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BONE MARROW MESENCHYMAL STEM CELLS ARE ABNORMAL IN MULTIPLE MYELOMA

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CJ and MK performed the experiments and participated in the writing of the paper
AM participated in the design of the research and the writing of the paper
GM contributed to new analytical tools and gave technical assistance with the experiments
HA collected bone marrow samples and clinical data
FS gave technical assistance with the experiments
DC and LP collected bone marrow samples and clinical data
KB participated in the design of the research and the writing of the paper
RT performed the bio-informatic studies and participated in the writing of the paper
BP participated to the design of the research and wrote the paper

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Abstract

Recent literature suggested that cell of the microenvironment of solid tumors could be abnormal as well. To address this hypothesis in multiple myeloma (MM), we studied bone marrow mesenchymal stem cells (BMMSCs), the only long-lived cells of the bone marrow microenvironment, by gene expression with Affymetrix arrays and phenotypic and functional study in 3 groups of individuals: patients with MM and those with monoclonal gamopathy of undefined significance (MGUS), and healthy aged-matched subjects. Gene expression profile independently classified the BMMSCs of these individuals in a normal and in a MM group. MGUS BMMSCs were interspersed between those 2 groups. Among the 145 distinct genes differentially expressed in MM and normal BMMSCs 46% were involved in tumor-microenvironment cross-talk. Known soluble factors involved in MM pathophysiologic features, (interleukin (IL)-6, IL-1 β , DKK1 and amphiregulin, were revealed and new ones found. In particular, GDF-15 was found to induce dose-dependant growth of MOLP-6, a stromal cell-dependent myeloma cell line. Functionally, MM BMMSCs induced an over-growth of MOLP-6, and their capacity to differentiate into an osteoblastic lineage was impaired. Thus, BMMSCs from MM patients could create a very efficient niche to support the survival and proliferation of the myeloma stem cells.

Introduction

The tumor-associated microenvironment constitutes a permissive milieu for the induction, selection and expansion of cancer cells¹⁻³. Conversely, neoplastic cells may modify the microenvironment through cell communication proteins, in particular growth factors. Genetic profiling of solid tumors has shown abnormal gene expression in both cancer cells and cells from the microenvironment⁴. The result is a vicious circle, whereby each cell type contributes to the growth of the tumor. Elucidating the role of the microenvironment is a major concern in finding ways to disrupt this vicious circle and induce cancer cell apoptosis.

Multiple myeloma (MM) is a B-cell neoplasia that affects 2000 new patients per year in France, 15 000 in Europe and 15 000 in the United States. This disease is still fatal, with a median survival of 4.6 years^{5,6}. MM is characterized by the proliferation of a malignant plasma cell clone, initially located in the bone marrow microenvironment (BMMe). This illness is unique among hematological malignancies in its capacity to cause great bone destruction, leading to pathologic bone fractures and intractable bone pain. This result is the consequence of an imbalance between osteoblastic and osteoclastic activity induced by MM cells⁷. Impaired osteoblastic function may be the consequence of a direct action on the osteoblasts, since MM cells enhance their apoptosis^{8,9}. MM cells may also block the differentiation of bone marrow mesenchymal stem cells (BMMSCs) into osteoblasts, by producing the Wnt inhibitors DKK1 or sFRP2^{10,11} or the EGF family member amphiregulin¹².

The BMMe is a complex network of extracellular matrix and at least 4 cell partners, namely osteoclasts, mesenchymal cells (BMMSCs and their progeny, osteoblasts, adipocytes), lymphoid cells and endothelial cells. Osteoclasts and endothelial cells were shown to provide efficient support for MM cell survival and proliferation. Indeed, BMMSCs strongly support MM cell growth¹³, producing a high level of interleukin (IL)-6, a major MM cell growth factor¹⁴. BMMSCs also support osteoclastogenesis and angiogenesis¹⁵⁻¹⁸. Previous study suggested that the BMMSCs in MM differ from those of healthy donors^{19,20}. In particular, MM BMMSCs expressed less CD106

and fibronectin and 2.8 times more IL-1 β as compared with normal BMMSCs²¹ and showed abnormal synthesis of hyaluronan^{22,23}.

Taken together, these data suggest that MM BMMSCs differ from their normal counterparts. To address this question we conducted studies in 3 complementary directions: gene expression profile (GEP) with microarray analysis, and phenotypic and functional studies. We compared 3 groups of individuals: MM patients, patients with monoclonal gamopathy of unknown significance (MGUS) and healthy age-matched subjects. GEP was able to discriminate BMMSCs in MM and healthy subjects, with MGUS BMMSC genes equally distributed between those 2 groups. We found 145 genes and 18 ESTs differentially expressed between normal and MM BMMSCs. Among the 127 genes annotated with Gene Ontology, 46% corresponded to molecules potentially involved in the tumor microenvironment. Functionally, MM BMMSCs were as potent as their normal counterparts in supporting the growth of hematopoietic stem/progenitor cells. They induced an over-growth of a stromal cell-dependent myeloma cell line. Finally, their capacity to differentiate into an osteoblastic lineage was impaired.

Patients, materials and methods

Subjects

Bone marrow samples of 22 patients with newly-diagnosed MM and 7 with MGUS were obtained after patients gave written informed consent. We selected 10 patients with stage-I and 12 with stage-III MM. Patients' characteristics are shown in Table 1. Eleven healthy people who were allogeneic bone marrow donors or were undergoing total hip replacement surgery and gave their informed consent, were also included. These healthy donors had the same age range as the patients with MM.

Cells

Bone marrow from patients was obtained by sternal puncture. Marrow from healthy individuals was obtained from filters used during the preparation of the allogeneic hematopoietic grafts or

from tiny bony fragments obtained during the hip surgery. For the primo-culture (P0), bone marrow cells were cultured (at 5×10^4 cells/cm²) in α MEM + 10% fetal calf serum + 10 μ g/ml of ciprofloxacin used as an antibiotic. After 3 days in culture, nonadherent cells were harvested, and medium was replaced by fresh medium. Then the cultures were fed 2 times a week until confluence or until 21 days. BMMSCs were detached by use of trypsin (Invitrogen, Cergy-Pontoise, France) and counted by use of trypan blue exclusion. They were cultured once, at 10^3 cells/cm², under the same conditions (first passage, P1). BMMSCs at P0 or P1 were used immediately or frozen until use.

To evaluate the frequency of colony-forming unit-fibroblast (CFU-F), fresh bone marrow cells were seeded at 2×10^4 cells/cm², P0 cells at 8 cells/cm² and P1 cells at 16 cells/cm² for 11 days as indicated above. Culture vessels were then washed with phosphate-buffered saline, fixed with methanol and stained with Giemsa (Oxoid, Dardilly, France), and colonies with more than 50 cells were counted. The CFU-F expansion at each passage was calculated as the total CFU-F at the end of the culture divided by that at culture start.

CD34⁺ cells from 2 granulocyte colony stimulating factor (G-CSF)-mobilized patients were purified (90% \pm 2% purity) with use of a MACS system (Miltenyi Biotec, Paris, France) according to the manufacturer's recommendations and frozen until use.

The XG-1 myeloma cell line²⁴ was cultured in RPMI-1640 medium with 10% FCS, ciprofloxacin and IL-6 (2 ng/ml, R&D Systems, Lille, France). The MOLP-6 cell line, a generous gift of Dr. Harashima²⁵, was grown on bone marrow stromal cells in RPMI-1640 with 10% FCS and ciprofloxacin.

Differentiation of BMMSCs into osteoblasts

BMMSCs (after P0 or P1) were seeded at 10^4 cells/cm² in 96-, 24- or 12-well culture plates in the culture medium plus 0.1 μ M dexamethasone and 50 μ g/ml ascorbic acid (Sigma, Lyon, France). For mineralization study, 3 mM of inorganic phosphate (Sigma, France) was also added. Medium was changed twice a week. Cultures were stopped after 21 days. Then, alkaline phosphatase

activity was assessed qualitatively and quantitatively by fast violet staining and use of Attophos (Promega, Charbonnières, France), respectively, and mineralization by von Kossa staining²⁶ and alizarine red staining, as described by Gregory²⁷, respectively.

