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ORIGINAL ARTICLE

CD56 expression in human myeloma cells derived from the neurogenic gene expression: possible role of the SRY-HMG box gene, *SOX4*

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Abstract CD56 is frequently detected on primary myeloma cells from more than 80% patients with overt myeloma. In order to clarify the possible mechanisms of CD56 expression in human myeloma, we underwent screening for potential targets by microarray analysis, where the CD56(+) myeloma cell lines showed markedly increased expressions of transcription factors involved in the neuronal cell lineage compared to the CD56(−) myeloma cell lines. Here, we show that among the SOX family of transcription factors, SOX4 was highly up-regulated and SOX1 was down-regulated in the CD56(+) myeloma cell lines as well as in primary myeloma cases as confirmed by the RT-PCR. ChIP analysis of the *CD56* promoter region showed specific bindings of SOX4 in the CD56(+) and SOX1 in the CD56(−) myeloma cell lines, respectively. shRNA against SOX1 failed to induce CD56 expression in CD56(−) myeloma cell line, U266. On the contrary, overexpression of SOX4 in the CD56(−) myeloma cell line could induce the CD56 expression. Silencing of SOX4 by shRNA transfection down-regulated CD56 expression and induced apoptosis to CD56(+) human myeloma cell line, AMO1. Thus, induction of *SOX4* gene expression might be responsible for the CD56 expression in human myeloma cells.

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1 Introduction

Multiple myeloma (MM) is a B-lineage cell malignancy characterized by the excess of bone marrow plasma cells, monoclonal proteins, osteolytic bone lesions, renal disease, and immunodeficiency. It has been shown that myeloma cells have the marked heterogeneity in surface marker expressions [1–3]. Especially, ectopic expression of CD56, also known as neural cell adhesion molecule (NCAM), one of the non-B cell lineage markers is frequently detected on primary myeloma cells from more than 80% patients with overt myeloma while normal bone marrow plasma cells show no expression of CD56 [4, 5].

CD56, a member of the immunoglobulin superfamily, was initially characterized in the cells of the nervous system, where its role in various neural processes is well established. However, CD56 is also expressed in a wide variety of non-neuronal cell types, where its function has remained largely elusive. Importantly, the expression of CD56 appears to be deregulated in many different cancer types, pointing to a possible role in tumor development and poor prognosis of the disease [5, 6]. To date, CD56 expression in MM correlates inversely with bone marrow infiltration and with the number of circulating tumor cells, and higher levels of CD56 are associated with lytic bone lesions as well [5]. We have corroborated the existence of CD56(+) primary myeloma cells that express neuronal cell marker genes such as *nse*, *β-tubulin III*, and *nestin* [1]. But, exact mechanisms behind these expressions are yet to be clarified.

SOX4 belong to the group C of SOX (SRY-related HMG box) transcription factor family, which is characterized as a highly conserved HMG box, DNA-binding domain and are found throughout the animal kingdom [7]. SOX4 plays critical roles in multiple major developmental processes, such as embryonic cardiac development, thymocyte development, and nervous system development [8–11]. SOX4 is very important transcription factor for early B-cell, late T-cell, endocrine pancreas, and osteoblast development [7]. Targeted deletion of SOX4 in the mouse led to embryonic lethality at day 14 post-coitum due to circulatory failure, with B-cell development being stalled at the pro-B-cell stage [11]. RNAi knockdown of SOX4 in chick embryo neural tubes blocked neuronal gene expression, while forced expression of SOX4 resulted in neuronal gene up-regulation [7]. Recently, increasing evidence has shown that the up-regulation of SOX4 is associated with many tumors such as medulloblastoma, bladder carcinoma, breast cancer, non-small cell lung tumors, colon cancer, prostate cancer, salivary gland cancer, and hepatocellular carcinoma [7, 12]. However, the precise mechanism by which SOX4 is involved in tumorigenesis remains largely unknown.

In this study, we have shown for the first time that SOX4 is highly up-regulated in the CD56(+) myeloma cell lines as well as CD56(+) primary myeloma cases along with other neuronal lineage genes expression. Analysis of the *CD56* promoter region indicates that SOX4 might mediate its transcriptional activities through direct binding to the *CD56* gene. Further studies demonstrated that over-expression of SOX4 in CD56(−) human myeloma cell line could induce the CD56 expression, and shRNA against SOX4 could down-regulate this expression. Therefore, these data suggested that SOX4 could be responsible for the up-regulation of CD56 in human myeloma cells.

2 Materials and methods

2.1 Myeloma cell lines, primary myeloma cells, and cell culture

Myeloma cell lines ILKM8 [13], NOP2 [14], AMO1 [15], U266, and B-cell line, Raji were cultured in RPMI1640 medium (Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; M. A. Bioproducts, Walkersville, MD, USA) at 37°C with 5% CO_2. IL-6-dependent human myeloma cell line, ILKM8 (13) were cultured with recombinant human IL-6 (2 ng/ml; Sawady Technology, Tokyo, Japan) as described previously [16]. Bone marrow mononuclear cells (BMMNC) were isolated from bone marrow aspirates of myeloma patients. All samples were obtained from the patients with appropriate informed consent. This study was performed according to the guidelines of the Internal Review Board of Hiroshima Red Cross Hospital.

