miR-200 Expression Regulates Epithelial-to-Mesenchymal Transition in Bladder Cancer Cells and Reverses Resistance to Epidermal Growth Factor Receptor Therapy

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Abstract

Purpose: The epithelial-to-mesenchymal transition (EMT) is a cell development-regulated process in which noncoding RNAs act as crucial modulators. Recent studies have implied that EMT may contribute to resistance to epidermal growth factor receptor (EGFR)-directed therapy. The aims of this study were to determine the potential role of microRNAs (miRNA) in controlling EMT and the role of EMT in inducing the sensitivity of human bladder cancer cells to the inhibitory effects of the anti-EGFR therapy.

Experimental Design: miRNA array screening and real-time reverse transcription-PCR were used to identify and validate the differential expression of miRNAs involved in EMT in nine bladder cancer cell lines. A list of potential miR-200 direct targets was identified through the TargetScan database. The precursor of miR-200b and miR-200c was expressed in UMUC3 and T24 cells using a retrovirus or a lentivirus construct, respectively. Protein expression and signaling pathway modulation, as well as intracellular distribution of EGFR and ERRFI-1, were validated through Western blot analysis and confocal microscopy, whereas ERRFI-1 direct target of miR-200 members was validated by using the wild-type and mutant 3′-untranslated region/ERRFI-1/luciferase reporters.

Results: We identified a tight association between the expression of miRNAs of the miR-200 family, epithelial phenotype, and sensitivity to EGFR inhibitors–induced growth inhibition in bladder carcinoma cell lines. Stable expression of miR-200 in mesenchymal UMUC3 cells increased E-cadherin levels, decreased expression of ZEB1, ZEB2, ERRFI-1, and cell migration, and increased sensitivity to EGFR-blocking agents. The changes in EGFR sensitivity by silencing or forced expression of ERRFI-1 or by miR-200 expression have also been validated in additional cell lines, UMUC5 and T24. Finally, luciferase assays using 3′-untranslated region/ERRFI-1/luciferase and miR-200 cotransfections showed that the direct down-regulation of ERRFI-1 was miR-200-dependent because mutations in the two putative miR-200-binding sites have rescued the inhibitory effect.

Conclusions: Members of the miR-200 family appear to control the EMT process and sensitivity to EGFR therapy in bladder cancer cells and the expression of miR-200 is sufficient to restore EGFR dependency at least in some of the mesenchymal bladder cancer cells. The targets of miR-200 include ERRFI-1, which is a novel regulator of EGFR-independent growth. (Clin Cancer Res 2009;15(16):5060–72)
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Translational Relevance

Inhibitors of the epidermal growth factor receptor (EGFR) are being evaluated for use in the treatment of urothelial cancers and other solid malignancies. Recent studies have reported that epithelial-to-mesenchymal transition (EMT) is implicated in resistance to EGFR therapy; however, the molecular mechanisms that regulate EMT and resistance to EGFR therapy remain elusive. Here we show that miRNA-200 (miR-200) family members control EMT and resistance to the EGFR inhibitors in human bladder cancer cells. Our data suggest that expression of miR-200 family members could be used to identify EGFR inhibitor–sensitive urothelial cancers, and reintroduction of miR-200 could be a strategy to reverse EGFR inhibitor resistance.

Materials and Methods

Cell lines and culture conditions. The UMUC series of urothelial carcinomas and 253J BV cells were isolated and fingerprinted and genotyped by the specimen core at the M. D. Anderson Genitourinary Specialized Program of Research Excellence in Bladder Cancer. KM7 cells were supplied by W. Benedict at M. D. Anderson. The cell lines were maintained at 33°C in modified Eagle's MEM supplemented with 10% fetal bovine serum, vitamins, sodium pyruvate, L-glutamine, penicillin, streptomycin, and nonessential amino acids.

