Mammary-Derived Growth Inhibitor Alters Traffic of EGFR and Induces a Novel Form of Cetuximab Resistance

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Abstract

Purpose: Only few predictive factors for the clinical activity of anti–epidermal growth factor receptor (EGFR) therapy are available. Mammary-derived growth inhibitor (MDGI) is a small cytosolic protein suggested to play a role in the differentiation of epithelial cells. Here, we have investigated the effect of MDGI expression on the EGFR signaling and cetuximab responsiveness of cancer cells.

Experimental Design: MDGI mRNA expression was investigated in clinical breast and lung cancer samples and in nontransformed and malignant cell lines. The effect of ectopic expression of MDGI on EGFR, ErbB2, and integrin function and traffic was investigated in breast and lung cancer cell lines using multiple methods. The effect of anti-EGFR agents on these cells were tested by cell proliferation measurements and by assessing tumor growth of breast cancer cells in cetuximab treated and control athymic nude mice.

Results: Here, we show that although MDGI is absent in cultured cell lines because of epigenetic silencing, MDGI mRNA is expressed in 40% of clinical breast carcinomas and 85% of lung cancers. Ectopic expression of MDGI rendered breast and lung cancer cells resistant to the anti-EGFR antibody cetuximab in vitro and in an orthotopic breast cancer xenograft model in vivo. When expressed in cancer cells, MDGI induces intracellular accumulation of EGFR, but not ErbB2, and the internalized receptor is phosphorylated and not degraded.

Conclusions: MDGI-driven inherent desensitization of cancer cells is a novel molecular mechanism for resistance to the anti-EGFR antibody therapy, and MDGI may be a biomarker for responsiveness to anti-EGFR antibody therapy.

Mammary-derived growth inhibitor (MDGI; also known as H-FABP and FABP-3) is a small 15-kDa cytosolic protein that belongs to the family of fatty acid–binding proteins. Expression of the fatty acid–binding proteins is relatively tissue specific. Fatty acid–binding proteins avidly bind hydrophobic ligands and mediate fatty acid metabolism (1). MDGI is expressed at least in 39 different tissues, including the heart, brain, lung, and breast (2). MDGI is involved in myocardial energy metabolism, and it has a role in the lactating mammary epithelium, but otherwise, relatively little is known about its molecular functions in tissues. MDGI has been suggested to have a tumor suppressor function in the mammary epithelium because it is apparently lost in breast cancer cell lines (3) and some primary tumors (4). However, there are contradictory data on the ability of MDGI to inhibit cell proliferation in cultured cells (3, 5), and mutations in the coding regions of the gene have not been found in breast cancer (4). Neither overexpression nor deletion of the gene encoding MDGI has yielded an overt phenotype in transgenic mouse mammary glands (6, 7).
Epidermal growth factor receptor (EGFR) regulates many cellular processes, including proliferation, differentiation, and migration (8). Perturbations in these biological processes can lead to malignant transformation, and numerous studies have documented the importance of EGFR signaling in cancer (9). EGFR is overexpressed or has an increased gene copy number in many solid tumors, including breast cancer and lung cancer (10, 11), and overexpression of the receptor has been linked with poor prognosis in several types of human cancer (12). Anti-EGFR antibodies (monoclonal antibody) and EGFR tyrosine kinase inhibitors have shown clinical benefit in the treatment of cancers of the colon and rectum, pancreas, lung, and head and neck. However, many patients diagnosed with one of these diseases may benefit little from anti-EGFR therapy, and predictive biomarkers for the clinical activity are under intensive investigation (13, 14).

Here, we report that MDGI expression of normal and cancer cells is lost under cell culture conditions because of epigenetic silencing. In contrast, MDGI is expressed in vivo in the normal mammary epithelium and in 40% of human breast carcinomas. MDGI is present in vivo also in normal lung tissue and in 85% of lung cancer specimens. We show that introduction of MDGI into cancer cells has a profound effect on EGFR localization and signaling. Expression of MDGI is sufficient to target EGFR to an intracellular compartment where the receptor is not degraded and remains active. MDGI expression modulates the sensitivity of breast and lung cancer cells to anti-EGFR antibodies in vivo and rendered them resistant to cetuximab treatment in vivo in an orthotopic xenograft model.

