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ORIGINAL ARTICLE

Niche-modulated and niche-modulating genes in bone marrow cells

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Bone marrow (BM) cells depend on their niche for growth and survival. However, the genes modulated by niche stimuli have not been discriminated yet. For this purpose, we investigated BM aspirations from patients with various hematological malignancies. Each aspirate was fractionated, and the various samples were fixed at different time points and analyzed by microarray. Identification of niche-modulated genes relied on sustained change in expression following loss of niche regulation. Compared with the reference ('authentic') samples, which were fixed immediately following aspiration, the BM samples fixed after longer stay out-of-niche acquired numerous changes in gene-expression profile (GEP). The overall genes modulated included a common subset of functionally diverse genes displaying prompt and sustained 'switch' in expression irrespective of the tumor type. Interestingly, the 'switch' in GEP was reversible and turned 'off-and-on' again in culture conditions, resuming cell–cell–matrix contact versus respread into suspension, respectively. Moreover, the resuming of contact prolonged the survival of tumor cells out-of-niche, and the regression of the 'contactless switch' was followed by induction of a new set of genes, this time mainly encoding extracellular proteins including angiogenic factors and extracellular matrix proteins. Our data set, being unique in authentic expression design, uncovered niche-modulated and niche-modulating genes capable of controlling homing, expansion and angiogenesis.

INTRODUCTION

In contrast to immortal cell lines, investigation of the authentic primary counterparts, including malignant bone marrow (BM) cells, is limited by the short survival of the latter *ex vivo*.[1] This obstacle reflects the strict dependence of primary BM cells on their niche, which contributes the space, supplies the nutrients and provides the conditions required for cell–cell interactions and soluble factor exchange.[1,2] Newer investigation tools, for example, molecular profiling of cell populations improved the discrimination between different subtypes of the same tumor and provided prognostic classifications to various hematological malignancies.[3–9]

As the loss of niche stimuli is one of the limiting factors in growing primary cells, we searched for the target genes in BM cells that respond to the niche stimuli and did it in the setting of BM malignancy. This selection was made in order to assist in designing new targeted therapy directed at critical niche signals required for growth and survival of the primary tumor cells. For this purpose, we analyzed the changes in gene-expression profile (GEP) following interruption of cell–niche interactions due to BM aspiration. The sustained changes in expression disclosed the subset of genes, which are sensitive to the BM microenvironment. The nature of the microenvironment stimuli regulating the BM cells was further specified using isolated culture conditions.

MATERIALS AND METHODS

Sample collections

BM aspiration was collected into heparinized syringe, and two drops ($\sim$100 µl) were transferred immediately into an Eppendorf tube and flash frozen with liquid nitrogen. Subsequent samples were frozen after longer incubation in the syringe pool (left in room temperature). Alternatively, two drops were transferred into tubes containing 1.2 ml RNAlater (Ambion Diagnostics, Austin, TX, USA).

Patients

Newly diagnosed patients with clinical and laboratory manifestations typical for multiple myeloma (MM) with a high tumor load (severe cytopenias, leukemic phase, extreme paraprotein levels, multiple lytic lesions and hypercalcemia) entered the study. Also, patients with acute leukemia presenting with severe cytopenias (grade 4) and no mature leukocytes seen in the peripheral blood were included. The samples from the leukemia cases were analyzed only if cytospine smears from the aspirates confirmed complete 'replacement' ($\sim$100%) by tumor cells. Another case had leukemic relapse of diffuse large B-cell lymphoma (DLBCL). The patients provided written informed consent. The study was approved by Assaf Harofeh Institute, Zerifin, Israel, review boards.

Long-term cultures

Residual BM aspirate was placed in a vertical tube for $\geqslant$30 min, and then the fluid layer above the red blood cell sediment was collected and 300 µl fractions (containing 20–100 $\times$ 10³ cell per µl) were seeded into 24-well

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plates (BD Falcon 24-well Multiwell Plate, Bactlab Diagnostics, Caesarea, Israel). After > 15 min incubation (room temperature, Bactlab Diagnostics, Caesarea, Israel), the cultures were supplemented with 1 ml RPMI1640 containing 2 mM L-glutamine (Sigma, Rehovot, Israel), 50 U/ml penicillin, 50 µg/ml streptomycin (Sigma) and 20% (v/v) heat-inactivated fetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained at 37 °C in a humidified 5% CO_2 incubator. Cultures were fed biweekly by replacing 50% of the medium with fresh supplement. The proportion of tumor cells was determined morphologically using cytospine smears. For RNA extraction, most of the medium was removed (leaving only 200–300 µl), and after vigorous pippetation, the released cells were collected into an Eppendorf tube. Fractions of ~70 µl were flash frozen at different time points, starting immediately following release. The cells were reincubated between freezing procedures.

RNA extraction:
Frozen samples were lyzed by adding 300 µl lysis buffer to tubes. Samples stored in RNAlater were fractionated and lyzed by adding 10 × volume (v/v) lysis buffer. RNA was isolated by MagNA Pure Compact RNA Isolation procedure using MagNA Pure Compact instrument (Roche). Integrity of RNA was examined by Agilent 2100 Bioanalyzer (Santa Clara, CA, USA).

Gene-expression profile
Biotin-labeled cRNA was generated from 200 ng total RNA, hybridized onto GeneChip Human Gene1.0 ST Array (Affymetrix, Santa Clara, CA,USA) and the data were processed with the Affymetrix GeneChip Scanner 3000 and Affymetrix Expression Console. Normalization was done by the Robust Multi-array Average (RMA) method, and fold-change results were calculated relative to the reference sample of each case (which was fixed without delay). The entire GEP data is accessible from NCBI Gene Expression Omnibus GSE36036 and GSE39184. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE36036. http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE39184.

Quantitative real-time PCR
cDNA was obtained by Transcriptor First strand cDNA synthesis kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instruction. Quantitative real-time PCR with various primers and

LightCycler 480 SYBR Green I Master kit (Roche Diagnostics) was done using LightCycler 480 Instrument II (Roche Diagnostics) according to the manufacturer's instructions. PCR primers and thermal cycling parameters were designed using Primer3 software (Supplementary Table 1). Standard curves were generated using 0.2, 0.5, 1, 2 and 5 ng/µl of control *GAPDH* cDNA.

