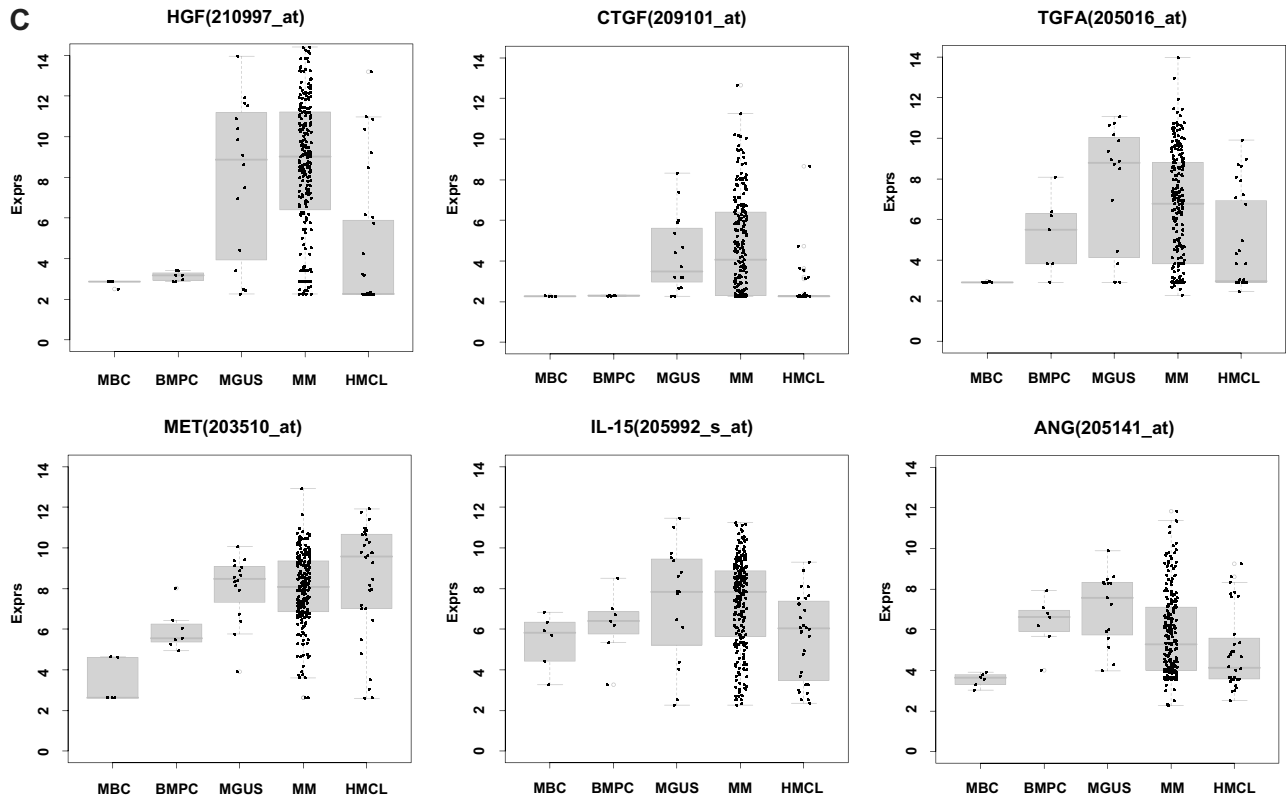


Figure 2. Continued

angiogenic genes. No correlation with the expression of (anti)angiogenic genes with those of D-type cyclins (*CCND1*, *CCND2*, *CCND3*) or clinical parameters (serum- $\beta$ 2-microglobulin (B2M), International Staging System stage, Salmon/Durie-stage, and serum albumin) could be found.

**Prognostic value of angiogenic gene expression**

Using Goeman global test, a significant association of the angiogenic gene expression (signature) could be found for EFS or OS within the VG and the Arkansas data (supplemental

**Table 2. Differential gene expression between bone marrow plasma cells and memory B cells within the validation group**

<b>A</b>					<b>B</b>				
Symbol	Probeset	Expr. BMPC	$\Delta$ MBC	P-Value MBC	Symbol	Probeset	Expr. BMPC	$\Delta$ MBC	P-Value MBC
CXCL12	209687_at	11.6	-8.6	<0.001	BMP6	206176_at	10.8	-8	<0.001
IGF1	209541_at	9.1	-6.5	<0.001	TIMP1	201666_at	11.6	-7.3	<0.001
RNASE4	213397_x_at	10.5	-5.7	<0.001	TIMP2	203167_at	12	-7	<0.001
JUN	201466_s_at	14.1	-4.8	<0.001	DAPK1	203139_at	7.6	-4.9	0.001
IL6R	205945_at	9.2	-4.6	<0.001	CALR	214315_x_at	9.1	-4.2	0.001
EPAS1	200878_at	7.9	-4.4	<0.001	FOXO3	204132_s_at	9.3	-3.9	<0.001
MDK	209035_at	6.9	-3.7	<0.001	JAG1	216268_s_at	7.8	-3.6	<0.001
IL6ST	212195_at	11.2	-3.5	<0.001	ERAP1	210385_s_at	10	-3.5	0.001
IGF2R	201393_s_at	9.4	-3.1	0.007	AKAP12	210517_s_at	5.6	-3.3	0.001
ELK3	221773_at	9.9	-2.9	0.001	ACVR1	203935_at	7.6	-3	<0.001
ANG	205141_at	6.3	-2.8	<0.001	EGR1	201693_s_at	8.2	-2.7	<0.001
MET	203510_at	6	-2.7	0.001	HEY1	44783_s_at	8.7	-2.6	<0.001
CCL2	216598_s_at	4.7	-2.5	0.03	ZFP36	201531_at	13.8	-1.2	<0.001
TGFA	205016_at	5.2	-2.3	0.008	WARS	200629_at	11.3	-0.9	0.03
AAMP	201511_at	7.5	-2.3	0.001	ACVR2A	205327_s_at	7.7	-0.5	0.01
VEGFA	210512_s_at	9.1	-2.1	0.002	SCYE1	202541_at	7.1	0.9	0.004
TEK	217711_at	5.9	-2	<0.001	ZFP36L1	211962_s_at	9.5	2	0.003
NRP1	212298_at	4.3	-2	0.02	COL4A3	222073_at	7.7	2.8	<0.001
CD40	215346_at	9.1	-1.2	0.001	HTATIP2	210253_at	5.3	3.2	<0.001
TNFAIP2	202510_s_at	3.3	-1	0.03					
HGF	210997_at	3.1	-0.3	0.01					
HDGF	200896_x_at	9.4	0.6	0.02					
ODC1	200790_at	13.6	0.7	0.02					
SRC	213324_at	5.9	0.9	0.03					
SMARCC1	201075_s_at	6.2	1.5	<0.001					
CTNNA1	201533_at	6.6	1.6	0.01					
PGF	215179_x_at	10.1	1.7	<0.001					
HIF1A	200989_at	11.2	1.9	0.001					
SOD2	215223_s_at	7.7	2.3	0.001					
ETS1	224833_at	9.9	2.7	0.001					
S1PR1	204642_at	3.2	3.2	<0.001					
PTPRJ	227396_at	6	4	0.001					
FGF9	206404_at	2.3	6.3	<0.001					

Genes with differential expression between normal BMPCs and MBCs as determined by EB statistics and Benjamini-Hochberg correction for multiple testing of genes expressed at least once in the training and validation group. (A) Proangiogenic and (B) antiangiogenic genes.

Figure 4). However, this signature is largely driven by expression of t(4;14) (eg, *FGFR3*) or proliferation-associated genes (eg, *BIRC5*). In a model including the presence of t(4;14) and the gene expression-based proliferation index as covariables, no association with survival could be found (on TG and VG only resulting from lack of t(4;14) data for the Arkansas group; supplemental Figure 5).

