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Epigenetic Silencing of SPINT2 promotes Cancer Cell Motility via HGF-MET Pathway Activation in Melanoma

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Abstract

Aberrant HGF-MET signaling activation via interactions with surrounding stromal cells in tumor microenvironment plays significant roles in malignant tumor progression. However, extracellular proteolytic regulation of HGF activation which is influenced by the tumor microenvironment and its consequential effects on melanoma malignancy remain uncharacterized. In this study we identified SPINT2: a proteolytic inhibitor of hepatocyte growth factor activator (HGFA), which plays a significant role in the suppression of the HGF-MET pathway and malignant melanoma progression. SPINT2 expression is significantly lower in metastatic melanoma tissues compared to those in early stage primary melanomas which also corresponded with DNA methylation levels isolated from tissue samples. Treatment with the DNA hypomethylating agent decitabine in cultured melanoma cells induced transcriptional reactivation of *SPINT2*, suggesting that this gene is epigenetically silenced in malignant melanomas. Furthermore, we show that ectopically expressed SPINT2 in melanoma cells inhibits HGF induced MET-AKT signaling pathway and decreases malignant phenotype potential such as cell motility, and invasive growth of melanoma cells. These results suggest that SPINT2 is associated with tumor suppressive functions in melanoma by inhibiting an extracellular signal regulator of HGF which is typically activated by tumor-stromal interactions. These findings indicate that epigenetic impairment of the tightly regulated cytokine-receptor communications in tumor microenvironment may contribute to malignant tumor progression.

Introduction

Hepatocyte growth factor/scatter factor (HGF/SF) and its receptor tyrosine kinase MET play critical roles in embryogenesis and wound healing (Birchmeier *et al.*, 2003). Upon ligand binding, MET activates various intracellular signaling cascades including PI3K-AKT,

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Conflict of Interest

The authors state no conflict of interest.

RAC1-CDC42, RAP1, and RAS-MAPK pathways for mitogenic and motogenic effects (Gherardi *et al.*, 2012; Jiang *et al.*, 2005). Cancer cells take advantage of the HGF/SF-MET exerted signaling pathways for promoting tumor progression and metastasis by stimulating cell proliferation, dissociation, migration, invasion, angiogenesis, and survival (Grant *et al.*, 1993; Rosen *et al.*, 1993; Straussman *et al.*, 2012; Sugawara *et al.*, 1997). As a major mechanism of cancer progression and resistance to therapy, functional crosstalk between HGF/SF activated MET and other receptor tyrosine kinases such as EGFR, ERBB2, IGFR as well as receptors of developmental signal pathways such as WNT and TGFR, has been reported (Bauer *et al.*, 2006; Bhowmick *et al.*, 2004; Khoury *et al.*, 2005; Klaus and Birchmeier, 2008; Monga *et al.*, 2002; Zhang *et al.*, 2010b). These new insights of HGF/SF-MET roles in cancer progression and therapy resistance also support the validity of HGF/SF-MET signaling pathway as potential targets in cancer therapy (Cepero *et al.*, 2010; Kwak *et al.*, 2010; Mueller *et al.*, 2008; Munshi *et al.*, 2010; Zhang *et al.*, 2010a).

Proteolytic activation of pro-HGF/SF to the active form of ligand (HGF/SF), which is primarily mediated by three distinctive forms of serine proteinase, HGF activator (HGFA), matriptase, and hepsin, is an important regulatory step (Owen *et al.*, 2010). In contrast to matriptase and hepsin which are membrane bound proteinase, HGFA is a soluble protein mainly secreted by the liver and circulates in plasma in the human body (Kataoka and Kawaguchi, 2010; Shimomura *et al.*, 1993) and is a potent activator of HGF/SF. HGFA converts inactive single chain pro-HGF to the active double-chain form which is known to bind and activate the MET signaling pathway. Proteolytic activation of HGF/SF by HGFA is tightly controlled by another layer of regulatory mechanism mediated by two known serine peptidase inhibitors Kunitz type 1 and type 2 (SPINT1 and SPINT2 respectively) (Kawaguchi *et al.*, 1997; Shimomura *et al.*, 1997). In cancer cells, balance between these two groups of proteases in the interface of stromal-tumor cells may determine HGF exerted malignant phenotypes; for example, decreased levels of SPINT1 and SPINT2 that were observed in aggressive cancer cells (Morris *et al.*, 2005) may lead to the higher levels of available proteolytic HGF activators (HGFA, matriptase, and hepsin) resulting in promotion of tumor growth and invasion (Klezovitch *et al.*, 2004; List *et al.*, 2005).

SPINT2 (also known as HAI2, HGF activator inhibitor 2) is a transmembrane protein with two Kunitz domains within its extracellular region. SPINT2 is suggested to play an important role during embryogenesis as deletion of SPINT2 is embryonic lethal in mice (Szabo *et al.*, 2009). Although decreased expression of SPINT2, which correlates with poor prognosis, has been shown to be epigenetically silenced in cancer via DNA hypermethylation (Bergum and List, 2010; Dong *et al.*, 2010; Nakamura *et al.*, 2011), physiological consequences of SPINT2 silencing in the context of malignant melanoma development have not been studied. Here, we report the malignant progression suppressive functions of SPINT2 by inhibiting the oncogenic MET-AKT pathway in melanoma.

