Thrombospondin-1 is part of a Slug-independent motility and metastatic program in cutaneous melanoma, in association with VEGFR-1 and FGF-2

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Introduction

Thrombospondin-1 (TSP-1), a matricellular glycoprotein, is a complex regulator of tumor progression (Kazerounian et al., 2008). Its structure, a string of different structural and functional domains, confers a multiplicity of functions on TSP-1. Its expression is cell type specific and is deregulated in pathological conditions, including tumor development and malignant progression (Bornstein and Sage, 2002).

Significance

In view of their developmental origins, melanoma cells have an intrinsic invasive ability. We show that the motility factor thrombospondin-1 (TSP-1) is produced by melanoma cells as part of a set of genes regulating melanoma motility and metastasis, together with FGF-2 and VEGF/VEGFR-1. The expression of these genes inversely correlated with Slug, a migration master gene implicated in melanoma invasiveness. We propose that melanoma cells, lacking the intrinsic Slug-driven motility program, might activate an alternative metastatic program, involving the coordinated expression of the pro-invasive genes TSP-1, VEGF, VEGFR-1, and FGF-2, which therefore represent potential targets for therapeutic interventions.

TSP-1 is a major endogenous inhibitor of angiogenesis, generally acting to suppress the tumor development (Adams and Lawler, 2011; Kazerounian et al., 2008). Its loss is instrumental in the 'angiogenic switch' of tumors, the acquisition of angiogenic phenotype by tumor cells that marks the transformation from a dormant tumor to vascularized, invasive cancer (Naumov et al., 2006).

TSP-1 has multifaceted effects on tumor metastatic dissemination (Adams and Lawler, 2011; Kazerounian et al., 2008; Tuszynski and Nicosia, 1996). On account of its antiangiogenic activity, TSP-1 acts as an inhibitor of metastasis (Rofstad et al., 2003). Conversely, owing to its pro-adhesive and pro-invasive properties (Taraboletti et al., 1987), TSP-1 can also promote tumor cell metastatic dissemination (Nucera et al., 2010; Yee et al., 2009).

In agreement with its angiostatic and tumor suppressive activity, the expression of TSP-1 is lower in most transformed cells than in the normal, non-transformed counterparts, as a result of genetic cues (oncosuppressor genes and oncogenes), growth factors and environmental factors such as hypoxia (Kazerounian et al., 2008; Lawler and Detmar, 2004; Vikhanskaya et al., 2001).

Cutaneous melanoma is an exception to this rule, because melanoma cells reportedly express TSP-1 (Straume and Akslen, 2001), whereas melanocytes, their normal counterparts, do not (Mcclenic et al., 1989). Melanoma is the most severe form of skin cancer, and its incidence is rising in Western populations. Because of the limited response to current therapies, metastatic malignant melanoma usually has a very poor prognosis (Gray-Schopfer et al., 2007).

Cutaneous melanoma originates from normal melanocytes. Tumorigenesis usually progresses through different phases, from common nevi to dysplastic nevi, radial growth-phase melanoma (RGP), and the more aggressive vertical growth-phase (VGP) melanoma (Miller and Mihm, 2006).

It has been proposed that the invasive propensity of melanoma cells has a developmental origin, deriving from the motility competence of melanocytes, necessary for the migration from the neural crest to the skin during embryonic development (Gupta et al., 2005). This process is regulated by components of the extracellular matrix, including TSP-1, that provide adhesive and motility cues to neural crest cells (Tucker et al., 1999), suggesting also a possible role for TSP-1 in melanoma invasiveness.

This study was designed to analyze whether the expression of TSP-1 in melanoma cell lines and patients' lesions is associated with the migratory, invasive, and metastatic phenotype, and its relationship with other genes involved in melanoma progression.

Results

Expression of TSP-1 in cell lines and samples from patients

TSP-1 expression was analyzed in melanocytes and melanoma cell lines obtained from patients at different stages of disease progression (Herlyn, 1990; Herlyn et al., 1985; Satyamoorthy et al., 1997). Expression of TSP-1, evaluated at mRNA and protein level, was below the detection limit in 4/4 melanocyte cultures and in the

