Enrichment of neural-related genes in human mesenchymal stem cells from neuroblastoma patients

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Abstract. Neuroblastoma (NB) is one of the most common pediatric solid tumors and, like most human cancers, is characterized by a broad variety of genomic alterations. Although mesenchymal stem cells (MSCs) are known to interact with cancer cells, the relationship between MSCs and metastatic NB cancer cells in bone marrow (BM) is unknown. To obtain genetic evidence about this interaction, we isolated BM-derived MSCs from children with NB and compared their global expression patterns with MSCs obtained from normal pediatric donors, using the Agilent 44K microarrays. Significance analysis of microarray results with a false discovery rate (FDR) <5% identified 496 differentially expressed genes showing either a 2-fold upregulation or downregulation between both groups of samples. Comparison of gene ontology categories of differentially expressed genes revealed the upregulation of genes categorized as 'neurological system process', 'cell adhesion', 'apoptosis', 'cell surface receptor linked signal transduction', 'intrinsic to membrane' and 'extracellular region'. Among the downregulated genes, several immunology-related terms were the most abundant. These findings provide preliminary genetic evidence of the interaction between MSCs and NB cancer cells in BM as well as identify relevant biological processes potentially altered in MSCs in response to NB.

Introduction

Neuroblastoma (NB), a poorly differentiated tumor derived from neural crest cells that affects mainly children, is the most common extracranial pediatric solid tumor. The origin of stroma in primary NB tumors, formed by Schwann cells, and whether the stroma is the cause or consequence of the maturation potential of tumor cells remain controversial. It has been hypothesized that crosstalk between Schwann cells and neuroblasts influences the biology and clinical behavior of NB tumors. However, little is known about the role of the NB microenvironment in metastasis localizations, especially in bone marrow (BM). Recent articles suggest that mesenchymal stem cells (MSCs) have a major role in maintaining stem cells niches in the BM (1), as well as in creating the tumor microenvironment (2). In this study we focused in BM-derived MSC from NB patients as a key factor in the development of metastasis.

During the past years several studies have used microarray-based high-throughput technologies to identify biological processes altered in NB cells. Hiyama *et al* (3) surveyed the differences in gene expression between unfavorable and maturing/regressing NB. Interestingly, in favorable NB, neuronal differentiation signals such as *CD44, IGF2, NTRK1* and *ANK1* were overexpressed in maturing tumors. Similarly, Kamei *et al* (4) identified genes that exhibited altered gene expression in NB tumors associated with a favorable outcome. More recently, Chen *et al* (5) performed parallel global protein and mRNA expression profiling on NB tumors and identified that cell adhesion, nervous system development and cell differentiation processes were downregulated in stage 4 *MYCN*-amplified NB tumors, suggesting a less mature neural and a more invasive phenotype of this type of cells. Finally, Abel *et al* (6), based on expression profiling, identified 4 molecular subgroups of NB that can be distinguished by a 6-gene signature. These studies show that microarray techniques are useful tools for gene expression profiling in NB tumors.

It is well known that the BM is a common site for metastasis in patients with high-risk NB. Although little is known about the control of NB tumor growth by the BM microenvironment, there has been an increasing interest in the role of MSCs and their BM niches in cancer (7,8). Some reports implicate MSCs having tumor-promoting effects whereas others show inhibition of tumor growth. Regarding the relationship between MSCs and NB, Ma *et al* (9) reported that MSCs in BM may enhance metastasis of NB via SDF-1/CXCR4 and SDF-1/CXCR7 signaling. Moreover, CXCR5 may be involved in the attraction of human metastatic NB cells to the BM (10). Despite these findings, the relationship between MSCs and NB cancer cells is still unknown, and research in this area would add new scientific knowledge and provides new therapeutic ideas and

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targets. Thus, we isolated MSCs from BM of NB patients and control donors and compared their global expression patterns using microarrays. Our findings provide preliminary genetic evidence of the interaction between MSCs and NB cancer cells in BM as well as identify relevant biological processes potentially altered in MSCs in response to NB.

Materials and methods

Mesenchymal stem cell isolation, culture and characterization. MSCs were isolated from 4 NB pediatric patients (with no amplification of the N-Myc gene) and 4 healthy donors. The study protocol was approved by the Ethics Committee of the Hospital. All patients and volunteers were informed about the purpose of the study and provided written consent, by the parents or legal guardians, regarding their participation in the study. BM-derived MSCs were obtained by adherence to plastic. Mononucleated cells were obtained after centrifugation using a Ficoll-Paque gradient. Cells were cultured at 37˚C with 5% CO_2 in DMEM (Lonza) supplemented with 10% fetal bovine serum (PAN-Biotech GmbH). MSCs cultures were characterized according the International Society for Cellular Therapy criteria (11).

