

SHORT COMMUNICATION

High expression of shMDG1 gene is associated with low metastatic potential of tumor cellsN Isachenko¹, N Dyakova¹, V Aushev¹, T Chepurnykh¹, K Gurova² and A Tatosyan¹¹*Viral and Cellular Oncogenes Laboratory, Cancer Research Center, Institute of Carcinogenesis, Moscow, Russia and* ²*Department of Molecular Genetics, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH, USA*

Metastasis is the primary cause of mortality associated with cancer. Molecular mechanisms leading to metastatic spread are poorly studied. To get a better understanding of this process, we compared the gene expression pattern of two isogenic cell lines, HET-SR and HET-SR1 (Rous Sarcoma Virus-transformed embryo hamster fibroblasts) with different metastatic activity using the differential display technique. A novel cDNA of hamster gene *shMDG1* (Syrian hamster homologue of microvascular differentiation gene 1), which had 94% homology with rat *MDG1* gene, was identified. Expression of *shMDG1* was increased in low metastatic HET-SR cell line in comparison to high metastatic HET-SR1. Sequence analysis of the ORF of *shMDG1* gene showed that it belongs to the DnaJ/heat-shock proteins of 40 kDa (HSP40) chaperones family, considered to function as a cochaperone of HSP70 family. In order to confirm involvement of *shMDG1* in metastasis, we injected parental and *shMDG1* overexpressed cells into animals. We showed that overexpression of the *shMDG1* gene significantly diminished the metastatic activity of both HET-SR and HET-SR1 cells. The *shMDG1*-induced repression of metastasis was not connected with alterations in cell proliferation and motility *in vitro*, but correlated well with a decrease in content of the Asn-linked β 1–6 branched oligosaccharides on cell surface.

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The process of metastasis is a complex series of stochastic events (Hanahan and Weinberg, 2000; Chambers *et al.*, 2002). Former metastatic cells detach from primary tumor mass, invade small vessels, survive the mechanical stresses of circulation, and finally extravagate and proliferate at a distant site. During this entire process, tumor cell evade or resist host defense

mechanisms, such as cytotoxic T-lymphocytes, macrophages, NK cells, and neutrophils. Only a very small percentage of cancer cells are capable of forming distant metastasis (Nicolson, 1991). Nevertheless, metastasis is the most fearsome aspect of cancer. The majority of patients with cancer die because of metastatic spread of primary tumor.

It is believed that metastasis formation involves an intricate interplay between altered expression of genes regulating cell adhesion, migration, lymph-/angiogenesis, and survival and proliferation in unusual microenvironment. Numerous gene products involved in the metastasis formation were identified, but this is still not enough for the complete understanding of the process (Chambers *et al.*, 2002).

To learn more about basic mechanisms that underlie metastasis, we compared two isogenic cell lines: HET-SR and HET-SR1 showing different metastatic activity (Deichman *et al.*, 1989). These cell lines were obtained by independent infections *in vitro* of primary hamster fibroblasts with two derivatives of Rous Sarcoma Virus Schmidt–Ruppin strain (RSV SR-D) containing several mutations in *v-src* gene (Tatosyan *et al.*, 1996). Previously we showed that substitution of the three amino acids (E₅₂₂→D, V₅₄₁→M, A₅₄₃→E) and one deletion (V₅₂₄) in the C-terminal regulation domain of *v-src* determinate the difference in the ability of HET-SR and HET-SR1 cell lines to form metastasis (Tatosyan *et al.*, 1996). Isoform of *v-src* from high metastatic line HET-SR1 was named as *v-srcHM* and from low metastatic line HET-SR was named as *v-srcLM*. We proposed that alteration of *v-src* structure reflects the difference in cell signaling and thereafter in metastasis formation. To define genes affecting metastatic activity, we compared the gene expression patterns of HET-SR and HET-SR1 cell lines using the RT–PCR-based differential display strategy (Liang and Pardee, 1992).