RESULTS

BM aspirations were obtained from an overall group of 27 patients with MM, 14 patients with acute leukemia and one case with leukemic phase of DLBCL. The aspirates remained in their syringes, and on each time point, a fraction was transferred into an empty tube and fixed (either by flash freezing or using RNAlater). Therefore, each case had its own reference sample fixed immediately following aspiration and many additional samples (6–40 samples) whose fixation was delayed (up to 24 h). GEP was analyzed from whole BM samples of the 10 MM cases, with the highest proportion of plasma cells (60–95%) and the best quality of purified RNA (RIN ≥ 6). The latter group of patients presented typical characteristics of symptomatic myeloma in terms of age distribution, gender, clinical and laboratory abnormalities (Table 1a). The relatively high proportion of tumor cells, despite skipping the usual cell-separation procedures, which would prevent the immediate fixation required to record the authentic 'in vivo' expression, was the consequence of strict patient selection and using of the initial aspiration fraction while the majority of residual normal cells were granulocytes. GEP was also analyzed from whole BM samples of four acute myeloid leukemia (AML) cases for whom the proportion of tumor cells was nearly 100% by morphology and from a case with leukemic relapse of testicular DLBC with 70% tumor cells (Table 1b).

Prompt 'switch' in GEP
The first participant had MM (case A), and the BM samples selected for microarray analysis were those frozen immediately following aspiration, at 17 min later and following 120 min spent

Table 1a.	MM patient characteristics					
Case	Age/sex	ISS	Paraprotein	CRAB symptoms	FISH abnormality	MM cells
A	68 ♂	II	IgGk 5.1 g/dl	R, A, B	13q-, t(11,14)	60%
B	67 ♀	III	IgAλ 6.41 g/dl	R, A, B	13q-	95%
C	85 ♂	II	Nonsecreting	C, R, A, B	t(4,14), P53-	95%
D	59 ♂	II	Nonsecreting	C, R, A, B; PCL	13q-, t(11,14)	70%
E	72 ♀	III	LCλ 3.9 g per day	A, B	13q-	90%
F	71 ♂	II	IgAk 4.87 g/dl	A	13q-, t(11,14)	90%
G	73 ♂	III	IgGk 6.75 g/dl	R, A, B	Not found	90%
H	67 ♂	III	LCk 0.5 g per day	C, R, A, B	N/A	80%
I	74 ♂	II	IgAλ 4 g/dl	R, A, B	t(11,14)	95%
J	61 ♀	III	IgAk 2.28 g/dl	C, R, A, B; PCL	t(4,14)	90%

Abbreviations: CRAB, calcium elevated, renal failure, anemia and bone disease; FISH, fluorescence *in situ* hybridization; ISS, international scoring system; LC, light chain; MM, multiple myeloma; N/A, not available; PCL, plasma cell leukemia.

Table 1b.	Other patient characteristics					
Case	Age/sex	Tumor	FAB	Cytogenetics	FISH abnormality	Tumor cells
A′	28 ♂	AML	M0	NK	Not found	100%
B′	70 ♂	DLBCL	—	N/A	Not assessed	70%
C′	81 ♀	AML	M3	t(15; 17) (q22; q21)	PML-RARα	100%
D′	59 ♀	AML	M3	t(15; 17) (q22; q21	PML-RARα	100%
E′	18 ♀	AML	M3	t(3; 17) (q21; q26), X0	RARα[a]	100%

Abbreviations: AML, acute myeloid leukemia; DLBCL, diffuse large B-cell lymphoma (leukemic relapse); FAB, French-American-British (FAB) classification; FISH, fluorescence *in situ* hybridization; NK, normal karyotype; N/A, not available. [a]Unknown partner gene in 3q26.

in the syringe pool. The comparison of the three GEPs accepted revealed prompt induction of numerous genes after 17 min ex vivo, which included upregulation of 363 genes >2-folds, 34 genes >3-folds, 10 genes >4-folds, 7 genes >5-folds and 3 genes >10-folds compared with the reference sample frozen immediately following aspiration. Moreover, after 120 min ex vivo, nearly two-third of the former upregulated genes (at 17 min) remained overexpressed and many of which displayed further increase in expression signals. Surprisingly, a very similar 'switch' signature was recorded from the aspiration samples of an AML patient (case A') after 20 min and 120 min spent out-of-niche compared with his reference GEP (Table 2). In both patients, downregulation was milder and involved fewer genes.

To trace the earliest onset of gene modulation and view the dynamic of changes, we used quantitative real-time PCR analysis, directed at mRNA levels of three inducible genes. The results revealed an almost immediate induction onset with progressive increase in transcript levels during the first 120 min ex vivo (Table 3).

The 'switch' in GEP is sustained

The annotation database revealed that many of the promptly induced genes in the aspirates from the first two cases examined are classified as 'immediate early genes' (FOS, FOSB, RGS1, DUSP1, JUN, CD69, EGR1, PTGS2 and NR4A2). This class of genes is characterized by rapid induction onset upon stimulation but transient expression pattern.[10] To follow the expression of these and related genes at more distant time intervals following aspiration, we analyzed BM samples from additional cases and extended the time-to-fixation delays. It was found that the 'switch' in expression of the above genes is still recognizable even after 11 h ex vivo (Figure 1). Furthermore, the majority of the early GEP changes (⩾2-folds) recorded during the first 120 min ex vivo persisted after 260 min (case C'), 420 min (case F), 480 min (case G) and 540 min (case B) spent in the syringe pool, and, frequently, the changes continued to increase (Figure 2). The 'switch' signature was also 'fixed' in a sense that no late-onset induction or downregulation of genes was evident during all our records, including caspases or other proapoptotic genes expected to be

rising. Finally, the tumor type had no visible landmarks on the 'switch' signature, and the top modulated genes were similar among different individuals whether diagnosed with MM, AML or DLBCL.

The 'switch' signature is reversible and contact dependent

In theory, the 'switch' in GEP could be secondary to any of the following insults: (1) Stress of aspiration and cell transfer to ambient conditions. (2) Loss of regulatory cues from the BM niche proper. (3) Loss of external inputs from habitual cell–cell interactions among adjacent BM cells due to cell spread, dilution with red blood cells and absence of extracellular matrix to adhere. To search for the real cause, we turned to isolated culture conditions. Our culture model was designed to enable recovery of the usual cell–cell contact relationships via seeding of intact BM samples (excluding red blood cells) on a matrix and adding the medium only after adherence of the whole sample in order to avoid cell dilution. In our experience, such conditions can delay apoptosis of primary MM cells for variable periods of time, although cell proliferation remains limited (as evident from the failure of cellular areas on the bottom well to fill adjacent empty areas).[11,12] Moreover, owing to the short life span of normal granulocytes, the purity of the MM cell populations increased over culture days and, eventually, the cultures became homotypic, excluding scattered macrophages (Supplementary Figure 1). As anticipated, the prolonged viability of the MM cell populations in our culture model was associated with near-complete regression of the changes in GEP, which evolved following aspiration. Specifically, in all analyzed cases: J, B and C, where their aspiration samples were cultured for 2, 7 and 29 days, respectively, the 'switch' in GEP fade over culture days except for IL8, which remained overexpressed and its signals even increased further (by 61.6-, 7.6- and 3.9-folds, respectively). Moreover, the 'switch' signature recurred after leaving the cultured cells in suspension for additional 60 min (case B) or 120 min (case C), following their release from the bottom well compared with the cells that were frozen immediately following release (Figure 3), and the mobilized cells died within 24 h. These observations indicate that the 'switch' phenomenon is reversible and basically contact dependent.