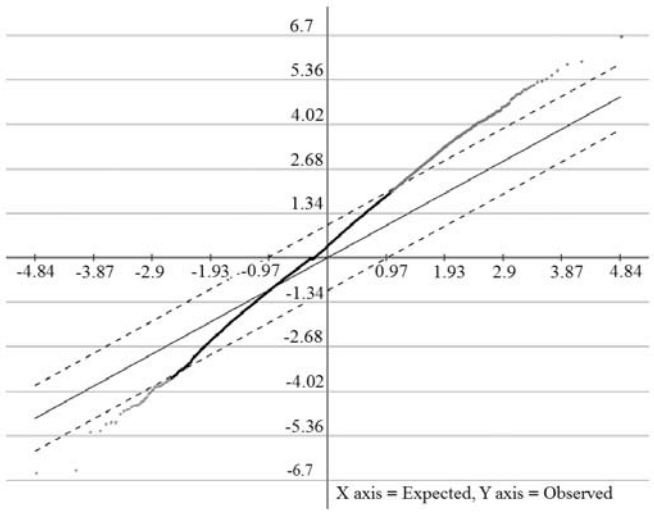

Figure 1. Significance analysis of microarrays (SAM) plot from the comparison of NB wih control. The plot identifies genes which are significantly different across the groups (2-fold upregulation or downregulation and FDR <0.05%). Spots above the upper dotted line indicate significantly upregulated genes and spots below the lower dotted line indicate significantly downregulated genes.

RNA isolation and cRNA labeling. MSCs were stabilized in PrepProtect™ (Miltenyi Biotec) and total-RNA was isolated using standard RNA extraction protocols (NucleoSpin® RNA II, Macherey-Nagel). RNA integrity and overall quality was checked via the Agilent 2100 Bioanalyzer expert software (Agilent Technologies). All RNA samples revealed an RNA Integrity Number (RIN) between 7.3 and 10. For the linear T7-based amplification step, 1 μg of each total-RNA sample was used. To produce Cy3-labeled cRNA, the RNA samples were amplified and labeled using the Agilent Low RNA Input Linear Amp kit (Agilent Technologies) following the manufacturer's protocol. Yields of cRNA and the dye-incorporation rate were measured with the ND-1000 Spectrophotometer (NanoDrop Technologies).

Microarray hybridization. The hybridization procedure was performed according to the Agilent 60-mer oligo microarray processing protocol using the Agilent Gene Expression Hybridization kit (Agilent Technologies). Briefly, 1.65 μg Cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight (17 h, 65˚C) to Agilent Whole Human Genome Oligo Microarrays 4x44K using Agilent's recommended hybridization chamber and oven. Finally, the microarrays were washed once with 6X SSPE buffer containing 0.005% N-lauroylsarcosine for 1 min at room temperature followed by a second wash with preheated 0.06X SSPE buffer (37˚C) containing 0.005% N-lauroylsarcosine for 1 min. The last washing step was performed with acetonitrile for 30 sec. Fluorescence signals of the hybridized Agilent microarrays were detected using Agilent's Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process the microarray image files.

Microarray data analysis. The microarray raw data have been deposited at the NCBI Gene Expression Omnibus under the accession number GSE35133 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=rdszfaigqkmgqdw&acc=GSE35133). Intensity values of flagged spots below background were filtered out, data were normalized using the array median and the mean values of 3 replicates for each biological sample were calculated. Statistical analysis of microarray significance (SAM) was performed to identify genes with significant changes in expression, and permutations were used to estimate the false discovery rate (FDR) (12). Genes were selected if they showed a 2-fold regulation and a FDR <0.05%. These statistical analyses were performed using TMEV and default parameters were used unless specified (13).

Gene ontology (GO) analysis. To better characterize the functionally-related genes which showed at least a 2-fold regulation in the microarray analysis, the genes were assigned to Gene Ontologies using a database for annotation, visualization and integrated discovery (DAVID) (14). We used the 3 ontologies produced by the GO consortium, namely 'biological process', 'cellular component' and 'molecular function'. GO terms were collected, redundant terms were excluded, and P-values were used to evaluate the significance of the terms.

Results

We isolated MSCs from NB patients (NB-MSCs) as well as from normal donors. All MSC cultures were characterized according to the International Society for Cellular Therapy criteria: morphology, positive/negative markers and differentiation properties (data not shown) (11). We analyzed the expression profiles of RNA from NB-MSCs compared to those from normal donors, using the Agilent Whole Human Genome Oligo Microarrays. The results from the SAM analysis (≥2-fold regulation and FDR <0.05%) revealed that 454 genes had transcript levels significantly higher in NB-MSCs, whereas 42 genes had transcript levels significantly lower in NB-MSCs (Fig. 1). A list of the genes showing the strongest upregulation

Table I. List of upregulated genes in hMSCs from NB patients.