We identified several differentially expressed transcripts (Chepurnykh *et al.*, 2000). One of them (365 bp length) was expressed in higher level in low metastatic cell line HET-SR compared with high metastatic cell line HET-SR1 (Figure 1a). Sequence analysis of the 365 bp fragment revealed significant homology with the rat *Mdg1* (microvascular differentiation gene 1), mouse (*mDj7*), and human (*DNJB9*) genes (Figure 1c) (Prols *et al.*, 2001; Shen *et al.*, 2002). We generated the full-length (981 bp) *shMDG1* (Syrian hamster homolog of

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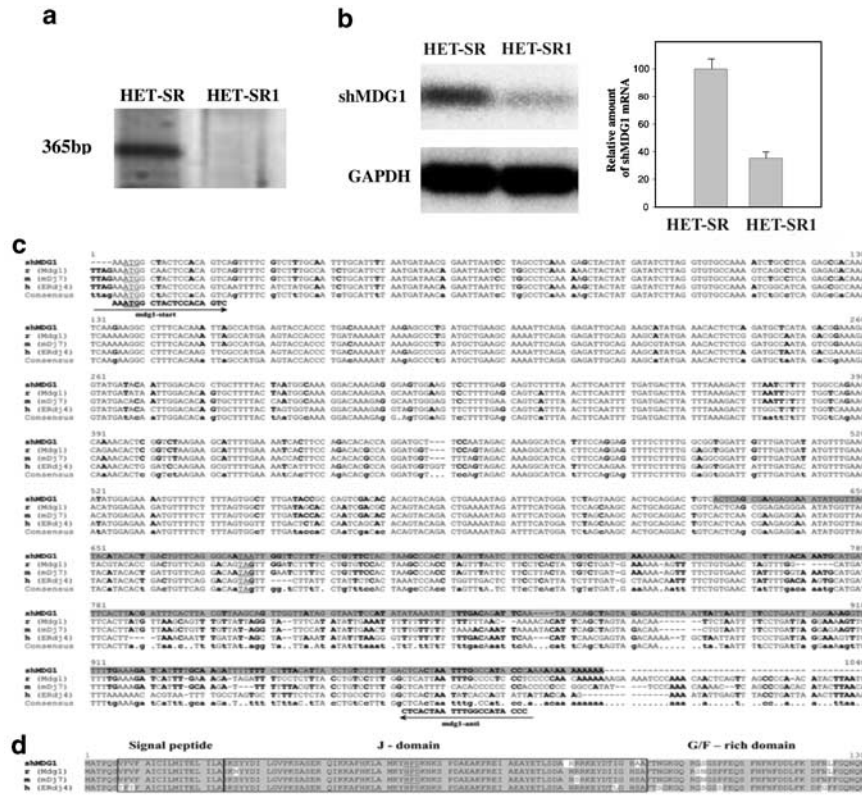