2.2 Viable cell assay and surface antigen expression

Cells were cultured and cell viability was measured both manually by the trypan blue exclusion assay on a hemocytometer and automatically by counting cells in 0.5 mL whole culture at a constant flow rate for 1 min using the cell sorter (Epics Elite ESP; Coulter, Hialeah, FL, USA) with forward and side scatters [17]. For surface expression, cells (1×10^5) were stained with fluorescein isothiocyanate (FITC)-labeled anti-CD38 and PC5 anti-IgG and PC5 anti-CD56 antibodies (Beckman Coulter, Marseille, France) and was subjected to a flow cytometer (Epics Elite ESP, Beckman Coulter, Hialeah, FL, USA) as described previously [17].

2.3 Microarray analysis

Total RNA was extracted from U266, ILKM8, AMO1, and NOP2 cells by using Nucleic Acid Isolation System, QuickGene-800 (Fuji film Co., Ltd., Tokyo, Japan) followed by reverse transcripted to cDNA with T7 oligo dT primer (Affymetrix, Inc. CA, USA). The cDNA synthesis product was used in an in vitro transcription reaction containing T7 RNA polymerase and biotinylated nucleotide analog (pseudouridine base). Then, the labeled cRNA products were fragmented, loaded onto GeneChip(R) Human Genome U133 Plus2.0 array (Affymetrix, Inc., CA, USA) and hybridized according to the manufacturer's protocol. Streptavidin–phycoerythrin (molecular probe) was used as the fluorescent conjugate to detect hybridized target sequences. Raw intensity data from the GeneChip array were analyzed by GeneChip Operating Software (Affymetrix, Inc., CA, USA).

2.4 Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from the cell lines and primary myeloma samples using an RNA extraction machine (Quickgene800, FujiFilm, Tokyo, Japan) according to the manufacturer's instructions. RT-PCR was performed as reported previously [1]. The oligonucleotide primers used were as follows: *GAPDH* forward: 5′-ACCACAGTCCATGCCATCAC-3′, reverse: 5′-TCCACCACCCTGTTGCTGTA-3′; *CALB1* forward: 5′-GACGGAAGTGGTTACCTGGA-3′, reverse: 5′-TGCCCATACTGATCCACAAA-3′; *CHGB* forward: 5′-ACCGTGTCCAAGAAAACCAG-3′, reverse: 5′-CAGCTGTGTGATGGGAGCTA-3′; *DBN1* forward: 5′-CTGTGGAAATGAAGCGGATT-3′,

reverse: 5′-GGGTCTCTTCCTCCTCATCC-3′; *DLX2* forward: 5′-TTCGGATAGTGAACGGGAAG-3′ reverse: 5′-GAAGCACAAGGTGGAGAAGC-3′; *ZIC2* forward: 5′-TCCGAGAACCTCAAGATCC-3′, reverse: 5′-TAGGG CTTATCGGAGGTG-3′; *CD56* forward: 5′-CCCGAATTC ATCCTTGTTCAAGC-3′, reverse: 5′-TCGGGATCCGGA CTGGCTGCGTCTT-3′; *PAX5* forward: 5′-GGGAAGGA GAGCTTGCTTTT-3′, reverse: 5′-GGGTGGAGCAGTCT TCTCAG-3′; *HOXB9* forward: 5′-GAGCAGGGCAAAG AGTAA-3′, reverse: 5′-CTTTCTCCTGACACCTAG-3′; *SOX1* forward: 5′-CAATGCGGGGAGGAGAAGTC-3′, reverse: 5′-CTCTGGACCAAACTGTGGCG-3′; *SOX4* forward: 5′-AAGCTTCAGCAACCAGCATT-3′, reverse: 5′-CCCTCTCTCTCGCTCTCTCA-3′; *PREP1* forward: 5′-AGGAGCAGACGCACCTGACTT-3′, reverse: 5′-GCA AAGACGCGCTTTAAGCTT-3′; *MEIS1* forward: 5′-ATG GCGCAAAGGTACGACGATCTAC-3′, reverse: 5′-TTAC ATGTAGTGCCACTGCCCCTCC-3′; *PBX2* forward: 5′-CTGGTTTGGCAACAAGAGGATTCGC-3′, reverse: 5′-TGGAGGTATCAGAGTGAACACTCCC-3′.

2.5 Chromatin immunoprecipitation (ChIP)

Chromatin from U266, NOP-2, and AMO1 cell lines were cross-linked, sheared by sonication and incubated overnight at 4°C with 1 µl anti SOX1 and anti SOX4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After addition of protein A-Agarose beads (Santa Cruz Biotechnology, Inc.) the immunocomplexes were collected, washed, and then genomic DNA was isolated by phenol extraction and ethanol precipitation before PCR detection of *CD56* (*NCAM1*) promoter region sequences as described previously [18]. For detection of the immunoprecipitated *CD56* promoter region, two primers, forward (5′-GTCTCTTCCCACCTCCTTCC-3′) and reverse (5′-AA CACAAGGATAGCCCAGGA-3′), specific for a 190-bp region in the *CD56* (*NCAM1*) promoter that encompasses the *SOX1* and *SOX4* binding sites were used for PCR amplification. Each PCR cycle comprised denaturation at 94°C for 1 min, primer annealing for 1 min at 60°C, and primer extension at 72°C for 1 min.