Translational Relevance

Understanding the molecular basis of resistance to anti–epidermal growth factor receptor (EGFR) therapy is of great importance. Mammary-derived growth inhibitor (MDGI) expression was identified as a determinant for cetuximab resistance and a novel modulator of EGFR trafficking in cancer cells. Thus, we predict that MDGI could be a biomarker to identify patients who will not benefit from therapy with anti-EGFR agents. Because MDGI expression is lost during cell culture, this resistance mechanism can only be studied in three-dimensional cell cultures and in vivo models.

Materials and Methods

Antibodies and reagents. Antibodies against the following antigens were used in Western blotting: MDGI (cFABP 16915, Abcam), Rab21 (15), green fluorescent protein (GFP; Molecular Probes), integrin β1 (MAB2252, Chemicon; Mab, BD Transduction Laboratories), and α-tubulin (12G10, Developmental Studies Hybridoma Bank). EGFR, pY1068-EGFR, pY1148-EGFR, pY992-EGFR, pY1248-ErbB2, pY1289-ErbB3, pY1284-ErbB4, pT308-Akt, pS473-Akt, pT202/pY204-p44/42 mitogen-activated protein kinase, and batinorserahid peroxidase were purchased from Cell Signaling Technology. ErbB2 (Neu), ErbB3, ErbB4, and Rab7 were purchased from Santa Cruz Biotechnology. EEA1 was purchased from the Interlab Cell Line Collection. HT-1080, SKBr3, MDA-MB-231, HeLa, and NCI-H358 cells were obtained from the American Type Culture Collection. MCF7 and MDA-MB-468 cells were obtained from the Interlab Cell Line Collection. HT-1080, SKBr3, MDA-MB-468, and HeLa were maintained in DMEM (Sigma) containing 4.5 g/L glucose and supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine. For MCF7, we used DMEM containing only 1.0 g/L glucose instead. MDA-MB-231 cells were cultured in DMEM containing 4.5 g/L glucose and supplemented with 10% inactivated fetal bovine serum, 1% nonessential amino acids, and 2 mmol/L L-glutamine. hTERT-HME1 and MCF10A were cultured in a 1:1 mixture of DMEM and F12 Ham (Gibco) supplemented with 5% horse serum, 2 mmol/L L-glutamine, 10 μg/mL insulin, 5 μg/mL hydrocortisone, 20 ng/mL EGF, and 100 ng/mL choleratoxin. NCI-H358 cells were cultured in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum and 2 mmol/L L-glutamine.

Expression vectors, transfection, and selection of stable cell lines. Human MDGI cDNA (IMAGE 3934295) was subcloned into pEGFP-C1 (Clontech) between Xhol and Xmal sites to create pEGFP-MDGI. DNA constructs were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. MDA-MB-468 cells were selected with 650 μg/mL G418 to establish stable cell lines MDA-MB-468 GFP (MDA-GFP) and MDA-MB-468 GFP-MDGI (MDA-MDGI). Both cell populations (lines 1 and 2) were generated identically but from independent transfections at different times. NCI-H358 cells were selected with 400 μg/mL G418 to establish stable cell lines NCI-H358 GFP (NCI-GFP) and NCI-H358 GFP-MDGI (NCI-MDGI). ERF-FLAG construct was a generous gift from Y. Daaka (16).

