Up-regulation of Flotillin-2 Is Associated with Melanoma Progression and Modulates Expression of the Thrombin Receptor Protease Activated Receptor 1

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ABSTRACT

Flotillin 2 (flot-2) is a highly conserved protein isolated from caveolae/lipid raft domains that tether growth factor receptors linked to signal transduction pathways. Flot-2 protein and mRNA were increased in tumorigenic and metastatic melanoma cell lines in vitro, and the immunostaining intensity increased substantially across a tissue array of melanocytic lesions. Flot-2 transfection transformed SB2 melanoma cells from nontumorigenic, nonmetastatic to highly tumorigenic and metastatic in a nude mouse xenograft model. SB2 cells stably transfected with the flot-2 cDNA (SB2-flot-2) cells proliferated faster in the absence of serum, and their migration through Matrigel was additionally enhanced by thrombin. When SB2-flot-2 cells were compared with SB2-vector–control cells on a cancer gene pathway array, SB2-flot-2 cells had increased expression of protease activated receptor 1 (PAR-1) mRNA, a transmembrane, G-protein–coupled receptor involved in melanoma progression. PAR-1 and flot-2 were coimmunoprecipitated from SB2-flot-2 cells. Up-regulation of PAR-1 was additionally confirmed in SB2-flot-2 cells and melanoma cell lines. SB2-flot-2 cells transfected with flot-2–specific small-interfering RNAs made substantially less flot-2 and PAR-1 mRNA. In conclusion, flot-2 overexpression is associated with melanoma progression, with increased PAR-1 expression, and with transformation of SB2 melanoma cells to a highly metastatic line. Flot-2 binds to PAR-1, a known upstream mediator of major signal transduction pathways implicated in cell growth and metastasis, and may thereby influence tumor progression.

INTRODUCTION

Although skin cancer represents 50% of all human cancers, only 4% are melanomas accounting for 79% of all skin cancer-related deaths. Melanoma has a propensity for early invasion and metastatic spread leading to poor overall survival (1). Cutaneous melanomas arise from epidermal neural, crest-derived melanocytes. Recent studies support both genetic and epidemiologic factors causing melanoma, including specific mutations in cell signal pathway or DNA repair genes after exposure to UV light (2, 3). In contrast, factors leading to melanoma progression (vertical growth or invasion and metastatic spread) are less well understood and require additional elucidation. Loss of dependency on growth factors, increased ability to adhere and invade, and ability to form new blood vessels and vascular slits have been recognized as key properties in transformation and metastatic behavior (4–9). Understanding biology of melanoma progression may accelerate the development of more effective treatments for metastatic melanoma.

Caveolae and lipid rafts are microdomains of the inner plasma membrane that function to organize growth factor receptors and modulate downstream signal transduction pathways for cell growth and malignancy (10). Lipid rafts were recently implicated in signal transduction initiated by integrins and mediated by the Rho family of GTPases (11). Flotillins and caveolins are the two major protein families isolated from lipid raft/caveolae (12, 13). Caveolins are putative tumor suppressors that are down-regulated by oncogenic transformation (14). Flotillin-2 (flot-2) is a very highly conserved, M41,7000 protein first isolated by our laboratory as a cDNA from an epidermal keratinocyte library (15–17). Flotillin-1 and flot-2 proteins isolated from lung caveolae (12) are homologues of goldfish proteins Reggii 2 and 1 isolated from regenerating goldfish optical neurons (18–20). Flot-2 lacks a transmembrane domain and is associated with the inner membrane through myristoylation and palmitoylation (21).

Normal melanocytes require growth factors to proliferate, but transformed melanocytes display loss of serum and growth factor dependency (22). Constitutive activation of mitogen-activated protein kinase (MAPK) signaling, as represented by phosphorylation of extracellular signal-regulated kinase (ERK)1/2, and mutations in b-raf kinase are found in transformed melanoma cells (8, 22). For melanoma cells to enter vertical growth phase (tumorigenic phase in the dermis) and metastasize, cells must adhere to and degrade matrix and create new blood vessels (angiogenesis; ref. 23).

DNA microarrays profiling gene expression patterns have identified key molecules and pathways in melanoma (24). In comparing a highly metastatic A375SM melanoma line cloned from pulmonary metastases in mice to its parental line, A375P, A375SM expressed increased levels of Rho-C, fibronectin, and thymosin B (25). G-protein–coupled receptors in the MAPK pathway signal through Rho-GTP–ases that control actin cytoskeleton organization influencing cell shape and mobility (26, 27). In previous studies, we showed that overexpression of flot-2 will induce filopodia in Cos epithelial, and others have confirmed this observation (17, 21). Thus, the ability of flot-2 to alter cell shape is hypothesized to influence Rho GTP–ases, such as Rho-C coupled to a G-protein–coupled receptor.

The thrombin receptor, PAR-1, is a transmembrane G-protein–coupled receptor recognized to play a central role in melanoma progression (28–30). PAR-1 is necessary and rate limiting for formation of thrombin-enhanced pulmonary melanoma metastases (28, 31). Of interest, PAR-1 transforms 3T3 cells through Rho–G-protein signaling (32, 33) and also transforms SB2, a known nonmutomogenic, nonmetastatic melanoma cell line, which has been studied extensively. Transformation by PAR-1 is associated with increased surface integrin expression and phosphorylation of focal adhesion kinase (34). PAR-1 is negatively regulated by the transcription factor AP2 that decreases with melanoma progression and promotes angiogenesis through increased interleukin 8 and vascular endothelial growth factor protein levels (30, 35).