Table 2. Spontaneous induction of numerous genes

	MM					AML				
Fold change	>2	>3	>4	>5	>10	>2	>3	>4	>5	>10
~20 min	363	34	10	7	3	325	43	12	4	1
120 min	235	56	21	15	4	267	46	30	15	7

Abbreviations: AML, acute myeloid leukemia; MM, multiple myeloma. BM aspiration samples (from MM case A and AML case A') were flash frozen at different time points following aspiration. GEPs analyzed from the samples revealed numerous changes in gene expression compared with the reference sample frozen immediately following aspiration.

Table 3. Induction onset (qRT-PCR)*

	Fold-change values						
Minutes post BM aspiration	3	10	17	28	40	80	120
FOS	1.2	4.9	37.7	112	223	309	277
FOSB	0.9	1.3	3.7	186	2385	6393	9614
MOP-1	1.67	3.4	9.4	17.7	32.8	24.3	23.5

Abbreviations: BM, bone marrow; qRT-PCR, quantitative real-time PCR. BM aspiration samples (from case A*) were flash frozen at a different time point following aspiration, and mRNA levels of three inducible genes were compared. Values for each gene were normalized to expression levels at 120 min, and fold induction at each time point was calculated relative to expression levels at time 0 (immediately following aspiration). Duplicates gave comparable results.

