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Differential expression of endoglin in human melanoma cells expressing the V3 isoform of versican by microarray analysis

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Abstract. Versican is a large chondroitin sulfate proteoglycan produced by several tumor types, including malignant melanoma, which exists as four different splice variants. The large isoforms V0 and V1 promote melanoma cell proliferation. We previously described that overexpression of the short V3 isoform in MeWo human melanoma cells markedly reduced tumor cell growth *in vitro* and *in vivo*, but favored the appearance of secondary tumors. This study aimed to elucidate the mechanisms of V3 by identifying differentially expressed genes between parental and V3-expressing MeWo melanoma cells using microarray analysis. V3 expression significantly reduced the expression of endoglin, a transforming growth factor-β superfamily co-receptor. Other differentially expressed genes were *VEGF* and *PPP1R14B*. Changes in endoglin levels were validated by qRT-PCR and Western blotting.

Introduction

Melanoma is one of the fastest rising malignancies of recent decades (1). Its progression involves an escape from processes regulating growth and differentiation, which are controlled by multiple signal transduction pathways, and are sometimes regulated by extracellular matrix (ECM) molecules. Versican belongs to the family of large chondroitin sulfate proteoglycans located within the ECM (2,3). Its overproduction is a common feature of several tumor types, and is usually related to a poor prognosis, for example in the case of prostate (4), breast (5), ovarian (6), lung (7) and oral (8) cancer, and melanomas (9,10).

Our previous study showed that the expression of the short isoform of versican, V3, in melanoma cells reversed the malignant phenotype, since V3-transduced cells resulted in a decrease in cell proliferation and an increase in cell adhesion to hyaluronan (11). Furthermore, our results showed that V3 overexpression reduced tumor growth rate in mice, but favored the appearance of secondary tumors (12).

Endoglin (ENG, CD105) is a cell surface component of the transforming growth factor (TGF)-β receptor complex (13), which is highly expressed in the tumor-associated vascular endothelium (14) and in tumor cells. The expression of endoglin appears to play an important role in cancer progression, influencing cell proliferation, motility, invasiveness and tumorigenicity (15).

In the present study, we used a high-throughput approach to gain further insight into the mechanism by which the V3 isoform exerts its effects on tumor progression. To this end, the gene expression profile of V3-overexpressing MeWo human melanoma cells was analyzed. In order to obtain a more complete picture of differentially expressed genes, the OncoChip cDNA array, which comprises 6,386 cancer-related genes, was used. We found that V3 expression significantly reduced endoglin expression together with other differentially expressed genes, including *VEGF* and *PPP1R14B*.

Materials and methods

Cell culture. MeWo human melanoma cells expressing the V3 isoform of versican (LV3SN) and the empty control vector (LXSN) were obtained as previously described (11). The cells were cultured in DMEM medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin and 100 μg/ml streptomycin (all from Gibco BRL/Life Technologies, Rockville, MD, USA) and grown in a humidified atmosphere at 37˚C with 5% CO_2.

RNA isolation. The RNeasy kit (Qiagen, Hilden, Germany) was used to isolate total RNA from the cell cultures according to the manufacturer's protocol. The cultures were used at 144 h after seeding, as this is when the maximal difference in cell proliferation was observed (11). RNA concentration and purity were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and an RNA LabChip® kit (Agilent Technologies). Only high-quality RNA was used in the subsequent analyses. Microarray and qRT-PCR analyses were performed on aliquots from identical RNA samples.

Hybridization. An aliquot of 13 μg of total RNA extracted from cultured cells was reverse-transcribed using an anchored

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oligo(dT) primer and CyScript reverse transcriptase in the presence of nucleotide mix (dATP, dCTP, dGTP) and aminoallyl-dUTP (CyScribe Post-labeling kit; GE Healthcare, Buckinghamshire, UK). The single strand cDNA was purified and labeled with CyDye post-labeling dye (GE Healthcare) for 1 h. Following a second purification as described above, the Cy5- and Cy3-labeled cDNAs were concentrated using a vacuum dryer and resuspended in 50% formamide, 5X SSC and 0.1% SDS. The Cy5 and Cy3 pools were individually mixed with 4 μg of salmon sperm DNA, 20 μg of mouse Cot1 DNA and 20 μg of polyA DNA.

