Plexin C1, A Receptor for Semaphorin 7A, Inactivates Cofilin and Is a Potential Tumor Suppressor for Melanoma Progression

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Melanocytes are progenitor cells for melanoma, which arises through step-wise progression from dysplastic to invasive, to metastatic tumor. Our previous data showed that semaphorin 7A (Sema7A), a protein involved in axon guidance, stimulates melanocyte adhesion and dendricity through opposing actions of β1-integrin and Plexin C1 receptors. We now show that Plexin C1 is diminished or absent in human melanoma cell lines; analysis of tissue microarrays of nevi, melanoma, and metastatic melanoma showed a decrease in Plexin C1 expression in metastatic melanoma, and an inverse correlation of Plexin C1 expression with depth of invasion. We examined the signaling intermediates of Sema7A and downstream targets of Plexin C1 in human melanocytes. Sema7A activated mitogen-activated protein kinase and inactivated cofilin, an actin-binding protein involved in cell migration. When Plexin C1 expression was silenced, Sema7A failed to phosphorylate cofilin, indicating that cofilin is downstream of Plexin C1. Further, Lim kinase II, a protein that phosphorylates cofilin, is upregulated by Sema7A in a Plexin C1-dependent manner. These data identify Plexin C1 as a potential tumor suppressor protein in melanoma progression, and suggest that loss of Plexin C1 expression may promote melanoma invasion and metastasis through loss of inhibitory signaling on cofilin activation.

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INTRODUCTION

Semaphorins are a large class of secreted and membrane-anchored proteins that are important in neuronal pathfinding and axon guidance in selected areas of the developing nervous system (Pasterkamp and Verhaagen, 2006). Semaphorins are subdivided into eight subfamilies, two found in invertebrates (classes 1 and 2), one in viruses (class 8), and five in vertebrates (classes 3–7). Semaphorins stimulate multiple signaling pathways, and all semaphorins have a conserved semaphorin (*sema*) domain in the amino terminus, which is also present in their receptors, the Plexins. The prototypical semaphorin, semaphorin 3A/collapsin-1, causes growth cone collapse of sensory neurons through remodeling of cytoskeletal proteins, and the majority of semaphorins described to date act as repellant signals to neurons (Giger *et al.*, 1998; Pasterkamp *et al.*, 1998a, b, 1999). Although their expression and function was originally described in the brain and spinal cord, semaphorins are now known to be widely expressed and have diverse functions. The protean effects of semaphorins include regulation of blood vessel development, modulation of the immune system, and drug resistance (Yamada *et al.*, 1997; Tamagnone and Comoglio, 2000; Ishida *et al.*, 2003; Moretti *et al.*, 2006). A role as tumor suppressors and tumor promoters, in part through regulation of tumor vessel formation, has also been described (Basile *et al.*, 2004, 2006; Bielenberg *et al.*, 2004; Kessler *et al.*, 2004).

Semaphorin 7A (Sema7A) is expressed at high levels in lymphoid organs, odontoblasts, bone cells, the nervous system of mice, and in human epidermal keratinocytes, fibroblasts, and in endothelial cells of blood vessels of the skin (Delorme *et al.*, 2005; Maurin *et al.*, 2005; Czopik *et al.*, 2006; Koh *et al.*, 2006; Suzuki *et al.*, 2007; Scott *et al.*, 2008). Sema7A binds to two distinct classes of receptors, β1-integrins and Plexin C1 (Pasterkamp *et al.*, 2003; Walzer *et al.*, 2005a, b). Integrins are transmembrane heterodimeric proteins that link the extracellular matrix with the cytoskeleton and are critical for cell adhesion and migration (Arnaout *et al.*, 2007; Huveneers *et al.*, 2007; Takagi, 2007). The cytoplasmic domain of integrins couples to vinculin, talin, and paxillin to form the focal adhesion complex, as well as with kinases such as focal adhesion kinase (FAK), which is phosphorylated upon integrin activation (Matsumoto *et al.*, 1995). Sema7A binds β_1-integrins, resulting in neurite extension, cytokine production, and migration in neurites, monocytes, and bone cells, respectively (Pasterkamp *et al.*,

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Abbreviations: Erk, extracellular-regulated kinase; FAK, focal adhesion kinase; FBS, fetal bovine serum; IS, intensity score; LIMK, LIM kinases; Map, mitogen-activated protein; Sema7A, semaphorin 7A

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2003; Delorme *et al.*, 2005; Suzuki *et al.*, 2007). We showed that Sema7A stimulates melanocyte adhesion and dendrite formation through β_1-integrins, by demonstrating that blocking β_1-integrin antibodies, and echistatin, a snake venom that inhibits integrins, abrogated effects of Sema7A on melanocyte adhesion and dendrite formation (Scott *et al.*, 2008). Therefore, integrin-mediated signaling appears to be a common mechanism by which Sema7A effects are controlled.

Plexins are a family of transmembrane receptors that bind to secreted and membrane-bound semaphorins (Artigiani *et al.*, 1999; Tamagnone *et al.*, 1999). Plexins were identified through their homology to the extracellular domain of the scatter factor receptors, and their cytoplasmic domains are highly conserved. Some of the effects of Plexin signaling are due to binding of guanosine triphosphate-binding proteins, and to inhibition of integrin and cofilin activation (Swiercz *et al.*, 2004; Pasterkamp, 2005; Walzer *et al.*, 2005a, b). Data suggest that Plexin C1 is a receptor for Sema7A in some, but not all cell types, suggesting additional ligands for Plexin C1 (Pasterkamp *et al.*, 2003; Walzer *et al.*, 2005a, b). There is very limited information on the signaling pathways stimulated by Plexin C1, however, in murine dendritic cells, Plexin C1 signaling inactivates cofilin, and inhibits integrins, resulting in decreased adhesion and migration (Walzer *et al.*, 2005a). We recently showed that human melanocytes express abundant Plexin C1 at the message and protein level, and that Plexin C1 is expressed in normal melanocytes in the skin *in vivo* (Scott *et al.*, 2008). The functional significance of Plexin C1 in mediating Sema7A effects in melanocytes was demonstrated through experiments in which silencing of Plexin C1 resulted in enhanced adhesion and spreading in response to Sema7A. Therefore, similar to murine dendritic cells, Plexin C1 signaling abrogates melanocyte adhesion, although the mechanisms have not been determined.

