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Tumorigenesis and Neoplastic Progression

Macrophage Inhibitory Cytokine-1 Regulates Melanoma Vascular Development

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Expression of macrophage inhibitory cytokine-1 (MIC-1), a member of the transforming growth factor-β family, normally increases during inflammation or organ injury. MIC-1 is also expressed at higher levels in melanomas; however, its role in tumorigenesis is unknown. This report identifies a novel function for MIC-1 in cancer. MIC-1 was overexpressed in ~67% of advanced melanomas, accompanied by fivefold to six-fold higher levels of secreted protein in serum of melanoma patients compared with normal individuals. Constitutively active mutant ^{V600E}B-Raf in melanoma regulated downstream MIC-1 expression. Indeed, small-interfering RNA-mediated targeting of MIC-1 or ^{V600E}B-Raf reduced expression and secretion by three-fold to fivefold. This decrease in MIC-1 levels reduced melanoma tumorigenesis by approximately threefold, but did not alter cultured cell growth, suggesting a unique function other than growth control. Instead, inhibition of MIC-1 was found to mechanistically retard melanoma tumor vascular development, subsequently affecting tumor cell proliferation and apoptosis. This role in melanoma angiogenesis was confirmed by comparing MIC-1 and vascular endothelial growth factor (VEGF) function in chick chorioallantoic membrane and matrigel plug assays. Similar to VEGF in melanomas, MIC-1 stimulated directional vessel development, acting as a potent angiogenic factor. Thus, MIC-1 is secreted from melanoma cells together with VEGF to promote vascular development mediated by ^{V600E}B-Raf signaling. ***(Am J Pathol 2010, 176:2948–2957; DOI: 10.2353/ajpath.2010.090963)***

The transforming growth factor-β (TGF-β) family of cytokines is a large multifunctional collection of proteins regulating cellular proliferation, migration, cell adhesion, immune surveillance, and angiogenesis.[1] TGF-β family members function by relaying signals from serine/threonine kinase receptors in the cell membrane to activate signaling cascades controlling transcriptional activators and repressors.[1,2] The role of TGF-β family members in cancer is an active area of research with specific roles dependent on stage of tumor development and cancer type.[1,2]

One member of the TGF-β family is the macrophage inhibitory cytokine-1 (MIC-1), which was originally identified as a factor overexpressed in activated macrophages to regulate inflammation.[3] Under normal physiological conditions, placenta is the only tissue expressing large quantities of MIC-1.[4] However, expression increases during inflammation or organ injury.[4,5]

A role for MIC-1 in cancer is starting to emerge, but its functional significance in tumorigenesis is unknown.[6,7] Expression of MIC-1 increases in carcinomas of the breast, colon, pancreas, and prostate.[4,7,8] Increased expression in cancer cells can be accompanied by elevated protein levels in the serum of patients.[8–13] Secreted MIC-1 also has no identified role in cancer development.

The mitogen-activated protein (MAP) kinase pathway is deregulated in ~60% of sporadic melanoma through mutation of *B-Raf* into a constitutively active V600E (^{V600E}B-Raf) form.[14,15] This activated pathway in turn regulates diverse processes aiding tumor development such as proliferation, apoptosis, metastasis, and angiogenesis.[16–19] MIC-1 has not been directly linked to the ^{V600E}B-Raf pathway in melanomas.

MIC-1 has been reported as being overexpressed in melanomas compared with benign lesions.[20–22] Furthermore, inhibition of MIC-1 using short hairpin RNA (shRNA) decreased melanoma tumor development, but the mechanism promoting tumorigenesis is unknown.[22] This study reveals that MIC-1 can be regulated through

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^{V600E}B-Raf signaling and that it plays a novel role in melanoma development. MIC-1 is shown to be overexpressed in ~67% of aggressive melanomas and accompanied by elevated protein levels in the serum of patients. Small-interfering RNA (siRNA)-mediated targeting of MIC-1 reduced expression and secretion thereby retarding vascular development, which decreased the tumorigenic potential of melanoma cells by 60% to 70%.

Materials and Methods

Cell Lines and Culture Conditions

Melanoma cell lines, UACC 903, C8161, and A375M, were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT). Melanocytes and melanoma cell lines WM35, WM3211, WM 98.1, WM115, and WM278 were maintained as described previously.[23]

SiRNA Targeting MIC-1

SiRNA (100 pmol) was introduced into 1.0×10^6 UACC 903 or A375M via nucleofection by using an Amaxa Nucleofector (Koeln, Germany) using Solution R/program K-17 or A-23, respectively.[16–18,23,24] Transfection efficiency was >95% with 80% to 90% cell viability.[17,23] After siRNA introduction into cells, cells were allowed to recover for 2 days and then replated in 96-well plates. Five days later, cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter 96 AQueous Cell Proliferation Assay; Promega, Madison, WI). Duplexed Stealth siRNA (Invitrogen) were used for these studies. The following siRNA sequences were used: *^{V600E}B-Raf*: 5′-GGUCUAGCUACAGAGAAAUCUCGAU-3′; *MIC-1#1*: 5′-GAUUCCGAGAGUUGCGGAAACGCUA-3′; *MIC-1#2*: 5′-CAAGAACUCAGGACGGUGAAUGGCU-3′; *MIC-1#3*: 5′-UCGGACCAACUGCUGGCAGAAUCUU-3′; and vascular endothelial growth factor (*VEGF*): 5′-GCACATAGGAGAGATGAGCTTCCTA-3′.

Ectopic Expression of ^{V600E}B-Raf Constructs

Five, 7.5, and 10 μg of hemagglutinin-tagged ^{V600E}B-Raf constructs[25] were introduced into C8161 cells with the Amaxa Nucleofector by using the K17 program. Transfection efficiencies were determined to be ~70% by using pMaxGFP (Koeln, Germany) as a green fluorescent protein expression plasmid control.