Reagents and chemicals. ImClone Systems generously provided cetuximab (C225), a selective anti-human EGFR antagonist monoclonal antibody in a stock concentration of 2 mg/mL. Iressa (AstraZeneca) and erlotinib/Tarceva (Genentech), which are specific EGFR kinase inhibitors, have been generously provided by the manufacturer or purchased from The University of Texas M. D. Anderson Pharmacy. The following antibodies were purchased from the manufacturer's list and used for Western blot analysis: EGFR (LabVision); E-cadherin and P-cadherin (BD Transduction Laboratories); actin and vinculin (Sigma-Aldrich); and ErbB receptor inhibitor-1 (ERFFI-1) and pEGFR (Tyr1068; Cell Signaling Technology). Pre-miRNAs and antagonirs were purchased from Ambion. The TaqMan gene expression assays, miRNA expression assays, and ERFFI-1, the short hairpin RNA against ERFFI-1 and nontargeting controls, as well as miR-200b RFP lentiviral system were all obtained from Open Biosystems-Thermo Scientific.

Cell proliferation assay. Cells (5 × 10^3) were plated in 96-well plates for 24 h, serum starved for 24 h in 2% FCS supplemented with MEM, and then treated with or without EGFR inhibitors at increasing concentrations in an EGF-nonsupplemented environment for 30 h. We measured the effect of cetuximab (C225), erlotinib (Tarceva), and Iressa on DNA synthesis by pulse-labeling cells with [3H]thymidine (MP Biologicals) for 2 h followed by lysis in 100 μL of 0.1 mol/L KOH. Cells were harvested onto fiberglass filters and incorporated tritium was quantified by β-counter.

Wound-healing assay. UMUC3 cells (4 × 10^3) were plated in 6-well plates for 24 to 48 h (until they reached confluence). A diametric scratch was done using a pipette tip followed by two culture medium changes. Cells were photographed in several pre-marked spots as time 0.

into account target expression and validation of molecular pathways. Growth factor receptors, such as the epidermal growth factor receptor (EGFR), fibroblast growth factor receptor, or platelet-derived growth factor receptor are differentially expressed by bladder cancer cell lines or tumors, making inhibitors to these molecules attractive therapeutic tools (3–5). Debate is ongoing as to whether EGFR expression or mutation in the tyrosine kinase domain correlates well with response to EGFR blockade by tyrosine kinase inhibitors and to survival. In previous studies, we have shown that mutations in the EGFR protein or gene amplifications are not as frequent in bladder cancer as in other cancers, for example, lung cancer (6, 7), suggesting that other predictors remain to be identified. Very recently, several groups reported that sensitivity to EGFR-directed therapy is associated with expression of E-cadherin and other properties of a typical “epithelial” tumor phenotype (8–13).

A phenomenon known as epithelial-to-mesenchymal transition (EMT) has been implicated as having a role in tumor invasion/migration and metastasis (14). Loss of E-cadherin expression is a hallmark of the EMT process, which is probably required for enhanced tumor cell motility. EMT is essential during mesoderm development or neural plate formation in vertebrates and it is thought to be important for wound healing and tumor metastasis. During EMT, down-regulation of E-cadherin allows epithelial cells to undergo changes in cell morphology and motility so that they adopt mesenchymal characteristics. Expression of E-cadherin is controlled by several transcriptional repressors, including Twist, Snail1, Snail2/Slug, E47, ZEB1/TCF8, and ZEB2/SIP1, which bind to E-boxes in the E-cadherin promoter (15).

Recent studies have shown that ZEB1 and ZEB2 are direct targets of microRNAs (miRNA) from the miR-200 family in human breast cancer cells (MDA-MB-231), canine kidney cells (MDCK), murine models, and a NCI-60 panel of cell lines (16–19). miRNAs are short noncoding RNAs that regulate protein production by pairing with appropriate complementary stretches in mRNAs (20, 21). Hurteau et al. (16), Gregory et al. (17), and Park et al. (19) all identified a new role for miR-200c, miR-141, miR-200b, and miR-205, all members of the miR-200 family of miRNAs, in EMT through the direct regulation of ZEB1 and ZEB2. Korpal et al. (18) also showed that ectopic expression of these miRNAs leads to reexpression of E-cadherin and epithelial phenotypes in NMuMG murine mammary model that undergone EMT on transforming growth factor (TGF)-β stimulation (18) and mesenchymal MDA-MB-231 cells (16). Hundreds of miRNAs are encoded in the human genome, with an estimated 30% of mRNAs possessing conserved miRNA-binding sites, suggesting that miRNA-based regulation is an integral component of the global gene expression program (22). The presence of diverse and often strong phenotypes, such as EMT or diseases associated with mutations or altered expression of miRNAs, suggests their importance in cell development processes and in the biology of cancers (23, 24).