## Discussion

### Current hypotheses about induction of angiogenesis in multiple myeloma

Several hypotheses have been formulated to explain the induction of angiogenesis in MM:

**Table 3. Differential gene expression between normal and malignant plasma cells as well as between early and late stage myeloma within the validation group**

Ai	Symbol	Probeset	Expr. BMPC	$\Delta$ MM	P-Value MM
	HGF	210997_at	3.1	5.4	<0.001
	ADM	202912_at	8.3	3.1	0.001
	MET	203510_at	6	2.1	0.002
	HDGF	200896_x_at	9.4	1.6	<0.001
	GPI	208308_s_at	10.4	1.2	0.01
	IGF1	209541_at	9.1	1.1	0.03
	IL6ST	212195_at	11.2	0.8	0.05
	TERT	207199_at	4.3	-0.7	0.03
	MYH9	211926_s_at	7.4	-1	0.01
	TF	203400_s_at	4	-1.4	0.003
	TEK	217711_at	5.9	-1.4	0.001
	CXCL2	209774_x_at	4	-1.4	0.002
	NRP1	212298_at	4.3	-1.6	<0.001
	IL1B	39402_at	5.1	-1.7	0.02
	CCL2	216598_s_at	4.7	-2.3	<0.001
	IL8	202859_x_at	7.6	-2.7	0.01
	PLAUR	210845_s_at	7.7	-2.8	<0.001
	HIF1A	200989_at	11.2	-2.8	0.006
	PPBP	214146_s_at	10.2	-4.3	<0.001
	CXCL12	209687_at	11.6	-5.2	<0.001

Aii	Symbol	Probeset	Expr. BMPC	$\Delta$ MM	P-Value MM
	PF4	206390_x_at	8.7	-2.2	0.01
	AKAP12	210517_s_at	5.6	-2	0.02
	TIMP2	203167_at	12	-1.9	0.04
	LAMA5	210150_s_at	8.7	-1.3	0.001
	SERPINF1	202283_at	3.2	-0.8	<0.001
	SCYE1	202541_at	7.1	0.9	0.01
	ACVR1	203935_at	7.6	1.2	0.003
	ACVR2B	220028_at	3.8	1.8	<0.001

Bi	Symbol	Probeset	Expr. Early	$\Delta$ Late	P-Value Late
	NCL	200610_s_at	12.7	0.6	<0.001
	IL6	205207_at	2.3	0.4	0.03
	YARS	212048_s_at	11.8	0.3	0.006
	PGF	215179_x_at	10.1	0.2	0.02
	PLAUR	210845_s_at	7.7	-0.5	0.02
	CAMP	210244_at	3.2	-0.6	0.002
	F13A1	203305_at	3.4	-0.7	0.002
	TNFSF13	210314_x_at	6.2	-0.9	<0.001
	NRP1	212298_at	4.3	-0.9	<0.001
	CXCL16	223454_at	5.9	-1.1	<0.001
	PPBP	214146_s_at	10.2	-1.3	0.005
	CXCL12	209687_at	11.6	-2.5	<0.001

Bii	Symbol	Probeset	Expr. Early	$\Delta$ Late	P-Value Late
	PF4	206390_x_at	8.7	-1.2	<0.001
	CXCL10	204533_at	2.9	-0.7	<0.001
	THBS1	201110_s_at	2.6	-0.3	0.001
	ERAP1	210385_s_at	10	0.5	0.005
	IFI16	208966_x_at	10.5	0.5	0.007

Ci	Symbol	Probeset	Expr. MGUS	$\Delta$ MM	P-Value MM
	NCL	200610_s_at	11.4	1.3	<0.001
	EGFL7	218825_at	2.9	-0.4	0.05
	PLAUR	210845_s_at	5.9	-0.9	0.01
	NRP1	212298_at	3.8	-1.1	<0.001
	TNFSF13	210314_x_at	8.2	-1.6	<0.001
	CXCL12	209687_at	8.5	-2.1	0.01
	PPBP	214146_s_at	8.1	-2.2	0.009

Cii	Symbol	Probeset	Expr. MGUS	$\Delta$ MM	P-Value MM
	PF4	206390_x_at	8.4	-1.9	0.002
	IFI16	208966_x_at	9.3	1.4	<0.001

Genes with differential expression between normal (BMPC) and malignant plasma cells (MMC) as determined by EB statistics and Benjamini-Hochberg correction for multiple testing of genes expressed at least once in the training and validation group. (Ai) Proangiogenic and (Aii) antiangiogenic genes. (Bi) Proangiogenic and (Bii) anti-angiogenic genes differentially expressed between early-stage (MGUS + MMI) and late-stage (MMII + MMIII) myeloma. (Ci) Proangiogenic and (Cii) antiangiogenic genes differentially expressed between BMPC and MGUS.

1. Angiogenesis in MM is the result of the tumor burden and mediated by proangiogenic factors appearing at the MGUS stage (expression of *VEGF*, *bFGF*, and their receptors at a similar level in MGUS, smoldering [SMM], and active MM).<sup>41</sup> However, *bFGF* is neither expressed by BMPCs nor a larger proportion of MMCs (Table 1); it is, however, expressed in all mesenchymal stromal cell samples (n = 19, data not shown); thus, lack of expression cannot be attributed to a defective probe set. *VEGFA*, in turn, is already expressed in BMPCs.
2. An "angiogenic switch" takes place at the MMC stage resulting from the expression of oncogenes (c-myc, c-fos, c-jun, ets-1) coding for angiogenic factors as a consequence of immunoglobu-

lin-translocations and genetic instability of plasma cells,<sup>42</sup> leading to an increased bFGF expression by MMCs. Whereas almost all MMCs show chromosomal aberrations,<sup>43</sup> c-myc, c-fos, and c-jun are already expressed at BMPC stage and do not show a significant up-regulation in MMCs (Table 1). Ets-1 is not expressed in any of the BMPC or MMC samples.

3. A loss of antiangiogenic activity mediated by down-regulation of antiangiogenic factors (in MMCs or indirectly the BMME) is necessary for switch MGUS to MM.<sup>41</sup> Jakob et al<sup>44</sup> had derived the hypothesis from the fact that the angiogenic potential of BM sera is not completely abrogated by antibodies against bFGF<sup>45</sup> or VEGF.<sup>46</sup> Further evidence

**Table 4. Differential gene expression between the whole bone marrow from normal donors (ND-WBM) and myeloma patients (MM-WBM) within the validation group**

<b>A</b>						
Symbol	Probeset	Expr. ND-WBM	$\Delta$ MM-WBM	P-Value MM-WBM	cor. with PCI	P-Value
IGF1	209541_at	2.1	2.7	0.01	0.35	0.01
IL15	205992_s_at	2.7	1.8	0.02	0.29	0.03
IL6ST	212195_at	7.4	1.7	0.005	0.41	0.002
ELK3	221773_at	6.3	1.3	0.01	0.27	0.04
EGFL7	218825_at	3	-0.5	0.02	0.06	0.7
GRN	216041_x_at	11.6	-0.8	0.05	-0.19	0.2
GPI	208308_s_at	11	-1	0.04	-0.33	0.02
MYH9	211926_s_at	8.7	-1.5	0.04	-0.15	0.3