2.6 siRNA transfection

Plasmid transfection was done using Nucleofector II (Amaxa Biosystem, Cologna, Germany) optimized program for U266 (program X005 and kit R). U266 cells (2×10^6) were transfected either with 5 µg shRNA *SOX1* or control shRNA (purchased from SuperArray Bioscience Corporation, MD, USA), and AMO1 cells (2×10^6) were transfected either with 2 µg of pGFP-V-RS SOX4 shRNA vector or control shRNA GFP (purchased from Origene Technologies Inc, MD, USA) and kept in RPMI1640 + 10% FBS for 48 h. Cells were harvested and analyzed for CD56 expression by flow cytometry (Epics Elite ESP, Coulter, Hialeah, FL, USA) as reported previously [17]. cDNA from the transfected cell population and control cells were subjected to RT-PCR to confirm *SOX1* and *SOX4* gene suppressions.

2.7 Gene transfer of SOX4 into myeloma cell line

Gene transfection was done using Nucleofector II (Amaxa Biosystems, Cologne, Germany) with a protocol specifically designed for the transfection of U266 by Amaxa Biosystems. The plasmid used for transfection was the expression vector pCMV6SOX4GFP (purchased from Origene Technologies Inc, MD, USA), or empty vector as the control. U266 cells transfected with the gene encoding green fluorescent protein (EGFP) served as a transfection control in which the efficiency of transfection was between 30 and 40%. U266 cells (approximately 2×10^6 cells) were transfected with 5 µg of the vector. After transfection, U266 cells were kept for 48 h at 37°C in 10% FBS supplemented RPMI1640. Subsequently, the expression of CD56 was analyzed using a PC5-CD56 antibody with a flowcytometer as reported previously [17]. cDNA of the transfected cell population and control cells were subjected to RT-PCR to confirm *SOX4* gene expression.

2.8 Statistical analysis

Statistical analysis was conducted using the Student's *t* test with the Statistical Package for the Social Science software package (SPSS Japan, Tokyo, Japan). Statistical significance was considered at P level of <0.05 ($^*P \leq 0.05$; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$, and *NS* not significant).

3 Results

3.1 Ectopic CD56 expression is observed in more than 80% of cases of multiple myeloma and two human myeloma cell lines

Among the different human myeloma cell lines, NOP-2 and AMO1 express CD56 strongly and the CD56 expression in ILKM8 was very weak (Fig. 1a), whereas the rest of the cell lines we checked do not express CD56. CD56 expression was also examined in primary myeloma cells from 105 myeloma patients in the CD38(++) regions of the cytogram of multicolor staining with FITC-labeled anti-CD38 and PC5-labeled anti-CD56 antibody, and about 80% of the patients showed the significant expression levels of CD56 in the CD38(++) plasma cell fractions [more than 10% of CD38(++)] (Fig. 1b). However, no

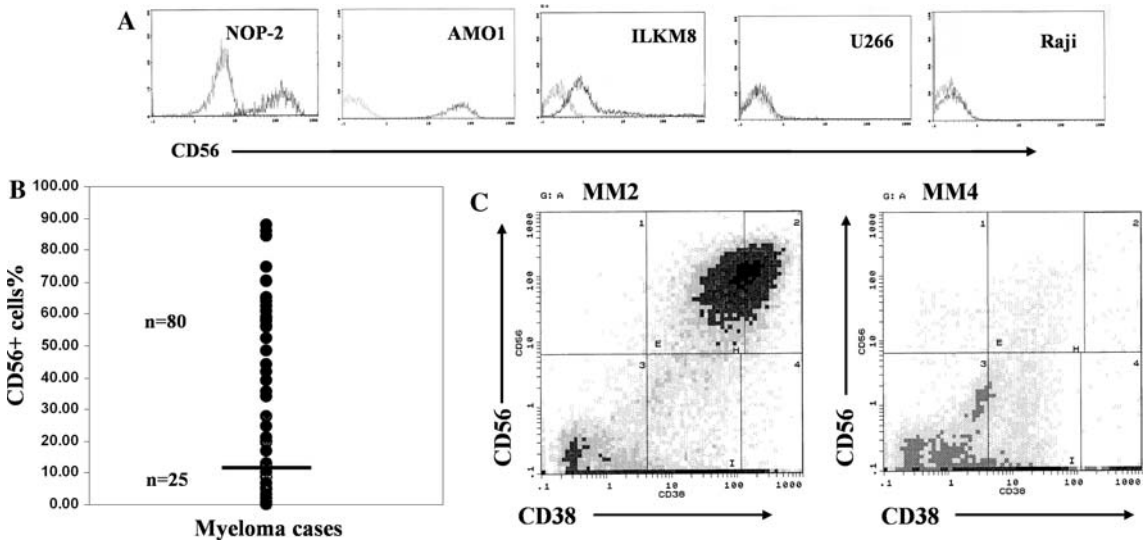

Fig. 1 CD56 expression in human myeloma as well as myeloma cell lines. **a** In opposite to the primary myeloma cases, only two of the human myeloma cell lines strongly express CD56 in their surface. **b** CD56 expression stained by PC5-CD56 was examined in 105 myeloma cases, and 80% cases showed positive staining for CD56 in the myeloma cells [CD38(++) fractions]. **c** Representative cases of [CD56(+) (MM2)] and [CD56(−) (MM4)] myelomas as shown in the cytograms of anti-CD38 and anti-CD56 antibody staining