Figure 1 (a) Analysis of gene expression in HET-SR and HET-SR1 cell lines by differential display. Differential display was performed using the RNAmapp kit (GeneHunter). Briefly, 2 μ g of total RNA were used as a template for reverse transcriptase reaction using 5 mM dT12MG (M can either be dA, dC, or dG), 20 mM dNTPs and 200 U MMLV reverse transcriptase. dT12MG (as a 3'-primer) and AP-1 (5'-AGCCAGCGAA-3'; as a 5'-primer) were used for the subsequent PCR reaction. PCR products were separated on a 6% polyacrylamide gel and bands were visualized by autoradiography. Fragments of interest were reamplified and sequenced. (b) Analysis of the *shMDG1* gene expression in HET-SR and HET-SR1 cell lines. Total mRNA was isolated from cells using Trizol Reagent (Gibco BRL) according to the manufacturer's instructions. In all, 30 μ g of each fraction per lane was loaded. Northern blot was performed using [α -³²P]labeled *shMDG1* fragment. Hybridized membrane was exposed for 30 days for *shMDG1* probe and 2 days for *GAPDH* probe. Hybridization with *GAPDH* was used as load control. Densitometric analysis revealed that the *shMDG1* expression level was about three-fold upregulated in low metastatic line HET-SR comparing with high metastatic line HET-SR1. (c) Sequence analysis of *shMDG1* cDNA. Comparison of *shMDG1* nucleotide sequence of human (*h*) *ERdj4*, mouse (*m*) *mDj7*, and rat (*r*) *Mdg1* genes. Gray boxes indicate 365 bp nucleotide sequence, which was obtained by differential display. *shMDG1* full-length cloning. In order to obtain the complete 5'-end of *shMDG1*, we performed RT-PCR using total RNA from HET-SR cells according to the protocol supplied by the manufacturer (Clontech). The template-specific primer used for the reverse transcriptase reaction was mdg1-start (5'-GAAATGGCTACTCCACAGTC-3') and mdg1-anti (5'-GGGTATGGCCAAATTAGTGAG-3'). Mdg1-start was chosen on the basis of alignment of the three genes: rat *Mdg1*, mouse *mDj7*, and human *DNJB9* genes. The PCR fragment was subcloned into TA-cloning vector pCR2.1 (Invitrogen) and sequenced using T7 or M13 reverse primer to obtain the full-length *shMDG1* cDNA. For the next experiments, we constructed pLV-shMdg1 by subcloning the open-reading frame of shMdg1-cDNA from pCR2.1 into pLV plasmid via *EcoRI*/*Apal* sites. pLV and pLV-EGFP (which was used as a control) plasmids were the generous gift of Dr Peter Chumakov (Lerner Research Institute, CCF, USA). (d) Comparison of shMDG1 amino-acid sequence with human (*h*) DNJB9, mouse (*m*) mDj7, and rat (*r*) Mdg1. Gray and white boxes indicate complete or partial homology, respectively. Boxed letters (7–23 amino acids) indicate the predicted signal peptide and the J domain (24–93 amino acids). The amino-acid and the nucleotide sequences were aligned using NClustalW (ver. 1.16.2.6., <http://srs.ebi.ac.uk>).

microvascular differentiation gene 1) that contains an open-reading frame of 669 bp (Figure 1c). The sequence of *shMDG1* is available in GenBank under Accession Number AY532644. Northern blot analysis revealed a significant decrease of *shMDG1* gene expression in high metastatic cell line HET-SR1 compared with low metastatic cell line HET-SR and thus confirmed the differential display data (Figure 1b).

shMDG1 belongs to the family of heat-shock proteins of 40 kDa – HSP40/DnaJ (Figure 1d). The HSPs

(chaperones) comprise a lot of proteins with different molecular weights (100, 90, 70, 60, 40, and 27 kDa) highly produced after exposure to a variety of cellular stresses such as heat, heavy metals, anoxia, and glucose starvation (Welch, 1990; Morimoto, 1991; Ohtsuka and Hata, 2000). HSP40/DnaJ proteins act as cochaperones and specific factors for HSP70 proteins (Yochem et al., 1978). The special feature of DnaJ protein family is a J domain, a highly conserved tetrahelical domain that binds to HSP70 chaperones and activates their ATPase

activity. HSP40/HSP70 system is involved in protein folding, protein degradation, assembling, and disassembling of multiprotein complexes and translocation of proteins across membranes.

It has been shown that rat homolog of shMDG1 protein was localized in the endoplasmic reticulum (ER) membrane and was upregulated only under ER stress conditions, but not by heat or other shocks (Prols *et al.*, 2001; Shen *et al.*, 2002). Human homolog was identified as a component of the ER-associated degradation system (Shen *et al.*, 2002). It may lead to the suggestion that shMDG1 plays an important role in either the protein refolding or/and the retrotranslocation of misfolded proteins including glycosylation enzymes.

It has been shown that human homolog of *shMDG1* is localized on chromosome segment 14q24.2–q24.3 (Prols *et al.*, 1997). The loss of this sequences correlate with colorectal metastasis formation and glioma progression (Thorstensen *et al.*, 2001; Dichamp *et al.*, 2004).