Reverse transcriptase-PCR. RNA was extracted from the indicated cell lines with RNeasy Mini Kit (Qiagen), and cDNA was synthesized by using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc.), according to manufacturer’s protocol. Normal human mammary gland QUICK-Clone cDNA (637231) was obtained from Clontech, and primary mammary epithelial cells RNA was a kindly gift from Prof. M. Poutanen. PCR was carried out in a Phusion reaction mix (Phusion RT-PCR kit, Finnzymes), according to the manufacturer’s protocol. Cycling was initiated with 30 s at 98°C, followed by 20 cycles of 10 s at 98°C, 15 s at 68°C, and 15 s at 72°C. When indicated, cells were stimulated with 10 ng/mL EGF for 15 min before fixing. Coverslips were fixed with cold 4% paraformaldehyde and permeabilized 20 min at room temperature with 10% horse serum/1% bovine serum albumin/0.1% Triton X-100/TBS, followed by blocking 1 h at room temperature with 10% horse serum/1% bovine serum albumin/TBS. Cells were stained with primary antibodies for overnight at 4°C, followed by incubation with 1:400 dilution of Alexa Fluor 555–conjugated secondary antibodies (Molecular Probes) for 1 h at room temperature. Coverslips were mounted in Mowiol mounting medium [100 mmol/L Tris-HCl (pH 8.5)/10% (w/v) Mowiol (Calbiochem)/25% (w/v) glycerol] supplemented with Vectashield containing DAPI (Vector Laboratories) and anti-fade reagent 1,4-diazabicyclo-2,2,2-octane (Sigma).

For immunohistochemical analysis, paraffin sections of human breast tissues were deparaffinized in xylene and rehydrated through...
a series of graded ethanol solutions. The endogenous peroxidase activity was blocked using 0.75% H2O2 for 30 min. The sections were incubated with the primary antibody diluted at 1:50 overnight at 4°C, followed by incubation of biotin-labeled secondary antibody diluted at 1:200 for 30 min at room temperature. Staining was detected using the Vector Elite ABC kit (Vector Laboratories) and the peroxidase substrate 3,3′-diaminobenzidine (Vector Laboratories), following manufacturer’s instructions. Slides were counterstained with Mayer’s hematoxylin and mounted in Aquamount.

Tumors from mouse experiments were embedded in paraffin and stained according to manufacturer’s protocol, with a total EGFR antibody. For fluorescence images, 1:200 diluted Alexa 448-conjugated avidin (Molecular Probes) was added after incubation of biotin-labeled secondary antibody, washed with PBS and distilled H2O2, and mounted in Moviol mounting medium. Hematoxylin and eosin staining was done using a standard procedure with an automated cartoon-type slide stainer Shandon Varistain 24-4.

Percoll gradients. This has been described elsewhere (17). Cells were grown on 10-cm plates. At the time of harvest, they were washed in Mowiol mounting medium. Hematoxylin and eosin staining was done using a standard procedure with an automated cartoon-type slide stainer Shandon Varistain 24-4.

Results

MDGI is expressed in vivo but not in cultured cells. To gain insight into the possible role of MDGI in the regulation of epithelial cells, we first investigated MDGI expression in nontransformed and cancer cells, and used a stable MDA-MDGI as a positive control. We found that MDGI protein was not expressed in seven cultured cell lines irrespective of their degree of malignancy (Fig. 1A). MDGI expression was not detected at the mRNA level in nontransformed mammary epithelial cell lines (HME1 and MCF10A) or in nonpassaged primary mammary epithelial cells (Fig. 1B). However, MDGI mRNA was present in histologically normal breast samples, 18 normal lung tissue samples, 553 human breast cancers, and 165 lung cancers, Gene Expression Omnibus (GSE2361, GSE3526, GSE2109, and GSE3494). MDGI mRNA expression levels were compared in normal versus cancer samples, as well as between different tumor grades, and the statistical significance of differences between the groups were determined by doing Wilcoxon rank sum test and t test on log 2 transformed data by using R (R Development Core team) and Bioconductor.8

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8 http://www.bioconductor.org

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normal and cancer cells with increasing concentrations of the methylation inhibitor 5-aza-2'-deoxycytidine. Interestingly, MDGI mRNA expression was reversed by this treatment in normal cells and in some, but not all, cancer cell lines (Fig. 1C), suggesting that cell culture conditions can induce epigenetic silencing of MDGI in cells that might express MDGI in vivo.