Melanoma is a deadly skin cancer that arises from melanocytes, or melanocyte stem cells, through multiple mechanisms. *De novo* expression of growth factors and constitutive activation or upregulation of receptor expression, stimulate pathways that result in melanoma initiation and progression (Klein *et al.*, 1991; Hamoen *et al.*, 2001; Haass *et al.*, 2005; Hess *et al.*, 2005). Semaphorins regulate cell migration and motility in virtually all cell types examined, so there is keen interest in the possible role of semaphorins in tumor progression (Artigiani *et al.*, 1999; Kreuter *et al.*, 2002; Bielenberg and Klagsbrun, 2007; Hu *et al.*, 2007). Investigation of the potential role of semaphorins in melanoma progression is limited, however, semaphorin 3F was identified as a tumor suppressor of melanoma through effects on melanoma proliferation (Bielenberg *et al.*, 2004). The present study shows that Plexin C1 expression is lost during melanoma invasion and metastasis, and that cofilin, an actin-binding protein involved in actin assembly, is a downstream target of Plexin C1 signaling in melanocytes. We propose that Plexin C1 is a previously undescribed tumor suppressor protein for melanoma progression, and that loss of Plexin C1 promotes melanoma progression through unopposed cofilin activation.

RESULTS

Plexin C1 expression is decreased at the protein and message level in human melanoma cell lines

Total cell lysates of six human melanoma cell lines and normal human melanocytes were blotted for Plexin C1 (Figure 1a). Plexin C1 is expressed in normal human melanocytes, is absent from cell lines WM115 and C32, and is decreased in WW165, YURIF, and YUSIK. YUMAC show increased Plexin C1 protein expression compared with normal melanocytes. Quantitative real-time PCR showed no Plexin C1 PCR product in the two cell lines that completely lacked Plexin C1 protein (Figure 1b; WM115 and C32). The YUMAC and YURIF cell lines expressed Plexin C1 PCR product at higher levels than melanocytes, despite lower levels of Plexin C1 protein in one of these cell lines (YURIF) compared with melanocytes. The WW165 melanoma cell line, which expressed lower Plexin C1 protein, also expressed less Plexin C1 mRNA. *De novo* production of growth factors are implicated in melanoma tumor progression, therefore, we determined cell-surface expression of Sema7A by FACs analysis of stained cells. Positive controls consisted of normal human fibroblasts, which we have shown express Sema7A (Scott *et al.*, 2008). None of the six human melanoma cell lines expressed detectable Sema7A (data not shown).

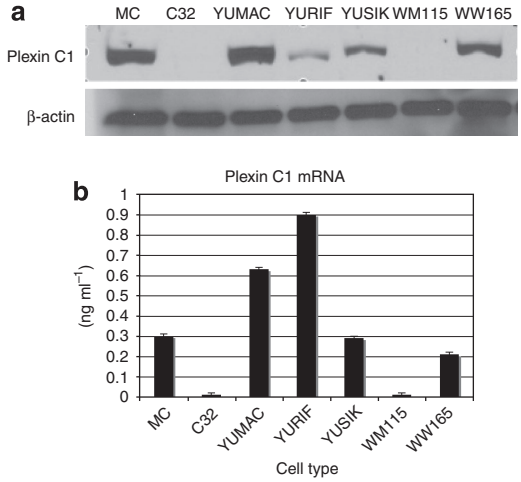

Figure 1. (a) Plexin C1 protein expression is decreased in human melanoma. Total cell lysates of melanocytes (MC) and six human melanoma cell lines were resolved on 7.5% SDS–PAGE and blotted with antibodies to Plexin C1. The lower part of the membrane was blotted for β-actin. Melanocytes express large amounts of Plexin C1. Plexin C1 is completely absent in cell lines WM115 and C32, is decreased in WM165, YURIF, and YUSIK, and is slightly increased in YUMAC. Results are representative of three separate experiments. (**b**) Plexin C1 message is decreased or absent in 3 of 6 human melanoma cell lines. Message RNA was reverse transcribed from melanocytes and six human melanoma cell lines. Quantitative real-time PCR of each sample was performed in triplicate against a standard curve of Plexin C1 PCR product to arrive at a quantitative Plexin C1 level. Three of the six cell lines either had no Plexin mRNA (C32 and WM115) or decreased Plexin C1 mRNA (WW165) compared with melanocytes. Plexin C1 message was increased compared with melanocytes in two cell lines (YURIF and YUMAC). Results are the averaged amount of Plexin C1 message (± SD) of three separate experiments.

Plexin C1 expression correlates with melanoma progression

Tissue microarrays were constructed from 26 cases of benign nevi, 27 cases of melanoma primary to the skin, and 24 cases of metastatic melanoma. For metastatic melanoma, 11 of 24 cases were metastatic to lymph nodes, 5 of 24 were metastatic to skin, and 8 of 24 cases were metastatic to organs including gastrointestinal system, lung, and bone. The percentage of cases from each group exhibiting no staining (0–1.5 intensity score, IS), moderate staining (1.6–2.5 IS) and strong staining (2.6–3.0 IS) is presented in Figure 2. A total of 66% of metastatic melanomas did not express Plexin C1, whereas all nevi showed either moderate or strong expression of Plexin C1. Expression of Plexin C1 was significantly different in nevi compared with metastatic melanoma, and between primary melanoma and metastatic melanoma ($P<0.001$). Nevi expressed more Plexin C1 than primary melanoma, and these differences were also statistically significant ($P<0.05$). Representative photographs of nevi, melanoma, and metastatic melanoma stained for Plexin C1 are shown in Figure 3a–d.

The average IS of the 26 nevi was 2.76 (±0.34 SD); 69% of nevi showed strong expression of Plexin C1 (IS between 2.6 and 3.0), 27% of nevi showed moderately strong Plexin C1 expression (IS between 1.6 and 2.5), and no cases were negative for Plexin C1 (IS between 0 and 1.5). Figure 3a shows representative cores of two cases of nevi stained for Plexin C1. Strong and diffuse membranous expression of Plexin C1 is observed. The lowest IS for a nevus was 2.3.

