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Adhesive Interactions Regulate Transcriptional Diversity in Malignant B Cells

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Abstract

The genetic profiling of B-cell malignancies is rapidly expanding, providing important information on the tumorigenic potential, response to treatment, and clinical outcome of these diseases. However, the relative contributions of inherent gene expression versus microenvironmental effects are poorly understood. The regulation of gene expression programs by means of adhesive interactions was studied here in ARH-77 human malignant B-cell variants, derived from the same cell line by selective adhesion to a fibronectin matrix. The populations included cells that adhere to fibronectin and are highly tumorigenic (designated "type A" cells) and cells that fail to adhere to fibronectin and fail to develop tumors *in vivo* ("type F" cells). To identify genes directly affected by cell adhesion to fibronectin, type A cells deprived of an adhesive substrate (designated "AF cells") were also examined. Bioinformatic analyses revealed a remarkable correlation between cell adhesion and both B-cell differentiation state and the expression of multiple myeloma (MM)–associated genes. The highly adherent type A cells expressed higher levels of NFκB-regulated genes, many of them associated with MM. Moreover, we found that the transcription of several MM-related proto-oncogenes is stimulated by adhesion to fibronectin. In contrast, type F cells, which display poor adhesive and tumorigenic properties, expressed genes associated with higher levels of B-cell differentiation. Our findings indicate that B-cell differentiation, as manifested by gene expression profiles, is attenuated by cell adhesion to fibronectin, leading to upregulation of specific genes known to be associated with the pathogenesis of MM. *Mol Cancer Res; 8(4); 482–93. ©2010 AACR.*

Introduction

The molecular and cellular diversity of cancerous cells is commonly attributed to genetic instability, leading to deregulated expression of genes associated with cell proliferation and survival (1-3). Such mechanisms are known to operate, for example, in B-lymphoid malignancies such as Hodgkin's lymphoma, where somatic hypermutation remains active throughout the progression of the tumors (4). In some instances, somatic mutations in non–Hodgkin's lymphomas could be attributed to sustained, activation-induced cytidine deaminase expression (5, 6). Major genomic alterations (e.g., chromosomal translocations) can result in the deregulation of oncogene expression, thereby contributing to the progression of B-cell malignancies. For example, a translocation involving the IgH promoter can induce expression of high levels of *maf, myc, cyclin D1*, and other genes associated with the development of multiple myeloma (MM; refs. 7, 8).

Although genomic alterations in the transformed cells play a key role in the establishment of malignant gene expression programs, transcriptional regulation in B-cell malignancies is also affected by the tumor microenvironment, including the surrounding mesenchymal cells, endothelial cells, and osteoclasts, as well as cytokines and components of the extracellular matrix (ECM). Both separately and collectively, these factors affect gene expression in the malignant cells. For example, it has been postulated that the aberrant overexpression of cyclin D1 in MM cells lacking IgH translocations may be driven by interactions of the malignant cells with the microenvironment (9). High levels of the *maf* oncogene can be found in ~50% of MM cases, but chromosomal translocations involving this oncogene were observed in only 5% to 10% of them (10), indicating that mechanisms other than major genetic alterations may be responsible for its overexpression. Cytokines and interactions with components of the ECM [e.g., interleukin (IL)-6, Wnt signaling factors, and fibronectin] can affect various physiologic functions of B cells (e.g., proliferation, apoptosis, drug resistance, and migration),

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including plasma cell dyscrasias (11, 12). Recent studies indicated that the survival of patients diagnosed with diffuse large B-cell lymphoma (DLBCL) and treated with cyclophosphamide-Adriamycin-vincristine-prednisone (CHOP) or rituximab-CHOP can be predicted by a trivariate model, characterized by a three-gene expression signature. Two of these (designated stromal-1 and stromal-2) originate from the stroma of these lymphomas, whereas the third signature (designated germinal center B cell) is composed of gene expression within the lymphoma cells (13). In B-cell chronic lymphocytic leukemia, stromal cells protect the malignant cells from spontaneous and fludarabine-induced apoptosis by increasing the Mcl-1 protein levels (14). These data indicate that the clinical outcome of B-cell malignancies may be established by both the intrinsic properties of the malignant cells ("tumor signature") and microenvironmental effects ("stromal signature").

Although molecular alterations form integral parts of common tumorigenesis schemes (15), the relative contribution of the microenvironment to the gene expression program of the cancer cell is far from clear. Because the microenvironment of malignant B cells is composed of highly diverse constituents, including both cellular and noncellular elements, we focused our analysis on the transcriptional programs of malignant B-cell variants, associated with their differential, long-term interactions with the ECM protein fibronectin. This ECM protein is present in the stroma of a subset of DLBCL (13) and can promote the survival, proliferation, and resistance to drugs of MM cells (16). Moreover, fibronectin is considered an important component of the premetastatic niche within the bone marrow (BM; refs. 17, 18).

In the present study, specialized subpopulations of malignant B cells with markedly different adhesive and motile properties were isolated from the ARH-77 human malignant B-cell line by means of differential, long-term interactions with fibronectin (19). One subpopulation, designated "type A," displays highly adhesive/low motile properties, whereas "type F" consists of poorly adhesive/highly motile cells. These two subpopulations differ widely in their malignant potential: Whereas type A cells are highly tumorigenic, causing MM-like disease when inoculated into nonobese diabetic/severe combined immunodeficient mice, tumor incidence with type F cells is low (20). Although these results suggest a strong correlation between the tumorigenic and adhesive phenotypes of malignant B cells, the molecular and cellular mechanisms underlying this phenomenon remain unclear.

We show herein that although both cell types share a common origin, type A and type F cells display distinct gene expression profiles. Some of these differences are primary (intrinsic), whereas others are triggered by the adhesion of the cells to fibronectin. In the highly tumorigenic type A cells, genes involved in transcriptional programs (e.g., NFκB-induced genes and immediate early genes) are highly expressed. In type F cells, on the other hand, genes characteristic of terminally differentiated plasma cells are prominent. We also found that growth factors and oncogenes, such as IL-6, vascular endothelial growth factor (VEGF), and *maf*, known to be associated with MM tumorigenesis, are predominantly expressed by type A cells, in agreement with their highly malignant potential (20). Taken together, these results indicate that adhesion-mediated signals can further modulate the gene expression program in malignant B cells, affecting genes associated with tumorigenesis.

