Milk Fat Globule EGF-8 Promotes Melanoma Progression through Coordinated Akt and Twist Signaling in the Tumor Microenvironment

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Abstract

The pathogenesis of malignant melanoma involves the interplay of tumor cells with normal host elements, but the underlying mechanisms are incompletely understood. Here, we show that milk fat globule EGF-8 (MFG-E8), a secreted protein expressed at high levels in the vertical growth phase of melanoma, promotes disease progression through coordinat ed αvβ3 integrin signaling in the tumor microenvironment. In a murine model of melanoma, MFG-E8 enhanced tumorigenicity and metastatic capacity through Akt-dependent and Twist-dependent pathways. MFG-E8 augmented melanoma cell resistance to apoptosis, triggered an epithelial-to-mesenchymal transition (EMT), and stimulated invasion and immune suppression. In human melanoma cells, MFG-E8 knockdown attenuated Akt and Twist signaling and thereby compromised tumour cell survival, EMT, and invasive ability. MFG-E8-deficient human melanoma cells also showed increased sensitivity to small molecule inhibitors of insulin-like growth factor 1 receptor and c-Met. Together, these findings delineate pleiotropic roles for MFG-E8 in the tumor microenvironment and raise the possibility that systemic MFG-E8 blockade might prove therapeutic for melanoma patients. [Cancer Res 2008;68(21):8889–98]

Introduction

The initiation and progression of cancer involves multiple interactions between transformed cells and normal host elements (1). Whereas cytotoxic lymphocytes may prevent or impede tumor growth in some cases, unresolved inflammation is a common and potent tumor promoter (2, 3). Early during carcinogenesis, innate immune cells, including macrophages, granulocytes, and mast cells, provide signals that regulate tumor cell growth and differentiation, whereas in later disease stages, stromal cell–derived cues foster angiogenesis, invasion, and metastasis (4, 5). These diverse pathogenic mechanisms are regulated in part by the mixture of soluble factors present in the tumor microenvironment. In particular, inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6 (II-6), and IL-1β, fuel disease progression through the activation of nuclear factor-κB (NF-κB), signal transducers and activators of transcription-3 (STAT-3), and MyD88-dependent pathways (6–9).

Granulocyte macrophage colony-stimulating factor (GM-CSF) is another cytokine frequently produced in the tumor microenvironment, but its precise role in carcinogenesis remains to be clarified fully. Vaccination with irradiated tumor cells engineered to secrete GM-CSF engenders protective immunity through improved tumor antigen presentation by dendritic cells and macrophages (10). Clinical testing of this vaccination scheme in patients with advanced solid and hematologic malignancies showed the consistent induction of intratumoral T-cell and B-cell infiltrates that effectuated extensive necrosis (11, 12). Further tumor destruction was accomplished through the subsequent administration of blocking antibodies to CTL-associated antigen-4 (13). Notwithstanding these therapeutic activities, tonic GM-CSF production in the tumor microenvironment is typically associated with disease progression, which may reflect in part the stimulation of myeloid suppressor cells that attenuate cytotoxic lymphocyte function (14–17).

Through an analysis of GM-CSF-deficient mice and irradiated GM-CSF-secreting tumor cell vaccines, we recently identified milk fat globule EGF-8 (MFG-E8) as a major determinant of GM-CSF function (18). MFG-E8 is a secreted phosphatidylinerine binding protein that engages αvβ3 and αvβ5 integrins (19, 20). Under steady-state conditions, GM-CSF triggers MFG-E8 expression in macrophages and dendritic cells, resulting in the efficient phagocytosis of apoptotic cells, the maintenance of FoxP3+ regulatory T cells (Treg), and the suppression of autoreactive Th1 and Th17 cells (18). However, under conditions of stress, Toll-like receptor agonists or necrotic cells down-regulate MFG-E8 levels, wherein GM-CSF evokes protective responses through an MFG-E8–independent mechanism. Together, these findings raise the possibility that the presence of MFG-E8 in the tumor microenvironment might skew GM-CSF activity toward disease promotion rather than inhibition. Consistent with this idea, we previously reported that infiltrating myeloid cells in diverse human cancers display strong MFG-E8 staining by immunohistochemistry (18).