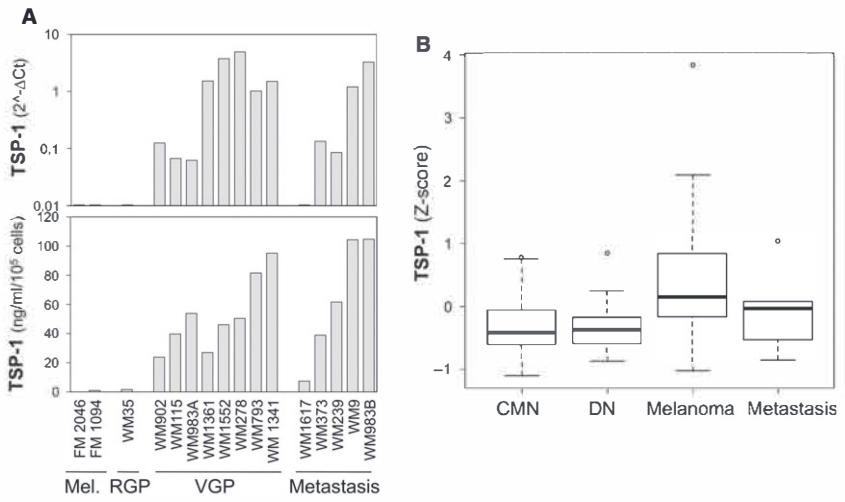

Figure 1. Expression of TSP-1 by melanoma cell lines (A) and lesions from patients (B) at different stages of disease progression. A) TSP-1 expression by 2 melanocyte primary cultures and 14 cultured melanoma cell lines was analyzed by RT-PCR (top panel, expressed as $2^{-\Delta Ct}$) or by ELISA of TSP-1 protein in the supernatant (lower panel, expressed as ng/mL/10^5 cells). B) Analysis of TSP-1 expression in 18 common nevi (CMN), 11 dysplastic nevi (DN), 21 melanomas, and 5 melanoma metastases. TSP-1 is expressed as the Z-score, calculated as described in Materials and Methods (mean of two TSP-1 probes). Boxes are delimited by the first and third quartile. The thick line represents the median. If the extreme values lie inside 1.5 times the interquartile range from the box, they are represented by whiskers, or otherwise by dots. The P-value of the two-class unpaired Student's t test applied to TSP-1 expression between 21 melanomas and 29 nevi (18 common nevi plus 11 dysplastic nevi) is < 0.05.

WM35 cell line derived from an RGP lesion, whereas it was variously detectable in cell lines derived from VGP melanomas or metastases (Figure 1A).

In human samples, TSP-1 mRNA expression was significantly higher in primary melanomas and melanoma metastases than in common and dysplastic nevi (Figure 1B), indicating that TSP-1 expression is acquired with the malignant progression of melanoma.

Regulation of TSP-1 expression in melanoma by FGF-2 and VEGF pathways

FGF-2 is a major factor associated with melanoma progression and an inducer of TSP-1 in different cell types (Donoviel et al., 1990). TSP-1 expression was higher in melanoma cell lines (Figure 2A) and in patient lesions expressing high FGF-2 (Figure 2B). FGF-2 stimulated TSP-1 production in four melanocyte cultures,

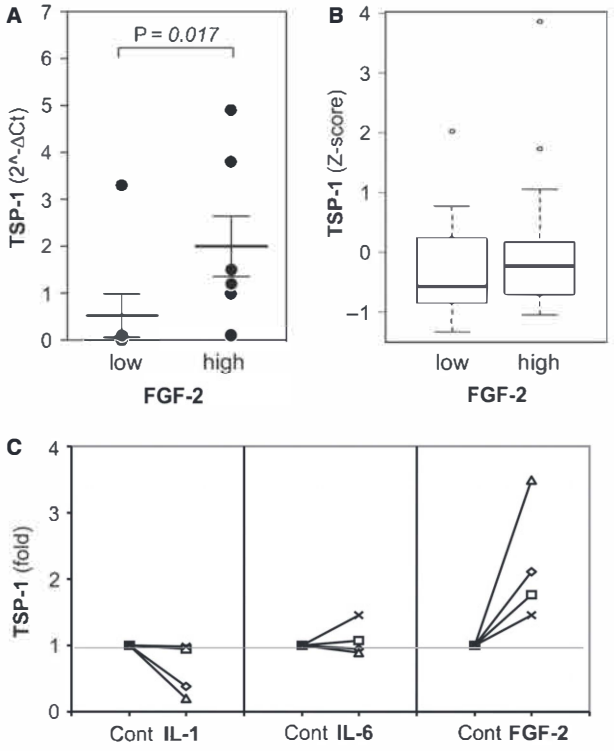

Figure 2. Role of FGF-2 in the regulation of TSP-1 production. Increased expression of TSP-1 in 14 melanoma cell lines (A) and patients' lesions (B) with higher expression of FGF-2. Samples with high FGF-2 expression were those with expression higher than the median of all FGF-2 expression data, used as the cutoff. Expression of both factors was assessed by RT-PCR (A) or microarray analysis (B). In A, TSP-1 is expressed as $2^{-\Delta Ct}$, each symbol representing a cell line, and the bar is the mean. Statistical analysis: values were analyzed by Mann–Whitney *U*-test. In B, TSP-1 is expressed as the Z-score (mean of two TSP-1 probes). C) Effect of a 24-h exposure to IL-1 (100 U/mL), IL-6 (100 U/mL), and FGF-2 (10 ng/mL) on TSP-1 production (measured by ELISA and expressed as the fold increase over untreated cells) by four primary cultures of melanocytes (each symbol and line represents a different melanocyte culture). Data, expressed as production of TSP in treated cells relative to untreated cells (Cont), are mean of two experiments.

