Tumorigenesis and Neoplastic Progression

Overexpression of Carcinoma and Embryonic Cytotrophoblast Cell-Specific Mig-7 Induces Invasion and Vessel-Like Structure Formation

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Molecular requirements for carcinoma cell interactions with the microenvironment are critical for disease progression but are poorly understood. Integrin $\alpha\beta 5$, which senses the extracellular matrix, is important for carcinoma cell dissemination *in vivo*. $\alpha\beta 5$ signaling induces Mig-7, a novel human gene product that is apparently carcinoma-specific. We hypothesized that Mig-7 expression facilitates tumor cell dissemination by increasing invasion and vasculogenic mimicry. Results show that embryonic cytotrophoblasts up-regulated Mig-7 expression before they acquired an invasive phenotype capable of pseudo-vasculogenesis. Mig-7 protein primarily co-localized with vasculogenic mimicry markers factor VIII-associated antigen, vascular endothelial-cadherin, and laminin 5 $\gamma 2$ chain domain III fragment in lymph node metastases. Overexpression of Mig-7 increased $\gamma 2$ chain domain III fragments known to contain epidermal growth factor (EGF)-like repeats that can activate EGF receptor. Interestingly, EGF also induced Mig-7 expression. Carcinoma cell adhesion to laminins was significantly reduced by Mig-7 expression. Remarkably, in two-dimensional and three-dimensional Matrigel cultures, Mig-7 expression caused invasion and vessel-like structures. Melanoma cells, which were previously characterized to invade aggressively and to undergo vasculogenic mimicry, expressed Mig-7. Taken together, these data suggest that

Mig-7 expression allows cells to sense their environment, to invade, and to form vessel-like structures through a novel relationship with laminin 5 $\gamma 2$ chain domain III fragments. (*Am J Pathol* 2007, 170:1763–1780; DOI: 10.2353/ajpath.2007.060969)

For more than 100 years, researchers have proposed that embryonic cytotrophoblast cells (CTBs) and cancer cells are one and the same.^{1,2} Although they may not be the exact same cells, embryonic CTBs are similar to cancer cells. They invade maternal tissues from the site of embryo implantation during placenta development. In addition, CTBs remodel maternal vasculature, a process known as vascular mimicry or pseudovasculogenesis, creating leaky vessels³ similar to those found in tumors.⁴ Invasion of these two cell types is initiated by receptor tyrosine kinase (RTK) and $\alpha\beta 5$ signaling cross talk.^{5–8}

Data suggest that highly invasive cancer cells can form conduits in tumors, a process some researchers call vasculogenic mimicry.^{9–11} Previous publications have described tumor cells lining the lumen of irregular and leaky tumor vessels (see review¹²). These tumor cells that form vessel-like structures and CTBs can masquerade as endothelial cells by expressing vascular endothelial (VE)-cadherin and factor VIII-associated antigen (FVIII assoc:ag; also known as von Willebrand factor), as well as other endothelial markers.^{10,11,13,14} Laminin 5 $\gamma 2$ chain promigratory fragments contribute to cell motility, invasion,^{15–19} and

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vessel-like structure formation by tumor cells.⁹ Invasive melanoma cells capable of forming vessels express significantly higher levels of laminin 5, a basement membrane component that is a heterotrimer of $\alpha 3$, $\beta 3$, and $\gamma 2$ chains. Matrix metalloproteinase-2 (MMP-2) and membrane type 1 (MT-1) MMP cooperate to cleave $\gamma 2$ chain into fragments that cause melanoma cell invasion leading to vasculogenic mimicry.⁹ *In vivo*, predominantly tumor cells rather than endothelial cells form vessels in the interior, more hypoxic regions of tumors, and are thought to provide new blood flow to the tumor causing renewed growth and dissemination.²⁰ This formation of tumor cell-lined vessels seems to be resistant to antiangiogenic therapies.²¹ In addition, invading tumor cells are resistant to current therapies.²² Because no specific marker expressed by tumor cells that invade or mimic endothelial cells has previously been discovered, these cells can evade detection. Understanding the regulation of tumor cell invasion and vessel formation is therefore of considerable importance for development of more efficient and effective targeted tumor cell detection as well as therapies.

Mig-7 is a cysteine-rich protein found in carcinoma cell membrane and cytoplasm protein lysate fractions by immunoblotting. Expression of Mig-7 seems to be restricted to carcinoma cells and putatively to early placenta, based on homology with expressed sequence tags (ESTs) isolated from this tissue. Human malignant tumors, blood, and metastatic sites from more than 200 cancer patients express Mig-7 regardless of tissue origin. Notably, Mig-7 is not detected in 25 different normal human tissues or in blood from normal patients.^{6,23} Signaling that initiates CTBs and tumor cell invasion from RTK c-Met, the hepatocyte growth factor/scatter factor (HGF/SF) receptor, and $\alpha v\beta 5$ integrin induces Mig-7 expression.^{6,8,24}

Based on these data, we hypothesized that Mig-7 expression plays a role in behaviors in common between CTBs and carcinoma cells, namely invasion and vascular cell mimicry. To test this hypothesis, Mig-7 expression by human CTBs was examined *in vitro* and *in vivo*. Tumor microenvironment extracellular matrix (ECM) and its growth factors important for these behaviors were used with endogenous Mig-7 and short interfering RNA (siRNA) stable knockdown cell lines, as well as overexpressed Mig-7 in two-dimensional and three-dimensional cultures, to determine Mig-7 biological relevance. Immunohistochemistry (IHC) of lymph nodes from our nude mouse model of tumor cell invasion determined localization of Mig-7 expression and topographical relationships *in situ* with VE-cadherin, laminin 5 $\gamma 2$ chain domain III fragments, and FVIII assoc.ag. Adhesion assays were also used to elucidate mechanisms by which Mig-7 expression contributes to tumor cell invasion and its potential role in vessel-like structure formation.

Materials and Methods

Cell Cultures

Human CTBs were isolated from second trimester placentas under institutional review board approval as de-

scribed previously.²⁵ CTBs were cultured on an ECM (Matrigel; BD Biosciences, San Jose, CA), which initiates their differentiation along the invasive pathway.²⁶ HEC1A, RL95-2 (RL95) endometrial carcinoma, and HT29 colon carcinoma cell lines (all from American Type Culture Collection, Rockville, MD) were cultured as described previously.^{6,23} Epidermal growth factor (EGF; Calbiochem, La Jolla, CA) or HGF/SF (R&D Systems, Minneapolis, MN) treatments (each at 20 ng/ml) were incubated for the indicated time with RL95 cells that had been serum-starved for 12 hours. Melanoma cell lines, MUM-2B, MUM-2C, C918, C8161, and A375P, were maintained in RPMI 1640 medium (Life Technologies, Inc., Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc., Invitrogen), 20 mmol/L HEPES, and 0.1% gentamicin sulfate. Cultures, invasive phenotypes, and ability to undergo vasculogenic mimicry are well characterized for melanoma cell lines.^{9,10,14,20,27,28} All cultures were maintained in a humidified incubator at 37°C in 5% CO₂ air.

For two-dimensional cultures, HT29, HEC1A, or RL95 cells were plated on Matrigel containing growth factors (GF⁺ Matrigel) or growth factor-reduced Matrigel (GFR Matrigel, >10 mg/ml lots; BD Biosciences) ~0.2 mm thick from 80% confluent cultures for indicated times before collecting protein lysates. Three-dimensional cultures were performed using 50- μ l domes of GF⁺ Matrigel (no dilution) allowed to polymerize for 30 minutes at 37°C in a humidified, 5% CO₂ incubator. Cells were removed nonenzymatically from their plates [2 mmol/L ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] and 2 μ l of single-cell suspensions (7.5×10^4) were injected into the dome of Matrigel. Images of two-dimensional and three-dimensional cultures were taken using a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) and Retiga 2000R digital charge-coupled device camera with QCapture 5.1 software (QImaging, Burnaby, BC, Canada).