In malignant melanoma, progression to the vertical growth phase is linked with the acquisition of competence for tumor cell invasion and distant dissemination (21, 22). In model systems, the enforced activation of Akt is sufficient to transform melanocytic lesions from the radial to the vertical growth phase (23). Clinicopathologic investigations delineated up-regulation of αvβ3 integrin expression on melanoma cells as the most consistent histologic marker for this stage of disease (24, 25). Melanoma cells thereby manifest an increased resistance to apoptosis and an
epithelial-to-mesenchymal transition (EMT), with down-regulation of E-cadherin and up-regulation of vimentin and N-cadherin (26–28). The enhanced release of vascular endothelial growth factor (VEGF), matrix metalloproteinase-2 (MMP-2), and MMP-9 at this stage may also stimulate angiogenesis and foster the breach of normal tissue planes (29–31). How these diverse mechanisms are orchestrated during melanoma progression, though, remains incompletely understood.

Based upon the increased expression of αvβ3 integrin on melanoma cells at the vertical growth phase and the ability of MFG-E8 to engage this receptor, we wondered whether MFG-E8 might play a role in melanoma biology. Here, we show that MFG-E8 production in tumor and infiltrating myeloid cells is associated with melanoma progression and that MFG-E8 acts as a potent tumor promoter through coordinated αvβ3 integrin signaling in the tumor microenvironment.

Materials and Methods

Mice. C57Bl/6 wild-type and GM-CSF–deficient mice backcrossed at least nine generations onto the C57Bl/6 strain were housed under specific pathogen-free conditions (32). All the experiments were conducted under a protocol approved by the Association for Assessment and Accreditation of Laboratory Animal Care International–accredited Dana-Farber Cancer Institute and Harvard Medical School Institutional Animal Care and Use Committee.

Histology and immunohistochemistry. Human melanocytic lesions from discarded tissues were embedded in paraffin, sectioned, and stained with H&E. Tissue sections were treated for antigen retrieval with a pressure cooker for 20 min, incubated with 5 μg/mL of mouse anti-human MFG-E8 primary antibody (R&D Systems) in 3% bovine serum albumin/PBS blocking solution for 16 h at 4°C, and developed with the corresponding secondary biotinylated antibody and the streptavidin-peroxidase complex from Vector Labs. The intensity of staining was graded from 0 to 3+ as follows: 0, no staining; 1+, faint, light beige staining; 2+, easily visible staining affecting most cells with a tan coloration; 3+, strong, diffuse brown staining of the cells.

Retroviral-mediated gene transfer. Full-length sequences encoding the open reading frames of murine MFG-E8 long splice form or the RGE mutant (which replaces the RGD sequence in the second epidermal growth factor domain with RGE) were introduced into the pMFG retroviral vector, high titer VSV-G-pseudotyped replication defective viral stocks prepared with 293-GPG cells and B16 cells transduced with the viruses, as previously described (18). Peritoneal macrophages were induced to replicate by culture in DME plus 10% FCS supplemented with 10 ng/mL M-CSF (R&D Systems). Viral supernatants were added to the cultured macrophages overnight in the presence of polybrene (8 μg/mL) to facilitate infection. The transduced cells were then washed and used for experiments 2 to 3 d later.

B16 melanoma model. Female C57Bl/6 mice (8–12 wk old) were injected s.c. in the flank with 2 × 10^5 live retrovirally transduced (GF, MFG-E8, and/or RGE) B16 cells, and the product of tumor diameters as a measure of tumor growth for 7 days. The efficacy of gene delivery was determined by Western blotting using antibodies against αvβ3 integrin (clone RMV-7; Millipore) yielding >95% pure population of NKp46 + CD3− cells, as well as CD3− NK cells. Expression of MFG-E8 in tumors was confirmed by immunohistochemistry using anti-MFG-E8 antibody followed by PE-labeled goat anti-IgG2 Ab (BD-PharMingen). For FoxP3 staining, lymphoid cells were labeled with anti-CD3 and anti-CD4 antibodies (BD PharMingen) and then stained with PE-labeled goat anti-IgG2 Ab (BD-PharMingen).

