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Original Article

Myelomatous plasma cells display an aberrant gene expression pattern similar to that observed in normal memory B cells

Alicia Báez, José I Piruat, Teresa Caballero-Velázquez, Luís I Sánchez-Abarca, Isabel Álvarez-Laderas, M Victoria Barbado, Estefanía García-Guerrero, África Millán-Uclés, Jesús Martín-Sánchez, Mayte Medrano, José Antonio Pérez-Simón

Department of Hematology, University Hospital Virgen del Rocío, Institute of Biomedicine of Seville (IBIS), CSIC, Universidad de Sevilla, Seville, Spain

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Abstract: Memory B cells (MBCs) remain in a quiescent state for years, expressing pro-survival and anti-apoptotic factors while repressing cell proliferation and activation genes. During their differentiation into plasma cells (PCs), their expression pattern is reversed, with a higher expression of genes related to cell proliferation and activation, and a lower expression of pro-survival genes. To determine whether myelomatous PCs (mPCs) share characteristics with normal PCs and MBCs and to identify genes involved in the pathophysiology of multiple myeloma (MM), we compared gene expression patterns in these three cell sub-types. We observed that mPCs had features intermediate between those of MBCs and normal PCs, and identified 3455 genes differentially expressed in mPCs relative to normal PCs but with a similar expression pattern to that in MBCs. Most of these genes are involved in cell death and survival, cell growth and proliferation and protein synthesis. According to our findings, mPCs have a gene expression pattern closer to a MBC than a PC with a high expression of genes involved in cell survival. These genes should be physiologically inactivated in the transit from MBC to PC, but remain overexpressed in mPCs and thus may play a role in the pathophysiology of the disease.

Keywords: Gene expression, plasma cells, cell survival, memory B cells, multiple myeloma

Introduction

B-cell differentiation into antibody-secreting cells constitutes the basis of the humoral adaptive immune system. Upon encountering a foreign antigen, naive B cells are activated and differentiated into antibody-secreting plasma cells (PCs) that mediate the primary humoral immune responses. In addition, some of them differentiate into memory B cells (MBCs) that drive the secondary humoral immune response upon re-exposure to the same antigen [1, 2]. Accordingly, during the differentiation from naive B cells to MBCs, B lymphocytes acquire a higher antigen binding affinity and, at the same time, change the expression patterns of multiple surface receptors and intracellular factors that contribute to increase their responsiveness [3]. Whereas PCs are terminally differentiated, MBCs are capable of undergoing consecutive phases of stimulation, expansion and generation of PCs [4-7]. Moreover, their detection more than 50 years after vaccination demonstrates that MBCs can remain viable in a non-proliferative state for decades [8]. In this regard, MBCs express high levels of genes involved in long-term survival, such as the transcription factors BCL6 and PAX5, which induce the expression of B-cell lineage genes [9], and anti-apoptotic factors, such as Bcl-2, A1 and Mcl-1, which promote cell survival [10]. These cells express low levels of genes associated with cell cycle regulation, including members of the Kruppel-like factor family, which endows them with the ability to enter quickly into division, thus facilitating enhanced secondary responses [11]. During the process of differentiation into PCs, several morphological, phenotypic and molecular changes occur. In this regard, the gene expression pattern of PCs is