Results

SPINT2 is epigenetically silenced in melanoma tissue samples and cell lines

In order to discover novel epigenetically silenced melanoma suppressor genes, we performed a comparative analysis of genome-wide DNA methylation profiles between

short-term cultured normal human primary melanocytes (HPM) and melanoma cell lines. We identified a gene signature of differential promoter DNA methylation (Figure 1a). Furthermore, to identify genes that are epigenetically silenced and functionally associated with metastatic melanoma development, we compared the methylation signature genes with a genome-wide expression profile of primary melanoma tissues obtained from two groups of patients (patients with recurrent metastasis within 4 years after initial diagnosis vs. patients without recurrences) (Winnepenninckx *et al.*, 2006) and identified *SPINT2* as the only gene in common between the two independent sets of signatures (DNA methylation and gene expression). We then validated the hypermethylation of *SPINT2* gene in melanoma cell lines compared to normal human primary melanocyte (HPM) using methylation-specific PCR (Figure 1b and Supplemental figure S1) and bisulfite sequencing analysis, showing that most CpG dinucleotides were hypermethylated in melanoma cell lines whereas aberrant methylation was significantly less in HPM cells (Figure 1c). Comparative measurement of *SPINT2* mRNA expression levels by semi-quantitative RT-PCR analysis revealed that melanoma cells express significantly lower levels of *SPINT2* mRNA compared to those of HPMs (Figure 2a), suggesting that DNA hypermethylation is a primary cause of *SPINT2* silencing in melanoma cells. Furthermore, treatment with a DNA hypomethylating agent (decitabine) in a panel of melanoma cell lines showed dose-dependent increased levels of *SPINT2* mRNA whereas no significant difference was seen in primary melanocytes (Figure 2b). Based on these observations along with potential biochemical function of *SPINT2* in inhibition of HGF/SF proteolytic activation, we hypothesized that epigenetic loss of *SPINT2* may contribute to malignant melanoma progression.

SPINT2 expression is significantly lower in clinically aggressive metastatic melanomas

We next examined whether tumors derived from clinically different stages of melanoma exhibit differential levels of *SPINT2* gene expression correlative to disease progression. *SPINT2* mRNA expression was assessed by quantitative RT-PCR from surgically removed clinical tissue samples of early stage primary and metastatic lesions of 24 melanoma patients (12 patients for each group). Differential expression of *SPINT2* mRNA levels was verified as shown in the significant decrease of *SPINT2* expression in metastatic melanoma tissue samples than that of primary melanoma samples (p-value=0.014) (Figure 3a). In order to correlate decreased *SPINT2* mRNA expression in metastatic melanoma with epigenetic silencing of the gene, specifically DNA hypermethylation, semi quantitative methylation specific PCR of the *SPINT2* gene was performed on bisulfite treated genomic DNA isolated from available clinical tissue samples. Two of the four primary melanoma samples failed to amplify whereas three of the four metastatic samples showed amplification (Figure 3b). The methylation specific amplification linear fold change of each sample was normalized to the lowest amplified primary melanoma and shows a statistically higher level of *SPINT2* gene methylation in metastatic tissue samples than primary. These results from clinical tissue samples suggest that abrogation in *SPINT2* expression by DNA hypermethylation may contribute to advancement in melanoma malignancy.

SPINT2 regulates proliferation and migration of melanoma cells

The observed silencing of *SPINT2* in aggressive clinical tissue samples suggests a potential metastasis suppressive role of *SPINT2* in malignant melanoma progression. To test this

hypothesis, stable melanoma cells over-expressing SPINT2 were generated using a lentiviral gene delivery system. SPINT2 over-expression was confirmed by immunoblot analysis (Figure 4a). Cell proliferation was assessed over a 72 hour period after seeding in which SPINT2 over-expression resulted in decreased growth compared to empty vector controls (Figure 4c). To obtain further evidence of decreased cell growth, cell cycle profile analysis was performed (Figure 4e). In melanoma cells over-expressing SPINT2, the percentage of the cell population in the G₀/G₁ stage increased and the percentage in the G₂/M stage decreased significantly compared to control cells; confirming the observed decrease in cell growth. SPINT2 expressing WM1552C cells (Figure 3c and d) were then chosen for lentiviral SPINT2 shRNA transduction for knockdown (Figure 4b) and showed cell proliferation significantly increased compared to scramble control (Figure 4d). These data suggest that SPINT2 may play an important role in regulating the cell cycle and that epigenetic silencing of SPINT2 may result in increased tumor cell growth in melanomas.

To determine the role of SPINT2 in regulating cell migration a wound healing assay was performed in melanoma cells with SPINT2 expression. In order to establish wound closure is dependent upon cell migration and not proliferation, cell growth in serum-free media was observed for 18 hours and no significant difference was seen between control and SPINT2 expressing cells (Figure 5c) which was consistent with the proliferation time course data (Figure 4c). Confluent monolayer of cells was scratched and wound closure was examined 18 hours later. As determined by percentage of wound closure SPINT2 over-expressing cells migrated significantly less compared to control cells with or without activated pro-HGF 18 hours after wounding (Figure 5a and b). Additional scratch wound healing assay performed in 1205Lu melanoma cell lines confirmed decreased wound closure at various time points independent of cell proliferation with SPINT2 over-expression (Supplementary Figure S2).

The effect of SPINT2 on anchorage independent tumor growth was also evaluated by soft agar colony formation assay. SPINT2 re-expression did not inhibit the ability of melanoma cell lines to form colonies in anchorage independent environments with or without pro-HGF stimulation (Figure 5d). However, stimulation with pro-HGF significantly increased colony size in control cells, whereas the colony size of melanoma cells with SPINT2 expression was significantly decreased (Figure 5e and f); suggesting SPINT2 re-expression may suppress invasive tumor growth even in the presence of pro-HGF stimulation. Together these data suggest epigenetic loss of SPINT2 expression in metastatic melanoma cell lines results in enhanced metastatic phenotypes such as tumor cell motility and invasive growth.