Flow cytometry. Tumor infiltrates were obtained from B16 challenge sites using a Nocorep (Axis-Shield) cell gradient separation. The cells were analyzed by flow cytometry using monoclonal antibodies (mAb) against CD3, CD4, CD11b, Gr-1, and Foxp3. For MFG-E8-stained, myeloid cells were pretreated with GolgiPlug (BD-PharMingen), stained with anti-CD11b and anti-GR1 mAb (BD-PharMingen), fixed, permeabilized with Cytofix/Cytoperm buffer (BD-PharMingen), and stained again with unlabelled FoxP3 mAb (Alexia) followed by PE-labeled goat anti-IgG2 Ab (BD-PharMingen). For FoxP3 staining, lymphoid cells were labeled with anti-CD3 and CD4 mAbs (BD PharMingen), washed, and then stained with PE-labeled anti-FoxP3 antibody using the FoxP3 staining set according to the manufacturer’s protocol (eBioscience). The frequency of each immune cell populations was determined by flow cytometry. Cell acquisition was done with a FW501 flow cytometer (Beckman-Coulter) and analyzed by FlowJo software (Tree Star).

Immunoblotting. Transduced B16 melanoma cells or wild-type B16 cells exposed to supernatants harvested from transduced macrophages were subjected to Western blotting using antibodies against MFG-E8 (MBL International), phosphorylated Akt, Akt, phosphorylated S6 kinase, S6 kinase, phosphorylated STAT-3 (Ser^277), STAT-3, phosphorylated STAT-1 (Ser^72), all from Cell Signaling Technology), Twist-1 (Santa Cruz Biotechnology), E-cadherin, Snail, and vimentin (BD Biosciences). β-Actin was used as a loading control to check the integrity of each sample.

Induction of apoptosis. B16 cells were treated with etoposide (10 μM/L) or anti-Fas antibody (1 μg/mL) overnight, visualized by phase contrast microscopy, and stained with Annexin V and propidium iodide for flow cytometric analysis of cell death. Caspase-3 activity was quantified with a colorimetric assay kit according to the manufacturer’s instructions (Invitrogen). In some experiments, blocking antibodies against αvβ3 integrin (clone RMV-7; Millipore) were used.

Immunofluorescence microscopy. 1 × 10^6 B16 cells were cultured on a glass chamber plate overnight, washed thrice to remove floating cells, and fixed with 20% methanol at −20°C for 5 min. The cells were stained with anti-E-cadherin or anti-vimentin mAbs, then AlexaFlor 488–labeled or Cy3-labeled IgG (H + L) as secondary antibodies and visualized using a TE2000-U inverted fluorescence microscope (Nikon).

In vitro transduction. The small interfering RNA (siRNA)–coding oligos against mouse Twist-1, Akt-1, and Akt-2 were designed using BLOCK-iT RNAi Designer (Invitorgen) and verified for specificity by blast search against the mouse genome. The sequences used were MFG-E8 ACAAAGACTAGGAACTCGTGTCCTT, Twist-1 AGCTGAG-CAGATTCAGACCTCCTCA, Akt-1 ACGAATTCTGATACCTGGAA, Akt-2 GAGACCTTCTACGTAGACTCTCA. The siRNA sequences (or control sequences that do not match any known murine cDNA) were cloned downstream of a U6 promoter in pCMMPp-eGFP, the siRNA cassette was introduced into the pMFG retroviral vector, and viral stocks were prepared with 293-GPG cells and used to transduce B16 cells. Akt-1 and Akt-2 knockdowns were performed concurrently. The efficiency of gene knockdown ranged from 50% to 90%, as determined by immunoblotting.

In vitro invasion assay. The effects of MFG-E8 on tumor cell invasion were determined using the BD BioCoat Tumor Invasion Assay System (BD Bioscience) according to the manufacturer’s instructions. Briefly, the upper chamber was precoated with 100 μL Matrigel, whereas serum-free media was used for the bottom chamber. 1 × 10^6 transduced B16 cells were seeded into the upper chamber, and 24 h later, cells that invaded into the bottom chamber were stained with 4 μg/mL Calcein AM in PBS at 37°C for 1 h and then counted with a fluorescence microscope.