To define whether *shMDG1* expression could affect metastatic activity of tumor cells, we overexpressed *shMDG1* in HET-SR and HET-SR1 cell lines by transduction of lentivirus containing the *shMDG1* gene. Overexpression of *shMDG1* in transduced cell lines was confirmed by Northern blot analysis (Figure 2a).

First, we studied the effect of *shMDG1* overexpression on tumorigenicity of HET-SR and HET-SR1 cells, that is, their ability to form tumors after injection into novel microenvironment. This test can reflect some aspects of metastasis. We measured the tumorigenicity index

(TrD₅₀) as a decimal logarithm of a number of cells causing tumors in 50% of hamsters after injection of different cell numbers (2×10^4 ; 2×10^3 ; 2×10^2 ; 2×10^1) into four legs of each animal (Deichman *et al.*, 1989). It was found that expression of the *shMDG1* increased the TrD₅₀ index for both HET-SR and HET-SR1 cells about three-fold (Mann–Whitney *U*-test, $P < 0.0001$). At higher cell dosages (2×10^4 cells/per leg), we found no

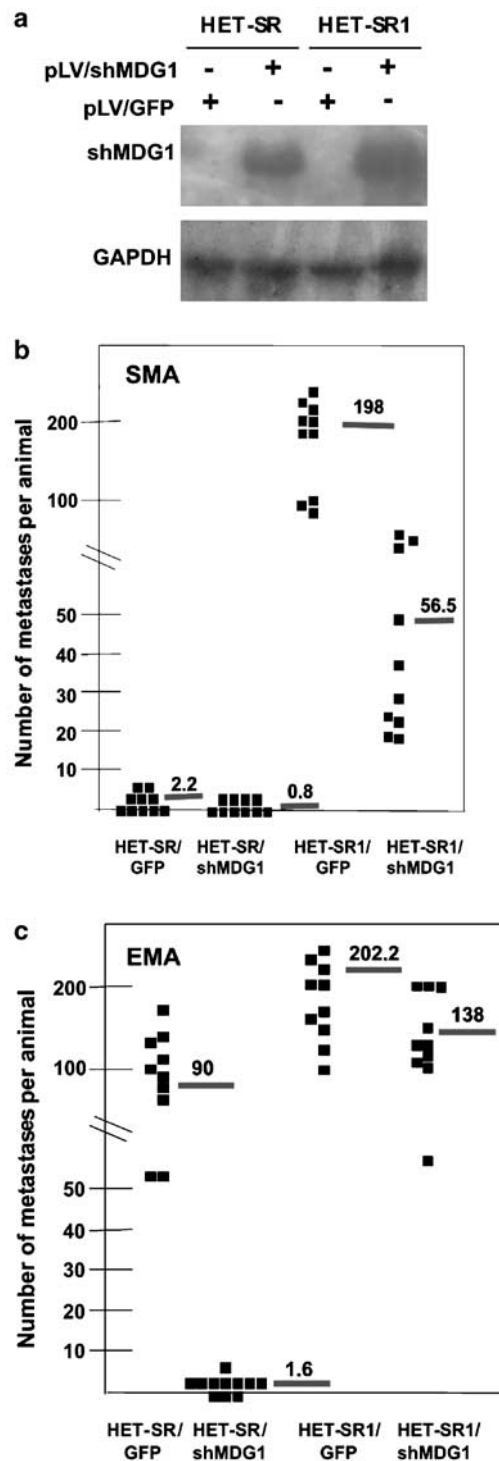


Figure 2 (a) Verification of *shMDG1* mRNA expression level in HET-SR/shMDG1 and HET-SR1/shMDG1 cell lines. HET-SR and HET-SR1 cells were infected by lentivirus bearing the *shMDG1* gene. Lentiviral vectors were generated as described previously (Blomer *et al.*, 1997). Briefly, A293 cells were transfected transiently by packaging plasmid, an envelope plasmid (vesicular stomatitis virus G protein (VSV-G)), and the expression vector pLV-shMDG1 (pLV-GFP was used as a control). In all, $4 \mu\text{g}$ of DNA per 100 mm Petri dish was used for Lipofectamine transfection technique as recommended by the manufacturer (Invitrogen). We harvested the condition media-containing virus after 48 and 72 h, filtered the medium through $0.45 \mu\text{m}$ filters, and used the virus containing media either fresh or as frozen aliquots. A total of 4×10^4 HET-SR and HET-SR1 cells per well were transduced in a six-well plate with 1 ml of the viral supernatant in the presence of $4 \mu\text{g}$ of Polybrene/ml. After 24 h, the medium was replaced by a fresh one. Transfectants were selected using 600 mg/ml of G418. Northern blot analysis was carried out as described above. Hybridized membrane was exposed for 2 days (for *shMDG1* and for *GAPDH* probes). (b) Distributions of lung metastases in spontaneous metastatic activity (SMA) test. All cell cultures used for injection were in log-phase growth. Tumor cells were injected subcutaneously (2×10^4 cells, 10 animals per group). After 60 days, the animals were killed and pulmonary metastases scored. (c) Distributions of lung metastases in experimental metastatic