MM 17 A	AML 20 A'	BCL 83 B'	AML 120 A'	AML 120 C'	AML 180 D'	AML 260 C'	AML 310 E'	MM 120 D	MM 120 B	MM 120 E	MM 120 H	MM 120 A	MM 120 G	MM 120 F	MM 180 I	MM 300 C	MM 420 F	MM 480 G	MM 540 B	MM 660 J	Case
10.3	2.8	5.9	11.9	20.9	24.2	27.0	15.2	14.1	9.3	8.3	9.4	29.6	39.0	19.3	22.4	11.5	15.0	37.7	10.9	41.7	FOSB
16.1	4.1	11.1	21.0	65.2	15.8	39.2	16.8	12.9	26.2	8.0	8.6	26.2	30.0	33.6	18.7	24.2	19.5	21.3	20.4	40.8	FOS
6.5	3.0	7.2	10.2	33.0	7.7	26.8	15.5	13.9	8.0	5.9	5.6	9.4	6.8	13.6	17.6	10.5	11.5	4.8	6.7	29.5	DUSP1
2.3	2.3	2.5	4.4	11.9	20.9	33.0	4.8	2.1	2.1	2.1	3.9	7.5	6.5	9.3	5.5	3.5	12.1	13.7	3.7	21.7	NR4A2
2.1	4.9	2.9	18.9	9.6	3.1	6.9	6.7	4.6	3.4	2.1	3.8	5.5	14.1	2.7	4.5	6.5	2.9	10.8	5.3	3.5	EGR1
2.4	4.4	7.0	6.6	5.0	4.1	5.6	2.4	2.9	7.4	4.3	6.5	5.0	13.2	114.3	25.9	6.7	115.9	30.2	8.6	19.4	RGS1
3.2	1.3	3.6	6.5	8.5	3.9	10.2	7.0	3.9	2.3	4.5	4.3	7.9	5.2	3.7	3.0	11.1	3.0	8.5	3.4	3.4	PTGS2
12.1	0.9	4.2	4.3	16.7	11.8	17.0	19.1	4.0	2.5	5.6	6.8	28.0	14.5	5.1	14.7	6.0	4.1	8.6	3.2	13.2	7919749
2.1	2.0	4.1	4.9	8.3	4.8	8.9	5.5	2.2	7.2	4.0	5.2	3.8	18.0	26.2	15.4	4.9	46.8	17.2	10.0	16.2	8017096
3.3	1.5	2.4	2.5	2.2	2.9	2.1	1.7	2.3	4.5	2.4	6.0	3.1	11.3	8.4	4.9	4.1	15.1	15.6	9.8	3.7	8015796
2.0	1.6	2.9	4.9	6.7	3.3	5.2	3.9	4.4	2.7	3.2	3.1	3.3	2.3	4.4	8.1	3.8	3.8	2.7	4.5	8.5	KLF6
1.3	1.5	6.5	19.7	19.3	89.0	88.1	11.3	2.7	1.8	2.9	7.7	4.2	20.2	45.7	3.3	6.0	70.3	48.0	4.6	48.0	AREG
1.3	0.9	1.6	2.1	1.9	1.8	2.1	2.1	2.3	2.2	2.6	2.2	2.4	1.9	2.0	3.2	2.1	2.7	2.0	3.1	4.4	YPEL5
4.9	12.0	8.2	32.5	33.4	20.8	42.3	3.1	3.0	3.3	1.3	6.9	6.1	18.8	61.5	9.7	6.4	58.0	18.2	7.4	20.1	MOP-1
3.2	2.6	5.0	11.8	12.9	12.8	15.4	7.9	1.8	5.9	3.1	4.4	6.8	6.5	5.3	7.0	4.9	7.4	6.7	5.0	4.6	CD69
1.2	1.1	2.3	1.4	1.8	1.4	2.6	2.5	2.7	3.4	2.2	3.2	2.8	2.0	2.2	4.6	5.0	1.9	2.3	5.1	6.4	TRIB1
1.7	2.4	2.8	4.5	5.6	3.8	4.7	3.4	1.8	3.4	4.2	2.8	2.9	9.4	14.2	7.0	4.0	26.9	6.8	5.4	8.2	8017098
1.2	1.4	1.0	2.3	2.4	1.9	3.2	1.7	0.6	3.4	2.6	3.2	2.2	4.3	8.7	9.0	2.3	9.6	5.0	5.5	2.5	RNU5F
1.3	1.4	1.4	2.3	3.0	2.4	2.9	0.7	3.1	2.4	3.1	2.1	1.6	2.5	5.5	3.0	3.4	10.3	3.1	5.8	4.3	7904572
1.1	1.4	4.2	10.0	10.1	62.1	41.1	6.3	1.9	1.7	2.6	5.6	2.6	10.8	31.2	2.1	4.5	48.9	24.6	4.6	33.9	AREG
1.7	1.3	1.2	2.3	1.3	1.7	2.0	2.1	2.6	1.7	2.2	2.4	2.5	11.6	7.4	1.8	5.6	8.2	7.4	2.9	5.0	8053646
3.3	1.4	3.5	1.9	7.6	4.0	4.4	2.0	3.6	1.3	2.1	2.9	9.5	2.3	1.7	3.2	4.3	2.4	3.0	1.3	7.1	MIR24-2
2.1	2.3	1.8	4.7	1.8	4.7	2.2	2.4	1.2	4.0	1.0	3.2	2.7	7.8	17.1	10.7	2.5	25.3	9.4	5.5	3.7	RNU5D
3.9	2.2	8.6	2.6	4.8	2.3	5.9	1.6	3.4	1.3	3.4	1.1	2.4	2.9	13.1	5.4	7.6	12.8	3.6	1.4	9.3	RGS2
0.8	0.8	0.9	1.6	2.0	1.4	1.9	2.1	0.9	3.2	3.6	2.3	1.7	3.9	7.3	3.4	3.5	11.3	5.3	3.8	4.7	7904715
1.3	1.1	1.1	1.7	2.2	1.8	1.7	1.2	2.3	2.9	2.4	0.9	1.6	9.3	9.8	3.1	3.2	12.4	9.6	3.8	6.7	7904967
1.1	0.8	1.1	1.1	1.9	1.3	1.9	1.9	3.5	4.0	1.9	2.7	1.6	12.1	7.7	6.7	2.8	12.7	12.5	5.3	4.7	7919560
1.0	0.7	1.1	1.0	1.9	1.3	1.9	1.9	3.5	4.0	1.8	2.8	1.5	11.5	8.0	5.9	2.7	13.1	12.3	5.4	4.8	7919556
1.7	1.5	5.5	4.4	5.9	8.1	14.7	2.0	1.6	2.4	1.4	3.6	4.6	2.6	10.6	6.2	2.0	16.9	8.8	10.2	8.6	8172082
1.7	1.3	2.0	2.5	3.0	2.7	3.1	2.2	1.8	2.2	2.5	1.8	2.7	2.0	2.2	2.8	3.4	2.4	2.7	2.9	9.0	PPP1R15A
1.5	1.4	1.8	4.4	4.0	2.9	4.3	2.8	2.3	1.5	2.1	1.8	2.0	2.6	3.0	4.1	3.1	2.8	3.0	2.8	4.0	NFKBIZ
2.8	2.2	4.4	2.8	1.6	4.0	3.2	1.0	2.7	2.3	1.5	3.2	2.5	1.5	11.0	2.4	1.5	16.8	8.1	3.3	4.4	7965836
0.6	1.2	1.5	1.9	3.7	5.2	5.6	2.4	1.7	1.9	2.0	2.6	1.2	2.2	2.4	3.0	3.0	2.4	3.0	2.9	7.8	ATF3
1.2	2.6	1.8	5.0	1.1	1.9	1.9	0.7	1.0	1.3	3.1	2.3	2.1	1.9	2.6	3.5	1.8	3.8	2.6	4.7	3.8	8116992
1.3	1.2	1.7	1.5	3.5	4.7	7.2	1.0	1.6	1.7	2.9	2.4	3.2	1.6	2.2	1.6	2.2	2.3	4.5	4.5	2.8	TNFAIP3
1.8	1.4	2.1	1.6	2.4	0.8	4.7	1.6	1.6	0.9	2.0	2.6	2.9	3.9	2.5	1.7	2.2	3.3	7.9	2.3	3.1	IL8
2.0	0.7	2.0	1.8	1.6	2.4	2.0	1.4	1.2	1.3	2.0	3.1	1.8	3.0	2.7	1.9	2.7	2.5	3.0	4.0	5.6	SNORA28
0.9	1.2	1.0	2.2	1.5	1.2	1.6	1.4	2.4	2.1	3.0	1.9	1.1	3.2	5.8	1.3	2.0	7.0	2.9	3.4	3.6	7919303
1.5	1.4	1.9	2.3	3.7	3.9	5.1	3.5	7.6	1.6	1.3	1.6	2.8	2.4	4.5	1.7	19.9	5.9	3.0	2.7	42.1	KLF4
1.4	1.2	0.8	1.6	1.5	0.3	1.4	1.8	1.2	1.8	2.2	1.5	1.1	3.6	5.1	3.6	2.2	8.4	5.9	2.1	2.9	RNU11
3.7	2.8	5.8	6.6	12.0	7.4	10.7	2.1	3.0	1.8	3.4	1.7	5.1	2.7	5.2	4.3	2.0	3.6	2.9	1.5	3.4	7919749
1.7	1.8	2.2	2.4	2.3	4.1	2.3	1.9	3.7	2.5	3.1	1.2	3.9	3.4	6.7	2.7	1.4	9.0	5.0	5.8	1.8	8165692
1.6	1.0	4.4	1.5	1.9	2.4	3.0	3.9	1.6	2.0	1.6	1.7	3.5	2.3	3.3	3.4	3.5	4.2	7.9	5.2	4.4	DUSP10
1.0	1.1	1.1	1.1	1.1	1.7	1.1	1.9	1.5	1.8	2.5	2.8	3.1	0.9	3.4	1.6	9.7	2.8	1.8	3.4	6.2	CCL3
2.2	2.4	3.1	3.9	7.7	2.3	14.6	2.0	1.7	1.4	3.5	2.6	2.1	2.2	2.5	3.1	1.3	6.9	7.2	5.8	2.0	8052231
1.2	1.9	1.7	2.7	1.4	1.8	2.0	1.8	1.6	1.5	1.8	2.0	2.0	2.6	2.7	4.2	7.5	3.8	3.7	3.1	8.3	OTUD1
2.5	2.7	3.0	1.9	1.1	2.3	0.7	0.7	8.6	1.8	5.0	1.9	3.7	5.0	5.4	3.9	0.7	2.7	6.5	2.6	1.6	7896748
1.2	1.3	2.8	2.5	2.1	2.3	3.2	2.2	3.1	1.9	1.6	1.7	3.1	2.3	4.3	3.9	2.6	4.5	2.6	1.9	6.2	RAB11FIP1
1.5	0.8	1.5	0.5	8.5	12.4	12.5	3.2	1.1	1.2	1.9	1.8	3.6	2.3	6.9	2.2	2.6	5.7	4.0	4.5	2.7	8037387
1.8	1.0	2.2	1.9	3.0	8.5	8.3	6.6	1.9	1.2	1.9	5.0	1.4	2.0	2.9	1.3	3.3	3.4	2.6	3.1		RASGEF1B
0.8	1.2	2.1	1.6	1.2	0.8	0.8	0.9	1.7	1.0	3.7	2.3	4.1	1.3	5.8	2.1	1.1	2.7	2.4	2.9	2.0	8102936
1.7	1.4	1.1	1.9	3.2	2.3	3.6	2.8	1.9	2.0	2.3	1.9	2.3	4.2	1.9	2.0	2.8	2.8	4.4	2.3	3.2	JUNB
1.2	1.0	1.5	1.2	1.3	1.8	1.7	1.2	1.5	1.5	2.2	4.1	1.5	8.3	4.3	3.2	7.5	2.2	1.8	3.7	9.7	8142979
1.6	1.2	1.8	2.4	2.3	2.8	2.9	3.0	1.3	1.3	2.5	1.7	3.1	1.8	6.2	3.2	1.8	6.5	3.3	2.1	9.7	TIPARP
1.4	0.7	1.9	1.3	2.9	2.5	4.7	2.7	2.2	1.5	2.3	1.8	1.9	1.1	2.2	2.6	1.4	3.0	2.1	2.1	2.4	PDE4B
0.8	0.5	0.2	0.3	0.3	0.4	0.2	0.2	1.1	0.7	0.5	0.9	0.5	0.3	0.6	0.3	0.4	0.6	0.4	0.4	0.4	8065990
1.1	0.5	0.5	0.4	0.4	0.2	0.3	0.4	0.3	1.0	0.4	0.6	0.6	0.4	0.3	0.3	0.6	0.3	0.3	0.3	0.5	8133623
1.1	0.5	0.5	0.4	0.4	0.2	0.3	0.4	0.3	1.0	0.4	0.6	0.6	0.4	0.3	0.3	0.6	0.3	0.3	0.3	0.5	8139975
0.9	1.4	0.8	0.7	0.5	0.2	0.5	0.1	0.6	0.3	0.6	0.4	0.3	0.3	0.5	0.3	0.5	0.3	0.2	0.2	0.2	MIR223
1.1	0.5	0.5	0.3	0.2	0.3	0.2	0.1	0.4	0.5	0.6	0.3	0.3	0.5	0.4	0.4	0.4	0.4	0.9	0.4	0.2	MIR15A
1.3	1.0	0.6	0.5	0.2	0.2	0.1	0.1	0.2	0.4	0.4	0.4	0.3	0.4	0.5	0.2	0.4	0.3	0.2	0.2	0.5	7928306