SPINT2 over-expression attenuates basal and pro-HGF/SF stimulated MET-AKT phosphorylation

Previous studies regarding pro-HGF/SF proteolytic activation have shown SPINT2 to bind and inhibit HGFA's proteolytic function of binding and processing pro-HGF/SF to the active form (Kataoka *et al.*, 2002). However, the inhibitory function of SPINT2 in MET phosphorylation and downstream signaling cascade activation has yet to be experimentally demonstrated. Basal MET phosphorylation levels in all the tested melanoma cell lines 1205Lu, WM983B WM902B, and A375 were minimal and there was no discernible effect of SPINT2 on MET phosphorylation (Figure 6a). However, when pro-HGF/SF was treated,

SPINT2 over-expression abrogated active HGF/SF induced MET phosphorylation compared to control cells. Consistent with decreased MET phosphorylation, SPINT2 over expressing cells exhibited decreased AKT phosphorylation during pro-HGF/SF stimulation. ERK phosphorylation was not affected with SPINT2 over-expression in melanoma cells, which may be attributed to the constitutional activation of BRAF in melanoma cell lines that harbor the BRAF^{V600E} mutation. However, pro-HGF/SF treatment in HeLa cells shows that SPINT2 over-expression within a BRAF wild type background is capable of blocking not only MET/AKT activation but also ERK signaling during pro-HGF/SF stimulation (Figure 6b). When SPINT2 expression was knocked down in WM1552C cells, pro-HGF stimulation increased MET phosphorylation in which one of the two SPINT2 knockdown cell lines exhibited increased total MET receptor protein levels which may enhance downstream signaling (Figure 6d). These data suggest SPINT2 abrogation of invasive growth and migration in cancer cell may be via inhibition of MET and downstream AKT activation.

Discussion

Transgenic mice models of melanoma have shown ectopic expression of HGF/SF and MET amplification play important roles in spontaneous melanoma tumorigenesis and acquisition of metastatic phenotype (Otsuka *et al.*, 1998; Vanbrocklin *et al.*, 2009). High expression of MET in metastatic progression of melanoma has also been previously documented (Natali *et al.*, 1993). These studies suggested that the HGF-MET pathway further plays crucial roles in metastatic progression. SPINT2 has been characterized as one of the prominent serine protease inhibitors to block pro-HGF activation by binding and inhibiting HGFA (Kawaguchi *et al.*, 1997). As shown in this study, epigenetic silencing of SPINT2 expression may directly contribute to tumor formation and malignancy progression by abnormal activation of the oncogenic HGF-MET pathway. In fact, ectopic expression of SPINT2 abrogates the pro-HGF stimulated MET and attenuates its downstream target AKT and ERK activation (Figure 6). Other proteases, such as matriptase (Bergum and List, 2010; Betsunoh *et al.*, 2007; Szabo *et al.*, 2009), have also been shown to activate pro-HGF and MET, and recent report by Tsai *et al.* suggests that matriptase is the major protease inhibited by SPINT2 in suppressing invasive growth and metastasis of prostate cancer (Tsai *et al.*, 2014). However, basal expression of matriptase was minimal in melanoma cells (Supplementary Figure S3) and does not seem to be the prominent activator of pro-HGF in melanoma although we cannot completely rule out a probable role of matriptase in metastatic phenotype development.

Influences of the tumor microenvironment in regulating metastasis, changes in tumor metabolism, and therapy resistance have been established (Denko, 2008; Joyce and Pollard, 2009; Straussman *et al.*, 2012; Wilson *et al.*, 2012). One example of this stromal-tumor interaction is the changes in tumor cell behaviors induced by the high level of HGF/SF secreted from cancer-associated fibroblasts (CAFs) into the tumor microenvironment which binds to MET receptor of the neighboring carcinoma cells. HGF/SF also has been shown to promote endothelial cell growth and migration as an angiogenesis factor (Bussolino *et al.*, 1992; Grant *et al.*, 1993) thus enabling cancer cells to establish a habitable tumor microenvironment. This paracrine communication of HGF/SF plays a critical role in invasive and metastatic characteristics of tumors (Matsumoto and Nakamura, 2006; Wels *et*

et al., 2008). In some cancers including melanoma (Li *et al.*, 2001), tumor cells express HGF/SF along with elevated levels of MET expression. In addition, HGFA is mainly secreted by the liver and circulates in plasma throughout the human body (Kataoka and Kawaguchi, 2010; Shimomura *et al.*, 1993). HGFA can also be expressed and secreted by tumor cells and activated in response to tissue injury and invasive tumor growth (Kataoka *et al.*, 2000; Moriyama *et al.*, 1995; Tjin *et al.*, 2006). Minimal levels of HGFA protein were expressed in melanoma cells compared to primary human liver cells (THLE-2) (Supplementary Figure S4) suggesting circulating HGFA from plasma is the primary source of HGFA in the tumor microenvironment of melanomas. These observations indicate that disruption of tightly regulated HGF/SF-MET signaling occurs at multiple levels and is an important factor for melanoma progression. Interestingly, recent studies characterizing acquired resistance to BRAF targeted therapy have suggested that HGF/SF secreted from CAFs causes innate resistance (Straussman *et al.*, 2012; Wilson *et al.*, 2012). The plethora of data indicating HGF as a prominent mediator of cancer metastasis and therapy resistance suggest pro-HGF regulator SPINT2 may play an important role maintaining homeostasis within the tumor microenvironment.