Gene symbol	Gene description	GenBank accession no.	Fold-change	Q-value
GPR128	G protein-coupled receptor 128	NM_032787	44.269	4.5173
TMEFF2	Transmembrane protein with EGF-like and two follistatin-like domains 2	NM_016192	18.655	0.7810
ANXA10	Annexin A10	NM_007193	13.700	1.2968
ITGA2	Integrin, α 2 (CD49B, α 2 subunit of VLA-2 receptor)	NM_002203	11.435	0.0000
A_32_P208076	Unknown	A_32_P208076	10.707	0.0000
A_24_P365349	Unknown	A_24_P365349	9.664	1.6333
THC2402993	Unknown	THC2402993	9.011	0.0000
NEF3	Neurofilament 3 (150 kDa medium)	NM_005382	8.468	1.0034
IGFBP1	Insulin-like growth factor binding protein 1, transcript variant 1	NM_000596	8.255	4.6632
WDR69	WD repeat domain 69	NM_178821	8.132	3.9950
C1orf110	Chromosome 1 open reading frame 110	BC040018	7.958	3.6213
TREM1	Triggering receptor expressed on myeloid cells 1	NM_018643	7.931	4.5173
FLJ37228	cDNA FLJ37228 fis, clone BRAMY2000411	AK094547	7.177	0.0000
KCTD4	Potassium channel tetramerisation domain containing 4	NM_198404	7.162	4.2453
CNIH3	Cornichon homolog 3	NM_152495	6.984	3.9107
ENST00000379108	Unknown	ENST00000379108	6.882	3.3557
CALB2	Calbindin 2, 29 kDa (calretinin)	NM_001740	6.503	4.3242
COL4A5	Collagen, type IV, α 5, transcript variant 2	NM_033380	6.438	2.1503
SLC6A15	Solute carrier family 6, member 15, transcript variant 1	NM_182767	6.234	0.9502
TSPAN8	Tetraspanin 8	NM_004616	5.955	1.4171
SLC7A14	mRNA for KIAA1613 protein	AB046833	5.889	0.0000
THC2438492	Unknown	THC2438492	5.588	0.0000
ENST00000222543	Similar to tissue factor pathway inhibitor 2 precursor (TFPI-2)	ENST00000222543	5.577	3.2311
PSG7	Pregnancy specific β-1-glycoprotein 7	NM_002783	5.553	2.6056
DCBLD2	Discoidin, CUB and LCCL domain containing 2	NM_080927	5.547	0.7810
HTR1F	5-hydroxytryptamine (serotonin) receptor 1F	NM_000866	5.539	1.6929
SLC7A14	Solute carrier family 7, member 14	NM_020949	5.424	1.2573
RNF128	Ring finger protein 128, transcript variant 1	NM_194463	5.387	4.2453
SHC3	SHC (Src homology 2 domain containing) transforming protein 3	NM_016848	5.329	2.0866
HLA-DR B	HLA class II DR-β	X12544	5.313	3.5143
SAMD3	Sterile α motif domain containing 3, transcript variant 2	NM_152552	5.254	0.0000
SEMA3E	Semaphorin 3E	NM_012431	5.227	2.0866
LOC284344	Similar to biliary glycoprotein 1 precursor	AK097672	5.197	4.8517
PSG4	Pregnancy specific β-1-glycoprotein 4, transcript variant 2	NM_213633	5.164	1.2496
RGS4	Regulator of G-protein signaling 4	NM_005613	5.151	1.4010
AREG	Amphiregulin (schwannoma-derived growth factor)	NM_001657	5.043	4.0968
DNER	Delta-notch-like EGF repeat-containing transmembrane	NM_139072	4.943	2.4280
AK094786	cDNA FLJ37467 fis, clone BRAWH2011920	AK094786	4.787	1.2384
HGD	Homogentisate 1,2-dioxygenase (homogentisate oxidase)	NM_000187	4.784	1.8692
RGS18	Regulator of G-protein signaling 18	NM_130782	4.752	4.6632
SAMD3	Sterile α motif domain containing 3, transcript variant 1	NM_001017373	4.749	1.1850
SLC24A3	Solute carrier family 24, member 3	NM_020689	4.736	3.4520
F2RL1	Coagulation factor II (thrombin) receptor-like 1	NM_005242	4.683	2.6953
CST1	Cystatin SN	NM_001898	4.625	1.6333
TMEM158	Transmembrane protein 158	NM_015444	4.618	3.4313
THC2455389	ORF2280 gene homolog	THC2455389	4.608	3.1260
AK127194	cDNA FLJ45259 fis, clone BRHIP2020695	AK127194	4.588	3.1367
PSCDBP	Pleckstrin, Sec7 and coiled-coil domains, binding protein	NM_004288	4.539	2.4280
CLGN	Calmegin	NM_004362	4.533	3.7978
RP11-138L21.1	Similar to contactin associated protein (Caspr)	AK054645	4.436	2.3612
BCAN	Brevican	BC005081	4.370	0.0000
NPTX1	Neuronal pentraxin I	NM_002522	4.290	2.8954
HLA-DRB5	Major histocompatibility complex, class II, DR β5	NM_002125	4.284	3.3557
SULT4A1	Sulfotransferase family 4A, member 1	NM_014351	4.246	2.5183
THC2335868	ALU5_HUMAN (P39192) Alu subfamily SC	THC2335868	4.231	0.0000
FATE1	Fetal and adult testis expressed 1	NM_033085	4.196	4.3477

Table II. List of downregulated genes in hMSCs from NB patients.