morphological changes were observed in the cells expressing CD56. Representative CD56(+) case (MM2) and CD56(−) case (MM4) were shown in Fig. 1c. Therefore, CD56(+) myeloma cells were confirmed to be detected in about 80% of multiple myeloma cases, and show no changes in the morphology.

3.2 CD56 expression in human myeloma cells might be derived from the neurogenic gene expressions

To elucidate the biological significance of CD56 expression in human myeloma cells, we carried out gene expression profiling among the CD56(+) and CD56(−) human myeloma cell lines. From a total of 54,675 genes analyzed by the Affymetrix microarray, 1,584 genes were more than two times (log-value) up-regulated and 2,110 genes were more than two times (log-value) down-regulated in the CD56(+) human myeloma cell lines, NOP-2 and AMO1 as compared to the CD56(−) human myeloma cell lines. To our surprise, the most significantly up-regulated and down-regulated genes ($P \leq 0.000002$) are the members of the neuronal lineage genes (Tables 1, 2). However, to rule out the preference of the NK cell lineage gene expressions, we further assessed the possible marker genes for both the Neuronal and NK cell lineages and found that the most of the marker genes belong to neuronal cell lineages were markedly expressed and up-regulated in the CD56(+) human myeloma cell lines than that of the NK cell lineage genes. As to confirm the microarray data, we carried out the expression of several important transcription factors for neuronal development and neuronal marker genes such as *CALB1*, *DBN1*, *CHGB*, and *DLX2* in the human myeloma cell lines as well as in primary myeloma cases and found high correlation by semi quantitative RT-PCR (Fig. 2). It is important to know that, among the HOX family of transcription factors, only *HOXB3* and *HOXB9* were highly correlated and we could only found good correlation in *SOX1* and *SOX4* expressions by RT-PCR, whereas the other important SOX transcription factors were not detected by both the microarray and RT-PCR (Fig. 2).

3.3 Possible binding of *SOX4* to the *CD56* promoter region to regulate CD56 expression in human myeloma cell lines

To explore the possible mechanisms of CD56 expression in human myeloma cell lines we analyzed the putative binding sites for transcription factors in the *CD56* promoter region (Fig. 3a) by EZ-Retrieve version 2.0 software. Among the various binding motifs, we have found almost nine binding sites specific to SOX group of transcription factors positioning from the +200 to −1,000-bp upstream region of the *CD56* promoter, of which six were specific to SOX4 (−75 to −523 bp) and four of the SOX4 binding motifs were overlapping (−523 to −551 bp) each other. As because *SOX4* was most significantly upregulated and SOX1 was down-regulated in the CD56(+) human myeloma cell lines as well as CD56(+) primary myeloma cases (Fig. 2), we assessed the binding of SOX4 and SOX1 to the *CD56* promoter region by ChIP analysis. We confirmed the binding of SOX4 to *CD56* promoter region in both the

Table 1 Most significantly over-expressed genes in CD56(+) human myeloma cell lines compared to CD56(−) myeloma cell lines by microarray analysis

Gene probe	Gene symbol	Gene description	Unigene ID	CD56(+) myeloma cell lines	
				NOP2 signal log ratio (fold change)	AMO1 signal log ratio (fold change)
201417_at	SOX4	SRY (sex determining region Y)-box 4	Hs.643910	9.6	8.7
215591_at	SATB2	SATB family member 2	Hs.516617	7.2	3.8
203556_at	ZHX2	Zinc fingers and homeoboxes 2	Hs.377090	6.9	6.5
231795_at	STON1	Stonin 1	Hs.44385	5.4	2.2
204779_s_at	HOXB7	Homeobox B7	Hs.436181	4.7	4.8
234393_at	HDAC9	Histone deacetylase 9	Hs.196054	4.7	3.3
243278_at	FOXP2	Forkhead box P2	Hs.282787	4.5	3.2
203132_at	RB1	Retinoblastoma 1 (including osteosarcoma)	Hs.408528	3.7	3.4
201331_s_at	STAT6	Signal transducer and activator of transcription 6, interleukin-4 induced	Hs.524518	3.3	4.2
207001_x_at	TSC22D3	TSC22 domain family, member 3	Hs.522074	3.2	3.4
213920_at	CUTL2	Cut-like 2 (Drosophila)	Hs.124953	3.1	2.1
1561038_at	ZNF81	Zinc finger protein 81	Hs.114246	3.1	2.1
228092_at	CREM	cAMP responsive element modulator	Hs.200250	2.9	1.5
233386_at	MEIS2	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	Hs.510989	2.9	3.3
233754_x_at	ZNF71	Zinc finger protein 71	Hs.301431	2.8	3.7
206578_at	NKX2-5	NK2 transcription factor related, locus 5 (Drosophila)	Hs.54473	2.7	4.6
207147_at	DLX2	Distal-less homeobox 2	Hs.419	2.6	1.7
201841_s_at	HSPB1	Heat shock 27 kDa protein 1	Hs.380923	2.3	1.2
	MEIS3	Meis1, myeloid ecotropic viral integration site 1 homolog 3 (mouse)			
226895_at	NFIC	Nuclear factor I/C (CCAAT-binding transcription factor)	Hs.170131	2.3	1.9
228904_at	HOXB3	Homeobox B3	–	2.3	2.2
236375_at	TCEA1	Transcription elongation factor A (SII), 1	Hs.344151	2.3	1.9
231953_at	BPTF	Bromodomain PHD finger transcription factor	Hs.444200	2.1	1.5
226461_at	HOXB9	Homeobox B9	Hs.463350	2.1	1.3