Co-culture of BMMSCs and CD34⁺ cells:

P1 frozen BMMSCs were thawed and put in 12-well plates in duplicate at 5000 cells/cm² in 1 ml of culture medium. After 24 h, the wells were emptied, and 4×10^4 CD34⁺ cells were seeded in each well in 2 mL Myelocult medium containing 10 μ M hydrocortisone (Stem Cell Technologies, Grenoble, France). At 7, 14, 21 and 28 days, half of the medium with nonadherent cells was replaced by fresh Myelocult medium with hydrocortisone. The cells in the supernatant were counted and assayed for hematopoietic progenitors in methyl-cellulose (Miltenyi Biotec, France). At the end of the co-culture (day 35), the cells in suspension and adhering to the BMMSCs were assayed for hematopoietic progenitors. The total number of clonogenic progenitors was calculated (number of colonies x total number of cells in the co-culture/number of seeded cells in methyl-cellulose).

Co-Culture of BMMSCs and myeloma cell lines

XG-1: BMMSCs were seeded in 12-well plates in quadruplicate at 11250 cells/cm² in 1 mL of culture medium. After 24 h of culture, the medium was eliminated, and 31250 XG-1 cells were added to each well in 1 ml of RPMI 1640 + 10% FCS. For each sample, 2 of the 4 wells received 10 μ g/mL of a neutralizing anti-IL-6 antibody²⁸. For each condition, the number of XG-1 cells was evaluated at days 4 and 7.

MOLP-6: BMMSCs were seeded in 12-well plates in quadruplicate at 11250 cells/cm² in 1 mL of culture medium. After 24 h of culture, the medium was eliminated and 20000 MOLP-6 cells were added in each well in 1 ml of RPMI 1640 + 10% FCS. The number of MOLP-6 cells was evaluated at days 4 and 7.

Flow cytometry

BMMSCs were phenotyped by flow cytometry with FITC- or phycoerythrin-conjugated anti-CD90, anti-CD45, anti-CD13 (Beckman-Coulter, Villepinte, France) and anti-CD73 (Becton-Dickinson, Le Pont de Claix, France) monoclonal antibodies (MoAb). Fluorescence was analyzed with use of a FACScan flow cytometer (Becton-Dickinson).

ELISA

The concentration of the following molecules was evaluated in the culture supernatant of BMMSCs by use of a commercial ELISA kit (R&D systems): IGF-1, IL-1 β , IL-6, GDF-15, SDF-1 and amphiregulin. DKK1 concentration was assessed with use of in-house ELISA with anti-mouse IgG-coated microplates. The capture DKK1 antibody (mouse MoAb to human DKK1, R&D Systems) was incubated for 1 h. After 3 washings, culture supernatants or standards (DKK1 concentrations ranging from 0 to 20 ng/ml) were incubated for 2 h. After 3 washings, the detection antibody (biotinylated goat anti-human DKK1) was incubated for 2 h. After 3 washings, the HRP-labeled streptavidin was added to each well and incubated for 20 min. After 3 additional washings, the substrate was added and incubated for 20 min in the dark, then 50 μ l of the stop solution was added and the plate was gently agitated. Optical density was read at 450 nm by use of a microplate reader.

Affymetrix microarrays

At the end of P1, the mRNA of BMMSCs from 7 healthy donors, 5 patients with MGUS and 6 with MM was extracted by use of the RNeasy Kit (Quiagen, Hilden, Germany). Biotinylated cRNA synthesis, hybridization to human U133 plus 2.0 GeneChip microarrays (54,675 probe sets for approximately 39,000 genes) and analysis were performed as previously described¹².

Real-time RT-PCR analysis

Complementary DNA was obtained from 2 μ g of total RNA with use of Superscript II reverse transcriptase (Life Technologies) and oligo d(T)¹²⁻¹⁸ (Amersham Pharmacia Biotech, Orsay, France) used as a primer. We used the assay-on-demand primers and probes and the TaqMan Universal Master Mix from Applied Biosystems (Courtaboeuf, France) according to the

manufacturer's instructions. Gene expression was measured by use of the ABI Prism 7000 Sequence Detection System. For comparative analysis of gene expression, data were obtained by the $\Delta\Delta C_t$ method derived from a mathematical approach. For each sample, the C_T value for the gene of interest was determined, normalized to its respective C_T value for GAPDH ($\Delta C_T = C_T - C_T \text{ GAPDH}$) and compared to one BMMSC sample used as a positive control. The formula used was: $1/2^{\Delta\Delta C_T} \text{ sample} - \Delta C_T \text{ control cell line}$. C_t values were collected during the log phase of the cycle. The results were expressed as the relative mRNA level to control cell mRNA.

Statistical analysis

Scanned data were uploaded in our bioinformatics facility. Probesets that were not expressed in at least one sample, using the Absent/Present "Call detection" algorithm (MAS5 software from Affymetrix²⁹), were discarded. To compare normal and MM BMMSCs, we performed an unsupervised classification with both principal component analysis (PCA) and hierarchical clustering³⁰. The principal component analysis involved original scripts based on the R statistics software through the RAGE bioinformatics facility (<http://rage.montp.inserm.fr>). In a two-scale representation, the separation between groups was indicated by the angle between sample vectors, which must be the smallest among classes for homogeneity but the highest between classes to ensure the best separation. Hierarchical clustering involved use of the Cluster and TreeView softwares (<http://rana.lbl.gov/EisenSoftware.htm>). GEP were analyzed with use of RAGE and confirmed with the Significance Analysis of Microarrays (SAM) software (<http://www-stat.stanford.edu/~tibs/SAM/>). Statistical comparisons involved the Mann-Whitney test, Kruskal-Wallis ANOVA, Spearman correlation and Student's *t*-test (CSS Statistica, Statsoft, Maisons-Alfort, France).

Results

BMMSC phenotype, growth characteristics and hematopoietic support

BMMSCs from 11 healthy donors were compared to those from 22 MM patients (10 stage I and 12 stage III). Normal and MM BMMSCs expressed the same density of CD13, CD73, and CD90 but lacked CD45 (results not shown). Because the BMMSC proliferation is correlated with age of donors, we chose normal donors who were age matched with the MM patients (Table 1). The CFU-F concentration in bone marrow did not differ between healthy donors and patients with MM, between patients with stage I or stage III MM, or between patients with or without bone lesions (Table 2). Cell density in the cultures at P0 and P1 did not differ among groups. However, CFU-F expansion was significantly reduced at the end of P0 for MM BMMSCs as compared to normal BMMSCs (1934-fold \pm 512 vs. 5588-fold \pm 2175, respectively, $P = 0.048$). No difference in CFU-F expansion at P0 was found between patients with stage I or stage III MM or patients with or without bone lesions (Table 2). BMMSCs from MGUS patients showed growth characteristics similar to those of MM patients. MM and normal BMMSCs did not differ significantly in their long-term ability to support hematopoietic stem/progenitor cell growth. As shown in Figure 1, the total number of GM-CFU generated in the cultures did not significantly differ when either normal or MM BMMSCs were used as hematopoietic stem cell support. Finally, MM BMMSCs did not carry the chromosome abnormalities found in MM cells by FISH (results not shown).

Genetic expression profiles distinguish normal BMMSCs from MM BMMSCs.

The genetic profile of 7 BMMSCs from normal donors, 6 BMMSCs from patients with stage III MM and 5 BMMSCs from MGUS patients was determined with Affymetrix U133 2 plus microarray (55000 probesets). We chose stage III MM patients to maximize the potential differences with use of normal subjects. Unsupervised analysis by PCA and hierarchical clustering first involved only normal and MM BMMSCs, with 2008 probesets expressed at least once (Present call) in all the samples and a high variation coefficient ($\geq 60\%$) between samples. Both unsupervised analyses delineated 2 homogeneous clusters of genes: an MM BMMSC and a normal

BMMSC cluster (Fig. 2A). When MGUS BMMSCs were considered, MGUS BMMSCs interspersed between the “MM cluster” and the “normal cluster” (Fig. 2B). These results suggest that microarrays could pinpoint differences between MM and normal BMMSCs.