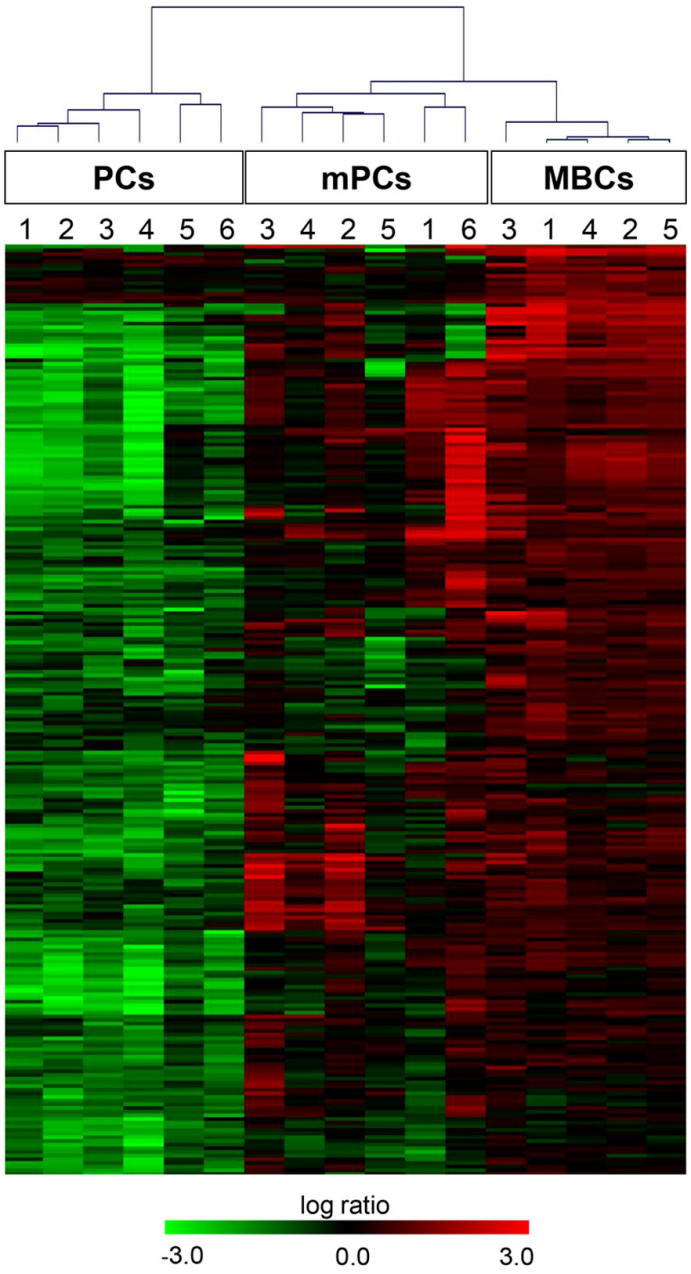

Figure 1. Gene expression pattern of mPCs versus normal PCs and MBCs. Unsupervised hierarchical cluster analysis performed with samples of myelomatous plasma cells (mPCs), normal plasma cells (PCs) and memory B cells (MBCs). Each numbered column represents an individual sample and each row represents a single gene. The panel contains a representative portion of the respective sets of genes. Red and green colors indicate gene expression levels on a logarithmic scale.

very different from that observed in MBCs. Among others, higher levels of expression of genes related to cell proliferation, such as PRDM1 and XBP1 [12, 13] and interferon regulatory factor IRF4, are apparent [14].

Multiple myeloma (MM) is characterized by the monoclonal expansion of malignant myelomatous PCs (mPCs) [15, 16]. Remarkably, these mPCs share characteristics of both MBCs and normal PCs. On one hand, they accumulate in bone marrow (BM) due either to failure in the mechanisms involved in apoptosis and/or to the abnormal overexpression of mechanisms responsible for long-term viability (similar to what occurs in MBCs). On the other hand, mPCs also show characteristics indicative of activated cell proliferation. In this regard, several reports have suggested that the mPC might be an abnormal MBC [17-19]. However, these findings have not been confirmed in other studies [20]. In fact, both hypotheses might be valid, i.e., mPCs could maintain features similar to MBCs. In turn, this would bestow long-term survival on this cell, which, at the same time, would acquire the functional, phenotypic and genetic characteristics of a PC in terms of activation. In the current study we compared the gene expression patterns of PCs and MBCs from healthy donors with that of mPCs with the dual aim of determining whether mPCs has an gene expression pattern intermediate between those of MBCs and PCs, and of identifying genes that may be associated with the physiopathology of the disease.

Material and methods

Samples

MBCs were isolated from 5 buffy coats from volunteer healthy donors. PCs were isolated from BM of 6 healthy donors and mPCS from BM of 6 patients diagnosed with MM. The local ethics committee of the University Hospital Virgen del Rocío provided institutional review board-approval for this study. Informed consent was obtained from all donors in accordance with the Declaration of Helsinki.