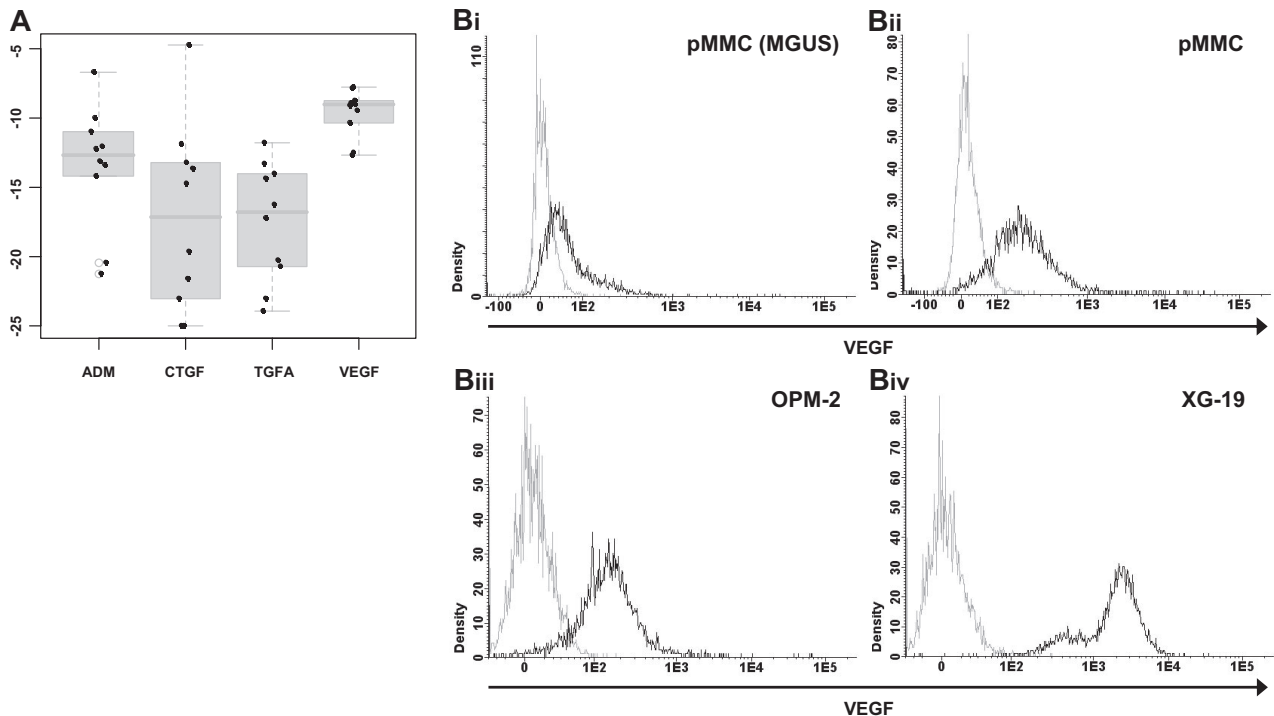
<b>B</b>						
Symbol	Probeset	Expr. ND-WBM	$\Delta$ MM-WBM	P-Value MM-WBM	cor. with PCI	P-Value
ACVR1	203935_at	3.2	1.1	0.04	0.38	0.005
ACVR2A	205327_s_at	3.1	1.1	0.02	0.56	<0.001
BMPR2	225144_at	3.9	1.4	0.02	0.39	0.004
BMP6	206176_at	2.7	3.2	0.005	0.45	<0.001

Expression as determined by EB statistics and Benjamini-Hochberg correction for multiple testing of genes expressed at least once in the training and validation group. Depicted as well is the correlation (cor) of these with plasma cell infiltration (PCI). (A) Proangiogenic and (B) antiangiogenic genes.

was indicated by a reported similar expression level of bFGF and VEGF by MMCs between MGUS, SMM, active MM,<sup>41</sup> and an in vitro inhibitory effect of MGUS samples compared with SMM or active MM.<sup>41</sup> However, in the latter case, no comparison was made to BMPC serum, and there has not been shown an inhibitory effect compared with medium. In

general, the expression level of antiangiogenic genes remains fairly constant, with a surplus of proangiogenic over antiangiogenic genes (Tables 1, 3).

4. A further discussion is whether the induction of angiogenesis (ie, MVD) correlates with tumor load (plasma cell infiltration [PCI] in the BM) or MMC proliferation.<sup>2,8</sup> Vacca et al



**Figure 3. Validation of gene expression by quantitative real-time PCR and flow cytometry.** Expression of *ADM*, *CTGF*, *TGFA*, and *VEGA* as determined by (A) quantitative real-time PCR. Shown are  $-dCt$  values (reference gene 18S RNA). (B) Flow cytometric analysis of intracellular VEGF in (i) one MGUS sample (pMMC, MGUS), (ii) one exemplary primary myeloma cell sample (pMMC), as well as the 2 myeloma cell lines (iii) OPM-2 and (iv) XG-19.

**Table 5. Secreted levels of VEGF, IGF-1, HGF, IL-15, and TGFA as measured by enzyme-linked immunosorbent assay**

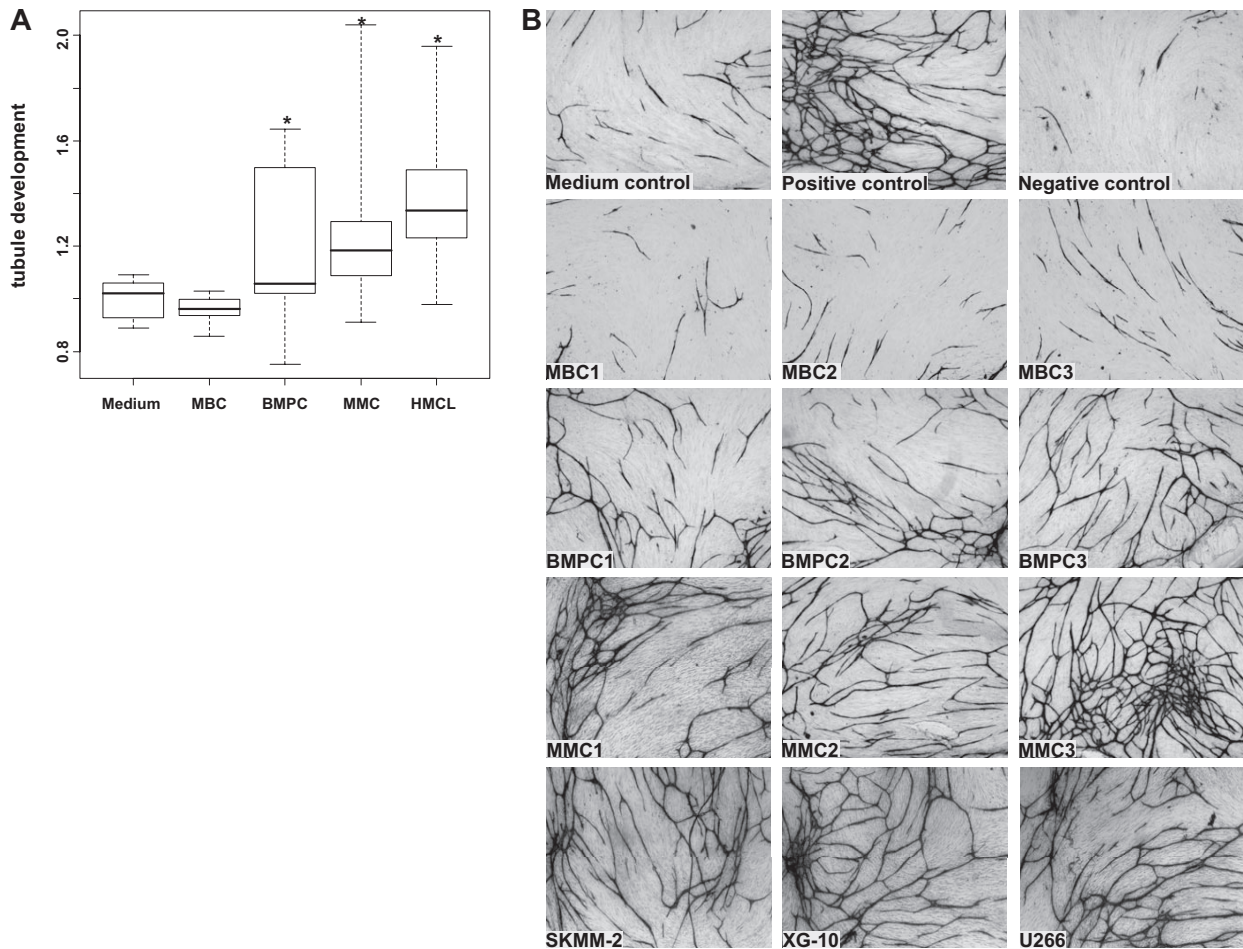
	HMCL		pMMC		BM serum (MM)		BM serum (ND)	
Samples	10		2		10		3	
VEGF (pg/mL)	9326 ±	4423	812 ±	1103	5008 ±	3830	2699 ±	1343
IGF-1 (pg/mL)	34 ±	41	28 ±	19	118943 ±	41985	144418 ±	47638
HGF (pg/mL)	1119 ±	1986	541 ±	596	2750 ±	1346	1258 ±	741
IL-15 (pg/mL)	1,4 ±	1,0	0,01 ±	0,01	21 ±	20	38 ±	19
TGFA (pg/mL)	40 ±	38	25 ±	35	70 ±	44	95 ±	48

Mean values ± SD are given.