Xenograft Mouse Model

The nude mouse model was used as described previously²³ under Institutional Animal Care and Use Committee approval. Briefly, 1×10^5 HEC1A or RL95 viable cells in 250 μ l of media were combined with 250 μ l of GF⁺ Matrigel and injected subcutaneously into the dorsal neck region of nu/nu athymic mice. Negative controls were mice injected with Matrigel alone (ie, no cells). Five animals per group were injected. After 6 weeks, animals were euthanized. Brachial and axillary lymph nodes were harvested, flash-frozen in OCT (Miles Laboratories, Elkhart, IN), and stored at -80°C until cryosectioned.

Expression of Mig-7 FLAG-Tagged Protein and Mig-7-Specific siRNA

Selection of transfected pooled clones ($n = 3$ each construct), as well as expression of 3XFLAG CMV Mig-7, were described previously.⁶ Endogenous Mig-7 expression was reduced with siRNA. In brief, three Mig-7-spe-

Table 1. siRNA Sequences Specific to Mig-7

Sequence <u>anti-sense-loop-sense</u>	Location in Mig-7 sequence (accession: DQ080207) in base pairs	Pooled clones number
5'-AAAGTTTCATTCTTCGACTTCAAGAGAGTCGAAGAAATGAACTTT-3'	379 to 398	1-3A
5'-AGATTTCCCTGTGATTTAAGTTCAAGAGACTTAAATCACAGGAAATCT-3'	728 to 746	2-3B
5'-CATGATCTGGATTGTAATCTTCAAGAGAGATTCAAATCCAGATCATG-3'	1275 to 1293	3-1A

cific siRNAs sequences (Table 1) were designed by Ambion (Austin, TX) with *Bam*HI and *Hind*III restriction enzyme sites at each 5' and 3', respectively, for directional cloning. No significant homology was found to any human sequence other than Mig-7 (accession no. DQ080207). Double-stranded DNA oligonucleotides that encode each Mig-7 siRNA were synthesized by Invitrogen (Carlsbad, CA), resuspended, quantified, annealed, cut with *Bam*HI and *Hind*III restriction enzymes, and ligated into the pSilencer vector (pSilencer 3.1-H1 neo) cut with the same enzymes according to the manufacturer's instructions (Ambion). After one optimal concentration of G418 for RL95 cell killing was determined, RL95 cells were transfected in triplicate for each construct at a ratio of 1 μ g of plasmid to 3 μ l of FuGene 6 (Roche, Indianapolis, IN). After G418 selection at 600 μ g/ml, levels of Mig-7 protein expression were determined for each pooled transfected cell line by immunoblotting and densitometry using β -tubulin as a normalizing gene expression.

Immunohistochemistry

Detection of Mig-7 protein was performed using Mig-7-specific affinity-purified antibody produced in rabbits immunized with KLH-conjugated Mig-7 peptide (MAASRC-SGL) representing the first nine amino acids of Mig-7 protein, as previously described.⁶ In brief, cryostat sections (10 μ m) of fresh-frozen lymph node from five HEC1A-, four RL95-, and five control (Matrigel-alone)-injected nude mice on Superfrost plus slides were washed two times in Dulbecco's phosphate-buffered saline (D-PBS; Hyclone, Logan, UT) and then treated with 50 mmol/L NH_4Cl (Sigma, St. Louis, MO) for 10 minutes at room temperature. Slides were washed two times in D-PBS and then permeabilized with 0.01% digitonin (Aldrich Chemical Co., Milwaukee, WI) in phosphate-buffered saline (PBS) at room temperature for 30 minutes. After washing two times in D-PBS, slides were blocked in 10% horse serum (Life Technologies, Inc.) in D-PBS for 30 minutes at room temperature. Primary antibodies, polyclonal rabbit anti-human Mig-7, monoclonal mouse, human-specific anti- γ 2 domain III (clone D4B5; Chemicon, Temecula, CA), polyclonal rabbit anti-VE-cadherin (Cayman Chemical, Ann Arbor, MI), and polyclonal rabbit, FVIII assoc:ag (DAKO, Carpinteria, CA) were diluted 1:50 and incubated on the tissue for 2 hours at room temperature and overnight at 4°C. Slides were washed two times in D-PBS then incubated for 20 minutes in 3% H_2O_2 in methanol. Slides were washed two times in D-PBS and then incubated for 30 minutes in 1:200 dilution

of goat anti-rabbit IgG-horseradish peroxidase (HRP) or goat anti-mouse IgG-HRP labeled antibody (Santa Cruz Biotechnology, Santa Cruz, CA) relevant to the primary antibody used in D-PBS containing 0.5% bovine serum albumin (catalog no. BP1600-100; Fisher Scientific, Fair Lawn, NJ). Slides were washed two times in D-PBS and then developed using 3,3'-diaminobenzidine (DAB) substrate kit (Vector Laboratories, Burlingame, CA) to detect HRP-labeled secondary antibodies until brown specific staining was detected by microscopy (less than 3 minutes). After washing in water for 5 minutes, slides were counterstained in Hematoxylin QS (Vector Laboratories). Slides were dehydrated in two incubations for 3 minutes each of 75, 95, and 100% ethanol, air-dried, and mounted in DPX mounting medium (BDH Laboratory Supplies, Poole, UK). Controls included sections without primary or secondary antibodies, as well as staining of normal (vehicle-injected animal) lymph node sections with each primary antibody.

Ten percent formalin-fixed, paraffin-embedded 5- μ m sections were used for basal plate placenta analyses. After deparaffinization with xylene for 10 minutes, rehydration through 100, 95, and 70% ethanol, and antigen retrieval for 2 seconds on ice in a microwave oven, these sections were processed as described above to detect Mig-7 or cytokeratin 7 (CK7). Mouse anti-human antibody for CK7 (DAKO) was used at 1:100. Images from low ($\times 40$) to high ($\times 1000$) magnifications were taken on a Nikon Microphot microscope with a Retiga 2000R digital charge-coupled device camera and analyzed with the QCapture 5.1 software program (both QImaging) and Canvas 8.0 (Deneba Systems Inc.). Measurement of vessels in lymph nodes invaded by HEC1A and RL95 cells was performed with the QCapture software program calibrated with a micrometer. At least four different tumors from four mice injected with each cell line (HEC1A or RL95) or Matrigel alone were analyzed.

Western Blot Analyses

Western blot analyses were performed as described previously with the following modifications.⁶ Cells grown on plastic or two-dimensional Matrigel (10 mg/ml lot) were homogenized in lysis buffer [2% sodium dodecyl sulfate, 100 mmol/L dithiothreitol, 0.01% bromophenol blue, 60 mmol/L Tris, 10% glycerol, 2 $\times$ protease inhibitor (Complete; Roche)] and quantitated using RC/DC protein assay (Bio-Rad, Hercules, CA). Equal amounts of protein were loaded onto a 12% polyacrylamide gel and run at constant 200 V for 30 to 40 minutes. Gels were semidry transferred (Boekel, Feasterville, PA) to polyvinylidene

fluoride membranes and blocked in Tris-buffered saline containing Tween 20 detergent (TBST; 0.1%) and 5% dry milk for 1 hour at room temperature. Endogenous or FLAG-tagged Mig-7 protein was detected using human-specific, affinity-purified Mig-7 antibody (1:2000) or the M2-peroxidase anti-FLAG antibody (1:100; Sigma), respectively. Mouse anti- β -tubulin monoclonal antibody (clone AA2; Upstate, Lake Placid, NY) or mouse anti- β -actin (clone AC-40; Sigma) served as loading controls. Antibody to laminin 5 γ 2 chain domain III was described in Immunohistochemistry. After washing in TBST, a HRP-labeled secondary anti-rabbit IgG antibody at a dilution of 1:40,000 was used to detect the Mig-7 antibody or goat anti-mouse IgG-HRP-labeled antibody (Santa Cruz Biotechnology) to detect the β -tubulin, actin, or laminin 5 γ 2 chain domain III antibodies. Chemiluminescence Plus Reagent (Amersham, Arlington Heights, IL) allowed detection of HRP-labeled antibodies once exposed to film. Densitometry was performed using the Bio-Rad imager and Quantity One analysis software program and comparing each protein of interest band intensity to its respective β -tubulin or actin band intensity. All raw signal intensities were corrected for background. Data analyses were performed with Prism 3.0 statistical software (GraphPad, San Diego, CA).