Although a significant number of genes associated with cancer metastasis has been reported, causality of epigenetic mechanism in metastasis development; particularly, silencing potential metastasis suppressor gene by hypermethylation of CpG islands has not been well established (Li and Chen, 2011; Lujambio and Esteller, 2009). Recent study conducted by Aryee *et al.* suggests that DNA methylation alterations have the potential for producing a selectable driver event in cancer progression (Aryee *et al.*, 2013). This finding indicates there is a strong association of epigenetic silencing of critical genetic loci by DNA hypermethylation with metastatic phenotype development in cancer. Identification of SPINT2 as an epigenetically silenced potential metastasis suppressor as shown in our current study supports the notion of the causal effect of epigenetic alterations in melanoma development and progression. Loss of SPINT2 expression and its association with poor disease prognosis in various cancer types was also reported previously (Dong *et al.*, 2010; Kataoka *et al.*, 2000; Morris *et al.*, 2005; Nagakawa *et al.*, 2005; Nakamura *et al.*, 2011). In addition, SPINT2 is shown to be epigenetically silenced via DNA methylation of the promoter CpG islands (Bergum and List, 2010; Dong *et al.*, 2010; Nakamura *et al.*, 2011), suggesting suppressive function of SPINT2 in malignant cancer progression is not limited to melanoma and epigenetic alteration is a pervasive mechanism of tumor development. Further studies should be directed to elucidate whether this epigenetic trait can be used as a biomarker for malignant progression and also as a therapeutic and prophylactic target for melanoma treatment.

Materials and methods

Cells culture

Six melanoma cell lines (WM35, SBC12, WM1552C, WM852, WM983B and 1205Lu) were obtained from Dr. Meenard Herlyn (The Wistar Institute, Philadelphia, PA). A375, HeLa, and THLE-2 cells were obtained from ATCC. These cell lines were maintained in Dulbecco's modified eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10%

fetal bovine serum, L-Glutamine (2 mM) and 1% penicillin/streptomycin. Human primary melanocytes (HPMs) were purchased from Life technologies and maintained in Medium 254 with human melanocyte growth supplements (Grand Island, NY).

Patients and tissue samples

This study was approved by the Institutional Review Board of Boston University School of Medicine. Clinical pathologic diagnosis of the patient biopsies was described in previous study (Rossi *et al.*, 2014).

SPINT2 knockdown

WM1552C cells were treated with SPINT2 shRNA or scramble containing lentiviral particles (Invitrogen, Carlsbad, CA). Cells were selected with puromycin 48 hours after transduction to create stable cell lines. SPINT2 knockdown was determined by quantitative RT-PCR.

Quantitative RT-PCR

Quantitative RT-PCR analysis of *SPINT2* expression in clinical tissue specimens and SPINT2 knockdown cells was performed using Taqman real-time assay method as previously described (Rossi *et al.*, 2014). Gene expression analysis of melanoma cell lines was performed using SYBR Green qRT-PCR method. Detailed descriptions are shown in supplementary information.

Genome-wide DNA methylation profiling

DNA (1 ug) from melanoma cells and normal melanocytes was processed in the Microarray Core Facility at the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins according to Illumina's protocols. Illumina Infinium Human Methylation27 Bead Chip was used to simultaneously interrogate promoter DNA methylation at 14,495 RefSeq genes. After initial data extraction and normalization, comparative analysis was performed using Significant Analysis of Microarrays to identify differentially methylated genes between normal human HPMs and melanoma cells as described previously (Ryu *et al.*, 2007). Top 15 genes most highly methylated and unmethylated genes in each group of samples were visualized as described in previous studies (Ryu *et al.*, 2007; Ryu *et al.*, 2011). The genome-wide methylation profile dataset is accessible (GSE53516) in a data repository site (www.ncbi.nlm.nih.gov/geo).

Methylation-specific PCR and bisulfite sequencing analysis of the SPINT2 gene

Genomic DNA was isolated from cells using DNeasy column purification and processed according to manufacturer's protocol using EpiTect Bisulfite Kit (Qiagen, Valencia, CA). 32 CpG sites from SPINT2 promoter region was amplified from bisulfite-modified DNA. Bisulfite DNA conversion PCR conditions are as follows: 99°C for 5 minutes, 60°C for 25 minutes, 99°C for 5 minutes, 60°C for 85 minutes, 99°C for 5 minutes, 60°C for 175 minutes and 20°C for indefinitely. Methylation specific PCR and bisulfite sequencing primers and conditions were as previously described (Dong *et al.*, 2010).

Plasmids and stable cell line generation

SPINT2 gene inserted lentiviral vector was created with pENTRTM221-SPINT2 and pDEST-FG12-CMV via Gateway[®] technology (Invitrogen, Carlsbad, CA). Lentiviral particles were produced in HEK293T cells according to manufacturer's instructions (Invitrogen, Carlsbad, CA) and stored at -80°C after $0.22\ \mu\text{m}$ filtration. Cancer cells were transduced with lentiviral particles overnight. Stably transduced cells were sorted by GFP expression via FACS Aria III cell sorter (BD Biosciences, Franklin Lakes, NJ) at Boston University Medical Center Flow Cytometry Core Facility.

Immunoblotting

Immunoblotting was performed as described in previous study (Ryu *et al.*, 2007; Ryu *et al.*, 2011). SPINT2 and matriptase protein was detected by mouse monoclonal antibody (R&D Systems, Minneapolis, MN). Total and phospho-MET, Akt and ERK were detected using rabbit polyclonal antibodies (Cell Signaling, Boston, MA).