The expression of Plexin C1 by melanoma primary to the skin was more heterogeneous (Figure 3b). The average IS for all cases ($n=27$) was 2.38 (±0.84 SD), which was significantly different from nevi (0.05), and ranged from 0 to 3. A total of 57% of cases showed strong staining, 25% showed moderately strong staining, and 17% showed no staining. Of note, a case of melanoma arising within a nevus showed strong staining of the nevic cells, but weak staining of the melanoma (Figure 3c). The depth of invasion of melanoma is a major prognostic indicator (Balch *et al.*, 1980; Patrick *et al.*, 2007). When Plexin C1 expression was analyzed in melanomas of different depths of invasion, a trend of diminished Plexin C1 expression with increasing depth of invasion was observed (Figure 3b). Melanomas with a depth <1 mm ($n=9$), which are considered thin melanomas with a near 100% 5-year survival, had an IS score for Plexin C1 of 3 (±0.10 SD). Melanomas with a depth of invasion between 1.01 and 2.0 mm ($n=8$) had an IS score of 2.5 (±0.53 SD). Melanomas with a depth of invasion between 2.01 and 4.0 mm ($n=6$) had an IS score of 2 (±0.51 SD) and melanomas with a depth of invasion of >4 mm ($n=4$), which are advanced tumors with a 50% 5-year survival rate, had an IS of 1.32 (±0.47 SD). Student's *t*-test showed that Plexin C1 expression was significantly different ($P<0.001$) between thin melanomas (depth of invasion <1 mm) and deeply invasive tumors (depth >4 mm).

A striking decrease in Plexin C1 expression was observed in metastatic melanoma (Figure 3d). The average IS for Plexin C1 in all cases of metastatic melanoma ($n=24$) was 1.0 (±0.97), which was significantly different from nevi ($P<0.001$) and melanoma primary to the skin ($P<0.001$). A total of 67% of metastatic melanomas did not stain at all for Plexin C1 and only 20% of cases showed moderately strong staining. Two of the five cases that showed strong staining (IS 2.6–3.0) for Plexin C1 were tumors metastatic to skin and bone.

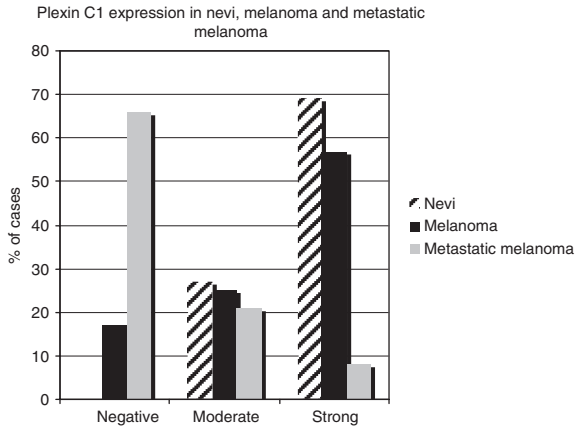

Figure 2. Loss of Plexin C1 is correlated with progression of primary melanoma to metastatic melanoma. Compiled data of Plexin C1 intensity scores (IS) of TMAs of nevi, melanoma primary to the skin, and metastatic melanoma. The percentage of cases of nevi, melanoma, and metastatic melanoma with no Plexin C1 staining, moderately strong staining, and strong staining is shown. A total of 66% of metastatic melanomas (pale gray bars) did not express Plexin C1, whereas all nevi (hatched bars) showed either moderate or strong expression of Plexin C1. Expression of Plexin C1 was significantly different in nevi compared with metastatic melanoma, and between primary melanoma (black bars) and metastatic melanoma ($P<0.001$). Nevi expressed more Plexin C1 than primary melanoma, and these differences were statistically significant ($P<0.05$).

Sema7A stimulates cofilin inactivation, and activates FAK and MAP kinase, in human melanocytes

To examine potential mechanisms by which loss of Plexin C1 leads to melanoma progression, we analyzed the signaling pathways stimulated by Sema7A in normal human melanocytes and the downstream targets of Plexin C1 signaling. The activity of cofilin is controlled through phosphorylation of the protein at Ser-33, which results in its inactivation (Moriyama *et al.*, 1996). Treatment of melanocytes with Sema7A stimulated the rapid phosphorylation of cofilin, as determined by western blotting with antibodies against cofilin phosphorylated on Ser-33 (Figure 4a). Phosphorylation of cofilin occurred within 5 minutes of treatment with Sema7A and began to diminish 30 minutes later. We have demonstrated previously that Sema7A stimulates melanocyte adhesion and dendrite formation through β1-integrin receptors. To determine if Sema7A stimulates integrin signaling, we examined the effect of Sema7A on the activation of the non-receptor protein tyrosine kinase FAK. FAK is rapidly phosphorylated upon integrin receptor binding, resulting in propagation of integrin signals (Richardson and Parsons, 1995; Hehlgans *et al.*, 2007). Sema7A stimulated the rapid phosphorylation of FAK in melanocytes, indicative of β1-integrin signaling (Figure 4a). A dose–response analysis showed that detectable