We present new evidence that overexpression of highly conserved flot-2 is associated with melanoma progression in cells and tissues, transforms SB2 cells, and is associated with expression of PAR-1.

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MATERIALS AND METHODS

Cell Lines and Culture. Human melanoma cell lines used are shown in Table 1; Fig. 1) and include SB2, A373P, A375SM, TXM13 (36), TXM18 (37), MeWo, WM 266-4 (35), WM793 (38), DX3 (36), Mel 501, and Mel 888 (5). Lines were previously characterized for tumorigenicity and metastases in the nude mouse model (36–38). Cells were grown in DMEM supplemented with 10% fetal bovine sera, sodium pyruvate, t-glutamine, nonessential amino acids, and penicillin-streptomycin.

Western Immunoblotting. Western blotting analysis was done as described previously (17). Cells were lysed in 20 mmol/L Tris (pH 7.5), 0.15 M NaCl, 1% Triton X-100, 60 mmol/L octyl-glucoside, and complete protease inhibitors cocktail (Roche Molecular Biochemicals, Indianapolis, IN). Soluble proteins were separated by centrifugation for 15 minutes at 14,000 rpm at 4°C. Ten micrograms of protein per lane were run on 10% SDS-PAGE, immunoblotted on nitrocellulose membranes, and blocked in 5% nonfat dry milk in PBS (pH 7.4) and 0.1% Tween 20. The membranes were incubated with flot-2 antibody (1:5000; BD PharMingen, San Diego, CA) in PBS (pH 7.4) and 0.1% Tween 20 for 2 hours at room temperature. The membranes were washed with PBS (pH 7.4) and 0.1% Tween 20, incubated with horseradish peroxidase-labeled goat antimouse IgG (1:5000; Jackson ImmunoResearch, West Grove, PA) for 1 hour at room temperature, washed again, and visualized with ECL-S Western blotting detection kit (Amersham, Arlington Heights, IL). Antibody to PAR-1 (WEDE 15, Beckman Coulter/Immunotech, Miami, FL) was used at 1:1000. Where applicable, membranes were stripped in 62.5 mmol/L Tris-HCl (pH 6.7), 2% SDS, and 100 mmol/L 2-mercaptoethanol for 30 minutes at 50°C and washed with PBS containing 0.1% Tween 20 before rehybridization. Equivalent loading was confirmed by immunostaining with actin antibody at 1:2000 (Santa Cruz Biotechnology, Santa Cruz, CA). The α-Ease Image analysis software was used to measure intensity of signals.

Quantitative Reverse Transcriptase-PCR mRNA Analysis. Total mRNA extracted from melanoma cells with Qiagen RNeasy kit (Qiagen Inc., Valencia, CA) was incubated with Dnase I in 4 mmol/L MgCl₂ at 37°C for 30 minutes with termination at 75°C for 10 minutes. Fluor-2 primers (F-CCCCA-GATTGGTCGCAAAA and R-TCCACTGAGGACCAAACTTCTCA) and fluorescein oligonucleotide probe (CGCTGCCCACTTACCAAGGTCG) were designed from the published sequence, accession no. M60922 (15) with Primer Express Software (Perkin-Elmer, Boston, MA). Primers and probes for measuring PAR-1, 36B4, and glyceraldehyde-3-phosphate dehydrogenase mRNA by quantitative reverse transcriptase-PCR were published previously (39, 40). PCR primers for flot-2 and PAR-1 were purchased from Sigma Genosys (Woodlands, TX). Fluorescent detection probes were purchased from Applied Biosystems (Foster City, CA).

RNA (1 μg) was reverse transcribed with MuMLV reverse transcriptase (Promega, Madison, WI) and random hexamers in a 20 μL total volume reaction mixture [1× PCR buffer (Perkin-Elmer), 4 mmol/L MgCl₂, and 500 μmol/L deoxynucleotide triphosphates] at 42°C for 15 minutes and terminated by incubation at 95°C for 5 minutes. PCR amplification was carried out in a total volume of 25 μL containing 1 μL reverse transcribed cDNA, 2.5 μL of 10× TaqMan buffer, 200 mmol/L of each forward and reverse primer, 100 mmol/L fluorescent probe, 3.5 mmol/L MgCl₂, and AmpliQag low 0.025 units/μL PCR conditions in the ABI 7000 PRISM Sequence Detector (Perkin-Elmer) were preheating for 95°C for 1 minute, followed by 50 cycles of melting (94°C for 15 seconds), and annealing and extension (60°C for 60 seconds). Glyceraldehyde-3-phosphate dehydrogenase or 36B4 mRNA was measured in the same reaction by quantitative reverse transcriptase-PCR for normalization standard.