HMCL indicates human myeloma cell line; pMMC, primary myeloma cells; BM, bone marrow; MM, multiple myeloma; and ND, normal donor.

reported a correlation of MVD with the labeling index (LI) but not with the PCI.<sup>2</sup> Rajkumar et al reported initially the same result<sup>8</sup>; in a larger series of patients, however, they found a correlation of MVD with PCI.<sup>47</sup> Niemoller et al found MVD to increase with disease progression and to correlate with PCI and B2M.<sup>48</sup> Both hypotheses sound convincing: Angiogenesis is needed for increased proliferation and infiltration, as both rely on

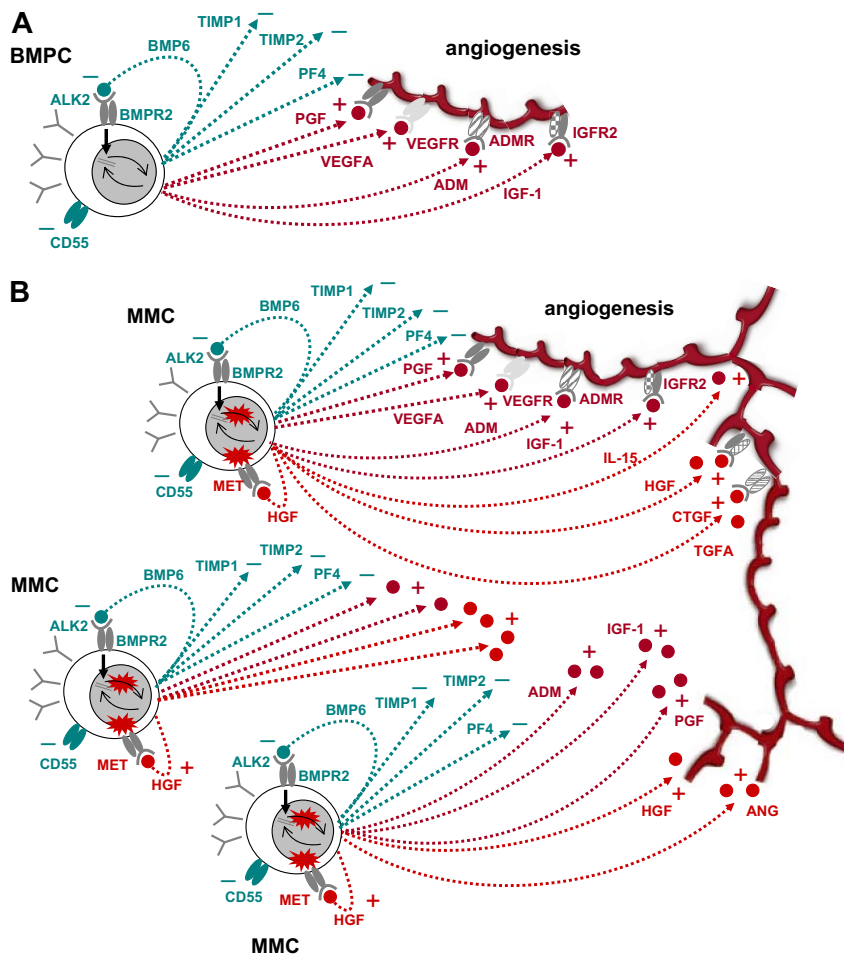
nutrition supply. In our data, however, the PCI does not significantly correlate with the expression of any proangiogenic or antiangiogenic gene, neither does the LI (n = 67) but for *BIRC5* (survivin), a gene associated with proliferation. A possible explanation is that increased LI and PCI are independent surrogates for advanced (vs MGUS or SMM) disease: PCI correlates with Salmon/Durie-stage, as does LI.<sup>49</sup>



**Figure 4. Induction of in vitro angiogenesis. Endothelial cell growth in the AngioKit model.** (A) Box plot summarizing the ELISA results. Unlike those of MBC supernatants of BMPCs, pMMCs and HMCLs show a significant induction of tubule formation compared with medium control. \*Significant difference compared with medium control ( $P < .05$ ). (B) Immunostaining with monoclonal anti-human CD31 antibody: medium control (RPMI 1640), positive control (VEGF), negative control (suramin) as well as supernatants of memory B-cell samples (MBC1-3), normal bone marrow plasma cell samples (BMPC1-3), primary myeloma cell samples (pMMC1-3), and the myeloma cell lines SKMM-2, XG-10, and U266. Original magnifications  $\times 40$ .

**Figure 5. Schematic representation of findings.**

(A) Interaction of normal BMPCs that produce proangiogenic (dark red) and antiangiogenic (dark green) factors, respectively. At a normal ratio, angiogenesis is restricted to the surrounding of BMPCs. (B) By proliferation and increasing number, aberrant as well as normal BMPC factors accumulate in the bone marrow microenvironment, inducing widespread angiogenesis.



### Angiogenesis in multiple myeloma: hypothetical model

Based on the data presented in this paper, we propose a new model of angiogenesis in MM comprising 3 hypotheses (H1-H3).

**H1: BMPCs induce controlled angiogenesis.** The function of BMPCs is to survive for several years and to produce huge quantities of antibodies (“antibody factories”). BMPCs therefore induce, in interaction with the BMME, a favorable microenvironment (“niche”), including blood-vessel supply, by producing and inducing the production of a slight surplus of proangiogenic over antiangiogenic factors. The concomitant expression of the latter allows (1) limiting the extent of the angiogenic stimulus to the vicinity of the BMPCs and (2) increasing angiogenesis in case of need by concomitant loosening of antiangiogenic breaks and increased production of proangiogenic factors.

Five lines of evidence support this hypothesis: (E1) BMPCs express proangiogenic factors, such as *VEGFA*, *ADM*, and *IGF-1*, and antiangiogenic factors, such as *TIMP1*, *TIMP2*, or *BMP6* (Table 1; Figure 2). (E2) BMPCs are found in close proximity to blood vessels in the BM,<sup>50</sup> indicating an interaction between the 2 cell types. (E3) Angiogenic factors are relevant for endothelial cell survival and maintenance of blood vessel integrity.<sup>51</sup> (E4) The strongest evidence is given by the fact that supernatants of BMPCs induce angiogenesis in our in vitro assay compared with medium controls (Figure 4). (E5) This induction cannot be seen for supernatants of MBCs and is therefore not a general characteristic of cells (of B-cell lineage). At gene expression level, MBCs show a significantly lower expression of several proangiogenic cytokines

compared with BMPCs, but likewise of antiangiogenic factors, such as *TIMP1* (Table 2; Figure 2). A possible explanation for the latter is the lack of a necessity for antiangiogenic regulation if no angiogenesis induction is present (see Figure 4).

**H2: An increase in the number of (BM) PCs increases angiogenesis.** Given a slight excess of production of proangiogenic over antiangiogenic factors in BMPCs (H1), it follows that an increase in plasma cell number yields an increase of the absolute surplus of proangiogenic factors produced in the BM. (Despite that the relative quantities remain the same, the absolute surplus in proangiogenic factors increases.) In this line of argumentation, it would not be necessary that accumulating MMCs show a differential expression of proangiogenic and antiangiogenic genes compared with BMPCs.