Cell cycle and proliferation analysis

Melanoma cells (5×10^5) were seeded in 6-well plates in 1% serum media. 24 hours after seeding cells were trypsinized and centrifuged ($1500 \times \text{rcf}$, 15 minutes at 4°C). Pellet was washed with cold PBS and centrifuged. Pellet was fixed with ice cold 70% ethanol overnight at 4°C . After additional PBS wash, cells were stained with PI solution ($50\ \mu\text{g/ml}$ PI, $100\ \mu\text{g/ml}$ RNase A in PBS) for 30 minutes in dark at room temperature. Cell cycle profile was obtained with FACScan System and analyzed via CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ) at Boston University Medical Center Flow Cytometry Core Facility. For cell proliferation analysis, PICO Green assay was performed at indicated time points after seeding 2×10^3 cells in 96-well plates (Life Technologies, Carlsbad, CA).

Cell migration and colony formation

Confluent monolayer was wounded and images were taken at indicated time points after wounding at $\times 100$ magnification (Nikon Eclipse TS100). WM983B monolayers were wounded and cell migration in serum free media was analyzed 18 hours post wounding. For 1205Lu cell line, cell migration in full growth media was quantified by calculating the difference between the wounded edges at each time point from time 0 hour and normalizing to control at 24 hours set at 100%. For soft agar colony formation assay 1.5×10^3 cells were seeded in 0.3% Noble agar (BD Biosciences, Franklin Lakes, NJ) overlaid on top of 0.6% agar layer. Cells were treated with $200\ \mu\text{l}$ of full growth media with or without $200\ \text{ng/ml}$ pro-HGF every 5 days. Colony number and size were analyzed with ImageJ from bright field images acquired from $\times 100$ magnification 20 days after seeding. Five independent image fields were taken per sample for analysis.

Statistical analysis

The values are presented as mean \pm Std Dev. The statistical analysis was conducted using the Student *t* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$). The number of independent replicates for each experiment was indicated in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

SPINT2	serine peptidase inhibitor Kunitz type 2
HGF/SF	hepatocyte growth factor/scatter factor
HGFA	hepatocyte growth factor activator
MET	met proto-oncogene
AKT	v-Akt murine thymoma viral oncogene

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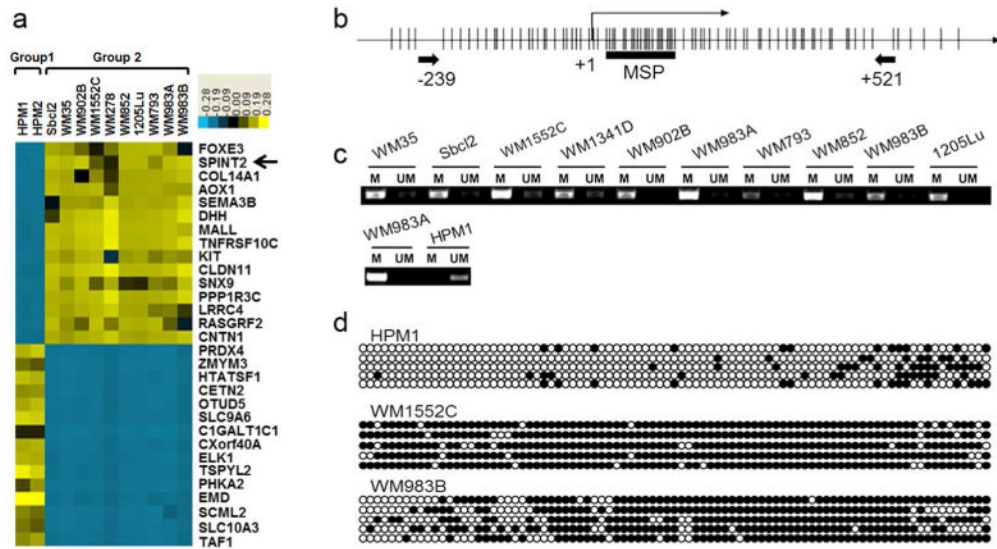


Figure 1. Identification of epigenetically silenced putative metastasis suppressor genes in melanoma

(a) Comparative analysis of global methylation profiles of melanoma cells and human primary melanocytes (HPMs). Heatmap shows top 30 differentially methylated genes between 10 melanoma cell lines and HPMs. (b) Schematic depiction of examined 85 CpG sites surrounding the transcription start site of *SPINT2* gene is shown. A region analyzed by methylation-specific PCR is indicated by black bar (MSP) below the CpG sites. Black arrows indicate location of bisulfite specific PCR primers. Image modified from (Dong *et al.*, 2010). (c) Methylation specific PCR analysis of *SPINT2* gene CpG islands in melanoma cell lines and HPMs. (d) Bisulfite sequencing analysis of *SPINT2* gene in melanoma cells and HPMs. Methylated and unmethylated CpG dinucleotides are shown by closed and open circles, respectively. Each line of circles represents analysis of a single cloned allele.