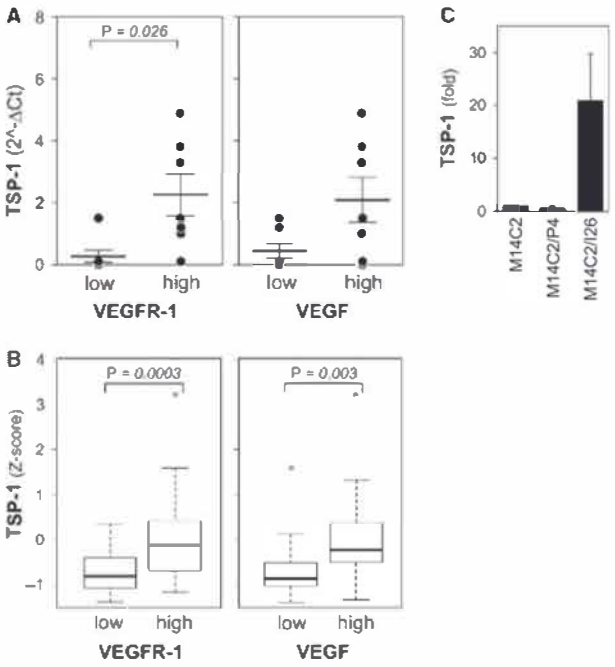

Figure 3. Involvement of the VEGF/VEGFR-1 pathway in TSP-1 production by melanoma. A) Expression of TSP-1 by 14 melanoma cell lines expressing high or low levels of VEGFR-1 or VEGF (the median of the expression data was used as the cutoff, as in figure 2). TSP-1 is expressed as $2^{-\Delta Ct}$, each symbol representing a cell line, and the bar is the mean. Statistical analysis: values were analyzed by Mann–Whitney *U*-test. B) Higher expression of TSP-1 in patients' melanoma lesions with higher expression of VEGFR-1 and VEGF. TSP-1 is expressed as the Z-score (mean of two TSP-1 probes). C) Production of TSP-1 (expressed as the fold increase over parental cells) by melanoma cells transfected to express VEGFR-1 (M14C2/I26) and parental cells (M14C2) or cells infected with the empty vector (M14C2/P24), lacking the receptor. Data are mean of three experiments.

whereas IL-1 and IL-6—other cytokines involved in melanoma progression—did not (Figure 2C).

TSP-1 expression also correlated with the expression of VEGFR-1 (FLT-1), a receptor expressed by melanoma cells and implicated in melanoma progression (Lacal et al., 2000) (Figure 3A). In cultured melanoma cell lines, the expression of TSP-1 showed a tendency to correlate (although not significantly) with the expression of VEGF, a VEGFR-1 ligand. A significant association between VEGFR-1 and VEGF and TSP-1 was also found in the lesions from patients (Figure 3B). Melanoma cells engineered to overexpress VEGFR-1 had greater expression of TSP-1 than VEGFR-1-deficient parental cells or cells transfected with empty vector (Figure 3C), conceivably as a response to endogenous VEGF produced by these cells (Lacal et al., 2000). The expression of TSP-1 did not correlate with VEGFR-2, in melanoma cell lines or in patient lesions (not shown).

These findings are indicative of the coexpression and involvement of the FGF-2 and VEGFR-1 pathways in the regulation of TPS-1 expression in melanoma.

TSP-1 is associated with a motility and metastatic phenotype in melanoma cells

We analyzed the biological properties of the genes whose expression mostly correlated or anticorrelated with TSP-1 expression in melanoma patients (80 transcripts, Pearson $P < 0.001$), using MetaCore for functional enrichment analysis. Five of the 11 process networks that were overrepresented ($P < 0.05$) were related to *cell adhesion*, *connective tissue degradation,* and *ECM remodeling* (Figure 4A). Nine transcripts were involved in *cell migration* (11%) and included the TSP-1 receptors CD47 (Pearson $P = 0.00031$) and the integrin $\beta 3$ chain (Pearson $P = 0.00019$).