Immune assays. Tumor infiltrating lymphocytes were obtained from B16 challenge sites using a Nocorep (Axis-Shield) cell gradient separation followed by CD3+ ‘T-cell purification with anti-CD3+–labeled magnetic beads (Miltenyi). Antigen-specific CD8+ responses against H-2b restricted epitides derived from TRP-2 (180–188, SVYDFFVWL) or E1A (234–243, CALAAGATTCAGACCCTAA, Akt-1 ACGAATTCTGATACCTGGAA, Akt-2 GAGACCTTCTACGTAGACTCTCA. The siRNA sequences (or control sequences that do not match any known murine cDNA) were cloned downstream of a U6 promoter in pCMMPp-eGFP, the siRNA cassette was introduced into the pMFG retroviral vector, and viral stocks were prepared with 293-GPG cells and used to transduce B16 cells. Akt-1 and Akt-2 knockdowns were performed concurrently. The efficiency of gene knockdown ranged from 50% to 90%, as determined by immunoblotting.

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and IFN-γ production were assessed against YAC-1 cells by flow cytometry. FITC-labeled CD107a mAb was added to the effector and target cell mixtures during 4 h of incubation, whereas PE-labeled anti–IFN-γ was added 2 h after brefeldin A treatment. For Treg stimulation assays, thioglycollate elicited peritoneal macrophages (treated with control, MFG-E8, or Twist-1 siRNAs) were exposed to apoptotic thymocytes (induced with 10 μmol/L dexamethasone for 6 h) and cocultured with wild-type syngeneic splenocytes. FoxP3 expressing Tregs were assayed by flow cytometry.

Human melanoma cells. Human melanoma cell lines were established from patients enrolled in vaccine studies, as described (11). The antibodies used for flow cytometry and immunoblotting were MFG-E8 (R&D Systems), FAK, Src, phosphorylated Akt, Akt, E-cadherin, N-cadherin (Cell Signaling Technology), Twist-1, and Snail (Santa Cruz Technology). The siRNA sequence for human MFG-E8 (CCTACAGCCTTAATGGACACGAATT) was designed using online design software (Invitrogen), synthesized as a double-strand oligonucleotide, cloned into the pENTRTM/U6 plasmid (Invitrogen), and then transfected into human melanoma cells. Insulin-like growth factor I (IGF-I; 10 μmol/L) receptor inhibitor (AG538) or c-Met inhibitor (SU11274; Sigma Aldrich) were applied to melanoma cells for 6 h, and cell death was determined by flow cytometry using Annexin V and propidium iodide (BD Bioscience). In vitro invasion assays were performed with Matrigel as above.

Statistics. The differences between two groups were determined with the Student’s t test or the two-sample t test with Welch correction. The differences among three or more groups were determined with a one-way ANOVA.

Results

MFG-E8 is associated with melanoma progression in patients and mice. To characterize MFG-E8 expression as a function of melanoma progression, we performed immunohistochemistry on 5 compound melanocytic nevi and 11 vertical growth phase melanomas (Fig. 1 A and Supplementary Table S1). Whereas minimal MFG-E8 was detected in normal skin, compound nevi, and the radial growth phase, strong expression was noted in the vertical growth phase.