The OncoChip arrays (CNIO, Madrid, Spain) were incubated in 5X SSC, 0.1% SDS and 1% BSA for 45 min at 42˚C, rinsed with distilled water and dried using an air gun. The labeled cDNA mixture was denatured at 95˚C for 3 min and cooled to 4˚C before being pipetted onto the array. The slides were incubated overnight at 42˚C in a hybridization chamber, then washed in 1X SSC/0.2% SDS solution for 8 min, in 0.1X SSC/0.2% SDS solution for 8 min and in 0.1X SSC solution for 8 min. The slides were then spun dry using a centrifuge.

Image analysis, normalization and statistics. The arrays were scanned using a ScanArray® 4000 scanner (PerkinElmer, Waltham, MA, USA) with adjusted settings in order to obtain a similar green and red overall intensity. The images were analyzed using Limma software (16), which is available from the Bioconductor project site (http://www.bioconductor.org). The Bioconductor packages use the free statistical programming environment R. Foreground intensities were background corrected using the 'normexp' background-correction method, and the spot log-ratios were normalized using global loess normalization (17). The genes were ranked in order of evidence of differential expression using the Empirical Bayes Statistics method.

Reverse transcription and real-time qRT-PCR. Single strand cDNA was synthesized from 2 μg of total RNA with oligo(dT) using the Expand Reverse Transcriptase (Roche Diagnostics GmbH, Mannheim, Germany). Real-time PCR was performed using the FastStart SYBR Green Master kit and SmartCycler System (Roche Diagnostics, East Sussex, UK) according to the manufacturer's protocol. Forward and reverse primers for real-time PCR (Table I) were designed by ProbeFinder software for Human, version 2.35. Data were normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and presented as the mean fold increase or decrease compared to the control. The PCR reaction consisted of a first step at 95˚C for 10 min and 45 cycles, each cycle including 15 sec at 95˚C, 30 sec at 55˚C and 30 sec at 72˚C. The primer concentration was 0.2 μM.

Protein extraction and Western blot analysis. Cell extracts were prepared by lysing the cells in 1% NP40, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 1 mM NaF. The amount of protein was normalized following MicroBCA™ Protein assay quantification (Pierce, Rockford, IL, USA). The samples were analyzed by SDS-PAGE on a 7.5% polyacrylamide gel under non-reducing conditions. Following electrophoresis, the proteins were transferred onto an Immobilon-P membrane (Millipore Corporation, Bedford, MA, USA). The blot was placed in a blocking solution consisting of 5% skim milk in TBS-0.05% Tween-20 and incubated for 1 h at room temperature. The membranes were incubated with the primary monoclonal antibody P4A4 against extracellular domain epitopes of human endoglin (18) at 4˚C overnight, washed, incubated with a HRP-labeled secondary antibody and visualized by chemiluminescence (ECL Plus System; GE Healthcare).

Table I. Primer sequences used for real-time PCR analysis.

Gene	Primer	Product size (bp)
ENG		
	F: 5'-CTTCCTGGAGTTCCCAACG-3'	70
	R: 5'-GGTGCCATTTTGCTTGGA-3'	
VEGF		
	F: 5'-GGTGCCATTTTGCTTGGA-3'	70
	R: 5'-TCTCCGCTCTGAGCAAGG-3'	
PPP1R14B		
	F: 5'-CAGCTCACGCGCCTCTAC-3'	66
	R: 5'-CTCATCCACGTCAATCTCCA-3'	
GAPDH		
	F: 5'-AGCCACATCGCTCAGACA-3'	66
	R: 5'-GCCCAATACGACCAAATCC-3'	

F, forward; R, reverse.

