Pericyte-Derived MFG-E8 Regulates Pathologic Angiogenesis

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Objective—MFG-E8 (also called lactadherin and SED1) is a secreted glycoprotein that has been previously implicated in enhancement of vascular endothelial growth factor–dependent angiogenesis. Major sources of MFG-E8 in vivo and precise mechanisms of MFG-E8 action remain undetermined. The objective of this study was to identify important sources of MFG-E8 in vivo and further elucidate the role(s) of MFG-E8 in the regulation of angiogenesis.

Methods and Results—We used knockout mice and anti-MFG-E8 antibodies to study MFG-E8 function in vivo. In melanomas and in retinas of mice with oxygen-induced retinopathy, MFG-E8 colocalized with pericytes rather than endothelial cells, and platelet-derived growth factor receptor β+ pericytes/pericyte precursors purified from tumors contained large amounts of MFG-E8 mRNA. Tumor- and retinopathy-associated angiogenesis was diminished in MFG-E8 knockout mice, and pericyte coverage of neovessels was reduced. Inhibition of MFG-E8 production by 10T1/2 cells (surrogate pericyte/pericyte precursors) using small interfering RNAs and short hairpin RNAs, or inhibition of MFG-E8 action with some anti-MFG-E8 antibodies, selectively attenuated migration in vitro. Significantly, the anti-MFG-E8 antibodies that inhibited 10T1/2 cell migration in vitro also inhibited pathological angiogenesis in vivo.

Conclusion—These studies strongly implicate MFG-E8 in pericyte/pericyte precursor function and indicate that MFG-E8-directed therapeutics may merit further development. (Arterioscler Thromb Vasc Biol. 2011;31:2024-2034.)

Key Words: angiogenesis ■ MFG-E8 ■ melanoma ■ oxygen-induced retinopathy ■ pericyte

Pathological neovascularization is an important component of several diseases, including cancer and proliferative retinopathies (both diabetic retinopathy and retinopathy of prematurity), and antiangiogenic therapy is commonly used in patients with these conditions. Most active agents act on endothelial cells (ECs) via inhibition of vascular endothelial growth factor (VEGF) or VEGF receptor tyrosine kinase signaling,1–3 but supporting cells such as pericytes (PCs) have also been targeted.3–5 PCs influence ECs by producing proteins including VEGF and angiopoietin 1, and these secreted growth factors promote EC survival, migration, proliferation, and formation of branching vessels in vitro.6–9 In vivo, PCs regulate vessel structure and permeability.6–9 ECs contribute to this dialog by producing platelet-derived growth factor (PDGF) that enhances PC/PC precursor recruitment, proliferation and differentiation.6–9 In mice, inhibition of PDGF/PDGF receptor β (PDGFRβ) signaling depletes PCs from tumor-associated vessels in the Rip1-Tag2 pancreatic cancer model, leading to reduced angiogenesis and tumor growth, especially when combined with anti-VEGF therapy.4 These data indicate that PCs play an important role in tumor-related angiogenesis and thus may represent a viable therapeutic target.

MFG-E8 (also called lactadherin and SED1) is a secreted glycoprotein that was initially identified as a component of milk fat globules (MFG) and that is also produced by various phagocytes including tingible-body macrophages and follicular dendritic cells in splenic germinal centers, macrophages in peripheral lymph nodes, activated peritoneal macrophages, and immature dendritic cells, including epidermal Langerhans cells.10–13 MFG-E8 is composed of 2 N-terminal epidermal growth factor–like domains, and 2 C-terminal discoidin-like domains (C1 and C2) that are homologous to blood coagulation factors V and VIII. One epidermal growth factor–like domain (E2) contains an RGD consensus integrin-binding sequence, and MFG-E8 binds to αvβ3,6 integrins. The carboxy-terminal domains bind to negatively charged and oxidized phospholipids (as do factors V and VIII).14 Because MFG-E8 binds avidly to phosphatidyl serine that is exposed on the surface of early apoptotic cells, MFG-E8 acts as an opsonin that targets apoptotic cells for uptake by αv integrin-expressing phagocytes.10,15 This process has been reported to...
influence regulatory T cell development in B16 melanoma tumors\(^{16}\) and to contribute to several other diseases, including autoimmunity, mastitis, sepsis, atherosclerosis, and Alzheimer’s disease.\(^{11,17-20}\) Interactions with \(\alpha_\nu\) integrins have also been previously implicated in regulation of angiogenesis and mammary gland branching.\(^{21,22}\) Interactions mediated via the C1 domain of MFG-E8 are important for sperm-egg binding and enhancement of collagen turnover.\(^{23,24}\)

With regard to angiogenesis, Silvestre et al have previously reported that MFG-E8 enhances revascularization in a mouse model of acute hindlimb ischemia.\(^{21}\) The angiogenesis-promoting activity of MFG-E8 was attributed to enhancement of VEGF-induced Akt phosphorylation and EC survival in an \(\alpha_\nu\beta_3/\alpha_\nu\beta_5\) integrin-dependent manner, and ECs were proposed to be the predominant source of MFG-E8. Our group subsequently demonstrated that MFG-E8 enhanced tumor-related angiogenesis and tumor growth in the Rip1-Tag2 mouse pancreatic tumor model, a model in which tumor progression is critically dependent on angiogenesis.\(^{25}\) The source (or sources) of MFG-E8 in tumors was not determined, and the mechanism by which MFG-E8 potentiated angiogenesis was not addressed. We have now additionally characterized the involvement of MFG-E8 in the regulation of angiogenesis in tumors, as well as in oxygen-induced retinopathy (OIR) in mice. Herein we demonstrate that PCs/PC precursors are important sources of MFG-E8 in vivo, that MFG-E8 may enhance angiogenesis via actions on PCs/PC precursors as well as ECs, and that MFG-E8 can be effectively targeted with therapeutic benefit.

**Materials and Methods**

Detailed descriptions of materials and methods used in this study are available in the supplemental materials, available online at http://atvb.ahajournals.org.

**Mice**

MFG-E8 knockout (KO) mice were generated as described previously\(^{25}\) and backcrossed to C57BL/6 mice as described in the Supplemental Data. Control animals were purchased from the National Cancer Institute/Frederick Animal Production Program unless otherwise indicated.

**Cells and Tumor Implantation Studies**

B16F10 melanoma cells (Tumor Cell Repository of the National Cancer Institute/Frederick Cancer Research and Development Program) were implanted subcutaneously into syngeneic mice as indicated and tumors sizes (width \(\times\) length) were determined with calipers. 10T1/2 cells were obtained from the ATCC.

**Antibodies and Flow Cytometry**

Monoclonal and polyclonal anti-mouse MFG-E8 antibodies (Abs) were generated and characterized as described in the Supplemental Data. Other monoclonal antibodies (mAbs) were obtained from commercial sources. Tumor cell subpopulations were obtained from single cell suspensions via preparative flow cytometry (BD FACSAria II Flow Cytometer, BD Biosciences).

**Immunofluorescence Microscopy and Image Analysis**

Frozen sections from tumors were stained as described in the Supplemental Data and visualized with an AxiolImager A1 conventional immunofluorescence microscope (Zeiss) or a LSM510 confocal laser-scanning microscope (Zeiss). Selected images were analyzed using Imaged software.

**Measurement of Vessel Permeability**

Tumor vessel permeability was quantified by determining the amount of dye that extravasated after intravenous injection (Miles assay; see Supplemental Data).