RNA Isolation, Relative Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), and Northern Blot Analyses

Total RNA isolation and relative RT-PCR, including optimization of cycle number to achieve mid-linear range, were performed as described previously.⁶ Placental RNA was obtained, with patient consent and institutional review board approval, as described previously.²⁹ PCR products were confirmed by Southern blot (Mig-7-specific cDNA probe) or by subcloning and sequencing. Northern blots were performed as described previously.⁶

Quantitative Real-Time RT-PCR (Q-PCR)

Reverse transcription of RNA was performed using the TaqMan Gold RT-PCR kit as described by the manufacturer (Applied Biosystems, Foster City, CA). Q-PCR, used the Applied Biosystems 9700HT sequence detection system. Each target was amplified in triplicate with a Mig-7-specific primer probe set (forward, 5'-CACCTGCCTCTGGTCGTTAGG-3'; reverse, 5'-TACTGGATTCCTCTAGC-TTTGGTGT-3'; probe, 5'-AAACTCTCAGTGATCTCT-3') or 18S primer probe set (Applied Biosystems). Primer pairs for HLA-G and integrin α 1 were as previously published.^{8,30} In brief, 5 μ l of cDNA target was added to a 20- μ l mix of 1 $\times$ TaqMan universal PCR master containing Amperase UNG and 1 μ l of primer/probe. Reactions were incubated at 50°C for 2 minutes and then at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. A fivefold titration of control template was included along with both RT-negative and no template controls. Relative mRNA levels were then cal-

culated using the standard curve method (ABI User Bulletin no. 2) using 18S as the endogenous control and 0 hour as the calibrator.

Adhesion Assays

Adhesion of stably transfected FLAG-Mig7-expressing, pooled clones, or empty vector HT29 colon carcinoma cells to laminins, fibronectin, vitronectin, collagen I, or collagen IV was tested using the CytoMatrix(5) screen kit (Chemicon) according to the manufacturer's instructions. In brief, after counting with a hemocytometer in the presence of trypan blue, 1000 viable cells of each cell line in 100 μ l of single cell suspensions per well were plated as indicated in six coated wells per ECM component plus six wells coated with bovine serum albumin as a control and incubated in a humidified CO₂ incubator at 37°C for 1 hour. After washing the plate three times with 200 μ l/well of PBS containing Ca²⁺ and Mg²⁺, cells were stained and fixed in 100 μ l of 0.2% Crystal violet in 10% ethanol for 5 minutes at room temperature. After washing three times to remove the stain with 300 μ l/well PBS, 100 μ l/well of a 50:50 mix containing 0.1 mol/L NaH₂PO₄, pH 4.5, and 50% ethanol was added with 5 minutes of gentle shaking at room temperature to solubilize and release the cell-bound stain. Absorbance readings were taken at 550 nm on a microplate reader (Bio-Rad). Median bovine serum albumin readings (background) for each cell line were subtracted from ECM readings, and resulting data were analyzed using GraphPad Prism 3.0.

Statistical Analyses

All experiments were performed two to four times as indicated in the figure legends. Student's *t*-test when comparing two experimental groups or one-way analysis of variance with Tukey-Kramer multiple comparisons post test for multiple group comparisons were used for statistical analyses in the GraphPad Prism statistical analyses software. A *P* < 0.05 was considered significant.

Results

Temporal Expression of Mig-7 in Placental Tissue and during Invasive CTB Differentiation

Mig-7 cDNA (accession no. DQ080207) is homologous to ESTs (N41315 and AI18969) isolated from 10-week gestation placenta.⁶ Even though our previous report suggests that Mig-7 is carcinoma cell-specific, this homology indicated that Mig-7 may be expressed by early placenta cells. Therefore, Mig-7 expression in placenta needed to be verified because behaviors of cancer cells have been compared with embryonic trophoblast cells,^{1,2} yet gene expression specific to these two cell types remains obscure.

Relative RT-PCR analyses of total, DNase-treated RNA from early and late gestation placenta detected Mig-7 expression strictly in early gestational tissue (Figure 1a).

Figure 1. Early gestation human placental tissue and invasive embryonic cytotrophoblasts express Mig-7. **a:** Representative relative RT-PCR analysis of Mig-7 mRNA expression in early and late gestation placenta. Mig-7 amplicon (501 bp) is present in early gestation placenta (7 weeks) but not in term placenta (38 weeks, term). Ribosomal 18S RNA amplicon (324 bp) served as loading control. RNA isolation and relative RT-PCR including optimization of cycle number to achieve mid-linear range were performed as described previously.⁶ **b:** RT-PCR of human placental basal plate RNA from second trimester and term shows expression of Mig-7 highest before 22 weeks gestation (16, 19.5 weeks), reduced at 23 weeks, and undetectable at term. RL95 cells served as positive control, and ribosomal 18S RNA served as loading control. **c:** Immunoblot of Mig-7 protein from cells of first, second, and third trimester placenta basal plate. Tubulin probe was used as a loading control. **d:** Densitometry of immunoblot in **c**. Note decrease of Mig-7 protein consistent with a decrease in Mig-7 protein mRNA shown in **a** and **b**. Ten basal plate placenta samples, two first trimester, two second trimester, two third trimester, and two term, were used. **e:** Quantitative (Q)-PCR analysis of Mig-7 mRNA in triplicate on reverse-transcribed total RNA isolated from human cytotrophoblasts before and 3, 12, and 36 hours after plating on Matrigel from four separate cytotrophoblast isolations (○, 13 weeks; ●, 13 + 15 weeks; △, 15 + 18 weeks; ▲, 14.5 + 19 weeks). Data are presented as mean ± SE. The mean 12-hour time point for all experiments was significantly different compared with 0 hour ($P = 0.001$) using the Student's *t*-test. **f:** Representative Q-PCR analysis of HLA-G and integrin $\alpha 1$ mRNA in triplicate on reverse-transcribed total RNA isolated from human cytotrophoblasts (13 weeks) before and 3, 12, and 36 hours after plating on Matrigel (○, integrin $\alpha 1$; ■, HLA-G). Data are presented as mean ± SE. **g:** Representative Western blot analysis of Mig-7 in second trimester-isolated CTBs plated on Matrigel for the indicated times. Human platelet (plt) lysate served as a negative control. Note that Mig-7 antibody detects a 46-kD band from CTBs plated on Matrigel in addition to the previously published 23-kD form from carcinoma cells plated on plastic. **h:** Representative IHC of a section (10% formalin-fixed, paraffin-embedded, 5 μ m) of 19-week gestation basal plate using antibody to cytokeratin 7 (CK7), an antigen specific to CTB. **i:** Representative IHC of cells in the basal plate (19 weeks of gestation) expressing Mig-7. Note that areas of staining (brown) are the same for both antigens (**h** and **i**) in these serial sections. Two different basal plates were examined from each of first, second, and third trimester placentas for each protein. CK7 and Mig-7 demonstrated the same areas of staining. Experiments were repeated at least twice (more than three times for Q-PCR). Scale bars = 20 μ m.