MIC-1 and VEGF Sandwich Enzyme-Linked Immunosorbent Assay

MIC-1 sandwich enzyme-linked immunosorbent assay (ELISA) was used for quantification of protein levels in cell culture media and blood samples with the DuoSet ELISA for human GDF15 (R&D Systems, Minneapolis, MN) following the manufacturer's protocol. After introducing siRNA by nucleofection, cells were replated and media were changed 24 hours later into fresh Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone). The next day, conditioned media were collected from the plates and analyzed. Assays were replicated at least twice. For VEGF, the human VEGF Quantikine kit (DVE00; R&D Systems) was used according to the manufacturer's instructions.

Western Blot Analysis

Western blot analysis was undertaken as described previously.[23,25,26] Blots were probed with antibodies according to each supplier's recommendations: antibodies to MIC-1 (NAG-1) from Upstate Inc. (Lake Placid, NY) and B-Raf, α-enolase, from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies conjugated with horseradish peroxidase were obtained from Santa Cruz Biotechnology. Immunoblots were developed by using the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Piscataway, NJ). Blots were normalized to α-enolase and quantified by using ImageJ software.[27,28] Analyses were replicated at least twice.

Animal Studies

All animal experiments were done according to protocols approved by the Institutional Animal Care and Use Committee at The Pennsylvania State University. Tumor formation was measured in athymic-Foxn1^{nu} nude mice purchased from Herlan Sprague Dawley (Indianapolis, IN). Five hundred picomoles of siRNA was nucleofected into 5.0×10^6 cells and after 48 hours of recovery, 1.0×10^6 cells were collected in 0.2 ml of 10% fetal bovine serum–Dulbecco's modified Eagle's medium to inject subcutaneously above both the left and right rib cages of 4- to 6-week-old female mice. Each group contained five mice, and each experiment was replicated twice. Dimensions of developing tumors were measured on alternate days by using calipers. Assays were replicated at least twice.

Studies Involving Human Melanoma Patient Tumors

All experiments using human material were undertaken according to protocols approved by the Institutional Review Board Committee at The Pennsylvania State University. Tissue samples were collected from 29 patients at surgery, immediately snap frozen in liquid nitrogen, and stored at −80°C until protein lysate collection. To collect protein for Western blotting, tumors were pulverized by using a mortar and pestle chilled in liquid nitrogen. Protein lysates were extracted from tumors as reported previously[23] and analyzed by Western blotting to measure levels of MIC-1. MIC-1 protein levels in tumors were normalized to an α-enolase loading control to quantify the relative quantity of MIC-1 expression by using ImageJ software. Fresh blood was collected from donors who were approved by the Biomedical Institutional Review

Board at The Pennsylvania State University (HY02-101EP-A), with informed consent provided according to the Declaration of Helsinki. For ELISA analysis, serum samples were obtained after removal of blood cells and stored at −80°C until analysis. Analyses were replicated at least twice.

Cell Proliferation, Apoptosis, and Vessel Density Analysis in Time-Size Matched Tumors

To generate tumors of the same size developing at parallel time points, 1.0×10^6 cells were injected into nude mice for UACC 903 cells nucleofected with either water control or scrambled siRNA and 2.0×10^6 cells injected for UACC 903 cells nucleofected with MIC-1 siRNA. Time and size matched tumors were harvested 9, 11, and 13 days later to assess changes in cell proliferation, apoptosis, and vessel density. Cell proliferation rates in formalin-fixed tumor sections were measured by using Ki-67 staining as described previously.[16,26] The number of Ki-67-stained cells was quantified as the percentage of total cells in tumors. Apoptosis rates were measured on formalin-fixed, paraffin-embedded tumor sections by using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling tetramethylrhodamine (TMR) red apoptosis kit from Roche (Mannheim, Germany).[16,26] Analyses were replicated at least twice.

Chick Chorioallantoic Membrane Assay

Day 0 fertilized White Leghorn eggs were provided by Hy-Line North America (Elizabethtown, PA) and used for the chorioallantoic membrane (CAM) assay according to published procedures.[29] Eggshells were cleaned with 70% ethanol and turned parallel in an incubator rack maintained at 37°C with 60% to 70% humidity. To prevent CAM adhesion to the inner eggshell, eggs were rotated frequently until albumen removal. Two milliliters of albumen was removed from the pointed end of each egg by using a 21 G syringe-needle on day 3. A second hole was made to puncture the air sac on the other end of the egg, causing the CAM to pull away from the shell membrane. A 1.0×1.0 cm square window was cut through the shell over the area from which the CAM had retracted with the use of a diamond-tipped glass slide marker. The window was sealed with transparent tape, and embryos were incubated to enable development of a full vasculature. On day 10, 1.0 mm^2 filter papers impregnated with varying concentrations of recombinant MIC-1 or media from cells nucleofected with siRNA were implanted on top of the CAM. Capillary tube formation was examined 3 days later, when the angiogenic response had peaked. CAM was fixed by injecting 2 ml ice-cold 4% paraformaldehyde-PBS into the egg and on top of the CAM that was left at 4°C overnight. The next day, the CAM was excised with small dissection scissors and forceps. It was placed in PBS and mounted on a microscope slide. Blood vessels entering the paper were photographed and counted. Assays were replicated at least twice.

Matrigel Plug Assay

Recombinant VEGF (200 pg/ml, VEGF165, R&D Systems) or MIC-1 (2.5 ng/ml or 5.0 ng/ml, GDF-15, R&D Systems) either alone or in combination was mixed with unpolymerized liquid Matrigel (BD Biosciences, San Jose, CA) containing 20 μg/ml heparin at 4°C. Matrigel (0.6 ml each) was injected subcutaneously into the abdominal region of C57/BL6 female mice by using a cold syringe. At body temperature, the Matrigel polymerized to form a solid gel plug. Each group contained four mice with two plugs per mice. After 10 days, gels from all groups were collected and fixed overnight in fresh 10% formalin and then paraffin embedded for H&E staining. Blood vessels in Matrigel plugs were quantified by using microscopy to score the area occupied by vessels over total area calculated by using the IP Lab imaging software program (San Jose, CA). Assays were replicated at least twice.