Materials and Methods

Cells

The ARH-77 EBV-transformed B-cell line was kindly provided by Hanna Ben-Bassat (Hadassah Medical School, Jerusalem, Israel; ref. 21). Cells were identified by their flow cytometric marker profile. Cells were cultured in RPMI 1640 supplemented with 1 mmol/L glutamine, 50 μg/mL streptomycin, 50 units/mL penicillin, and 20% heat-inactivated bovine serum (Biological Industries) at 37°C in a humidified incubator in an atmosphere of 5% CO_2 and 95% air. ARH-77 cells were plated on tissue culture plates (Corning, Inc.) coated with fibronectin (15 μg/mL; Sigma-Aldrich Israel Ltd.). The adherent and nonadherent cells were then separated from the original tissue culture dish until stable, highly adhesive (type A) and poorly adhesive (type F) phenotypes were established, as described by Nadav et al. (19). Briefly, adherent and nonadherent cells were separated from the original tissue culture dish. Then, the adherent cells were repeatedly transferred for 6 wk twice weekly into new dishes, discarding cells that did not adhere, until a stable, highly adhesive (type A) phenotype was established. The floating cells were repeatedly isolated from the medium of fibronectin-coated dishes and plated on new dish without transferring cells that adhered to the previous fibronectin-coated dish. The cells were repeatedly transferred for 6 wk twice weekly until a stable, poorly adhesive (type F) phenotype was established. RNA extracted from these two variants (see below) at the end of this 6-wk separation process was further used in this study. AF cells were obtained by plating type A cells on nonadhesive bacterial dishes for 1 wk, and then RNA was extracted from these cells as described below.

RNA Purification and Microarray Analysis

Total RNA was isolated from ARH-77 cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions. The separation between populations and microarray-based analyses was repeated five times each for type A and type F cells and three times for AF cells. A 10-μg sample of total RNA was used to generate double-stranded cDNA, and the resulting cDNA was used as a template for *in vitro* transcription. A 10-μg sample of cDNA was loaded onto each array, and washing, staining, and scanning were done according to the manufacturer's instructions.

Gene expression profiles were measured on 13 Affymetrix U133A arrays, normalized by MAS5. Probe sets assigned a label of "absent" by an Affymetrix detection call