**Assessment and Treatment of OIR-Associated Angiogenesis**

Angiogenesis associated with OIR was studied in neonatal MFG-E8 KO and littermate control mice as previously described,\(^{27}\) and neovascularization was quantified as described in the Supplemental Data. Mice were exposed to 75% \(\text{O}_2\) from postnatal day 7 (P7) to P12 and 20% \(\text{O}_2\) from P12 to P17. In treatment studies, rabbit anti-MFG-E8 polyclonal antibody (pAb), mouse anti-MFG-E8 mAb and appropriate control proteins were injected intraperitoneally (50 \(\mu\)g per injection) on days P12, 14, and 16 (normoxic period). On day P17, retinal neovascularization was quantified.

**MFG-E8 Knockdown Experiments and Quantification of MFG-E8 mRNA and Protein**

MFG-E8 production by 10T1/2 cells was inhibited by small interfering RNAs (siRNAs) and short hairpin RNAs as described in the Supplemental Data. MFG-E8 mRNA levels were determined using quantitative reverse transcription–polymerase chain reaction and MFG-E8 protein production was quantified by ELISA (see Supplemental Data).

**10T1/2 Cell Migration, Proliferation and Differentiation Studies**

PDGF-dependent 10T1/2 cell migration, proliferation, and transforming growth factor-\(\beta1\) (TGF-\(\beta1\))–dependent differentiation was assessed as described in the Supplemental Data. Migration assays were carried out in Transwell plates.

**Statistics**

Probability values were calculated using the Student \(t\) test (2-sided) or by analysis of 1-way ANOVA followed by Bonferroni’s post test as appropriate. Survival differences were calculated using a log-rank test. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.

**Results**

**Production of MFG-E8 by Tumor Vessel–Associated Pericytes**

We previously reported that, in the Rip1-Tag2 model of pancreatic \(\beta\)-cell tumorigenesis, MFG-E8 was predominantly vessel-associated, and we suggested, based on preliminary immunofluorescence studies, that MFG-E8 appeared to colocalize with PCs.\(^{25}\) To definitively address the origin, and to gain insight into the mode of action, of MFG-E8 in tumors, we developed improved reagents and carried out additional experiments. High-titer, affinity-purified, monospecific anti-MFG-E8 pAbs were raised in rabbits, purified, characterized as described in the Supplemental Methods and Figure 4A, and then used to localize MFG-E8 protein in tissue sections of rapidly growing, nonnecrotic (1 cm diameter) B16F10 melanoma tumors that had been implanted subcutaneously in the flanks of C57BL/6 mice. The intensity of staining that we observed was much greater than that seen with the mouse anti-mouse MFG-E8 mAbs that we previously described,\(^{25}\) allowing for more precise localization of MFG-E8 protein in tumors in situ using confocal laser immunofluorescence...
microscopy. Double staining of tumor sections for MFG-E8 and the EC marker CD31 revealed that MFG-E8 staining occurred primarily around, and in close proximity to, CD31+ blood vessels (Figure 1A and Supplemental Figure IA and IB, arrows). Interestingly, the perivascular distribution of MFG-E8 protein was primarily external to ECs and was much more coincident with PDGFRβ+ and NG2+ PCs (Figure 1A and Supplemental Figure IB to ID). Note that, in B16 tumors, there was almost complete correspondence of PDGFRβ and NG2 perivascular staining (Supplemental Figure ID). Analysis of digital images with ImageJ revealed that whereas only 27% of the CD31+ area overlapped with perivascular MFG-E8-specific immunofluorescence, 66% of the PDGFRβ and 54% of the NG2 staining colocalized with MFG-E8 (Figure 1B). These results suggested that in established B16 tumors, only a minority of MFG-E8 protein was melanoma cell derived and, further, that PDGFRβ+ NG2+ PCs might be a more important source of MFG-E8 than ECs.

However, in addition to MFG-E8-producing perivascular cells (Supplemental Figure IB, IE, and IF, arrows), we identified small numbers of PDGFRβ+ MFG-E8+ stromal cells that were not obviously associated with blood vessels in B16 tumors (Supplemental Figure IE, arrowheads). We also detected PDGFRβ+ MFG-E8+ spindle cells near the periphery of tumors (Supplemental Figure IF, arrowheads). The identities of these minor subpopulations are unknown, but the former may represent PC precursors and the latter may be fibroblasts. Somewhat surprisingly, we did not observe MFG-E8 positive leukocytes (CD45+) or macrophages (CD68+) in melanomas (data not shown).

To test the hypothesis that intratumoral PCs produced large amounts of MFG-E8, we assessed the levels of mRNA encoding MFG-E8 in tumor cell subpopulations derived from B16 melanomas. To accomplish this, we prepared single cell suspensions from tumors and used preparative flow cytometry to purify leukocytes (white blood cells; CD45+), ECs (CD31+), PDGFRβ, and cells enriched in B16 melanoma cells (CD45−, CD31−, PDGFRβ−) (Figure 1C). Note that we cannot exclude contamination of tumor cell fraction with lineage marker-negative stromal cells. We also cannot rule out the possibility that CD45−, CD31−, PDGFRβ+ cells include small number of non-PCs. RNA was extracted from unsorted and sorted cells, and MFG-E8 mRNA levels were determined using quantitative real-time polymerase chain reaction. Relative to unfractonated tumor cells (MFG-E8 mRNA abundance=1), levels of MFG-E8 mRNA were decreased in melanoma cells (0.7) and white blood cells (0.4) and increased in ECs (1.3) and PCs (3.9) (Figure 1D). These results, considered in conjunction with our in situ localization studies, indicate that PCs can produce MFG-E8 in tumors and that, on a per-cell basis, PCs have a greater capacity to produce MFG-E8 protein than all other tumor-associated cells.

**Influence of Host-Derived MFG-E8 on Tumor-Associated Vessels and Melanoma Growth**

Because MFG-E8 protein localized primarily around tumor blood vessels (Figure 1 and Supplemental Figure I), we assessed the involvement of MFG-E8 in development and function of tumor vessels and tumor growth. The vascularity of B16 melanomas growing in the subcutis of C57BL/6 MFG-E8 KO mice was ~50% of that in tumors growing in wild-type (WT) mice as assessed by quantifying the relative
areas of CD31 immunofluorescence staining using ImageJ (Figure 2A and 2B and Supplemental Figure II). Quantification of tumor vessel-associated PCs using an analogous approach revealed a significantly reduction in KO tumors, and the level of PC coverage which was assessed by PC/EC ratio in 1-cm B16 melanomas growing in wild-type (WT) and MFG-E8 KO mice. **P<0.01. Values were determined in 10 random confocal microscopic fields in n=4 tumors per genotype. C, Quantification of vessel permeability (Evan’s blue dye extravasation) in 1-cm B16 melanomas growing in WT and MFG-E8 KO mice. **P<0.01; n=10 to 12 mice in each group. D, Retardation of tumor growth and prolongation of survival in MFG-E8 KO mice after implantation of B16 melanoma cells. *P<0.05, **P<0.01. Data are representative of n=3 experiments (n=10 mice in each group). E, Diminished proliferation of B16 cells without increased apoptosis in MFG-E8 KO as compared with WT mice. **P<0.01. Values were determined in 10 random microscopic fields in n=3 tumors per genotype. NS indicates not significant; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling.
mice persisted after treatment with either of the mAb alone or in combination (Supplemental Figure III). These results indicate that it is unlikely that anti-MFG-E8 immunity was responsible for delayed tumor growth in MFG-E8 KO mice, and suggest that the effect that we observed reflects a role for MFG-E8 in the regulation of angiogenesis.

**Accumulation and Distribution of MFG-E8 in Melanomas in WT and MFG-E8 KO Mice**

The relative importance of malignant melanoma cells as a source of MFG-E8 in tumors was additionally analyzed by localizing MFG-E8 protein in B16 tumors growing in MFG-E8 KO mice via immunofluorescence microscopy. As expected, we observed much less intense MFG-E8-specific staining in tumors in KO mice than in WT mice (Figure 3). Interestingly, however, the perivascular staining pattern that we observed in tumors in WT mice also predominated in MFG-E8 KO mice (Figure 3B). These results indicate that although most of the MFG-E8 protein that is present in tumors is derived from nonmalignant cells (predominantly PCs and ECs), tumor cells also produce MFG-E8. Furthermore, even melanoma cell–derived MFG-E8 protein accumulates in areas where PCs localize.