Statistical Analysis

One-way analysis of variance or nonparametric one-way analysis of variance Kruskal-Wallis test followed by Dunnett's or Bonferroni multiple comparison tests were used to establish statistical significance. Results were considered significant at a P value of <0.05.

Results

MIC-1 Protein Expression and Secretion Are Elevated in Melanoma Patients

Although MIC-1 is known to be overexpressed in melanomas, it is unknown whether it is secreted into the serum of melanoma patients.[22] Figure 1A shows that 66.7% (18 of 27) of melanoma patients have advanced metastatic tumors that express MIC-1 protein at higher levels than that observed in normal human melanocytes, confirming prior reports. To extend this prior knowledge, serum from 10 metastatic melanoma patients was examined and found to have MIC-1 levels ranging from 0.5 to 3.2 ng/ml compared with healthy blood donors having concentrations ranging from 0.2 to 0.4 ng/ml. Patients who had elevated expression of MIC-1 in tumors also had high levels of MIC-1 in their blood serum (Figure 1, A and B, labeled a, b, and c). Eighty percent of patients had serum MIC-1 levels higher than 1 ng/ml (Figure 1B). Thus, metastatic melanoma patients having elevated MIC-1 expression in tumors also had high levels of MIC-1 in blood serum.

MIC-1 Protein Is Not Expressed in Normal Skin Cells but Is Overexpressed in Melanoma Cell Lines

Levels of MIC-1 protein were measured in a variety of melanoma cell lines derived from primary tumors at the radial (RGP; WM35 and WM3211), vertical (VGP; WM115, WM98.1, and WM278) and metastatic (MM; UACC 903)

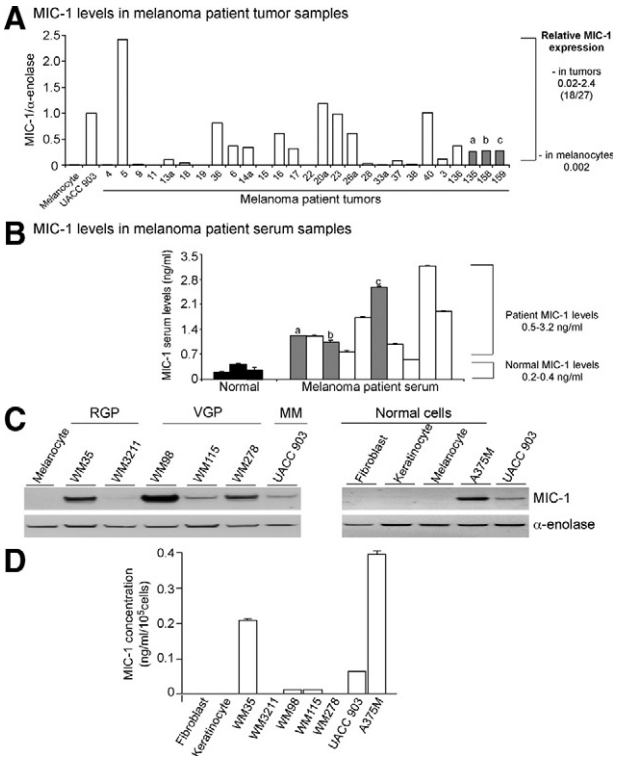

Figure 1. MIC-1 is overexpressed in and secreted from melanomas. **A:** MIC-1 protein was expressed at higher levels in melanoma patient tumors compared with normal human melanocytes. α-Enolase served as a control for equal protein loading. **B:** MIC-1 concentrations are elevated in the blood serum of melanoma patients compared with normal individuals. MIC-1 concentrations in blood serum of melanoma patients were measured by ELISA. Values are presented as mean ± SEM. **C:** MIC-1 is overexpressed in the majority of melanoma cell lines. Expression of MIC-1 was examined in melanoma cell lines established from primary tumors at the radial (RGP; WM35 and WM3211), vertical (VGP; WM115, WM98.1, and WM278), and metastatic (MM; UACC 903) stages of melanoma progression. Only WM3211 did not express MIC-1 at levels higher than that observed in melanocytes. Normal skin melanocytes, fibroblasts, and keratinocytes did not contain detectable levels of MIC-1 protein by Western blotting. α-Enolase was used as a control for equal protein loading. **D:** MIC-1 was secreted from metastatic melanoma cell lines but not at significant levels from RGP or VGP cell lines. MIC-1 concentrations in media were measured by ELISA. Values are presented as mean ± SEM.

stages of melanoma progression and compared with normal skin cells. All melanoma cell lines, except for WM3211 cells, expressed MIC-1 levels higher than occurs in normal human melanocytes (Figure 1C). Furthermore, MIC-1 expression and secretion was also not detectable in normal human fibroblasts or keratinocytes found in skin (Figure 1, C and D). Barely detectable levels of MIC-1 were found in conditioned media collected from RGP (except WM35) and VGP cell lines; however, high levels were observed in metastatic UACC 903 and A375M melanoma cell lines (Figure 1D). WM35 seems to be an aberration among the group because the majority of RGP and VGP cells lines express but do not secrete the protein at high levels. In contrast, both metastatic melanoma cell lines and serum from metastatic melanoma patients express and secrete significant levels of MIC-1 (Figure 1). Thus, MIC-1 is overexpressed in and secreted from metastatic melanomas, but although present in the majority of RGP or VGP cells lines, it is generally not secreted to very high levels from them.