CD56(+) human myeloma cell lines NOP-2 and AMO1 as well as SOX1 binding in the CD56(−) human myeloma cell line, U266 (Fig. 3b).

3.4 Over-expression of SOX4 could induce CD56 expression in the CD56(−) human myeloma cell line, U266

To clarify the possible role of SOX4 protein in *CD56* gene and CD56 protein expression in human myeloma cell lines, a full length cDNA of *hSOX4* gene in pCMV6GFP vector was introduced transiently into CD56(−) U266 cell line by Amaxa Nucleofector. The transfection efficiencies were reproducibly around 30–40% in U266 cells, evaluated from the control data of transfection with a GFP-control vector. As shown in Fig. 4a, CD56 expression was detected in U266 cells after 48 h of transfection with the *SOX4* plasmid, where cells were weekly CD56(+) as analyzed by the flow cytometry. Moreover, the induction of *CD56* gene was observed in the U266 cell line transfected with SOX4 (Fig. 4b), while the unrelated other genes such as *PBX2* and *PAX5* remained unchanged. Furthermore, we can

Table 2 Most significantly under-expressed genes in CD56(+) human myeloma cell lines compared to CD56(−) myeloma cell lines by microarray analysis

Gene probe	Gene symbol	Gene description	Unigene ID	CD56(+) myeloma cell lines	
				NOP2 signal log ratio (fold change)	AMO1 signal log ratio (fold change)
218486_at	KLF11	Kruppel-like factor 11	Hs.12229	−9.4	−6
1560763_at	DIP2C	DIP2 disco-interacting protein 2 homolog C (*Drosophila*)	Hs.432397	−8	−8.6
209348_s_at	MAF	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	Hs.134859	−6.4	−5.6
221530_s_at	BHLHB3	Basic helix-loop-helix domain containing, class B, 3	Hs.177841	−4.7	−1.9
236313_at	CDKN2B	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	Hs.72901	−4.3	−4.2
238997_at	POU5F1	POU domain, class 5, transcription factor 1	Hs.249184	−4.1	−4.9
210306_at	L3MBTL	l(3)mbt-like (Drosophila)	–	−4.1	−1.8
206118_at	STAT4	Signal transducer and activator of transcription 4	Hs.80642	−4	−7.2
1562529_s_at	RORA	RAR-related orphan receptor A	Hs.569497	−3.7	−1.5
243771_at	PPP1R16B	Protein phosphatase 1, regulatory (inhibitor) subunit 16B	Hs.45719	−3.4	−3.2
240713_s_at	SOX1	SRY (sex determining region Y)-box 1	Hs.202526	−3.2	−3
208436_s_at	IRF7	Interferon regulatory factor 7	Hs.166120	−2.9	−2.6
242637_at	SGK2	Serum/glucocorticoid regulated kinase 2	Hs.300863	−2	−1.1
205170_at	STAT2	Signal transducer and activator of transcription 2, 113 kDa	Hs.530595	−1.7	−1.3
205398_s_at	SMAD3	SMAD family member 3	Hs.36915	−1.5	−1.4
244535_at	FOXP1	Forkhead box P1	Hs.431498	−1.4	−3.1
206940_s_at	POU4F1	POU domain, class 4, transcription factor 1	Hs.493062	−1.2	−3.7
243455_at	TEAD1	TEA domain family member 1 (SV40 transcriptional enhancer factor)	Hs.153408	−1.1	−1.8