Gene symbol	Gene description	GenBank accession no.	Fold-change	Q-value
IFI27	Interferon, α-inducible protein 27	NM_005532	0.0487	4.052
CRIP1	Cysteine-rich protein 1 (intestinal)	NM_001311	0.0994	4.324
CCL8	Chemokine (C-C motif) ligand 8	NM_005623	0.1021	3.438
LSP1	Lymphocyte-specific protein 1, transcript variant 3	NM_001013254	0.1188	1.462
CDCA7	Cell division cycle associated 7, transcript variant 1	NM_031942	0.1509	0.000
ENST00000372045	cDNA clone CS0DI016YJ18 (CR623913)	ENST00000372045	0.1757	2.385
IL21R	Interleukin 21 receptor, transcript variant 2	NM_181078	0.1975	4.052
C2	Complement component 2	NM_000063	0.2005	4.448
MT1JP	MTB	AF348994	0.2014	4.517
IFITM1	Interferon induced transmembrane protein 1 (9-27)	NM_003641	0.2049	3.995
ENST00000313624	cDNA clone DKFZp667P0410 (AL831953)	ENST00000313624	0.2118	3.438
FAM70A	Family with sequence similarity 70, member A	NM_017938	0.2231	2.385
ISG20	Interferon stimulated exonuclease gene 20 kDa	NM_002201	0.2419	2.385
DKFZP761M1511	cDNA FLJ39342 fis, clone OCBBF2018873	AK096661	0.2459	3.995
MBOAT1	cDNA FLJ16207 fis, clone CTONG2019822	AK131269	0.2475	0.000
JPH2	Junctophilin 2, transcript variant 1	NM_020433	0.2524	1.448
C1R	Complement component 1, r subcomponent	NM_001733	0.2639	4.724
ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin), transcript variant 1	NM_006209	0.2789	1.462
TMEM119	Transmembrane protein 119	NM_181724	0.2826	2.385
E2F2	E2F transcription factor 2	NM_004091	0.2863	2.385
OLFML2B	Olfactomedin-like 2B	NM_015441	0.2879	2.854
EXO1	Exonuclease 1, transcript variant 3	NM_003686	0.2881	2.487
CCNE2	Cyclin E2, transcript variant 1	NM_057749	0.2951	1.448
C12orf46	Chromosome 12 open reading frame 46	NM_152321	0.3028	2.385
ECGF1	Endothelial cell growth factor 1 (platelet-derived)	NM_001953	0.3067	1.462
SLC2A12	Solute carrier family 2 (facilitated glucose transporter), member 12	NM_145176	0.3321	4.724
NAV2	Steerin3 protein, alternative exon 1b	AJ488202	0.3387	3.145
A_24_P927205	Unknown	A_24_P927205	0.3480	4.052
IL7	Interleukin 7	NM_000880	0.3811	4.463
ENST00000270031	Unknown	ENST00000270031	0.3965	4.448
GBP1	Guanylate binding protein 1, interferon-inducible, 67 kDa	NM_002053	0.4015	2.385
RAB42	RAS oncogene family	NM_152304	0.4214	4.517
HELLS	Helicase, lymphoid-specific	NM_018063	0.4240	4.448
BARD1	BRCA1 associated RING domain 1	NM_000465	0.4244	3.699
RAD51AP1	RAD51 associated protein 1	NM_006479	0.4256	2.392
FLJ39660	cDNA clone DKFZp434P055	AL834537	0.4292	2.576
HIRA	Histone cell cycle regulation defective homolog A	NM_003325	0.4327	4.448
THC2376015	Unknown	THC2376015	0.4385	4.168
POLE2	Polymerase (DNA directed), ε 2 (p59 subunit)	NM_002692	0.4623	2.854
GBP2	Guanylate binding protein 2, interferon-inducible	NM_004120	0.4708	4.183
ATAD2	ATPase family, AAA domain containing 2	NM_014109	0.4838	4.069

in NB-MSCs is shown in Table I whereas the downregulated genes are shown in Table II.

To illustrate the differences between NB and normal samples the genes whose expression was induced or repressed by at least 2.5-fold in NB samples are shown as heat map in the Fig. 2, where NB and normal samples are clearly differentiated. Interestingly, several of the genes are known to play roles in NB (ANXA10, ITGA2, COL4A5 and SHC3) or other types of cancer (TMEFF2, TSPAN8, DCBLD2, PSCDBP and BCAN) (Table I). Table V lists the genes involved in neuronal processes that were >2.0-fold upregulated.