Targeting ^{V600E}B-Raf in the MAP Kinase-Signaling Cascade Decreased MIC-1 Expression

It is unknown as to which aberrant pathways promote MIC-1 deregulation in melanomas. Therefore, to test whether it might be regulated by ^{V600E}B-Raf signaling, siRNA was used to decrease ^{V600E}B-Raf protein levels and effect on MIC-1 expression examined. SiRNA targeting ^{V600E}B-Raf or three different regions of MIC-1 were introduced into UACC 903 or A375M via nucleofection, and Western blotting was used to show significant reduction of each respective protein (Figure 2, A and B). In UACC 903 cells, all three siRNAs targeting MIC-1 similarly inhibited protein expression by >80% compared with control cells nucleofected with buffer or scrambled siRNA (Figure 2A). However, in A375M melanoma cells, only siMIC-1 number 1 and siMIC-1 number 3 reduced MIC-1 protein expression by ~80% (Figure 2B). The latter results were not unusual because normal sequence variation can affect the efficacy of siRNA in certain but not other cell lines.[28,30] Importantly, decreased ^{V600E}B-Raf protein levels in both cell lines reduced expression of MIC-1, suggesting that it lies downstream in the MAP kinase pathway. In contrast, targeting MIC-1 by using siRNA did not alter B-Raf protein expression (Figure 2, A and B). Thus, MIC-1 expression in melanomas appears to be mechanistically regulated through ^{V600E}B-Raf signaling.

Targeting MIC-1 or Upstream ^{V600E}B-Raf Decreased Secretion from Melanoma Cells

Next, siRNA was introduced into melanoma cells and effect on secreted MIC-1 was measured (Figure 2, C and D). ELISA showed that siRNA-mediated targeting of MIC-1 reduced secreted levels by 3.5-fold from 3 to 0.8 ng/ml for UACC 903 cells (Figure 2C) and by eightfold from 17 to 2 ng/ml for A375M cells (Figure 2D). SiRNA-mediated targeting of ^{V600E}B-Raf similarly reduced secreted MIC-1 concentrations (Figure 2, C and D). Thus, decreasing cellular levels of MIC-1 or ^{V600E}B-Raf also reduced MIC-1 levels secreted into culture media.

Decreasing MIC-1 Protein Levels in Melanoma Cells Does Not Alter Growth in Culture

Because MIC-1 was found to lie downstream of ^{V600E}B-Raf signaling, which is known to regulate cellular proliferation, effects on cell growth were examined after siRNA-mediated targeting of MIC-1 or ^{V600E}B-Raf.[16,25,28] Although targeting ^{V600E}B-Raf inhibited cultured melanoma cell growth by ~80% (Figure 3A), reducing MIC-1 protein levels had no effect on UACC 903 or A375M cell growth compared with controls, suggesting a function other than growth control (Figure 3A). Thus, even though MIC-1 lies downstream of ^{V600E}B-Raf, it does not regulate melanoma cells growth.

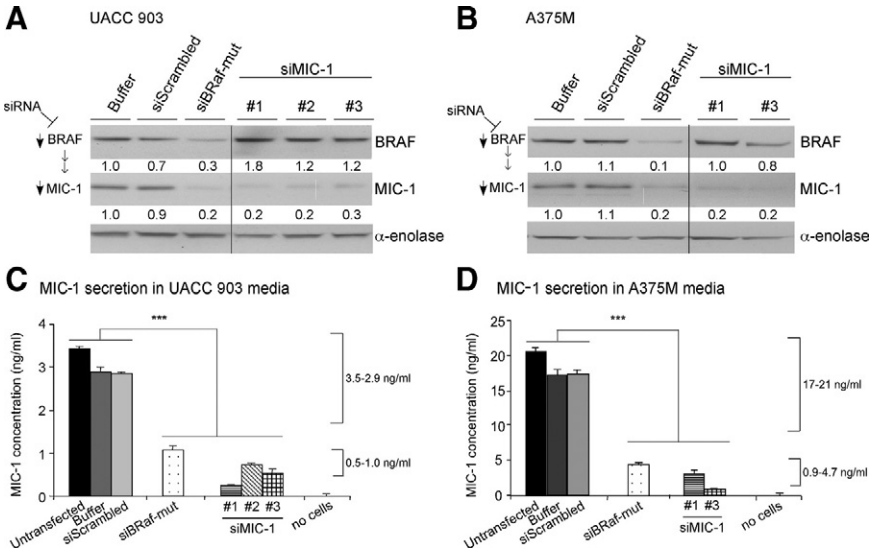

Figure 2. Targeting mutant *^{V600E}B-Raf* reduced levels of MIC-1 protein expression in and secretion from melanoma cells. SiRNA-mediated inhibition of mutant *^{V600E}B-Raf* significantly reduced MIC-1 protein expression in UACC 903 (**A**) and A375M (**B**) melanoma cells compared with untransfected controls or cells nucleofected with buffer or scrambled siRNA. Reduction of mutant ^{V600E}B-Raf protein expression decreased MIC-1 levels similar to that observed after inhibition of MIC-1 expression by using siRNA. α-Enolase was used as a control for equal protein loading. Inhibition of mutant *^{V600E}B-Raf* reduced MIC-1 secretion from UACC 903 (**C**) and A375M (**D**) melanoma cells. Media alone were used as a negative control. Values are presented as mean ± SEM. $^{***}P < 0.001$ compared with controls.

Ectopic Expression of ^{V600E}B-Raf in Melanoma Cells Containing Wild-Type B-Raf Increased MIC-1 Secretion

To confirm that constitutively active ^{V600E}B-Raf protein regulates MIC-1 expression and secretion, HA-tagged ^{V600E}B-Raf was transiently transfected into C8161 melanoma cells lacking the mutation and having low pathway activity. Cells ectopically expressing HA-^{V600E}B-Raf had ~50% higher levels of secreted MIC-1, compared with vector-transfected control cells (Figure 3B, $^{*}P < 0.05$, one-way analysis of variance). Thus, MIC-1 secretion can be regulated by ^{V600E}B-Raf signaling in melanomas.