Human CTBs invade maternal decidua and vasculature during early placental development, with peak invasion and vascular remodeling occurring between 14 and 22 weeks of gestation. This invasive capacity is greatly reduced by term.^{2,25} Therefore, placental basal plate tissue, the location of cytotrophoblast invasion and vascular remodeling, was isolated from first, second, and third trimester as well as term placentas. Mig-7 mRNA expression was highest in the second trimester samples before

22 weeks of gestation, was reduced at 23 weeks, and was absent at term (Figure 1b). Immunoblot analysis of Mig-7 protein in lysates from basal plates of the indicated stage in gestation is shown in Figure 1c. Densitometry analyses (Figure 1d) showed a 10-fold decrease in 38-week basal plate expression of Mig-7 protein relative to β -tubulin compared with that of first trimester basal plate.

Isolated embryonic CTBs plated on GF⁺ Matrigel differentiate in a manner that recapitulates that of invasive

basal plate CTBs.³ Therefore, we analyzed Mig-7 expression in isolated human CTBs cultured on GF⁺ Matrigel. Mig-7 mRNA expression increased 6- to 15-fold by 12 hours after plating on Matrigel (Figure 1e). To confirm that the primary CTBs differentiated down the invasive pathway, mRNA levels of CTB invasive markers HLA-G³⁰ and integrin $\alpha 1^8$ were also measured and found to increase at least 15-fold by 12 hours on Matrigel as expected (Figure 1f).

Immunoblot analyses demonstrated that the up-regulation of Mig-7 protein also occurred by 3 hours and peaked at 12 hours. The previously reported 23-kd Mig-7 band when carcinoma cells were plated on plastic⁶ was detected along with a novel ~46-kd band (Figure 1g). Lysates from platelets that served as negative controls lacked detectable Mig-7 protein (Figure 1g). IHC of basal plates from first, second, or third trimester ($n = 2$ each stage) using CTB-specific cytokeratin-7³¹ (CK7) antibody (Figure 1h) or antibody to Mig-7 (Figure 1i) were performed. In serial sections, cells stained for Mig-7 were in the same areas that stained for CK7.

Tumor Microenvironment Growth Factors Induce Expression of Mig-7 that Leads to Laminin 5 $\gamma 2$ Chain Fragmentation in Carcinoma Cells

Mig-7 is detected in tumor cells from all types of solid cancers and is induced by RTK c-Met activation. Preincubation of serum-starved endometrial carcinoma cells with well-characterized blocking antibodies to $\alpha v\beta 6$, $\beta 1$, or $\alpha v\beta 5$ integrins before HGF treatment has demonstrated that $\alpha v\beta 5$ ligation is required in cross-talk signaling with RTK c-Met to initiate Mig-7 expression.⁶ EGF-induced invasion of another $\alpha v\beta 5$ -positive cell line, FG pancreatic carcinoma cells, as well as CTBs, also requires this cross-talk signaling.^{7,32} Therefore, we tested whether EGF can induce Mig-7 in $\alpha v\beta 5$ -positive RL95 endometrial carcinoma cell line from which we originally isolated Mig-7.⁶

After serum starvation for 12 hours, RL95 endometrial carcinoma cells were treated with 20 ng/ml of either HGF or EGF. By Northern blot analyses, Mig-7 mRNA was increased 2.5 hours after treatment with HGF, consistent with our previous findings with this growth factor.⁶ EGF also induced Mig-7 mRNA levels 2.5 hours after treatment (Figure 2a). Densitometry analyses revealed a 3-fold (HGF) and 3.5-fold (EGF) increase at 2.5 hours of growth factor treatment over 12-hour serum-starved RL95 cells (Figure 2b).

To explore further the effects of tumor microenvironment growth factors, we used plating of endogenous Mig-7-expressing human cell lines, RL-95 and HEC1A endometrial epithelial carcinoma,⁶ on GF⁺ or GFR Matrigel. The RL95 cell line is isolated from a moderately differentiated adenosquamous carcinoma of the endometrium.³³ HEC1A cells were isolated from a stage IA endometrial cancer.³⁴ Both cell lines are invasive *in vitro*³⁵ and *in vivo*.²³ Matrigel is a commercially available

basement membrane isolated from Engelbreth-Holm-Swarm mouse tumor whose major matrix components are laminins (56%), collagen IV (31%), and entactin (8%).^{36,37} Greater than 10 mg/ml of GF⁺ Matrigel ECM contains approximate concentrations of EGF, 1.3 ng; basic fibroblast growth factor, 0.2 pg; nerve growth factor, 0.2 ng; platelet-derived growth factor, 48 pg; insulin-like growth factor-I, 24 ng; and transforming growth factor- β , 4.7 ng (all per ml).^{36,37}

Immunoblot analyses detected Mig-7 as the known 23-kd and a more predominant, novel ~63-kd protein in lysates from RL95 or HEC1A cells plated on GF⁺ Matrigel but not from cells plated on GFR Matrigel (Figure 2c). Lysates from Matrigel alone cultures did not possess these bands (data not shown). Densitometry of these immunoblots comparing intensities of Mig-7 to β -actin bands revealed that levels of this upper 63-kd Mig-7 band in HEC1A cells were significantly 40% greater than the 63-kd bands in RL95 cells, whereas the levels of the 23-kd Mig-7 band were not significantly different (Figure 2d). Plating endometrial carcinoma cell lines on GF⁺ Matrigel also induced production of laminin 5 $\gamma 2$ fragments that were different from those found from cells plated on GFR Matrigel. An ~63-kd $\gamma 2$ chain domain III fragment was detectable in the GF⁺ Matrigel-plated cells and not in the GFR Matrigel-plated cells (Figure 2e).

To confirm the Mig-7 ~63-kd band and the $\gamma 2$ chain fragment of 63 kd, we used our 3XFLAG CMV Mig-7 or empty vector stably transfected, pooled clones of a cell line that does not endogenously express Mig-7, HT-29 colon carcinoma.⁶ HT-29 cells are from a human stage I colon tumor. On plastic, we previously detected 26-kd Mig-7 bands (an increase of ~3 kd is attributable to the 3XFLAG of 24 amino acids) from each pooled clones A, B, and C⁶; these bands were not detected in vector alone cells plated on GF⁺ Matrigel (Figure 2f). However, plating HT29 transfectants on GF⁺ Matrigel revealed, by immunoblotting with anti-FLAG antibody, the additional ~66-kd Mig-7 band (again, an increase of ~3 kd is attributable to the 3XFLAG of 24 amino acids) (Figure 2f). Fragmentation of $\gamma 2$ chain was predominantly detected at the same size, ~66 kd, as the upper band of Mig-7 only in the HT29 cell lines expressing Mig-7 (Figure 2f). This upper band was previously seen in cells endogenously expressing Mig-7 (Figure 2e). No 80-kd $\gamma 2$ chain fragment and very low levels of ~66-kd as well as 40-kd sizes were seen by immunoblotting of lysates from empty vector cells plated on GF⁺ Matrigel, even though equal levels of protein were loaded as determined by protein assays and by β -tubulin antibody (Figure 2f).

Mig-7 Expression Causes Cell Spreading on Two-Dimensional GF⁺ Matrigel

Laminin 5 $\gamma 2$ chain fragmentation is required for carcinoma cell invasion^{19,38} and for vasculogenic mimicry.^{9,20} Domain III $\gamma 2$ chain fragments, detected by the $\gamma 2$ antibody used in the previous immunoblots, activate