To further examine the differences in the expression profiles between NB-MSCs and normal donors, the 496 significantly up or downregulated genes were analyzed with the DAVID software and classified into the 3 main GO domains. For the upregulated genes, in the gene ontology 'biological process' we identified 138 terms, in 'cellular component' 23 terms, and in 'molecular function' 17 terms. The highest ranked terms

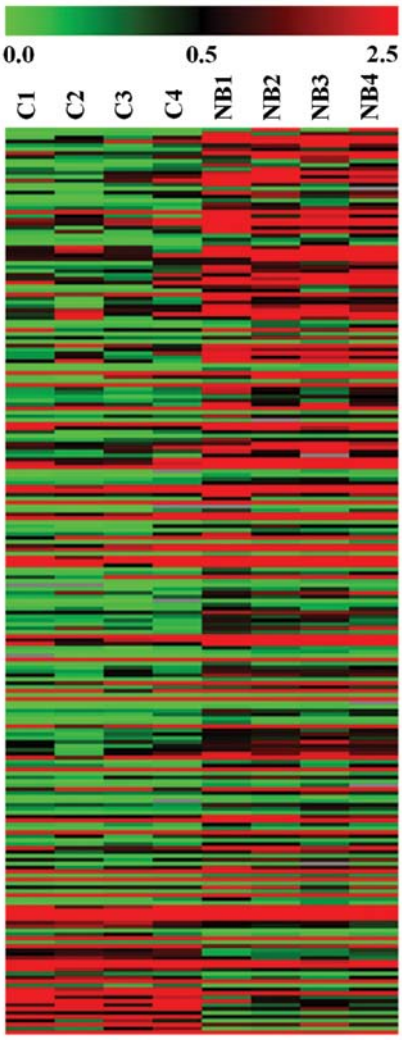

Figure 2. Heat map analysis of microarray data by SAM. Only genes undergoing a 2.5-fold (up or down) differential expression between control (C) and neuroblastoma (NB) samples (FDR <0.05%) are shown.

are shown in Table III. For the downregulated genes, in the gene ontology 'biological process' we identified 24 terms, in 'cellular component' 1 term, and in 'molecular function' 9 terms. The highest ranked terms are shown in Table IV.

Discussion

The role of MSCs in tumor progression is unclear as it has been suggested that MSCs may promote or suppress tumor growth (8). Therefore, identifying potential genes regulated in BM-derived MSCs by NB cancer cells would be of great importance to assess their role in determining disease outcome. Previously, Hahn *et al* (15) studied the effect of conditioned medium of BM cultures in NB cell growth *in vitro* (15). They showed that BM cultures may stimulate the proliferation and differentiation suppression of NB cells. In this model, monocytes seem to be the mediators of these effects. However, there is no data on how NB cells modify the characteristics of BM-resident cell populations. In this study, we analyzed for the first time the expression profiles of BM-derived MSCs from NB patients and report the identification of 496 genes with more than a 2-fold increase or decrease transcript levels. Our findings suggest that NB cancer cells may have an impact on several processes of MSCs localized in BM.

Interestingly, our microarray analysis revealed that some of the top ranked upregulated genes in NB-MSCs (ANXA10, ITGA2, COL4A5 and SHC3) have been previously reported to have a potential role in NB (Table I). For instance, Annexin A10 (ANXA10) has been identified in a microarray analysis of human NB stem cells as a gene associated with malignancy (16). Similarly, integrin upregulation has been reported as a marker of NB cell differentiation (17). The same study identified the overexpression of COL4A5 in unfavorable NB. Finally, a distinct role of ShcC (SHC3) docking protein in the differentiation of NB has been proposed (18). In addition, we identified several genes reported in other studies to be regulated in different types of cancer: TMEFF2 (19,20), TSPAN8 (21,22), DCBLD2 (23,24). Taken together, these observations suggest that the interaction between MSCs and NB cancer cells in the BM microenvironment induces changes in the expression of cancer-related genes in the MSCs. An unlikely explanation would be the cellular fusion of NB cells and MSCs, in a similar manner to Rizvanov *et al* (25) who observed rare *in vitro* cell fusion in co-cultures of NB tumor cells and MSCs.

The GO functional classification analysis through DAVID showed a number of mainly affected categories further suggesting that NB-MSCs are altered (Table III). Overall, we noted that in the 'cellular component' domain, NB upregulated a large number of genes encoding proteins 'intrinsic to membrane' (n=98) and localized to the 'extracellular region' (n=54), suggesting that NB cancer cells may exert a large repertoire of changes in these MSCs compartments. It is likely that functional relationships between NB and MSCs are mostly mediated through these proteins. Analysis of the category 'biological process' revealed effects on MSCs in terms previously described in NB cancer cells in the literature. Most importantly, we remark on the upregulation of genes in the term 'neurological system processes'. Despite of the wide variety of proteins encoded by these genes, they may provide insights into potential neurological functions altered in NB-MSCs. Interestingly, regulation of neural-related genes has also been shown in previous microarray analysis of NB tumors. Thus, Hiyama *et al* (3) reported that in favorable NB neuronal differentiation signals were overexpressed in maturing tumors whereas Chen *et al* (5) found that in NB tumors of stage 4^{+}, proteins with functions in nervous system development were downregulated, suggesting a less mature neural and a more invasive phenotype of these tumors.