Immunostaining of Melanocytic Lesion Tissue Array for Flot-2. A human melanocytic tissue array of paraffin-embedded clinical specimens was constructed as described previously (41). The array contained 182 sections from 96 different melanocytic lesions, including 19 benign nevi, 21 dysplastic nevi, 25 primary, radial growth phase melanomas, and 31 metastases (12 visceral and 19 nodal). Three slides were immunoreacted with antifl-2 antibody (1:50) and detected by immunohistochemistry with a tyramine catalyzed amplification system (DAKO, Carpinteria, CA) and azure B counter-stain for melanin. The numbers of melanocytes expressing flot-2 were graded as 0 (<5%), 1 (5 to 25%), 2 (25 to 75%), or 3 (>75 to 100%). Immunoreactivity intensity was graded with a semiquantitative scale: 0 = no staining; 1 = moderate staining; and 5 = most intense possible staining. Average staining intensities for each group are shown in Table 2A. Random sampling with replacement among the records within each tissue sample was repeated to generate 1,000 data sets, each with 96 observations. To suit the analysis purpose, two cutoff points equally spaced from 1 to 5 were used to divide the intensity readings into three groups based on cutoffs of low (1 to 2.33), medium (2.34 to 3.67), and high (3.68 to 5.0). Fisher’s exact test was used for comparing the readings among the patient groups on the 1,000 data sets (Table 2B). The median of 1,000 Ps for each comparison was recorded. To avoid the reporting bias from the multiple comparisons, the adjusted Ps with the step-down Bonferroni method of Holm were reported.

Overexpression of Flotillin-2 in SB2 Cells. Full-length flot-2 mouse cDNA was ligated into the mammalian expression vector p-EGFP/C2 (Clontech, Palo Alto, CA) driven by a cytomegalovirus (CMV) promoter (16, 17). SB2 cells (2×10⁶) were transfected with 1.5 μg of DNA in 10 μL LipofectAMINE reagent (Life Technologies, Inc., Gaithersburg, MD). Transfections were done in parallel with enhanced green fluorescent protein (EGFP)-flot-2, EGFP neovector, and no DNA in 1 mL of OPTIMEM (Life Technologies, Inc.) for 5 hours. Cells were selected for 2 weeks in media containing 800 μg/ml neomycin (G418). Several independent neoreistant colonies were isolated by tryp신ization and established by limiting dilution in culture. Expression of EGFP-flot-2-fusion protein was confirmed by Western blotting (17).

Xenograft Model. Male athymic BALB/c nude mice were purchased from the Animal Production Area of National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD) housed in American Association for Accreditation of Laboratory Animal Care approved facilities in laminar flow isolators under specific pathogen-free conditions. Animals were fed ad libitum at 8 weeks of age in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and NIH.

Tumorigenicity experiments were conducted with previously published methods (36, 42). Briefly, 1×10⁶ flot-2–EGFP-transfected SB2 cells (SB2-flot-2) or empty vector control SB2 cells (SB2-V) were injected subcutaneously to each of five BALB/c nude mice for each group, and the size of the subcutaneous tumor nodule was monitored weekly until day 60.

Experimental Lung Metastasis in Nude Mice. Cells (1×10⁶) from each line were injected into the lateral vein of groups of five nude mice as described previously (36). Mice were sacrificed after 60 days. Lungs were removed, washed in water, and fixed with Bouin’s solution for 24 hours to facilitate counting of tumor nodules by dissecting microscope. Lung sections were also stained with H&E to confirm that the nodules were melanoma and to monitor the presence of micrometastases.

Microvessel Density Assay. Immunohistochemistry for microvessel density was done as described previously (42) on 8- to 10-μm sections of subcutaneous tumors embedded in OCT compound on positively charged Superfrost slides (Fisher Scientific, Houston, TX). Slides were fixed in −20°C acetone for 10 minutes, washed in PBS (pH 7.5), and blocked with PBS supplemented with 1% normal goat serum and 5% normal horse serum for 20 minutes at room temperature. Incubation with rat antimouse monoclonal CD31/platelet/endothelial cell adhesion molecule 1 antibody (PharMingen; 1:800) was overnight at 4°C in protein blocking solution. Slides were washed with PBS and incubated for 10 minutes in protein blocking solution before the

Table 1 Melanoma cell lines of variable tumorigenicity

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Derivation</th>
<th>Tumor/mets in nude mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>SB2</td>
<td>Lymph node metastasis</td>
<td>Very low (36)</td>
</tr>
<tr>
<td>A373P</td>
<td>Lymph node metastasis</td>
<td>Low (36)</td>
</tr>
<tr>
<td>A375SM</td>
<td>A375P pulmonary mets in mice</td>
<td>High (36)</td>
</tr>
<tr>
<td>TXM13</td>
<td>Brain metastasis</td>
<td>High (36)</td>
</tr>
<tr>
<td>TXM18</td>
<td>Brain metastasis</td>
<td>Low intermediate (37)</td>
</tr>
<tr>
<td>MeWo</td>
<td>Lymph node metastasis</td>
<td>Low intermediate (35)</td>
</tr>
<tr>
<td>WM266-4</td>
<td>Thigh skin</td>
<td>High (35)</td>
</tr>
<tr>
<td>WM793</td>
<td>Sternum</td>
<td>VGP (38)</td>
</tr>
<tr>
<td>DX3</td>
<td>Cutaneous melanoma</td>
<td>Low (36)</td>
</tr>
<tr>
<td>Mel 501</td>
<td>Metastasis</td>
<td>Low (5)</td>
</tr>
<tr>
<td>Mel 888</td>
<td>Dissociated metastatic tumor cells</td>
<td>Low (5)</td>
</tr>
</tbody>
</table>