Figure 2. Tumor microenvironment RTK ligands, HGF, and EGF induce Mig-7 in RL95 endometrial carcinoma cells. **a:** Representative Northern blot analyses show that after 12 hours of serum starvation, EGF induces Mig-7 similarly to HGF treatment, the growth factor used to isolate Mig-7. **b:** Northern blot densitometry analyses with Mig-7 band intensities normalized to those for respective 18S bands stained with methylene blue revealed that EGF induced Mig-7 mRNA at least if not more so than does HGF. Error bars represent SEM. **c:** Representative immunoblot of serum-starved (48 hours) HEC1A and RL95 cells that endogenously express Mig-7 plated on Matrigel containing growth factors, unless indicated as reduced growth factor Matrigel (GFR). Previously reported 23-kD Mig-7 was detected in addition to a higher ~63-kD band not seen in lysates from cells plated on plastic. No Mig-7 was detected in lysates from either cell line plated on GFR Matrigel. **d:** Densitometry and statistical analyses of these immunoblots revealed that the larger Mig-7 kD band was significantly greater in HEC1A cells. Error bars represent SEM. **e:** Representative Mig-7 and laminin 5 γ2 chain domain III fragment immunoblot analysis of protein lysates from serum-starved (48 hours) RL95 cells plated for 17 hours on GF⁺ or GFR Matrigel. Blots were stripped and re-probed with anti-γ2 domain III antibody or new immunoblots run and probed first with anti-γ2 antibody to confirm that this was not residual Mig-7 antibody detection at ~63 kD. Detection of β-actin at the last probing served as loading control. **f:** Representative immunoblot detection of FLAG-Mig-7 and laminin 5 γ2 chain domain III fragments in lysates from three pooled clones, stable transfectants (lanes A, B, and C), and from empty vector-transfected HT29 colon carcinoma cells. All cells were plated on GF⁺ Matrigel. Note predominant γ2 chain fragment ~66-kD bands in Mig-7-expressing HT29 cells. Detection of β-actin at the last probing of each blot served as loading control. Experiments were repeated three times in triplicate.

the EGF receptor.^{39,40} Because we detected different Mig-7- and γ2 chain-specific bands when either endogenous or FLAG-Mig-7 HT29 cells were cultured on GF⁺ Matrigel compared with when cultured on plastic, we examined morphology of these cell lines on plastic to those cultured in two-dimensional and in three-dimensional GF⁺ Matrigel cultures. When cultured on a thick layer (no dilution) of GF⁺ Matrigel, FLAG-Mig-7-expressing cells were found to be similar in morphology to vector alone cells (Figure 3, a and d) after 17 hours of plating. In contrast, after 10 days in culture, Mig-7-expressing HT29 cells were predominantly spread out over the Matrigel (Figure 3b), whereas cells transfected with empty vector remained primarily in

colonies comprised of densely piled cells (Figure 3e). On tissue culture plastic (no Matrigel), the morphology of FLAG-Mig-7-expressing HT29 cells (Figure 3c) was indistinguishable from that of HT29 cells transfected with empty vector (Figure 3f).

Invasion and Vessel-Like Structure Formation in Three-Dimensional GF⁺ Matrigels Caused by Mig-7 Overexpression in HT29 Cells

Cells cultured in three-dimensional matrices can more accurately recapitulate the *in vivo* microenvironment, based on different morphologies, signal transduction,

Figure 3. HT29 Mig-7 overexpression causes cell spreading on GF⁺ Matrigel but not on plastic. **a:** HT29 cells overexpressing FLAG-Mig-7 at 17 hours. There is no morphological difference observed at this time period compared with empty vector HT29 cells (**a** and **d**). **b:** After 10 days on GF⁺ Matrigel, HT29 cells overexpressing FLAG-Mig-7 spread over the matrix in contrast to HT29 nonexpressing cells that primarily form colonies and do not spread over the matrix (compare **b** and **e**). **c:** HT29 cells overexpressing FLAG-Mig-7 plated on plastic did not appear different from empty vector HT29 cells on plastic (**c** and **f**). Empty vector HT29 cells after 17 hours on GF⁺ Matrigel (**d**), 10 days on GF⁺ Matrigel (**e**), or on plastic (**f**). Experiments were repeated three times in triplicate. Scale bars: 100 μ m (**a**, **d**); 150 μ m (**b**); 20 μ m (**c**, **f**).

and microfilaments than the same cells plated on rigid substrates or plastic.^{41–45} Therefore, we used a GF⁺ Matrigel three-dimensional culture system to determine effects of Mig-7 expression on cell behavior via interaction with this microenvironment.

Domes of 50- μ l GF⁺ Matrigel can be formed on plastic because polymerization initiates as soon as the cold Matrigel comes in contact with room temperature plastic, thereby building up Matrigel instead of it spreading over the surface. Media was added to the dish to cover the dome of Matrigel. No other cells were co-cultured with the transfected HT29 cells in these three-dimensional domes. After injection, the single cells started to coalesce during days 1 and 2. Empty vector HT29 cells formed colonies by days 4 and 5. Throughout 10 days of culture, equal numbers of HT29 empty vector stably transfected cells formed discrete, dense colonies in three-dimensional cultures (Figure 4a). In stark contrast, Mig-7-overexpressing HT29 cells invaded and formed vessel-like structures in the Matrigel domes (Figure 4, b and c). Photomicroscopy through four planes of view (z-dimension) demonstrated that cords of invading cells were formed by Mig-7-expressing HT29 (Supplemental data images

were taken through three-dimensional Matrigel; available at <http://ajp.amjpathol.org>). Because of the branching morphology, cells that were in that plane are in focus; the rest are out of focus.

To further examine the possibility that Mig-7 expression leads to vasculogenic mimicry, we obtained melanoma cell lines previously characterized to invade aggressively and to undergo vasculogenic mimicry (C918, C8161, MUM2B), as well as melanoma cell lines that do not possess these behaviors (A375P, MUM2C, generously provided by Dr. Mary Hendrix, Northwestern University, Chicago, IL). The MUM-2B and MUM-2C cell lines were derived from a metastatic uveal melanoma explant within the liver from the MUM-2 heterogeneous cell strain.²⁸ C8161 cells are a cutaneous melanoma cell line from a metastatic site, whereas C918 (uveal) and A375P (cutaneous) are from primary sites (for a review of these cell lines see Hess et al¹⁰). As determined by immunoblotting of lysates from these melanoma cell lines plated on plastic, 23-kD Mig-7 protein expression was limited to melanoma cell lines C918, C8161, and MUM2B. In contrast, A375P and MUM2C did not express Mig-7 (Figure 4d).

Figure 4. Mig-7 overexpression caused vessel formation in Matrigel three-dimensional cultures. **a:** HT29 cells with empty vector form discrete colonies in three-dimensional GF⁺ Matrigel cultures. **b:** In contrast, HT29 cells overexpressing FLAG Mig-7 invade the three-dimensional Matrigel and appear to form vessel-like structures. **c:** Inset from **b** at higher magnification. **d:** Representative immunoblot analysis of Mig-7 expression in melanoma cells that aggressively invade and undergo vasculogenic mimicry (C918, C8161, MUM2B). A lack of Mig-7 protein is found in melanoma cell lines that are poorly invasive and fail to form vessel-like structures (A375P, MUM2C). Stripping and reprobing with β -tubulin antibody was used to confirm equal amounts of protein loaded for each cell lysate. Experiments were repeated three times in triplicate (three-dimensional cultures) or with each cell line (**d**). Scale bars: 150 μ m (**a**, **b**); 100 μ m (**c**).

Decrease of Endogenous Mig-7 Causes Colony Formation and Less Invasion in Three-Dimensional Cultures

Because the behaviors of empty vector and Mig-7-transfected HT29 cell lines were so strikingly different, we developed Mig-7-specific siRNA stably transfected RL95 cell lines to validate these differences. Sequences for each siRNA construct are shown in Table 1. In Figure 5a, immunoblotting revealed that two of three siRNA constructs efficiently decreased levels of Mig-7 protein. Densitometry comparing band intensities of Mig-7 to β -tubulin bands for each sample confirmed a 3.8-fold reduction in Mig-7 protein levels in the 1-3A and 2-3B cell lines, whereas the 3-1A siRNA cell line showed no reduction in Mig-7 protein as compared with parental RL95 cells (Figure 5b).

Morphologies of RL95 siRNA cell lines 1-3A, 3-1A, and parental were not discernibly different when plated on tissue culture plastic with no Matrigel (Figure 5, c–e). However, when equal numbers of cells were grown for 10 days in three-dimensional GF⁺ Matrigel, the RL95 parental cell line and the 3-1A siRNA cell line that express endogenous levels of Mig-7 were similar in invasive behavior with no dense colony formations observed (Figure 5, f and h). In contrast, the siRNA Mig-7 knockdown expressing 3.8-fold less Mig-7 protein formed dense colonies (Figure 5g).