TSP-1 is a motility factor for a variety of tumor types, including melanoma (Taraboletti et al., 1987). The motile and invasive properties of melanoma cells were indeed significantly associated with TSP-1 production (Figure 4B), confirming that endogenous TSP-1 was associated with a more invasive phenotype.

A relation was also found between the production of TSP-1 by melanoma cells and their potential to colonize the lung after i.v. injection. Of the five melanoma lines producing high levels of TSP-1, four were able to generate lung colonies, whereas only one of the five melanomas with low TSP-1 produced lung colonies (Figure 5A). Silencing of TSP-1 by shRNA resulted in a partial inhibition of TSP-1 (51% reduction of mRNA and 54% reduction of secreted protein, Figure 5B). Although not completed, silencing of TSP-1 in WM983A cells reduced the retention of labeled cells to the lung 24 h after injection (Figure 5C) and the long-term formation of macroscopic lung colonies (Figure 5D, E). The reduction did not reach statistical significance ($P = 0.06$ for score). Nonetheless, the biological significance of this finding lays in the fact that the reduction of TSP-1 in the WM983A melanoma diminished lung colonies, instead of favoring tumor growth, as described for most tumors.

We next investigated whether this entire set of genes (TSP-1, FGF-2, and VEGF/VEGFR-1) might be regulated by Slug (SNAI2), a cell migration master gene responsible for the invasive program of melanocyte and melanoma cells (Gupta et al., 2005). Surprisingly, Slug expression in

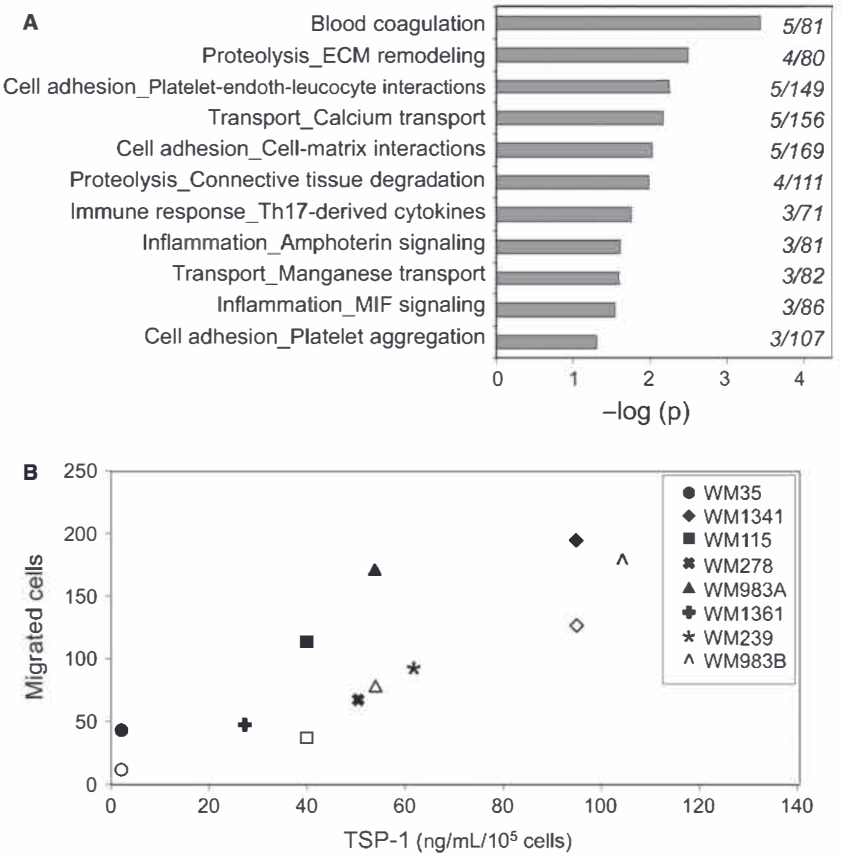

Figure 4. A) Statistically significant enriched GeneGo process networks ($P < 0.05$) in the list of genes mostly correlated or anticorrelated with TSP-1 in melanoma patients. On the *x*-axis, –log(P-values) are shown. Values alongside bars indicate the ratios of the number of genes within the list to the number of genes involved in each process network. B) Motility (black symbols) and invasiveness (white symbols) of the indicated melanoma cell lines correlated with TSP-1 production (ELISA analysis of TSP-1 in the conditioned media). Motility and invasiveness were assessed in the Boyden chamber, as described in Materials and Methods. Data (mean of 2–3 experiments) are the number of migrated cells. (Pearson $P = 0.029$ for chemotaxis and $P = 0.049$ for invasion).