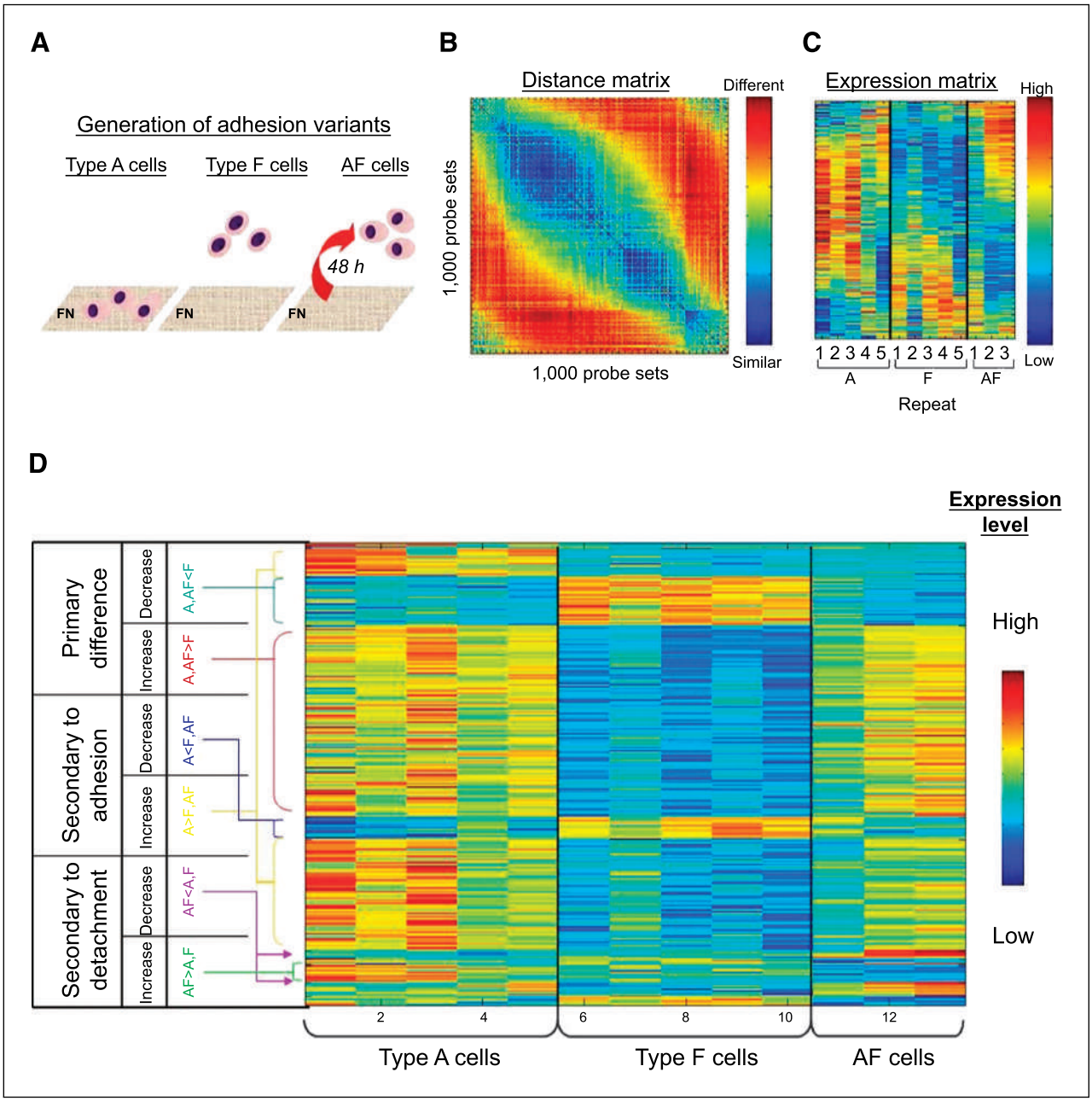

FIGURE 1. Gene expression profiling of adhesion variants from ARH-77 cells. A, fractionation of the B-cell line ARH-77 was done by separating the adherent and nonadherent cells from original cultures. The adherent cells were repeatedly transferred into new dishes until a stable, highly adhesive type A phenotype was established. Floating cells were repeatedly isolated from the medium of fibronectin (FN)–coated dishes and plated on new dishes until a stable, poorly adhesive type F phenotype was established. AF cells were constituted from type A cells that were grown on bacterial plates but not allowed to adhere to them. B and C, gene expression profiling was done five times for type A cells, five times for type F cells, and three times for AF cells. The 1,000 probe sets with the highest SDs over all the samples were analyzed. B, distance matrix. C, expression matrix of these genes. Each row represents a gene, and each column represents an independent sample. The expression level of each gene from the probe sets chosen is color coded: each value represents the difference from the mean expression value of the gene over the 13 samples, divided by the SD. Blue, gene expression at levels less than the mean; red, gene expression at levels greater than the mean. The magnitude of the expression values is reflected by the degree of color saturation (see color scale). D, gene expression patterns in ARH-77 adhesive variants. One-way ANOVA was done for each of the 1,000 probe sets, with a FDR of 10%, to compare gene expression profiles in the A, F, and AF populations. The number of probe sets that passed the FDR criterion was 275. The ANOVA was followed by a secondary step analysis, implemented by the multicompare function of Matlab, to determine which populations significantly differed. Based on the results of this analysis, the 275 probe sets were then separated into six gene groups. The six groups (indicated in the figure) include the following: primary (intrinsic; adhesion-independent) difference: increased (1) or decreased (2) in type A cells compared with type F cells; secondary (adhesion-dependent) difference: increased (3) or decreased (4) in type A cells compared with type F cells; secondary (detachment-induced) difference: increased (5) or decreased (6) in AF cells compared with type A and type F cells. As shown, the expression levels of most genes increase in an adhesion-independent (primary) or adhesion-dependent (secondary) manner.

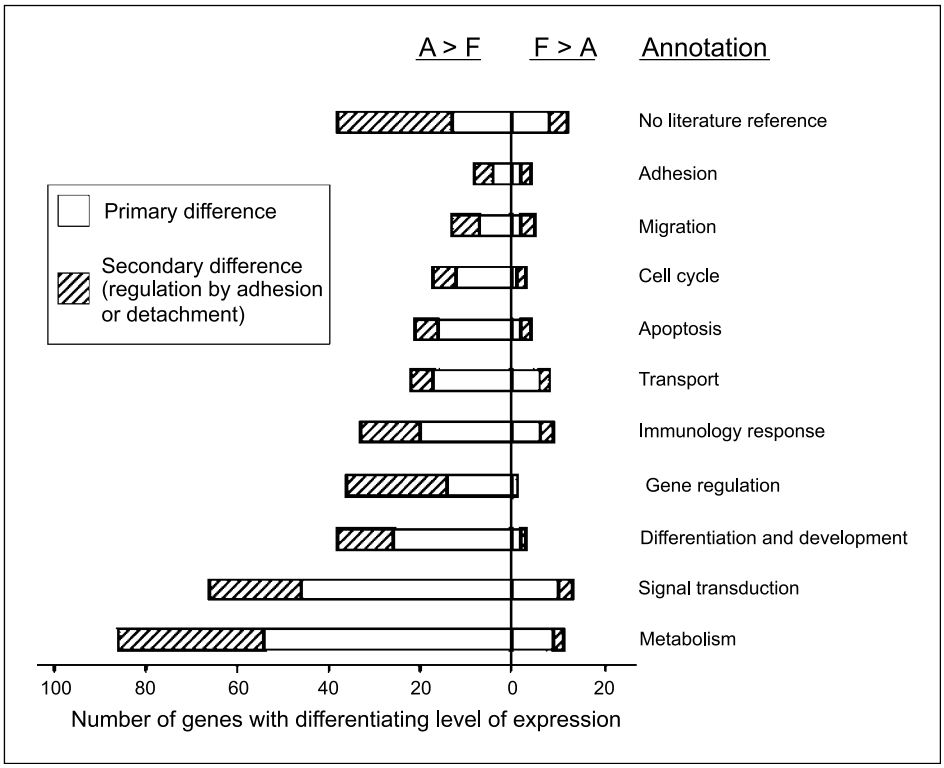

FIGURE 2. Classification of the genes into different functional groups. The differentially expressed genes were chosen according to criteria described in detail in Materials and Methods. Briefly, after setting an expression threshold, a $\log_2$ transformation was done, and only the 1,000 probe sets with the highest SD over all samples were analyzed. One-way ANOVA was done for each probe set, comparing the A, F, and AF populations, and only 275 genes passed the FDR criterion and those genes were grouped according to their expression into six groups described in Fig. 1. Those genes were further separated into functional groups according to their Gene Ontology annotation in the Affymetrix database. The internal division of the different functions varies in each gene group: some of the differences are primary and others are secondary to adhesion or detachment. As illustrated in the figure, most of the genes differentially expressed are highly expressed in type A cells, and some of the genes differentially expressed are adhesion regulated. Of note, the majority of gene expression regulators that are highly expressed in type A cells are adhesion regulated. For detailed gene description, see Table 1. A > F, all genes highly expressed in type A compared with type F; F > A, all genes highly expressed in type F compared with type A.

in all 13 arrays were removed. A threshold of $T = 30$ was set due to the relatively high additive noise levels at low expression levels. All expression values below T were set to have the value T. A $\log_2$ transformation was then done, and only the 1,000 probe sets with the highest SD over all samples were analyzed. One-way ANOVA was done for each probe set using the Matlab function "anova1" to compare the A, F, and AF populations. The resulting list of P values was then submitted to false discovery rate (FDR) analysis (22). To overcome the multiple comparison problem for probe sets identified as differentially expressed at a FDR of 10%, a secondary test was used to determine which of the three populations significantly differed from the other two (as indicated by the "multicompare" function of Matlab). Promoter analysis was then done in a given group of genes using Searching Transcription factors of Promoters (STOP) software.[5] STOP uses a score threshold specific for each transcription factor (TF); each sequence with a score higher than the threshold is considered a "hit" (i.e., a putative binding site). Using extensive expression data, score thresholds were determined based on the assumption that if a group of genes is regulated by a given TF, its average expression level will differ from those of genes in the remainder of the genome. STOP searches any selected group of genes (e.g., genes highly expressed in type A cells) for putative targets of each TF in the Transfac database (23). For such a gene group and a TF of interest, STOP calculates the fraction of genes with a hit and compares it with its corresponding fraction in the rest of the genome. STOP then produces a hypergeometric P value (for each TF and each gene group of interest) for enrichment of TF targets in the given gene group. The FDR method is then applied to the P values of the TF to overcome the multiple comparisons problem and to identify those TFs that are statistically significant. The A-F difference is calculated as the absolute value of sum (Ai-Fi), where Ai and Fi are the expression levels of type A and type F genes in repeat number i (after $\log_2$).