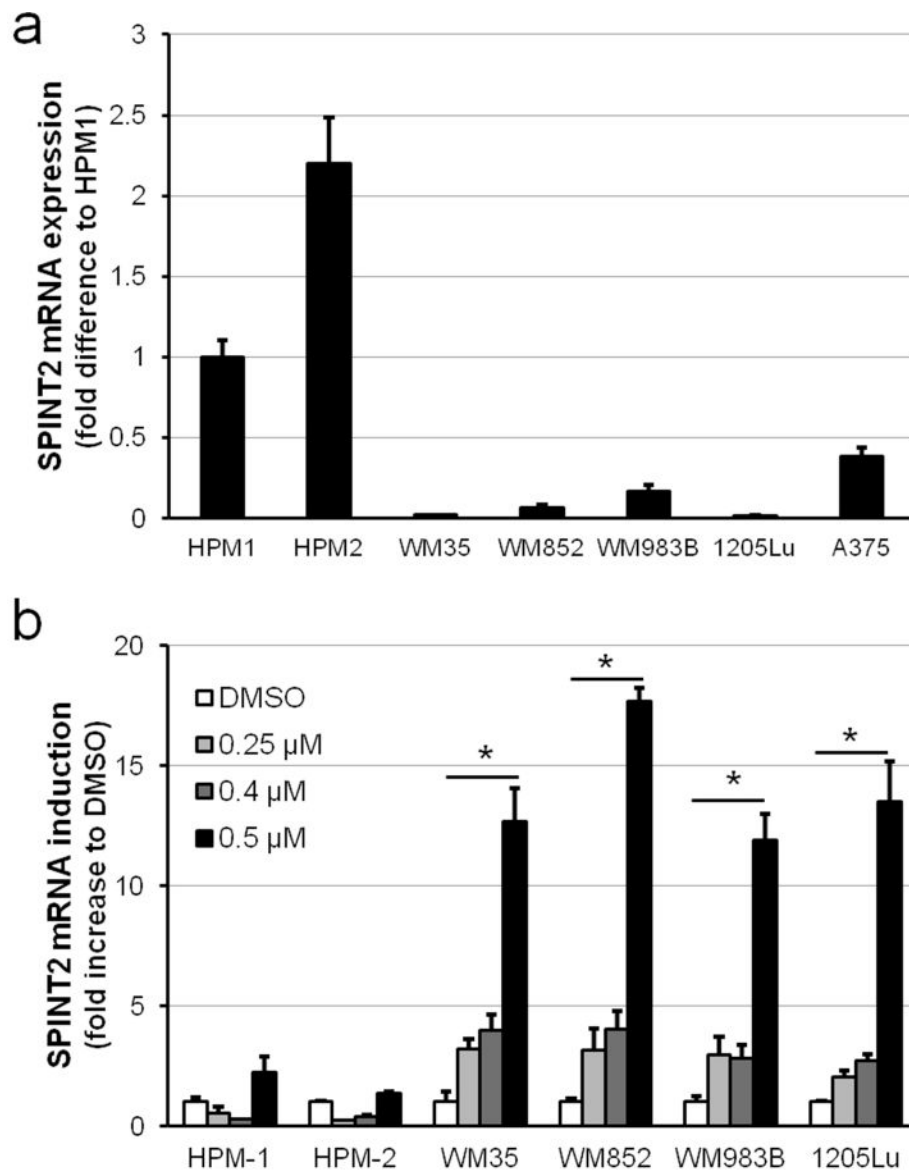


Figure 2. Decreased expression of SPINT2 gene in melanoma compared to melanocyte cells and transcriptional re-activation by a DNA hypomethylating agent (decitabine) treatment in melanoma cells

(a) Relative transcript levels of SPINT2 were examined in melanoma cell lines compared to normal melanocytes (HPM-1 passage # 2 and HPM-2 passage # 10). (b) Normal melanocytes (HPM-1 and HPM-2) and a panel of melanoma cell lines were treated with 0, 0.25, 0.4 and 0.5 μ M of 5-aza-dC (decitabine) for 72 hours. Isolated total RNAs from treated cells were used to examine SPINT2 transcript levels via RT-PCR. Asterisk denotes a statistically significant difference between DMSO and 5-aza-dC treated samples ($p < 0.05$).