Figure 1. MFG-E8 is linked with melanoma progression in patients and mice. A, immunohistochemistry for MFG-E8 expression was performed on human melanocytic lesions (n = 16). NI, normal skin, compound nevus; RGP, radial growth phase; VGP, vertical growth phase. Magnification, 200× for normal skin, 100× for all others. B, B16 tumors growing s.c. were processed to single cells and the infiltrates stained for CD11b, Gr-1, and MFG-E8. Spleens are shown for comparison. C, C57Bl/6 mice were injected s.c. with 2 × 10⁵ B16-GFP, B16-MFG-E8, or B16-RGE cells (four per group), and tumor growth was determined. Similar results were observed in three experiments. D, GM-CSF–deficient mice were reconstituted with the indicated transduced bone marrow (four per group) and, 2 mo later, injected s.c. with wild-type B16 cells as above (MFG-E8 versus GFP, P = 0.032). Similar results were observed in a second transplant experiment.
growth phase of most lesions, with staining in melanoma cells and/or infiltrating myeloid cells. MFG-E8 levels were significantly higher in melanomas compared with nevi (8 of 11 versus 1 of 5, \( P = 0.049 \)). MFG-E8–positive tumors included all nodular and superficial spreading types examined and an unclassified case, although no expression was observed in two acral lentigious and one lentigo maligna melanoma. The percentage of positive cells varied from <5% to 90%, with an average of 40%. Staining intensity ranged from 2 to 3+ and was diffused in five cases, deep in two, and superficial in one.

Because \( \alpha_\beta_3 \) integrin expression is similarly increased in the vertical growth phase of melanoma (24, 25), our histologic findings raise the possibility that MFG-E8 derived from tumor and/or infiltrating myeloid cells might affect disease progression through autocrine or paracrine mechanisms. To explore this issue, we first evaluated the B16 murine melanoma model, as these cells express \( \alpha_\beta_3 \) integrins (not shown) and are highly virulent when injected into syngeneic C57Bl/6 hosts. Although B16 cells did not display MFG-E8 by immunoblotting or flow cytometry (not shown), growing B16 tumors elicited myeloid cell infiltrates that expressed MFG-E8 (Fig. 1B). Single-cell suspensions prepared from s.c. lesions revealed strong MFG-E8 staining in both tumor-associated macrophages (CD11b‘Gr1\(^-\)) and myeloid suppressor cells (CD11b‘Gr1\(^+\)). Increased proportions of MFG-E8 expressing CD11b‘Gr1\(^-\) and CD11b‘Gr1\(^+\) cells were also evident in the spleens of tumor-bearing mice compared with control littermates.

To determine the effect of tumor-derived MFG-E8 in this model, we used retroviral-mediated gene transfer to engineer B16 cells to secrete MFG-E8 constitutively. In addition, a high titer virus encoding a previously described MFG-E8 mutant, in which the

![Figure 2](image-url)
RGD sequence mediating integrin engagement was modified to RGE, was constructed for comparison (33). This protein retains the capacity to bind phosphatidylserine but cannot signal through αvβ3 integrins. The MFG-E8 expression achieved with these vectors is comparable with levels found in several human melanoma cell lines (Supplementary Fig. S1). Although wild-type MFG-E8 and the RGE mutant did not significantly influence the growth of B16 cells in vitro (not shown), MFG-E8 secreting B16 cells manifested increased tumorigenicity in vivo after s.c. inoculation into wild-type C57Bl/6 mice (Fig. 1C). B16-GFP and wild-type B16 cells showed comparable growth under these conditions (not shown).

To delineate the contribution of myeloid cell–derived MFG-E8 in this model, we used GM-CSF–deficient mice, which show markedly decreased MFG-E8 expression in macrophages and dendritic cells (18). GM-CSF–deficient bone marrow was infected with retroviral vectors encoding MFG-E8, RGE, or green fluorescent protein (GFP) and then used to reconstitute lethally irradiated GM-CSF–deficient recipients. Two months after transplantation, mice were challenged with wild-type B16 cells. Mice that received MFG-E8 expressing bone marrow displayed enhanced B16 growth compared with GFP controls, whereas recipients of RGE expressing marrow evidenced a modest decrease in tumor growth (Fig. 1D). This latter inhibition might reflect increased levels of circulating IL-12 and IFN-γ, which we previously showed, were due to the RGE blockade of phagocyte uptake of apoptotic cells (18). B16 growth was similarly enhanced in C57Bl/6 wild-type mice reconstituted with bone marrow transduced with MFG-E8 compared with GFP or RGE vectors (not shown). Taken together, these experiments indicate that MFG-E8, derived from either tumor or host myeloid cells, promotes B16 melanoma growth in vivo.