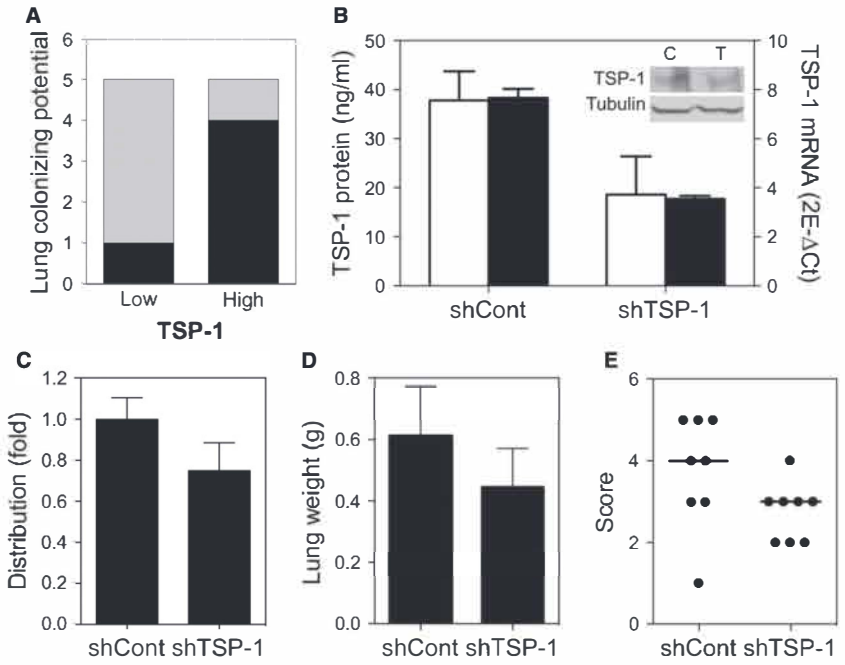

Figure 5. Association of TSP-1 expression with the lung colonizing ability of melanoma cells. Melanoma cells were injected intravenously in immunodeficient mice, and pulmonary colonies were evaluated after 3 months. A) Lung colonization potential of 10 melanoma cell lines with low or high production of TSP-1. As in Figure 2, the median of TSP-1 production (measured by ELISA) was used as the cutoff. Data are the number of metastatic (black) and non-metastatic melanomas (gray), as assessed here or in previous studies (MacDougall et al., 1999; Peng et al., 2007; Silini et al., 2010). B) Silencing of TSP-1 was verified by real-time PCR (white columns), ELISA (black columns), and Western blot analysis (insert), in cells transfected with control (shCont) or TSP-1 shRNA (shTSP-1). C) Effect of TSP-1 silencing on lung retention of labeled WM983A cells. The presence of melanoma cells in the lung 24 h after injection was measured as described in Materials and Methods. Data are the fold retention compared with controls (shCont). D, E) Lung colonization ability of WM983A silenced for TSP-1 (shTSP-1), in comparison with control cells (shCont). Colonization potential is expressed as lung weight (D) or arbitrary score of lung nodules (E).

patients was not only correlated, but also actually anticorrelated ($P \leq 0.02$) with that of TSP-1, VEGFR-1, VEGF, and FGF-2 (Figure 6A). A similar pattern was observed when analyzing TSP-1, VEGFR-1, and FGF-2 (RNA and protein) in the melanoma cell lines, although it reached statistical significance only in the case of FGF-2 protein (Figure 6B).

These findings indicate that TSP-1, with FGF-2 and VEGF/VEGFR-1, forms a Slug-independent set of genes involved in the invasive behavior of melanoma.

Discussion

This study reports that increased expression of TSP-1 is associated with an invasive and metastatic phenotype of melanoma, as part of a Slug-independent motility program that includes the melanoma-related VEGF/VEGFR-1 and FGF-2 pathways.

Unlike other tumor types in which TSP-1 expression is lost during the angiogenic switch that marks malignant transformation (Adams and Lawler, 2011; Kazerounian et al., 2008; Naumov et al., 2006), malignant melanoma cells acquire the ability to produce TSP-1, not expressed by normal melanocytes or in early RGP melanoma. Indeed, previous studies showed that TSP-1 produced by melanoma cells significantly correlates with increased tumor thickness, tumor cell proliferation, and shorter survival of patients with VGP melanoma (Straume and Akslen, 2001). Concordant data were also found in the Oncomine database (www.oncomine.org), by searching for TSP-1 expression in cutaneous melanomas versus normal skin (Riker et al., 2008) and in melanomas versus non-neoplastic nevi or melanoma precursors (Haqq et al., 2005; Talantov et al., 2005), or by performing cancer outlier profile analysis (COPA score >2 in 5 datasets).