activity (EMA) test. Tumor cells were injected intravenously (1×10^5 cells per animal, 10 animals per group). After 30 days, animals were killed and pulmonary metastasis were counted by direct visualization of surface colonies under a dissecting microscope. All experiments were performed twice and conducted under the auspices of and in accordance with the sanitary and ethical rules on the equipment and maintenance of experimental biological clinics stated by the Main State health officer of Russian Federation (Order No. 1045-73 from April, 6, 1973).

differences in tumor growth between control and *shMDG1*-expressing cells. The finding of decreased tumorigenic ability of small amounts of the *shMDG1*-expressing cells is in agreement with the idea of repressive effect of *shMDG1* on metastasis.

To prove the influence of *shMDG1* expression on metastatic potential, we used two specific tests: SMA (spontaneous metastatic activity) and EMA (experimental metastasis activity). SMA was estimated by counting of tumor foci in the lung 8 weeks after subcutaneous injection of 2×10^4 cells (at this cell dosage all tested cell lines formed subcutaneous tumors of approximately equal size in 100% of animals). In agreement with previous report (Tatosyan *et al.*, 1996), original HET-SR and HET-SR1 cell lines showed mainly different levels of their SMA: HET-SR1 was highly metastatic cell line, forming about 200 foci in the lung of 100% of animals, while HET-SR cells either did not form any metastasis or formed less than five metastases in the animal's lung in about 50% of injected animals (Figure 2b) ($P < 0.001$). The introduction of the *shMDG1* gene significantly diminished spontaneous metastatic activity of HET-SR1 cell line (amount of metastases between two groups was compared using Mann-Whitney *U*-test, $P < 0.05$). Metastatic potential of HET-SR cells was not significantly altered upon *shMDG1* overexpression (Figure 2b).

Similar results were obtained using the EMA test (Figure 2c). In EMA test, cancer cells were injected directly into blood circulation of a hamster to simulate the later phase of the metastatic process. (In SMA test, the cells were injected subcutaneously and formed the primary tumor prior to metastasis formation (Deichman *et al.*, 1989).) Previously, we reported that HET-SR and HET-SR1 cells were similar in EMA test (Deichman *et al.*, 1989). Overexpression of the *shMDG1* considerably decreased experimental metastasis activity of HET-SR cell line about 60-fold (Figure 2c) ($P < 0.001$). HET-SR1/*shMDG1* cells to minor extent lost their experimental metastasis activity in comparison with parental HET-SR1 cells (Figure 2c).