Comparison of gene expression between malignant and normal BMMSCs

We then used supervised analysis to investigate genes differentially expressed between the MM and normal BMMSCs. Signal data from the 2 classes were filtered according to the ratio of the means of expression values in each sample group and a Mann-Whitney non-parametric rank test as previously described³¹. A total of 183 probesets was differentially expressed between normal and MM BMMSCs (at least one presence, ratio of means of expression ≥ 2 or ≤ 0.5 , $P \leq 0.01$). Among them, 103 showed high expression in the MM BMMSCs. We also performed a supervised analysis using SAM software on the same samples with one-presence and 2-ratio filters and 100 permutations, which resulted in a list of 255 probesets, with a false discovery rate of 7.6%. This list encompassed all of the 183 (100%) probesets selected through the first supervised comparison. This 183 probeset list is available as supplementary Table I and represents 145 distinct genes and 18 ESTs (163 genes/ESTs). 20 noteworthy genes are shown in Table 3. A total of 79 genes/ESTs were overexpressed in MM BMMSCs. Use of Gene Ontology annotation with the Fatigo platform (<http://fatigo.bioinfo.cnio.es/>) allowed annotating 127 of these 163 genes/ESTs. Of note, 46% (59 out of 127) were classified in a “tumor environment” category (Figure 3). These 46% tumor-environment genes encode for cell communication proteins (27%), receptor signalization molecules (11%) and extracellular matrix or secreted molecules (9%). The other functions were metabolism, nuclear function, cytoskeleton, protein synthesis, cell cycle, and apoptosis (Figure 3). Some of these genes encode for growth factors reported to be involved in MM. Three examples are amphiregulin (AREG), a member of the EGF family, and IL-1 β , which were overexpressed in MM BMMSCs as compared to normal BMMSCs, in a ratio of 2.8 and 3.5, respectively. These data were validated at the mRNA level by real-time RT-PCR (Table 4). We performed validation at the protein level by ELISA. Although IL-1 β secretion was 16 times higher for MM BMMSCs, the

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difference was not significant, probably because of the low number of samples studied and the low level of secretion. AREG concentration was under the sensibility threshold for our ELISA test. The *DKK1* mRNA level was increased by 2.4 times in MM BMMSCs and the protein was increased by 1.4 times in the culture supernatant. Two other examples are IGF-1 and SDF-1, which were underexpressed in MM BMMSCs as compared to normal BMMSCs, in a ratio of 5.5 and 3, respectively. Data were validated with real-time RT-PCR. In addition, the concentration of SDF-1 in the supernatant of MM BMMSCs represented 40% of the level found in normal BMMSC supernatant. The level of secreted IGF-1 was under the sensitivity threshold of our ELISA test.

We also discovered some genes never described in MM, such as growth and differentiation factor-15 (GDF-15), increased in MM BMMSCs. Both GDF-15 mRNA and secreted protein levels were 4-fold higher in MM BMMSCs as compared to normal BMMSCs (Table 4). The effect of GDF15 was tested on the growth of the stromal cell-dependent MOLP-6 myeloma cells. Without the addition of stromal cells, MOLP-6 cells progressively underwent apoptosis. GDF-15 could replace stromal cells, supporting long term MOLP-6 growth in a dose-dependent manner ($P = 0.04$) (Fig. 4). A concentration of 100 ng/ml of GDF15 increased 3 fold the MOLP-6 cell number in 5 day cultures. GDF15 had no effect on XG-1 cell line even associated with a suboptimal dose of IL-6.

We also tested the concentration of IL-6 in culture supernatant, because this molecule is important in MM pathophysiologic features and its mRNA level is increased in BMMSCs, although the level (1.9) did not reach our limit of two-fold increase. MM BMMSCs produced larger amounts of IL-6 than normal BMMSCs, with good correlation between the *IL-6* mRNA level measured by microarray and the IL-6 concentration produced by BMMSCs in vitro (Table 4).

Increased proliferation of the MOLP-6 myeloma cell line cocultured with MM BMMSCs as compared to normal BMMSCs

Because normal BMMSCs have a distinct genetic profile as compared with MM BMMSCs, we investigated their ability to support MM cell growth using the MOLP-6 stromal cell-dependent myeloma cell line and the XG-1 stromal cell-independent but IL-6-dependent cell line. Of interest,

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MM BMMSCs supported MOLP-6 better than normal BMMSCs after 7 days of culture (3.3 ± 0.4 -fold vs. 2.2 ± 0.3 -fold amplification, $P = 0.04$). This effect could be due to GDF15 that is highly produced by MM BMMSCs compared to normal ones. But we could not investigate it directly because no commercially available anti-GDF15 antibodies are available presently. The proliferation of the stromal cell-independent but IL-6-dependent XG-1 myeloma cells was supported equally efficiently by normal and MM BMMSCs (Table 5) and was abrogated by adding an anti-IL-6 monoclonal antibody. Although MM BMMSCs produced higher amounts of IL-6 than normal BMMSCs, this lack of difference in supporting XG-1 growth is understandable because normal BMMSCs produced enough IL-6 (Table 4) to induce maximal proliferation of XG-1 cells that are highly sensitive to IL-6.

BMMSC osteoblastic differentiation

Because osteoblastic function is impaired in advanced myeloma, we checked the differentiation of BMMSCs into the osteoblastic lineage. Because qualitative assessment of phosphatase activity did not differ between normal and MM BMMSCs (data not shown), we used a quantitative technique to measure phosphatase activity. After 21 days of differentiation culture, the activity ratio for normal BMMSCs did not differ significantly from that of MM BMMSCs (2.5 ± 0.3 vs. 3 ± 0.5). However, matrix mineralization of MM BMMSCs, as assessed by von Kossa staining, was qualitatively less abundant than that obtained with normal BMMSCs (Fig. 5). To assess this difference, we quantified matrix mineralization for 2 normal and 3 MM BMMSCs, with a striking reduction in matrix mineralization revealed for MM BMMSCs (Fig. 5).

Discussion

The aim of this study was to investigate the differences in BMMSCs from normal donors and from patients with newly-diagnosed myeloma. The bone marrow of MM patients showed a normal frequency of CFU-F, whose expansion ability was reduced in 6-week cultures. The ability to support the growth of hematopoietic stem cells did not differ between normal and MM BMMSCs.

A remarkable finding is that MM BMMSCs had a distinctive GEP on microarray analysis. Indeed 2 unsupervised methods could classify genes in BMMSCs from MM patients into an MM cluster and those from normal age-related donors into a normal cluster. Even more remarkable is that these BMMSCs had been allowed to expand for 6 weeks in culture, with about 16 to 20 doublings, still retaining this specific gene signature. Although study of solid tumors has shown cells from a microenvironment to keep their specific features even after culture³², such findings have not yet been reported for hematopoietic cancers.

Our data suggest that mesenchymal stem cells in the bone marrow of MM patients differed from normal cells and that these differences were retained throughout the expansion culture. This difference may be due to a selection by the malignant clone of a mesenchymal stem cell subpopulation in vivo that may favor the malignant plasma cell growth. We cannot exclude genetic abnormalities occurring in the patients' mesenchymal stem cells themselves, but no gross abnormalities could be found by conventional genetic analysis. Finally, this specific MM BMMSC genetic profile could be characteristic of a population at increased risk for monoclonal gamopathy. Of note, genes for MGUS BMMSCs could be classified into normal or MM clusters. Of major interest is investigating whether patients with MGUS with a MM-specific BMMSC genetic profile are at increased risk for MM.

145 genes were differentially expressed between normal and MM BMMSCs. Study of their ontology revealed that almost half may participate in the support of malignant plasma cells; thus, we called them "tumor microenvironment" genes. The cell communication molecules represented 27% of the genes and are likely involved in the association with myeloma cells. The factors IL-1 β , IGF-1, SDF-1, AREG, IL-6, and DKK1 had already been described to play a role in the biology of myeloma³³. Bone marrow myeloma cells produce the pro-inflammatory cytokine IL-1 β , which contributes to the high IL-6 production in the MM tumor environment. However, controversy has surrounded the IL-1 producer cells in MM³⁴⁻³⁶. Here we show that the expression of IL-1 β in BMMSCs is, on average, 3.5-fold higher in MM BMMSCs as compared to normal BMMSCs²¹.