**MFG-E8 and Susceptibility to OIR**

To determine whether MFG-E8 modulated angiogenesis only in tumors or whether this effect was more general, we next characterized the involvement of MFG-E8 in pathological neoangiogenesis associated with OIR in mice. This condition mimics retinopathy of prematurity, is thought to model some aspects of diabetic retinopathy and is a nontumor experimental system in which PC function can be studied in detail.27 In the OIR model, P7 pups are exposed to 75% oxygen for 5 days (P7 to P12), and then returned to room air until P17 (when experiments are terminated). Exposure of neonatal mice to hyperoxia (75% O2) leads to regression of retinal vessels. The relative retinal hypoxia that ensues when mice are placed in a normoxic (20% O2) atmosphere induces neovascularization. Neovascularization can be readily quantified and antiangiogenic interventions can be attempted during the P12 to P17 “hypoxic” period.

In normal retinal whole mounts from WT mice, most MFG-E8 colocalized with PDGFRβ and NG2+ PCs, whereas some MFG-E8 was CD68+ macrophage associated (Figure 4A). The lack of staining of retinas from MFG-E8 KO mice confirmed the high specificity of the anti-MFG-E8 pAb that we generated (Figure 4A). In mice subjected to OIR, numerous pathological neangiogenic tufts were observed at P17, as expected (Figure 4B, arrows). These tufts were encased with PDGFRβ+ PCs and were coated with MFG-E8 protein (Figure 4B). Scattered CD68+, MFG-E8+ macrophages were more numerous in these preparations than they were in retinas from untreated mice (data not shown), and some of these macrophages were located in close proximity to the angiogenic tufts (Figure 4B). We compared neovascularization in the retinas of WT and MFG-E8 KO mice in the OIR model and determined that the response was blunted considerably in MFG-E8-deficient animals (Figure 4C and Supplemental Figure IV). Quantification of retinal vascularity using a standard morphometric approach confirmed this and also revealed that heterozygous mice could not be distinguished from WT mice (Figure 4D). These results indicate that MFG-E8 derived from PCs/PC precursors, and perhaps macrophages, promotes neovascularization in the OIR mouse model.

**Cell Autonomous Actions of C3H 10T1/2 Cell-Derived MFG-E8**

Previous reports demonstrated that MFG-E8 potentiates VEGF action on ECs.21 The striking colocalization of
MFG-E8 with PCs that we observed suggested that PC-derived MFG-E8 might also act on PCs. To test this hypothesis, we used C3H 10T1/2 cells as surrogate PCs/PC precursors in in vitro studies. 10T1/2 cells express the PC markers smooth muscle actin, NG2, desmin, and PDGFRβ and have previously been demonstrated to promote formation of cord-like structures when cocultured with bovine ECs, suggestive of PC-like function, and to be PDGF responsive. We confirmed that 10T1/2 cells produced both MFG-E8 mRNA and protein using quantitative reverse transcription–polymerase chain reaction and ELISA, respectively, and determined that 10T1/2 cells expressed 25-fold more MFG-E8 mRNA than B16 melanoma cells (Supplemental Figure VA and VB).

To examine the functional consequences of MFG-E8 production by 10T1/2 cells, we manipulated MFG-E8 mRNA accumulation and protein production with retroviruses encoding short hairpin RNAs that target MFG-E8 mRNA as well as corresponding siRNAs. MFG-E8 siRNAs inhibited mRNA and protein expression in 10T1/2 cells by 70% to 80% depending on the sequence of the siRNA and the experiment (Supplemental Figures VB and VIA). Similar levels of inhibition were obtained with some, but not all, short hairpin RNAs (Supplemental Figure VIIA). In initial experiments, we tested the ability of 10T1/2 cells that had been transfected with siRNAs, or infected with retroviruses producing MFG-E8 and control short hairpin RNAs, to close “wounds” in cell monolayers in “scratch” assays. We observed that 10T1/2 cells that produced less MFG-E8 migrated more slowly than cells that produced normal amounts of MFG-E8 and that exogenous MFG-E8 could restore the migratory capacity of 10T1/2 cells with diminished MFG-E8 production (Supplemental Figure VIB and VIIB).

Because EC-derived PDGF-B and PDGF-B/PDGFRβ signaling is critical for PC/PC progenitor cell recruitment during angiogenesis, the involvement of MFG-E8 in PDGF-stimulated 10T1/2 cell migration was also studied using a Transwell migration assay. After confirming that 10T1/2 cells expressed PDGFRβ by immunofluorescence staining, flow cytometry, and reverse transcription–polymerase chain reaction (data not shown), we depleted MFG-E8 mRNA and protein in 10T1/2 cells and assessed PDGF-induced cell migration. In these experiments, MFG-E8 mRNA expression in MFG-E8 siRNA-transfected cells was inhibited by 55% to 70% relative to that in control siRNA-transfected cells (Figure 5A), and the ability of 10T1/2 cells depleted of MFG-E8 with PCs that we observed suggested that PC-derived MFG-E8 might also act on PCs.

Figure 4. Characterization of the role of MFG-E8 in angiogenesis induced during oxygen-induced retinopathy in mice. A, Localization of MFG-E8 in relation to pericytes (PCs) (PDGFRβ+, NG2+) in normal retina from wild-type (WT) and MFG-E8 knockout (KO) mice using rabbit anti-mouse MFG-E8 polyclonal antibody. Scale bars=20 μm. B, Fluorescence photomicrographs depicting localization of MFG-E8 in relation to neovascular tufts (arrows, lectin+), PCs (PDGFRβ+), and macrophages (CD68+) (postnatal day 17 [P17] after initiation of hyperoxia in WT mice) Scale bars=50 μm. C, Photomicrograph of neovascular tufts (arrows) extending into the vitreous in littermate control and MFG-E8 KO mice on day P17 (formalin-fixed, paraffin-embedded sections stained with periodic acid Schiff and hematoxylin). D, Quantification of retinal neovascularization which is the number of vessel-associated nuclei extending above the inner limiting membrane into the vitreous on day P17 after initiation of oxygen-induced retinopathy protocol. **P<0.01; n=16, 12, and 16 mice for WT, heterozygous (Het), and MFG-E8 KO mice, respectively.

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MFG-E8 by RNA interference to migrate was also markedly inhibited (Figure 5B). In addition, as we observed in the scratch assays (see above), adding recombinant MFG-E8 into the medium reversed the migration inhibitory effects of MFG-E8 mRNA depletion (Figure 5B). These results strongly implicate 10T1/2-derived MFG-E8 as an autocrine regulator of cell migration.