Legend: <0.5 | <0.75 | >1.5 | >2

Figure 1. Sustained 'switch' in GEP following BM aspiration. BM samples from 10 patients with MM, 4 with AML and 1 case with DLBCL (BCL) were fractionated and fixed (either by flash freezing or using RNAlater) at different time points following aspiration. The samples were analyzed by microarray, and the common changes in gene expression ($\geqslant$2-folds) are displayed as fold changes relative to the signals recorded from the corresponding reference samples (fixed immediately following aspiration).

Another source of pure MM cell population was malignant pleural effusion that developed in case C. The pleural fluid obtained formed thick sediment on the bottom tube, which by morphology contained only sheets of MM cells without normal cells. On that occasion, the GEP comparison was between sediment cells that were flash frozen after respreading and maintenance in suspension for additional 270 min versus intact sediment cells flash frozen without any spread. Once again, the spread of the crowding tumor cells into suspension reinduced their 'contactless' signature, which already started to regress when the cells were allowed to cluster together on the bottom tube (Figure 3).

'Contactless switch' during remission

To clarify if the 'contactless switch' is tumor related or actually represents a more generalized phenomenon, we repeated the GEP comparison during remission. The patient selected (case B) entered complete remission following treatment with bortezomib and dexamethasone (with drop in her monoclonal protein from 6.41 g/dl to undetectable levels). It was found that the switch in GEP recorded before treatment recurred following aspiration during remission, although many of the modulated genes were discordant. Consequently, the overall GEP changes could be categorized into three different groups: (1) Genes that were modulated during remission only; in this group, the top

modulated gene was *CXCL12* (data not shown) which has a major role in homing of myeloid progenitors. (2) Genes modulated in pretreatment samples only (Figure 4, lower field); in this group, the top cited gene in the context of MM was the homing receptor *CXCR4*.[13] (3) Concordant genes which were modulated in both disease stages. The latter category included 22 genes, with many of which being recognizable as immediate early genes (Figure 4, upper field).

Unannotated niche-modulated genes

In addition to known genes, the 'contactless' signature consisted of many nameless and unannotated genes (Figure 1). The prominent member in this category was *MOP-1*, showing remarkable induction in all 'contactless' situations. Other genes (that is, Affymetrix probe sets 8017096 and 8015796) were only induced in BM samples that were largely 'replaced' by tumor cells. The top unannotated genes induced in the aspirates from the 10 MM cases are presented on Supplementary Table 2. This list likely includes newer immediate early genes waiting for validation.

Niche-modulating genes

Interestingly, the regression of the 'contactless' switch during culture was followed by induction of a new set of genes, this time mostly encoding extracellular proteins (Figure 5); among which were angiogenic factors (*IL8* and *CXCL5*) and extracellular matrix proteins (*SPP1* and *FN1*), as well as additional chemokines and

growth factors (*CXCL5, CCL2, CCL20* and *IL6*). This adaptive response uncovered inducible genes becoming overexpressed permanently, presumably due to failure of the tumor cells to
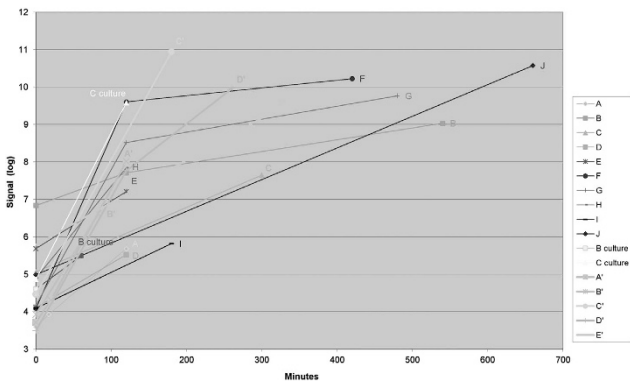
Figure 2. Induction of *AREG*. The signal intensities are depicted against the time to RNA fixation following BM aspirations obtained from patients with MM (cases A–J), leukemic phase of DLBCL (case B') and AML (cases A', C'–E') or alternatively following mobilization from the bottom well into suspension of cultured BM cells from cases B and C (see details in the next section).

Figure 3. The 'switch' in GEP is contact dependent. Cultured BM cells (CC) were flash frozen at different time points following their mobilization from the bottom well into suspension. Pleural fluid (PF) cell sediment containing pure MM cell population was split with one part left in the bottom tube and the second was transferred into another tube and maintained in suspension after redilution with pleural fluid. Both parts of the sediment were flash frozen at 270 min after the split. The frozen samples were analyzed by microarray, and the differences in signal intensities are displayed as fold changes relative to the GEPs recorded from the reference samples (fixed immediately following mobilization into suspension). Unannotated genes are denoted by their Affymetrix probe set ID number. The results showed similar changes in GEP following cell mobilization and maintenance in suspension as evolved following BM aspiration, indicating that the switch in GEP is related to the loss of cell–cell–matrix contact relationships.