The overall goal of this study was to investigate the mechanisms underlying the EMT process and MET-induced resistance to EGFR-directed therapy in human bladder cancer cells. Our results revealed a close, direct association between expression of miRNAs of the miR-200 family, E-cadherin expression, and sensitivity to the EGFR antagonists. We also showed that ZEB1 and ZEB2 are involved in the EMT process and identified a new target (ERRFI-1/Mig6) that also plays an important role in mediating EGFR inhibitor resistance. These results should help us to prospectively identify bladder tumors that will be most susceptible to EGFR-directed therapy.

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Fig. 1. Identification of miRNAs that are differentially expressed in an epithelial versus a mesenchymal bladder cancer cell line. A, volcano plots showing all miRNAs detected in the two cell lines (left) or only the miRNA with significantly different expression (right, red dots) as opposed to nonsignificant levels of differential expression (right, blue dots). Bayesian log odds of differential expression are plotted against log2 (expression in UMUC5 divided by expression in KU7 cells). B, heat map of differentially expressed miRNA clustered by cell type (left, low expressed miRNAs in KU7 cells; right, high expression of the same miRNAs in UMUC5 cells). C to G, quantification of miRNAs in a panel of nine bladder cancer cell lines as measured by TaqMan real-time RT-PCR. Mean of triplicate RT-PCR assays.
Multiple photographs were then taken at 24 and 48 h in the same spots for comparison.

**Plasmid construction and luciferase reporter assays.** 3′-Untranslated region (3′-UTR) reporter plasmids for miR-200c were constructed via insertion of miR-200c seed sequence into the XbaI restriction site 3′ to luciferase gene in the pGL3-control plasmid (Promega). An ERFI-1 3′-UTR segment of 648 bp was amplified by PCR from human cDNA and inserted into the pMIR-REPORT (Ambion) with MluI and SpeI sites.

**Fig. 2.** Identification of miR-200 direct targets in a panel of bladder cancer cell lines. A, *Renilla* reporter plasmid harboring a miR-200c site with miR-200c precursor or the unrelated miR-26b (control) precursor were transiently cotransfected into KU7, UMUC3, UMUC5, and UMUC13 cells along with a firefly luciferase reporter (pGL3 control) for normalization. Mean ± SE of separate transfections (*n* = 6), shown as the ratio of *Renilla* activity to firefly. B to E, measurement by real-time RT-PCR of epithelial and mesenchymal markers: ZEB1, ZEB2, E-cadherin, and P-cadherin. Mean of measurements from triplicate experiments. F, heat map of six differentially expressed putative targets of miR-200c in a panel of nine bladder cancer cell lines using the Illumina gene profiling array platform.
For point mutations, we used the QuickChange II Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer’s instructions. The primers for point mutations (U1C) are mutant 1-F: 5′-ctctggtgttcggcttcagg-caggttagtgcttacagttgagc-3′, mutant 1-R: 5′-aactcataagctcgtttagaag-agtcttagcagtc-3′, mutant 2-F: 5′-ctgtccctggttgctgctgctc-3′, and mutant 2-R: 5′-aactcataagctcgtttagaag-agtcttagcagtc-3′. The sequences of the recombinant plasmids were confirmed by DNA sequencing.

Reporter vector transfection was done using Lipofectamine 2000 reagent (Invitrogen) as described previously (25). miRNA transfections were done using 20 nmol/L Lipofectamine 2000. Plasmid transfections were carried out similarly but with 50 nmol/L reporter plasmid in 2-well plates plus 0.02 μg cytomegalovirus-Remilla.

Real-time reverse transcription-PCR to quantify mature miRNAs. Total RNA was extracted using a Mirvana extraction reverse transcription kit (Applied Biosystems) and 10 ng total RNA along with miR-specific primer miRNA were used for expression analysis. cDNA was synthesized using TaqMan miRNA specific kit (Ambion-Applied Biosystems).