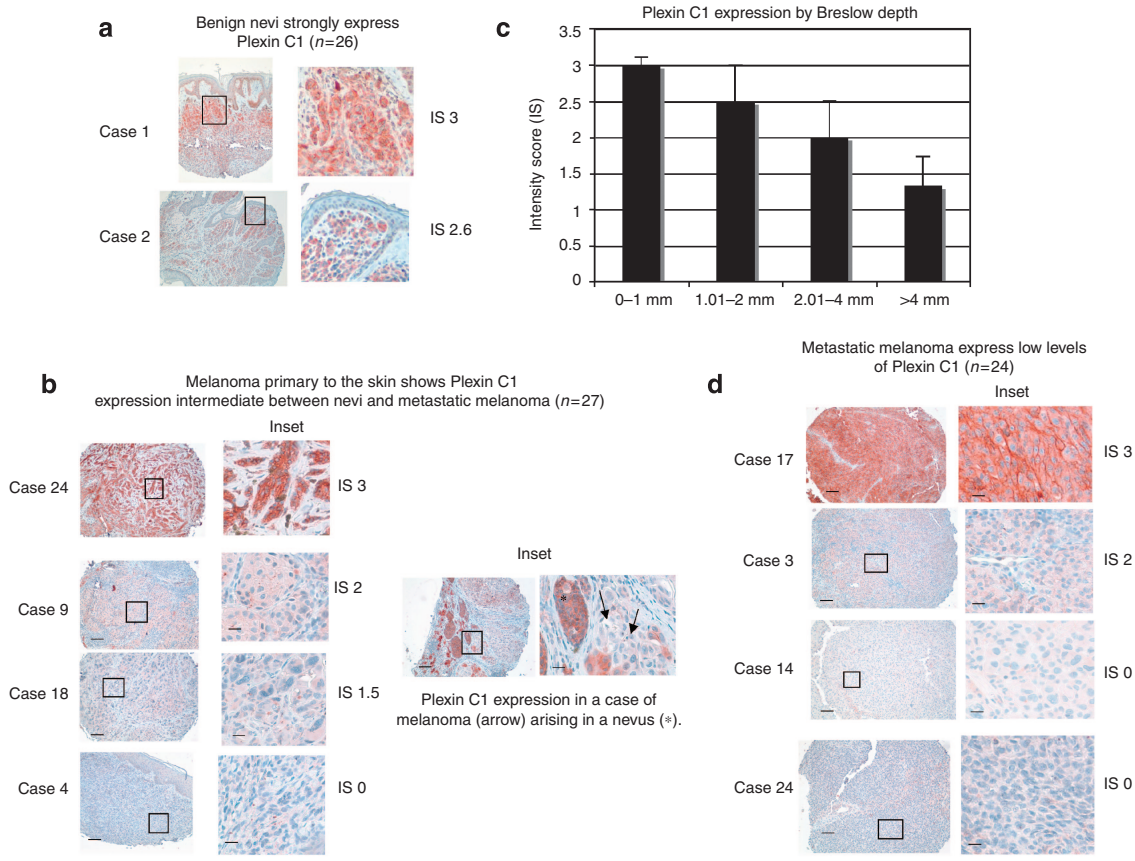

Figure 3. Plexin C1 is lost during melanoma progression *in vivo*. (**a**) Benign nevi strongly express Plexin C1. Two representative cases of benign nevi from TMA stained for Plexin C1 are shown. One core from each case, with a high power inset, is shown, along with the assigned intensity score (IS). The nevic cells in virtually all nevi showed moderate to strong expression of Plexin C1. Mag = 0.4 mm; inset Mag = 200 μm. (**b**) Primary melanomas show Plexin C1 expression intermediate between nevi and metastatic melanoma. Four representative cases of melanomas stained for Plexin C1 from TMA are shown. One core from each case, with a high-power inset, along with the assigned intensity score (IS) is shown. Of note, one case showed melanoma arising within a nevus. In this case, strong expression of Plexin C1 within the nevic cells (*) is present, juxtaposed with melanoma cells with weaker Plexin C1 expression (arrows). Mag = 0.4 mm; inset Mag = 200 μm. (**c**) Plexin C1 expression decreases with increasing depth of invasion. When cases were separated into groups of different depths of invasion, a clear trend of decreasing Plexin C1 expression with increasing depth of invasion is present. Thin melanomas (0.1–1 mm) had a significantly higher IS for Plexin C1 compared with melanomas 4 mm or thicker (IS of 3 and 1.33, respectively, $P<0.001$). (**d**) Metastatic melanomas express low levels of Plexin C1. Four representative cases of metastatic melanoma from TMA stained for Plexin C1 are shown. One core from each case, with a high-power inset, along with the assigned intensity score (IS) is shown. The vast majority of metastatic melanomas did not express Plexin C1. Mag = 0.4 mm; inset Mag = 200 μm.

cofilin and FAK phosphorylation occurred at 10 ng ml^{-1} Sema7A, and peaked at 50 ng ml^{-1} (Figure 4b). Sema7A activates mitogen-activated protein (MAP) kinase in neurons (Pasterkamp *et al.*, 2003; Huang *et al.*, 2007; Li *et al.*, 2007; Yee *et al.*, 2008). To determine if Sema7A activates MAP kinase in melanocytes, cells were treated with Sema7A (doses ranging from 1 to 50 ng ml^{-1}) for 5 minutes, and total cell lysates were blotted for phosphorylated extracellular-regulated kinases (Erks) 1 and 2 (Figure 4c). Sema7A stimulated the rapid phosphorylation of Erk1/Erk2 at a dose of 10 ng ml^{-1}, which peaked at a dose of 25 ng ml^{-1}, which was inhibited by the selective MAP kinase kinase inhibitor PD 98059 (Figure 4d). To determine if Sema7A-dependent phosphorylation of FAK and cofilin requires Erk1/Erk2 activation, melanocytes were treated with Sema7A (50 ng ml^{-1} for 5 minutes) in the presence or absence of PD 98059. Pretreatment of melanocytes with PD 98059 for 2 hours did not block phosphorylation of FAK or cofilin in response to Sema7A, suggesting that cofilin and FAK phosphorylation are not downstream of Erk1/Erk2 activation (data not shown).

Plexin C1 signaling stimulates cofilin phosphorylation/inactivation in melanocytes

To determine the relationship between Plexin C1 signaling and cofilin phosphorylation, Plexin C1 was silenced in melanocytes using siRNAs, and silenced cells were treated with Sema7A (50 ng ml^{-1}) for 5 and 15 minutes. Partial (>75%) silencing of Plexin C1 was achieved in the samples from the 5-minute treatment condition; complete silencing was achieved in the 15-minute treatment samples (Figure 5a). Phosphorylation of cofilin in response to Sema7A directly correlated with levels of Plexin C1 expression. At the 5-minute time point, in which residual Plexin C1 was present,