Mig-7 Expression Causes Decreased Adhesion to Tissue Culture Plastic and to Laminins

Laminin 5 γ 2 fragments allow migration and invasion, apparently through less stable adhesion to basement membrane and up-regulation of MMP-2.^{15,46} During selection of stable 3XFLAGCMVMig-7 HT29 cells and before pooling clones, we noticed that Mig-7-expressing cell colonies but not empty vector colonies could be suctioned off during media aspiration (Figure 6a). Various cell types are known to lay down laminin 5 and fragment γ 2 chains differently, because of the type of protease they produce that can cleave γ 2. Some cells form less stable adhesions to basement membrane, of which laminin is a major component.^{9,15,17–19} Therefore, we performed adhesion assays with HT29 transfected cell lines. Mig-7-expressing HT29 cells were significantly less adherent to laminin-coated wells. In contrast, adhesion to other ECM components (fibronectin, vitronectin, collagen I, or collagen IV) was not significantly affected by Mig-7 expression (Figure 6b).

Mig-7 Protein and FVIII assoc:ag Co-Localize to Vessel-Like Structures in Lymph Node

Carcinoma cells use vessels to travel and metastasize to distant sites. Once at the distant site, tumor cells must acclimate or die. Mig-7 has been detected at primary and secondary sites, as well as in the blood of cancer pa-

Figure 5. Mig-7 siRNA reduces Mig-7 protein levels and invasion. **a:** Representative immunoblot analysis of parental and Mig-7-specific siRNA stably transfected, pooled clones RL95 cell lines 1-3A, 2-3B, and 3-1A. Cells were grown for 4 days before harvesting cell lysates. Stripping and reprobing with β -tubulin confirmed equal loading and transfer of 20 μ g of protein each sample. **b:** Densitometry analyses demonstrated a consistent 3.5- to 3.8-fold, statistically significant ($P < 0.01$), reduction of Mig-7 protein in RL95 1-3A and 2-3B siRNA stably transfected cells. Experiments were repeated three times. Error bars represent SEM. **c:** Stably transfected RL95 siRNA 3-1A on plastic. **d:** Stably transfected RL95 siRNA 1-3A on plastic. **e:** Parental (no transfection) RL95 on plastic. No difference in morphology was observed on plastic in normal growth media (compare **c**, **d**, and **e**). **f:** RL95 cells stably transfected with 3-1A Mig-7 siRNA invaded (**arrows**) GF⁺ Matrigel and did not form dense colonies (**arrow**) after 10 days. **g:** In contrast, RL95 cells stably transfected with 1-3A Mig-7 siRNA, which reduced levels of Mig-7 $\times 3.8$ -fold, predominantly formed dense colonies (**arrows**) after 10 days. **h:** Parental RL95 cells that expressed the same level of Mig-7 protein as those transfected with 3-1A construct invaded and did not form dense colonies (**arrows**) in GF⁺ Matrigel after 10 days. Scale bars = 100 μ m.

tients, but not in cells of or blood from normal patients.^{6,23} To determine HEC1A and RL95 cell fate in brachial and axillary lymph nodes, to which these human endometrial carcinoma cells had invaded in a xenograft nude mouse model, we performed IHC to detect Mig-7 and FVIII assoc:ag, an endothelial cell marker that is also found in

plasma.⁴⁷ In serial sections, areas of Mig-7 staining colocalized with areas of staining for FVIII assoc:ag in vessel-like structures that are irregular in shape and sometimes adjacent to one another in lymph nodes from HEC1A-injected nude mice (Figure 7, a-f) or RL95-injected nude mice (Figure 7, g-l). Staining for FVIII asso-

Figure 6. Mig-7 expression causes decreased adhesion to tissue culture plastic and to laminin. **a:** Image of selected colonies in which the top well contained empty vector-transfected HT29 cells, and the bottom well contained 3XFLAG cytomegalovirus (CMV) promoter vector containing Mig-7 encoding region transfected cells. Note the area denuded (dotted line) after suctioning off media in Mig-7-expressing cells. **b:** HT29 Mig-7-expressing cells (black columns) were >30% less adherent than HT29 cells transfected with empty vector (gray columns) to a mix of laminins 1, 2, 3, 6, 8, and 10 in a statistically significant manner ($P < 0.001$). Experiments were performed three times in replicates of six wells per matrix component and bovine serum albumin as control. One-way analysis of variance was used in the GraphPad Prism statistical analysis software. $P < 0.05$ was considered significant. Error bars represent SEM. Experiments in this figure were repeated three times.

c:ag appeared to be more blurred and outside of vessel-like structures than Mig-7 staining (Figure 7, a–l). Overlays of FVIII assoc:ag-stained sections onto Mig-7 serial sections using Canvas 8.0 imaging software revealed that the staining patterns for both antigens were in the same regions (data not shown). No similar staining above background or vessel-like structures were observed in sections from normal lymph nodes stained for Mig-7 (Figure 7, m–o) or for FVIII assoc:ag (Figure 7, p–r). Controls also included no primary antibody-processed sections of lymph nodes from HEC1A-injected animals and no specific brown staining was detected (Figure 7, s–u).

Because HEC1A cells are known to be more invasive *in vitro* than RL95 cells,³⁵ the number of vessel-like structure hot spots, arbitrarily defined as more than three vessel-like structures per area positive for Mig-7, were quantitated and statistically analyzed. At least four lymph nodes from four different nude mice injected with either cell line were analyzed. There was a significant fourfold increase in vessel-like hot spots in lymph nodes from HEC1A-injected animals as compared with lymph nodes from RL95-injected animals (Figure 7v).

Tumor vessels, especially those lined by tumor cells, are small in diameter.^{4,13} QCapture imaging program calibrated to a micrometer was used to measure the diameter of these Mig-7-positive vessel-like structures. Median diameter size was not significantly different between vessels lined with RL95 cells or HEC1A cells as determined by human Mig-7 staining; median diameters ranged from 9 to 13 μm (Figure 7w).

Mig-7- and FVIII assoc:ag-Positive Vessel-Like Structures in Lymph Node Stain for $\gamma 2$ Chain Domain III Fragment as Well as VE-Cadherin

To extend our studies of the possible biological relevance of Mig-7 expression in tumors,^{6,23} we examined expression of Mig-7 and known vasculogenic mimicry-associated proteins, FVIII assoc:ag, VE-cadherin, and laminin 5 $\gamma 2$ chain domain III fragment, at high magnification ($\times 1000$). All of these markers were found in vessel-like structures. At high magnification, Mig-7-positive cells lining vessel-like structures appeared flattened and spindle shaped rather than endometrial epithelial cuboidal in shape (Figure 8, a and e).

In lymph nodes from RL95-injected nude mice, Mig-7 and FVIII assoc:ag co-localized predominantly to cells lining vessel-like structures in both the membrane and cytoplasm (Figure 8, a and b; arrows). Again, FVIII assoc:ag staining appeared to spread away from the vessel-like structures (Figure 8b, asterisks). Laminin 5 $\gamma 2$ chain domain III also localized to cells lining vessel-like structures, mostly in the cytoplasm (Figure 8c, arrows). VE-cadherin-specific staining was observed in membranes of cells lining vessel-like structures (Figure 8d, arrows). As shown in Figure 8, a–d, vessel-like structures are located adjacent to one another or as single structures that also contain cytoplasmic projections or membranous extensions that appear to cross the lumen.