[5] L. Hertzberg, S. Izraeli, E. Domany. Bioinformatics. 2007;23:1737–43.

Table 1. Differentially expressed genes in plasma cell adhesive variants

Gene symbol	Gene title	A-F difference	ANOVA *P*
A. Representative genes differentially expressed in type A cells (primary difference, adhesion vs floating phenotypes)			
Highly expressed in type A cells			
CCL20	Chemokine ligand 20	29.1	0.00211
SPRR2B	Small proline-rich protein 2B	25.8	0.00113
IL1R2	IL-1 receptor, type II	21.1	0.0001
SV2B	Synaptic vesicle glycoprotein 2B	20.9	0
STK32B	Serine/threonine kinase 32B	19.6	0.00028
SULF1	Sulfatase 1	14.9	0.00002
FBN1	Fibrillin 1	14.5	0.0031
ANXA1	Annexin A1	13.9	0.00006
RGS2	Regulator of G protein signaling 2	13.2	0.00048
Decreased expression in type A cells			
CXCL13	Chemokine ligand 13	21	0.00018
CFH	Complement factor H	17.8	0.00116
Ig	IgH chain VDJ	13.5	0.00051
ABLIM1	Actin-binding LIM protein 1	9.6	0.00052
ZDHHC14	Zinc finger, DHHC-type containing 14	9.1	0.00019
Ig	Ig-rearranged H-chain	8.8	0.00157
GIMAP6	GTPase, IMAP family member 6	8.7	0.00004
IGHV1-69	Immunoglobulin heavy variable 1-69	7.8	0.00506
CLSTN2	Calsyntenin 2	7.7	0.00092
FUCA1	Fucosidase, α-L-1, tissue	5.8	0.00017
B. Examples of genes modulated in response to adhesion (secondary difference)			
Genes whose expression increases in response to adhesion			
FOS	v-*fos* viral oncogene homologue	29	0.00211
EGR1	Early growth response 1	21.3	0.00012
FOSB	Viral oncogene homologue B	20.1	0.01072
EGR3	Early growth response 3	13.4	0.00005
KEL	Kell blood group	13	0.00301
MYCN	v-*myc*	12.8	0.00589
JUN	v-*jun*	10	0.0001
EGR2	Early growth response 2	8.1	0.00062
INHBE	Inhibin, βE	7.9	0.00716
MAFF	v-maf musculoaponeurotic fibrosarcoma oncogene homologue F (avian)	8.6	0.00114
Genes whose expression decreases in response to adhesion			
IGJ	Immunoglobulin J polypeptide	24	0.01987
LSP1	Lymphocyte-specific protein 1	13.6	0.01319
IGH	Immunoglobulin heavy locus constant	8.2	0.00506
IGHM	Immunoglobulin heavy constant μ	7.9	0.00308
HIST1H1C	Histone 1, H1c	7.8	0.01613
CCR10	Chemokine receptor 10	7.2	0.0012
C1orf41	Chromosome 1 open reading frame 41	6.4	0.02432
TXNDC5	Thioredoxin domain containing 5	5.6	0.00411
ATXN1	Ataxin 1	5.2	0.0127
C. Examples of genes modulated in response to detachment (secondary difference)			
Genes whose expression increases in response to detachment			
SLC16A3	Solute carrier family 16	2.6	0.01791
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3	1.2	0.00114

(*Continued on the following page*)

Table 1. Differentially expressed genes in plasma cell adhesive variants (Cont'd)

Gene symbol	Gene title	A-F difference	ANOVA *P*
C. Examples of genes modulated in response to detachment (secondary difference)			
TUBB2	Tubulin, β 2	0.1	0.01881
HIG2	Hypoxia-inducible protein 2	0.8	0.02449
PDK3	Pyruvate dehydrogenase kinase	2.9	0.01288
HK1	Hexokinase 1	3.1	0.02264
Genes whose expression decreases in response to detachment			
ADAM28	ADAM metallopeptidase domain 28	3.7	0.00335
ADAM23	ADAM metallopeptidase domain 23	2.8	0.01656
SLC7A6	Solute carrier family 7	2.2	0.01649
TSGA10	Testis specific, 10	2	0.0186
PSEN2	Presenilin 2	2.9	0.01288
RSL1D1	Ribosomal L1 domain containing 1	1.8	0.01558

D. Regulation of expression of adhesion- and motility-related genes in type A cells

Gene group	Increase	Decrease
Adhesion-related genes		
Primary difference (adhesion vs floating phenotypes)	*NCAM1, catenin* (δ), *PLEKHC1, BTK, TSPAN12, LCK, TUBB2, KRT7, CLDN16, CCL4*	*CLSTN2, ITGAL, ABLIM1, WASF1*
Secondary to adhesion (adhesion regulated)	*TNF*	*C1orf41*
Secondary to detachment	*JUP, DDR2*	*ADAM23, SSH1*
Motility-related genes		
Primary difference (adhesion vs floating phenotypes)	*ENPP2, PTGS2, ANXA1, TUBB2, CCL20, XCL2, CCL4, PLAU, PLD1, CXCL11*	*CXCL13, WASF1, F2R, TGAL*
Secondary to adhesion	*APBB2, MYO1E, PAX3*	*LSP1*
Secondary to detachment	None	None

Reverse Transcription-PCR

Validation of microarray data for selected genes was done by means of reverse transcription-PCR (RT-PCR) as follows: cDNA samples were prepared from 5 μg of total RNA isolated from type A, type F, and AF cells using the SuperScript II reverse transcriptase kit (Invitrogen). Oligo(dT)$_{12\text{-}18}$ (Promega) was used as primer. The cDNA was mixed with primers to the following genes: *fucosidase*, 5′-GGATTTGGTTGGTGAATTGGCCAACAG-CAAGAAGCCTTTC-3′; *IgJ*, 5′-TCCAGG-ATCATCCGTTCTTCGGTTAAGGCTGTTTCCACCA-3′; *sulfatase*, 5′-CCACCTACCACTGTCCGAGTCAGAA-AGATCCCAGGTTCCA-3′; *EGR1*, 5′-CTGCGACATCTGTGGAAGAATGTCCTGGG-AGAAAAGGTTG-3′; and *GAPDH*, 5′-GAGTCAACGGA-TTTGGTCGTTGTGGTCATGAGTCCTTCCA-3′.