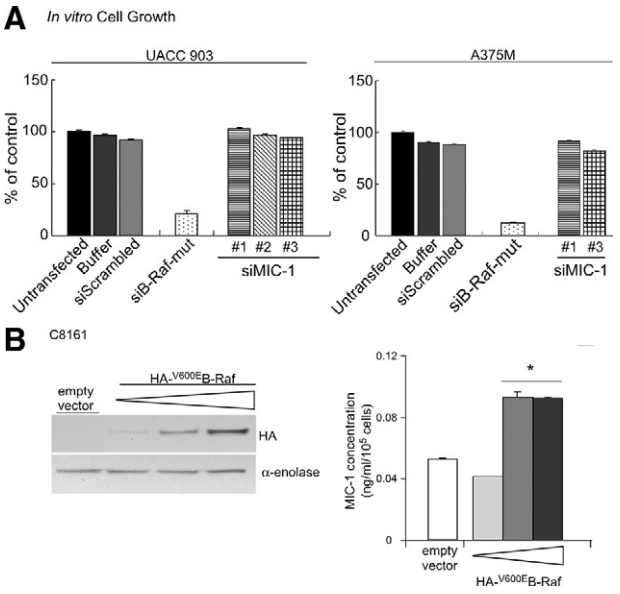

Figure 3. Decreasing MIC-1 expression does not alter the growth of cultured melanoma cells. **A:** SiRNA-mediated knockdown of MIC-1 protein expression did not affect the growth of cultured melanoma cells. UACC 903 or A375M cell viability was measured by using an MTS assay after siRNA-mediated knockdown of MIC-1. Values are presented as mean ± SEM. ^{V600E}B-Raf protein knockdown was used as a positive control for inhibition of cell growth. **B:** Ectopic expression of HA-tagged-^{V600E}B-Raf led to a 50% increase in secretion of MIC-1 from melanoma cells. HA-tagged-^{V600E}B-Raf (5.0, 7.5, and 10 μg of construct) was ectopically expressed in C8161 melanoma cells lacking ^{V600E}B-Raf. Western blotting shows expression of HA-tagged protein. α-Enolase was used as a control for equal protein loading. ELISA showed a 50% increase in MIC-1 secretion from cells expressing HA-tagged-^{V600E}B-Raf compared with controls. Values are presented as mean ± SEM. $^{*}P < 0.05$ compared with controls.

Decreased MIC-1 Protein Expression Stunts Melanoma Tumor Development

To identify the functional role played by MIC-1 in melanoma development, an established published approach was used in which siRNA was introduced into UACC 903 and A375M and subsequently injected in nude mice.[16,26] Developing tumor sizes were measured on alternate days up to 19.5 days. SiRNA-mediated targeting of MIC-1 in both UACC 903 (Figure 4A) and A375M (Figure 4B) melanoma cells reduced tumorigenesis by 60% to 70%. Tumors initially grew to ~100 mm^3 for UACC 903 and ~50 mm^3 for A375M cells followed by a prolonged period of no or minimal growth. Serum collected from control mice containing A375M xenografted tumors at day 19.5 was analyzed for MIC-1 levels by ELISA and showed the presence of 50% to 75% less secreted protein in tumors in which MIC-1 expression had been reduced by using siRNA compared with controls (Figure 4C). Thus, throughout this experiment, MIC-1 levels in blood serum remained low compared with controls mediated by the siRNA introduced into the cells.

Targeting MIC-1 Retards Melanoma Vascular Development

To identify the underlying mechanism by which MIC-1 inhibition impedes melanoma tumor development, an established published approach was used.[16,26] It involves quantifying the rates of tumor angiogenesis (using CD31 stating), cell proliferation (using Ki-67), and apoptosis (using terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) in time and size matched tumors

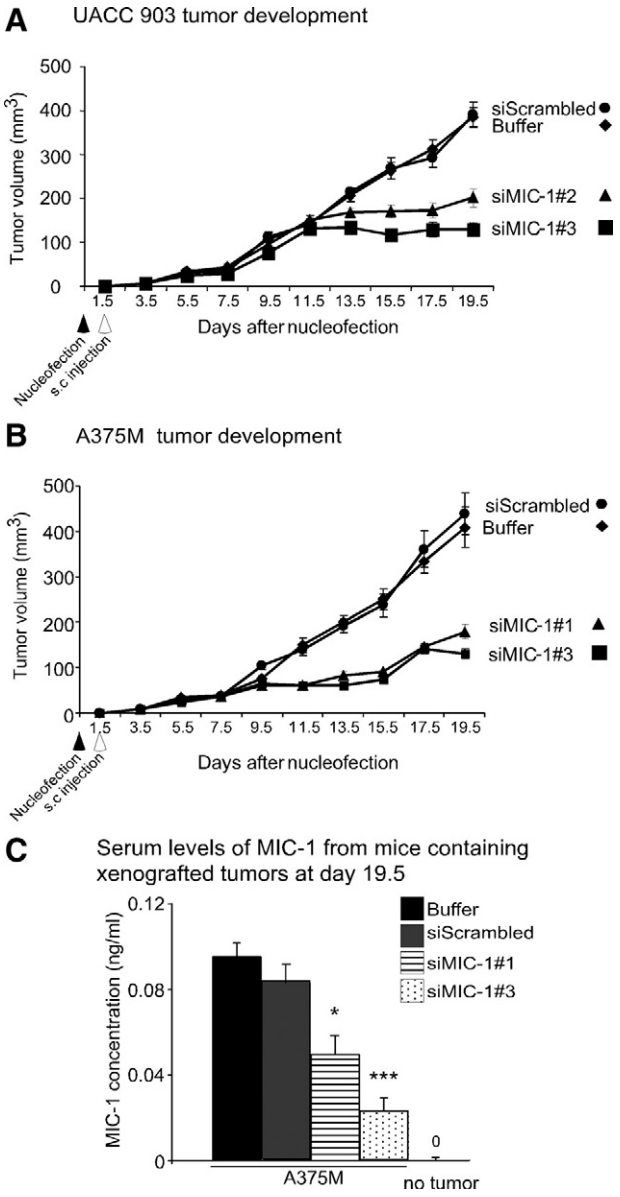

Figure 4. SiRNA-mediated inhibition of MIC-1 retarded the tumorigenic potential of melanoma cells. Targeting MIC-1 by using siRNA inhibits melanoma tumor development. SiRNA targeting MIC-1, siScrambled, or buffer controls were nucleofected into UACC 903 (**A**) or A375M (**B**) cell lines, and 36 hours later, cells were subcutaneously injected into nude mice. Tumor size was measured on alternate days up to day 19.5. Values are presented as mean ± SEM. **C:** siRNA-mediated inhibition reduced MIC-1 levels in mouse blood serum by 50%. At day 19.5 after nucleofection of siRNA targeting MIC-1, levels secreted from A375M tumors were measured from five separate mice by ELISA. Mice lacking xenografted tumors were used as negative control. Values are presented as mean ± SEM. $^{*}P < 0.05$; $^{***}P < 0.001$ compared with controls.