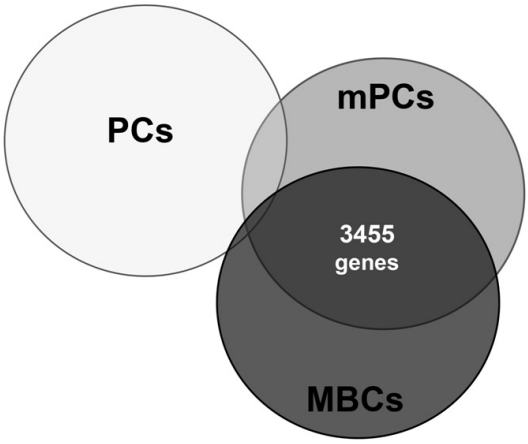

Figure 2. Flow-chart of analysis. Diagram of the method used to compare gene expression between the grouped samples. The number of genes from myelomatous plasma cells (mPCs) with different expression levels from those of normal plasma cells (PCs) and similar levels to those of memory B cells (MBCs) are shown. For more details see the methods section.

Isolation of MBCs

Peripheral blood mononuclear cells from buffy coats were isolated by density gradient centrifugation using Ficoll-Paque solution (Amersham Biosciences, Uppsala). The isolation of MBCs was performed in a two-step procedure by immunomagnetic separation in an AutoMACS pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany) using the Memory B cell isolation kit human (Miltenyi Biotec, Bergisch Gladbach, Germany). MBCs were isolated by depletion of non-B cells and subsequent positive selection with anti-CD27 conjugated MicroBeads. Firstly, we performed an indirect magnetic labeling of non-B cells with Biotin-Antibody Cocktail and Anti-Biotin MicroBeads and we retained the negative fraction. The second step was a direct magnetic labeling of CD27+ MBCs with CD27 MicroBeads. The purity of the isolated MBCs was higher than 95% in all cases as demonstrated by flow cytometry.

Isolation of PCs and mPCs

Mononuclear cells were isloated from BM using ammonium chloride. The CD138+ cells from both healthy donors and patients were isolated in AutoMACS pro separator (Miltenyi Biotec, Bergisch Gladbach, Germany) by positive inmunomagnetic selection using the CD138 MACS microbead Human Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the isolated CD138+ cells was higher than 95% in all cases.

RNA extraction

Total RNA was extracted using the AllPrep DNA/RNA mini Kit (Qiagen, Hilden, Germany). The quality and integrity of the RNA was verified by a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). A RNA integrity number (RIN) higher than 7.5 was required for further analyses of gene expression profiling.

Gene expression

We analyzed the expression profile of whole genome in each sample using the Whole Human Genome Oligo microarray kit 4x44K (Agilent Technologies, Santa Clara, CA). Total RNA (200 ng) was transcribed into cRNA and labeled with the Low Input Quick Amp Labeling Kit (Agilent Technologies, Santa Clara, CA). The quality and integrity of the cRNA was verified by a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). All experimental samples were hybridized with a pool of samples of B cells as reference sample. Microarrays were scanned in a GenePix reader (Molecular Devices, Sunnyvale, CA).

Validation of significant genes

The expression of significant genes was validated by quantitative real-time PCR using Quantitec Primer Assays and the Quantitec SYBR green Kit (both from Qiagen, Hilden, Germany) in a 7900 HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA). Data were normalized to the housekeeping gene *GAPDH*. The relative gene expression levels were calculated by the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Unsupervised hierarchical clustering of gene expression data was performed using the average linkage and the Euclidean distance. To identify differentially expressed genes between experimental groups we applied a non parametric Mann-Whitney test. The differences in expression with a p value < 0.05 were considered significant. To obtain positive and negative expression values data were transformed to logarithmic scale. All analyses were performed

Table 1. Biological processes involving genes differentially expressed in myelomatous plasma cells (mPCs)

Top Biological Functions	Molecules
Molecular and cellular functions	
Cell death and survival	384
Cellular growth and proliferation	360
Protein synthesis	155
Cellular compromise	59
RNA post-transcriptional modification	56
Physiological system development and functions	
Lymphoid tissue structure and development	106
Tumor morphology	83
Embryonic development	83
Organ development	73
Connective tissue development and function	73

using the Multi-experiment Viewer 4.7.1 software. The functional analysis of the genes of interest was performed with the Ingenuity Pathways Analysis (http://www.ingenuity.com) and Gene Ontology (http://www.geneontology.org/) software.

Results

Gene expression in PCs, mPCs and MBCs

The unsupervised hierarchical clustering of the expression data from all the samples clearly identified two groups, one including normal PCs samples and the other containing the mPC and MBC samples (**Figure 1**). Interestingly, mPCs were not only grouped with MBCs, but also clustered and located closer to the PC samples, implying that mPCs display an intermediate gene expression pattern between MBCs and normal PCs, although with a profile closer to that of MBCs.