Figure 3. MFG-E8 induces EMT in melanoma cells. A, engineered B16 cells were assayed with antibodies against E-cadherin and vimentin by confocal microscopy. B, immunoblotting of indicated B16 populations for transcription factors related to EMT. C, vimentin and E-cadherin levels after shRNA-mediated Twist knockdown in MFG-E8 secreting B16 cells. Representative of three experiments.
MFG-E8 stimulates melanoma cell resistance to apoptosis and triggers EMT. The up-regulation of αvβ3 integrins and activation of Akt in vertical growth phase melanoma cells is associated with enhanced resistance to apoptosis (23). To examine whether MFG-E8 promotes tumor cell survival, we initially characterized relevant signaling pathways by immunoblotting. Both MFG-E8 secreting B16 cells and wild-type B16 cells exposed to supernatants from MFG-E8 expressing peritoneal macrophages (generated by retroviral transduction) showed more phosphorylated Akt, phosphorylated S6 kinase, and phosphorylated STAT-3, but less phosphorylated STAT-1, compared with GFP controls and the RGE mutant (Fig. 2A). Moreover, MFG-E8 increased the resistance of B16 cells to etoposide and fas ligation, as revealed by morphology, Annexin V/propidium iodide staining, and caspase 3 processing (Fig. 2B and C). These effects were antagonized with blocking antibodies to αvβ3 (Supplementary Fig. S2), consistent with the activation of integrin signaling. Knockdown of Akt with short hairpin RNAs (shRNA) restored B16 cell sensitivity to etoposide (Fig. 2D), delineating a contribution of this pathway to MFG-E8–mediated survival.

Other characteristics of vertical growth phase melanoma cells include reduced E-cadherin but increased N-cadherin and vimentin levels compared with earlier stage melanocytic lesions (27–29). This expression profile is indicative of EMT, although the factors regulating this program in vivo are not well defined (34). To investigate whether MFG-E8 contributes to these effects, we performed confocal microscopy on the engineered B16 populations. MFG-E8–expressing B16 cells showed diminished E-cadherin but robust vimentin expression compared with wild-type and RGE-expressing B16 cells (Fig. 3A), whereas antibodies to αvβ3 integrins blocked these effects (Supplementary Fig. S3). MFG-E8 also stimulated the production of Twist and Snail (Fig. 3B), transcription factors previously linked with the induction of EMT (35–37). Indeed, knockdown of Twist with shRNAs antagonized the changes in E-cadherin and vimentin, thereby establishing the participation of Twist in MFG-E8–triggered EMT (Fig. 3C).

MFG-E8 enhances melanoma invasion, metastatic seeding, and angiogenesis. EMT is critical for tumor cell invasion and metastasis (35–37). In this context, MFG-E8 secreting B16 cells manifested greater invasion in Matrigel assays compared with GFP and RGE-expressing B16 cells, whereas this advantage was ameliorated by Twist but not Akt shRNA-mediated knockdown (Fig. 4A). Melanoma cell invasion at the vertical growth phase is also promoted by the increased generation of MMPs, which

Figure 4. MFG-E8 enhances melanoma invasion and metastasis. A, the indicated B16 cells were evaluated for in vitro invasion in Matrigel assays. Columns, mean for triplicates, with similar results in three experiments; bars, SD. B, peritoneal macrophages were exposed to supernatants from engineered B16 cells and assayed for MMP-2 and MMP-9 levels with ELISAs. Columns, means for triplicates, with similar results in three experiments; bars, SD. C, MFG-E8 secreting B16 cells were treated with Twist-1 or control siRNAs and injected in the tail vein of wild-type mice. Fourteen days later, lungs were harvested and subjected to pathologic analysis. The findings are representative of eight mice per group. Magnification, 100×. Arrows denote melanin in the B16 cells.
degrade extracellular matrix components and some membrane proteins (38). Accordingly, supernatants from MFG-E8 secreting B16 cells stimulated greater production of MMP-2 and MMP-9 by macrophages in vitro compared with supernatants from GFP or RGE expressing B16 cells (Fig. 4A).