treated with siRNA targeting MIC-1 compared with controls. Cells were subcutaneously injected into nude mice, and on days 9, 11, and 13, size-matched tumors were removed from animals. Decreased MIC-1 protein expression of 50% was observed from day 13 for UACC 903 and A375M xenografted tumors, which were normalized against α-enolase (Figure 5A). Blood serum collected at day 13 from mice containing tumors in which MIC-1 expression has been reduced by using siRNA showed a statistically significant 50% decrease compared with controls (Figure 5B, $^{*}P < 0.05$, $^{**}P < 0.01$ one-way analysis of variance). Size and time matched tumors at each time were then compared with the first statistically quantifiable difference in vascular development, proliferation, or apoptosis to identify the key event through which MIC-1 regulated tumorigenesis.[16,26] At day 9, a statistically significant 50% reduction in vessel development was observed after MIC-1 targeting but not in cellular proliferation or apoptosis rates compared with buffer or scrambled siRNA controls (Figure 5C, $^{*}P < 0.05$, $^{**}P < 0.01$, one-way analysis of variance). There was a significant difference in proliferation between control cells and those nucleofected with siMIC-1 number 3 at day 9; however, because this difference was not observed with siMIC-1 number 2, it was considered as an inconsistency and not the major cause of tumor inhibition. Similar significant differences in vascular development, cellular proliferation, and apoptosis were detected in all tumors compared with controls at days 11 and 13, suggesting that lack of development of an effective vascular network subsequently decreased cell proliferation and triggered apoptosis (Figure 5C, $^{**}P < 0.01$, one-way analysis of variance). Thus, inhibiting MIC-1 secretion by tumor cells retarded tumor angiogenesis, subsequently decreasing cellular proliferation and increasing apoptosis.

Directional Vessel Growth Occurs Toward MIC-1 in the Chick Chorioallantoic Membrane Vessel Stimulation Assay

One technique to demonstrate that a secreted protein functions as an angiogenic factor measures vessel development in the direction of the protein by using the chick chorioallantoic membrane vessel stimulation assay.[29] Filter papers (1 mm^2) were soaked with physiological concentrations of recombinant MIC-1 ranging from 2.5 to 500 ng/ml and placed on developing chick chorioallantoic membranes. The number of blood vessels growing toward recombinant MIC-1 in the filter paper was photographed and quantified 3 days later (Figure 6A). Compared with filter paper soaked with PBS, recombinant MIC-1 caused 100% vessel growth toward the filter paper and also led to a twofold increase in vessel number from 2.5 to 10 ng/ml, which plateaued at concentrations >10 ng/ml (Figure 6A). Thus, secreted MIC-1 stimulated directional vessel development, demonstrating it can act as an angiogenic factor.

Co-Stimulation of VEGF and MIC-1 Promotes Blood Vessel Formation

Because VEGF secretion is also regulated by ^{V600E}B-Raf in malignant melanomas,[16] MIC-1 and/or VEGF were targeted by using siRNA to reduce secretion of each respective protein to compare the role of each in melanoma (Figure 6B). Filter papers (1 mm^2) soaked with conditioned media collected from cells nucleofected with siRNA targeting MIC-1 and/or VEGF were placed on de-