PCRs consisted of 22 cycles (94°C for 30 s, 56°C for 30 s, and 72°C for 30 s). PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

RT-PCR and Quantitative Real-time PCR

Total RNAs were extracted from F and F/A cells with RNeasy Mini kit (Qiagen), and TURBO DNA-free kit (Ambion) was used to remove any contaminating DNA, if present. Reverse transcription of purified RNA was done using oligo(dT) priming and Verso cDNA kit (Thermo Fisher Scientific-ABgene) according to the manufacturer's instructions. The quantification of all gene transcripts was done by quantitative PCR using the Absolute Blue QPCR SYBR Green ROX mix (Thermo Fisher Scientific-ABgene) and a Rotor-Gene RG-6000 apparatus (Corbett Research).

The primer pairs used for human *GAPDH* gene were 5′-ATGGGGAAGGTGAAGGTCG-3′ and 5′-GGGGTCATTGATGGCAACAATA-3′. The primer pairs used for the human *c-myc* gene were 5′-TGCTCCAT-GAGGAGACA-3′ and 5′-CCTCCAGCAGAAGG-TGA-3′. The primer pairs used for the human *c-fos* gene were 5′-CTCGGGCTTCAACGCAGACTA-3′ and 5′-GGAATGAAGTTGGCACTGGAGAC-3′.

Results

Differential Gene Expression Profiles of Malignant B-Cell Subpopulations

Type A and type F cells were isolated from the parental ARH-77 B-cell line, as previously described (see also Fig. 1A; ref. 19). To identify genes whose expression is directly modulated by cell adhesion (attachment or detachment), a third cell type was generated: type A cells that were devoid of adhesion for 1 week, designated "AF cells." Both the isolation of RNA from the adhesion variants and the gene array profiling were repeated five times each for type A and type F cells and three times for AF cells. The probe sets were ordered by means of the Sorting Points Into Neighborhood (SPIN) algorithm (24), which places probe sets with similar expression profiles near each other, as seen in the ordered distance matrix (Fig. 1B). As shown in the expression matrix (Fig. 1C), the unsupervised ordering generated by SPIN reveals distinct gene expression profiles that characterize most of the type A, type F, and AF cell samples.

The primary analysis identified 275 genes that passed the FDR of 10% and, hence, distinguished between the highly malignant type A cells and the far less malignant type F cells. It was then necessary to identify the primary genes (i.e., those genes whose expression levels are stable) independent of the adhesion status of the variant cells. This group includes genes whose expression levels differ in type A and type F cells, yet their expression levels in AF cells are similar to those seen in type A cells (Fig. 1D). Among these genes, we distinguished between those expressed at higher levels in type A and AF cells as opposed to type F cells and those expressed at higher levels solely in type F cells. As shown in Fig. 2, most of the genes are highly expressed on type A, and in some of them, the increment is adhesion regulated. Most of the gene expression regulators that are increased in type A are adhesion regulated. The adhesion- and motility-related genes are also significantly adhesion regulated but comprise a rather small group of genes from the entire group of genes (275) that are significantly different between type A and type F cells. As shown in Table 1A, the groups of genes stably expressed at high levels by type A or type F cells are quite heterogeneous and include genes associated with metabolism and signal transduction. Several genes that belong to the immunoglobulin family are highly expressed in type F cells (Table 1A).

Several genes and cellular programs are altered in MM cells compared with normal plasma cells (8, 25). Notably, many of these genes were found to be highly expressed in the type A subpopulation (Table 2). These include cytokines [IL-6, tumor necrosis factor (TNF) α, IL-8, and IL-15], growth factors [VEGF and epidermal growth factor (EGF)], and Wnt signaling components. In line with these findings, promoter analysis was done using the STOP software, indicating that the probe sets that are higher in type A cells are enriched for NFκB binding sites in the 1,000 bp upstream of their transcription start site, relative to all genes on the chip (Fig. 3; Table 3). Examples of such genes are cell cycle regulators (e.g., cyclin D1), cytokines (IL-6), or chemokines (CXCL11 and CCL20). The enrichment of NFκB-responsive genes in type A cells was accompanied by high levels of NFκB1 and NFκB2 expression in these cells (Fig. 3; Table 3). All of these were identified as "primary genes" (i.e., genes whose expression was unaffected by the adhesion status of the variant cells).

Adhesion-Mediated Effects on Gene Expression in Malignant B Cells

Given the fact that type A cells are more malignant, as well as highly adherent, in nature, we chose to examine the adhesion-dependent regulation of genes in these cells, specifically those genes involved in B-cell malignancies. This group included genes whose expression levels differ in type A and type F cells, yet their expression levels in type AF and type F cells are similar. Expression of these genes was considered to be either induced (A > F, AF) or repressed (A < F, AF) by adhesion to fibronectin. Another group included genes whose levels of expression were similar in type A and type F cells but differed in AF cells. Upregulation or downregulation of these genes seems to be related to the detachment of the cells from the fibronectin matrix. As shown in Fig. 2, large numbers of regulators of gene expression were induced by fibronectin adhesion, including several genes known to be associated with MM (e.g., *c-myc*; Table 1B).

Using the STOP software (details in Materials and Methods) promoter analysis, we found that the probe sets whose values are higher in type A cells are also enriched for binding sites to serum response factor (SRF) in the 1,000 bp upstream of their transcription start site. SRF is a TF and has known target genes that are highly expressed in type A cells. These genes are often hyperexpressed in these cells in response to their adhesion to fibronectin (marked with an asterisk in Fig. 3B). Examples include proto-oncogenes that have known binding sites to SRF, such as *c-fos, c-fosB, c-JunB*, EGR1, EGR2, and CRIP1 (cysteine-rich protein 1). As shown in Fig. 3, no differences were found in the expression levels of SRF, in and of itself, among the various cell types. We found that the levels of MM-associated oncogenes (e.g., c-*fos* and c-*myc*) are downregulated by cell adhesion of type A cells (Table 1B). To further study the link between cell adhesion and oncogene expression, we assessed the levels of c-*fos* and c-*myc* expression in type F cells that were reattached to fibronectin for several days. As shown in Fig. 5A, reattachment upregulated the levels of c-*fos* expression in type F cells, but the levels of c-*myc* were not modified.