The Mann-Whitney test identified 5159 genes significantly differentially expressed in mPCs compared with normal PCs ($p < 0.05$). Of these, we were able to select 3455 genes with a similar (i.e., not significantly different) expression level to that observed in MBCs (**Figure 2**). Of this subset of genes, 1349 displayed a greater than 1.5-fold difference in their expression levels with respect to normal PCs, comprising 1062 upregulated and 287 downregulated genes in mPCs. The functional analysis using Ingenuity Pathway Analysis software showed these genes to be involved in several biological processes such as cell death and survival, cellular growth and proliferation, protein synthesis, and RNA post-transcriptional modification. Likewise, these genes appear to be involved in the development and function of various physiological systems, such as lymphoid tissue structure and development, and tumor morphology (**Table 1**).

The functional analysis showed that some of these 1349 differentially expressed genes play a precise role in several canonical pathways, the most significant being EIF2 signaling, mTOR signaling, regulation of eIF4 and p70S6K signaling, mitochondrial dysfunction and the antigen presentation pathway (**Table 2**).

Validation of significant genes

To validate our high-throughput screening, we confirmed the expression of several of the identified genes, analyzing *AKT1*, *BMI1*, *CAV1*, *COX5A*, *GTF2I*, *HNRNPA3*, *IRF2BP2*, *NDUFA1*, *PCNA1*, *RPS15*, *RPS27*, *SDHD* and *SF3B1*. Most of these genes were chosen because they are representative of the canonical pathways that are differentially expressed in mPCs and MBCs compared with PCs. *AKT1*, *RPS15* and *RPS27* are involved in EIF2 signaling, mTOR signaling and regulation of eIF4 and p70S6K signaling. *COX5A*, *NDFUA1* and *SDHD* encode proteins of several mitochondrial complexes. *BMI1*, *CAV1*, *GTF2I*, *HNRNPA3*, *IRF2BP2*, *PCNA1* and *SF3B1* were selected because they are well known pro-survival genes, and have already being implicated in the pathophysiology of MM. The results obtained confirmed that the level of expression for most of these genes was higher in mPCs and MBCs than in normal PCs (**Figure 3**), suggesting that the previously described molecular pathways are activated. The expression levels of some genes in mPC were intermediate between those of the other cell types, whereas for others, levels were even higher than those observed in MBCs.

Discussion

Many genetic aberrations have been described in MM, some of which may contribute to the pathogenesis of the disease, while others are secondary translocations [21, 22]. In fact, it is a matter of intense debate whether these alter-

Table 2. Most significantly affected pathways in myelomatous plasma cells (mPCS)

Canonical Pathways	p	Ratio*	Genes
EIF2 signaling	2E-09	62/201 (0.308)	AKT1, EIF1, EIF5, EIF1AX, EIF2AK2, EIF3A, EIF3C, EIF4A1, EIF4A2, EIF4G2, FAU, GSK3B, PABPC1, PPP1CC, RPL3, RPL4, RPL5, RPL6, RPL8, RPL10, RPL14, RPL15, RPL17, RPL19, RPL21, RPL23, RPL24, RPL27, RPL29, RPL32, RPL34, RPL35, RPL39, RPL10A, RPL23A, RPL27A, RPL35A, RPL36A, RPL36AL, RPL37A, RPL7A, RPLP0, RPLP1, RPS3, RPS6, RPS7, RPS10, RPS13, RPS15, RPS16, RPS18, RPS19, RPS20, RPS24, RPS25, RPS27, RPS28, RPS3A, RPS4X, RPSA, RRAS, UBA52
mTOR signaling	2.22E-09	36/213 (0.169)	AKT1, ARHGAP8, DDIT4, EIF3A, EIF3C, EIF4A1, EIF4A2, EIF4B, EIF4G2, FAU, HMOX1, MAPKAP1, PPP2R1B, PRKCD, RAC1, RHOA, RHOC, RPS3, RPS6, RPS7, RPS10, RPS13, RPS15, RPS16, RPS18, RPS19, RPS20, RPS24, RPS25, RPS27, RPS28, RPS3A, RPS4X, RPSA, RRAS, ULK1
Regulation of eIF4 and p70S6K signaling	8.34E-09	30/175 (0.171)	AKT1, EIF1, EIF1AX, EIF3A, EIF3C, EIF4A1, EIF4A2, EIF4EBP2, EIF4G2, FAU, PABPC1, PPP2R1B, RPS3, RPS6, RPS7, RPS10, RPS13, RPS15, RPS16, RPS18, RPS19, RPS20, RPS24, RPS25, RPS27, RPS28, RPS3A, RPS4X, RPSA, RRAS
Mitochondrial dysfunction	3.25E-06	26/201 (0.129)	ATP5A1, ATP5B, ATP5D, ATP5E, CASP3, CASP8, COX5A, COX5B, COX6A2, COX6C, COX7A2L, MT-COI, NDUFA1, NDUFA4, NDUFA9, NDUFA11, NDUFA12, NDUFB7, NDUFB8, NDUFB10, NDUFV1, PRDX5, SDHD, UQCR11, UQCRH, VPS9D1
Antigen presentation pathway	1.03E-05	11/42 (0.262)	B2M, CANX, CIITA, HLA-A, HLA-B, HLA-C, HLA-F, NLRC5, PDIA3, TAP1, TAPBP