Immunoblot. Cells were treated with cetuximab (C225) for 3 h, harvested at ~75% to 80%, and lysed. Protein concentration was assayed using the Bio-Rad protein assay reagent (Bio-Rad Laboratories). To prepare cell extracts, cells were washed three times with PBS and then lysed with radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5) 150 mmol/L NaCl, 0.5% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, protease inhibitor cocktail (Roche Applied Science), and 1 mmol/L sodium orthovanadate] for 20 min on ice. To measure the activity (phosphorylation) of EGFR, we used an anti-EGFR—specific antibody (Cell Signaling Technology) antibodies and then treated with Alexa 488 labeled goat anti-rabbit and Alexa 546 labeled goat anti-mouse secondary antibodies (Molecular Probes). Confocal analysis was carried out using a Zeiss laser-scanning confocal microscope and established methods involving processing of the same section for each detector (two excitations corresponding to 546 and 488). Colocalization of the two proteins (EGFR and ERRFI-1) was indicated by the presence of yellow color as a result of overlapping red and green pixels.

RNA isolation, microarray platform, and statistics. All transcriptome data were generated from duplicates of the cell lines. Cells were plated and total RNA was isolated independently using Trizol reagent (Roche) followed by cleanup with RNeasy Mini kit (Qiagen). RNA was used for the synthesis of biotin-labeled cRNA, which was prepared using the Illumina RNA amplification kit (Ambion). Briefly, 500 ng total RNA from each specimen was converted to cDNA then to cRNA by in vitro transcription. After purification, 1.5 μg cRNA was fragmented and hybridized to Illumina human-6 v2 (Illumina) chip. After being washed, the slides were scanned with Bead Station 500+ (Illumina), and the signal intensities were quantified with Bead Studio (Illumina). Quantile normalization was used to normalize the data.

Statistics. Each experiment was done at least twice and at least one replicate mRNA data normalization was done by VSN. All calculations including statistical analysis were done by one-way or multi-way ANOVA. miRNA target prediction and associated mRNA pathway analysis were done using Ingenuity Pathway Analysis and TargetScan.

miRNAs of the miR-200 family are expressed in bladder cancer cell lines that display an “epithelial” phenotype. To identify miRNAs that might be important for regulating E-cadherin expression, we compared global miRNA expression patterns in two bladder cell lines: an EGFR-sensitive, E-cadherin-positive cell line (UMUC5) and an EGFR-resistant, E-cadherin-negative, and vimentin-positive cell line (KU7). We used miRNA array analysis that contained a data set composed of all known miRNAs biologically shown or predicted through bioinformatics (Assuragen, MiRInform, December 2007). Triplicate samples of untreated and cetuximab (C225)—treated UMUC5 and KU7 cells were analyzed using this platform. A diagram showing the total number or miRNA species expressed by the two cells without treatment is shown in Fig. 1A (left). All miRNAs were then ranked according to the differences in expression between untreated epithelial and mesenchymal cells with a cutoff of a
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Fig. 4. miR-200c expression induces an EMT phenotype in UMUC3 bladder cancer cell line. A, phase-contrast microscopy of UMUC3 cells transduced with a miR-200c containing retrovirus (UMUC3/200c) or with an empty, control, retroviral construct (UMUC3-E). Bar, 100 μm. B, measurement of in vitro cell migration by "wound-healing" assay. Representative pictures for same single spot. The experiment was done twice in triplicate experiments. Bar, 400 μm. C, quantification of miR-200c in empty virus-transduced (UMUC3-E) and miR-200c-transduced (UMUC3/200c) cells. Levels of miR-200c in UMUC5 cells are plotted for comparison. D, measurement by real-time RT-PCR of the epithelial and mesenchymal markers E-cadherin, ZEB1, and ZEB2 in empty virus-transduced cells and miR-200c-transduced UMUC3 cells. E, confocal microscopy analysis of the UMUC3 series costained for ZEB1 or ZEB2 (red pixels) and nuclear DNA (green pixels). Note the down-regulation of nuclear ZEB1 and ZEB2 in UMUC3 clones expressing the miR-200c. F, measurement of ERRFI-1 and E-cadherin protein levels by Western blot. Actin reprobing served as internal (loading) control. Bottom, absorbance relative values expressed as ratios between actin (internal control) and ERRFI-1. Note the up-regulation of E-cadherin and down-regulation of ERRFI-1 protein levels on miR-200c expression.
2-fold difference between the three replicate samples of the cells (Fig. 1A, right, red dots). We then performed a cluster analysis in which we grouped the known (validated) and the predicted miRNAs by their level of differential expression. One example of such a cluster is shown in Fig. 1B. Six miRNAs were selectively expressed at very high levels in the epithelial UMUC5 cells compared with mesenchymal KU7 cells. These miRNAs were miR-200a/141, miR-200b/c/429, and miR-205, all of which are part of the miR-200 family.