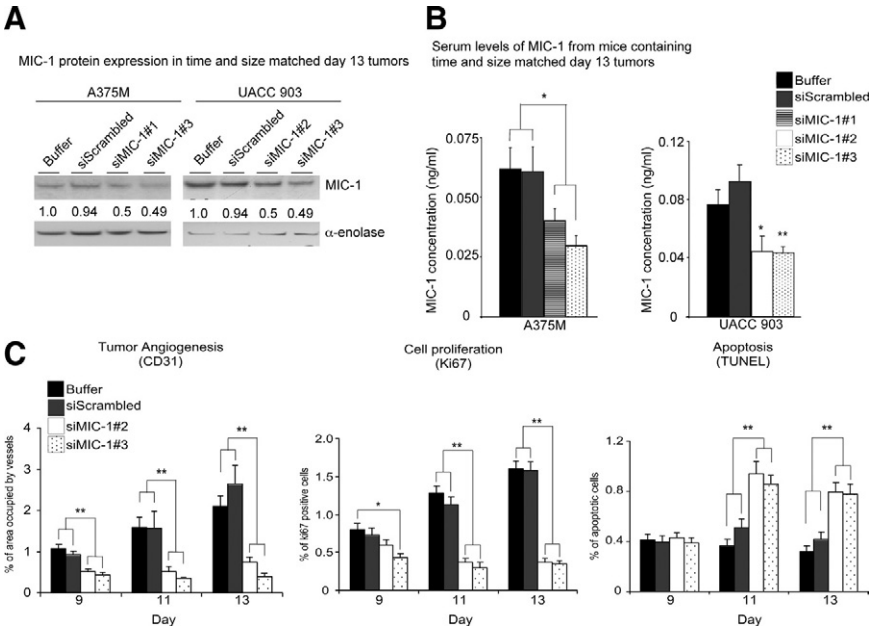

Figure 5. Targeting MIC-1 reduced the angiogenic potential of melanoma cells to decrease melanoma tumor development. **A:** SiRNA-mediated inhibition of MIC-1 in A375M or UACC 903 tumors reduced protein expression by 50% in time and size matched tumors. **B:** siRNA-mediated inhibition of MIC-1 in A375M or UACC 903 tumors reduced serum levels of the protein in mice bearing day 13 time and sized matched tumors by 50% (*$P < 0.05$; **$P < 0.01$ compared with controls). **C:** SiRNA-mediated inhibition of MIC-1 reduced vascular development to decrease tumor development. Comparison of vascular development, proliferation, and cellular apoptosis rates in size and time matched UACC 903 tumors differing in MIC-1 protein expression. Change in vascular development was the first statistically significant difference (**$P <$ 0.01) observed in size and time matched tumors in which MIC-1 was targeted by using siRNA. Reduced cell proliferation (*$P < 0.05$; **$P <$ 0.01) and increased apoptosis (**$P < 0.01$) were consistently observed starting at day 11. Columns represent means of 12 fields analyzed from four tumors per group; bars represent ± SEM.

veloping chick chorioallantoic membranes. The number of blood vessels growing toward the proteins was photographed and quantified 3 days later (Figure 6B). Compared with control filter paper soaked with conditioned media from buffer and scrambled siRNA controls, conditioned media from cells having decreased MIC-1 or VEGF secretion reduced vessel number by 40% (Figure 6B). Reducing levels of secreted MIC-1 and VEGF cooperatively reduced vessel to levels observed with media that had not been exposed to the cells. Thus, melanoma cells secrete MIC-1 to promote directional blood vessel development in a manner similar to VEGF.

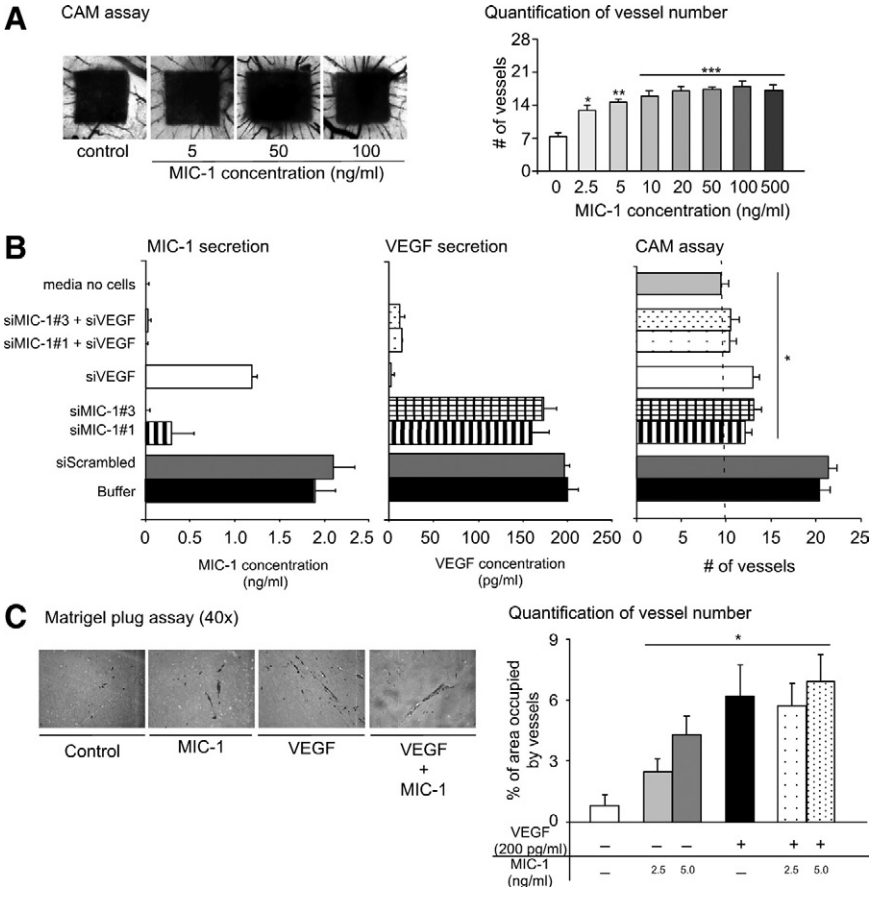

Figure 6. MIC-1 and VEGF promote melanoma vascular development. **A:** MIC-1 increased blood vessels formation in the direction of filter paper soaked with MIC-1. Quantification of newly formed blood vessels showed MIC-1 promoted angiogenesis in the CAM assay. A minimum of 20 filter papers were photographed (original magnification, ×40) and quantified to determine blood vessel number. PBS served as negative control. Values are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. **B:** Targeting MIC-1 and/or VEGF suppressed blood vessel formation compared with controls in the chick chorioallantoic membrane assay. SiRNA was used to decrease secreted levels of MIC-1 and/or VEGF and conditioned media used on a CAM assay. Values are presented as mean ± SEM. **C:** MIC-1 and VEGF stimulation alone and in combination increased formation of blood vessels in the matrigel plug assay. Matrigel was mixed with 2.5 or 5.0 ng/ml of MIC-1 and/or 200 pg/ml VEGF and injected subcutaneously into mice. After 10 days, matrigel plugs were removed, fixed, and photographed (original magnification, ×40). Blood vessels formed in the gels were quantified. Values are presented as mean ± SEM. *$P < 0.05$ compared with controls.

To further validate the angiogenic potential of MIC-1, an *in vivo* Matrigel plug assay was performed in which matrigel containing MIC-1 (2.5 ng/ml or 5.0 ng/ml), VEGF (200 pg/ml), or a combination of both were injected subcutaneously in C57/BL6 mice. After 10 days, subcutaneous gel plugs were removed from mice and blood vessel development was quantified as the percentage of area occupied by vessels (Figure 6C). MIC-1 treatment led to a threefold to sixfold increase in blood vessel area compared with controls (Figure 6C). MIC-1 and VEGF together produced a 7.5-fold increase in vessel development, which was greater that MIC-1 alone but not more than VEGF alone, suggesting the response had been maximized (Figure 6C). Thus, MIC-1 alone was sufficient to induce blood vessel formation, which could be enhanced in combination with VEGF.