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AREG, a member of the EGF family, is overexpressed in MM BMMSCs. We have previously shown that AREG is expressed by myeloma cells and can be both an autocrine factor for the myeloma cells themselves and a paracrine factor, stimulating the production of IL-6 by the BMMSCs¹². Thus, the expression of AREG, at the same time by the cells of microenvironment and the malignant plasma cells, could ensure the survival of the tumor cells. Of note, IGF-1, a major myeloma growth factor³⁷, was underexpressed in MM BMMSCs as compared to normal BMMSCs and was weakly expressed in MM and normal samples, which suggests that IGF-1 may be secreted by other cells in MM. We also observed an underexpression of SDF-1 at the mRNA and protein level in MM BMMSCs, which demonstrates that BMMSCs are not responsible for increased SDF-1 concentration observed in MM. In agreement with this finding, Zanettino showed that SDF-1 was secreted by MM cells themselves and it supports the mobility and activation of osteoclasts³⁸. The gene coding for IL-6, a major MM cell growth factor (ref 14), was also significantly overexpressed in MM BMMSCs as compared to normal BMMSCs, but at a ratio below 2 (Table 4). Regarding IL-6 protein, MM BMMSCs produced twice more IL-6 than normal BMMSCs, with good correlation between RNA and protein data. MM BMMSCs secreted high levels of IL-6, about 0.5 pg/cell, approximately 20-fold higher than that reported by Wallace²¹. *DKK1*, coding for another remarkable inhibitor of the Wnt pathway in MM, was also significantly overexpressed in MM BMMSCs. Gregory et al. demonstrated that DKK1 is secreted by BMMSCs and enhances their proliferation³⁹. As well, Tian et al. found that DKK1 could be secreted by MM cells and the increased secretion was associated with the early stage of osteolytic lesions¹⁰. The authors demonstrated that DKK1 inhibits osteoblastic differentiation of C2C12 cell lines. Recently, Gunn et al. showed that DKK1 could enhance IL-6 secretion by osteoblasts⁴⁰. We have confirmed DKK1 secretion by MM and normal BMMSCs.

Of interest, we also identified genes coding for proteins that have never been directly implied in MM. One group could be, in part, responsible for the development of angiogenesis by increasing the imbalance between the pro- and anti-angiogenic factors⁴¹. Angiopoietin-like 4 (ANGPTL4),

plasminogen activator inhibitor 1 (PAI-1) and secretogranin II (SCG2) were overexpressed 4.5, 2.6 and 2.6 times, respectively, by the MM BMMSCs. ANGPTL4 is an angiogenic factor, independent of VEGF, whose production is mediated by PPAR-gamma⁴². PAI-1 has been reported to control key aspects of angiogenesis and, in turn, tumor progression through its anti-proteolytic activity⁴³. Stromal cell production of PAI-1 has been reported to be more important for its action than cancer cell production⁴⁴. SCG2 belongs to a family of the chromogranins and is the precursor of secretoneurin, which is both a pro-angiogenic and a chemotactic factor for monocytes and endothelial cells⁴⁵. In vivo, secretoneurin mobilizes the endothelial progenitors and supports their integration in the newly formed vessels. IL-17D, recently discovered, belongs to a family of inflammatory cytokines, which includes 6 members (IL-17A to IL-17F). We found that the MM BMMSCs overexpressed *IL-17D*. IL-17D induces the secretion of IL-6 by endothelial cells and inhibits, in vitro, the proliferation of the myeloid progenitors⁴⁶. Another group of molecules, underexpressed by MM BMMPCs as compared to normal BMMSCs, are involved in bone physiology and may explain in part the osteoblast anomalies. We observed a 3.6-, 3- and 2-fold decrease in mRNA expression of the C receptor of natriuretic peptide (NPR3), Wnt-1-induced secreted protein 1 (WISP-1) and endothelial differentiation gene 2 (EDG2), respectively. NPR3 is considered responsible for the clearance of the ANPs and thus, the modulation of their effects. NPR3-knockout mice show abnormalities of the skeleton, in particular a defect of endochondral ossification⁴⁷. WISP-1 belongs to the CCN family and has been shown to support BMP-2-induced osteoblastic differentiation⁴⁸. In vivo, in a model of fracture, WISP-1 is expressed in mesenchymal cells that surround the site of the injury. EDG2 is one receptor for lysophosphatidic acid (LPA), which is involved in osteoblast proliferation and prevents their apoptosis.^{49,50} EDG2 mutant mice displayed skeletal anomalies⁵¹.

Finally, we have shown for the first time, both at the mRNA and protein level, that GDF15 expression was higher in MM BMMSCs than in normal BMMSCs and that GDF15 is a novel MM cell growth factor, supporting the growth of the MOLP-6 stromal cell-dependant myeloma cell

line. GDF-15 is a divergent member of the TGF β superfamily⁵². In normal human subjects, it is highly expressed in the placenta and its serum concentration increases during pregnancy⁵³. GDF15 concentration is increased in the serum of patients with prostate, colorectal or pancreatic cancers⁵⁴⁻⁵⁶ and was recently described as one of the 20 best biomarkers of numerous tumours⁵⁷. In vitro, GDF15 prevents the apoptosis of cerebellar granule neurons via the PI3K/Akt pathway⁵⁸ or induces apoptosis and inhibits the proliferation of several tumor cell lines⁵⁵. Interestingly, the 2 MGUS BMMSCs located in the “myeloma cluster” on genetic profiling secreted twice more GDF-15 than the 3 MGUS BMMSCs located in the “normal cluster”.

In agreement with the GEP of normal and MM BMMSCs, the 2 cell populations also showed functional differences. (i) MM BMMSCs showed a reduced matrix mineralization capability, which might be linked to the DKK1 upregulation in those cells as compared to normal BMMSCs and the downregulation of WISP-1, NPR3 and EDG-2. (ii) MM BMMSCs can support the growth of the stromal cell-dependant myeloma cell line MOLP-6 better than the normal and MGUS BMMSCs. We could not investigate whether GDF15 was involved in part in this effect because no neutralizing anti-GDF15 antibody is commercially available.

In conclusion, our study reveals that BMMSCs from patients with MM show a specific gene expression profile, with an overexpression of genes coding for proteins involved in MM cell growth, angiogenesis, and osteoblast differentiation. Thus, these BMMSCs could create a very efficient niche to support the survival and proliferation of the myeloma stem cells and protect them from chemotherapy-induced apoptosis.

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Table 1 : Subjects' characteristics

	Multiple myeloma	MGUS	Healthy persons
	n=22	n=7	n=11
Age, yr \pm SEM	61 \pm 11	62 \pm 13	58 \pm 11
Sex (% Male)	58%	30%	60%
Disease stage	I = 10 III = 12		
Bone lesions	Absence = 11 Presence = 11		

Table 2 : BMMSC growth culture characteristics

Normal donors			MGUS patients		MM patients			
			All	Stage I	Stage III	without bone lesions		with bone lesions
<u>Number of subjects</u>			11	7	22	10	12	11
CFU-F Frequency								
Bone marrow			69.8 ± 15.8	59.4 ± 7.9	57.7 ± 9.6	55.8 ± 13.3	59.3 ± 14.1	64.6 ± 14.9
Primo-culture			0.22 ± 0.04	0.21 ± 0.06	0.18 ± 0.03	0.22 ± 0.05	0.14 ± 0.03	0.2 ± 0.05
1st passage			0.11 ± 0.03	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01	0.06 ± 0.03	0.04 ± 0.01
Cell densities								
Primo-culture			3 10 ⁴ ± 4.5 10 ³	2.2 10 ⁴ ± 4.7 10 ³	2.4 10 ⁴ ± 4.2 10 ³	2 10 ⁴ ± 4.7 10 ³	2.8 10 ⁴ ± 6.8 10 ³	2.4 10 ⁴ ± 5.6 10 ³
1st passage			2.6 10 ⁴ ± 3.3 10 ³	28.9 10 ⁴ ± 6.4 10 ³	2.3 10 ⁴ ± 3.4 10 ³	2.5 10 ⁴ ± 5.6 10 ³	2.2 10 ⁴ ± 4.3 10 ³	2.5 10 ⁴ ± 5.1 10 ³
CFU-F expansions								
Primo-culture			5.6 10 ³ ± 2.2 10 ³	1.7 10 ³ ± 0.6 10 ³	1.9 10 ³ ± 0.5 10 ³ §	1.8 10 ³ ± 0.5 10 ³	2 10 ³ ± 0.8 10 ³	1.7 10 ³ ± 0.5 10 ³
1st passage			15.5 ± 6.7	3.9 ± 2.3	6.9 ± 1.8	7.5 ± 2.1	6.4 ± 3	6.6 ± 2