We found a causative association between VEGF/VEGFR-1 and FGF-2 pathways and TSP-1 expression, supported by the finding that the production of TSP-1 was boosted by exogenous FGF-2 or by overexpression of VEGFR-1. These factors are major regulators of tumor angiogenesis. Although angiogenesis is a relevant event and potential therapeutic target in melanoma (Helfrich and Schadendorf, 2011; Trapp et al., 2010; Zaki et al., 2012), our study indicates that VEGF/VEGFRs, FGF-2, and TSP-1 have a role in melanoma progression that extends beyond the regulation of angiogenesis, through a direct pro-invasive effect on tumor cells. This is further supported by the observation that (i) the reduction in the number of TSP-1-silenced cells retained in the lung is observed 24 h after injection, a short time that rules out

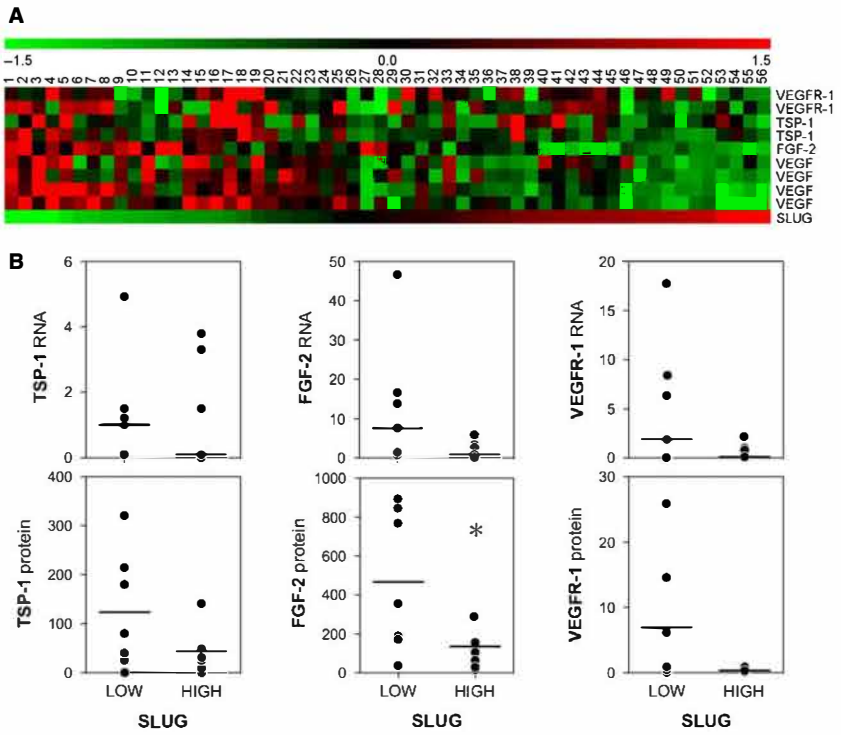

Figure 6. Anticorrelation between the expression of Slug and of TSP-1, VEGF, VEGFR-1, and FGF-2. A) Heatmap of Z-scores for Slug, TSP-1, VEGFR-1, VEGF, and FGF-2 across 55 biopsies from common melanocytic nevi (CMN), dysplastic nevi (DN), primary radial growth-phase melanomas (RGPM), primary vertical growth-phase melanomas (VGPM), and melanoma metastases. A green-to-red gradient was used to indicate the level of down- or up-regulation for each gene. Replicated probes for VEGF, VEGFR-1, and TSP-1 were present on the array used for gene expression profiling. Pearson $P = 0.01$ for TSP-1, $P < 0.0001$ for VEGF, $P = 0.02$ for VEGFR-1, and $P < 0.0001$ for FGF-2 (mean of the replicate probes). B) Expression of TSP-1, FGF-2, and VEGFR-1 RNA (top) and protein (bottom) in the 14 melanoma cell lines expressing high or low Slug. The median of Slug RNA or protein expression data (see Figure S1) was used as the cutoff, as in Figure 2). *$P < 0.05$.

the contribution of the effect of TSP-1 on angiogenesis, and (ii) silencing of TSP-1 did not significantly affect the blood vessel density in this model (CD31-positive vessels were 8.9 ± 1.1 and 9.2 ± 0.2 in control and TSP-1 silenced tumors, respectively). In agreement with these findings, no correlation between TSP-1 or VEGF expression and microvessel density was found in melanoma (Straume and Akslen, 2001).