Discussion

Fibroblast, keratinocytes, and melanocytes which are cells constituting the normal skin layer have undetectable levels of MIC-1, whereas ~67% of melanoma patient tumors had measurable concentrations. This study shows that MIC-1 secreted from tumors can regulate an important process in melanoma development. Factors secreted by tumors are known to alter the surrounding environment by regulating immune, fibroblasts, or endothelial cells' activities to promote tumor development.[31,32] Decreasing MIC-1 expression and secretion from melanoma cells did not alter cultured cell survival but significantly decreased tumor development, suggesting it could be regulating an extracellular process important in tumor development. Decreased tumor vascular development was observed in time and size matched tumors in which siRNA had been used to reduce MIC-1 protein expression compared with control tumors. Involvement of MIC-1 in melanoma angiogenesis was subsequently confirmed by showing that it could regulate angiogenesis in chick chorioallantoic membrane vessel stimulation and matrigel plug assays. Physiologically relevant concentrations of MIC-1 and not abnormally high levels were used in these models, demonstrating that MIC-1 could double directional blood vessel formation thereby confirming that it could play a role in angiogenesis. Collectively, these observations led to the conclusion that the mechanistic basis for tumor reduction after targeting MIC-1 was retardation in vascular development. However, it is possible that MIC-1 can also regulate other processes involved in tumor development or that other secreted factors could also cooperate in this process.

MIC-1 is a member of the TGF-β family, whose proteins perform a wide variety of complex and contradictory roles dependent on cancer type, tumor stage, interaction with tumor microenvironment, or genetic status of cyclin dependent kinase inhibitors such as $p15^{INK4b}$, $p21^{WAF1/CIP1}$, or Smads of the tumor cell.[1,7] MIC-1 promotes tumor development under one circumstance and prevents it under another, making its function dependent on a particular tumor type.[7,33] Although this is the first report indicating a role for MIC-1 in melanoma angiogenesis, it has also been documented to aid cancer development by up-regulating transcription factors playing key roles in tumorigenesis such as nuclear factor-κB, early growth response factor-1, and microphthalmia-associated transcription factor in gastric cancer and melanomas.[7,22,34] Increased MIC-1 expression also aids tumor development by activating epidermal growth factor receptor 2 tyrosine kinase in human breast and gastric cancer cells, which induced hypoxia inducible factor-1α through PI3 and MAP kinase pathways.[35] MIC-1 can increase expression of urokinase type plasminogen activator and the urokinase type plasminogen activator receptor as well as decreasing *RhoE* and *catenin δ1* gene expression to reduce cell adhesion, thereby regulating cell migration through autocrine signaling.[34,36] Additionally, MIC-1 can be inhibitory to tumor development as observed when p53 up-regulates MIC-1 expression to inhibit tumor growth by inducing cell cycle arrest and apoptosis.[7,37–40] MIC-1 overexpression has also been found to increase $p21^{WAF1/CIP1}$ levels, which in turn led to G1 phase cell cycle arrest.[37] Whether MIC-1 regulates other important process in addition to angiogenesis in melanomas remains to be determined.

Constitutive activation of the MAP kinase pathway by *B-Raf* mutation is important for melanoma development leading to regulation of tumor cellular proliferation, apoptosis, vascular development, and metastasis.[16,17,25] However, downstream genes regulating each of these essential tumorigenic processes remain to be fully identified.[41–43] This report identifies MIC-1 as being one of these downstream proteins since decreased ^{V600E}B-Raf expression reduced MIC-1 levels in and secretion from melanoma cells and ectopic expression of ^{V600E}B-Raf in melanoma cells lacking this mutant protein increased MIC-1 secretion. The exact mechanism by which ^{V600E}B-Raf up-regulates MIC-1 expression remains to be fully elucidated but is likely to involve activation of transcription factors such as microphthalmia-associated transcription factor[44,45] or nuclear factor-κB,[18,46] which play key roles in melanocyte differentiation as well as melanoma survival and proliferation.[18,22,44,45] The *MIC-1* promoter contains binding sites for these transcriptional regulators that could enhance its expression.[22]

VEGF is another angiogenic factor regulated by ^{V600E}B-Raf signaling in melanomas[16]; 2.5 to 5.0 ng/ml MIC-1 and 200 pg/ml VEGF stimulation alone or in combination in this report increased blood vessel formation compared with controls. Other authors have shown that 100 ng/ml MIC-1 inhibits angiogenesis when co-stimulated with 100 ng/ml VEGF in the matrigel plug assay.[47] This discrepancy is likely due to use of concentrations higher than the normal physiological range seen in melanomas (0.5 to 3.4 ng/ml) and other cancers such as pancreatic cancer (2.0 to 2.5 ng/ml).[11] It is well documented that proteins can be stimulatory at one concentration and inhibitory at a higher one as seen with the angiogenic properties of Hepatocyte Growth Factor (HGF).[48] This possibility is further supported in this report by observation that concentrations higher than 10 ng/ml of MIC-1 led to a plateau effect in blood vessel formation in the chick chorioallantoic membrane vessel stimulation

assay; 3.2 ng/ml was the highest concentration measured in serum from melanoma patients, suggesting higher amounts might not be more effective. Furthermore, 2.5 to 5.0 ng/ml MIC-1 and 200 pg/ml VEGF maximized vessels development in the matrigel plug assay again supporting this possibility.

In conclusion, ^{V600E}B-Raf signaling is one pathway regulating MIC-1 expression, leading to secretion that stimulates angiogenesis and development of a vascular network enabling an increase in tumor size. Without MIC-1, melanoma tumor development can be stunted due to lack of vascular development, which in turn decreases cell proliferation and triggers apoptosis. Thus, MIC-1 is a useful prognostic marker, a regulator of angiogenesis, and a potentially important therapeutic target for melanoma.

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