Analysis of MDGI expression in human tissue samples, using an antibody specific for MDGI in cells (Supplementary Fig. S1A), confirmed that it is expressed in histologically normal human mammary epithelium (Fig. 1D, arrow). Interestingly, low to normal levels of MDGI protein were found in malignant epithelial cells in breast cancer specimens (Fig. 1D, arrow), suggesting that MDGI is not lost in all breast cancers. To investigate the clinical significance of MDGI expression, we carried out an in silico meta-analysis of MDGI mRNA expression levels of microarray studies consisting of eight samples containing normal breast epithelium, 18 normal lung tissue samples, 553 breast cancers, and 165 lung cancers (18–20). Five percent (n = 26) of breast cancers and 2% (n = 4) of lung cancers showed increased MDGI mRNA expression compared with the normal tissue samples, and in 40% of breast tumors (n = 223) and in 85% of lung cancers (n = 141), MDGI mRNA...
was expressed at similar levels (defined as mean expression in normal samples ± 1.96 × SD) as in the normal tissue samples (Fig. 1E). However, in breast cancer the median expression of MDGI was significantly lower than in the normal breast tissue (median expression; P = 2 × 10^-16; Wilcoxon rank sum test), suggesting that MDGI may have different biological roles in different cancer types. Taken together, the results from this meta-analysis revealed that expression of MDGI is usually retained in breast and lung cancer in vivo.

**MDGI alters EGF traffic.** To assess whether MDGI expression imposes phenotypic alterations to transformed cells, we expressed MDA-GFP and MDA-MDGI cells. We did not observe any significant changes in the cell proliferation rate, or the rate of apoptosis induced by serum withdrawal between MDA-GFP and MDA-MDGI cells (Supplementary Fig. S1B and C). Next, we investigated the effect of MDGI expression on several proteins, such as EGFR, focal adhesion kinase, and β1-integrin, that have been shown to play an important role in cancer progression. No difference was detected in focal adhesion kinase phosphorylation, total EGFR protein levels, or cell surface expression of β1-integrin between the MDA-GFP and MDA-MDGI cell lines (Fig. 2A and B; data not shown). However, analysis of EGFR expression with FACS revealed a 36 ± 4% reduction in EGFR cell surface levels in MDA-MDGI cells compared with MDA-GFP cells (Fig. 2B). In addition, less labeled EGF bound to the surface of MDA-MDGI cells maintained on ice as compared with MDA-GFP cells (Supplementary Fig. S2). Thus, MDGI induces changes in EGFR localization to the plasma membrane in breast cancer cells.

The mechanism underlying the differential cell surface EGFR levels might be associated with alterations in receptor trafficking. We found that MDA-MDGI cells showed increased accumulation of endocytosed biotin-labeled, cell surface-derived EGFR as compared with MDA-GFP cells (Fig. 2C). Based on the kinetics and obvious accumulation of the internalized EGF in MDGI-MDGI cells, this could be a result of reduced receptor recycling.

No change was observed in endocytosis of labeled transferrin (Supplementary Fig. S3) or in cell surface levels of β1-integrin (Fig. 2B). These data suggest that MDGI expression does not alter the traffic of all receptors but may specifically influence EGFR traffic.

EGFR and other members of the ErbB receptor family have disparate trafficking patterns. Whereas EGFR can be endocytosed constitutively as an empty receptor or rapidly in a ligand induced manner, the other members of the ErbB family signal to regulate EGFR localization through alterations in other ErbB family proteins. Caveolin-1, EEA1, Rab7, and Rab21 on the limiting membrane. There is also limited overlap with LAMP1, but no obvious colocalization is detected with clathrin or Rab5A (Fig. 3B). Biochemical analysis of MDA-GFP and MDA-MDGI cells showed high EGFR phosphorylation in nonstimulated and stimulated conditions (Fig. 3C). Importantly, in nonstimulated cells, phosphorylated EGFR (pY1068) was detected mainly on the membrane in GFP expressing control cells, whereas in MDGI-expressing cells, phosphorylated EGFR was detected within the cell (Supplementary Fig. S4A), suggesting that the receptor is active in both subcellular localizations. Furthermore, MDGI was able to regulate the subcellular localization of coexpressed exogenous EGFR in HeLa and COS7 cells (Supplementary Fig. S4B; data not shown). These data show that MDGI is able to regulate EGFR localization in several cell types. Subcellular fractionation analyses showed that, in MDA-GFP cells, EGFR was detected mainly in the light plasma membrane containing fractions, and only a minor amount of the receptor was detected internalized in the endosomal fractions (Fig. 3D).