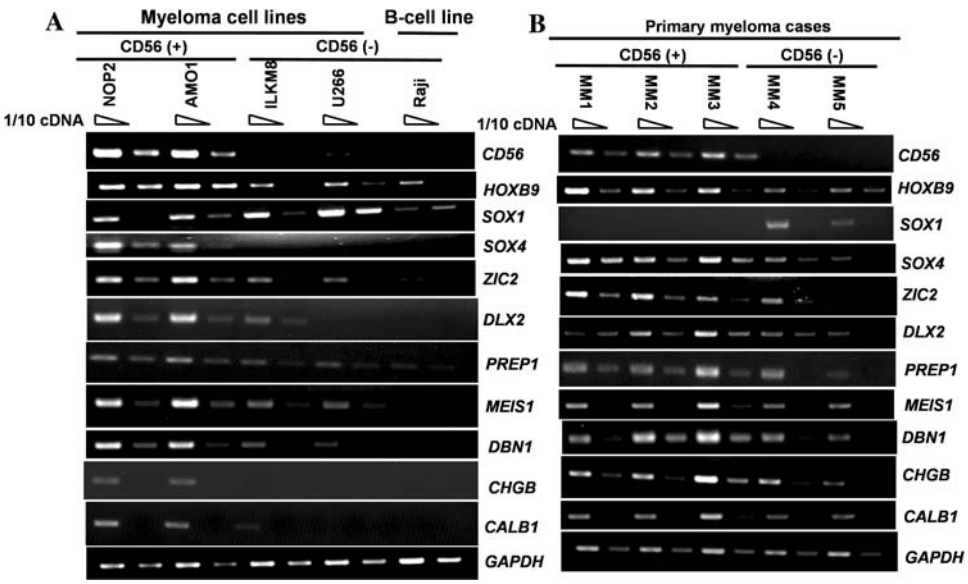

Fig. 2 Positive correlation of the up-regulated genes expression by microarray in the CD56(+) in human myeloma cells. Two dilutions of cDNA (1 and 10 times) from primary myeloma cells as well as myeloma cell lines were subjected to RT-PCR assays for the expressions of the indicated genes. **a** Expressions of genes in the human myeloma cell lines. **b** As for primary myeloma cells, 5 representative cases (MM1–MM5) which showed more than 85% of myeloma cells in the BMNNC are shown; 3 cases were CD56(+) and 2 cases were CD56(−)

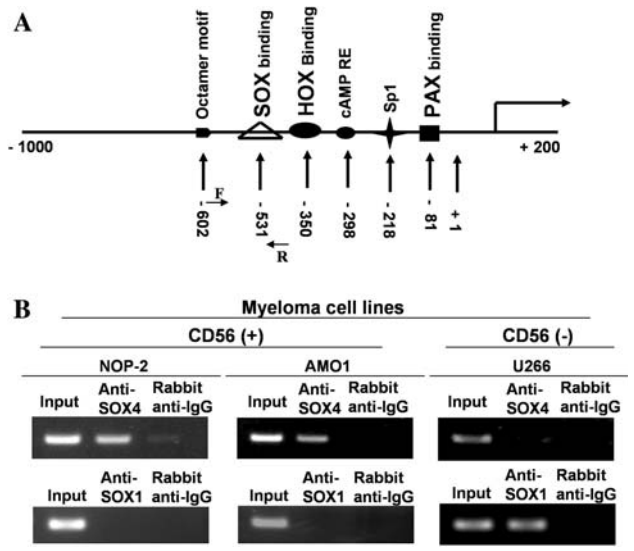

Fig. 3 Possible binding of SOX4 to the *CD56* promoter region. **a** Schematic representation of the upstream promoter region of the *CD56* gene and putative transcription factor binding sites were shown as analyzed by the EZ-Retrieve version 2.0 software. *Arrow* indicates the forward and reverse primer positions for ChIP assay. **b** CD56(+) AMO1 and NOP-2, and CD56(−) U266 human myeloma cells were cultured and subjected to ChIP assays using anti-SOX4 and anti-SOX1 antibody followed by PCR with specific primers for the proximal promoter region of the *CD56* gene

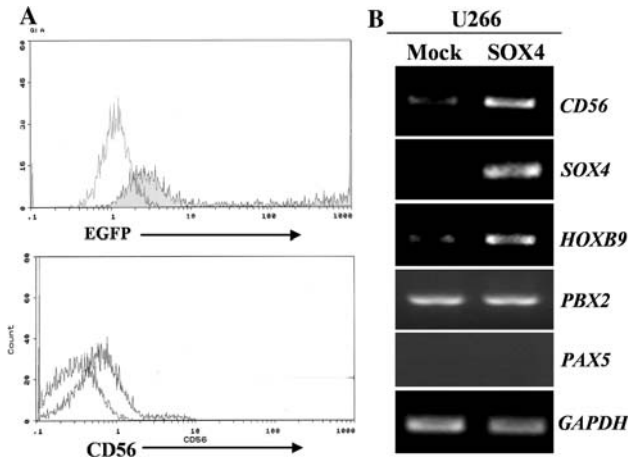

Fig. 4 Induction of *CD56* gene by SOX4. U266 cells were transfected with either empty vector (mock) or pCMV6SOX4GFP plasmid, and after 48 h the cells were examined for CD56 and GFP expression using a flow cytometer (**a**). **b** Cells were harvested after 48 h of transfection with pCMV6SOX4GFP plasmid or empty vector (mock), and RNA was extracted followed by the RT-PCR analysis. The expression levels of *SOX4* and *CD56* were analyzed parallel to the other unrelated genes such as *PAX-5* and *PBX2*

detect the up-regulation of *HOXB9* in the SOX4-transfected U266 cell line (Fig. 4b), and no change was observed in the *SOX1* expression (data not shown). However, as the CD56(−) human myeloma cell line express comparatively higher levels of SOX1 and the down-regulation of SOX1 can also induce neuronal lineage gene induction [19, 20], we checked the effect of SOX1 knockdown in the U266 and failed to detect the *CD56* induction after shRNA-mediated knockdown of SOX1 (data not shown). Therefore, these findings suggested that the augmentation of *SOX4* gene expression could be responsible for the CD56 expression in myeloma cells as well as myeloma cell lines.