Results

Determination of differential gene expression status of LV3SN MeWo melanoma cells. The OncoChip array was used to survey the differential changes in gene expression between V3-expressing MeWo melanoma cells (LV3SN) and their control counterparts (LXSN). RNA from LXSN and LV3SN MeWo melanoma cells cultured for 144 h were hybridized on OncoChip arrays, in which 6,386 tumor progression-related genes were represented. A 2-fold change cut-off and $p<0.05$ were used as restriction parameters. A large proportion of the genes were equally expressed in the LXSN and LV3SN cells. Nevertheless, differential expression of several genes was observed between the cell lines, as listed in Table II. Notably, the key difference was found in the endoglin gene (*ENG*), which was decreased in the LV3SN MeWo melanoma cells by >3-fold compared to their cellular counterparts (Fig. 1A).

Also of particular note were the signaling-related genes *PPP1R14B* [regulatory (inhibitor) subunit 14B of PP1] and *VEGF* (vascular endothelial growth factor). Both were inversely regulated, as *PPP1R14B* expression was decreased by >2-fold and *VEGF* expression was increased by >2-fold in the LV3SN MeWo melanoma cells compared to the LXSN control cells (Fig. 1A).

Verification of microarray data. To verify the microarray data, real-time qRT-PCR for the *ENG* gene was performed.

Table II. Gene expression profile analysis of LV3SN cells. RNA samples were extracted from cells cultured for 144 h and hybridized on an OncoChip cDNA microarray.

ID	Name	M	A	p-value	adj. p-value	B	Molecular function
hAA2258	*ENG*	-1.70	9.4	6.10E-05	0.044346	1.97	Protein binding
hAC0028	*PPP1R14B*	-1.70	9.6	1.06E-08	5.36E-05	8.32	
hAB9497	*KYNU*	-1.40	9.1	1.89E-07	0.000522	6.50	Hydrolase activity
hAB6803	*HIST1H4F*	-1.40	8.5	4.65E-07	0.000714	5.86	
hAC9756	*MRPS30*	-1.40	11.0	1.04E-07	0.000358	6.90	Structural constituent of ribosome
hAB5226	*SFRP2*	-1.40	12.0	1.15E-08	5.36E-05	8.27	Receptor activity
hAC5609	*WBP1*	-1.20	12.0	3.34E-06	0.003554	4.37	
hAB7244	*MMP2*	-1.00	9.2	2.06E-06	0.002376	4.74	Metal ion binding
hAC5580	*ASRGL1*	-1.00	7.9	0.00031	0.124761	0.55	Hydrolase activity
hAA1140	*FKBP2*	1.56	10.0	0.00074	0.161520	-0.23	Drug binding
hAA1129	*FN1*	1.46	11.0	0.00271	0.278494	-1.41	Extracellular matrix constituent
hAB7104	*CYR61*	1.42	10.0	0.00079	0.162998	-0.29	Protein binding
hAA1107	*MGST1*	1.15	7.4	0.00040	0.132650	0.31	Transferase activity
hAA1477	*PRG1*	1.12	9.5	0.00849	0.447711	-2.44	
hAA2245	*FGFR4*	1.08	8.7	0.00071	0.161520	-0.20	Receptor activity
hAE7978	*VEGF*	1.05	8.3	0.00026	0.115560	0.71	Receptor Activity
hAE8154	*A2M*	1.00	7.4	0.00371	0.309357	-1.69	Protein binding
hAA2309	*CTGF*	1.00	7.4	0.00066	0.160995	-0.13	Protein binding