In contrast, in MDA-MDGI cells, a higher proportion of EGFR was detected in denser endosomal fractions (Fig. 3D; see Supplementary Fig. S5A for localization of β1-integrin, EEA1, Rab7, and Rab21 on the fractionations), thus further confirming that MDGI expression in cells results in relocalization of a large pool of EGFR to an intracellular compartment.

Finally, we analyzed receptor turnover using a cycloheximide, which blocks protein biosynthesis. EGFR turnover was similar in MDA-GFP and MDA-MDGI cells in the presence or absence of EGF (Supplementary Fig. S5B). This is consistent with the notion that EGFR protein levels are not altered by MDGI expression (Figs. 2A and 4C) and that ubiquitination of EGFR was not increased in MDGI cells (data not shown). Taken together, these data indicate that MDGI targets a large fraction of active EGFR to an intracellular compartment where the receptor is not degraded.

**MDGI induces accumulation of intracellular EGFR in three dimensions.** Three-dimensional culture systems provide an experimental model that takes into account the critical interaction of cells with their surroundings in addition to the intracellular signals that govern cell behavior (23), which prompted us to investigate the phenotype of MDGI expressing cancer cells in a three-dimensional culture. In contrast to two dimensions (Supplementary Fig. S1B and C), MDA-MDGI cells showed 50% ± 10% reduction in cell growth in Matrigel as compared with GFP cells (Fig. 4A). In addition, the cell surface expression of β1-integrin was comparable between MDA-GFP and MDA-MDGI cells cultured in Matrigel for 3 days (Fig. 4B). The cell surface expression of EGFR, however, was dramatically different between the two cell types. MDA-MDGI cells expressed 91% ± 1% less EGFR on the cell surface compared with GFP.
cells (Fig. 4B). This was most likely due to altered receptor traffic because a Western blot analysis of cells isolated from the same Matrigel culture showed that total EGFR protein levels were not lower in MDA-MDGI cells as compared with MDA-GFP cells under basal conditions or following 15 minutes EGF stimulation (Fig. 4C). Conversely, three-dimensional Matrigel induced a 3.3 ± 0.15-fold increase in the cell surface expression of ErbB2 (Fig. 4D), which was also detected at the protein levels (Fig. 4D). Thus, these data suggest that expression of MDGI specifically targets a large fraction of EGFR to an intracellular compartment in cells cultured in three dimensions.

MDGI expressing cells are resistant to cetuximab in vitro and in vivo. The observed differences in EGFR localization between MDGI- and GFP-expressing cells implied the possibility that MDGI expression alters accessibility of EGFR to the function blocking antibodies of the receptor. Thus, we tested the ability of EGFR inhibitors to influence proliferation of GFP- and MDGI-expressing cancer cells growing in Matrigel. In MDA-GFP control cells, an anti-EGFR antibody LA1 inhibited cell proliferation by 65% ± 23% and anti-EGFR antibody cetuximab by 58% ± 12%. Proliferation of MDGI cells in Matrigel was reduced compared with GFP cells (Fig. 5A). Importantly, neither antibody
inhibited proliferation of MDGI cells further. Similar results were obtained using independently generated GFP- and MDGI-expressing cell populations (Fig. 5A, line 2). Conversely, membrane permeable anti-EGFR tyrosine kinase inhibitor (AG1478) inhibited proliferation of MDA-GFP and MDA-MDGI cells by 50% ± 1% and 38% ± 1%, respectively (Fig. 5B). These data are in line with the notion of MDGI induced intracellular localization of a large fraction of EGFR (24) as an underlying mechanism of the observed resistance to cetuximab.