3.5 shRNA against SOX4 down-regulated the CD56 expression in CD56(+) human myeloma cell line, AMO1

In order to confirm the SOX4-mediated CD56 expression in human myeloma, we transiently transfected pGFP-V-RS SOX4 shRNA vector or control shRNA GFP to the CD56(+) human myeloma cell line, AMO1. The transfection efficiency was reproducibly around 20% (Fig. 5a), and suppression of SOX4 in this cell line induced the down-regulation of the *CD56* as shown in Fig. 5b, whereas the other unrelated genes such as *PAX5* and *PBX2* remained unchanged (Fig. 5b). Furthermore, we found that the knock-down of SOX4 strongly suppressed the cell viability as the viable cell number decreased to 10% in 72 h compared to the control cells transfected with the mock vector (Fig. 5c). There was a little difference in the 24-h transfection, but cell viability was drastically decreased in 48 h compared to the mock-transfected cells. These data suggest that SOX4 is not only responsible for the CD56 expression but also contributes to the malignant phenotype of myeloma cells by promoting the cell survival.

4 Discussion

Multiple myeloma is highly heterogeneous; here, we confirmed that about 80% of myeloma patients expressed CD56 on CD38(++) myeloma cell fractions, and these CD56(+) myeloma cells showed no change in the morphology (Fig. 1) as reported earlier [1, 2]. In this study, we have shown for the first time that *SOX4* was highly up-regulated approximately ninefold in the CD56(+) human myeloma cell lines as well as CD56(+) primary myeloma cases along with other neuronal lineage genes (Tables 1, 2; Fig. 2) and SOX4 is responsible for CD56 expression in human myeloma as SOX4 binds to the CD56 promoter region (Fig. 3) and could induce CD56 expression (Figs. 4, 5). However, up to now, we do not know the biological meanings of CD56 expression in myeloma cells; we speculate that CD56 might facilitate the anchorage between plasma cells and bone marrow stromal structures in the bone marrow micro-environment as well as it might play roles in the pathogenesis of the disease although exact molecular mechanisms remained to be clarified.

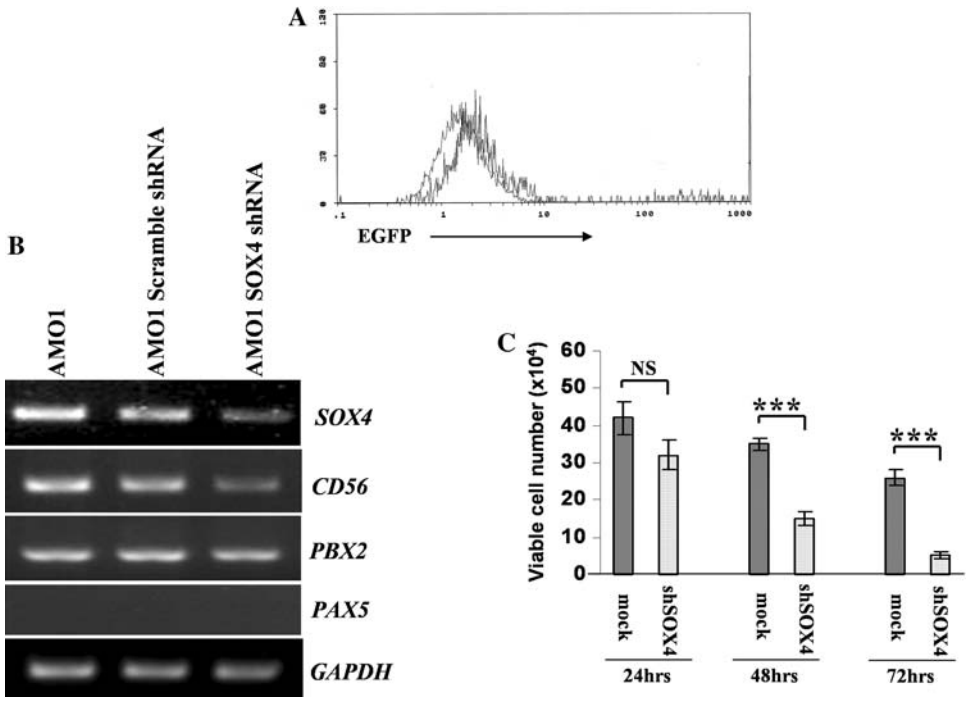

Fig. 5 Knockdown of SOX4 could suppress the *CD56* gene expression. **a**, **b** AMO1 cells transfected with 2 μg SOX4 shRNA-EGFP plasmid (SOX4 shRNA) or control-EGFP plasmid (mock, control shRNA) were cultured, and after RNA extraction cells was subjected to flow cytometry and RT-PCR analysis for *CD56*, *SOX4*, and other non-related gene expression. **c** Viable cell numbers of the SOX4 shRNA-transfected cells were estimated after 24, 48, and 72 h both manually by the trypan blue exclusion assay on a hemocytometer and automatically by flowcytometer. Statistical analysis by the 2-tailed Student's *t* test indicated that the viable cell numbers of the shSOX4-transfected AMO1 was significantly different from that of the mock-transfected controls in 48 and 72 h (***$P \leq 0.001$), whereas the 24-h time point was not significant (NS)