Importantly, the overexpression of *shMDG1* gene led to better survival of the animals with subcutaneously injected HET-SR1 (about four-fold) and intravenously injected HET-SR and HET-SR1 cells (all animals were alive in 30 days, while 10 and 40% of original HET-SR

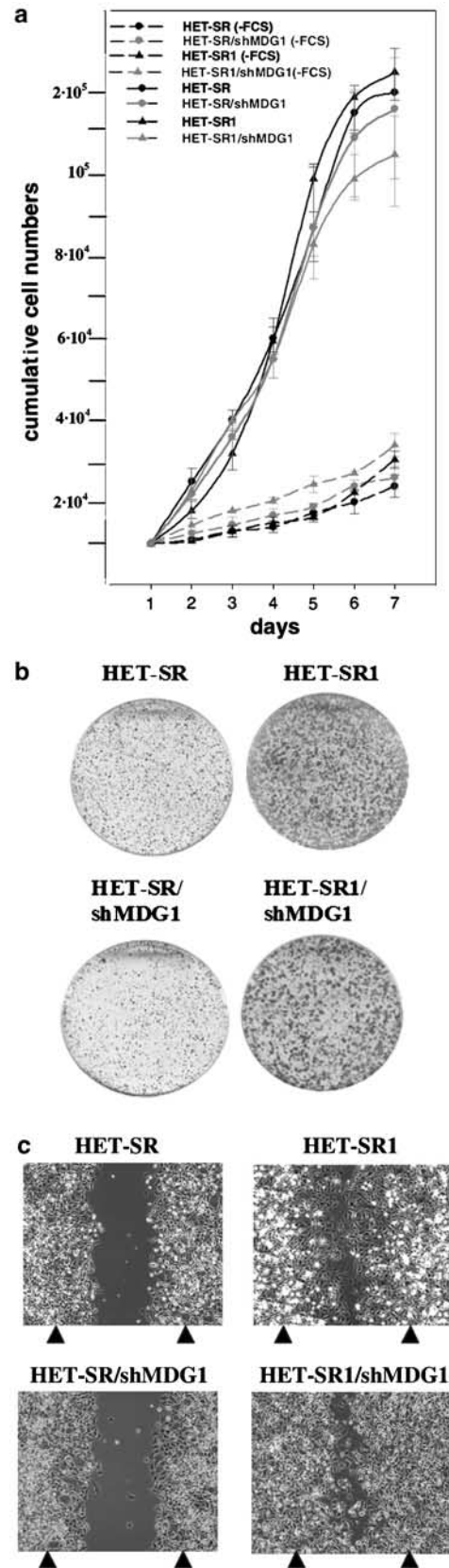


Figure 3 Comparison of HET-SR and HET-SR1 cell lines with their derivatives overexpressed *shMDG1*. **(a)** Proliferation kinetics of HET-SR1/*shMDG1* (gray triangles) and HET-SR/*shMDG1* (gray circles) compared to parental HET-SR1 (black triangles) and HET-SR (black circles) cells. The same experiment was done with serum withdraw (dashed curves). A total of, 10^4 cells was plated in each well of 24-well plate. Then, the cells were counted daily using a Neubauer hemacytometer. **(b)** For clonogenic assay, 500 cells per six-well plate of each studied lines were grown for 10 days. **(c)** Wound healing assay was carried out on confluent HET-SR, HET-SR1, HET-SR1/*shMDG1*, and HET-SR/*shMDG1* cell lines by the pipette tip (arrowheads show the size of initial wound). After incubation for 24 h cells were fixed. To estimate cell migration, the cultures were stained by Hoechst 33258. The number of nuclei of cells migrated into the wound were counted.