The ability of MDGI to induce resistance to cetuximab was investigated further in lung cancer cells because cetuximab is undergoing clinical trials in lung cancer. Like other cultured cells, NCI-H358 lung bronchioalveolar carcinoma cells do not express endogenous MDGI (Fig. 5C). These cells are cetuximab sensitive in vitro, albeit they have an activating K-RAS mutation. Stable expression of GFP or GFP-MDGI did not alter the protein expression of EGFR or β1-integrin (Fig. 5C). Whereas cetuximab inhibited proliferation of GFP expressing cells by 19% ± 2% (P < 0.05), proliferation of MDGI-expressing cells was not significantly reduced (5% ± 6%; Fig. 5D). An anti-EGFR tyrosine kinase inhibitor inhibited proliferation of both cell lines efficiently (Fig. 5D), in line with our previous results.
observations in breast cancer cells. The observed differences in sensitivity of MDA-MDGI and MDA-GFP cells to anti-EGFR antibodies but not EGFR tyrosine kinase inhibitor were also reflected in EGFR-dependent signal transduction. Basal phosphorylation of EGFR in MDA-GFP and MDA-MDGI cells seemed to vary significantly under nonstarved culture conditions, and thus, the effect of EGF on EGFR phosphorylation was different between experiments. However, activation of ERK and Akt was clearly EGF inducible in both cell lines (Fig. 5E and F). As expected, anti-EGFR neutralizing antibody LA1 inhibited EGF-induced activation of ERK and Akt in MDA-GFP but not MDA-MDGI cells (Fig. 5E). Conversely, the effect of EGFR tyrosine kinase inhibitor AG1478 on EGFR downregulation and EGF-induced signaling was similar in MDA-GFP and MDA-MDGI cells (Fig. 5F). These results suggest that MDGI expression renders EGFR less sensitive to the anti-EGFR antibodies without dramatically altering receptor phosphorylation. There are two possible explanations to the ineffectiveness of anti-EGFR antibodies in MDA-MDGI cells. One likely reason is the reduced amount of EGFR on the surface of MDA-MDGI cells. However, the fact that the receptor is endocytosed and accumulates inside MDA-MDGI cells argues that additional mechanism might be involved. To test this, we analyzed endocytosis and recycling of biotinylated cetuximab in MDA-GFP and MDA-MDGI cells. Interestingly, biotinylated cetuximab is internalized by both cell lines; however, in MDA-GFP cells, the antibody recycles back to the membrane (seen as loss of biotinylation of the antibody after 15 minutes of recycling), whereas in MDA-MDGI cells, cetuximab is retained to a larger extent inside the cells (Fig. 5G). Because of the low pH of the endosomal compartment, it is possible that cetuximab retained inside the cells will dissociate from EGFR, and thus, inhibited recycling might contribute to the ineffectiveness of cetuximab in MDA-MDGI cells.

To evaluate the in vivo relevance of our findings, we investigated tumor growth of orthotopically inoculated MDA-GFP and MDA-MDGI cells in nude mice. We found that 10 days after inoculation, the tumors formed by MDA-GFP and MDA-MDGI cells were similar in size and grew at the same rate (Fig. 6A).
Next, we used this orthotopic model to assess the efficacy of an anti-EGFR antibody cetuximab in inhibiting tumor growth. Cetuximab has antitumor activity in vivo and in vitro, and it has been approved for clinical use in advanced colon cancer, as well as head and neck cancer (25). We used a previously defined dose of cetuximab (26, 27) and administered the drug biweekly starting 10 days after tumor cell inoculation. Treatment with cetuximab efficiently inhibited growth of control

Fig. 5. MDGI expressing cells are resistant to cetuximab in vitro. A, two independent lines (1 and 2) of MDA-GFP and MDA-MDGI cells were seeded inside Matrigel and allowed to proliferate for 72 h in the presence or absence of anti-EGFR antibodies cetuximab or LA1 (5 μg/mL). The number of live cells (mean ± SE; line 1, n = 4; line 2, n = 2) was determined using WST-1 reagent. *, P < 0.05; **, P < 0.01; ***, P < 0.001; two-tailed Student's t test.