TSP-1 has multiple roles in tumor progression. Usually acting as a tumor suppressor inhibitor of angiogenesis, it can also promote tumor progression and metastatic dissemination. This is through different mechanisms, including immunosuppression (Baumgartner et al., 2008; Kudo-Saito et al., 2009), promotion of cell–cell and cell-platelet interactions (Tuszynski et al., 1987), and induction of cell adhesion, motility, and invasiveness (De Fraipont et al., 2004; Firlej et al., 2011; Kawataki et al., 2000; Yee et al., 2009). Moreover, TSP-1 effect on metastasis is organ dependent, due to the different regulation of TSP-1 antiangiogenic activity by cleaving enzymes, such as ADAMTS1, in different organs (Lee et al., 2010). We previously reported that exogenous TSP-1 enhances melanoma cell motility by acting both as a soluble (chemotactic) and as a matrix-associated (haptotactic) attractant (Taraboletti et al., 1987). Here, we further demonstrate that TSP-1 can act in an autocrine manner, because the endogenous production of TSP-1 by melanomas correlates with their motility, invasive, and lung colonizing ability. This suggests that during malignant progression, melanoma cells, intrinsically able to respond to the migratory signals of TSP-1, also acquire the ability to produce the attractant, triggering an autocrine invasive and metastatic process.

Several genes, functionally associated with the 'motility' phenotype, were coexpressed with TSP-1 in melanoma patients, including the two TSP-1 receptors CD47 and the integrin $\beta 3$ chain, and the matrix metalloproteinases MMP1 and MMP3, suggesting that they are part of the TSP-1 invasive program in melanoma.

Our search for factors regulating TSP-1 production in melanoma ruled out B-Raf^{V600E} (not shown), but unexpectedly indicated a negative correlation between TSP-1 and Slug, a member of the Snail family of transcription factors responsible for the epithelial-to-mesenchymal transition in epithelial tumors. Slug, a master regulator of neural crest migration, is expressed in melanocytes and is responsible for the metastatic behavior of melanomas (Gupta et al., 2005). The inverse correlation between Slug and TSP-1/VEGFR-1/VEGF/FGF-2 suggests that these factors become expressed in melanoma cells

lacking Slug. TSP-1 has not been reported as a direct target of Slug (Dhasarathy et al., 2011; Gupta et al., 2005; Newkirk et al., 2008); however, we cannot rule out an indirect role of Slug in the regulation of TSP-1 expression, or the presence of other factors, upstream Slug, that regulate the expression of both factors.

Interestingly, Slug and SNAI1, another member of the Snail family, are differentially regulated in melanocytes and melanoma cells (Shirley et al., 2012). SNAI1 induces TSP-1 expression in melanoma (Kudo-Saito et al., 2009). The mutual functional regulation between members of the Snail family in regulating TSP-1 production and the consequences in terms of invasive properties of melanoma cells warrant further investigation.

This study indicates that melanoma cells, lacking the intrinsic Slug-driven motility program (Gupta et al., 2005), might activate an alternative metastatic program, involving the coordinated expression of the pro-invasive genes TSP-1, VEGF, VEGFR-1, and FGF-2, which therefore represent potential targets for therapeutic interventions.

Methods

Melanocytes and melanoma cell lines

Human melanocytes and melanoma cell lines were kindly provided by M. Herlyn (Wistar Institute, Philadelphia, PA). Melanocytes were cultured in MCDB153 and L-15 (4:1, GIBCO Paisley, UK) with 5% FBS. Of the 14 melanoma cell lines, WM35 was derived from a RGP melanoma: WM115, WM278, WM793, WM902, WM983A, WM1341, WM1361, and WM1552 were established from VGP primary melanoma lesions, while WM9, WM239, WM373, WM983B, and WM1617 were derived from distant melanoma metastasis. Melanoma cells were grown in RPM1 1640 with 5% FBS. Their origin, DNA fingerprinting, and molecular and biological properties have been described (Herlyn, 1990; Herlyn et al., 1985; Satyamoorthy et al., 1997).

To generate melanoma cells expressing VEGFR-1, M14C2, a clone of the human melanoma cell line M14 (Lacal et al., 2000), was transfected with the empty pIRES2 vector or pIRES-VEGFR-1hs-GFP (generous gifts from K. Ballmer-Hofer, PSI, Zurich) as described (Lacal et al., 2005). After selection with G418, VEGFR-1-expressing subclones were identified by RT-PCR analysis. Receptor function was demonstrated by the chemotactic response to VEGF or PlGF of the VEGFR-1-expressing cells (M14C2/MF26) but not control cells (M14C2/P4) (not shown).

To prepare conditioned media, subconfluent cells were incubated in serum-free medium for 4 h and then resupplied with fresh serum-free medium. Where indicated, FGF-2 (10 ng/mL), IL-1, and IL-6 (both at 100 U/mL) were added. After 24 h, the conditioned medium was collected and centrifuged. The remaining cells were counted.