We next examined the influence of anti-MFG-E8 Ab on PDGF-induced cell migration to determine whether MFG-E8 might be a relevant therapeutic target. PDGF-induced 10T1/2 cell migration was inhibited by rabbit anti-MFG-E8 pAb that recognized the N terminus of MFG-E8, and by inhibitory anti-α5 integrin mAb to similar extents and the combination of anti-MFG-E8 and anti-α5 integrin Ab was no more inhibitory than either Ab alone (Figure 5C). Addition of recombinant MFG-E8 reversed the inhibition caused by anti-MFG-E8 pAb but not that due to anti-α5 integrin mAb (Figure 5D). Mouse anti-MFG-E8 mAb B10C7 that recognized the RGD motif in the E2 domain (or a nearby epitope) also inhibited PDGF-induced 10T1/2 cell migration like anti-MFG-E8 pAb, whereas several other mAbs were less effective (C1 domain-reactive mAb B18A9 and 1H6) or were ineffective (B1F10, a mAb reactive with an epitope in the E1, E2 or L domains) (Figure 5E). Both rabbit anti-MFG-E8 pAb and mouse anti-MFG-E8 mAb B10C7 inhibited cell migration in a dose-dependent manner, with maximal (~50%) inhibition occurring at 20 to 50 μg/mL for each Ab (Supplemental Figure VIII). In the aggregate, these results suggest that MFG-E8 enhances 10T1/2 cell migration via binding to α5 integrin on the surfaces of 10T1/2 cells, and that MFG-E8 may be the major 10T1/2 cell-derived α5 integrin ligand that is involved in migration. The lesser, but also significant,
inhibition that we observed with anti-MFG-E8 mAb B18A9 and 1H6 suggests that the C1 domain might also contribute to cell migration, perhaps by tethering MFG-E8 to the extracellular matrix.24

To determine whether effects of MFG-E8 on 10T1/2 cell migration were secondary to global alterations in cell physiology, we assessed PDGF-BB and serum-induced proliferation in control and MFG-E8 siRNA-treated 10T1/2 cells. Data depicted in Supplemental Figure IX failed to document altered cell growth in MFG-E8 knockdown cells. We also sought to determine whether MFG-E8 might regulate TGF-β1-induced 10T1/2 cell differentiation. TGF-β1 induces 10T1/2 cells to acquire characteristics of the smooth muscle cell/PC lineage, and TGF-β1 is required for formation of capillary-like structures by ECs and 10T1/2 cells.28,30 As previously reported,28 TGF-β1 induced α-smooth muscle actin expression in 10T1/2 cells (Supplemental Figure X). TGF-β induced α-smooth muscle actin expression by 10T1/2 cells was not altered by MFG-E8 knockdown using siRNA or by anti-MFG-E8 pAb treatment (Supplemental Figure X). In the aggregate, these results suggest that MFG-E8 regulates PC/PC precursor migration rather than proliferation or differentiation.

Inhibition of OIR by Anti-MFG-E8 Ab

The OIR mouse model has previously been used to test several antiangiogenic agents that are now widely used in the clinic or are in development, including agents that target VEGF/VEGF receptor signaling and integrin antagonists.32–35 Having demonstrated that anti-MFG-E8 Ab could inhibit 10T1/2 cell migration in vitro and that neangiogenesis in the OIR model was attenuated in MFG-E8 KO mice, we characterized the activities of anti-MFG-E8 Ab in the OIR model in vivo. We administered anti-MFG-E8 Ab that inhibited 10T1/2 cell migration in vitro to neonatal mice during the normoxic period (50 μg of Ab or control IgG injected intraperitoneally on P12, P14, and P16) and quantified angiogenesis on P17. We determined that anti-MFG-E8 Ab inhibited pathological neangiogenesis, and observed that the relative potencies of the various reagents were similar in the in vitro and the in vivo assays (Figure 6). Interestingly, the magnitude of maximal inhibition of angiogenesis that we observed (≈50%) was very similar to the degree of OIR attenuation that occurred in MFG-E8 KO mice. These results suggest that in experimental animals, anti-MFG-E8 Ab can completely inhibit this aspect of MFG-E8 function and that analogous agents may have therapeutic potential in patients.

Discussion

MFG-E8 is a glycoprotein that has multiple domains and binds to multiple ligands.26–39 At this juncture, MFG-E8 has been implicated in a number of mouse models that have relevance to several important human diseases,18–20,24,25,40 and in normal physiology as well.17,22,23,41 Because MFG-E8 is secreted and thus does not necessarily remain associated with the cells that produce it, and because MFG-E8 is produced by a variety of cells, determining where MFG-E8 is produced in vivo and how it acts is not trivial. This problem is also challenging because MFG-E8 production can be regulated. Thus, cells that produce MFG-E8 in the setting of stress or injury,42 may or may not produce significant amounts of protein at baseline. The pleiotropic effects of MFG-E8 may also relate, at least in part, to the fact that the α5β1 and α6β4 integrins are the best characterized cell surface receptors for MFG-E8. These integrins are widely distributed, are promiscuous with regard to the extracellular matrix proteins that they bind to, and are important regulators of a number of biological activities that are basic and required for normal function of many cells (eg, migration, survival, and proliferation).

We identified PDGFRβ+ PCs, PC precursors, or both as a major source of MFG-E8 in B16 melanoma tumors. Using a newly developed anti-MFG-E8 pAb that is much more sensitive than the mAb that was used in a previous report from our group,25 we determined that MFG-E8 accumulation in tumors was primarily perivascular and that MFG-E8 staining colocalized with PCs more than with ECs. We also found that amounts of MFG-E8 mRNA in PDGFRβ+ PCs/PC precursors and ECs from tumors exceeded those in malignant cells and infiltrating white blood cells and that MFG-E8 mRNA levels were 3-fold higher in PCs/PC precursors than in ECs. Finally, we observed much less intense staining of MFG-E8 in tumors growing in MFG-E8 KO mice than in WT mice. We conclude that in untreated B16 tumors, PCs/PC precursors and ECs are likely to be the most relevant sources of MFG-E8. These results are consistent with and extend the results of previous studies.

Figure 6. Inhibition of pathological angiogenesis occurring secondary to oxygen induced retinopathy by anti-MFG-E8 antibodies. A, Representative photomicrographs of histological sections from retinas in wild-type (WT) mice at postnatal day 17 [P17] in the oxygen induced retinopathy protocol (arrows highlight neovascularization). Mice were treated with 50 μg of the indicated antibody or control IgG on days P12, 14, and 16. B, Quantification of retinal neovascularization, which is the number of vessel-associated nuclei above the inner limiting membrane into the vitreous on day P17 after initiation of oxygen-induced retinopathy protocol. **P<0.01; n=4 to 5 mice per treatment. mAb indicates monoclonal antibody; pAb, polyclonal antibody.
from our group and from other groups in which MFG-E8 was detected in association with adventitial microvessels, vascular smooth muscle cells in the media of the aorta, some luminal ECs, and PCs.19,21,23,41,43

Although MFG-E8 staining was drastically reduced in B16 tumors growing in MFG-E8 KO mice, the perivascular staining pattern that we observed in tumors in WT mice also predominated in tumors in MFG-E8 KO mice. This indicates that MFG-E8 that was secreted by malignant cells continued to accumulate near PCs. In normal blood vessels, PCs and ECs are surrounded by basement membrane that contains laminin, collagen type 4, nidogen, and heparin sulfate proteoglycans.6–8 We also detected perivascular laminin and collagens type 1 and 4 in B16 tumors in close proximity to PCs (not shown). It has been demonstrated that MFG-E8 binds to collagen type 1 via the C1 domain,24 providing a plausible explanation for its distribution in tumors.

Assessment of vascular structures in B16 tumors in control and MFG-E8 KO mice revealed significant differences that were consequential. Vessel density and PC coverage were reduced, and vascular permeability was increased in tumors in MFG-E8 KO mice. These alterations were associated with decreased malignant cell proliferation and delayed tumor growth. The involvement of MFG-E8 in B16 tumor growth has been studied previously,16,40,42 but the experimental approaches that were used differed from those that we used. The previously published studies clearly indicate that MFG-E8 can have an immunomodulatory role when incorporated into melanoma cell–based vaccines, that MFG-E8 can enhance tumorigenesis when overexpressed in malignant cells, and that MFG-E8 production by malignant cells (including B16 cells) is increased by exposure to cytotoxic agents. We did not overexpress MFG-E8 in B16 cells and did not identify an important role for T cell immunity in attenuation of melanoma growth in MFG-E8 KO mice. We believe that our results implicating MFG-E8 in regulation of tumor vascularity are complementary to those that have previously been published16,40,42 rather than inconsistent with them, reflecting the multiple and varied functional activities of this protein.