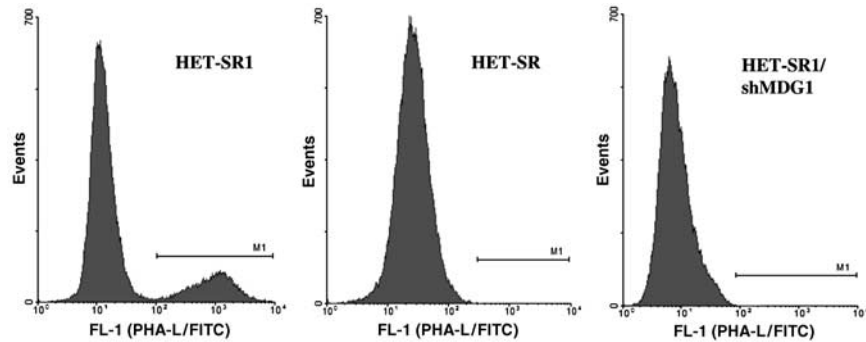


Figure 4 Flow cytometry analysis of PHA-L binding to the cell membrane of HET-SR1, HET-SR, and HET-SR1/shMDG1 cells. Detection of β 1–6 branched oligosaccharides has been performed using the FITC-labeling lectin *Phaseolus vulgaris* (PHA-L), which specifically recognizes the 2,6-branched tri- and tetra-antennary complex (Osawa and Tsuji, 1987). HET-SR1 cells expressed a higher number of ligands for the lectins PHA-L on their cell membrane in comparison to HET-SR and HET-SR1/shMDG1 cells. One representative experiment out of four is shown. Flow cytometry studies were performed with an EPICS ELITE flow cytometer (Coulter, Hialeah, FLA, USA). The 488 nm line of an argon laser was used as an excitation beam for the fluorochrome FITC ($\lambda_{\text{ex}} = 488 \text{ nm}$; $\lambda_{\text{em}} = 520 \text{ nm}$) and propidium iodide (Sigma) ($\lambda_{\text{ex}} = 488 \text{ nm}$; $\lambda_{\text{em}} = 620 \text{ nm}$). The data were analysed from 10 000 cells, recorded at a flow rate of 500 cells/s. Nonviable cells in each sample were detected by the addition of propidium iodide (0.5 $\mu\text{g/ml}$) to each cell sample immediately before flow cytometry analysis. Cells stained strongly with propidium iodide represented dead cells and were excluded for data acquisition purposes. Cell viability ranged from 95 to 98% in all samples.

and HET-SR1 cells, respectively, died at this time point) (log-rank test, $P < 0.05$ and $P < 0.01$, respectively). To reveal possible mechanism underlying the ability of shMDG1 to modulate metastatic potential, we performed a set of assays estimated proliferation, clonogenesis, and migration *in vitro*. As measured by daily cell counts, low metastatic HET-SR and high metastatic HET-SR1 cell lines transduced by shMDG1 exhibited the same proliferation kinetics as parental cells that showed an estimated doubling time of about 30 h at exponential growth (Figure 3a). Serum withdrawn did not elicit any difference in cells proliferation under constrained condition (doubling time of about 40 h at exponential growth). The overexpression of shMDG1 also did not affect the clonogenic ability of studied cells (Figure 3b). The same results were obtained in analysis of anchorage-independent growth of HET-SR, HET-SR/shMDG1 and HET-SR1, HET-SR1/shMDG1 cells in soft agar (data not shown). In addition, we did not find any difference in actin cytoskeleton organization (data not shown) and migration abilities *in vitro* (Figure 3c) of studied cultures. And finally, the activities of matrix metalloproteases 2 and 9 were the same in parental and shMDG1 overexpressed cell lines (data of zymography; not shown). These data indicate that the shMDG1-induced repression of metastasis is not connected with changes in cell proliferation and migration abilities that are manifesting in cell cultures *in vitro*. However, we could not exclude the possibility that the

expression of shMDG1 influence on these processes *in vivo*.

The mechanism by which the shMDG1 expression affects on metastatic activity remains to be established. It was shown that the acquisition of metastatic activity was accompanied by alteration of surface glycosylation pattern (Pierce and Arango, 1986). Numerous studies have revealed that β 1,6-GlcNAc branching on N-glycans, a product of UDP-GlcNAc α -D-mannoside β 1,6-N-acetylglucosaminyl transferase-V (GnTV, EC 2.4.1.155), is one of the key structure associated with tumor metastasis and malignant transformation (Dennis *et al.*, 1987; Gu *et al.*, 1993; Demetriou *et al.*, 1995). Using flow cytometry analysis, we found that overexpression of shMDG1 leads to the decreased expression of Asn-linked β 1–6 branched oligosaccharides on the surface of highly metastatic HET-SR1 cells (Figure 4) ($P < 0.01$). Further experiments should answer the question whether such changes are critical for modulation of metastatic potential and which cell features are changed by expression of Asn-linked β 1–6 branched oligosaccharides.

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