Abbreviation: VGP, vertical growth phase.
addition of 1.5,000 horseradish peroxidase-conjugated goat antirat mouse F(ab')2 (Jackson Research Laboratories, West Grove, PA). After incubation for 1 hour at room temperature, slides were washed and incubated with 3,3-diaminobenzidine (Vector Laboratories, Burlingame, CA). The sections were washed three times with distilled water and counterstained with Gill’s hematoxylin (Sigma, St. Louis, MO). The slides were mounted with Universal Mount (Research Genetics, Huntsville, AL) and examined in a bright-field microscope. Controls included omission of primary antibody, incubation with normal mouse serum, or incubation with normal mouse IgG. Images of ten 0.159 mm² fields at 100× magnification were digitized and stored for additional analysis. The number of blood vessels was counted with Scion program (Scion Corp., Frederick, MD), and microvessel density was expressed as the mean number of blood vessels/100× field.

Matrigel Tube Formation Assay. SB2, SB2-flot-2, and SB2-V cells were plated at 2 × 10⁵/well in triplicate on 24-well plates coated with 200 μL Matrigel (Becton Dickinson, San Diego, CA) as described previously (43). Cells were examined 24 hours later for tube formation under light microscopy (100×). Images were captured with a digital camera and analyzed using Optimas imaging software (Silver Springs, MD). Tube formation was quantified by manual counting of triplicate wells on low-power fields at 40×.

Cell Proliferation Assay. Cells were plated at 2 × 10⁵/well in triplicate on 24-well plates for 48 hours in media with or without serum. The cells were trypsinized and counted by trypan blue exclusion method.

Matrigel Invasion Assay. The in vitro ability to invade Matrigel coated inserts was compared between SB2-flot-2 and SB2-V cells as described previously (44). Briefly, 8-μm pore transwell inserts (Costar, Cambridge, MA) coated with Matrigel in cold serum-free medium were seeded with 2 × 10⁵ cells per well and incubated in 5% CO₂ at 37°C for 2 hours. Cells on the upper surface of the filter were removed by wiping with a cotton swab, and cells that had migrated to the lower surface of the membrane were stained with Hema-3 stain kit (Biochemical Science Inc., Swedesboro, NJ). Cells in five predetermined fields were counted under microscopy. Data were expressed as the average number of cells migrated through the filters.

Differential Gene Expression by SB2-Flot-2 Transfected Cells with DNA Microarray. Total RNA was extracted from SB2-flot-2 cells and from EGFP-neovector control cells with RNA easy kit (Qiagen). RNA was converted to cDNA and labeled with Cy3 green (SB2-flot-2) or Cy5 red (SB2-vector). Labeled cDNAs (100 μg) were hybridized with a DNA cancer pathway array on poly-l-lysine-coated slides composed of 75 bp oligomers representing 1,100 unique genes (45). Signals were detected with a laser
scanning and quantified with Array Vision (Imaging Research, Inc., St. Catherine’s, Ontario, Canada).

Immunoprecipitation. Confluent SB2-float-2 cells in a 60-mm dish culture were solubilized for 1 hour on ice with 500 µL of TBS (10 mmol/L Tris (pH 8.0) and sodium glycolate, complete protease inhibitors cocktail (Roche Molecular Biochemicals), 1 mmol/L sodium vanadate, 1 mmol/L EDTA, and 1 mmol/L EGTA. Cell lysates were centrifuged for 15 min at 15,000 g at 4°C. Supernatants (800 µg protein) were precleared with Protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and then incubated with 3 µg PAR-1 (WEDE 15) primary antibody (1:1,000) at 4°C overnight. The immunocomplex was captured by incubation with Protein A/G PLUS-Agarose for 1 hour to overnight at 4°C. The immunoprecipitate was pelleted by centrifugation at 2,500 rpm for 5 min at 4°C and washed four times in ice-cold lysis buffer. The pellet was resuspended in 40 µL of SDS sample buffer, boiled for 3 min, and analyzed by Western blotting with float-2 antibody (1:1,000).

Small-Interfering mRNA Treatments. A cocktail of four small-interfering RNAs designed for float-2 (SMARTpool) and nontumor control small-interfering RNAs were purchased from Dharmacon (Lafayette, CO) and used according to the manufacturer’s protocols. One × 10⁶ SB2-float-2 cells were plated in triplicate on 12-well plates in antibiotic-free DMEM (10% fetal bovine serum, 1% sodium pyruvate, 1% essential amino acids, 1% l-glutamine, and 1% HEPES buffer). One hundred microliters Opti-MEM containing 100 µmol float-2 small-interfering RNA SMARTpool from Dharmacon, Lafayette, CO, and 100 µL Opti-MEM containing 2 µL LipofectAMINE were mixed and allowed to complex for 25 min at room temperature. Cells were rinsed in Opti-MEM, and 200 µL of small-interfering RNA-LipofectAMINE complexes were added for 6 h at 37°C before being replaced with antibiotic-free DMEM for another 48 h. RNA was isolated from each well with RNeasy Mini Kit (Qiagen). Amount of mRNA after treatment with float-2 or control small-interfering RNAs was analyzed by quantitative reverse transcriptase-PCR for float-2 mRNA (A) or PAR-1 mRNA (B) with an ABI 7000 PRISM Sequence Detection System (Applied Biosystems). Values were normalized to the 36B4 standard. Data were expressed as mean of triplicate wells treated with small-interfering RNA-flot2 versus control small-interfering RNA-treated cells as percent. Student’s t test was used to compare the data for each condition.