Cell Adhesion Regulates Genes Associated with B-Cell Differentiation

We next examined the expression of genes associated with B-cell differentiation in the adhesive variants. The type A cell subpopulation expressed genes that positively regulate cell proliferation. In this connection, we found markedly higher levels of immunoglobulin-related genes in type F cells compared with type A cells (Fig. 4). In

Table 2. Differential expression of genes known to be related to MM pathogenesis in type A cells

Gene program	Upregulated genes in type A cells
Survival and proliferation	*IL-6, VEGF, EGF, TNF, TNFR, IL-8, IL-15*
Wnt signaling	*Wnt, Frizzled, catenin, CCND1*
Oncogenes	*v-maf*,* *v-fos*,* *v-fosB*,* *v-jun*,* *v-myc**

NOTE: Screening of the genes listed in the six groups as shown in Fig. 1 revealed that in type A cells, there are primary differences in gene expression, manifested by increased expression of ILs and growth factors known to be involved in the survival and proliferation of MM cells, and genes belong to the Wnt signaling pathway. Oncogenes known to play a role in MM were also highly expressed in type A cells, but their activation was adhesion dependent.
*Secondary to adhesion.

addition, type A cells expressed high levels of genes typically expressed at earlier stages of B-cell differentiation compared with type F cells (Fig. 4). Most of these genes were upregulated by cell adhesion (Table 1B) rather than downregulated by cell detachment (Table 1C).

In the Supplementary Data, we list each of the 275 genes and the group to which they belong and provide annotations referencing them in the literature (Supplementary Table S1). Representative genes from each of the six gene groups are shown in Table 1A (genes differentially expressed in type A cells—primary difference, adhesion versus floating phenotypes), Table 1B (genes modulated in response to adhesion—secondary difference), and Table 1C (genes modulated in response to detachment—secondary difference). To validate the microarray screening results, RT-PCR analysis was done on four representative genes from several gene groups, among them *sulfatase 1, fucosidase, EGR1*, and *IgJ*. As shown in Fig. 5B, the RT-PCR results confirmed the microarray screening results for the selected genes.

Discussion

Gene regulation in tumor cell variants is controlled by two parallel mechanisms: intrinsic, lineage-imprinted transcriptional programs and external microenvironmental cues. Because the expression signatures detected in the malignant tissues are attributable both to malignant cells proper and to the nonmalignant stroma, it is important to correlate the changes in the "malignant gene expression profile" with particular cell populations within the tumor. A recent study indicated that in DLBCL, some of these signatures (e.g., stromal-1 and stromal-2) originate in the stroma, whereas another (designated the germinal center B-cell signature) characterizes gene expression within the lymphoma cells (13).

However, genes expressed within the malignant cells can be either intrinsically regulated or affected by microenvironmental interactions. To assess the specific contribution of each of these mechanisms to the diverse transcriptional programs controlling the development of malignant B cells, we studied differences in gene expression between malignant B-cell variants that are either intrinsically adhesion independent (primary genes) or dependent on adhesion to, or detachment from, fibronectin (secondary genes whose expression is regulated by adhesion). Transcriptional differences between these variants would be expected at two levels: (*a*) primary differences that independently affect both adhesion and tumorigenesis and (*b*) primary differences that affect cell adhesion followed by secondary adhesion-dependent differences that affect the behavior of the cells *in vivo*.

Based on the data described herein, the latter view seems most likely. Most of the differentially expressed genes are highly expressed in type A cells, either in a "primary" or in an "adhesion-regulated" manner, emphasizing the critical role played by cell adhesion in the control of gene expression in malignant B cells. In contrast, cell detachment plays a relatively minor role in gene regulation in such cells, as reflected by the small size of the group of "detachment-dependent" genes. It is interesting to note that when cells detach from fibronectin, the transcription and differentiation machineries become less active, with no apparent involvement of motility-related genes, a finding that can be explained by their physiologic state as floating cells.

As expected, highly expressed genes in the strongly adhesive type A cells are associated with the cell-ECM adhesion responses or cellular motility (see Table 1D). For example, NCAM (also designated CD56), which is an aberrant marker in MM (26), is highly expressed in type A cells in an adhesion-independent manner. This finding is in line with our previous findings, showing high levels of CD56 on the surface of type A cells compared with type F cells (19), thus indicating that the differences in CD56 transcription are translated to the protein level. The higher levels of CD56 in type A cells may be associated with the increased capacity of these cells to cause lytic bone lesions in mice (20), in agreement with similar findings in human MM (26).

Although differences in the expression of genes regulating adhesion and motility in adhesive cell variants are to be expected, we herein revealed a remarkable correlation

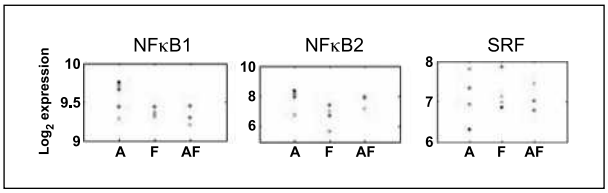

FIGURE 3. Promoter analysis of B-cell variants. The expression of the probe sets representing NFκB and SRF is shown and indicates that NFκB itself is expressed in type A cells at higher levels, whereas no such correlation exists with SRF. Most of the immediate early genes are activated in an adhesion-dependent (secondary) manner.