The concept that PC/PC precursor-derived MFG-E8 is important in pathological angiogenesis is also supported by our studies in the OIR model. We determined that neovascular tufts were ensheathed with PCs and coated with MFG-E8 in retinal whole mounts and that neoangiogenesis was markedly inhibited in MFG-E8 KO mice. Several previous studies examined the role of PCs in OIR. NG2-deficient mice exhibited decreased PC/EC ratios in retinal neovascular tufts and decreased neovascularization in OIR.44 In contrast, PDGF-B heterozygous mice, which also exhibit reduced PC coverage of retinal blood vessels showed increased neovascularization in OIR.45 Although we could not quantify PC coverage in retinal vessels in our OIR studies, there is an apparent unresolved contradiction between these latter results and our results. Previous studies documented that microglia/macrophages were increased in the retinas of mice with OIR.46 We also observed scattered macrophages, some in close approximation to neoangiogenic tufts, in OIR, and most of these CD68+ cells also expressed MFG-E8. Thus, our results clearly indicate that MFG-E8 regulates neoangiogenesis in OIR and suggest that both PCs and macrophages may be relevant sources.

A previous study reported that MFG-E8 promoted ischemia-induced angiogenesis and demonstrated that MFG-E8 enhanced VEGF-induced Akt phosphorylation and proliferation in ECs via binding to αvβ3 integrins.21 The striking colocalization of MFG-E8 with PCs/PC precursors that we observed led us to hypothesize that MFG-E8 might act on PCs/PC precursors as well as on ECs. We studied C3H 10T1/2 cells as surrogates for PCs/PC precursors. Derived from mouse embryos almost 40 years ago, 10T1/2 cells have been variously termed fibroblasts, PC-like cells, mesenchymal stromal cells, mesenchymal precursor cells, smooth muscle precursor cells, and mesenchymal stem cells.28–30,47–51 Although their precise relationship to normal PCs is uncertain, 10T1/2 cells and PCs share some surface characteristics, are both capable of influencing EC characteristics, and both exhibit multilineage potential under some circumstances. We used 10T1/2 cells in the experiments described herein because they produce MFG-E8, they were known to be PDGF responsive (like PCs), and they could be readily propagated and manipulated.

We observed that depletion of MFG-E8 from 10T1/2 cells by RNA interference markedly inhibited basal and PDGF-induced migration and that adding recombinant MFG-E8 reversed these effects. We also determined that 10T1/2 cell migration was inhibited by rabbit anti-MFG-E8 pAb and mouse anti-MFG-E8 mAb (B10C7) that recognized the N terminus of MFG-E8 and by inhibitory anti-αv integrin Ab to similar extents and that the combination of anti-MFG-E8 and anti-αv Ab was no more active than either alone. Interestingly, inhibition of MFG-E8 production or action did not attenuate 10T1/2 cell proliferation or obviously alter 10T1/2 cell differentiation. Thus, MFG-E8 may exert some of its proangiogenic effects by facilitating recruitment of PCs, PC precursors, or both to perivascular locations where they can support EC function.

Our results suggest that MFG-E8 enhances 10T1/2 cell migration via binding to αv integrins on the cell surfaces and that (at least in vitro) MFG-E8 is an important autocrine αv ligand. Cell movement requires synergy between PDGF-and integrin-mediated signaling,52 and we speculate that MFG-E8 facilitates cell migration by promoting this interaction. However, 10T1/2 cell migration was also inhibited to some extent by mouse anti-MFG-E8 mAbs (B1A8A9 and 1H6) that recognize the C terminus of MFG-E8. It has previously been reported that MFG-E8 enhanced the migration of mouse intestinal epithelial cells in vitro and in vivo.53 This effect has been attributed to MFG-E8–phosphatidyl serine binding and resulting cytoskeletal reorganization, but the C terminus of MFG-E8 also has other ligands. A recent study indicated that the C1 domain of MFG-E8 mediates binding and uptake of collagen type 1.24 We detected collagen type 1 production by 10T1/2 cells in vitro, documented pericellular collagen deposition by immunofluorescence staining (unpublished observations), and we thus propose that binding of MFG-E8 to collagen type 1 via the C1 domain and engagement of αv integrins via the E2 domain of MFG-E8 may facilitate...
PDGF-induced 10T1/2 cell migration. Although the mechanism by which this may occur remains to be elucidated, cooperation between α5 integrins and growth factor receptors with tyrosine kinase activity is known to occur.54

Finally, we demonstrated that selected mAbs and pAbs that reacted with MFG-E8 and that were potent inhibitors of 10T1/2 cell migration in vitro also inhibited angiogenesis in vivo. Abs that recognized the E1 and E2 domains (pAb) or an RGD-dependent epitope in the E2 domain of MFG-E8 (mAb B10C7) were effective inhibitors of OIR, whereas the mAbs reactive with C-terminal domains were not. We conclude that PCs/PC precursor-derived MFG-E8 promotes pathologic neoangiogenesis in several settings, and that MFG-E8 may enhance angiogenesis via actions on PCs or PC precursors as well as ECs. Our results also indicate that MFG-E8 is a relevant therapeutic target in several important disease models characterized by unwanted neoangiogenesis, and we suggest that Abs that react with the N terminus (especially the RGD sequence-containing E2 domain) may be particularly interesting. Extrapolating our results to patients, anti-human lactadherin (MFG-E8) Abs that bind to the single N-terminal epidermal growth factor–like domain that contains the integrin binding site may warrant deliberate development. Abs with this characteristic may represent useful therapeutics when used alone or in conjunction with other agents (including other antiangiogenic agents) in the treatment of diseases such as diabetic retinopathy or cancer.

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Disclosures

None.

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Pericyte-Derived MFG-E8 Regulates Pathologic Angiogenesis
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Supplemental Material

Detailed Methods

Mice

MFG-E8 KO mice were generated as described previously. Heterozygous founders were speed backcrossed to C57BL/6NCr mice to generate animals with appropriate and uniform genetic backgrounds using the Speed Congenics Services of the NCI-Frederick Center for Cancer Research Laboratory Animal Sciences Program. This resulted in mice that were ~99% identical to C57BL/6NCr based on the distribution of highly polymorphic microsatellite markers and equivalent to N10 backcrosses to C57BL/6NCr using a standard approach. MFG-E8–deficient mice were generated by interbreeding homozygous animals carrying the targeted MFG-E8 allele. Adult female WT C57BL/6NCr mice (8-12 weeks old) were obtained from the NCI-Frederick Animal Production Program. Mice were bred and housed in a pathogen-free environment and used in experiments in accordance with institutional guidelines.

Cells and Tumor Implantation Studies

B16F10 cells (Tumor Repository of the NCI/Frederick Cancer Research and Development Center) were maintained in RPMI 1640 (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 10 mM HEPES-NaOH (pH 7.4), penicillin (100 U/ml), streptomycin (100 µg/ml), and 1 mM sodium pyruvate (RPMI 1640 complete medium). Tumors were initiated by subcutaneous injection of 10⁵ washed melanoma cells into the flanks of MFG-E8 WT and KO mice. Tumor dimensions were measured 3x per week using engineer’s calipers and tumor sizes reported represent length x width (mm²). Tumor-bearing mice were euthanized when maximal tumor diameters reached 2 cm or humane endpoints incorporated into the protocol prospectively were reached. Mouse C3H/10T1/2 cells (ATCC) were maintained in BME medium (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and used before passage 10.
Antibodies

Abs and their sources were as follows: rat anti-mouse CD31 mAb (MEC13.3; BD Bioscience-Pharminingen), rabbit anti-mouse NG2 pAb (Chemicon), rat anti-mouse PDGFRβ mAb (eBioscience), rat anti-mouse CD68 mAb (AbD Serotec), mouse anti-α-smooth muscle actin (SMA) mAb (Sigma). Alexa 488-, Alexa 568-, Alexa 647-conjugated secondary Abs and Zenon Rabbit IgG Labeling Kits were obtained from Molecular Probes (Invitrogen). Hybridomas producing rat anti-mouse CD4 mAb (clone GK1.5) and CD8 mAb (clone 2.43) were from ATCC. Purified mAb were prepared from Hybridoma-SFM Media (Invitrogen) that had been conditioned in CL1000 bioreactors (Argos Technologies) populated with hybridoma cells that were grown to exhaustion using protein G-Sepharose (GE Healthcare Biosciences).