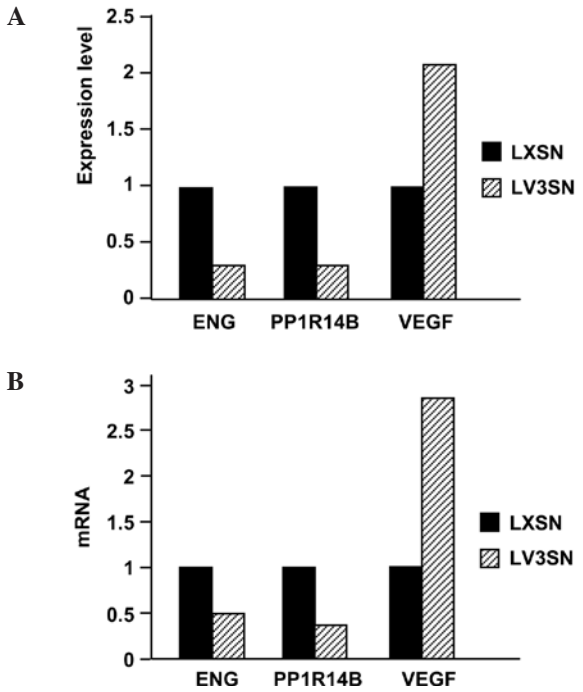

Figure 1. (A) Gene expression of *ENG*, *PPP1R14B* and *VEGF* analyzed by cDNA microarrays. RNA samples were extracted from cells cultured for 144 h and hybridized on OncoChip cDNA microarray; *p<0.05 (n=2). (B) Results were verified by real-time qRT-PCR. Data were normalized to the reference gene GAPDH and are presented as the mean fold increase or decrease compared to control (n=2).

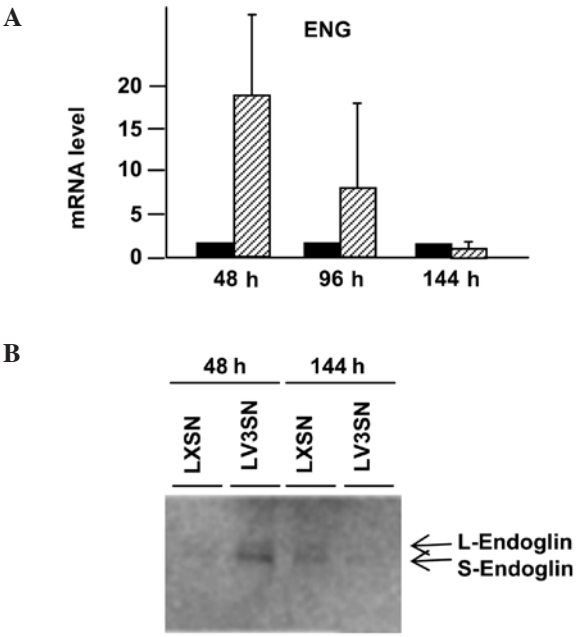

Figure 2. (A) Expression of endoglin transcripts determined by real-time qRT-PCR. RNA samples were extracted from the cell cultures at indicated times. Data were normalized to the reference gene GAPDH and are presented as the mean fold increase or decrease compared to the control (n=3). (B) Expression of endoglin protein was determined by Western blotting. Protein extracts were obtained from cultured cells at indicated times.

As shown in Fig. 1B, the expression levels of *ENG* observed by real-time qRT-PCR were consistent with the microarray analysis, verifying the changes in the LV3SN MeWo cells identified by the microarray. The results for *PPP1R14B* and *VEGF* were also confirmed by real-time qRT-PCR (Fig. 1B).

V3 overexpression in the MeWo melanoma cells decreased cell proliferation *in vitro* and *in vivo*, as previously described (11,12). To analyze whether *ENG* expression changed during

cell proliferation, RNA samples from cells at different time points during culture were obtained, and a real-time qRT-PCR analysis was performed. The results indicate that the *ENG* mRNA level was higher in the LV3SN MeWo melanoma cells after 48 h of proliferation, but decreased over time up to 144 h, when its expression was lower than in LXSN cells (Fig. 2A). Finally, the endoglin protein level was analyzed by Western blotting. Two different alternatively spliced isoforms of endoglin [long (L)-endoglin and short (S)-endoglin] are expressed in human and mouse tissues (18,19). The two isoforms were detected in MeWo melanoma cells using the P4A4 antibody, as shown in Fig. 2B. Furthermore, the LV3SN MeWo melanoma cells presented higher endoglin protein levels at 48 h of proliferation than the control cells, whereas this inversely changed at 144 h of proliferation, consistent with the real-time qRT-PCR results.