The absence of NB markers in our MSC cultures pants to the absence of a tumor cell contamination. Then, it is tempting to speculate whether our findings reflect that MSCs in the BM microenvironment of NB patients redirect toward neuronal lineage. Therefore, MSCs have been proposed to adopt neural cell phenotypes, although this occurs at a very low frequency (26). In this sense, it has been proposed that NB cells would induce MSCs differentiation into Schwann cells (27). However in our data we did not observe an increase of classical Schwann-markers such as S100, Egr-1 or Egr-2 in NB-MSCs. On the other hand, MSCs would suffer a dedifferentiation process since a neuroectodermal origin of fetal MSCs localised in BM has been proposed (28).

Table III. Main GO terms of the different GO categories enriched in upregulated genes in hMSCs from NB patients.

GO term	Count	P-value	Genes
Biological process			
GO:0007166. Cell surface receptor linked signal transduction	33	0.09	GPR128, CDK5R1, TACR3, EDN1, F2RL1, PTPN22, OXTR, OPN1SW, LGR6, OR4F4, DNER, TGM2, HHIP, SHC3, HTR1F, PTPRC, RET, VAV3, GPR135, ITGA2, RGS18, GPR132, OR10J5, CCND1, GPR34, OR12D3, OR5AK2, HIPK2, GPR56, CRH, STC1, IGFBP1, AREG
GO:0050877. Neurological system process	27	0.01	RTP3, SYT1, UTS2, SNCA, SLC6A4, OXTR, OPN1SW, OR4F4, ESPN, DFNB31, NPTX1, DMD, SHC3, HAP1, HTR1F, DTNA, PRKCA, COCH, ITGA2, OR10J5, PRKCG, OR12D3, OR5AK2, CRH, USH1C, CLN8, TCF15
GO:0007242. Intracellular signaling cascade	25	0.05	DCBLD2, EDN1, OXTR, TLR7, TGM2, SHC3, KNDC1, HTR1F, PRKCA, PTPRC, RET, VAV3, PRKCG, RGNEF, CCND1, UACA, CNIH3, RGS4, C7ORF16, HIPK2, ASB1, TREM1, ABL1, GRB14, DUSP6
GO:0042981. Regulation of apoptosis	21	0.01	PRKCA, PTPRC, IER3, CDK5R1, VAV3, SNCA, SOX9, GDNF, ACVR1C, CDKN2A, UACA, TNFRSF10D, HIPK2, CRH, TGM2, BIK, CTSB, ABL1, CLN8, PHLDA1, ANGPTL4
GO:0007155. Cell adhesion	17	0.03	DCBLD2, PTPRC, OLFM4, CDK5R1, RET, PCDH10, BCAN, ITGA2, MGP, CDH2, PCDH7, SOX9, BTBD9, GPR56, CNTNAP3, ABL1, CDH10
GO:0010605. Negative regulation of macromolecule metabolic process	17	0.04	PRKCA, PTPRC, SNCA, EDN1, PRKCG, SOX9, NR0B1, PKIA, PROX1, CDKN2A, HIPK2, RNF128, HES2, ASB1, PRDM1, CLN8, C1D
GO:0007610. Behavior	13	0.02	PRKCA, SNCA, OXTR, PRKCG, GDNF, ESPN, C7ORF16, HIPK2, CRH, SERPIND1, SHC3, CLN8, TCF15