RESULTS

Float-2 Protein Expression Is Low in Nontumorigenic and High in Metastatic Melanoma Cell Lines in Vitro. Float-2 is a ubiquitously expressed, highly conserved protein previously shown in many cell lines (15, 16, 28). In surveying benign and malignant melanoma cell lines by Western blotting (Fig. 1), low float-2 protein level was found in a previously well-characterized, nontumorigenic and nonmetastatic cell line, SB2 (36). Float-2 and PAR-1 (thrombin receptor) protein levels normalized to actin were measured from eleven cultured melanoma cell lines selected to represent a range of tumorigenicity and metastatic behavior (Table 1: Fig. 1, A–C). All nonaggressive cell lines (SB2, DX3, Mel-501, and Mel-888) had lower levels of both float-2 and PAR-1 than other cell lines that are known to form subcutaneous tumors and metastases in nude mice (TXM13, TXM 18, and WM66-4). Of interest, the most aggressive line, A375SM, derived from pulmonary metastases of parental A375P, exhibited higher levels of both float-2 protein and mRNA than did the parental A375P.

To confirm the protein data, we measured float-2 mRNA levels by a quantitative assay (Fig. 1D). Float-2 mRNA was more abundant in the most aggressive tumor cell lines (A375SM, TXM13, and WM66-4) compared with poorly tumorigenic lines (SB2, A375P, MeWo, DX3, Mel 501, and Mel 888). These data first suggested transcriptional regulation of float-2 expression in melanoma cell lines as well as possible correlation between biological behavior and float-2 expression. One exception was the WM793 line, originally derived from a vertical growth phase melanoma and known to exhibit high tumorigenicity with low metastatic behavior. Although WM793 had high PAR-1 protein and float-2 mRNA levels, its expression of float-2 protein was low.

Overexpression of Flotillin-2 Is Significantly Associated with Progression of Human Melanoma in Vivo. To verify the in vitro observation in cells, we next stained for float-2 on a melanocytic lesion tissue array (41). The clinical lesions were divided into four major
groups (all nevi, primary melanoma, invasive melanoma or nodal metastases, and visceral metastases). Representative samples are shown in Fig. 2, A–D. The average staining intensities for each group are shown in Table 2A. Cytoplasmic staining for flot-2 in epidermal and follicular keratinocytes was less intense than in nests of melanocytes, dendritic cells, and endothelium. The intensity of flot-2 did not distinguish between benign (Fig. 2A) or dysplastic nevi (Fig. 2B). In nevi, flot-2 expression was often more intense in epidermal melanocyte nests than in dermal melanocytes. In contrast, very strong homogenous expression was observed in almost all of the melanoma cells from invasive melanomas and metastases (Fig. 2, C and D). By four-way comparisons of staining intensity, there was a highly significant difference between overall comparison of lesion groups (P < 0.0001; adjusted; Table 2B). Metastatic melanomas from nodal or visceral sites were significantly higher in flot-2 staining than all nevi and primary melanomas.

**Overexpression of Mouse Flotillin-2 cDNA in Nontumorigenic SB2 Cells Induces Tumorigenicity and Metastases.** To examine the hypothesis that flot-2 overexpression leads to melanoma progression, we selected the SB2 line with low flot-2 protein levels and known poor tumorigenicity in the nude mouse xenograft model for forced overexpression (36). A construct containing mouse flot-2 cDNA in a CMV-EGFP neovector (17) and empty EGFP-neovector plasmid DNA were transfected into SB2 cells. Two independently derived stable SB2-flot-2 clones and one vector control line (SB2-V) were selected for additional characterization of in vivo tumor and metastasis formation. SB2-flot-2 lines in culture had normal morphology and expressed 2- to 3-fold more green fluorescent flot-2 fusion protein of M̀ r 69,000 than endogenous flot-2 of M̀ r 41,700 (Fig. 3A, Lanes 2 and 3). Overexpression of recombinant protein in SB2 cells (Fig. 1A, lane 2; Fig. 3A) did not alter the expression of native flot-2 protein compared with the vector control (Fig. 3A, Lane 1).

The time course of tumors formed by subcutaneous injections of 1 × 10⁶ SB2-vector or flot-2–transfected cells are plotted in Fig. 3B. Only 1 of 5 mice injected with SB2-vector cells formed a small palpable tumor by day 36 in contrast to injections of each SB2-flot-2 clone that formed rapidly growing tumors by day 20 in 3 of 5 mice. The mean tumor volume over the time course showed higher tumor volumes from two SB2-flot-2 lines compared with the SB2-vector control. There was no significant difference in the tumor volumes between the two SB2-flot-2 clones at day 24 and day 36 (P = 0.52 and P = 0.09, respectively).