In lymph nodes from HEC1A-injected nude mice, when slides were processed in parallel, IHC staining for Mig-7 and FVIII assoc:ag seemed to be more intense than those found in RL95 vessel-like structures. Mig-7 staining was both membrane and cytoplasm localized in cells lining vessel-like structures (Figure 8e, arrows). FVIII assoc:ag was localized to cells lining vessel-like structures (arrows) and other sites (asterisks) near these vessels (Figure 8f). In Figure 8f, laminin 5 $\gamma 2$ chain domain III staining appeared predominantly membrane localized with some residual cytoplasmic staining mostly in cells of vessel-like regions. VE-cadherin staining was specific for the membranes of cells lining these vessels (Figure 8g, arrows).

Figure 7. *In situ*, Mig-7 protein co-localized primarily to vessel-like structures in lymph nodes from xenograft nude mouse models of metastasis. **a–c:** Representative IHC of lymph nodes from HEC-1A cell-injected nude mice detecting Mig-7 protein localization (DAB, brown stain) (**a**), **arrows** show representative hot spots of vessel-like structures. **d–f:** Representative IHC of lymph nodes from HEC-1A cell-injected nude mice detecting FVIII assoc:ag protein localization (DAB, brown stain) (**d**), **arrows** show representative hot spots of vessel-like structures. **g–i:** Representative IHC of lymph nodes from RL95 cell-injected nude mice detecting Mig-7 protein localization (DAB, brown stain) (**g**), **arrows** show representative hot spots of vessel-like structures. **j–l:** Representative IHC of lymph nodes from RL95 cell-injected nude mice detecting FVIII assoc:ag protein localization (DAB, brown stain) (**j**), **arrows**

(*Legend continues*)

show representative hot spots of vessel-like structures. **m–o**: Representative IHC of normal lymph nodes from nude mice to detect Mig-7 protein. Note that no vessel-like structures or specific DAB staining was observed. **p–r**: Representative IHC of normal lymph nodes to detect FVIII assoc:ag. Note that no vessel-like structures or specific DAB staining was observed. **s–u**: Representative no primary antibody IHC of HEC-1A cell-injected nude mice. Note that no specific DAB staining was observed. Experiments were repeated four times with four to five animals per treatment group. Counterstain was hematoxylin. **v**: Median number of vessel-like hot spots in lymph nodes from HEC1A or RL95 cell line-injected nude mice. $P < 0.05$ was considered significant. Error bars represent SEM. **w**: Median diameter of vessel-like structures in lymph nodes from HEC1A or RL95 cell line-injected nude mice. Error bars represent SEM. Scale bars: 100 μm (**a, b, d, e, g, h, j, k, m, n, p, q, s, t**); 20 μm (**c, f, i, l, o, r, u**). Original magnifications: $\times 40$ (**a, d, g, j, m, p, s**); $\times 100$ (**b, e, h, k, n, q, t**); $\times 400$ (**c, f, i, l, o, r, u**).

Figure 8. Localization of endothelial markers and laminin 5 $\gamma 2$ to cells lining vessel-like structures in lymph nodes of RL95 or HEC1A cell line-injected nude mice. In lymph nodes from RL95 cell line-injected nude mice Mig-7-specific staining in vessel-like structure (**a**, **arrows**); FVIII assoc:ag-specific staining in vessel-like structures (**arrows**) and at sites (**asterisks**) outside these structures (**b**); laminin 5 $\gamma 2$ chain staining (**arrows**) in vessel-like structures (**c**); and VE-cadherin-specific staining (**arrows**) in vessel-like structure (**d**). In lymph nodes from HEC1A cell line-injected nude mice Mig-7-specific staining in vessel-like structure (**e**, **arrows**); FVIII assoc:ag-specific staining in vessel-like structures (**arrows**) and at sites (**asterisks**) outside these structures (**f**); laminin 5 $\gamma 2$ chain staining (**arrows**) in vessel-like structures (**g**); and VE-cadherin-specific staining (**arrows**) in vessel-like structure (**h**). **i**: No specific staining was observed in no primary antibody processed lymph nodes from carcinoma cell-injected nude mice. Vessel-like structures or positive staining for Mig-7 (**j**), FVIII assoc:ag (**k**), laminin 5 $\gamma 2$ chain domain III fragment (**l**), or VE-cadherin (**m**) was detected in normal lymph nodes from nude mice. Experiments were repeated four times with four to five animals per treatment group. Counterstain was hematoxylin. Scale bars = 10 μ m.

Again, in HEC1A-injected animal lymph nodes, vessel-like structures were located adjacent to one another or as single structures that also contain cytoplasmic projections or membranous extensions that appeared to cross the lumen (Figure 8, e–h) such as those observed in RL95 cell-injected animal lymph nodes (Figure 8, a–d). No specific staining was detected in sections processed in parallel with no primary antibody (Figure 8i). Staining of Mig-7, FVIII assoc:ag, $\gamma 2$ chain, or VE-cadherin in normal lymph nodes was not specific above background, and no vessel-like structures were observed (Figure 8, j–m).

Discussion

The present study sought to determine the biological relevance and related mechanisms of Mig-7 during invasion by using overexpression and expression knock-down, cell culture, and *in vivo* analyses. Specificity of

Mig-7 antibody in these studies is established by immunoblot, IHC, and detection of RNAi-specific knockdown of Mig-7 protein. Detection of same-sized bands with anti-FLAG antibody also supports that our Mig-7 antibody is specific. Confirmation by RT-PCR, immunoblot, and IHC of Mig-7 expression in CTBs is important because these embryonic cells possess aggressive invasion and vessel-remodeling capabilities found in carcinoma cells that metastasize and can undergo vasculogenic mimicry. In addition, because normal tissues previously studied do not express Mig-7,^{6,23} it is important that cells of a normal physiological process express Mig-7.

We found that expression of Mig-7 affected the invasiveness of carcinoma cells and cleavage of laminin 5 $\gamma 2$ chain fragments. These effects seemed to be dependent on ECM, modification and attachment to laminins, as well as the availability of growth factors. Human, Mig-7-expressing endometrial carcinoma cells injected into nude

mice localized to vessel-like structures to which these cells invaded. FVIII assoc:ag staining in and around these vessels co-localized and spread away from the vessels, suggesting that these are leaky vessels typically found in tumors and in the spiral arteries that CTBs remodel. The morphology of these vessel-like structures support that they are the leaky vessels previously described by Hashizume and coworkers.⁴ Specifically, the vessels are adjacent to one another, unlike normal vessels, are of small diameter, and contain cytoplasmic projections or membranous extensions that appear to cross the lumen.⁴ Other endothelial cell markers localized to these vessel-like structures suggest that these human endometrial epithelial carcinoma cells are masquerading as endothelial cells.

Biological Relevance of Mig-7 in CTBs and Carcinoma Cell Functions

CTBs and carcinoma cells require $\alpha v\beta 5$ ligation for induction of their invasive and vessel-modifying behaviors.⁵⁻⁸ Plating carcinoma cells or CTBs on GF⁺ Matrigel induces Mig-7 expression, whereas lysates from platelets, which served as negative controls, lack detectable Mig-7 protein, consistent with our previous reports that platelets lack detectable Mig-7 mRNA even with sensitive RT-PCR.^{6,23} Mig-7, HLA-G, and integrin $\alpha 1$ expression decrease between 12 and 36 hours on GF⁺ Matrigel. These data are consistent with a depletion of Matrigel growth factors because Mig-7 mRNA decreases with a single dose of HGF in serum-deprived endometrial carcinoma cell line cultures.⁶

We detected the previously reported Mig-7 23-kd band in common between these carcinoma and CTB types but different sized larger bands of Mig-7 between CTBs (~46 kd) and carcinoma cells (~63 kd) when lysates were from cells plated on Matrigel. This finding is not unusual because other cysteine-rich proteins form dimers migrate aberrantly in reducing gels.^{46,48,49} Mig-7 protein possesses 10% cysteine residues in the first 91 amino acids (accession no. DQ080207). In addition, there may be a difference in protease expression between carcinomas and CTBs plated on Matrigel.³¹ Importantly, in carcinoma cells the larger sized band is detected with either Mig-7, $\gamma 2$ domain III, or FLAG antibody, strongly suggesting that Mig-7 is interacting with itself or with another protein depending on the cell type and presence of ECM. Regardless, these data strongly suggest that invading CTBs from early placenta express Mig-7. This novel finding is significant because invasion and vascular remodeling are behaviors in common between CTBs and carcinoma cells. Our data suggest that Mig-7 plays a role in the plasticity of carcinoma cells to invade and to mimic endothelial cells.