*The ratio is of the number of molecules affected in the study to the total number of molecules involved in the pathway.

ations really contribute to the transit from a normal PC to an mPC, or if they are secondary events. Consequently, various studies have attempted to identify the origin of the myelomatous stem cell. The true nature and phenotype of cancer stem cells in MM remain unclear and controversial. Based on the expression of antigens such as CD19 or CD27, some authors have suggested that the neoplastic clone originates from a MBC [23] or even from a more immature stage B cell [24]. In fact, *in vivo* studies have shown that clonotypic B cells isolated from an advanced myeloma patient can generate disease in NOD/SCID mice [25], while CD138+ plasma cells fail to engraft NOD/SCID mice following tail vein injection [26, 27]. Conversely, other authors have reported that mPCs display extensive somatic hypermutations of rearranged Ig-genes [28], indicating that the neoplastic clone originates from a B cell that has undergone antigen selection [29], and subsequent differentiation into a PC [30]. Furthermore, several studies have failed to demonstrate the presence of clonotypic B cells in patients diagnosed with MM [31]. Against this background, we hypothesized that mPCs might simultaneously gain the activation pattern acquired by a normal PC during its physiological maturation while not losing the gene expression pattern displayed by the MBCs that confer long-term viability on these cells. Thus, mPCs would aberrantly share both activation and anti-apoptotic gene expression patterns from the normal PCs and MBCs counterparts, respectively. If this hypothesis were correct, mPCs would be expected to display an intermediate gene expression pattern between those of the two normal subpopulations.

With this idea in mind, the current study compared the gene expression profile of mPCs and normal PCs obtained from healthy donor BM compared with MBCs obtained from healthy donor peripheral blood. An unsupervised hierarchical clustering showed that mPCs did have an intermediate gene expression pattern between normal PCs and MBCs that was closer to the expression profile observed in MBCs than to the normal PC counterparts. We also attempted to identify genes differentially expressed in mPCs compared with normal PCs but with a similar expression pattern to that observed in MBCs. Using a cut-off value of 1.5 above or below the difference of their expression levels with respect to normal PCs, we identified 1349 genes that were involved in several biological processes, such as cell death and survival, cellular growth and proliferation, protein synthesis and RNA post-transcriptional modification. These differentially expressed genes affect several canonical pathways, such as EIF2 signaling, mTOR signaling, regulation of eIF4 and p70S6K signaling, antigen presentation pathways and mitochondrial function. EIF2 and mTOR pathways are essential for modulating proliferation, growth and survival of mPCs [32, 33]. Other canonical pathway differentially expressed in mPCs involved protein synthesis, a crucial biological process affecting cell growth, survival and proliferation. In eukary-