Consistent with these in vitro effects, MFG-E8 enhanced metastatic seeding in vivo. After tail vein injection, MFG-E8 secreting B16 cells formed multiple pulmonary metastases that were readily identified on histopathologic examination, but GFP expressing cells yielded only minimal lesions under the conditions tested (Fig. 4C). Moreover, the knockdown of Twist with shRNAs abrogated the development of lung nodules, revealing a role for this transcription factor in MFG-E8, induced tumor spread.

MFG-E8 mediates immune suppression. We previously showed that MFG-E8 inhibited vaccine-stimulated tumor immunity through the induction of Tregs (18). To determine whether MFG-E8 modulated Treg responses during tumor development, we performed flow cytometry on single-cell suspensions prepared from engineered B16 tumors and stained for CD4 and FoxP3. Similar results were observed in four mice per group. B, tumor-infiltrating lymphocytes were isolated and stimulated with B16 cells and IL-2 for 72 h and then tested for IFN-γ production against splenocytes pulsed with TRP-2 or E1A derived peptides by ELISPOT. Columns, means for triplicates, with similar results in three experiments; bars, SD. C, NKp46+CD3 - NK cells were isolated by flow cytometry from spleens of mice harboring the indicated tumors. IFN-γ production and CD107a mobilization were determined by intracellular staining after exposure to Yac-1 cells. Similar results were observed in three experiments. D, the indicated macrophages were exposed to apoptotic thymocytes and cocultured with wild-type syngeneic splenocytes. FoxP3-expressing Tregs were assayed by flow cytometry. Similar results were observed in three experiments.

To explore the mechanism by which MFG-E8 stimulated Tregs, we cocultured syngeneic wild-type CD4+ T cells with apoptotic cell loaded peritoneal macrophages and then analyzed FoxP3 expression. Consistent with our earlier studies using GM-CSF–deficient macrophages (18), knockdown of MFG-E8 in macrophages impaired their ability to elicit Tregs in this assay (Fig. 5D). Furthermore, Twist knockdown similarly inhibited Treg induction, establishing a role for this transcription factor in MFG-E8–mediated immunoregulation.

Autocrine MFG-E8 signaling in human melanoma cells. To determine whether the effects of MFG-E8 on melanoma cell biology, as revealed in the B16 murine model, were also operative in patient specimens, we characterized six human melanoma cell lines, including four established from subjects participating in our clinical immunotherapy trials (11, 39). All samples tested showed robust MFG-E8 expression by flow cytometry (Supplementary Fig. S3), and two lines (K008 and K029) were selected for detailed study. Knockdown of MFG-E8 sensitized human melanoma cells to small molecule inhibitors of IGF-I receptor and c-Met (Fig. 6B), whereas the increased killing was reversed with the addition of recombinant MFG-E8 to the cultures (not shown). Susceptibility to MEK and
ERK inhibitors, in contrast, was not consistently enhanced (not shown). MFG-E8 was also required for the expression of Twist and Snail (Fig. 6A). MFG-E8 knockdown cells displayed an attenuated EMT phenotype, with increased E-cadherin but decreased N-cadherin levels (Fig. 6C). Moreover, MFG-E8 knockdown cells showed impaired invasion in Matrigel assays (Fig. 6D). Together, these findings highlight the broad effect of MFG-E8 on human melanoma cell biology.

**Discussion**

Carcinogenesis involves the dynamic interplay of transformed cells and normal host elements. Cancer cells that have evaded immune-mediated destruction exploit factors present in the tumor microenvironment to accomplish disease progression. Our studies show that MFG-E8, a secreted protein expressed at high levels in the vertical growth phase, contributes to melanoma development through coordinated αβ3 integrin signaling in tumor and myeloid cells. MFG-E8 triggers Akt and Twist-dependent pathways to stimulate tumor cell survival, EMT, invasion, and immune suppression. In the B16 murine melanoma model, these effects cooperate to enhance tumorigenicity and metastatic seeding, whereas in human melanoma cells, autocrine MFG-E8 production is required for full resistance to apoptosis, EMT, and invasive capacity. Collectively, these findings delineate pleiotropic functions for MFG-E8 in melanoma pathogenesis.