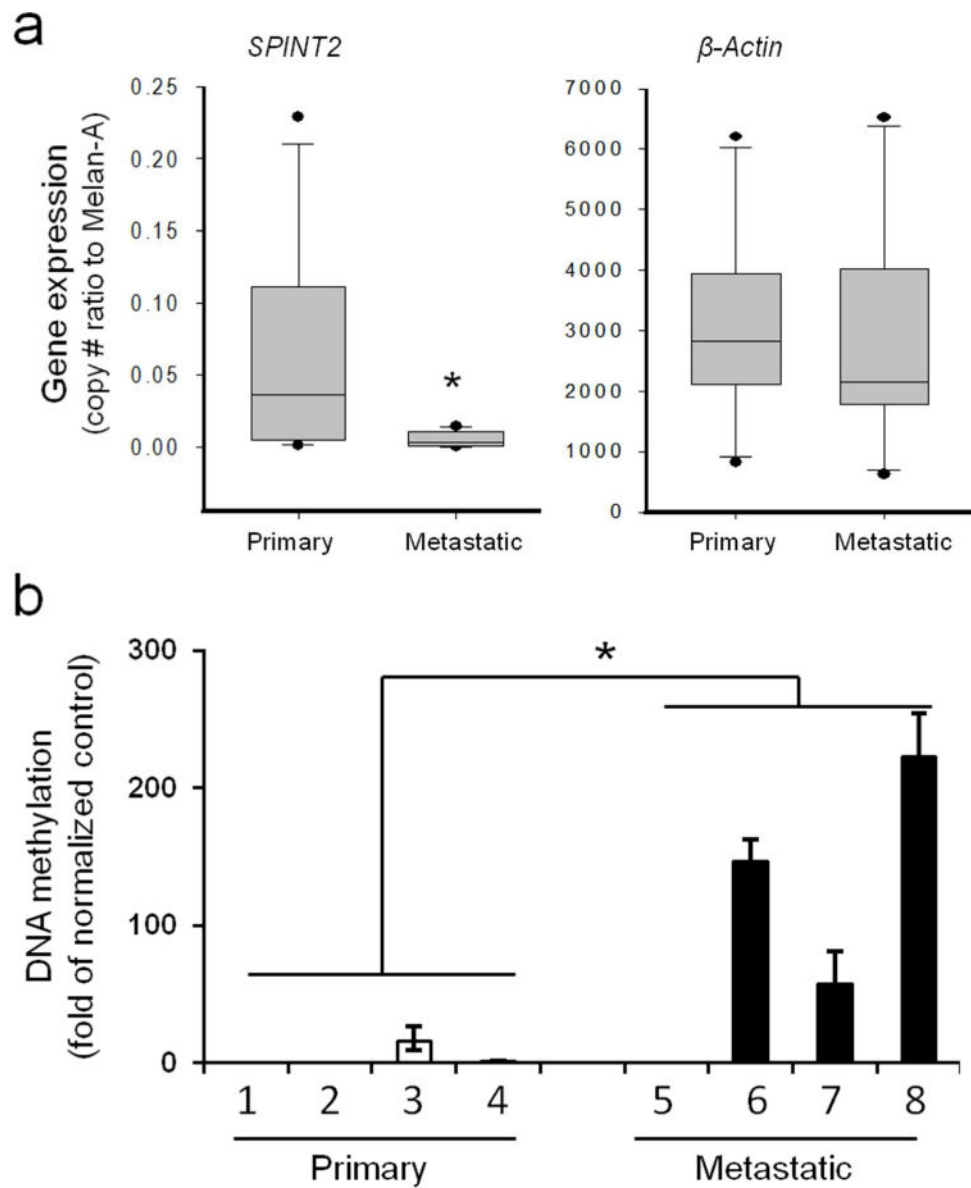


Figure 3. Transcriptional SPINT2 mRNA expression level in metastatic melanoma tissue is less than primary tumor

(a) Quantitative RT-PCR was performed for SPINT2 gene expression in early stage primary and metastatic melanoma tissue samples, β -actin serves as a negative control. Data are represented as box plot. Asterisk denotes a statistically significant difference between primary and metastatic tissue samples ($p < 0.015$). (b) Semi quantitative methylation specific PCR of SPINT2 gene in early stage primary and metastatic melanoma tissue samples. $n = 4$ for each primary and metastatic group. Fold change in methylation was normalized to primary melanoma tissue sample 4. Asterisk denotes a statistically significant difference between primary and metastatic tissue samples ($p < 0.05$).

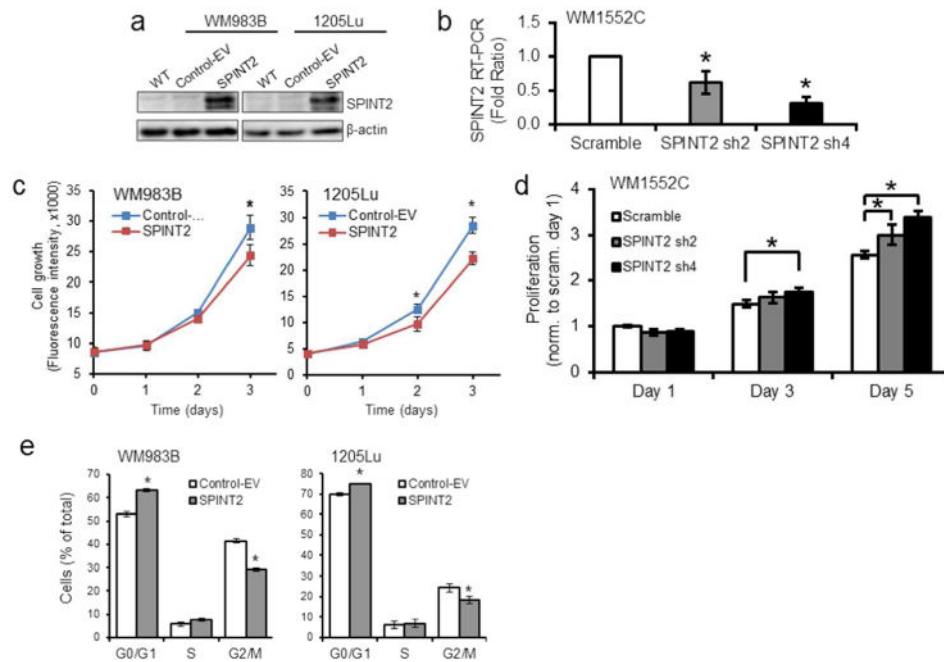


Figure 4. SPINT-2 over-expression in metastatic melanoma cell lines inhibits cell proliferation (a) Immunoblot image of SPINT2 from WM983B and 1205Lu total cell lysates. Wild type (WT), empty vector control (Control-EV) and SPINT2 over-expressing cells (SPINT2). β -actin is shown as loading control. (b) RT-PCR of SPINT2 mRNA from WM1552C treated with scramble or SPINT2 shRNA. Asterisk denotes a statistically significant difference between scramble control and SPINT2 shRNA samples ($p < 0.05$). (c) PICO green proliferation assay of SPINT2 over-expressing WM983B and 1205Lu cells 24, 48 and 72 hours after seeding. Data shown are mean \pm Std Dev from triplicate wells of two separate experiments. Asterisk denotes a statistically significant difference between empty vector control and SPINT2 over-expressing samples ($p < 0.05$). (d) PICO green proliferation assay of SPINT2 knockdown WM1552C cells 24, 72 and 120 hours after seeding. Asterisk denotes a statistically significant difference between scramble control and SPINT2 shRNA samples ($p < 0.05$). (e) Cell cycle analysis of SPINT2 over-expressing WM983B and 1205Lu cells. Data shown as percentage of total cell population at indicated cell cycle phase and from duplicate samples of three separate experiments. Asterisk denotes a statistically significant difference between empty vector control and SPINT2 over-expressing samples ($p < 0.05$).