B, MDA-GFP and MDA-MDGI cells were seeded inside Matrigel and allowed to proliferate for 72 h in the presence or absence of tyrosine kinase inhibitor Tyrphostin AG1478 (2.5 μmol/L). The number of live cells (mean ± SE; n = 2) was determined using WST-1 reagent. *, P < 0.05; **, P < 0.01; two-tailed Student's t test. C, NCI-GFP or NCI-MDGI was harvested, and lysates were resolved on SDS-PAGE and blotted with the indicated antibodies. D, NCI-GFP and NCI-MDGI cells were cultured on plastic for 4 d in the presence or absence of AG1478 (2.5 μmol/L) or cetuximab (10 μg/mL). The number of live cells (mean ± SE; n = 8) was determined using WST-1 reagent. *, P < 0.05; ***, P < 0.001; two-tailed Student's t test. n.s., not significant.

E, Western blot analysis of EGF induced signalling in MDA-GFP and MDA-MDGI cells on plastic. Cells were pretreated with anti-EGFR neutralizing antibody LA1 5 μg/mL for 15 min, then stimulated with 10 ng/mL EGF for 15 min, and lysates were resolved on SDS-PAGE and blotted with the indicated antibodies. F, MDA-GFP and MDA-MDGI cells were treated with two different concentrations of AG1478 for 30 min, lysates were harvested, resolved on SDS-PAGE, and blotted with the indicated antibodies (top). Bottom, MDA-GFP and MDA-MDGI cells were plated on 5 μg/mL type I collagen for 30 min. When indicated, the cells were pretreated in suspension with 2.5 μmol/L AG1478 for 10 min or 10 ng/mL EGF was added directly onto plates for 30 min. Lysates were resolved on SDS-PAGE and blotted with the indicated antibodies. G, internalization and recycling of biotinylated cetuximab. MDA-GFP and MDA-MDGI cells were incubated in the presence of cleavable biotinylated cetuximab at +4°C for 30 min and then allowed to internalize cetuximab for 15 or 30 min. Biotin remaining on the cell surface was cleaved. Two samples (lanes 4 and 9) were first allowed to internalize cetuximab for 30 min; cell surface biotin was cleaved, and cells were allowed to recycle for 15 min, followed by a second cleavage of cell surface biotin. Protein G beads were added on lysates for immunoprecipitation, and samples were then resolved on nonreducing SDS-PAGE. Biotinylated cetuximab was first detected with an anti-biotin antibody followed by stripping and reprobing with a horseradish peroxidase-conjugated anti-human IgG.
tumors. MDA-MDGI xenografts were strikingly resistant to anti-EGFR therapy and grew significantly faster as compared with the control tumors (Fig. 6B). The postmortem weights of the MDGI tumors were 45% ± 7% higher than those of GFP tumors (mean ± SE, n = 20 tumors per group; P < 0.001), and histologic examination of the tumors confirmed these findings (Fig. 6C).

Increased ubiquitination and subsequent downregulation of the EGFR receptor (28) and high levels of EGFR in conjunction with marked increase in MAP kinase phosphorylation (29) have been proposed as mechanisms of cetuximab resistance. We found no difference in EGFR protein levels or in ERK phosphorylation in our xenograft tumor lysates (Fig. 6D) that clearly retained the expression of GFP and GFP-MDGI (Fig. 6D). This
suggests that a decrease in receptor protein was not the underlying mechanism for the observed cetuximab resistance.

Finally, the subcellular localization of EGFR was analyzed in GFP and MDGI tumors using confocal microscopy. In line with the in vitro data, MDGI tumors had more intracellular EGFR (Fig. 6E, arrows) and less intensive plasma membrane EGFR staining when compared with GFP tumors (Fig. 6E). It is conceivable that the endocytosed fraction of EGFR is sufficient for sustained EGFR signaling, although residual EGFR remaining on the plasma membrane is accessible to cetuximab also in MDGI cells. Taken together, these data suggest that MDGI-induced redistribution of EGFR is one of the underlying mechanisms for cetuximab resistance of MDGI expressing tumors in vivo.