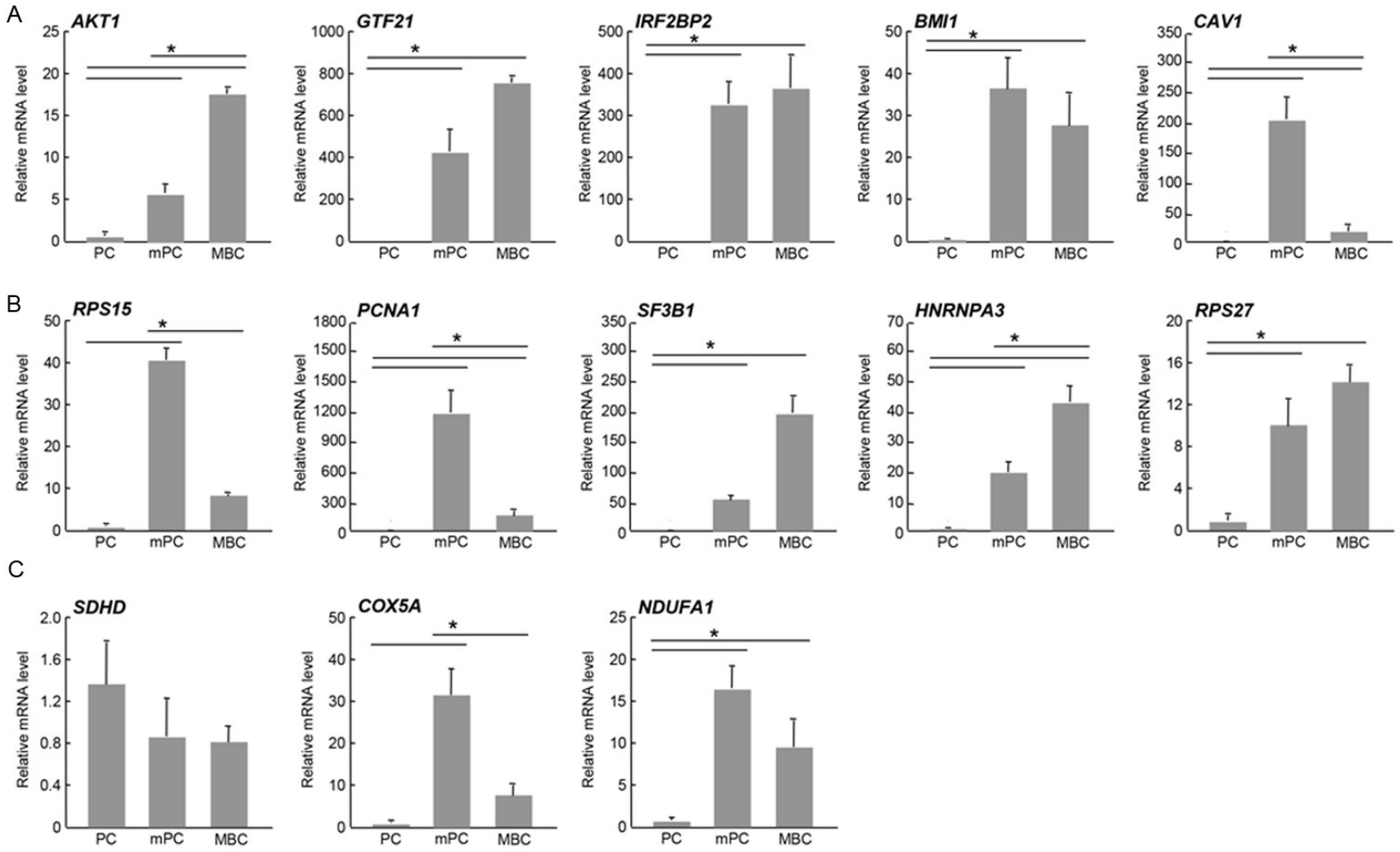

Figure 3. Validation of significant genes. Expression levels of genes differentially expressed in myelomatous plasma cells (mPCs) versus normal plasma cells (PCs) and memory B cells (MBCs). Genes implicated in (A) cell survival, proliferation and differentiation, (B) DNA replication, RNA transcription, splicing, and protein translation, and (C) mitochondrial dysfunction, as determined by quantitative real-time PCR. *; $p < 0.05$.

otes, translation initiation is facilitated by multiple protein factors collectively known as eIFs. Deregulated translational control plays an important role in oncogenic transformation. In fact, in various types of human cancers, eIFs are either overexpressed or ectopically activated by Ras-MAPK and PI3K-mTOR signaling cascades, resulting in increased survival and accelerated proliferation [34]. The antigen presentation pathway is also affected in MM and might contribute to the immune escape of mPCs from T-cell recognition due to the impaired antigen processing-presenting machinery [35, 36]. In contrast to these previously identified pathways, information about mitochondrial dysfunction in myeloma is very limited, making the corresponding findings of the current study of particular interest, which might allow targets to be identified of use for developing new therapeutic approaches [37].