Rabbit anti-mouse MFG-E8 pAb were generated by immunizing and boosting rabbits with a fusion protein comprised of the N-terminal 148 amino acids of MFG-E8 (including the EGF–like domains (E1 and E2) and the linker region (L) in frame with the Fc region of human IgG1 (250 μg per injection in complete (x1) and incomplete Freund’s adjuvant (x3) at 3 week intervals). Monospecific pAb were prepared from high titer antisera using serial affinity chromatography that first removed reactivity with human IgG and then selected for binding to MFG-Ig. Ab specificity was verified by ELISA, western blotting and immunofluorescence microscopy with relevant proteins and tissues from control and MFG-E8 KO mice (see Figure 4A). Mouse anti-mouse MFG-E8 were generated by immunizing MFG-E8 KO mice with an MFG-E8-encoding plasmid via gene gun immunization and boosting immunized mice with MFG-E8 protein and/or MFG-E8 producing 293T cells. Splenocytes and lymphocytes were isolated from immunized mice and fused with SP2 myeloma cells. Drug-resistant hybridoma clones producing mAb that reacted with MFG-E8 were identified by ELISA and 4 clones (1H6, B1F10, B10C7, B18A9) were ultimately expanded and utilized. The mAb produced by these clones were purified from culture supernatants via protein G-Sepharose chromatography and their reactivity was characterized by ELISA and immunoblot using recombinant MFG-E8 and MFG-Ig fusion proteins produced in 293-F cells (FreeStyle 293 Expression System) as well as MFG-E8 protein fragments produced in bacteria. mAb
reactivities are as follows: 1H6 - C1 domain; B1F10 - E1, E2 or linker domain; B10C7 - RGD-dependent epitope in E2 domain and B18A9 - C1 domain (data not shown).

**Preparative Flow Cytometry**

Single cell suspensions were prepared from 100 mm² (1 cm diameter) B16 melanoma tumors growing subcutaneously in flank skin. Tumors were minced in 5 mg/ml collagenase D (Roche) in RPMI with 5% FCS and DNase, incubated at 37°C for 2 hours and erythrocytes were lysed (ACK buffer). Prior to flow sorting, tumor cells were incubated with rat anti-mouse FcγR mAb (2.4G2, reactive with CD16/CD32), washed and stained consecutively at 4°C with Alexa 488-conjugated anti-CD45 mAb (BioLegend), PE-conjugated anti-PDGFRβ (eBioscience) and Alexa 647-conjugated anti-CD31 mAb (BioLegend). Stained cells were sorted via a BD FACSARia™ II Flow Cytometer (BD Biosciences).

**Lymphocyte Depletion**

To deplete T lymphocytes in tumor-bearing mice, mice were injected i.p. on days -3 and -2 before B16 cell inoculation with 200 μg anti-CD4 mAb (clone GK1.5) or anti-CD8 mAb (clone 2.43) alone or in combination. Depletion efficiency was monitored by determining CD4 and CD8 T cell frequencies in peripheral blood, and mAb injections were repeated approximately every 10 days to maintain depletion levels of >95%.

**Immunofluorescence Microscopy and Image Analysis**

Tumors (100 mm²) were excised from flank skin and 14 μm frozen tissue sections were prepared and fixed in 4% PFA in PBS or cold acetone. After blocking with 3% dry milk-PBS (Bio-Rad) supplemented with 5% normal donkey serum or 5% normal goat serum (as appropriate) for 1 hour at room temperature, sections were stained with Ab of interest followed by Alexa 488-, Alexa 568-conjugated secondary Ab or they were stained with Alexa 488-, Alexa 568-conjugated Ab and control proteins that were prepared using Zenon Labeling Kits (Invitrogen). Sections were counterstained with 4,6-diamidino-2-phenylindole.
(DAPI) to visualize nuclei, mounted in ProLong Gold antifade reagent (Invitrogen) and examined. To visualize proliferating cells in tumors, sections were stained with rabbit anti-Ki-67 pAb (Abcam) and counterstained with DAPI. Percentages of Ki-67 and DAPI double positive nuclei were analyzed using Image J software (NIH). Apoptotic cells were identified by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling (TUNEL) using an in situ cell death detection kit (Roche) according to the manufacturer’s protocol. Percentages of TUNEL and DAPI double positive nuclei were analyzed using Image J software. All immunofluorescence images were collected and visualized with an AxiosImager A1 conventional immunofluorescence microscope (Zeiss) or a LSM510 confocal laser-scanning microscope (Zeiss).

**Measurement of Vessel Permeability**

Tumor vessel permeability was quantified by determining the amount of dye that extravasated after i.v. injection (Miles assay). Mice were injected with Evans blue dye (1% stock; Sigma) at a dose of 30 mg/kg. After 30 minutes, animals were euthanized and perfused with 20 mL of 0.9% NaCl solution using a peristaltic pump. Tumors were removed and weighed, and then incubated in formamide for 18 hours at 37°C. A $\text{A}_{620}$ of tumor lysates was determined spectrophotometrically. The amount of extravasated dye was calculated and reported after normalization (μg dye per g tumor).

**Assessment and Treatment of Oxygen-Induced Retinopathy-Associated Angiogenesis**

Angiogenesis associated with oxygen-induced retinopathy (OIR) was studied as previously described. Litters of pups born to MFG-E8 heterozygous parents were exposed to 75% O$_2$ for 5 days (P7-P12) with nursing mothers. Mice were returned to ambient air (20% O$_2$) on P12, and on day P17 retinal neovascularization was examined in retinal whole mounts or axial sections. Retinal whole mounts were obtained from enucleated eyes, fixed with 4% PFA at 4°C overnight, washed, permeabilized and blocked with 0.5% Triton-X supplemented with 1% BSA/PBS and 5% goat serum for 2 hours at room temperature. Prior to immunofluorescence microscopy, retinas were stained with FITC-conjugated *Bandeiraea*
simplicifolia isolectin B4 (Sigma-Aldrich), anti-NG2 pAb, anti-MFG-E8 pAb and rat anti-CD68 Ab (clone FA-11; Serotec) as indicated. Thereafter, retinas were stained with Alexa 568-conjugated secondary Abs and visualized with an AxioImager A1 immunofluorescence microscope (Zeiss) equipped with a digital camera.

To quantify neovascularization, 6 μ paraffin-embedded axial sections of retinas were stained with periodic acid Schiff (PAS) and hematoxylin and the number of vessel-associated nuclei that protruded above the inner limiting membrane into the vitreous were counted. The data presented represent the mean of nuclear counts in 10 sections (every third serial section) per eye and were obtained by an observer (S.M.) who was blinded with respect to mouse genotype. In treatment studies, rabbit anti-MFG-E8 pAb, mouse anti-MFG-E8 mAb and appropriate control proteins were injected i.p. on days P12, 14 and 16 (50 μg per injection). On day P17, retinal neovascularisation was quantified.