This hypothesis is supported by several lines of evidence: (E1) Comparing the percentage of plasma cells in the normal BM of approximately 0.5% with the infiltration rates seen in advanced MM of more than 50% and the increase in BM cellularity, the amount of plasma (myeloma) cells can be estimated to be a factor of at least 100 times higher compared with that in normal BM. Hence, a surplus in proangiogenic over antiangiogenic stimulation (on the basis of H1) would follow. (E2) If an increased rate of angiogenesis is the result of a slight excess of production of proangiogenic over antiangiogenic factors present already in BMPCs, all MM patients should show increased BM angiogenesis, which is the case. (E3) MMCs do not show a significantly higher median number of expressed proangiogenic (45) or antiangiogenic

(31) genes, and neither a single factor nor a factor combination is aberrantly expressed in all MMCs (Table 1; see also point E1 in “H3”). (E4) The BMME mirrors presence of (malignant) plasma cells, evidenced by ND-WBM clustering together (Figure S2) and the predictability of “being” ND- versus MM-WBM with an error rate of 9% (Table S5B). Indeed, of 12 genes differentially expressed between ND- and MM-WBM, 10 are already expressed by BMPCs and 7 of these correlate positively with PCI (Table 4). (E5) There is no significant association with chromosomal aberrations detected by iFISH of a single proangiogenic or antiangiogenic factor but for the association of *FGFR3* expression and t(4;14). (E6) There is no association with surrogates of tumor mass, such as B2M or International Staging System-stage, or clinical parameters. (E7) Despite a well-known increase of surrogates of MVD or BM perfusion as assessed by dce-MRI,<sup>4</sup> no association with angiogenic gene expression could be found.

**H3: Aberrantly expressed angiogenic factors by MMCs further increase BM angiogenesis in MM and might lead to different angiogenic patterns.** Evidence is given by the following observations: (E1) Despite the lack of a single aberrantly expressed factor or factor combination, 89% to 97% of MMC samples in different cohorts (TG, VG) show an aberrant expression of at least one of the angiogenic factors *HGF*, *IL-15*, *ANG*, *APRIL*, *CTGF*, or *TGFA* (Table 1). (E2) Based on expression of (anti)angiogenic genes, “being” MBC/BMPC/MMC/HMCL can be predicted fairly well (error rate: 3% TG, 3% VG) and populations separate in an unsupervised clustering (Figure 2D, supplemental Figure 1D; supplemental Table 5A) denoting a characteristic expression difference. (E3) Supernatants of MMCs and HMCLs induce higher in vitro angiogenesis compared with BMPCs (Figure 4). HMCLs here retain the proangiogenic pattern of MMCs, in analogy with HMCLs conserving signatures of BM dependence or independence.<sup>26</sup>

Based on these observations, angiogenesis seems to be a general feature of MM, not an (additional) risk factor per se for patients treated with HDT and ASCT. Another subject for further studies given these findings is whether angiogenesis may not be critical for MM pathogenesis, but just an epiphenomenon driven by the accumulation of (malignant) plasma cells and a production of proangiogenic cytokines that have a dual role as growth and survival factors for MMCs, eg, IGF-1.<sup>52</sup> Likewise, aberrant expression of the HGF receptor MET by 70% of MMCs (Table 1; Figure 2) might allow these to make use of the HGF levels present within the BM (Table 1). This possibility would arguably explain the lack of major differences in gene expression in contrast to the striking angiogenesis induction seen in the myelomatous BM compared with normal persons. A further question is if or to what extent inflammatory cells (ie, macrophages, mast cells, lymphocytes) may also contribute substantially to angiogenesis induction in myeloma.

In conclusion, in contrast to MBCs, BMPCs express a surplus of proangiogenic over antiangiogenic genes transmitting to induction of in vitro angiogenesis. Thus, already an accumulation of BMPCs can induce a basal level of angiogenesis. Aberrant expression of proangiogenic genes and down-regulation of antiangiogenic genes

by MMCs further increase the angiogenic stimulus already induced by BMPC genes, together explaining the presence of BM angiogenesis at various degrees in all myeloma patients (Figure 5). Chromosomal aberrations and changes in gene expression driving the evolution to MGUS and further to active MM thereby lead to the slow but progressive accumulation of plasma cells/MMCs, which “draw” their own supply with blood and nutrients by an induced and increased angiogenesis. This leads to a gradual change of the BMME (“BMME-switch,” Figure 5), providing in turn supply (nutrition, O<sub>2</sub>) and increased growth factor stimulation (proangiogenic cytokines with dual role) that help progressively overruling cell cycle breaks on the basis of altered D-type cyclin expression characteristic for MM.

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## Authorship

Contribution: D.H. designed research, wrote the paper, and participated in the microarray experiments; J.M. participated in the analyzing of the data; T. Meissner and A.B. performed statistical analysis; A.S. performed experiments and participated in the writing of the paper; H.G. participated in the analyzing of the data and in the writing of the paper; K.M., T.R., and M.C. participated in the analyzing of the data; J.H. collected bone marrow samples as well as clinical data and contributed in performing the dce-MRI experiments and analyzing of the data; M.H., U.B., and J.-F.R. collected bone marrow samples and clinical data; J.D.V. participated in the microarray experiments; A.J. contributed in performing the iFISH experiments; and B.K. and T. Möhler participated in the analyzing of the data and in the writing of the paper.

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<b>Characteristic</b>	<b>TG n=48</b>	<b>VG n=159</b>	<b>Arkansas n=345</b>
Age	58,5 [37-72]	57 [27-73]	57 [25-77]
Monoclonal protein			
IgG	25	97	193
IgA	11	35	93
Bence Jones	10	24	47
Asecretory	2	2	6
IgD	0	1	3
NA	0	0	3
Myeloma in Durie and Salmon stage			
I	4	16	NA
II	5	27	NA
III	39	116	NA
Myeloma in ISS stage			
I	15	80	189
II	26	50	86
III	7	26	70
NA	0	3	0
Serum $\beta$ 2-microglobulin	3.55 [1.3-11.9]	2.9 [1.3-53.6]	2.9 [1.0-38.7]
Plasma cells in bone marrow	45 [5-100]	38 [1-100]	42 [4-98]

Analysis	Details				n	
GEP	MMC	HM-group	TG	U133A+B	65	
			VG	U133 Plus 2.0	235	
					<b>300</b>	
	GEP	Arkansas-group	U133 Plus 2.0		345	
					7	
				MBC	7	
				BMPC	7	
				MGUS	7	
			HMCL	20		
				<b>41</b>		
other populations	HM-group			MBC	6	
				BMPC	7	
			MGUS	16		
			HMCL	32		
				<b>61</b>		
		WBM	WBM-ND	7		
			WBM-MM	57		
				<b>64</b>		
				<b>811</b>		
dce-MRI	HM-group			MGUS	7	
				MM	57	
				<b>64</b>		
iFISH	MMC	HM-group		TG	VG	
			t(4;14)(p16.3;q32.3)	65	167	232
			t(11;14)(p16.3;q32.3)	65	169	234
			1q21	57	162	219
			4p16	8	135	143
			6q21	41	88	129
			8p21	45	124	169
			9q34	44	108	152
			11q13	56	169	225
			11q23	57	169	226
			13q14	65	171	236
			14q32	17	162	179
			15q22	46	112	158
			17p13	58	168	226
			19q13	46	131	177
			22q11	45	89	134
	Arkansas-group	1q21				
		any (MCG)		<b>344</b>		
Survival (HDT)	MMC	HM-group	TG		48	
			VG		159	
					<b>207</b>	
		Arkansas-group			345	
					<b>552</b>	