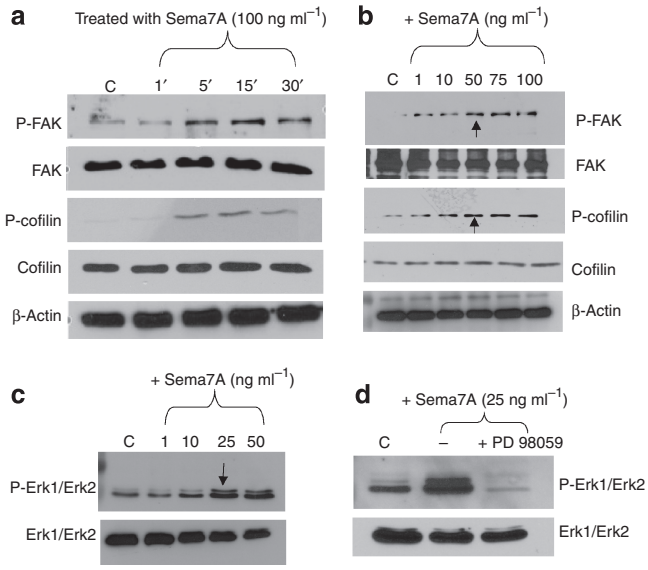

Figure 4. Sema7A actuates FAK and Erk1/Erk2, and inhibits cofilin. (**a**) Sema7A stimulates the phosphorylation of FAK and cofilin in human melanocytes. Melanocytes were placed in basal medium (MCDB-153) for 8 hours and were treated with recombinant Sema7A (100 ng ml^{-1}) for the indicated time points; controls were treated with vehicle. Total cell lysates were resolved on 10% SDS–PAGE and blotted for phosphorylated focal adhesion kinase (FAK) and phosphorylated cofilin. Sema7A stimulated the rapid (within 5 minutes) phosphorylation of FAK, indicative of integrin activation. Levels of inactive (phosphorylated) cofilin were also rapidly increased in melanocytes upon Sema7A treatment, which peaked at 15 min. Results are representative of three separate experiments performed on pooled cultures of human melanocytes. (**b**) A dose–response analysis of FAK and cofilin phosphorylation in response to Sema7A was performed. Melanocytes were placed in basal medium (MCDB-153) for 8 hours and were treated with recombinant Sema7A at doses ranging from 1 to 100 ng ml^{-1}, for 5 min. Controls were treated with vehicle. Total cell lysates were resolved on 10% SDS–PAGE and blotted for phosphorylated FAK and phosphorylated cofilin. Maximal FAK and cofilin phosphorylation was observed at 50 ng ml^{-1} (arrow), and in the case of FAK, occurred at concentrations of Sema7A as low as 1 ng ml^{-1}. Cofilin phosphorylation was first detected at a dose of 10 ng ml^{-1} of Sema7A. Results are representative of two separate experiments performed on pooled cultures of human melanocytes. (**c**, **d**) Sema7A stimulates MAP kinase activation in melanocytes. Melanocytes were placed in basal medium (MCDB-153) for 8 hours and were treated for 5 min with recombinant Sema7A at doses ranging from 1 to 50 ng ml^{-1}; controls were treated with vehicle. Total cell lysates were resolved on 10% SDS–PAGE and blotted for phosphorylated Erk1/Erk2; the blot was stripped and reblotted for total Erk1/Erk2. A dose-dependent increase in P-Erk1/Erk2 was observed in response to Sema7A, with a peak response seen at a dose of 25 ng ml^{-1} (arrow). Results are representative of two separate experiments. (**d**) The specificity of MAP kinase activation was tested in melanocytes by the inclusion of the selective p42/44 MAP kinase inhibitor PD 98059 (10 μM) in cells treated with Sema7A (25 ng ml^{-1}) for 5 minutes. Cell lyates resolved on SDS–PAGE and blotted for P-Erk1/Erk2 are shown. The blot was stripped and reblotted for total Erk1/Erk2. As expected, Sema7A stimulated phosphorylation of Erk1/Erk2 within 5 minutes of treatment. Cells treated with PD 98059 for 1 hour before treatment with Sema7A showed no induction of Erk1/Erk2 phosphorylation. Results are representative of two separate experiments.

phosphorylation of cofilin in response to Sema7A was diminished, but not absent. At the 15-minute point, in which Plexin C1 levels were fully silenced, phosphorylation of cofilin in response to Sema7A was completely abrogated. In contrast with cofilin phosphorylation, silencing of Plexin C1 expression had no effect on Erk1/Erk2 phosphorylation in response to Sema7A (Figure 5b).

Cofilin is a direct downstream target of LIM kinases (LIMK), which phosphorylate cofilin on Ser-33. There are two isoforms of LIMK; LIMKI is expressed in neural cells, whereas LIMKII is expressed more widely (Bernard, 2007; Scott and Olson, 2007). To determine which isoform of LIMK is expressed in human melanocytes and our panel of melanoma cell lines, total cell lysates were blotted for LIMK1 and LIMKII (Figure 5c). LIMKII, but not LIMKI, was expressed by melanocytes, and by 3 of 5 melanoma cell lines. Of interest, the two cell lines that completely lack LIMKII expression (WM115 and C32) also lack Plexin C1, and levels of LIMKII roughly correlate with Plexin C1 expression in melanoma cell lines (Figure 1a).

To determine if Plexin C1 regulates LIMKII expression, Plexin C1 was silenced in melanocytes, and 24 hours later silenced cells, and cells transfected with scrambled siRNA, were treated with Sema7A (50 ng ml^{-1}). Controls consisted of cells treated with supernatants of COS-1 cells that do not stable express Sema7A (Figure 5d). Twenty-four hours after the second treatment with Sema7A (72 hours after silencing), cells were lysed. Lysates were resolved on 6% SDS–PAGE, transferred to nitrocellulose membranes, and the membrane was cut and blotted for Plexin C1, LIMKII, and β-actin. Melanocytes transfected with scrambled siRNA (which have normal Plexin C1 levels) and treated with Sema7A, showed a marked increased LIMKII expression, compared with cells treated with COS-1 supernatants. The increase in LIMKII in response to Sema7A was abrogated in cells in which Plexin C1 was silenced, confirming that Plexin C1 is involved in the effect of Sema7A on LIMKII expression. These data indicate that LIMKII expression is regulated, at least in part, by Sema7A-dependent Plexin C1 signaling, suggesting that the Plexin C1–LIMKII–cofilin pathway is tightly controlled in melanocytes.

DISCUSSION

We recently showed that Sema7A is expressed in the skin, where it controls the dendricity and adhesion of human melanocytes through the opposing actions of Sema7A-dependent β1-integrin and Plexin C1 signaling (Scott *et al.*, 2008). An interesting observation that emerged from that initial study was that silencing of Plexin C1 enhanced the effect of Sema7A on melanocyte adhesion and dendricity, suggesting that Plexin C1 signals downstream to targets that inhibit actin assembly or turnover. Gain or loss of receptor expression, or *de novo* expression of growth factors, contributes to melanoma initiation and progression (Herlyn *et al.*, 1988; Easty and Bennett, 2000; Meier *et al.*, 2003; Haass *et al.*, 2005; Kwong *et al.*, 2007). In six human melanoma cell lines, Plexin C1 protein expression was completely absent in two, was decreased in three, and was slightly increased in one. Levels of Plexin C1 mRNA generally correlated with protein expression, however, the YURIF melanoma cell line, which expressed less Plexin C1 protein