Upon validating by quantitative real-time PCR some of the 1349 genes differentially expressed in mPCs that are involved in the previously mentioned pathways (*AKT1*, *RPS15*, *RPS27*, *HNRNPA3*, *BMI1*, *CAV1*, *GTF2I*, *IRF2BP2*, *PCNA1*, *SF3B1*, *COX5A*, *NDUFA1* and *SDHD*) we confirmed the intermediate gene expression profile of mPCs between that of normal PCs and MBCs. Specifically, *AKT1* is upregulated in mPCs, resulting in sustained overexpression of the receptor activator of NF-κB [38]. *RPS15*, *RPS27* and *HNRNPA3* encode ribosomal proteins involved in translation processes [39-41]. *BMI1*, *CAV1*, *GTF2I*, *IRF2BP2*, *PCNA1* and *SF3B1* are also upregulated in mPCs and MBCs relative to PCs. *BMI1* is a member of the Polycomb group family of proteins involved in the epigenetic silencing of genes governing self-renewal, differentiation, and proliferation, and regulate the growth and clonogenic capacity of MM cells [42]. *CAV1* mediates growth and survival of MM cells, thereby representing a potential novel therapeutic target [43]. *GTF2I* is a multifunctional transcription factor that coordinates changes in the transcriptional program in response to developmental and proliferative signals [44]. *IRF2BP2* is a transcriptional repressor of P53 involved in an apoptotic mechanism that maintains cell growth arrest, and is recognized as a potential prognostic marker of MM [45]. *PCNA1* is an important DNA replication factor in eukaryotic cells [46]. Finally, *SF3B1* is a critical component of the splicing machinery. Several studies have identified mutations in *SF3B1* in chronic lymphocytic leukemia, and various lines of evidence suggest that such mutations might be linked to genomic stability and epigenetic modification [47]. We also found several genes that are abnormally expressed in mPCs and involved in mitochondrial function. Since no studies have previously been reported concerning this aspect, we attempted to validate several of these genes (*COX5A*, NDFUA1 and SDHD). *COX5A*, which was upregulated in mPCs in our study, encodes the Va subunit of the human mitochondrial respiratory chain enzyme. Several groups have reported that high levels of *COX5A* activity and mitochondrial respiration in tumor cells lead to the overexpression of Bcl-2, thus promoting survival of cancer cells [48]. *NDFUA1*, which is also upregulated in mPCs, codes for an essential component of complex I of the respiratory chain, which transfers electrons from NADH to ubiquinone. Mutations in this gene lead to the development of several diseases [49]. Finally, *SDHD* was downregulated in mPCs and MBCs with respect to normal PCs in this study. *SDHD* encodes a member of complex II of the respiratory chain, which is responsible for the oxidation of succinate, and acts as a tumor suppressor. Several studies have shown that mutations in this gene are also associated with the formation of tumors [50]. Taken together, these data suggest that the activation and resting processes faced by an MBC during its life span could lead to a "derailment" mediated by the failure or wastage of the mechanisms regulating gene expression, leading to malignant transformation in mPCs.

In summary, we conclude that the expression of genes involved in cell survival, which should be normally inactivated in the transit from MBC to PC, is maintained in mPCs while simultaneously acquiring genes related to cell activation and proliferation, such as occurs in normal PCs. Accordingly, they have a gene expression pattern between those of normal PCs and MBCs. Moreover, mPCs display a high level of expression of survival and anti-apoptotic genes closer to that of an MBC than of a normal PC.

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Disclosure of conflict of interest

None to disclose.

Address correspondence to: Dr. Jose Antonio Pérez-Simón, Department of Hematology, Hospital Universitario Virgen del Rocío, Instituto de Biomedicina de Sevilla (IBIS)/CSIC/Universidad de Sevilla, Avenida de Manuel Siurot s/n 41013, Seville, Spain. Tel: +34 955 013 260; Fax: +34 955 013 265; E-mail: josea.perez.simon.sspa@juntadeandalucia.es

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