To determine whether flot-2 transfection also induces metastasis formation, SB2-flot-2 and SB2-V cells were injected intravenously into mice (Table 3). Although 3 of 5 mice injected with neovector-control transfected SB2 cells developed metastases in the lungs, the numbers were very low (mean = 1.5 lung metastasis per animal). In contrast, all five of the animals injected with each SB2-flot-2–transfected cell line formed >100 lung metastases by day 60. Differences between SB2-flot-2 and SB2-vector control lines were highly significant (F = 109.61; P < 0.01; ANOVA). In addition, large intracardiac metastases arose in mice injected with SB2-flot-2 cells but not with SB2-V controls (data not shown).

**Overexpression of Flot-2 in SB2 Cells Is Associated with Increased Tumor Microvessel Density and with Increased Cellular Proliferation, Vasculogenic Mimicry.** We observed that flot-2 staining was prominent on the vascular endothelium of melanomas (Fig. 2), and thus flot-2 may be associated with angiogenesis. Frozen sections of subcutaneous tumors from mice were stained for CD31 and analyzed as described previously (42). SB2-flot-2–derived tumors had significantly increased microvessel density compared with SB2-V tumors (P ≤ 0.01; Fig. 4, A–C). We also investigated vasculogenic mimicry defined as the ability of cancer cells to spontaneously form vascular slits previously associated with aggressive ocular melanomas (46). To test vasculogenic mimicry tube formation, cells were plated on Matrigel-coated membranes and observed under light microscopy. Both SB2-flot-2 clones formed significantly more elongated three-dimensional tubular structures than the parental line or vector control cells (P ≤ 0.001; Fig. 4, D and E).

**SB2-Flot–2 Cell Proliferation Is Higher in Serum-free Media.** To determine whether flot-2 overexpression promotes cell proliferation, we compared one SB2-flot-2 line to the control cells. Although proliferation was similar in the presence of 10% fetal bovine serum, in the absence of serum, SB2-flot-2 cells proliferated significantly faster than control SB2 cells (P = 0.001; Fig. 4F). Although metastatic melanomas acquire invasive properties mediated through induction of metalloproteases-2, no difference in metalloproteases-2 was seen in SB2-flot-2 cells by zymography (data not shown).

**Overexpression of Flot-2 in SB2 Cells Is Associated with Increased Expression of the Thrombin Receptor, PAR-1.** To study whether flot-2 transformation of SB2 cells induced the expression of any new genes, we compared the differential mRNA expression to that of SB2-V cells with an oligonucleotide DNA cancer pathway microarray (45). PAR-1, the thrombin receptor, was the only gene
nude mice were stained with antibody to CD31/platelet/endothelial cell adhesion molecule (PECAM-1) to perform immunohistochemistry staining of tumors for CD31. Frozen sections of day 60 s.c. tumors formed in vivo were measured with Quantimet 570 (Leica, Bannockburn, IL). Microvessel density was determined by dividing the number of vessels per 100× field by the number of field. As shown in Fig. 1C, PAR-1 levels were higher in SB2-flot-2-transfected cells and aggressive melanoma lines, with the exception of WM793, compared with low tumorigenic lines.

Small-interfering RNAs for Flot-2 Decrease Expression of Both Flotillin-2 and PAR-1. To additionally study the relationship between flot-2 and PAR-1 expression, we used a cocktail of four small-interfering RNAs against flot-2 compared with control small-interfering RNAs to transiently transfect SB2-flot-2 cells in triplicate. After 48 hours, mRNAs were extracted and flot-2 and PAR-1 mRNA signals were measured by quantitative reverse transcriptase-PCR. Flot-2 small-interfering RNA treatment was associated not only with significantly decreased expression of flot-2 mRNA (P = 0.044) but also of PAR-1 mRNA levels (P = 0.022; Fig. 5, D and E).

Flot-2 Is Coimmunoprecipitated with PAR-1 in SB2-Flot-2 Cells. Immunoprecipitation of SB2-flot-2 cell extracts with antibodies to flot-2 (data not shown) and to PAR-1 coprecipitated both proteins, as detected by Western blotting (Fig. 5F). A physical association between flot-2 and PAR-1 proteins is therefore likely within SB2-flot-2 cells.

Matrigel Invasion Is Higher in SB2-Flot-2 Cells and Additionally Enhanced by Thrombin Activation. Because PAR-1 activation can enhance cell migration, and this property is important for tumor cell metastasis, we next studied SB2-flot-2 cells versus control cells plated on Matrigel-coated filters. The SB2-flot-2 cells had significantly higher migration detected at 2 hours, compared with the control SB2 cells (Fig. 5G; P = 0.007), and was significantly enhanced by pretreatment with 1 unit/ml thrombin for one hour before plating (P = 0.002). There was no significant difference in migration of SB2 cells after treatment with thrombin.

DISCUSSION

We demonstrate for the first time that overexpression of flotillin-2, a novel and highly conserved caveolae/lipid raft-associated protein, alters the phenotype of SB2 melanoma cells and is associated with up-regulation of the G-protein-coupled receptor for thrombin, PAR-1. Although flot-2 is also expressed in normal melanocytes (15), these data suggest that the levels of flot-2 increase with melanoma progression in cell lines and in melanocytic lesions. The A375 lines showed increased flot-2 expression in the more metastatic derivative. To test the hypothesis that overexpression of flot-2 facilitates melanoma initial growth, we transfected SB2 cells with flotillin-2 initially found to be up-regulated by >2-fold in flot-2-transfected SB2 cells (data not shown). Western blotting (Fig. 5, A and B) and quantitative reverse transcriptase-PCR (Fig. 5C) measurements also confirmed increase in PAR-1 protein and mRNA expression in SB2-flot-2 cells compared with SB2-V control cells. PAR-1 protein expression was at least 2-fold higher in SB2-flot-2 lines than parental or vector-control cells, whereas PAR-1 mRNA levels were increased by 3-fold. As shown in Fig. 1C, PAR-1 levels were higher in SB2-flot-2-transfected cells and aggressive melanoma lines, with the exception of WM793, compared with low tumorigenic lines.