GO:0006928. Cell motion	12	0.06	PRKCA, KLF7, CDK5R1, RET, VAV3, ANK3, DNER, PRSS3, ITGA2, CDH2, GDNF, TNP2
GO:0060341. Regulation of cellular localization	11	0.01	PRKCA, SYT1, CDKN2A, UACA, SNCA, EDN1, CRH, OXTR, PKIA, GDNF, ACVR1C
GO:0048584. Positive regulation of response to stimulus	10	0.01	PRKCA, PTPRC, HIPK2, F2RL1, CRH, TGM2, PTPN22, ITGA2, PRKCG, TLR7
Cellular component			
GO:0031224. Intrinsic to membrane	98	0.04	GPR128, SYT1, SLC9A7, KLRC2, SLC6A4, F2RL1, TSPAN8, TLR7, PAPPA, CREB3L3, HTR1F, TMEFF2, LRRC3, RET, GPR135, PLXNB3, TMEM132B, GPR132, CYP2E1, PTPRO, HLA-DQA1, SLITRK1, GPR56, NEU3, RTP3, DCBLD2, OPN1SW, RIC3, HLA-DRB5, MFAP3L, CACNA2D1, MLC1, PCDH10, ITGA2, SLC6A15, NCKAP1L, SLAMF8, REEP2, KCTD4, P2RX5, GPR34, OR12D3, CLGN, OR5AK2, CNIH3, AREG, CD200, SLC45A3, KCNJ16, SLC16A14, IER3, SLC5A4, MSR1, TACR3, SLC20A1, DPP10, BCAN, CDCP1, LGR6, ACVR1C, ST6GALNAC5, SLC24A3, CNTNAP3, HHIP, CEACAM3, PPAP2C, MMP16, OR10J5, PCDH7, SLC7A14, PSG8, PSG7, TNFRSF10D, PSG4, TREM1, CLN8, GPAM, KCNH5, TMCO2, FATE1, MFSD4, TMEM158, OXTR, CDH2, C12ORF53, OR4F4, LOC151162, DNER, TMEM35, RNF128, HS6ST3, PTPRC, KIAA1244, TMPRSS9, TMEM51, GDPD1, CYBB, SLC17A3, BIK, CDH10
GO:0005576. Extracellular region	54	$6x10^{-5}$	UTS2, MSR1, EDN1, BCAN, CDCP1, GDNF, PSG11, SERPINA9, PAPPA, SEMA3E, SERPINE1, CNTNAP3, HHIP, ANGPT2, TFPI2, PRSS35, COCH, TMEFF2, SCUBE3, CA11, CRISP1, CST2, MGP, PSG3, CST1, MMP16, PSG1, C17ORF69, MMP12, PSG9, PSG8, PSG7, PSG4, TFPI, STC1, CTSB, TREM1, GPHA2, CA2, OLFM4, DEFB126, MIA2, DEFB114, PRSS2, PRSS3, ANGPTL4, COL4A5, TSLP, IGSF21, UACA, MCFD2, CRH, AREG, IGFBP1, SERPIND1, TLL2
GO:0042995. Cell projection	18	0.04	SYT1, CDK5R1, FBXO2, SNCA, OXTR, ITGA2, PRKCG, CDH2, OPN1SW, ESPN, DFNB31, PPP1R9A, GPR34, ANK3, DNER, DRP2, USH1C, CA2
Molecular function			
GO:0005509. Calcium ion binding	32	$6x10^{-7}$	SYT1, CDK5R1, SNCA, CDH2, CALB2, NPTX1, SLC24A3, PRSS2, DNER, DMD, PRSS3, TGM2, DTNA, PRKCA, CACNA2D1, RET, SCUBE3, PCDH10, MGP, ITGA2, PRKCG, MMP16, PCDH7, PADI1, MMP12, CLGN, ANXA10, EFHB, DRP2, MCFD2, TLL2, CDH10
GO:0004857. Enzyme inhibitor activity	14	$5x10^{-5}$	SNCA, CST2, CST1, PKIA, SERPINA9, CDKN2A, PPP1R1C, C7ORF16, SERPINE1, TFPI, SERPINB4, SERPIND1, TFPI2, ANGPTL4