We then evaluated the expression of the miR-200 family of miRNAs in a panel of nine bladder cancer cells that display varying sensitivities to EGFR inhibitor–induced growth arrest. The panel contained four epithelial cell lines (UMUC6, UMUC9, UMUC16, UMUC5, and UMUC6), five mesenchymal cell lines (UMUC2, T24, UMUC3, UMUC13, and KU7), and one cell line that contained markers of both phenotypes (253J BV). Importantly, the latter cell line was derived from 253J cells through five cycles of in vitro selection, as described previously (26), with the goal of producing a more invasive phenotype. The resultant 253J BV cells were highly invasive in vitro and in vivo and some of the epithelial characteristics of the mother cell were lost (12). We observed increased expression of miR-200b/c, miR-205, miR-429, and miR-141 in all “epithelial” cells (E-cadherin positive), including the UMUC5 cells, whereas expression levels were very low in the “mesenchymal” cells, including KU7 cells (Fig. 1C-G). The 253J BV cells, which express low levels of E-cadherin and some vimentin (12) and are considered to be part of the EGFR-sensitive group, displayed coexpression of three of the miR-200 family members (Fig. 1D-G), suggesting that miR-200 family members are important regulators of EMT in bladder cancer cells. miR-141 was coexpressed with miR-205 in the UMUC5, UMUC6, UMUC9, and UMUC16 cells; however, the epithelial UMUC9 cell line lacked miR-205, whereas 253J BV cells lacked miR-141 (Fig. 1E and G). A comparison of miRNA expression patterns in UMUC5 and KU7 cells incubated with or without the EGFR antagonist C225 revealed that miR-200c was the only miRNA species induced substantially in the EGFR-sensitive UMUC5 cells, whereas no miRNA species, known or predicted, was modulated substantially by treatment with cetuximab (C225) in the EGFR-resistant KU7 cells (data not shown). These observations, corroborated by our personal observation that C225 treatment induced up-regulation of E-cadherin in UMUC5 cells, indicated that miR-200 is the best candidate to use in a study of the relationship between the EMT and EGFR signaling.

Because miR-200c, miR-200b, and miR-429 target the same sequences in their target miRNAs and because miR-200c seemed to be associated with the epithelial phenotype and with responsiveness to the EGFR inhibition by C225, we next decided to use a luciferase-based reporter system to measure the functionality of miR-200 family in our panel of “epithelial” bladder cancer cell lines. These constructs had the seed* sequence of miR-200c inserted downstream of the luciferase gene of the pGL3 vector. As an example, the UMUC5 epithelial cell line showed low baseline luciferase activity as opposed to the mesenchymal cell lines in which the baseline luciferase activity was very high although further suppressed by the transient expression of a miR-200c precursor (Fig. 2A) and not by the unrelated miR-26b, showing that miR-200b/c/429 expressed by E-cadherin-positive cells are also functional. Further real-time reverse transcription-PCR (RT-PCR) analysis in the same panel of bladder cancer cell lines revealed that the expression of miR-200 family members was inversely correlated with the expression of ZEB1 and ZEB2 (Fig. 2B and C, respectively). The expression of E-cadherin suppressors ZEB1 and ZEB2 was inversely correlated with the E-cadherin expression (Fig. 2D), suggesting that the epithelial phenotype of bladder cancer cells may also be regulated by miR-200 family members as it has been recently described in breast cancer cells (16), NCI-60 cell lines (19), and colon cancer cells (17) or in a murine model of EMT (18). We looked for other classical cadherins that are involved in homotypic interactions and found that P-cadherin was also associated with the expression of miRNAs of the miR-200 family (Fig. 2E). However, P-cadherin did not correlate with EGFR sensitivity because it was not expressed by 253J BV (Fig. 2D compared with Fig. 2E).