2-EGFP cells (100× magnification). C, Graph of average number of vessels per 100× tumor calculated from digitized images and Scion program. SB2-flot-2 cell tumors had significantly more microvessel density than tumors from SB2-vector control cells (Student’s t test, P < 0.01). D and E, SB2-flot-2 cells plated on Matrigel show increased tube formation. D, Tube formation by living parental SB2 cells, vector transfected controls, versus two clones of flot-2 transfected SB2 cells was observed at 24 hours by light microscopy (100× magnification). Cells were plated in triplicate at 20,000 cells per well on Matrigel-coated wells. Images were captured by Optimas TEC-470 camera and digitized with Optimas imaging software (Silver Spring, MD). E, Number of tubes were counted in triplicate wells at low-power fields (magnification 40×) and expressed as percentage cell elongation (% of) resulting in tube formation. SB2-flot-2 clones had significantly higher tube formation than either SB2 parental or SB2 vector (Student’s t test, P < 0.001). F, Cell proliferation in serum-free media is higher in SB2-flot-2 cells. SB2-vector versus SB2-flot-2 cells were plated in triplicate at 2 × 10³ cells on 24-well plates and grown in media with or without serum for 48 hours. Cells were trypsinized and counted by trypan blue exclusion. In serum-free media, proliferation of SB2-flot-2 cells was higher than controls (data are express as triplicates; bars, ±SD, P = 0.001).

Fig. 4. SB2-flot-2 cells make tumors with increased microvessel density have increased tube formation in vitro and proliferate in sera-free media. A and B, immunohistochemistry staining of tumors for CD31. Frozen sections of day 60 s.c. tumors formed in nude mice were stained with antibody to CD31/platelet/endothelial cell adhesion molecule 1 (1:800) A, tumors formed by SB2-vector control cells. B, tumors formed by SB2-flot-2 EGFP cells (100× magnification). C, Graph of average number of vessels per 100× tumor calculated from digitized images and Scion program. SB2-flot-2 cell tumors had significantly more microvessel density than tumors from SB2-vector control cells (Student’s t test, P < 0.01). D and E, SB2-flot-2 cells plated on Matrigel show increased tube formation. D, Tube formation by living parental SB2 cells, vector transfected controls, versus two clones of flot-2 transfected SB2 cells was observed at 24 hours by light microscopy (100× magnification). Cells were plated in triplicate at 20,000 cells per well on Matrigel-coated wells. Images were captured by Optimas TEC-470 camera and digitized with Optimas imaging software (Silver Spring, MD). E, Number of tubes were counted in triplicate wells at low-power fields (magnification 40×) and expressed as percentage cell elongation (% of) resulting in tube formation. SB2-flot-2 clones had significantly higher tube formation than either SB2 parental or SB2 vector (Student’s t test, P < 0.001). F, Cell proliferation in serum-free media is higher in SB2-flot-2 cells. SB2-vector versus SB2-flot-2 cells were plated in triplicate at 2 × 10³ cells on 24-well plates and grown in media with or without serum for 48 hours. Cells were trypsinized and counted by trypan blue exclusion. In serum-free media, proliferation of SB2-flot-2 cells was higher than controls (data are express as triplicates; bars, ±SD, P = 0.001).
tumors and metastases, we established and characterized two independently derived SB2-flot-2 cell lines in the mouse model and showed significantly increased tumorigenicity and metastases.

We do not attribute the findings to clonal differences among SB2 cells, as the SB2 line has been well characterized as a very low tumorigenic line (34), 37). On the basis of our data, it may not be a coincidence that overexpression of PAR-1 also transforms SB2 cells so that activation of the PAR-1 receptor by thrombin appears to be synergistic with overexpression of flot-2 in SB2-flot-2 cells so that activation of the PAR-1 receptor by thrombin appears to be synergistic with overexpression of flot-2 in SB2 melanoma cells (34). PAR-1 has not previously been linked to flot-2. Thrombin treatment enhanced invasion of SB2-flot-2 cells and additionally enhanced by thrombin activation. SB2 parental and SB2-flot-2 cells grown in serum-free medium for 24 hours were left untreated or were activated by 1 unit/ml thrombin (Sigma) for 1 hour. Cells were trypsinized, resuspended in serum-free medium, and plated at 2 \times 10^6 cells on triplicate Matrigel-coated inserts (44). After 2 hours, cells from five separate fields on lower surface of the membrane were counted and plotted as the average number of cells migrating through filters. SB2-flot-2 cells showed significantly increased tumorigenicity and metastases. SB2-flot-2 cells grown in serum-free medium for 24 hours were left untreated or were activated by 1 unit/ml thrombin (Sigma) for 1 hour. Cells were trypsinized, resuspended in serum-free medium, and plated at 2 \times 10^6 cells on triplicate Matrigel-coated inserts (44). After 2 hours, cells from five separate fields on lower surface of the membrane were counted and plotted as the average number of cells migrating through filters. SB2-flot-2 cells showed significantly increased tumorigenicity and metastases.
The ability to transform SB2 cells has been shown to involve the cell trafficking pathways that are not fully elucidated (48). Because PAR-1, COOH terminus (ref. 48; Fig. 6). PAR-1 can also be recycled through phosphorylation or modification of key sequences at the cytoplasmic terminus. The cytoplasmic COOH domain contains sequences implicated in degradation and in transformation. Both flot-2 and Ras are attached to the membrane through modifications. PAR-1 can signal through Rho-Ras family of GTPases, activating the mitogen-activated protein kinase pathway. The intermediate b-Raf kinase is commonly mutated in melanomas. PAR-1 can also signal through other pathways including tyrosine kinases (focal adhesion kinase) and IP3.