**A**

Gene Symbol	AAAMP	ADM	AGGF1	ALOX12	AMOT	AMOTL1	ANG	ANGPT1	ANGPT2	ANGPT4	ANGPTL3	ANGPTL4	ANGPTL6	ANPEP	APOLD1	BIRC5	CALCRL	CAMP	CCL2	CD40	CDX2	CEACAM1	CITED1	CITED2	CMA1	CSF2	CSF3	CSH1	CSPG4	CTGF	CTNNB1	CXCL1	CXCL12	CXCL16	CXCL2	CXCL3	CXCL5	CXCL6	DKK4	DLL1	EDIL3	EDN1	EFNA1	EGF	EGFL7	EGFR	ELK3	ENPEP	ENPP2	EPAS1	ETS1	F11R	F13A1	F2	F3	FGF1	FGF2	FGF4	FGF6
FGF9	FGFBP1	FGFR1	FGFR2	FGFR3	FGFR4	FGG	FIGF	FLT1	FLT4	FN1	FOS	GNLY	GPI	GPR182	GRN	HAND2	HBEGF	HDOG	HGF	HIF1A	HPR	HPSE	HSP90AA1	ID1	IGF1	IGF1R	IGF2R	IGFBP7	IHH	IL15	IL17A	IL18	IL1A	IL1B	IL6	IL6R	IL6ST	IL8	IL8RA	IL8RB	JUN	KDR	KLF5	LEP	LOX	MDK	MET	MMP13	MMP14	MMP2	MMP7	MMP9	MYC	MYH9	NCL	NFKB1	NRP1	NRP2	NTN1
ODC1	PDGFA	PDGFB	PDGFD	PDPN	PGF	PLAT	PLAU	PLAUR	PLXNB1	PPBP	PROK1	PROK2	PTGES	PTPRJ	RAMP1	RAMP2	RAMP3	RLN1	RNASE4	ROBO4	RUNX2	S100A7	S1PR1	SCG2	SEMA3C	SEMA4D	SERPINE1	SHB	SHH	SIRT1	SLIT2	SMARCC1	SMOC2	SOD2	SPP1	SRC	TEK	TERT	TF	TGFA	TGFB1	TGFB2	TGFB3	TIE1	TNF	TNFAIP2	TNFSF12	TNFSF13	TPSAB1	TYMP	UNC5B	UTS2	VEGFA	VEGFB	VEGFC	VEZF1	VHL	YARS	

**B**

Gene Symbol	ACVR1	ACVR2A	ACVR2B	ACVRL1	ADAMTS1	ADAMTS8	AGT	AKAP12	ANGPTL1	ANGPTL7	APP	BA1	BA2	BA3	BMP10	BMP6	BMPRI1A	BMPRI1B	BMPRI2	BPI	CALR	CD55	COL15A1	COL18A1	COL4A1	COL4A2	COL4A3	CXCL10	CXCL11	CXCL14	CXCL9	DAPK1	EGR1	ERAP1	FBLN5	FGA	FOXO1	FOXO3	GC	GDF2	GHRL	HEY1	HRG	HSD17B11	HSPG2	HTATIP2	IFI16	IFNA1	IFNB1	IFNG	IGFBP4	IL12A
IL17F	IL24	IL4	ING4	JAG1	JUND	KNG1	LAMA5	LECT1	LTA	MMP19	MMP3	NAB2	NDRG1	NOTCH4	NRG2	PF4	PLG	PRL	PTEN	PTH1H	PTN	SCYE1	SEMA3A	SEMA3F	SERPINA4	SERPINB2	SERPINB5	SERPINF1	SLURP1	SP100	SPARC	SPON1	SPRY1	THBS1	THBS2	THBS3	THBS4	TIMP1	TIMP2	TIMP3	TIMP4	TNFRSF12A	TNFSF15	TNMD	TP53	TP73	VASH1	VASH2	WARS	WARSZ	ZFP36	ZFP36L1

**C**

Gene Symbol	ABCC8	ADM2	ADRA1B	ARD1A	ARHGAP22	ATP1F1	BAMBI	BCL9	C1GALT1	C6orf66	CANX	CCM2	CD80	CD86	CDC42BP3	CDX4	CHM	CRKL	CUL7	CYP21A2	CYSLTR1	CYSLTR2	DAPK2	DHCR7	DLL3	DLL4	E2F1	EGR2	EMCN	EMP3	EREG	FAAH	FMR1	FNDC1	FOXA2	FOXO3	FOXF1	GALR1	GALR2	GBX2	GCK	GD2	GJA5	GLMN	GMPS	GNA13	GNAI2	GPR137B	GPRC5A	GFSM3	GSC	HEY2	HIST1H2AB	HIST1H2BJ	HLX	HMGAI	HMGCR	HOXC4	IGHG1
KRT16	KRT81	LACTB	LTC4S	MAGEF1	MCL1	MIB1	MST1R	MYH7B	NARG1	NAT13	NAT5	NEBD9	NFEZL2	NOP5NOP58	NUS1	OAZ2	PADI4	PDIHX	PKM2	PLA2G2E	PLXDC1	PPAP2B	PRDM2	C2orf13	PROKR2	PROP1	PRSS23	PTPN1	PTPRC	RAB6A	RAB9A	RAC1	RASA1	RB1	RBBP9	RHOB	SDCCAG10	SELE	SEMA5A	SH2D2A	SLC22A18AS	SMO	SP1	SP3	SPG20	SPHK1	SPHK2	SREBF1	STAB1	STAB2	TBX20	TCP10	TFAP2A	THY1	TLE3	TMPPRSS6	TOB2	TSPO	UBASH3A

**A1**

Symbol	Probeset	Expr. BMPC	$\Delta$ MBC	p-Value MBC
CXCL12	209687_at	8.8	-5.9	<0.001
IGF1	209541_at	7.2	-4	<0.001
JUN	201466_s_at	12.3	-3.3	<0.001
RNASE4	213397_x_at	7.1	-3	<0.001
EPAS1	200878_at	6.4	-3	<0.001
IL6R	205945_at	7.1	-2.6	<0.001
IL6ST	212195_at	10.8	-2.3	<0.001
ELK3	221773_at	8.7	-2	0.001
VEGFA	210512_s_at	7.6	-1.8	0.006
IGF2R	201393_s_at	6.3	-1.4	0.01
TEK	217711_at	5.9	-1.3	<0.001
MDK	209035_at	6.7	-1.3	<0.001
TGFA	205016_at	4.1	-1.2	0.01
NRP1	212298_at	4.9	-1.1	0.02
CCL2	216598_s_at	4.9	-1.1	0.005
ANG	205141_at	3.7	-1	0.001
SRC	213324_at	7.4	-0.9	0.006
MET	203510_at	5.5	-0.7	0.01
AAMP	201511_at	6.4	-0.6	0.009
TNFAIP2	202510_s_at	5.1	-0.4	0.01
HGF	210997_at	3	-0.4	0.001
CD40	215346_at	7.5	0.5	0.03
ODC1	200790_at	11.6	0.6	0.006
HDGF	200896_x_at	9.3	0.6	0.004
CTNNB1	201533_at	5.7	0.9	<0.001
PGF	215179_x_at	8.4	1.2	<0.001
S1PR1	204642_at	4.1	1.2	0.02
SMARCC1	201075_s_at	5.3	1.3	0.003
PTPRJ	227396_at	5.1	1.8	0.03
FGF9	206404_at	3.4	1.8	0.007
HIF1A	200989_at	9.8	2.4	<0.001
SOD2	215223_s_at	6.9	2.8	<0.001
ETS1	224833_at	6.3	3	0.02