All data are expressed as mean ± standard error of the mean

CFU-F frequencies in bone marrow are expressed as colonies/10⁶ cells

§ different from the mean value with normal donors (*P* = .048 with a Student's *t*-test

Table 3 : Differentially noteworthy expressed genes

GenBank	Gene	Ratio	Localization	Description
Gene overexpressed in MM BMMSC				
NM_001657	AREG	5.2	4q13-q21	amphiregulin (schwannoma-derived growth factor)
NM_016109	ANGPTL4	4.5	19p13.3	angiopoietin-like 4
AF003934	GDF15	4.1	19p13.1-13.2	growth differentiation factor 15
NM_002575	SERPINE2	4	18q21.3	serpin peptidase inhibitor; clade B (ovalbumin); member 2
AF063591	CD200	3.2	3q12-q13	CD200 antigen
NM_000576	IL1B	2.9	2q14	interleukin 1; beta
NM_003469	SCG2	2.6	2q35-q36	secretogranin II (chromogranin C)
AL574210	SERPINE1	2.6	7q21.3-q22	plasminogen activator inhibitor type 1
AI093492	DKK1	2.4	10q21.1	Dickopf 1 Homolog
AI669535	IL17D	2.1	13q12.11	Interleukin 17D
Gene underexpressed in MM BMMSC				
NM_001401	EDG2	0.5	9q31.3	endothelial differentiation gene 2
NM_003882	WISP1	0.32	8q24.1-q24.3	WNT1 inducible signaling pathway protein 1
AK026829	LAMA2	0.3	6q22-q23	laminin; alpha 2 (merosin; congenital muscular dystrophy)
NM_000609	CXCL12	0.29	10q11.1	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)
NM_000908	NPR3	0.29	5p14-p13	natriuretic peptide receptor C
BF432648	TNFRSF19	0.26	13q12.11-q12.3	tumor necrosis factor receptor superfamily; member 19
X17406	AGC1	0.25	15q26.1	aggrecan 1
Z95331	FBLN1	0.21	22q13.31	fibulin 1
AU144912	IGF1	0.16	12q22-q23	insulin-like growth factor 1 (somatomedin C)
J04177	COL11A1	0.1	1p21	collagen; type XI; alpha 1

Table 4 : Expression of mRNA and protein secretion by normal or MM BMMSCs

	IL-1β	GDF-15	SDF-1	IL-6	DKK1	AREG	IGF-1
mRNA	Normal	88.4 ± 18.9	183.5 ± 64.9	926.8 ± 88.6	1197 ± 116	130 ± 18	57.1 ± 16.7
	Myeloma	309.6 ± 83.3	749 ± 90.9	275.3 ± 59.1	2300 ± 402	310 ± 30.4	119 ± 37.2
	Ratio	3.5	4.1	0.3	1.9	2.4	5.2
	P value	0.05	0.0003	0.0001	0.02	0.004	0.017
Protein (pg/cell)	Normal	2.1 10 ⁻⁶ ± 5.7 10 ⁻⁶	1 10 ⁻⁴ ± 1.9 10 ⁻⁵	3.4 10 ⁻² ± 5.8 10 ⁻³	3 10 ⁻¹ ± 2.7 10 ⁻²	10 ⁻³ ± 2.2 10 ⁻⁴	B.D.L.
	Myeloma	3.2 10 ⁻⁵ ± 1.6 10 ⁻⁵	4.3 10 ⁻⁴ ± 1.4 10 ⁻⁴	1.3 10 ⁻² ± 4.7 10 ⁻³	7.3 10 ⁻¹ ± 1.7 10 ⁻¹	1.4 10 ⁻³ ± 3.6 10 ⁻⁴	B.D.L.
	Ratio	16	4.3	0.4	2.4	1.4	B.D.L.
	P value	0.09	0.04	0.02	0.03	0.36	B.D.L.
Correlation		R = 0.94, P < 0.001	R = 0.53, P < 0.01	R = 0.9, P < 0.001	R = 0.86, P < 0.001	R = 0.65, P < 0.004	
qRT-PCR	Normal		473.4 ± 110.4		125.8 ± 46.5	33.3 ± 12.4	2378.8 ± 1130.2
	Myeloma		98.8 ± 30.3		215.2 ± 52.1	219.4 ± 117	36.5 ± 18.8
	Ratio		0.21		1.7	6.6	0.02
	P value		0.011		0.22	0.11	0.08

All data are expressed as mean ± S.E.M. (n=7 for normal subjects and n=6 for MM patients)

Ratio = Myeloma/Normal BMMSCs

B.D.L. = Below the detection level of ELISA

Table 5 : Myeloma cell lines and BMMSC Co-culture

		XG1		MOLP-6	
		Normal	Myeloma	Normal	Myeloma
		BMMSCs (7)	BMMSCs (6)	BMMSCs (7)	BMMSCs (8)
Day 4	-anti-IL6	3.2 ± 0.4	3.2 ± 0.3	1.6 ± 0.2	1.8 ± 0.1
	+anti-IL6	1.9 ± 0.2	1.8 ± 0.2	N.D.	N.D.
Day 7	-anti-IL6	11.5 ± 2.2	13.5 ± 2.8	2.1 ± 0.3	3.3 ± 0.4 §
	+anti-IL6	5.1 ± 0.9	5.6 ± 1.5	N.D.	N.D.

All data are expressed as mean ± S.E.M. of cell expansions.

Numbers in parenthesis = number of BMMSCs from different individuals.

N.D. = Not Done

- anti-IL-6 = culture without anti-IL-6 monoclonal antibody

+anti-IL-6 = culture with anti-IL-6 monoclonal antibody

§ The mean value is significantly different from that with normal BMMSCs ($P = .047$) using a Student's *t*-test

Figure 1: Co-culture of CD34⁺ cells with BMMSCs. CD34⁺ cells ($4 \cdot 10^4$) were cultured on normal or MM BMMSCs ($5 \cdot 10^3/\text{cm}^2$) in 12-well plates. The co-culture was performed in Myelocult medium with 10 μM of hydrocortisone as described in Materials and Methods. At days 7, 14, 21 and 28, nonadherent cells were counted and a clonogenic test was performed. At day 35, nonadherent and adherent cells were harvested, counted and assayed in the clonogenic test.

Figure 2: Unsupervised data analysis of BMMSCs of the 3 groups of subjects. A: Principal component analysis (PCA) of BMMSCs from 6 myeloma patients (●) and from 7 normal individuals (●). Data were obtained from U133 2plus microarrays (Affymetrix) as described in Materials and Methods. Analysis was performed on 28746 probe sets declared as present by the call detection algorithm. The variation coefficient (VC), defined as the ratio of the SD to the mean of signal values for the considered probe set, was used to select genes incorporated in the PCA. By varying VC from 0% to 100%, the normal and MM BMMSC clusters can be more or less separated. At a VC of 60% the separation was the best and includes $\sim 2,000$ genes. The boxed scheme corresponds to the cluster analysis performed on the same set of data with Cluster and TreeView softwares (<http://rana.lbl.gov/EisenSoftware.htm>) B: MGUS BMMSC (●) were added to the analysis.

Figure 3: Differentially expressed genes classification. The genes found to be differentially expressed between normal ($n=7$) and MM BMMSCs ($n=6$) were classified by use of gene ontology through the Fatigo platform (<http://fatigo.bioinfo.cnio.es/>) into 7 classes. The tumor microenvironment class comprised genes that belong to the intercellular communications (27%), to receptor signalization (9%) and to extracellular matrix and other secreted molecules (9%).