Thrombin has a multitude of actions, many of which relate to its ability to activate the PAR-1 (31). If flot-2 transformation is mediated through PAR-1 interaction, this would be a key discovery in understanding PAR-1-mediated signal transduction. PAR-1 interacts with several signal transduction pathways including MAPK with Rho-GTPase intermediates (8, 31, 47, 48). Constitutive activation of MAPK through mutations in the kinase b-raf and phosphorylation of ERK1/2 was previously implicated in melanoma progression (2, 8).

When thrombin cleaves the NH2-terminal extra-cellular tail of PAR-1, a tethered ligand activates the receptor, distinguishing it from growth factor receptors activated by extracellular ligands (31). PAR-1 must then be inactivated, and this is thought to occur through phosphorylation or modification of key sequences at the cytoplasmic COOH terminus (ref. 48; Fig. 6). PAR-1 can also be recycled through cell trafficking pathways that are not fully elucidated (48). Because the ability to transform SB2 cells has been shown to involve the cytoplasmic carboxyl tail of PAR-1 (34), it is possible that flot-2 binds to this region of the receptor with functional consequences. Unlike PAR-1, flot-2 contains no transmembrane domain but interacts with the lipid membrane through myristoylation and palmitoylation (21). Flot-2, attached to the inner membrane in lipid domains or caveolae, should be in a position to interact with the cytoplasmic COOH-terminal domain of PAR-1 that is critical for SB2-PAR-1-induced transformation. Flot-2 binding might stabilize PAR-1 by blocking inactivation or degradation of an activated PAR-1 receptor. Cytoplasmic flot-2 might also modulate PAR-1 trafficking within the cell or stabilize PAR-1 mRNA. Any of these activities by flot-2 might promote PAR-1-induced constitutive signaling through MAPK or other signal transduction pathways.

PAR-1 induces phosphorylation of focal adhesion kinase and integrin expression in SB2 cells (34). Focal adhesion kinase is a tyrosine kinase that also modulates cytoskeletal reorganization. Previous studies have shown that flot-2 and its homologue Reggie 1 alter cell morphology and induce filopodia formation after transfection (17, 21). Changes in cell shape result from changes in actin stress fibers and focal adhesions and are mediated through the Rho family of GTPases (27). Rho-Ras proteins are found downstream to PAR-1 as intermediates of the ERK1/2 MAPK pathway (26, 49) implicated in melanoma progression (8, 50).

The transforming and angiogenic properties of PAR-1 are thought to be mediated through MAPK signaling (32, 33, 47). The transcription factor AP2 decreases with melanoma progression and negatively regulates PAR-1, increasing interleukin-8 and vascular endothelial growth factor levels (30, 35). SB2-flot-2 melanoma cells like SB2-PAR-1 transfected cells displayed enhanced angiogenic properties, including increased microvessel density and vasculogenic mimicry, that have been associated with aggressive melanomas and expression of VE-cadherin (46). SB2-flot-2 cells were more invasive than control SB2 cells, and their ability to invade Matrigel membranes was enhanced by thrombin. This additionally supports a functional cooperation between PAR-1 and flot-2 overexpression in melanoma that may relate to increased angiogenic properties of the transformed cells (Fig. 6).

In conclusion, these data suggest the existence of an important and novel relationship between the highly conserved, lipid raft/caveolae associated protein flot-2 and the G-protein coupled receptor, thrombin receptor, PAR-1. PAR-1 is upstream to the b-raf- MAPK-ERK pathway implicated in melanoma progression (8). Why overexpression of either flot-2 or PAR-1 can transform SB2 cells and how flot-2 overexpression regulates PAR-1 transcription are not yet known. Others have reported that transforming and regulatory sequences of PAR-1 reside in the COOH-terminal cytoplasmic domain (48). As shown in Fig. 6, flot-2 is well positioned to interact with the cytoplasmic tail of PAR-1, which could stabilize and prevent degradation of the activated-PAR-1 molecule. We would also expect that flot-2 may influence other G-protein coupled receptor, so that modulation of PAR-1 by flot-2 may have significance for signal transduction beyond one specific tumor model. Additional studies are underway to elucidate the relationship between flot-2 and PAR-1 and to determine the general significance to cancer biology as well as to melanoma.

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