**A2**

Symbol	Probeset	Expr. BMPC	$\Delta$ MBC	p-Value MBC
BMP6	206176_at	8.6	-5.2	<0.001
DAPK1	203139_at	8	-4.7	<0.001
EGR1	201693_s_at	8.4	-3.7	<0.001
TIMP2	203167_at	8.7	-3.7	<0.001
AKAP12	210517_s_at	6.1	-2.8	<0.001
TIMP1	201666_at	8.2	-2.7	<0.001
FOXO3	204132_s_at	8.2	-2.3	<0.001
HEY1	44783_s_at	7.3	-2.3	<0.001
ERAP1	210385_s_at	9.2	-1.8	0.002
JAG1	216268_s_at	5.4	-1.8	<0.001
CALR	214315_x_at	8.9	-1.6	<0.001
ACVR1	203935_at	6.1	-1	0.02
ZFP36	201531_at	11.9	-0.9	0.001
WARS	200629_at	9.4	-0.7	0.008
ACVR2A	205327_s_at	7.8	-0.5	0.006
SCYE1	202541_at	6	1.8	<0.001
ZFP36L1	211962_s_at	8.8	1.9	<0.001
HTATIP2	210253_at	4.5	2.4	<0.001
COL4A3	222073_at	7.6	2.5	<0.001

**B1**

Symbol	Probeset	Expr. BMPC	$\Delta$ MM	p-Value MM
HGF	210997_at	3	4.9	<0.001
ADM	202912_at	8.1	2.2	<0.001
GPI	208308_s_at	6.9	1.8	<0.001
MET	203510_at	5.5	1.3	0.02
IGF1	209541_at	7.2	1	0.01
IL6ST	212195_at	10.8	0.9	0.03
HDGF	200896_x_at	9.3	0.6	0.02
TERT	207199_at	5.5	-0.3	0.04
MYH9	211926_s_at	7.2	-0.4	0.01
TF	203400_s_at	3.4	-0.9	0.03
TEK	217711_at	5.9	-0.9	<0.001
NRP1	212298_at	4.9	-1.1	<0.001
CCL2	216598_s_at	4.9	-1.1	<0.001
IL1B	39402_at	6.7	-1.2	0.02
PLAUR	210845_s_at	6.9	-1.5	<0.001
CXCL2	209774_x_at	5.5	-1.7	0.003
HIF1A	200989_at	9.8	-1.9	0.04
PPBP	214146_s_at	6.6	-2.4	0.01
IL8	202859_x_at	9.4	-2.5	0.002
CXCL12	209687_at	8.8	-4.4	<0.001

**B2**

Symbol	Probeset	Expr. BMPC	$\Delta$ MM	p-Value MM
PF4	206390_x_at	5.7	-1.9	0.01
TIMP2	203167_at	8.7	-1.5	0.02
AKAP12	210517_s_at	6.1	-1.4	0.03
SERPINF1	202283_at	4	-0.8	0.002
LAMA5	210150_s_at	6.7	-0.4	0.01
SCYE1	202541_at	6	1.1	0.001
ACVR1	203935_at	6.1	1.2	0.004
ACVR2B	220028_at	3.4	1.6	<0.001

**C1**

Symbol	Probeset	Expr. Early	$\Delta$ Late	p-Value Late
IL6	205207_at	3.5	1	0.03
NCL	200610_s_at	11.1	0.5	0.001
YARS	212048_s_at	8.6	0.4	0.009
PGF	215179_x_at	8.4	0.3	0.03
CAMP	210244_at	3.1	-0.5	0.04
NRP1	212298_at	4.9	-0.6	<0.001
PLAUR	210845_s_at	6.9	-0.7	0.001
F13A1	203305_at	3.5	-0.7	0.001
CXCL16	223454_at	3.2	-0.7	0.005
TNFSF13	210314_x_at	5.1	-0.9	0.006
PPBP	214146_s_at	6.6	-1.4	0.04
CXCL12	209687_at	8.8	-2.3	<0.001

**C2**

Symbol	Probeset	Expr. Early	$\Delta$ Late	p-Value Late
PF4	206390_x_at	5.7	-1.4	0.01
CXCL10	204533_at	3.3	-0.6	0.03
THBS1	201110_s_at	5.1	-0.3	0.008
ERAP1	210385_s_at	9.2	0.5	0.02
IFI16	208966_x_at	9.4	0.6	0.04

**C3**

Symbol	Probeset	Expr. BMPC	$\Delta$ MGUS	p-Value MGUS
HGF	210997_at	3	3.1	0.008
TNFSF13	210314_x_at	5.1	1.3	0.02
MET	203510_at	5.5	1	0.04
FGF2	204421_s_at	3.7	0.7	0.03
IL8	202859_x_at	9.4	-1.5	0.04
HIF1A	200989_at	9.8	-2.4	0.01

**C4**

Symbol	Probeset	Expr. BMPC	$\Delta$ MGUS	p-Value MGUS
ZFP36	201531_at	11.9	-0.6	0.03
BMP6	206176_at	8.6	0.8	0.03
HTATIP2	210253_at	4.5	0.8	0.001



**C5**

Symbol	Probeset	Expr. MGUS	$\Delta$ MM	p-Value MM
NCL	200610_s_at	10.8	0.7	<0.001
NRP1	212298_at	4.6	-0.8	<0.001
EGFL7	218825_at	5.4	-0.9	0.001
PLAUR	210845_s_at	6.4	-1.1	<0.001
TNFSF13	210314_x_at	6.4	-1.3	0.004
PPBP	214146_s_at	6.8	-2.6	0.007
CXCL12	209687_at	7.9	-3.4	<0.001

**C6**

Symbol	Probeset	Expr. MGUS	$\Delta$ MM	p-Value MM
PF4	206390_x_at	5.7	-1.8	0.02
IFI16	208966_x_at	9.1	1.1	0.009

**D1**

Symbol	Probeset	Expr. BMPC	$\Delta$ HMCL	p-Value HMCI
BIRC5	202095_s_at	3.2	5.8	<0.001
GPI	208308_s_at	6.9	3.7	<0.001
RUNX2	232231_at	3.5	2.7	0.02
HPSE	219403_s_at	2.1	2.5	<0.001
HDGF	200896_x_at	9.3	2.2	<0.001
MET	203510_at	5.5	2	0.001
IGF2R	201393_s_at	6.3	1.6	0.001
YARS	212048_s_at	8.6	1.5	<0.001
SMARCC1	201075_s_at	5.3	1.5	<0.001
SEMA3C	203789_s_at	3	1.3	0.003
HBEGF	203821_at	2.5	1.2	0.004
ODC1	200790_at	11.6	0.8	0.01
AAMP	201511_at	6.4	0.7	0.003
APOLD1	221031_s_at	5.1	0.5	0.03
TF	203400_s_at	3.4	-1.2	0.003
IGF1	209541_at	7.2	-1.2	0.02
CCL2	216598_s_at	4.9	-1.2	<0.001
TNFSF13	210314_x_at	5.1	-1.4	0.001
NRP1	212298_at	4.9	-1.4	<0.001
MDK	209035_at	6.7	-1.5	<0.001
TEK	217711_at	5.9	-1.6	<0.001
CD40	215346_at	7.5	-1.7	<0.001
PLAUR	210845_s_at	6.9	-2.2	<0.001
IL1B	39402_at	6.7	-2.4	<0.001
CXCL2	209774_x_at	5.5	-2.5	<0.001
EPAS1	200878_at	6.4	-2.6	<0.001
RNASE4	213397_x_at	7.1	-2.8	<0.001
CITED2	207980_s_at	6.6	-2.8	<0.001
PPBP	214146_s_at	6.6	-4.1	<0.001
IL8	202859_x_at	9.4	-5.8	<0.001
CXCL12	209687_at	8.8	-5.9	<0.001
JUN	201466_s_at	12.3	-6	<0.001
FOS	209189_at	10	-6.9	<0.001