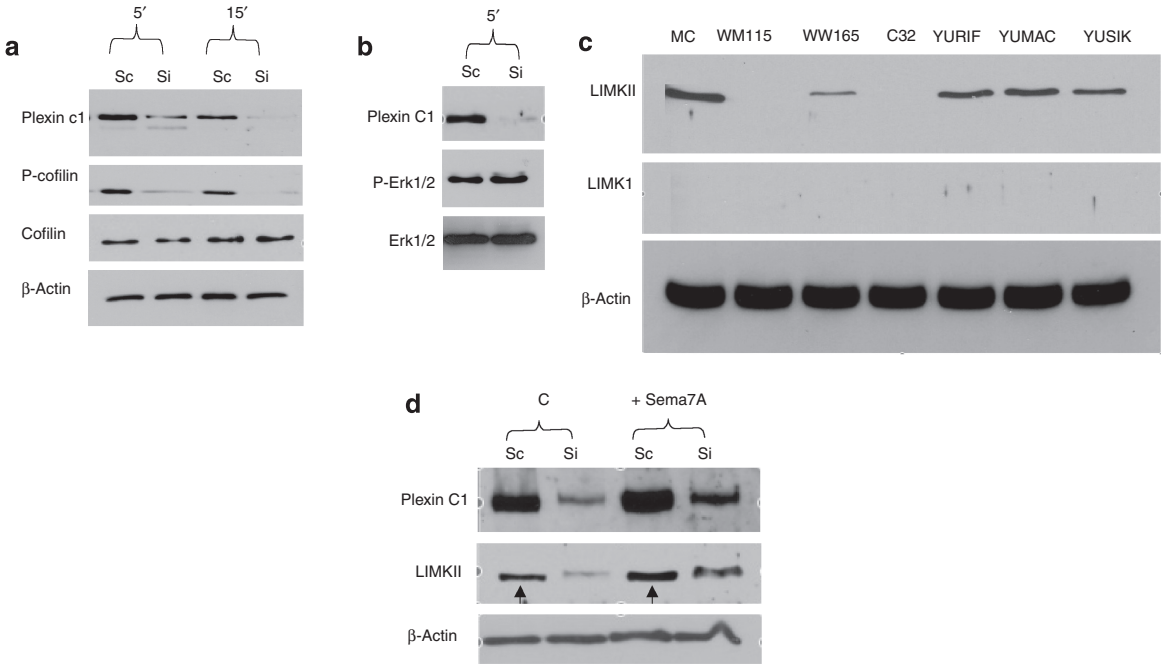

Figure 5. Plexin C1 signaling regulates cofilin activation and LIMKII expression. (**a**, **b**) Silencing of Plexin C1 abrogates cofilin phosphorylation/inactivation, but has no effect on MAP kinase activation. Melanocytes were plated onto four 60 mm dishes, and two dishes were transfected with siRNAs to Plexin C1 (Si) and two dishes were transfected with scrambled siRNAs (Sc). At 48 hours later, cells were placed in basal medium for 8 hours and then treated for 5 or 15 minutes with Sema7A (50 ng ml^{-1}). Total cell lysates were resolved on 7.5% SDS–PAGE. The top of the membrane was blotted for Plexin C1, and the bottom was blotted for phospho-cofilin. (**a**) Phosphorylation of cofilin, in response to Sema7A, correlated with levels of Plexin C1 expression; at the 5 minute time point, in which Plexin C1 was silenced >50%, there was a >50% reduction in phosphorylated cofilin. In the 15 minutes sample, in which total silencing of Plexin C1 was achieved, there was no detectable phosphorylation of cofilin. The different degree of silencing of Plexin C1 likely reflects differences in silencing efficiency between the dishes. Results are representative of three separate experiments. (**b**) Melanocytes were transfected with siRNAs to Plexin C1 (Si) or scrambled siRNAs (Sc) as described above and treated for 5 minutes with Sema7A (25 ng ml^{-1}). Total cell lysates were resolved on 7.5% SDS–PAGE. The top of the membrane was blotted for Plexin C1, and the bottom was blotted for phospho-Erk1/2. Knockdown of Plexin C1 did not affect phosphorylation of Erk1/Erk2 in response to Sema7A. Results are representative of three separate experiments. (**c**) Human melanocytes and melanoma cells express LIMKII. Lysates (40 μg) of human melanocytes (MC), WM115, WW165, C32, YURIF, YUMAC, and YUSIK were resolved on 10% SDS–PAGE and blotted with antibodies that recognize LIMKI or LIMKII. None of the cells express LIMKI. Melanocytes, and 4 of 6 melanoma cell lines, express LIMK II. Interestingly, the expression of LIMKII correlates with Plexin C1 expression (Figure 1a). Results are representative of two separate experiments. (**d**) Plexin C1 signaling regulates LIMKII expression. Melanocytes were plated onto four 60 mm dishes, and two dishes were transfected with siRNAs to Plexin C1 (Si) and two dishes were transfected with scrambled siRNAs (Sc). At 24 and 48 hours after silencing, cells were treated with either Sema7A (50 ng ml^{-1}) or COS-1 supernatants from nontransfected cells (''C''). At 24 hours following the second treatment (72 hours after silencing), cells were lysed and resolved on 6% SDS–PAGE. The top of the membrane was blotted for Plexin C1, the middle was blotted for LIMKII, and the bottom was blotted for β-actin. Melanocytes treated with Sema7A show a marked increased LIMKII expression, compared with cells treated with COS-1 supernatants. Silencing of Plexin C1 abrogated the effect of Sema7A on LIMKII expression. Representative results of three separate experiments are shown.

than melanocytes, had increased levels of Plexin C1 message. The reason for this is unclear, but may reflect a longer mRNA half-life, or increased degradation of the protein. The mechanism by which Plexin C1 is lost in melanoma remains to be determined. Loss of heterozygosity contributes to *Apaf-1* deletion, a gene linked to Plexin C1 on chromosome 12, that is also lost during melanoma progression (Soengas *et al.*, 2001). The complete absence of Plexin C1 message in 2 of 6 melanoma cell lines suggests that gene deletion may be involved in loss of Plexin C1. We are currently examining genomic DNA from metastatic and primary melanomas to determine if Plexin C1 loss is due to deletion of part or the entire gene.

We examined the expression of Plexin C1 by immunohistochemical staining of TMA of benign nevi, melanoma primary to the skin, and metastatic melanoma. Tissue microarrays allow analysis of multiple samples on the same slide that are uniformly stained (Simon *et al.*, 2004). Our data indicate a striking loss of Plexin C1 expression in metastatic melanoma, and an inverse correlation between Plexin C1 expression and tumor invasiveness in primary melanomas. Nevic cells in virtually all subjects showed strong homogenous expression of Plexin C1. In contrast, the majority of metastatic melanomas showed weak or no staining for Plexin C1. The expression of Plexin C1 in primary melanoma was intermediate between nevi and metastatic tumors, and Plexin C1 expression was progressively lost in melanomas of increasing depth. Very deep melanomas (>4 mm) showed an average IS of 1.3, compared with thin melanomas (<1 mm) that showed an average IS of 3. Particularly informative was the juxtaposition of a benign nevus and melanoma, in which strong expression of Plexin C1 was

evident in the nevic cells and was absent in the melanoma cells (Figure 3b). *In toto*, these data support a role for Plexin C1 as a tumor suppressor protein for melanoma progression. Because expression of Plexin C1 in benign nevi and superficially invasive melanoma (<1 mm depth of invasion) was essentially the same, loss of Plexin C1 is likely to promote tumor progression (invasion and metastasis) rather than tumor initiation. Studies in which Plexin C1 expression in melanomas are correlated with matched metastatic tumors, are currently being performed to determine if loss of Plexin C1 expression in primary tumors is correlated with metastatic disease in individual patients. If so, Plexin C1 expression has the potential to be useful as a prognostic indicator for melanoma metastasis.