Table 3. Examples of genes highly expressed in Type A with NFκB and SRF binding sites

Promoter analysis	
NFκB signalling	*C3, CARD15, CCL20, CCND1, CXCL11, IL-6, TNFRSF9, BCL2A1, TGS2, DUSP1,* SOX9*
Immediate early genes	*v-fos*, v-fosB*, v-jun*, EGR1*, EGR2*, CRIP1*

NOTE: Results of promoter analysis using the STOP software done by identifying binding sites enriched in the 1,000 bp upstream of the transcription start site in the given group of genes. The probe sets that are expressed at higher levels in type A cells are enriched for the appearance of NFκB and SRF binding sites in the 1,000 bp upstream of their transcription start site.
*Secondary to adhesion.

between cell adhesion and either B-cell differentiation states or transcriptional programs known to be associated with MM. In type A cells, we found genes whose enhanced expression is typical of less-differentiated B cells. These genes include positive regulators of B-cell proliferation such as tissue inhibitor of metalloproteinase-1 (27), B-cell chronic lymphocytic leukemia/lymphoma 6 (zinc finger protein 51; ref. 28), and IL-6 (29). Other genes expressed in less-differentiated B cells include TNF family members and their receptors (TNFR), which play critical roles in determining splenic architecture and subsequent B-cell development (30).

Type A cells also express high levels of *c-myc*, which is repressed during cell differentiation, causing cessation of the cell cycle in plasma cells (31), as well as genes such as *Btk* that are involved in B-cell signaling and whose levels decrease during plasma cell differentiation (32). When plasma cells differentiate, there is a marked increase in steady-state levels of immunoglobulin heavy, light, and J chain mRNA (33), a finding that is in line with the increased expression of immunoglobulin-related genes seen in type F cells. Some of the genes are regulated in an adhesion-dependent (secondary) manner, which could suggest that plasma cell differentiation might be restricted or attenuated when cells adhere to the fibronectin matrix. Although the role played by cell-ECM adhesion (or detachment) in B-cell differentiation and maturation remains unclear, our results suggest that such regulation exists.

Furthermore, we found that known MM-related growth factors and cytokines are highly expressed in type A cells. Prime examples include cytokines such as IL-6, TNFα, and VEGF, all known to play key roles in MM by inducing cell proliferation and survival, as well as bone lesion formation and angiogenesis (34). Additional genes

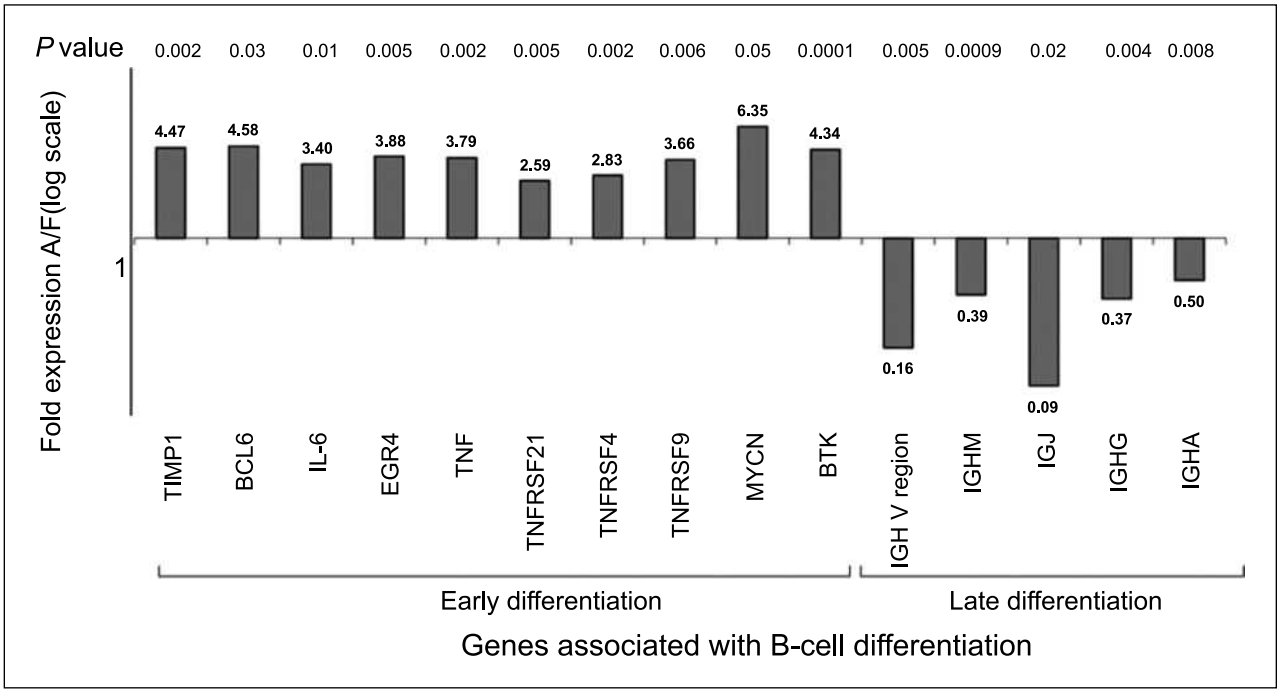

FIGURE 4. Candidate genes that bear relevance to B-cell differentiation. Expression of genes associated with B-cell differentiation in the adhesive variants was examined. According to the literature, some genes are expressed in early B-cell differentiation processes in the spleen, whereas others are expressed in terminally differentiated plasma cells. For each gene, we calculated the fold expression in type A compared with type F (A/F); genes associated with early stage of differentiation had >1 ratio, whereas the immunoglobulin genes that are a marker of mature cells had a ratio <1. The indicated *P* values were calculated according to one-way ANOVA and then submitted to FDR analysis.