Quantitative real-time polymerase chain reaction

Total RNA was isolated with RNeasy® Mini Kit (Qiagen, Milan, Italy), DNase digested during purification, and converted to cDNA with random hexamers (Archive Kit; Applied Biosystems, Life Technology, Monza MB, Italy). Gene expression was analyzed by real-time PCR as already described (Ghilardi et al., 2008) using TaqMan® Gene Expression Assay (Applied Biosystems) for TSP-1 (Assay Hs00170236_m1), VEGF (Hs00173626_m1), VEGFR-1 (Hs00176573_m1), FGF-2 (Hs00266645_m1), Slug (SNAI2, Hs00161904_m1), and 18s (Hs99999901_s1), the latter used as an endogenous control gene to normalize the level of gene expression by calculating the deltaCt (ΔCt) values.

ELISA and Western blot analysis

TSP-1 was analyzed by ELISA in the supernatant of cells as previously described (Vikhanskaya et al., 2001) or with an ELISA kit (R&D Systems, Minneapolis, MN, USA). FGF-2 and VEGFR-1 were measured in the cell lysate with Luminex Performance Assay and DuoSet IC assay, respectively (R&D Systems). Slug protein was analyzed in the whole lysate of confluent cells by Western blot analysis, with anti-Slug antibody (Cell Signaling Technology, Leiden, The Netherlands).

Gene expression profiling

A total of 55 excisional biopsies from common melanocytic nevi (18), dysplastic nevi (11), primary melanomas (21), and melanoma metastases (5) were analyzed as previously described (Scatolini et al., 2010) (GEO dataset GSE12391). Expression values were Z-score-transformed by subtracting means and dividing the result by standard deviations (Cheadle et al., 2003).

Functional enrichment analysis

MetaCore™ version 6.9 (https://portal.genego.com) was used for functional annotation enrichment analysis. Process networks were used to calculate enrichment in the ontology of prebuilt and manually created functional processes.

Motility and invasion assays

Cell migration and invasion were assayed in Boyden chambers, as previously described (Silini et al., 2010), using 8-μM pore-size, PVP-free polycarbonate membranes (Whatman, Nucleopore), coated with 0.1% gelatin (for chemotaxis) or Matrigel (0.5 mg/mL; Becton–Dickinson, Heidelberg, Germany, for invasion), and NIH3T3 fibroblast-conditioned medium as a general chemoattractant.

TSP-1 silencing

To obtain a durable silencing, required by the length of the in vivo lung colonization assays, WM983A cells were stably transfected with SureSilencing shRNA plasmids for human THBS1 (KH00799N; insert sequence: CAAGCAGGACTGTCCAATTGA) or control shRNA (GGAA TCTCATTCGATGCATAC, all from SABioscience, Qiagen) with Lipofectamin (Invitrogen, Life Technology). Cells were selected in a medium containing 800 μg/ml of G418 (Invitrogen). Silencing was verified by real-time RT-PCR and ELISA analysis as above or by Western blot analysis with anti-TSP-1 antibody (Thermo Scientific, Fremont, CA, USA).

Cell distribution analysis

Cells were labeled overnight with NP-DiR fluorescent nanoparticles (0.5 mg/ml), prepared as described in Methods S1. Labeled cells (2×10^6) were injected into the tail vein of SCID mice (Harlan, Bresso, Italy). After 24 h, lungs were collected and homogenized, and the presence of NP-DiR-labeled tumor was evaluated by measuring fluorescence at 750 nm with Tecan Infinity 200Pro reader (Tecan, Männedorf, Switzerland) and expressed as the percentage of fluorescence values of total injected cells.

Experimental metastasis

Cells (2×10^6) were injected into the tail vein of 6–8-week-old female SCID or athymic nude mice (Harlan) (Silini et al., 2010). Preliminary studies indicated that melanoma cells had a similar

behavior in the two strains. After 3 months, mice were sacrificed, lungs were fixed in Bouin's fixative (BioOptica, Milan, Italy), and tumor nodules were counted. Where indicated, lung colonization was quantified by arbitrary score, from 1 (few metastatic nodules) to 5 (>300 metastasis) or as weight (in g) of tumor-colonized lungs, subtracted the mean weight of normal lungs.

In vivo experiments were conducted in conformity with institutional regulation, in compliance with national law (D.lgs 26/2014, authorization n.19/2008 issued on March 6, 2008, by the Ministry of Health), EU directives (EEC Council Directive 2010/63/UE), and international guidelines (National Research, 2011).

Statistical analysis

Statistical analyses were performed with PRISM 5 software (GraphPad Software, La Jolla, CA, USA), using Pearson's correlation analysis. For comparison of two groups, the nonparametric Mann–Whitney U-est was used for cell lines and Student's t test for biopsy data.

Acknowledgements

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