Gene expression profile of the CD56(+) human myeloma cell lines revealed that the most significantly over-expressed genes preferentially belong to the neuronal lineage than that of the NK cell lineage, and we could further confirm the expression of some of these genes by the RT-PCR analysis in the myeloma cell lines as well as primary myeloma cases (Fig. 2). To look for the potential candidate responsible for CD56 in human myeloma, we analyzed the up-stream promoter region of the gene by the EZ-Retrieve software and found that along with other important transcription binding sites for HOX, PAX, cAMP, and Octamer motif as reported earlier [21], there are nine SOX binding sites of which, six of these were specific to group C SOX4 heptamer motif 5-AACAAA-3 [22], and interestingly we could detect four overlapping SOX4 binding motifs in between the −523 and −551 bp (Fig. 3a). Recently, as increasing evidence has suggested the up-regulation of SOX4 in many different tumors [7, 12], we checked the binding of SOX4 by ChIP assay and found that SOX4 can bind to *CD56* promoter region in both the CD56(+) human myeloma cell lines (Fig. 3b). Although more critical experiments are absolutely required to clarify the exact position of SOX4 binding to its motif, we can set aside the possibility of the binding of other SOX factors to the C*D56* promoter, as the expression of other *SOX* genes were mostly negative, especially the other group C SOX such as *SOX11* and *SOX12* as confirmed by the microarray analysis and RT-PCR.

As we have detected up-regulated expression of neuronal lineage genes in the CD56(+) human myeloma cell lines and *SOX1* was significantly down-regulated in both the CD56(+) human myeloma cell lines, we first tried to decipher the role of *SOX1* in CD56 expression. *SOX1* belongs to the group B1 and are expressed by most precursor cells, act to maintain the expression of progenitor identities and thus preserve cells in undifferentiated state [20]. As the down-regulation of *SOX1* could induce neuronal lineage genes [19, 20], we successfully suppressed *SOX1* by shRNA in the U266, but could not observe the induction of CD56 in this cell line (data not shown). So we assume that the down-regulation of SOX1 is not important for the CD56 expression in human myeloma. However, as SOX4 can specifically bind to the CD56 up-stream promoter region and also significantly up-regulated in the CD56(+) myeloma cells, we over-expressed SOX4 to the CD56(−) human myeloma cell line, U266. Over-expression of SOX4 could induce CD56 expression in protein (Fig. 4a) and mRNA level (Fig. 4b), and most interestingly we could

found the up-regulation of homeobox *HOXB9* in the SOX4 over-expressed U266 cells. The Hox family of homeodomain transcription factors are involved in numerous cellular processes, including organogenesis, cellular differentiation, cell adhesion and migration, cell cycle and apoptosis [23], and it has been shown that mouse Hoxb9 can directly bind and up-regulate CD56 [23]. Growing evidence suggested that SOX family of transcription factors exert their effect on transcription via cooperative binding to DNA with transcription factor partners [22]. For example, SOX11 has been shown to cooperatively bind and synergize with the homeobox POU factor Brn-1 [22], and SOX2 forms complexes with the homeodomain factor Oct1 [24]. As we have found the up-regulated expression of *HOXB9* and its cofactors *MEIS1/3* and *PREP1* [25] in the CD56(+) human myeloma cell lines as well as CD56(+) primary myeloma cases and *HOXB9* promoter region also has the specific binding region for the SOX4, it is tempting to speculate that SOX4 and HOXB9 might cooperate to modulate CD56 expression in human myeloma cells.

To further confirm the SOX4-mediated CD56 expression, we suppressed SOX4 by the shRNA in CD56(+) human myeloma cell line, AMO1 and observed the down-regulation of CD56 in this cell line (Fig. 5b). Moreover, we have also found that SOX4 is very important for the growth and survival of myeloma, as SOX4 shRNA-transfected myeloma cells were prone to apoptosis (Fig. 5c) as has been reported for SOX4 to other cancers [7]. SOX4 is a transcription factor that affects expression of many genes, some of which promote cell survival, and the balance of the proapoptotic and antiapoptotic SOX4 target may determine cellular survival. Although, more severe approaches should be taken to clarify the precise role of SOX4 in the CD56 expression in human myeloma as CD56 expression results in the heterogeneity in myeloma and cause poor prognosis [5]; this study collectively shows for the first time, the enhanced expression of SOX4 in myeloma as well as its potential role in the induction of CD56 in human myeloma. Our data also suggested that SOX4 plays a vital role in the growth and survival of myeloma that expresses CD56.

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