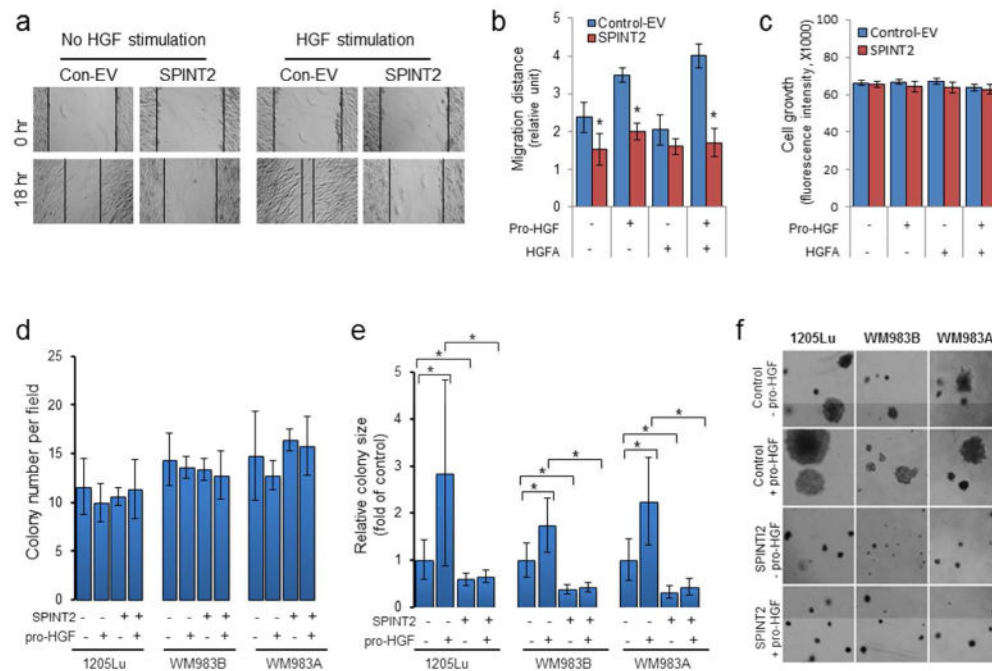


Figure 5. Stable SPINT2 expression in metastatic melanoma cells suppresses metastatic phenotypes of migration and invasive growth

(a) Representative images of control and SPINT2 over-expressing WM983B cell migration 18 hours after wounding (migrating edge denoted by black line) with or without pro-HGF (50ng/ml) treatment. (b) Quantification of SPINT2 over-expressing WM983B cell migration with or without pro-HGF. Data shown from three separate experiments. Asterisk denotes a statistically significant difference between empty vector control and SPINT2 over-expressing samples ($p < 0.05$). (c) PICO green proliferation assay of SPINT2 over-expressing WM983B cells 18 hours after seeding with or without pro-HGF (50ng/ml) treatment in serum free media. Data shown from triplicate experiment. (d) Anchorage independent colony formation assay of malignant melanoma cell lines (1205Lu, WM983B and WM983A). Cells are treated either with or without pro-HGF (100ng/ml). Data shown are colony number per field from a triplicate sample assay of two independent experiments. (e) Quantification of colony size related to respective empty vector controls of no pro-HGF treatment groups. Data shown are mean from a triplicate assay of two independent experiments. Asterisk denotes a statistically significant difference between empty vector control and SPINT2 over-expressing samples ($p < 0.05$). (f) Representative images of colonies in soft agar colony formation assay.

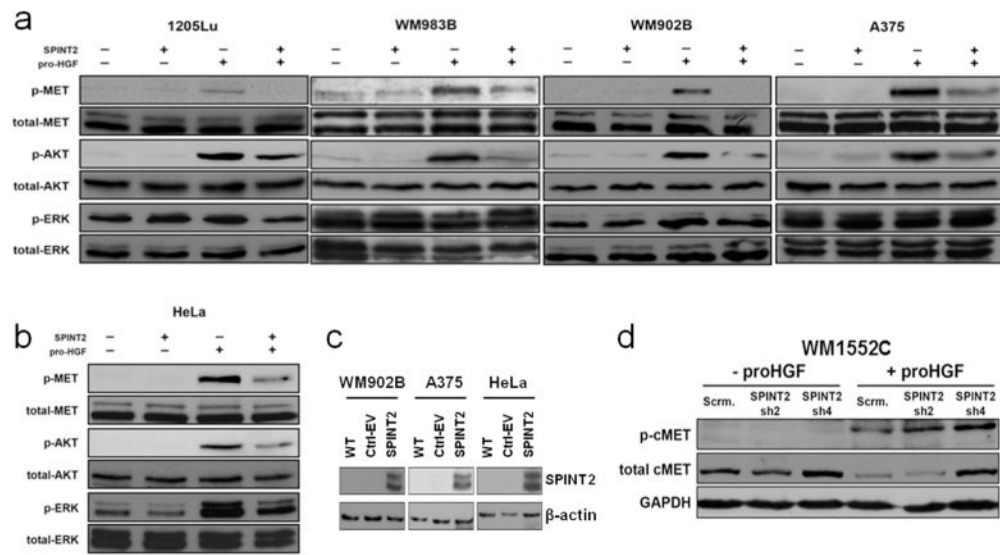


Figure 6. SPINT2 over-expression attenuates pro-HGF/SF induced MET and downstream target phosphorylation

(a) Immunoblot images of MET/AKT/ERK phosphorylation with or without pro-HGF (50ng/ml) in 1205Lu, WM983B, WM902B and A375 cells constitutively expressing SPINT2. (b) Immunoblot image of MET/AKT/ERK phosphorylation pro-HGF/SF treatment in BRAF wild type background HeLa cells. (c) Immunoblot of SPINT2 over-expression validating constitutive expression of SPINT2 in melanoma and HeLa cell lines. (d) Immunoblot image of MET phosphorylation in SPINT2 knockdown WM1552C cell line.