Increased expression of αβ3 integrins on melanoma cells is the most consistent histopathologic marker for the vertical growth phase, and previous work established an important role for this receptor in melanoma biology (24, 25). Whereas vitronectin, an extracellular matrix component, serves as a major ligand under steady-state conditions, our investigations reveal a mechanism by which melanoma cells may capitalize on autocrine or paracrine MFG-E8 secretion to maintain constitutive αβ3 integrin signaling after detachment from the basement membrane. This pathway likely provides a selective advantage to melanoma cells during invasion and dissemination. Although additional studies are required to determine the potential contributions of other αβ3 ligands, such as Del-1, thrombospondin, and osteopontin (41, 42), our gene transfer analysis in the B16 model together with the knockdown experiments in human melanoma cells implicate a critical function for MFG-E8.
Melanoma cells acquire MFG-E8 through two complementary strategies. In one approach, infiltrating myeloid cells release the factor in a paracrine fashion. This mode is dominant in the B16 melanoma but is similarly operative in other transplantable tumors, including RENCA renal cell carcinoma, Lewis lung carcinoma, and CMS fibrosarcoma, as well as the Her2/neu transgenic breast carcinoma model (not shown). MFG-E8 expression in myeloid cells may be triggered by GM-CSF and perhaps other cytokines generated in the tumor microenvironment that may also mediate myeloid cell recruitment (18). Nonetheless, we previously showed that Toll-like receptor agonists or necrotic debris diminish MFG-E8 levels in myeloid cells. Autocrine MFG-E8 production by melanoma cells thereby constitutes an alternative scheme that might be more resistant to varying local conditions. Whereas the specific pathways that regulate MFG-E8 expression in cancer cells remain to be elucidated, p63 may be involved in some cases (43).

Myeloid cells are a major component of the host reaction to cancer, and their contributions to tumorigenesis are under active investigation (44). Tie-2 positive macrophages and myeloid suppressor cells are recruited to the developing vasculature, wherein they promote angiogenesis through the release of growth factors and MMPs (45, 46). Previous studies indicated that MFG-E8 derived from myeloid cells may be a critical cofactor for VEGF-stimulated angiogenesis (47, 48). Consistent with these results, we found that MFG-E8 production engendered intense tumor vascularity with elevated levels of VEGF and MMP-9 in situ, which might reflect the coordination of endothelial and myeloid cell responses (not shown). Moreover, the vascular remodeling and inflammatory environment might further cooperate with the MFG-E8 induced EMT to foster metastatic seeding (47, 49). Indeed, the ability of MFG-E8 released by myeloid cells to trigger Twist and Snail expression in melanoma cells highlights the intricacies of myeloid tumor cell cross-talk.

Myeloid suppressor cells attenuate protective T-cell immunity through elaborating reactive oxygen and nitrogen species and modulating arginine metabolism (17). Our investigations establish the stimulation of Tregs by MFG-E8 as an additional mechanism of immune suppression that attenuates both innate and adaptive antitumor cytotoxicity. A role for Twist in macrophage immunomodulation is consistent with previous reports, indicating that this transcription factor functions as a negative regulator of NF-κB (50). Whether Twist induces transforming growth factor-β will directly require further investigation, but the repression of proinflammatory cytokines is likely critical for Treg development and inhibition of protective Th1 responses. It is also noteworthy that MFG-E8 elicits STAT-3 activation, as this transcription factor similarly promotes tumor cell survival and immune suppression (7).

Finally, the multiple activities of MFG-E8 in the tumor microenvironment suggest several possibilities for combinatorial cancer therapies. MFG-E8 knockdown sensitized melanoma cells to small molecule inhibitors of IGF-I receptor and c-Met, consistent with the interplay of integrin and growth factor receptor signaling (40). MFG-E8 blockade might also enhance the efficacy of antiangiogenic strategies, especially those targeting VEGF. Finally, MFG-E8 inhibition might antagonize Tregs and thereby increase immune-mediated tumor destruction, particularly in conjunction with immunostimulatory approaches, such as vaccines and anti-CTLA-4 antibodies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Milk Fat Globule EGF-8 Promotes Melanoma Progression through Coordinated Akt and Twist Signaling in the Tumor Microenvironment

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