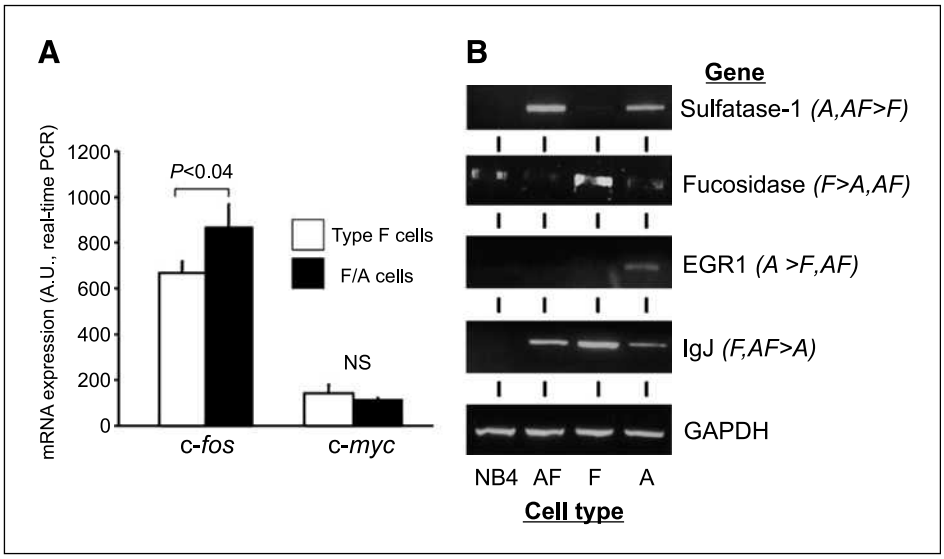

FIGURE 5. Assessment of gene expression in B-cell variants by PCR. A, c-*fos* and c-*myc* expression in type F cell revertants. F/A cells were obtained by plating type F cells on nonadhesive bacterial dishes for 1 wk. Then, adhesive cells were collected and RNA was extracted. The levels of c-*fos* and c-*myc* in these cells and in type F cells were evaluated by quantitative real-time PCR. B, validation of microarray results by means of RT-PCR analysis of selected genes. Gene expression levels were validated using RT-PCR of selected genes representative of the four main gene groups. The NB4 human acute promyelocytic leukemia cell line served as a negative control for lymphocyte-related genes.

upregulated in type A cells include cytokines (e.g., *IL-8* and *IL-15*) and EGF family members, which induce the survival and proliferation of MM cells (35). In type A cells, we also noted increased expression of Wnt signaling components whose regulation was adhesion independent, indicating intrinsic upregulation of this pathway in this cellular variant. Recently, it was shown that Wnt signaling is upregulated in MM cells (36).

Oncogenes associated with B-cell malignancies, mainly MM, are often regulated by genetic modifications (e.g., translocations; ref. 37). We found that in type A cells, oncogenes such as *myc, fos*, and *fosB*, known to play a role in B-cell malignancies (38), are expressed at higher levels. *c-Jun*, for example, is involved in the activation of IL-6 by NFκB (39). We also determined that expression of *c-maf*, as well as other oncogenes related to MM, is induced in malignant plasma cells on adhesion to fibronectin (Table 2; Fig. 3; ref. 10). Taken together, these results indicate that cell adhesion may stimulate the concerted expression of oncogenes that drive the progression of MM, independent of physical genomic alterations. The levels of c-*fos* expression seem to be more susceptible to adhesion-mediated regulation compared with those of c-*myc* because the reattachment of type F cells resulted in increase in c-*fos* expression, whereas levels of c-*myc* were not modified (Fig 5A). Although the expression of these oncogenes is transient, we show that continuous adhesion of the malignant B cells to fibronectin can sustain their expression for prolonged periods of time.

The large numbers of genes that participate in normal and/or aberrant (e.g., malignant) developmental processes are coordinated within the framework of transcriptional programs that may be identified by promoter analyses of their targets. The promoter analysis used in this study revealed that those probe sets that are higher in type A cells are enriched for NFκB and SRF binding sites. NFκB is known to play a critical role in MM by regulating the transcription of genes that influence cell growth, cell adhesion, and protection from apoptosis (e.g., CCND1, IL-6, and CXCL11; ref. 40). SRF is a TF that is required for the expression of many genes, including immediate early genes (41). SRF is activated in response to extracellular signals by associating with a diverse set of coactivators in various cell types, one of them being the myocardin-related MKL family of proteins that includes MKL1 and MKL2 (42). It has been shown that the induction of gene expression by SRF may be either dependent on, or independent of, MKL family coactivators (42). It is interesting to note that expression of all the SRF-responsive genes in type A cells (*v-fos, v-fosB, v-jun*, EGR1, EGR2, and CRIP1) was shown to be MKL independent and increased in an adhesion-dependent manner (Table 3). Taken together, these findings indicate that the early growth response genes are activated in malignant B cells on adhesion to fibronectin. Our study indicates that the SRF and EGR signaling pathway may be involved in mediating microenvironmental cues affecting malignant B cells.

In contending with nongenetic, intratumoral diversity in instances of B-cell malignancies, our findings point toward novel concepts that could explain the underpinnings of gene regulation in such cells. We conclude that diversity in gene expression is controlled by two parallel mechanisms: internal cues and microenvironmental cues; this study constitutes the first work that systematically dissected the relative contributions of each. Furthermore, most of the

alterations in gene expression, including those associated with B-cell differentiation, are due to cell adhesion to fibronectin rather than to detachment from it (Table 1). Oncogenes, in their proto-oncogenic form, may be activated by microenvironmental cues, not necessarily by intrinsic activation. In that connection, we note that oncogenes in the poorly differentiated type A cells were highly expressed, a finding that is compatible with previous data showing that MM M4 patients with poor prognoses are characterized by gene expression profiles clustered with tonsil B cells that are poorly differentiated in nature (43). Our recent study showed that on removal from their microenvironment (*ex vivo* cultivation), primary MM cells significantly upregulate their differentiation markers (44). These cells do not attach to fibronectin and grow as floating cells in a similar manner to type F cells (data not shown). This observation supports the hypothesis that microenvironmental factors (e.g., cell adhesion) can maintain low level of cellular differentiation in MM cells (44). MM cell that may display the type A phenotype should be isolated from the solid textures of the BM (e.g., BM biopsies, ongoing study).

In this work, we studied the difference in gene expression profiles between two subpopulations of malignant B cells, established by differential adhesive interactions with fibronectin. We have recently characterized the disease manifestations of these subpopulations. We found that type A cells are highly tumorigenic and caused MM-like disease in nonobese diabetic/severe combined immunodeficient mice, accompanied by the occupation of the BM, lytic bone lesions, and neuropathies (20). In contrast, type F cells exhibited a low tumorigenic potential (20). Although it is too early to draw conclusions, this finding might suggest a correlation between gene expression and clinical findings. If proven to be the case, this information may point toward a potential therapeutic approach, as previously suggested (45), namely, disrupting cellular adhesion or downstream signaling through key point mediators. One novel mediator that might thus be targeted is the immediate early gene pathway that was shown in this analysis to be regulated by adhesion. Consequently, such therapeutic efforts should perhaps be focused on specific, less-differentiated, and potentially more aggressive subpopulations of malignant B cells.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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