Figure 4: Myeloma cell line and BMMSC co-culture. MOLP-6 cells were cultured in RPMI-1640 medium with 10% FCS at 2×10^4 cells/well in 24-well plates with or without an increasing concentration of GDF-15 (10, 50 and 100 ng/ml). After 7 days, the cells were counted by trypan blue exclusion, and the expansion coefficient was calculated ($\text{d7 cell number}/\text{d0 cell number}$).

MOLP-6 expansion with 100 ng/ml of GDF-15 was 2 times greater than that without GDF-15 (n=7, R=0.38 and p=0.048)

Figure 5: Osteoblastic differentiation of BMMPCs. The cells were cultured in osteoblastic medium as described in Materials and Methods during 21 days. (A) Matrix mineralization was then revealed by von Kossa staining, and cells were counter colored by use of giemsa. Pictures were taken on an Olympus IX71 inverted microscope (magnification 100X) and underwent analysis with Analysis B software. (B) Plates were stained with alizarin red and extensively washed in distilled water. The intensity of the staining was quantified according to Gregory²⁷.

Figure 1

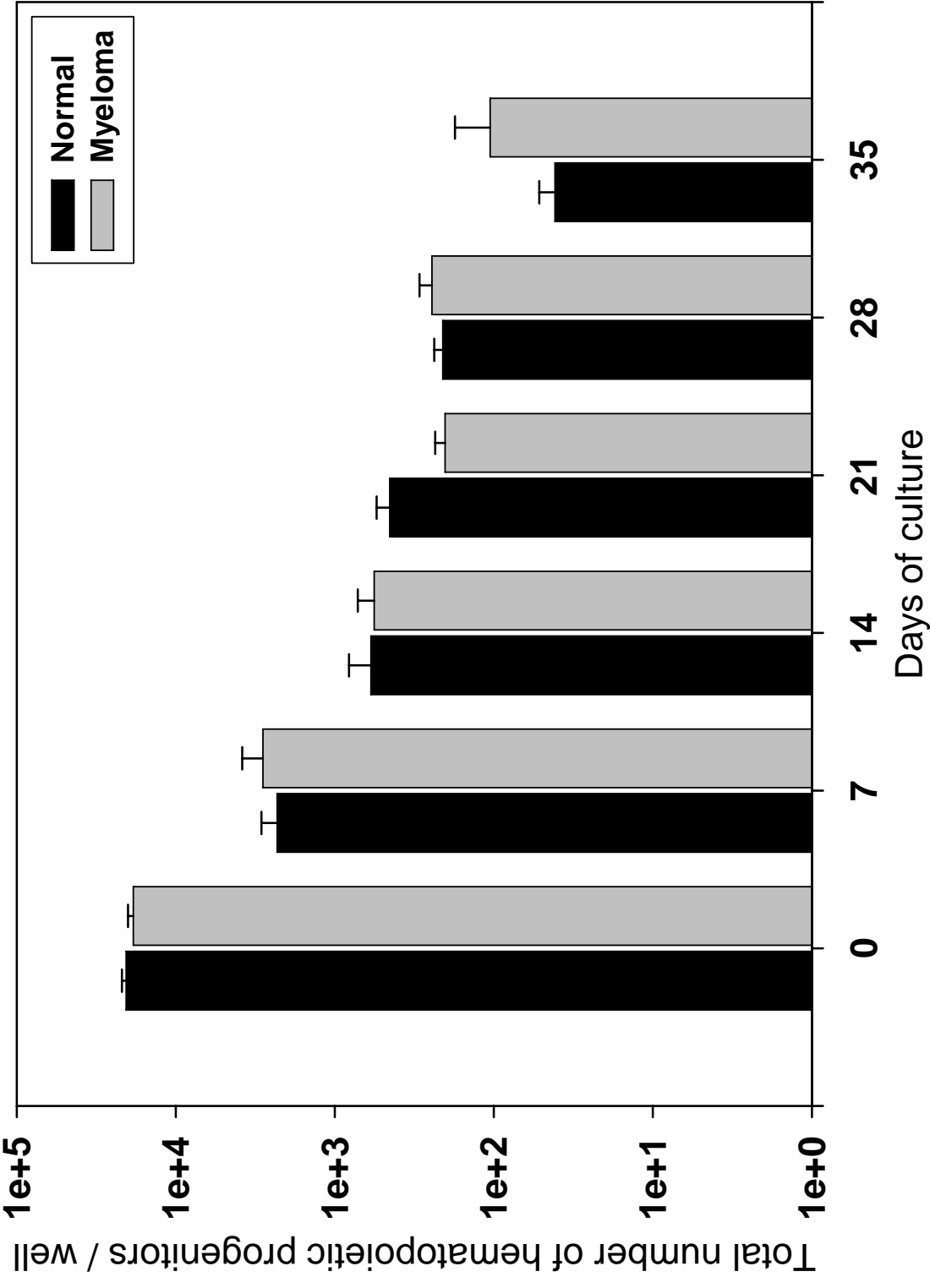


Figure 2a

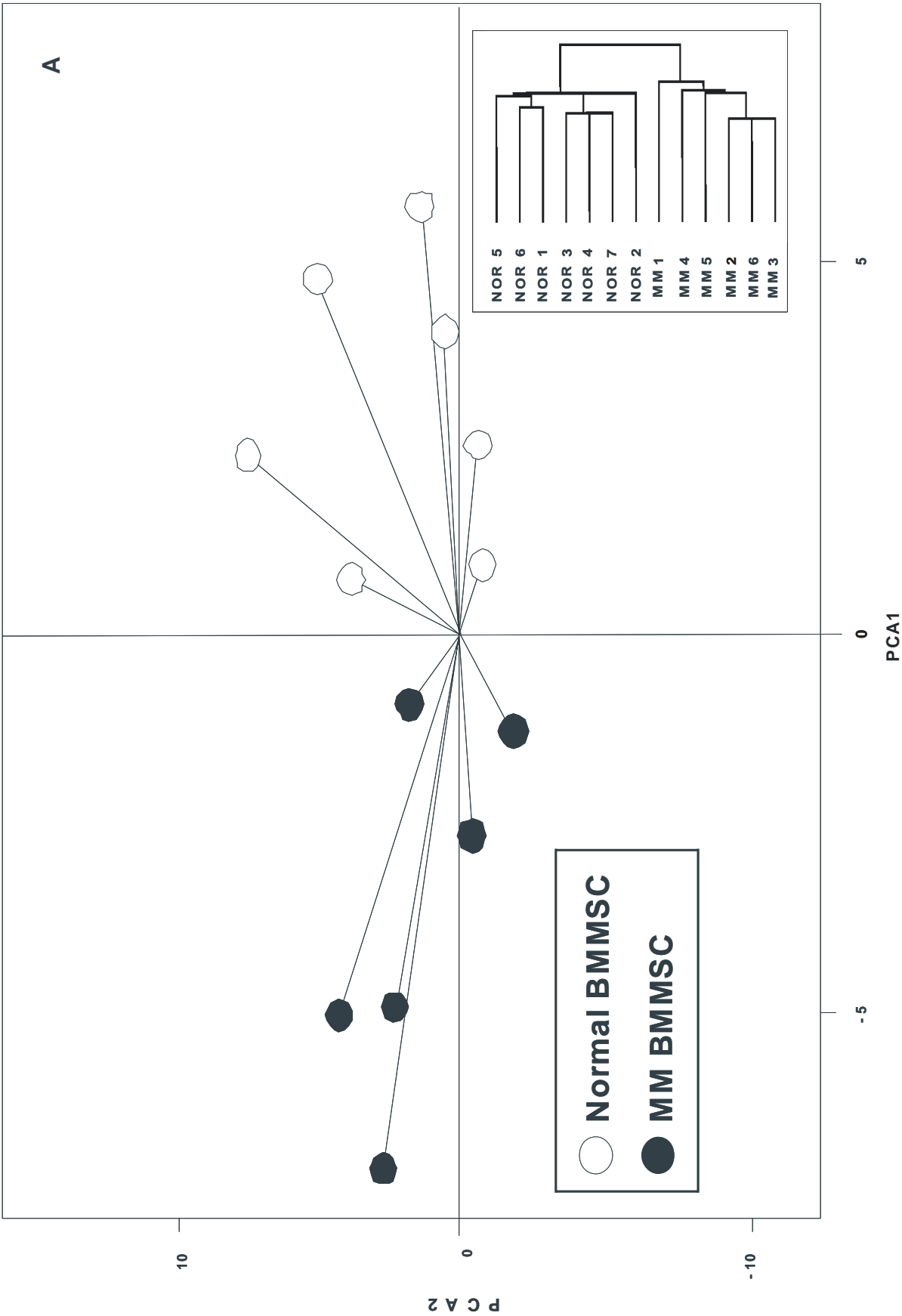


Figure 2b

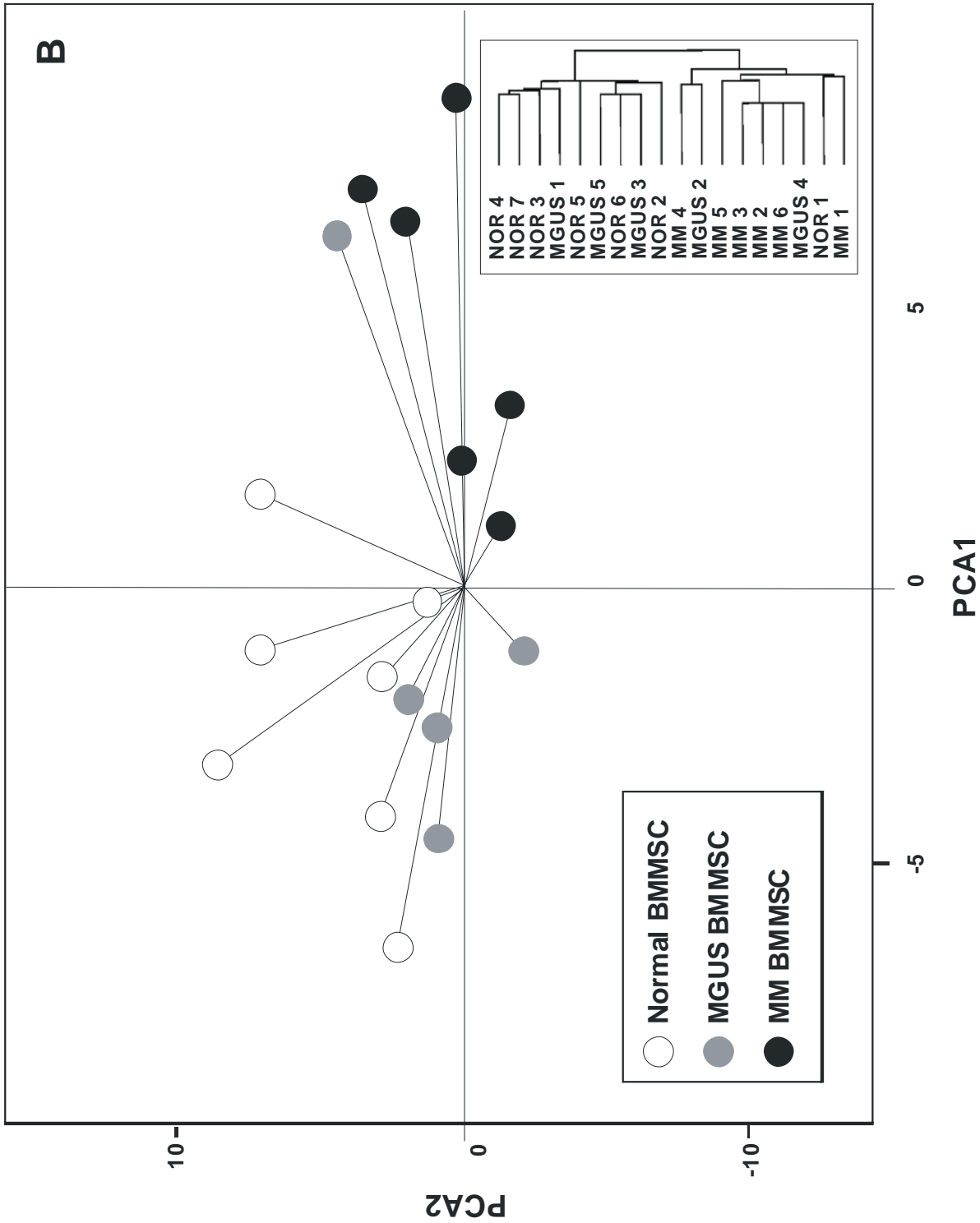


Figure 3

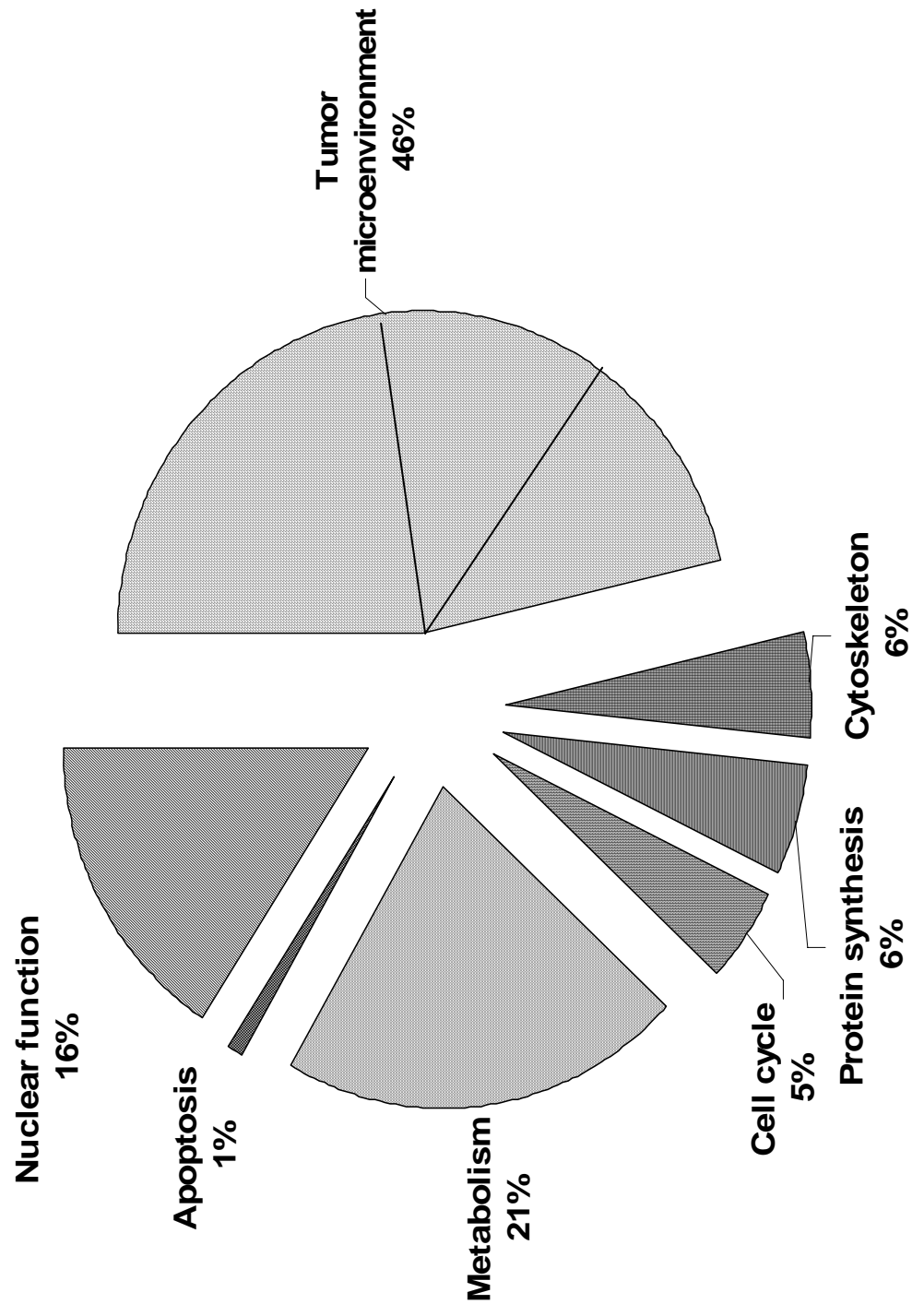


Figure 4

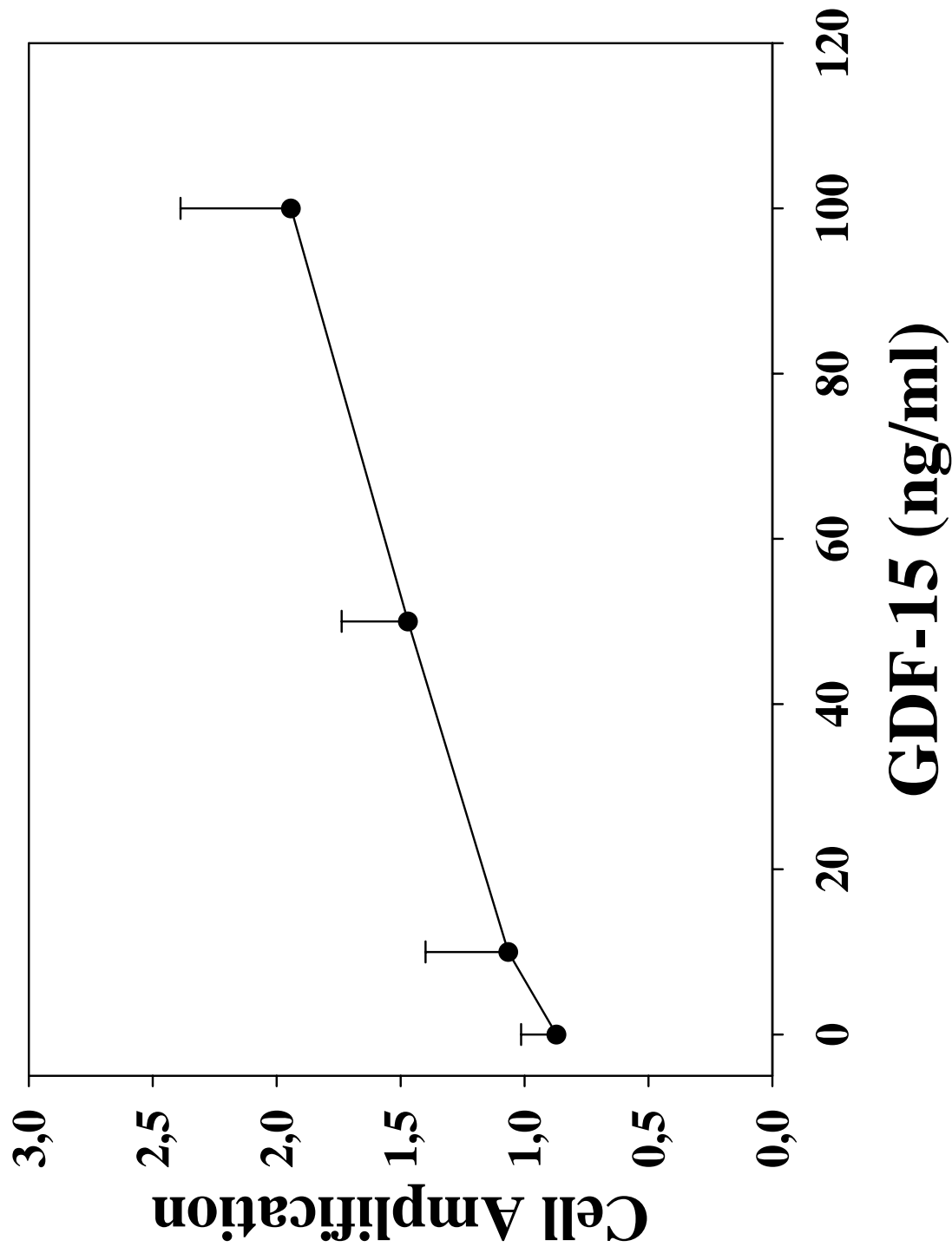
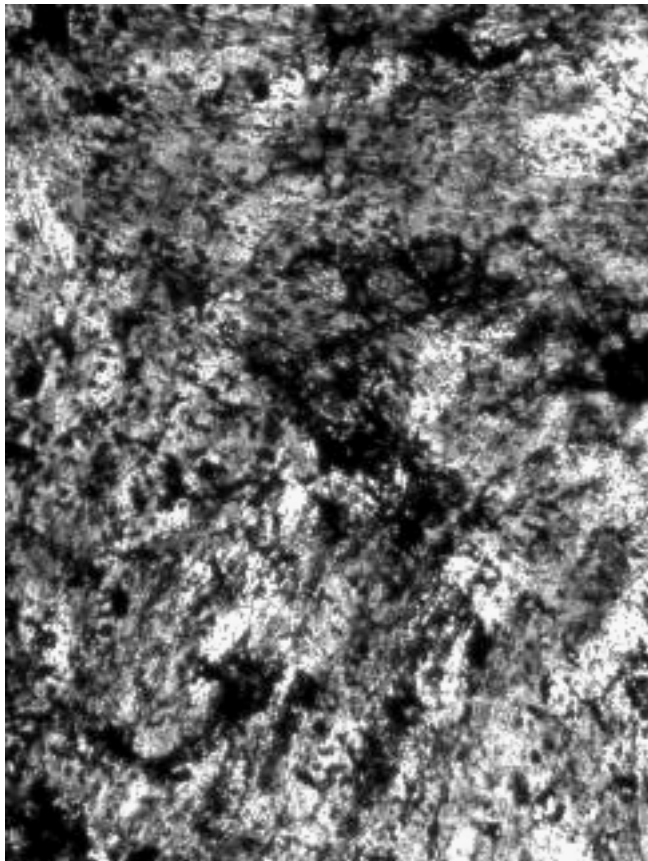


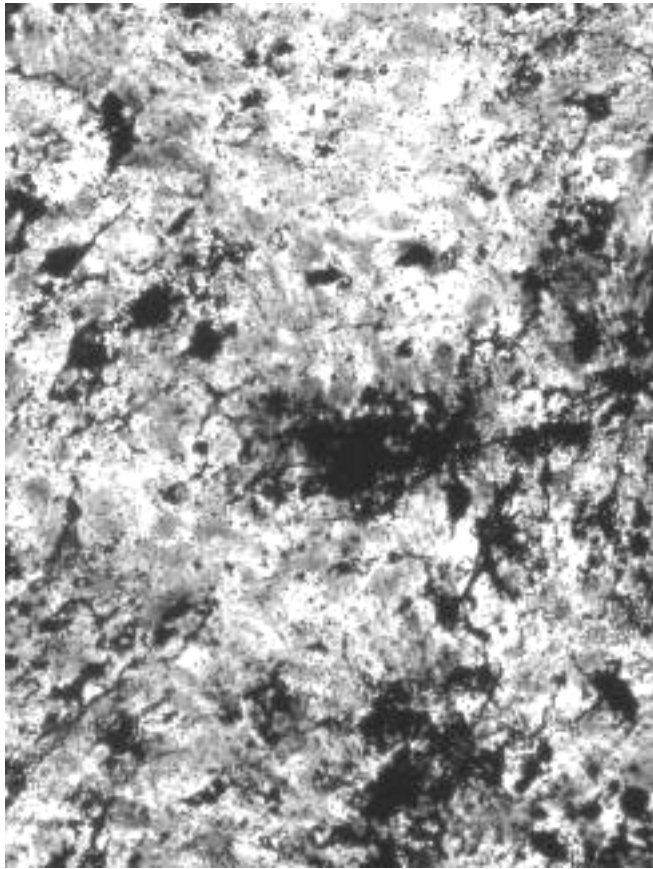
Figure 5

A

Normal



Myeloma



B