## D2

Symbol	Probeset	Expr. BMPC	$\Delta$ HMCL	p-Value HMCL
EGR1	201693_s_at	8.4	-4.6	<0.001
PF4	206390_x_at	5.7	-3.3	<0.001
JUND	203752_s_at	13.2	-3.2	<0.001
ZFP36	201531_at	11.9	-3.1	<0.001
AKAP12	210517_s_at	6.1	-2.5	<0.001
ZFP36L1	211962_s_at	8.8	-2.3	<0.001
BMP6	206176_at	8.6	-1.9	<0.001
CD55	201926_s_at	9.7	-1.9	<0.001
SP100	202863_at	8.1	-1.5	<0.001
FOXO3	204132_s_at	8.2	-1.2	0.03
HSD17B11	217989_at	9	-1.1	0.02
CALR	214315_x_at	8.9	-0.8	0.04
LAMA5	210150_s_at	6.7	-0.8	<0.001
IFNG	210354_at	2.8	0.5	0.05
TP53	201746_at	2.6	0.7	0.02
PTEN	225363_at	9.2	0.9	0.03
ING4	218234_at	3.7	1.2	<0.001
PTN	211737_x_at	3.6	1.2	0.01
ACVR2B	220028_at	3.4	1.6	<0.001
SCYE1	202541_at	6	1.6	<0.001
HTATIP2	210253_at	4.5	2.5	<0.001

## A1

Nr	Gene Symbol	Probeset			
1	BIRC5	202095_s_at	67	NFKB1	209239_at
2	FOS	209189_at	68	TIMP1	201666_at
3	JUN	201466_s_at	69	IL15	205992_s_at
4	BMP6	206176_at	70	IFI16	208966_x_at
5	IGF1	209541_at	71	SEMA3F	209730_at
6	CITED2	207980_s_at	72	HBEGF	203821_at
7	CXCL12	209687_at	73	PDGFB	216061_x_at
8	JUND	203752_s_at	74	AAMP	201511_at
9	ADM	202912_at	75	MDK	209035_at
10	SOD2	215223_s_at	76	TGFA	205016_at
11	ZFP36	201531_at	77	SIRT1	218878_s_at
12	IL6ST	212195_at	78	RUNX2	232231_at
13	COL4A3	222073_at	79	PTN	211737_x_at
14	EGR1	201693_s_at	80	CTGF	209101_at
15	SP100	202863_at	81	MYH9	211926_s_at
16	DAPK1	203139_at	82	VEZF1	202173_s_at
17	ZFP36L1	211962_s_at	83	EGFL7	218825_at
18	IL6R	205945_at	84	THBS1	201110_s_at
19	IL8	202859_x_at	85	TF	203400_s_at
20	GPI /// LOC100133951	208308_s_at	86	MYC	202431_s_at
21	HIF1A	200989_at	87	AGGF1	222661_at
22	VEGFA	210512_s_at	88	BMPR2	225144_at
23	IGF2R	201393_s_at	89	ACVR2A	205327_s_at
24	HGF	210997_at	90	SRC	213324_at
25	ELK3	221773_at	91	NCL	200610_s_at
26	HDGF	200896_x_at	92	SMOC2	223235_s_at
27	F13A1	203305_at	93	CXCL14	222484_s_at
28	PLAUR	210845_s_at	94	SERPINF1	202283_at
29	CD55	201926_s_at	95	CALR	214315_x_at
30	ACVR2B	220028_at	96	GRN	216041_x_at
31	PPBP	214146_s_at	97	TIMP3	201150_s_at
32	HEY1	44783_s_at	98	AMOTL1	225450_at
33	ERAP1	210385_s_at	99	SPP1	209875_s_at
34	CXCL2	209774_x_at	100	ENPP2	209392_at
35	FOXO3	204132_s_at	101	AGT	202834_at
36	HPSE	219403_s_at	102	WARS	200629_at
37	JAG1	216268_s_at	103	NRG2	206879_s_at
38	EPAS1	200878_at	104	PTEN	225363_at
39	NRP1	212298_at	105	BMPR1B	229975_at
40	ACVR1	203935_at	106	LAMA5	210150_s_at
41	SMARCC1	201075_s_at	107	ANG	205141_at
42	HTATIP2	210253_at	108	CXCL16	223454_at
43	TIMP2	203167_at	109	ODC1	200790_at
44	CD40	215346_at	110	CTNNB1	201533_at
45	PF4	206390_x_at	111	SEMA4D	203528_at
46	RNASE4	213397_x_at	112	PGF	215179_x_at
47	MET	203510_at	113	FN1	211719_x_at
48	YARS	212048_s_at	114	IFNG	210354_at
49	TNFSF13	210314_x_at	115	PTPRJ	227396_at
50	TEK	217711_at	116	TNF	207113_s_at
51	IL1B	39402_at	117	IGF1R	225330_at
52	AKAP12	210517_s_at	118	APOLD1	221031_s_at
53	SCYE1	202541_at	119	PROK2	232629_at
54	ING4	218234_at	120	BAI3	205638_at
55	ID1	208937_s_at	121	SPARC	200665_s_at
56	HSP90AA1	210211_s_at	122	TNFAIP2	202510_s_at
57	FGF9	206404_at	123	VEGFC	209946_at
58	S1PR1	204642_at	124	PTHLH	211756_at
59	HSD17B11	217989_at	125	NRP2	225566_at
60	SEMA3C	203789_s_at	126	CAMP	210244_at
61	CCL2	216598_s_at	127	BMPR1A	213578_at
62	IL6	205207_at	128	APP	200602_at
63	ETS1	224833_at	129	CALCRL	234996_at
64	VEGFB	203683_s_at	130	TYMP	204858_s_at
65	KLF5	209212_s_at	131	FGFR3	204379_s_at
			132	TP53	201746_at
			133	PDGFD	219304_s_at

**A2**

	MBC	BMPC	MM	HMCL	Class Error rate
MBC	7	0	0	0	0
BMPC	0	7	0	0	0
MM	0	3	0	62	0.05
HMCL	0	0	0	20	0
Overall error rate = 0.03					

**A3**

	MBC	BMPC	MM	HMCL	Class Error rate
MBC	6	0	0	0	0
BMPC	0	3	4	0	0.6
MM	0	2	231	2	0.02
HMCL	0	0	0	32	0
Overall error rate = 0.03					

**B1**

Nr	Gene Symbol	Probeset
1	BMP6	206176_at
2	IGF1	209541_at
3	IL6ST	212195_at
4	IL15	205992_s_at
5	ELK3	221773_at
6	BMPR2	225144_at
7	MYH9	211926_s_at
8	CAMP	210244_at
9	ACVR2A	205327_s_at
10	KLF5	209212_s_at
11	ACVR1	203935_at
12	GPI	208308_s_at
13	BIRC5	202095_s_at
14	MMP9	203936_s_at
15	MET	203510_at
16	GRN	216041_x_at
17	RNASE4	213397_x_at
18	CTGF	209101_at
19	IGF1R	225330_at
20	BPI	205557_at
21	LAMA5	210150_s_at
22	CD40	215346_at
23	EPAS1	200878_at
24	TNFSF13	210314_x_at
25	MDK	209035_at
26	SIRT1	218878_s_at
27	TIMP2	203167_at
28	IFI16	208966_x_at
29	HTATIP2	210253_at
30	EGFL7	218825_at
31	HEY1	44783_s_at
32	VEGFC	209946_at
33	BMPR1A	213578_at
34	JUN	201466_s_at
35	ODC1	200790_at
36	PDGFD	219304_s_at
37	RUNX2	232231_at
38	SERPINF1	202283_at
39	IGF2R	201393_s_at
40	NRG2	206879_s_at
41	ANG	205141_at
42	PLAUR	210845_s_at
43	COL4A3	222073_at
44	F11R	223000_s_at
45	ACVR2B	220028_at
46	HGF	210997_at
47	PTH1H	211756_at
48	PDGFB	216061_x_at
49	IL6R	205945_at

**B2**

	<b>ND-WBM</b>	<b>MM-WBM</b>	<b>Class Error rate</b>
<b>ND-WBM</b>	7	0	0
<b>MM-WBM</b>	6	51	0.11
<b>Overall error rate = 0.09</b>			