**MFG-E8 Knockdown Experiments and Quantification of MFG-E8 mRNA and Protein**

Short interfering RNAs (siRNA) specific for mouse MFG-E8 mRNA was designed using the QIAGEN GeneGlobe Search Center. The targeting sequences were as follows; MFG-E8 siRNA #1 (5’-AAGCGGTGGAGACAAGGAGTT-3’), MFG-E8 siRNA #2 (5’-AAGGCTGAATAACTAGGGCAA-3’), and MFG-E8 siRNA #3 (5’-AAAGCAATGGAACCTGCGTGC-3’). siRNAs and AllStars negative control siRNA were purchased from QIAGEN. To inhibit MFG-E8 production, 10T1/2 cells (5 x 10^5 cells per 60 mm plate) were transfected with 10 nM siRNA using HiPerFect Transfection Reagent (QIAGEN). After 48 hours, MFG-E8 mRNA and protein levels were assessed by quantitative RT-PCR and ELISA. MFG-E8-depleting shRNAs were also designed. Two plasmids containing shRNA oligonucleotides targeting the MFG-E8 sequences [GGCTGGATAATCAGGGCAA (MFG-E8 shRNA#1) and AGACATGGAACCTGCGTGC (MFG-E8 shRNA#2)] were constructed. Corresponding synthetic oligonucleotides were annealed and cloned into the pSUPER.retro.puro vector (Oligoengine) at Bgl II and Xho I restriction sites. The resulting plasmids were characterized by restriction enzyme digestion and DNA sequencing. MFG-E8 shRNA or the control empty vector plasmids were transfected into Phoenix-
Ampho cells (from Dr. Gary Nolan, Stanford University), and virus-containing supernatants were collected and used to infect 10T1/2 cells. Stable MFG-E8 shRNA transductants were established by selection with 2 μg/ml puromycin for 1 week.

MFG-E8 mRNA levels were determined using quantitative RT-PCR. Total RNA was isolated with RNeasy Kits (Qiagen) and reverse transcribed with SuperScriptIII First-Strand Synthesis System for RT-PCR (Invitrogen). Quantitative RT-PCR was performed using the following primers: MFG-E8 (forward, 5’-ATCTACTGCTCTGCCCCTGA-3’; reverse, 5’-ACACAGAGGAGCGGAATC-3’) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward. 5’-ACCCAGAGACTGTGGATGGGAGG-3’; reverse, 5’-CACATTGGGCGGTAGGAACAC-3’). PCR products were generated and quantified with SYBR Green PCR Master Mix (Applied Biosystems) using CFX96 Real-Time PCR Detection System (Bio-Rad) equipped with CFX Manager™ Software version 1.5.

MFG-E8 protein was quantified by ELISA. Mouse anti-mouse MFG-E8 mAb 1H6 (reactive with the C1 domain) was adsorbed (100 ng/well) onto Maxisorp ELISA plates (Nunc) via overnight incubation at 4°C. After blocking with 2%BSA/PBS, conditioned medium was added and incubated for 90 minutes at 37°C. After washing, plates were incubated with rabbit anti-mouse MFG-E8 pAb (reactive with the N-terminus) for 60 minutes at 37°C. Washed plates were then incubated with HRP-conjugated goat anti-rabbit IgG (Jackson) for 60 minutes, developed with TMB substrate solution (Pierce) and enzymatic reactions were halted with 1 N H2SO4 before determining the A450. Absolute concentrations were determined using appropriate standard curves.

10T1/2 Cell Migration Studies

10T1/2 cells were incubated in low serum (0.5% FCS)-containing media overnight and cell migration was subsequently assayed using FluoroBlok™ 24-Multiwell Insert System transwell culture plates (3 μ pore size; BD Biosciences). Cells were stained with Calcein AM (BD Biosciences), resuspended in 0.5% FCS-containing BME medium, and 10^5 cells were seeded into upper compartments of the transwell
apparatus. Medium containing 0.5% FCS and 50 ng/ml PDGF-BB (PeproTech) was added to the lower compartments. After 4 hours incubation at 37°C, images of cells that were attached to the undersurfaces of transwell filters were acquired with an inverted phase fluorescent microscope equipped with a digital camera. Areas covered by migrated cells in each well were quantified using Image J.

To assess the involvement of MFG-E8 in 10T1/2 cell migration in the transwell assay, MFG-E8 production by 10T1/2 cells was inhibited with siRNAs prior to assessment of migratory ability. To accomplish this, 10T1/2 cells were transfected with appropriate siRNA oligonucleotides, and transfected cells were used for transwell migration assays 42 hours later. As indicated, siRNA transfected 10T1/2 cells and control cells were preincubated with or without 100 ng/ml recombinant MFG-E8 (R&D systems) for 15 minutes prior to addition to the upper chamber to reconstitute the effect of MFG-E8 depletion. The involvement of MFG-E8 in migration in transwell assays was also assessed using anti-MFG-E8 Ab. In these experiments, 10T1/2 cells were incubated for 20 minutes with anti-MFG-E8 mAb (1H6, B1F10, B10C7, B18A9), rabbit anti-MFG-E8 pAb, anti-αV integrin mAb (RMV-7; Chemicon) or with corresponding control Abs (all at 20 μg/ml unless otherwise specified) before migration assessment. After treatment with Abs, 10T1/2 cells were incubated with or without 100 ng/ml recombinant MFG-E8 for 15 minutes prior to addition to the upper chamber. Ab and control proteins were also added into the media that was placed in the lower chambers of transwell plates to avoid Ab dilution.

The involvement of MFG-E8 in 10T1/2 cell migration was also assessed in an in vitro wound healing (“scratch”) assay. In these experiments, MFG-E8 knockdown and control 10T1/2 cells were incubated in 0.5% FCS-containing medium overnight, the confluent cell monolayer was scratched with a pipette tip and displaced cells were removed with washing. “Wounded” monolayers were incubated for 16 hours, images of wounded areas were acquired at identical locations at the beginning and the end of the assay and the areas of residual “wounds” were determined using Image J. Both MFG-E8 shRNA and siRNA transfected 10T1/2 cells were used for scratch assays. In studies involving siRNA transfected cells, the scratch assay was performed 32 hours after transfection. As indicated, the ability of MFG-E8 to reverse of effects of MFG-E8 knockdown with shRNA was tested by adding recombinant MFG-E8 (100
ng/ml; R&D Systems) into the medium at the beginning of the 16 hour recovery period.

**Proliferation assay**

Cell proliferation was measured using the MTS assay. 10T1/2 cells were transfected with siRNA oligos (MFG-E8 siRNA or control siRNA). Sixteen hours after transfection, cells were treated with trypsin-EDTA and plated at a density of 5,000 cells per well with 0.5% FCS containing medium in 96 well plates. Twenty four hours after transfection, cells were stimulated with PDGF-BB (5, 10, 50, 100 ng/ml) or 10% FCS medium as a positive control. After 48 hours incubation at 37°C, 20 µl of CellTiter 96 AQueous One Solution Reagent (Promega) was added. After an additional incubation at 37°C for 2 hours, the absorbance at 490 nm was measured using an ELISA plate reader.