Cofilin activation is strongly linked to tumor progression, although the relationship between cofilin activation and tumor progression is complex (Paavilainen *et al.*, 2004; Huang *et al.*, 2006). Loss of cofilin expression in metastatic tumor cell lines is associated with decreased cell turning and chemotactic sensitivity to epidermal growth factor (Sidani *et al.*, 2007) and the cofilin activation pathway is a major determinant of breast cancer metastasis (Wang *et al.*, 2007). Although there are abundant data to suggest a role for cofilin activation in carcinogenesis, data on the role of cofilin and melanoma progression is more limited. Knockdown of cofilin and actin depolymerizing factor in B16F1 melanoma cells inhibited cell migration primarily through diminished actin filament depolymerization rates (Hotulainen *et al.*, 2005). In K1735 murine melanoma cells, expression of wild-type (nonphosphorylatable) cofilin increased melanoma invasion and migration (Dang *et al.*, 2006). Although the mechanisms by which loss of Plexin C1 promotes melanoma progression are likely multifactorial, our data indicate a potential link between loss of Plexin C1 and cofilin activation in melanoma.

Sema7A stimulated the rapid phosphorylation of cofilin and FAK at low doses (10 ng ml^{-1}) in human melanocytes. Phosphorylation of FAK, which occurs upon integrin activation, is consistent with our prior data showing that Sema7A-dependent melanocyte adhesion occurs through the engagement of β1-integrins (Scott *et al.*, 2008). MAP kinase was also activated by Sema7A in normal human melanocytes, and was unaffected by Plexin C1 silencing. It is likely that MAP kinase activation is secondary to Sema7A-dependent integrin activation, as has been reported in neural cells, bone cells, and monocytes (Pasterkamp *et al.*, 2003; Delorme *et al.*, 2005; Suzuki *et al.*, 2007). To address whether Plexin C1 regulates cofilin phosphorylation, we silenced Plexin C1 in human melanocytes, and examined the effect of Sema7A on cofilin phosphorylation. When Plexin C1 was silenced, Sema7A-dependent cofilin phosphorylation was lost or attenuated, indicating that Plexin C1 regulates cofilin phosphorylation. The LIMK family of proteins consists of two members, LIMKI, and LIMKII, which phosphorylate cofilin at Ser-33 (Bernard, 2007; Scott and Olson, 2007). LIMKI is primarily expressed in the central nervous system whereas LIMKII is widely expressed (Scott and Olson, 2007). Normal melanocytes, and 4 of 6 melanoma cell lines examined, expressed LIMKII, but not LIMKI, making LIMKII a likely downstream target of Plexin C1 signaling in melanocytic cells. Examination of cofilin regulation in melanocytes expressing kinase dead mutants of LIMKII will be needed to address the role of LIMKII in Plexin C1-dependent cofilin regulation. Of interest was the precise correlation between levels LIMKII and Plexin C1 expression: melanoma cell lines lacking Plexin C1 (C32 and WM115) also lacked LIMKII, and levels of LIMKII protein closely paralleled Plexin C1 levels. Because treatment of melanocytes with Sema7A upregulated the expression of LIMKII, and because silencing of Plexin C1 blocked Sema7A-dependent LIMKII upregulation, we propose that Plexin C1 signaling stimulates either the synthesis or stability of LIMKII. Therefore, loss of Plexin C1 by melanoma cells may result in additional changes in cytoskeletal regulatory proteins, such as LIMKII, which are important for cell motility. It will be of interest to examine potential coordinated loss of Plexin C1 and LIMKII in melanoma TMAs.

In summary, we have demonstrated that Plexin C1, a receptor for Sema7A, is decreased or lost during melanoma progression *in vivo*, and that Plexin C1 signaling in normal human melanocytes results in phosphorylation and inactivation of cofilin. These data are the first to define the signaling intermediates of Sema7A in human melanocytes, and point to a role for Plexin C1 as a previously undescribed tumor suppressor protein for melanoma progression, potentially through loss of inhibition of cofilin activation. Future studies examining the role of Plexin C1 in melanoma migration, metastasis, and interaction with integrin receptor signaling, are being planned to define the effect of loss of Plexin C1 in critical determinants melanoma progression and metastasis.

MATERIALS AND METHODS

Reagents

Rabbit polyclonal antibodies to β-actin, goat polyclonal antibodies to Plexin C1, and rabbit polyclonal antibodies to LIMKI and LIMKII were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit polyclonal antibodies to cofilin phosphorylated on Ser-33, rabbit polyclonal antibodies to phospho-p44/42 MAP kinase, and mouse MAbs to p42 MAP kinase were purchased from Cell Signaling Technology (Danvers, MA), mouse MAbs to FAK phosphorylated on Y397 were purchased from Chemicon International (Temecula, CA). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse antibodies were purchased from Sigma Co. (St. Louis, MO). FITC-conjugated mouse anti-human Sema7A (CDw108) and mouse IgM conjugated to FITC for flow cytometry were purchased from Serotec (Kidlington, Oxford, UK). Full range rainbow molecular weight markers were purchased from Amersham Life Sciences (Arlington Heights, IL). Silencing RNAs (siRNA) to human Plexin C1, Silencer Negative Control no. 1 siRNA, and lipofectamine were purchased from Ambion (Austin, TX). Sema7A, in frame with human Fc fragment, in pcDNA3 vector was a generous gift from Dr Ruslan Medzhitov (Section of Immunobiology, Yale University, New Haven, CT) and has been described previously (Czopik *et al.*, 2006). The selective p42/44 MAP kinase inhibitor PD 98059 was purchased from Sigma Co.

Cells and cell culture

Neonatal foreskins were obtained according to the University of Rochester Research Subjects Review Board guidelines and were the source of cultured human melanocytes. Human melanocytes were cultured in MCDB 153 supplemented with 0.5% fetal bovine serum (FBS), bovine pituitary extract (15 $\mu g\,ml^{-1}$), phorbol ester (10 nM), basic fibroblast growth factor (1 $ng\,ml^{-1}$), insulin (5 $\mu g\,ml^{-1}$), and hydrocortisone (500 $ng\,ml^{-1}$). All supplements were purchased from Sigma Co., except FBS, which was purchased from Mediatech (Manassas, VA). The following human melanoma cell lines were purchased from the Yale University Cell Core Facility: WW165, YURIF, YUSIK, YUMAC, and were maintained in Opti-MEM + 5% FBS, except WW165 which was maintained in Opti-MEM + 5% FBS and IBMX 0.1 mM. The C32 and WM115 human melanoma cell lines were obtained from ATCC (Manassas, VA) and were maintained in Eagle minimal essential media + 10% FBS and 1 mM sodium pyruvate. The WW165, WM115, and C32 cell lines were derived from melanomas primary to the skin; the other cell lines were derived from metastatic melanoma.