CC	BM	BM	CC	PF	BM	Source
60	120	540	120	270	300	Minutes
B	B	B	C	C	C	Case
3.7	9.3	10.9	8.7	4.8	11.5	FOSB
6.7	26.2	20.4	13.7	2.0	24.2	FOS
2.9	8.0	6.7	4.2	2.8	10.5	DUSP1
2.0	2.1	3.7	14.1	2.8	3.5	NR4A2
6.2	3.4	5.3	4.2	2.2	6.5	EGR1
2.4	7.4	8.6	6.2	2.9	6.7	RGS1
1.7	2.3	3.4	4.6	4.1	11.1	PTGS2
1.7	2.5	3.2	4.2	1.9	6.0	JUN
7.7	7.2	10.0	1.4	2.3	4.9	8017096
2.8	4.5	9.8	0.4	3.3	4.1	8015796
1.6	2.7	4.5	1.8	1.3	3.8	KLF6
1.8	1.8	4.6	26.3	1.7	6.0	AREG
1.3	2.2	3.1	1.6	1.9	2.1	YPEL5
3.6	3.3	7.4	4.4	2.6	6.4	MOP-1
2.2	5.9	5.0	3.7	0.8	4.9	CD69
2.1	3.4	5.1	2.4	1.5	5.0	TRIB1
5.9	3.4	5.4	0.8	3.4	4.0	8017098
2.5	3.4	5.5	0.6	1.2	2.3	RNU5F
3.9	2.4	5.8	0.6	1.4	3.4	7904572
1.2	1.7	4.6	16.0	1.6	4.5	AREG
3.0	1.7	2.9	0.6	1.4	5.6	8053646
1.0	1.3	1.3	1.3	1.1	4.3	MIR24-2
2.1	4.0	5.5	0.8	1.9	2.5	RNU5D
3.1	1.3	1.4	10.8	1.4	7.6	RGS2
4.5	3.2	3.8	0.8	3.2	3.5	7904715
5.8	2.9	3.8	1.1	2.3	3.2	7904967
6.0	4.0	5.3	1.0	2.8	2.8	7919560
5.9	4.0	5.4	1.0	2.8	2.7	7919556
0.9	2.4	10.2	1.2	1.7	2.0	8172082
1.5	2.2	2.9	2.2	3.4	3.4	PPP1R15A
2.6	1.5	2.8	1.5	2.4	3.1	NFKBIZ
1.6	2.3	3.3	0.8	1.2	1.5	7965836
1.6	1.9	2.9	1.5	2.6	3.0	ATF3
3.5	1.3	4.7	1.4	2.0	1.8	8116992
1.1	1.7	4.5	1.4	3.1	2.2	TNFAIP3
2.3	0.9	2.3	2.1	2.0	2.2	IL8
2.3	1.3	4.0	1.2	1.7	2.7	SNORA28
3.2	2.1	3.4	0.6	1.6	2.0	7919303
1.2	1.6	2.7	2.5	5.6	19.9	KLF4
3.3	1.8	2.1	1.0	2.3	2.2	RNU11
0.7	1.8	1.5	0.6	1.6	2.0	7919749
1.2	2.5	5.8	0.8	1.2	1.4	8165692
1.7	2.0	5.2	2.3	1.9	3.5	DUSP10
4.2	1.8	3.4	7.7	1.5	9.7	CCL3
1.7	1.4	5.8	1.1	0.9	1.3	8052231
1.3	1.5	3.1	5.6	1.7	7.5	OTUD1
1.3	1.8	2.6	0.2	3.6	0.7	7896748
1.2	1.9	1.9	1.6	1.6	2.6	RAB11FIP1
1.9	1.2	4.5	1.1	0.5	2.6	8037387
1.1	1.2	2.6	3.4	2.5	1.3	RASGEF1B
1.1	1.0	2.9	1.1	2.2	1.1	8102936
1.4	2.0	2.3	1.6	1.6	2.8	JUNB
1.3	1.5	1.8	1.5	1.1	3.2	8142979
1.1	1.3	2.1	1.4	1.7	1.8	TIPARP
1.5	1.5	2.1	1.4	0.9	1.4	PDE4B
0.9	0.7	0.4	1.5	0.8	0.4	8065990
0.7	1.0	0.3	1.2	0.5	0.6	8133623
0.7	1.0	0.3	1.2	0.5	0.6	8139975
0.6	0.3	0.2	1.3	0.9	0.5	MIR223
0.6	0.5	0.4	0.4	1.6	0.4	MIR15A
0.6	0.4	0.2	0.7	0.7	0.4	7928306

<0.5	<0.75	>1.5	>2

recruit the stimulated niche components (for example, vascular vessels and extracellular matrix scaffold). Some of the above niche-modulating genes can be considered therefore attractive therapeutic targets, especially *SPP1* (or its product osteopontin) and *IL8* because of their strongest induction. Likewise, inhibitors to niche-modulated gene products, such as amphiregulin (encoded by *AREG*) and COX-2 (encoded by *PTGS2*), may have a role in reducing the tumor expansion in certain BM malignancies. Conversely, suppression of niche-modulated genes like *JUN*, *EGR1* and *KLF4* may improve the culture success.

DISCUSSION

This study exhibited pitfalls in gene-expression profiling when RNA fixation is not executed immediately following interruption of homeostasis. In addition, the changes in GEP recorded following aspiration from the whole BM samples of a heterogeneous group of patients with diverse hematological malignancies, and disease stages were integrated into a uniform 'switch' signature that was recognizable as early as few minutes following aspiration, persisted for many hours and regressed only after cell–cell–matrix contact was resumed. The latter observation indicates that cell–cell contact *per se* controls the expression of numerous genes in BM cells, while interruption of the contact relationships relieves cell-to-cell regulation and permits full expression of the 'default' genetic program inherent to each individual cell.