Table IV. Main GO terms of the different GO categories enriched in downregulated genes in hMSCs from NB patients.

GO term	Count	P-value	Genes
Biological process			
GO:0006955. Immune response	9	0.00003	EXO1, IL7, ENPP2, CCL8, RSAD2, C1R, C2, GBP2, GBP1
GO:0006259. DNA metabolic process	7	0.003	EXO1, CCNE2, RAD51AP1, POLE2, HELLS, ISG20, BARD1
GO:0006952. Defense response	5	0.02	LSP1, CCL8, RSAD2, C1R, C2
GO:0007049. Cell cycle	5	0.06	EXO1, CCNE2, E2F2, HELLS, BARD1
GO:0006959. Humoral immune response	4	0.0004	EXO1, IL7, C1R, C2
GO:0002252. Immune effector process	4	0.002	EXO1, RSAD2, C1R, C2
GO:0046649. Lymphocyte activation	4	0.01	EXO1, IL7, IL21R, HELLS
GO:0006281. DNA repair	4	0.01	EXO1, RAD51AP1, POLE2, BARD1

Table V. List of neuronal-related genes upregulated in hMSCs from NB patients.

Gene symbol	Gene description	GenBank accession no.	Fold change	Q-value
ITGA2	Integrin, α 2 (CD49B, α 2 subunit of VLA-2 receptor)	NM_002203	11.435	0.0000
HTR1F	5-hydroxytryptamine (serotonin) receptor 1F	NM_000866	5.539	1.6929
SHC3	SHC (Src homology 2 domain containing) transforming protein 3	NM_016848	5.329	2.0866
NPTX1	Neuronal pentraxin I	NM_002522	4.290	2.8954
DFNB31	Autosomal recessive deafness type 31 protein 2	AK056190	3.487	0.0000
SYT1	Synaptotagmin I	NM_005639	3.452	4.6886
RTP3	Receptor transporter protein 3	NM_031440	3.282	2.3923
OXTR	Oxytocin receptor	NM_000916	3.274	3.5862
SNCA	Synuclein, α (non A4 component of amyloid precursor)	NM_007308	2.980	2.8954
ESPN	Espin	NM_031475	2.518	1.5910
CRH	Corticotropin releasing hormone	NM_000756	2.496	3.3557
CLN8	Ceroid-lipofuscinosis, neuronal 8 (epilepsy, progressive with mental retardation)	NM_018941	2.479	1.0034
COCH	Coagulation factor C homolog, cochlin	NM_004086	2.420	3.6986
DMD	Dystrophin (muscular dystrophy, Duchenne and Becker types)	NM_004010	2.408	4.0521
DTNA	Dystrobrevin, α, transcript variant 1	NM_001390	2.330	2.4868
HAP1	Huntingtin-associated protein 1 (neuroan 1), transcript variant 1	NM_003949	2.311	2.1529
OR12D3	Olfactory receptor, family 12, subfamily D, member 3	NM_030959	2.301	1.0207
OR5AK2	Olfactory receptor, family 5, subfamily AK, member 2	NM_001005323	2.293	0.7810
TCF15	Transcription factor 15 (basic helix-loop-helix)	NM_004609	2.151	2.5183
PRKCG	Protein kinase C, γ	NM_002739	2.138	3.4520
PRKCA	Protein kinase C, α	NM_002737	2.132	1.3963
OR4F4	Olfactory receptor, family 4, subfamily F, member 4	NM_001004195	2.104	1.6012
OPN1SW	Opsin 1 (cone pigments), short-wave-sensitive (color blindness, tritan)	NM_001708	2.069	1.1850
UTS2	Urotensin 2, transcript variant 1	NM_021995	2.067	1.3372
OR10J5	Olfactory receptor, family 10, subfamily J, member 5	NM_001004469	2.052	1.0034
USH1C	cDNA: FLJ21290 fis, clone COL01954	AK024943	2.011	1.0034
SLC6A4	Solute carrier family 6 (neurotransmitter transporter, serotonin), member 4	NM_001045	2.001	4.5173

The term 'cell surface receptor linked signal transduction' included several members of the G protein-coupled receptor family (GPR128, GPR135, GPR132, GPR56 and GPR34). Indeed, GPR128 was the strongest upregulated gene in our microarray analysis (Table I). The G protein-coupled receptor (GPCR) superfamily has long been proposed to have vital dual roles in cellular adhesion and signaling (29). One of the best described GPCRs is GPR56. In addition to its role in neural progenitor cell migration (30), a role in suppression of tumor growth by the microenvironment have been investigated (31). By interacting with an extracellular matrix ligand, TG2 (transglutaminase 2), GPR56 seems to suppress tumor growth and metastasis *in vivo*; conversely, reduced expression is associated with tumor progression. In addition, it is overexpressed

in many human glioblastomas and functions in tumor cell adhesion (32). SHC3, another component of this GO term, has been described in the literature. Miyake *et al* (18) observed a significantly higher level of ShcC protein in NBs with poor prognostic factors and indicated that the expression of ShcC potentially has a function in inhibiting the differentiation of NB cells (18).

Upregulation of 'cell adhesion' genes supports the hypothesis that NB-MSCs may undergo changes in their extracellular matrix and cell adhesion properties. Previous research has shown the modulation of NB cell differentiation by the extracellular matrix (33). In this study, the authors showed how extracellular matrix rigidity potentiates NB cell differentiation and decreases cell proliferation; and, as we mentioned above, the receptors of extracellular matrix molecules have been reported as markers of NB cell differentiation (17). Similarly, Chen *et al* (5) reported that suppression of cell adhesion proteins in NB tumors of stage 4^+ indicates the metastatic nature of this kind of NB tumors.

In addition, increased transcript levels of a number of relevant genes involved in cell-cycle regulation or apoptosis (e.g., CCND1, CDKN2A (p14/p16), RET and GDNF) suggest that there may be alterations in these processes in NB-MSCs. Similarly, the number of upregulated genes in the 'calcium ion binding' category may indicate that intracellular calcium is likely involved in the response of MSCs to NB. ANXA10 belongs to the annexin super-family of closely related calcium and membrane-binding proteins, and many studies have shown their potential role in tumor development and progression (34). An alternative explanation would be based in the evidence that NB cells stimulate osteoclasts to generate osteolytic lesions and set free calcium, in which interactions of NB cells with BM-derived MSCs play a critical role (35).

Finally, most of the downregulated GO terms contained genes encoding immune-related proteins. However, our results also showed upregulation of HLA-DRB5 and HLA-DOA1, which encode MHC class II molecules. In this sense, Johann *et al* (36) showed that NK cell cytotoxicity was significantly impaired after co-culturing NB cells with NB-MSCs, compared with MSCs of normal donors. Further study is needed to assess the impact of NB cancer cells on the immune response of MSCs.

In summary, we present initial data of a genome-wide analysis of MSCs from NB patients. Our data suggest that the microarray approach is a useful tool to identify deregulated genes in cultured MSCs isolated from NB patients. We provide preliminary genetic evidence of the interaction between MSCs and NB cancer cells in BM. Furthermore, we identifed relevant biological processes potentially altered in MSCs in response to NB. Future studies are necessary to connect these and other differentially expressed genes into their biological roles.

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