Purification of Fc-tagged Sema7A

Fc-tagged Sema7A was isolated from culture supernatant of stable transfectants of COS-1 cells expressing Fc-Sema7A, on HiTrap protein A HP columns (General Electric Healthcare, St. Giles, UK) as per manufacturer's instructions. Eluent was resolved on a 7.5% SDS–PAGE and blotted for Sema7A to verify its identity as previously described (Scott *et al.*, 2008). Coomassie-stained gels showed a single band corresponding to the molecular weight of Sema7A at a protein concentration of 1 $mg\,ml^{-1}$.

Construction, staining, and analysis of tissue microarrays

The project received IRB exemption from the Human Subjects Review Board at the University of Rochester: category 4 (45 CRF 46.101): secondary use of preexisting data. A total of 26 cases of benign nevi, 27 cases of melanoma primary to the skin, and 24 cases of metastatic melanoma were chosen from formalin-fixed, paraffin-embedded archival material from Strong Memorial Department of Pathology. One of the authors (GS) diagnosed each case during clinical duties as Director of Dermatopathology at Strong Memorial Hospital, Rochester, NY. The slides from each case were reexamined and three representative areas from each slide were chosen. Three 1 mm cores corresponding to these areas were removed from the paraffin block and placed in a recipient block. All benign nevi were dermal nevi with a minimal junctional component, primary melanomas had depths of <1 mm ($n=9$), between 1.01 and 2.0 mm ($n=8$), between 2.01 and 4.0 mm ($n=6$), and >4 mm ($n=4$). For metastatic melanoma, 11 of 24 cases were metastatic to lymph nodes, 5 of 24 were metastatic to skin, and 8 of 24 cases were metastatic to organs including gastrointestinal system, lung, and bone.

Staining of TMA for Plexin C1

Sections were deparaffinized through graded series of alcohol, rehydrated in water, and endogenous peroxidase was quenched by incubation in 3% H_2O_2 for 3 min 2×. Nonspecific staining was blocked by incubation in nonprotein blocking solution (Dako, Carpenteria, CA) for 15 min at room temperature. Antigen retrieval was performed by incubating the slides in Target Retrieval Buffer (Dako) at 98 °C for 15 minutes. Slides were incubated with polyclonal antibodies against Plexin C1 in antibody diluent (1/100; Dako) for 1 h at room temperature. Sections were then incubated with biotin-conjugated rabbit anti-goat antibodies for 30 minutes at room temperature followed by streptavidin horseradish-peroxidase (Vector Laboratories, Burlingame, CA) for 20 minutes. The reaction was developed with amino-ethyl carbizol and the slides were counterstained in Mayer's hematoxylin. Negative controls consisted of goat IgG (Sigma Co.) instead of the primary antibody.

Analysis of TMAs

The intensity of staining of each 1 mm core was rated on a scale of 0–3, with 0, negative; 1, weak; 2, moderate, and 3, strong. For all cases, when staining was present, it was diffused, with all nevi cells or melanoma cells expressing uniform staining for Plexin C1. Each core was examined under a light microscope by one of the authors (GS) and separately scored, and the staining intensity of the cores averaged to arrive at an IS. Cores with $<50\%$ of designated tissue present were disregarded. Cases with an average IS score of 0–1.5 were considered negative; those with an IS score between 1.6 and 2.5 were considered moderately positive, and those with an IS score of 2.6–3 were considered strongly positive.

Statistical evaluation of data

Data were summarized in terms of their mean and standard deviation. Tissue expression of Plexin C1 was analyzed using Fisher's exact test to determine statistically significant difference in expression between nevi, primary melanoma, metastatic melanoma, and in melanomas of different Breslow depths (0.1–1, 1.01–2.0, 2.1–4, and >4.0 mm). A P-value of <0.05 was considered significant.

Western blotting and flow cytometry

For western blotting, cells were lysed in radioimmunoprecipitation assay buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl) with protease inhibitors (Boehringer Mannheim GmbH, Mannheim, Germany) and phosphatase inhibitors (GBiosciences, St. Louis, MO) and protein was quantified using BSA as standard (Bio-Rad Laboratories, Hercules, CA). Visualization of the immunoreactive proteins was accomplished with an enhanced chemiluminescence reaction (Pierce Chemical, Rockford, IL). For analysis of cell surface Sema7A expression in melanoma cell lines by flow cytometry, cells were placed in suspension in phosphate-buffered saline/1% BSA and stained with FITC-conjugated antibodies against Sema7A for 30 minutes at room temperature. Negative controls consisted of cells incubated with FITC-conjugated IgM. Positive controls consisted of normal human fibroblasts. After washing in phosphate-buffered saline, cells were resuspended in 0.5% formalin in phosphate-buffered saline/1% BSA and analyzed using a FACS Caliber Machine (Becton Dickinson) at the flow cytometry core facility at the University of Rochester.

Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini Kit (QIAGEN, Valencia, CA) according to manufacturer's instructions. Reverse transcription was performed using 0.75 µg of total RNA with SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA). PCR

was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories) on the Applied Biosystems ABI prism 7700 sequence-detection system (Bio-Rad iCycler). Primers used for amplification of Plexin C1 were: forward: 5′-AACCATTGCACTGCAACC-3′; reverse: 5′-GATTCCATCTTCAAGAATCACG-3′. The conditions were: 95 °C, 3 minutes (1 cycle); 95 °C 15 seconds, 54.5 °C, 30 seconds, 72 °C, 40 seconds (40 cycles). Primers used for amplification of β-actin were: forward: 5′-CACGCACGATTTCCC GCTCGG-3′; reverse: 5′-CAGGCTGTGCTATCCTGTAC-3′. The conditions were 95 °C, 3 minutes (1 cycle); 95 °C 15 seconds, 54.5 °C, 30 seconds, 72 °C, 40 seconds (40 cycles). The PCR product was resolved on 1% agarose gels, sequenced, and verified. Quantification of PCR product was determined by analyzing a standard curve of known amounts of Plexin C1 PCR product to unknown samples. Samples were then normalized to β-actin.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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