**10T1/2 differentiation assays**

To assess the possible role of MFG-E8 in the differentiation of 10T1/2 cells, expression of α smooth muscle actin (SMA) by TGF-β1-treated 10T1/2 cells was analyzed via Western blot assays and immunofluorescence microscopy. To induce differentiation of 10T1/2 cells into pericytes/vascular SMC, 10T1/2 cells were cultured with TGF-β1 (1 ng/ml; R&D Systems) for 24 hours. To assess the involvement of MFG-E8 in 10T1/2 cell differentiation, MFG-E8 production by 10T1/2 cells was inhibited with siRNA prior to induction of differentiation by TGF-β1. 10T1/2 cells were transfected with appropriate siRNA oligonucleotides, and transfected cells were cultured with TGF-β1 for an additional 24 hours. The involvement of MFG-E8 in differentiation was also assessed using neutralizing anti-MFG-E8 pAb. In these experiments, 10T1/2 cells were incubated for 1 hour with rabbit anti-MFG-E8 pAb or with corresponding control Ab (20 µg/ml) before TGF-β1 treatment. After treatment with Ab, 10T1/2 cells were incubated with or without 1 ng/ml TGF-β1 for 24 hours. After washing with ice-cold PBS, cells were disrupted in lysis buffer (20 mM Tris-HCl (pH 7.6) 140 mM NaCl, 1% Nonidet P-40) containing a protease inhibitor cocktail (Roche) and phosphatase inhibitor (Roche) (complete lysis buffer) on ice. Cell
lysates were centrifuged at 10,000xg for 15 minutes at 4°C, and the resulting supernatants were subjected to SDS-PAGE. Ten μg of total protein was loaded per lane. Immunoblot analysis utilized mouse anti-αSMA mAb and anti-mouse HRP-conjugated secondary antibodies (Jackson) in conjunction with ECL (Pierce). Densitometric analysis of exposed films was accomplished using Image J software.

**Statistics**

*P* values were calculated using the Student’s *t*-test (two-sided) or by analysis of one-way ANOVA followed by Bonferroni’s post test as appropriate. Survival differences were subjected to statistical analysis using a log-rank test. Error bars represent standard errors of the mean, and numbers of experiments (n) are as indicated.
**Supplemental References**


Supplemental Figure I. MFG-E8 localizes in close proximity to pericytes in tumors.
(A) Localization of MFG-E8 in relationship to EC (CD31+) in low magnification view of B16 melanoma (Bar=500 μ).  
(B) Localization of MFG-E8 in relationship to EC (CD31+) in B16 melanoma (Arrow: MFG-E8+ CD31- cells, Bar=50 μ).  
(C) Localization of MFG-E8 in relationship to PC (NG2+) in B16 melanoma (Bar=50 μ).  
(D) Localization of PDGFRβ+ and NG2+ PC in B16 melanoma (Bar=50 μ).  
(E) Localization of MFG-E8 in relationship to PC (PDGFRβ+) and EC (CD31+) in B16 melanoma (Arrow: PDGFRβ+ MFG-E8+ PC, Arrowhead: PDGFRβ+ MFG-E8+ stromal cells, Bar=50 μ).  
(F) Localization of MFG-E8 in relationship to PDGFRβ+ stromal cells or PDGFRβ+ PC in the periphery of B16 melanoma (Arrow: PDGFRβ+ MFG-E8+ PC, Arrowhead: PDGFRβ+ MFG-E8- spindle cells, Bar=50 μ).
Supplemental Figure II. Tumor-associated blood vessels in wild type and MFG-E8 knockout mice.
Composite views of tumor-associated vessels in 1 cm B16 melanomas growing in WT and MFG-E8 KO C57BL/6 mice (CD31 immunofluorescence staining in red and NG2 staining in green).
Supplemental Figure III. Melanoma growth delay and extended survival in lymphocyte-depleted wild type and MFG-E8 knockout mice. WT and KO mice were treated with lymphocyte-depleting anti-CD4 mAb and/or anti-CD8 mAb such that depletion of circulating lymphocytes was maintained at >95%. Tumor sizes were determined as indicated (A) and mice were euthanized when humane endpoints were reached (B) (*p<0.05; **p<0.01; data are representative of n=2 experiments (n=8-10 mice in each group)).
Supplemental Figure IV. Angiogenesis associated with oxygen-induced retinopathy in wild type and MFG-E8 knockout mice Composite views of retinal axial sections obtained from littermate control and MFG-E8 KO mice on day P17 after initiation of the oxygen induced retinopathy protocol described in Methods (* identifies optic nerve).
Supplemental Figure V. Expression of MFG-E8 mRNA and protein by 10T1/2 cells and modulation by siRNAs.

(A) Comparison of MFG-E8 mRNA levels in 10T1/2 and B16 melanoma cells using quantitative RT-PCR (normalized to GAPDH mRNA levels in each cell type). (B) Effects of MFG-E8 and control siRNAs on MFG-E8 mRNA (measured using quantitative RT-PCR), and protein secreted into the media during a 24 hour incubation (assessed via ELISA) in a single experiment 48 hours after transfection of 10T1/2 cells.
Supplemental Figure VI. Inhibition of 10T1/2 cell migration by MFG-E8 siRNAs.

(A) Documentation of inhibition of MFG-E8 mRNA expression 48 hours after transfection of 10T1/2 cells using quantitative RT-PCR (**p < 0.01 relative to siRNA control; Values determined in 3 independent “scratch assay” experiments). (B) Inhibition of 10T1/2 cell migration in a “scratch” (in vitro wound healing) assay as documented in phase contrast photomicrographs (bar= 0.5 mm) and quantified via analysis of digital images using Image J software (**p<0.01, representative of n=3 experiments).
**Supplemental Figure VII. Inhibition of 10T1/2 cell migration by MFG-E8 shRNAs and reversal with recombinant MFG-E8.** (A) Documentation of inhibition of MFG-E8 expression in a single experiment after selection of long-term 10T1/2 transductants expressing MFG-E8 shRNAs using quantitative RT-PCR and ELISA. (B) Inhibition of 10T1/2 cell migration in “scratch” assays as documented in photomicrographs (bar= 0.5 mm), quantification via analysis of digital images using Image J software and reversal with recombinant MFG-E8 protein (100 ng/ml) (**p<0.01, representative of n=2 experiments).
Supplemental Figure VIII. Dose-dependent of inhibition of 10T1/2 cell migration by anti-MFG-E8 Ab.

PDGF-B-induced 10T1/2 cell migration was assessed in a FluoroBlok™ migration assay in the presence and the absence of anti-MFG-E8 mAb and pAb or appropriate control IgG (20 μg/ml) as indicated. Ab were added into lower and upper chambers 20 minutes prior to addition of PDGF-B (50 ng/ml) and migration was quantified 4 hours later via image analysis (*p<0.05; **p<0.01).
Supplemental Figure IX. Lack of effect of MFG-E8 on 10T1/2 cell proliferation. PDGF-induced proliferation of 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA. Control siRNA transfected cells cultured in medium without PDGF for 48 hours were assigned a value of 1. Data are means ± SEM from n=3 experiments performed in triplicate.
Supplemental Figure X. Lack of effect of MFG-E8 on 10T1/2 cell differentiation. (A) Documentation of inhibition of MFG-E8 mRNA expression 48 hours after transfection of 10T1/2 cells with siRNA using quantitative RT-PCR (**p < 0.01 relative to siRNA control; Values determined in 3 independent “differentiation” experiments). Immunofluorescence photomicrographs depicting TGF-β-induced αSMA expression in 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA (Bar=40 µ). TGF-β-induced expression of αSMA protein in 10T1/2 cells transfected with siRNA targeting MFG-E8 or control siRNA (Western Blot). Quantification of αSMA expression in digitized images using Image J (Histogram). The amount of αSMA in control siRNA transfected cells cultured in medium without TGF-β was assigned a value of 1 (***p<0.01; values determined in 3 independent experiments, NS = not significant). (B) Immunofluorescence photomicrographs depicting TGF-β-induced αSMA expression in 10T1/2 cells treated with control Ig or anti-MFG-E8 pAb (Bar=40 µ). TGF-β-induced expression of αSMA protein in 10T1/2 cells treated with control Ig or anti-MFG-E8 pAb (Western Blot). Quantification of αSMA expression in digitized images using Image J (Histogram). The amount of αSMA in cells cultured in medium without Ab and without TGF-β was assigned a value of 1 (***p<0.01; values determined in 3 independent experiments, NS = not significant).