The resuming of contact that suppressed the 'contactless' switch' signature contributed in our culture model to increased viability of the tumor cells. Such protective effect may enhance tumor cell crowding in niche, and the contact may also serve to distribute vital signals from unreachable niche components like osteoclasts, vascular vessels and stromal cells, thereby overcoming the limited access to these regulators. The survival advantage for tumor cells gained by the over-whelming cell density was balanced, however, by stasis and lack of any expansion features, suggesting contact inhibition of cell cycle combined. Indeed, tumor expansion is more likely favored by the 'contactless' genetic program with its overexpressed oncogenes (*FOS, JUN),* homing genes (*RGS1, CD69*), angiogenic genes (*IL8, AREG*) and growth-promoting genes (*PTGS2*), whereas suppression of these genes presumably stimulates cell migration and inhibits proliferation. Nevertheless, the real physiological set up in which switch to the 'contactless' signature is allowed remains obscured. One possible scenario is that the dual function of contact, which delays apoptosis of crowding tumor cells but also inhibits their proliferation combined together with contact suppression of homing genes, ultimately, directs cell spreading and tumor expansion toward less populated niche regions where the 'contactless' gene signature is supposed to be less suppressed.

In data analysis of the niche-modulated genes based on the Ingenuity software (Redwood, CA, USA), the top function annotation category was "cancer" (25 molecules) followed by "cell death" (23 molecules) and "proliferation of cells" (20 molecules) (Supplementary Table 3). However, "proliferation of vascular tissue" yielded only one molecule (*PTGS2*), whereas our literature search yielded six molecules. Further analysis of the data from a

Figure 4. 'Switch' in GEP during remission versus before treatment. A comparison between the top post-aspiration changes in GEP (⩾2-folds) evolved in the BM samples from case B during complete remission (CR) (right column) versus the changes recorded before treatment (MM). The results are displayed as fold changes relative to corresponding reference samples (fixed immediately following aspiration). The lower field demonstrates the genes modulated in the pretreatment samples only. The upper field demonstrates overlapping genes modulated in both disease stages. Unannotated genes are denoted by their Affymetrix probe set ID number.

MM 120	MM 540	CR 120	Source Minutes
3.3	7.4	8.3	MOP-1
26.2	20.4	7.9	FOS
3.4	5.3	6.7	EGR1
9.3	10.9	6.4	FOSB
0.9	2.3	4.8	IL8
2.1	3.7	4.4	NR4A2
2.5	3.2	3.5	JUN
1.8	4.6	3.5	AREG
8.0	6.7	3.2	DUSP1
3.4	5.5	3.2	RNU5F
5.9	5.0	2.5	CD69
2.4	10.2	2.3	8172082
2.3	3.4	2.3	PTGS2
7.4	8.6	2.1	RGS1
2.4	5.8	1.9	7904572
3.4	5.1	1.8	TRIB1
2.5	5.8	1.8	8165692
3.4	5.4	1.7	8017098
2.3	5.1	1.5	8088126
0.3	0.2	0.4	MIR223
0.5	0.4	0.2	MIR15A
0.4	0.2	0.1	7928306
7.2	10.0	1.3	8017096
2.2	2.9	1.1	PPP1R15A
2.7	4.5	1.0	KLF6
2.9	3.8	0.9	7904967
2.2	3.2	1.1	CXCR4
4.0	5.4	0.9	7919556
3.2	3.8	0.6	7904715
4.0	5.3	0.8	7919560
2.2	3.1	1.2	YPEL5
2.3	3.3	1.3	7965836
2.7	3.7	0.9	ANXA1
4.0	5.5	1.0	RNU5D
4.5	9.8	1.4	8015796
2.1	3.4	1.0	7919303
2.1	1.6	0.6	7919747
2.0	5.4	1.1	PMAIP1
2.3	3.1	1.0	7904953
2.9	2.7	0.8	PTCH2
0.5	0.4	1.2	7959572
0.5	0.4	1.4	C5orf54
0.3	0.2	1.6	8093037
0.5	0.5	1.1	MIR148B
0.5	0.5	1.0	SNORD80
0.2	0.2	1.5	SCARNA15
0.2	0.5	1.6	MIR148A