Importance of the Tumor Microenvironment in Mig-7 Effects

Our data show that the tumor microenvironment growth factors, HGF/SF⁶ and EGF, as well as GF⁺ Matrigel,

induce carcinoma-specific Mig-7 in $\alpha v\beta 5$ integrin-positive cells. This redundancy suggests that Mig-7 expression is important for the invasive capabilities of tumor cells. It is well known that these growth factors promote tumor progression and metastasis. The FG pancreatic carcinoma cell line requires signaling from $\alpha v\beta 5$ in conjunction with EGF receptor activation for migration on vitronectin.⁷ Interestingly, EGF also acts as a chemoattractant for tumor cell intravasation into blood vessels, a process required for tumor metastasis.²² $\alpha v\beta 5$ integrin ligation is also required for cytotrophoblast invasion⁸ as well as for tumor dissemination *in vivo*.^{5,7} These results, indicating that multiple tumor-microenvironment growth factors induce Mig-7, may account for its expression in virtually all cancer samples ($n > 200$) irrespective of tissue of origin.^{6,23}

Laminin 5 $\gamma 2$ promigratory fragments are involved in tumor cell invasion and vasculogenic mimicry.^{9,19,38} Intriguingly, cleavage of laminin 5 $\gamma 2$ chain releases domain III consisting of several EGF-like repeats that activate EGF receptor stimulating downstream signaling (eg, Erk activation), MMP-2 gene expression, and cell migration.^{15,46} Another link to $\gamma 2$ fragments and invasion is provided by our data that show carcinoma cells expressing Mig-7 fragment $\gamma 2$ differently than cells that do not express Mig-7 when plated on Matrigel. It is tempting to speculate that Mig-7 and $\gamma 2$ domain III fragments, which range in size from 20 to 40 kd, interact in a nonreducible manner attributable to both Mig-7 (accession no. DQ080207) and $\gamma 2$ domain III possessing 10% cysteine residues. In support of these two cysteine-rich proteins interacting, we observed that Mig-7-expressing cells were significantly less adherent to a mix of laminins but not to other ECM components. In addition, recombinant $\gamma 2$ domain III fragments form dimers on reducing gels.⁴⁶ Because carcinoma cells consistently invaded when Mig-7 was expressed, these data are consistent with a novel relationship between Mig-7, $\gamma 2$ fragments, invasion, and vasculogenic mimicry.

Overexpression of Mig-7 in HT29 colon carcinoma cells in GF⁺ Matrigel causes invasion, branching, and cords of cells with a vessel-like appearance. In stark contrast, empty vector HT29 cell morphology is comprised of well-defined colonies with no visible invasion. These are the same differences in morphology found by Folberg and colleagues⁵⁰ when aggressive melanoma cells were compared with their nonaggressive counterparts; in addition, these differences in morphology are detected solely on thick or three-dimensional Matrigel, not on tissue culture plastic. Our findings show that carcinoma cells expressing Mig-7 invade and form vessel-like structures in the context of ECM that mimics the tumor microenvironment but not when plated on plastic.

Because three-dimensional cultures were initiated with the same number of cells and cultured for the same duration of time, it seems that Mig-7-expressing HT29 were more invasive than proliferative. This is consistent with recent data demonstrating that growth arrest of keratinocytes occurs after initiation of migration hypermotility in a $\gamma 2$ chain-dependent manner.⁵¹ It has long been noted that the cells leading re-epithelialization to close a

wound are less mitotically active than those behind them.⁵² Importantly, melanoma cells that undergo vasculogenic mimicry decrease expression of proliferation-related genes GDF15 and p21.⁵⁰

A Role for Mig-7 in Vasculogenic Mimicry

Vasculogenic mimicry has been reported in other tumors such as ovarian, Ewing sarcoma, uveal melanoma, and hepatocarcinomas.^{10,11,13,53,54} Plasticity of tumor cells that form vessel-like structures has been termed vasculogenic mimicry and correlates with poor patient outcome.⁵³ These vessels are capable of being perfused and can carry blood to tumors.⁵⁵

The formation of laminin 5 γ 2 promigratory fragments is important for the process of vasculogenic mimicry.²⁰ Laminin 5 is the only laminin that contains the γ 2 chain. As previously discussed, when cleaved into promigratory fragments the domain III region that contains EGF repeats causes EGF-like effects, including up-regulation of MMP-2, a protease shown to be important in vasculogenic mimicry via γ 2 chain fragmentation.⁹ As we have shown in this report, Mig-7 mRNA is induced by EGF.

As determined by immunoblotting, Mig-7 protein expression was limited to melanoma cells that aggressively invade and undergo vasculogenic mimicry (C918, C8161, MUM2B¹⁰). In contrast, melanoma cell lines that are poorly invasive and fail to form vessel-like structures (A375P, MUM2C¹⁰) do not express Mig-7. These data further support a role for Mig-7 in carcinoma cell-specific behaviors of aggressive invasion and vasculogenic mimicry.

Mig-7 protein is localized to CTBs and carcinoma cells as well as to cells of vessel-like structures in lymph nodes to which human carcinoma cells had spread in nude mice. Mig-7 protein co-localizes with endothelial markers FVIII assoc:ag, VE-cadherin, and γ 2 chain domain III fragment by IHC analyses. VE-cadherin is expressed by the same aggressively invasive melanoma cell lines that can undergo vasculogenic mimicry and that express Mig-7.⁵⁶ Taken together with Mig-7 induction of invasion and vessel-like structure formation in three-dimensional cultures and Mig-7 expression in melanoma cells that undergo vasculogenic mimicry, these data strongly suggest that Mig-7 plays an important role in these processes.

Identity of Cells Lining Vessel-Like Structures

Endothelial markers such as FVIII assoc:ag (also known as von Willebrand factor) have been previously localized to tumor vessels.⁴⁷ FVIII assoc:ag is a plasma protein produced by endothelial cells and platelets.⁵⁷ In serial sections, areas of Mig-7 staining co-localized with areas of staining for FVIII assoc:ag in vessel-like structures that are irregular in shape found with tumor cell-formed vessels during vasculogenic mimicry.^{13,55} These vessel-like structures are adjacent to one another, unlike normal vessels, are of small diameter, and contain cytoplasmic projections or membranous extensions that appear to

cross the lumen as previously described for leaky tumor vessels.⁴ Frequently, Mig-7-expressing tumor cells that line vessel-like structures are more spindle shaped. This morphology is consistent with melanoma cells undergoing vasculogenic mimicry changing to a spindle shape.⁵⁰ Because CTBs form leaky vessels in the maternal vasculature of the placenta³ and because we did not detect vessel-like structures or Mig-7 in nude mice normal lymph nodes, these data strongly suggest that these vessels are formed by Mig-7-expressing tumor cells that were injected.

Our data suggest that Mig-7 expression affects tumor cell interaction with the tumor microenvironment in a growth factor-dependent manner. Mig-7 expression causes invasion and vasculogenic mimicry. It has been proposed that finding a protein specific to vasculogenic mimicry would be an excellent anti-cancer target because this process does not occur in any normal tissue in children or adults. Vasculogenic mimicry is prognostic of a poor patient outcome¹³ and is not reduced by anti-angiogenic therapies.²¹ In addition, invading cancer cells are resistant to cancer therapies that typically target cell growth.²² Therefore, therapies directed specifically to invading cancer cells are needed. Mig-7 may be a carcinoma-specific target to inhibit invasion and vasculogenic mimicry *in vivo*, thereby preventing growth of tumors and dissemination of tumor cells.

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