No significant changes in *PPP1R14B* or *VEGF* mRNA levels during the cell culture period were observed in either of the genes (data not shown).

Discussion

We previously demonstrated that V3 overexpression decreases human melanoma cell proliferation *in vitro* (11) and *in vivo* (12). This study aimed to provide a comprehensive analysis of gene expression changes in LV3SN MeWo melanoma cells compared to their control counterpart LXSN cells in order to gain further insight into the mechanism by which V3 regulates cell proliferation and tumor progression. These changes were analyzed in proliferating melanoma cells cultured for 144 h, at which time the maximal differences in cell proliferation were observed (11).

The most markedly differentially expressed gene was endoglin, which was down-regulated in V3-overexpressing MeWo melanoma cells. Endoglin, also known as CD105, is a type III TGF-β receptor. It was originally identified as a human endothelial cell marker, but further studies demonstrated that this cell surface antigen is also expressed by other non-endothelial cell lines, including macrophages and fibroblasts. Endoglin is expressed in solid neoplasias including breast, prostate and colorectal carcinomas, where it has been proposed to be an indicator of a poor prognosis (14,20). Likewise, endoglin is expressed by normal and neoplastic cells of melanocytic lineage (27). Besides its pro-angiogenic role (14,22,23), recent studies have indicated that endoglin is a positive regulator of tumor cell proliferation, since it has been described that the addition of a neutralizing anti-endoglin mAb down-regulates the growth of human melanoma cells (20). Thus, a reduction in endoglin levels contributes to a lower proliferation rate of V3-expressing MeWo cells.

Nevertheless, a negative correlation between *ENG* expression and metastatic ability has also been suggested in prostate cancer, where *ENG* was found to be down-regulated among 4,000 evaluated genes during the detachment of metastatic cells (24), while the overexpression of endoglin suppressed cell migration and metastasis (25). Moreover, endoglin-haploinsufficient mice (Eng+/-) experience reduced lung tumor growth compared to their control littermates (Eng+/+), whereas VEGF levels were slightly increased in Eng+/- compared to Eng+/+ mice (26). Notably, we previously described that V3 overexpression shows this dual behavior, since it decreases tumor cell growth while increasing the formation of secondary tumors in mice (12). These findings are in accordance with our results, since the LV3SN MeWo melanoma cells presented higher expression of another metastasis-related gene, *VEGF*, as well as *ENG* down-regulation. Furthermore, *VEGF* is up-regulated by CYR61 (27), a gene that is also increased in LV3SN cells (Table II). CYR61 is a member of the cysteine rich 61/connective tissue growth factor/nephroblastoma overexpressed family of growth regulators, and was recently described as a tumor suppressor gene in melanoma, serving to inhibit cell motility, invasion and angiogenesis (28).

On the other hand, *PPP1R14B* expression was significantly lower in the LV3SN MeWo melanoma cells compared to the LXSN control cells. This protein inhibits multiple PP1 holoenzyme forms and may link various signaling pathways to the regulation of PP1 (29). Available evidence suggests that PP1 activity is required for cell cycle progression, since the retinoblastoma protein (RB) is one of its substrates (30). Thus, lower *PPP1R14B* expression may inhibit cell cycle progression and inhibit the proliferation rate. Correspondingly, V3-overexpressing MeWo melanoma cells present a delay in cell cycle entry (11).

In conclusion, V3 expression in MeWo melanoma cells alters their gene expression profile, specifically endoglin expression. Our current model for a V3-mediated mechanism in melanoma cells (unpublished data) involves the participation of CD44, the cell membrane receptor for hyaluronan (31). Interestingly enough, a spatial relationship was recently found between CD44 and the TGF-β receptor system (32). Taken together, we suggest that endoglin is another piece in this model system. Further studies are required to fully understand the molecular mechanism underlying the role of endoglin in V3-expressing MeWo human melanoma cells.

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