<0.5	<0.75	>1.5	>2

Case	J	J	B	B	B	B	C	C	C	C	
Source	BM	TC	BM	BM	TC	TC	BM	TC	PF	TC	
hours	11	24	2	9	1	168	5	2	3	696	
Probe	**Gene**										
8096301	SPP1	0.4	9.5	1.1	1.3	0.9	66.5	0.9	1.1	0.2	3.5	extracellular
8095680	IL8	3.1	61.6	0.9	2.3	2.3	7.6	2.2	2.1	2.0	3.9	extracellular
8021635	SERPINB2	5.7	37.2	1.2	1.1	1.0	15.0	1.7	1.1	0.7	11.2	extracellular
8100977	CXCL5	1.2	31.2	0.9	1.0	1.1	19.6	1.0	1.0	1.2	4.8	extracellular
8145317	ADAMDEC1	1.0	17.0	1.0	1.1	1.2	14.6	1.1	0.9	1.1	10.0	extracellular
8006433	CCL2	1.7	16.0	1.4	1.6	1.7	8.1	1.1	0.9	1.1	8.3	extracellular
8058765	FN1	0.4	19.4	1.2	0.9	0.8	3.5	0.9	0.5	0.3	8.1	extracellular
7910385	---	1.2	13.4	2.1	1.8	1.0	6.5	1.2	0.8	1.0	5.4	---
8111210	---	1.2	15.6	2.1	1.6	1.1	6.3	1.4	0.8	1.0	2.9	---
8054722	IL1B	1.0	19.0	1.0	0.7	1.8	2.7	1.3	0.9	0.7	2.3	extracellular
8048864	CCL20	1.4	17.2	1.1	1.4	1.3	3.7	0.7	0.7	0.8	2.9	extracellular
8126784	PLA2G7	0.5	13.7	1.0	0.8	0.8	3.0	1.2	0.8	1.0	6.6	extracellular
8131844	GPNMB	0.7	7.4	1.3	1.0	0.9	7.8	1.0	1.0	0.3	5.8	membranal
8156228	CTSL1	0.8	7.2	1.0	1.2	0.9	9.8	1.2	1.0	0.4	2.7	extracellular
8051583	CYP1B1	0.6	2.5	0.9	0.5	0.8	12.1	1.1	0.9	0.7	4.7	membranal
7928308	DDIT4	1.4	4.2	0.9	0.4	0.9	11.6	1.7	1.1	0.6	3.2	intracellular
8151540	---	1.7	9.3	1.8	1.6	1.0	6.4	0.9	0.9	1.0	2.2	---
8147461	SDC2	1.1	4.2	1.2	1.1	1.1	10.7	0.9	0.7	1.0	2.0	membranal
8033257	C3	1.2	8.3	0.8	1.1	1.0	4.5	1.2	0.9	0.7	3.6	extracellular
8088180	WNT5A	1.1	9.5	1.0	0.8	0.8	2.7	1.3	0.6	1.0	3.8	extracellular
8013660	ALDOC	1.1	3.6	1.3	1.1	1.1	8.0	1.1	1.1	0.8	3.6	intracellular
7982597	THBS1	1.8	5.4	0.8	1.7	1.4	6.1	1.2	0.7	1.1	3.6	extracellular
7968883	C13orf31	0.9	4.1	1.0	1.0	0.8	5.8	1.0	0.7	1.4	4.6	---
8160297	PLIN2	1.1	7.4	0.9	1.0	1.0	3.1	1.0	0.9	1.2	3.0	extracellular
8108217	TGFBI	0.9	6.7	1.0	0.9	1.1	3.2	0.9	1.0	1.0	3.5	extracellular
7913694	FUCA1	1.0	2.3	1.1	1.0	1.3	5.5	1.2	1.0	1.0	5.5	intracellular
8001531	MT1G	8.3	6.2	1.1	1.1	0.9	3.8	1.0	1.9	1.2	2.9	---
8048432	CYP27A1	0.6	3.9	1.0	1.0	1.0	5.0	0.8	0.8	1.0	3.8	intracellular
8011713	CXCL16	1.1	6.2	1.2	2.8	1.0	3.8	1.4	1.1	1.1	2.3	extracellular
8030171	FTL	1.2	6.4	1.1	1.0	1.1	3.1	1.2	1.4	0.9	2.3	intracellular
8114612	CD14	0.9	3.7	1.7	1.0	1.3	5.7	1.1	1.2	0.4	2.0	extracellular
8114572	HBEGF	1.5	2.2	1.3	2.2	1.4	6.0	1.5	0.9	1.0	3.3	extracellular
8162531	---	7.3	6.4	1.3	1.2	0.9	2.5	1.0	1.6	1.1	2.5	---
7953532	ENO2	0.9	3.0	1.2	1.0	0.9	5.1	1.1	1.1	0.8	2.4	intracellular
8075462	SELM	1.2	3.2	1.1	0.8	1.0	3.5	0.8	1.4	1.0	3.8	intracellular
8030866	FPR3	0.9	2.1	1.2	0.9	0.9	4.1	1.2	0.8	0.9	3.3	membranal
7896748	---	1.6	2.9	1.8	2.6	1.3	2.2	0.7	0.2	3.6	4.2	---
7940996	PRDX5	1.2	2.9	1.2	1.2	1.2	2.5	1.0	0.8	1.0	3.7	intracellular
7989501	CA12	1.1	4.5	0.9	0.9	1.2	2.5	0.8	0.8	1.0	2.0	membranal
8149330	CTSB	0.6	2.7	2.2	1.1	1.2	3.8	1.1	1.0	0.4	2.5	extracellular
8004510	CD68	0.9	3.1	1.0	1.0	0.9	2.9	1.1	1.3	0.5	2.3	membranal
8018864	SOCS3	2.5	2.7	1.5	1.9	1.1	2.8	2.5	1.3	0.7	2.8	intracellular
7982868	CHAC1	1.2	3.3	1.1	1.0	1.5	2.3	1.7	0.7	1.6	2.7	intracellular
7945666	CTSD	0.9	2.8	1.4	0.8	1.0	2.4	1.0	1.2	0.9	3.0	extracellular
8060738	---	1.5	3.2	1.2	1.1	1.0	2.7	1.0	0.9	1.1	2.2	---
7990151	PKM2	0.9	2.0	1.0	1.0	1.1	2.8	1.0	1.1	0.7	2.3	intracellular
7945663	LOC402778	1.2	2.3	1.0	1.0	0.9	2.3	1.0	1.4	0.7	2.4	membranal
8138602	DFNA5	1.0	2.1	0.9	0.8	0.8	2.3	1.0	0.9	1.0	2.1	---
8131803	IL6	1.9	7.2	1.0	1.1	1.0	2.0	1.7	0.8	1.3	3.4	extracellular

Figure 5. Niche-modulating genes. The top induced genes in cultured BM samples from three patients with MM are listed . The changes were recorded after culture for 1, 7 and 29 days and are presented as fold changes (green) relative to the signal intensities recorded from corresponding fresh BM samples flash frozen immediately following aspiration. The genes induced during culture were totally different from the 'contact-modulated genes' except for IL8, which was also induced following aspiration.

niche-regulation perspective reasoned classification of the niche-modulated genes (Figure 1) into four functional categories:

Homing
Both RGS1 and CD69 regulate leukocyte retention in niche. RGS1 attenuates migration and retains cells in niche by regulation of G-protein signaling and reduction of B-cell response to chemokines such as CXCL12.[14] Overexpression of CD69 retains immature B-cells in the BM and reduces their number in blood.[15]

Angiogenesis and stromagenesis
Several niche-modulated genes, for example, AREG, PTGS2, RGS1, RGS2, NR4A2 and IL8 support angiogenesis and recruitment of stromal cells.[16–24]

Expansion of BM cells

Amphiregulin (encoded by *AREG*) also supports tumor cells directly and promotes MM cell growth.[25,26] The upregulation of *FOS, JUN, NR4A2, TRIB1* and *CCL3* and the downregulation of *MIR15A* can also enhance leukocyte expansion and tumorigenesis.[27–32]

Suppression of BM cells

As pointed above, the reduced viability of primary tumor cells out-of-niche was not preceded during all our records by induction of any specific proapoptotic gene like caspase. Still, constitutive overexpression of master regulatory genes like *JUN,* tumor-suppressor genes like *EGR1* and *KLF4* and less-defined genes like *FOSB, CD69, PPP1R15A, TRIB1* and *NFKBIA* can be lethal by itself,[10,33–39] unless contact is resumed on time before translation/protein activation.

In reviewing the literature, we were unable to find any other series with the same GEP design. Still, the study by Chen *et al.*[36] who observed downregulation of *EGR1* and *JUN* in coculture of purified MM cells with osteoclasts compared with culture without osteoclasts is consistent with our data concerning the downregulation of both genes when contact is resumed. Irrespective of this, Chen *et al.*[36] also found correlation between the outcome of MM patients to *JUN* and *EGR1* expression by their tumor cells. The latter observation, however, did not consider the timing of RNA fixation, which in our series strongly influenced *JUN* and *EGR1* expression. Furthermore, in most published GEP data sets analyzing purified MM or AML cells, the expression of the inducible contact-modulated genes was generally high as opposed to the 'silence' of these genes when recorded during homeostasis (immediately following aspiration). For example, in GDS531,[40] the signal intensities recorded from *FOSB, FOS, DUSP1, NR4A2, EGR1* and *RGS1* approached the levels of GAPDH (Supplementary Table 4). Therefore, expression analysis may need some standardization in the future.

In conclusion, despite the limited number of cases analyzed in the present study, the serial methodology used and the reproducibility of results substantiated our contact regulation model. The major questions left are: (1) Who is the sensor of contact? and (2) How is the message transmitted and how does it regulate transcription of so many genes simultaneously?

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