Discussion

MDGI has been suggested to function as a tumor suppressor for more than a decade (30). Here, we describe that this is an incomplete view. We show that (a) MDGI is expressed in 40% of breast cancers and 85% of lung cancers but is lost in cell culture conditions because of epigenetic silencing; (b) MDGI expression induces the redistribution of EGFR into an intracellular pool where the receptor is active but in a compartment that renders anti-EGFR antibody therapy inefficient; (c) MDGI expression in breast and lung cancer cells facilitates escape from cetuximab-induced growth inhibition in vitro and in vivo; and (d) in an orthotopic xenograft model, MDGI expressing cells are resistant to cetuximab. Taken together, these data suggest that MDGI regulates responsiveness of cancer cells to anti-EGFR antibody therapy.

We found that a large proportion of breast cancers and lung cancers express MDGI at levels similar to those found in the corresponding normal epithelia. In addition, based on our analysis, MDGI is also expressed at mRNA level in colorectal cancer specimens (Supplementary Fig. S6; ref. 20), suggesting that MDGI expression is silenced by methylation under cell culture conditions, it is likely that studies investigating cultured cells cannot properly address regulation of EGFR by MDGI. The present data, therefore, reveal a fundamentally important and previously unrecognized aspect of EGFR biology, which may be highly relevant for in vivo conditions.

The most important biological determinants for cancer responsiveness to anti-EGFR therapy remain elusive. EGFR activating mutations have not been described in breast cancer (32), and unlike with anti-ErbB2 therapy, EGFR expression levels have not been shown to correlate well with cancer responsiveness to anti-EGFR therapy (14). In contrast, in non–small cell lung cancer the presence of somatic mutations within the tyrosine kinase domain of EGFR are associated with response to EGFR tyrosine kinase inhibitors (13), and in colorectal cancer, the efficacy of cetuximab treatment is limited to those cancers that express a nonmutated K-RAS gene (33). Several mechanisms of acquired resistance to anti-EGFR therapy have been described. These include downregulation of the EGFR receptor with concomitant upregulation of compensatory signaling pathways, leading to activation of Akt, Src, and ERK (14). Upregulation of other members of the ErbB family has been described in resistant cells (34). Here, we show that introduction of MDGI to cancer cells results in inherent cetuximab resistance because of the alteration of EGFR traffic. Interestingly, a very recent study on MDA-MB-468 cells shows that the subcellular localization of EGFR either on the plasma membrane or inside the cells (achieved by artificially coupling EGFR to the particles that cannot enter cells) dictates the biological response of these cells to EGFR stimulation (35).

Three recent studies have described how alterations in EGFR traffic influence receptor function and susceptibility to EGFR inhibitors in lung cancer. One of the studies shows how oxidative stress induces aberrant phosphorylation of the EGFR and relocation of the receptor into a caveolin-1–positive intracellular compartment where the receptor is not degraded (22). This is interesting because we show that caveolin-1–positive membranes seem to be in close proximity with intracellular EGFR in MDA-MDGI cells. Another study suggested that upregulation of ErbB2 and ErbB3 receptors results in reduced endocytosis and increased cell surface expression of EGFR (36), and in the third study, acquired resistance to gefitinib was found to involve increased endocytosis of EGFR through a nondefined mechanism (37). We show here that MDGI-expressing cancer cells are inherently cetuximab resistant, and in these cells, EGFRs are increasingly endocytosed into an intracellular compartment where they are active and not degraded (Fig. 6F).

In conclusion, we describe a role for MDGI expression in the regulation of EGFR subcellular localization and sensitivity to cetuximab and other antibodies targeted to EGFR. Tumors with retained expression of MDGI may have an increased rate of EGFR endocytosis in vivo, which could influence the inherent susceptibility of cancer cells to anti-EGFR therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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