ZEB1, ZEB2, and ERRFI-1 are inversely associated with miR-200c expression. We inquired the TargetScan and PictarVert databases for potential direct targets of miR-200b/c/429 that would associate to an EMT phenotype and targets involved in the EGFR pathway. Several targets were identified according to their ranking in terms of conservation of miR-200 sites and their expression pattern in our panel of bladder cancer cell lines as a result of gene expression profiling of the nine cell lines. This list included ZEB1, ZEB2, ERRFI-1, MSN (a modulator of the cytoskeleton), and an unknown zinc finger transcription factor (ZNF532). A heat map showing the relative differences between the cell lines with respect to these potential miR-200 direct targets is shown in Fig. 2F. Further scrutiny for known direct modulators of EGFR-related signaling eliminated all of the targets, except ERRFI-1. Real-time RT-PCR analysis revealed that ERRFI-1 mRNA levels were very low in three of five epithelial cell lines and high in all three resistant cell lines (Fig. 3A), suggesting that it is a good candidate target of miR-200 and that its higher baseline expression may be a component of EGFR resistance.

Analysis of the 3′-UTR of ERRFI-1 and ZEB1/ZEB2 revealed that although ZEB1 and ZEB2 displayed at least five potential binding sites for the miR-200 family members (miR-200a/b/c/429/141 and 205), ERRFI-1 displayed two such conserved sites, with one additional miRNA conserved site for miR-148/152, which was not found in either ZEB1 or ZEB2 3′-UTR. We analyzed the expression pattern of the miR-148 in all nine cell lines and found no correlation with an epithelial or a mesenchymal phenotype, which underscored its role as a primary ERRFI-1 regulator (data not shown).

Because an intriguing property of ERRFI-1 is its ability to bind more tightly to activated EGFR than to the unligated receptor, we next determined which EGFR ligands are expressed by our cell lines. Gene expression array analysis revealed that three of the E-cadherin-positive cell lines, 253J BV, UMUC16, and UMUC9, also expressed TGF-α, whereas none of the mesenchymal cell lines expressed this gene (Fig. 3B). In addition, these “autocrine-positive” and E-cadherin positive cells displayed slightly higher levels of the ERRFI-1 protein (Fig. 3A), suggesting that the “feedback” mechanism of ligand-induced ERRFI-1 up-regulation is present in these cells as described previously (27). Importantly, UMUC5, which is E-cadherin positive but do not express the TGF-α, have almost undetectable

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levels of ERRFI-1 protein (Fig. 3A and B). An evaluation of cell growth inhibition by the cetuximab (C225) treatment and expression of ERRFI-1 and TGF-α revealed that U17, UMUC2, UMUC13, and UMUC3 (high expressers of ERRFI-1 and E-cadherin-negative) were resistant for up to 1 μmol/L cetuximab (C225) concentrations, whereas UMUC9, 253J BV, and UMUC16 (moderate ERRFI-1 expressers and high TGF-α expressors) or UMUC5 and UMUC6 (no detectable expression of ERRFI-1 protein or TGF-α mRNA) cell proliferation was inhibited up to 40% by 1 μmol/L cetuximab (C225; Fig. 3C). Importantly, these assays were done in EGF-unstimulated or “baseline” growth conditions in the presence of DMEM supplemented with 2% FCS and reflect the “lower inhibitory efficiencies” for the ligand antagonist cetuximab (C225).

miR-200c expression induced a mesenchymal-to-epithelial transition phenotype. To more directly define the role of miR-200c in maintaining the epithelial phenotype and EGFR dependency in bladder cancer cells, we stably transduced the mesenchymal UMUC3 cells with a retroviral vector containing the miR-200c precursor as shown previously (16). Stable expression of miR-200c in fibroblast-like mesenchymal UMUC3 cells resulted in morphologic changes suggestive of epithelial differentiation in that the cells grew in a more compact fashion and displayed cobblestone-like cell morphologies (Fig. 4A). These changes were associated with a reduced invasive phenotype as measured by in vitro wound-healing assays, where the miR-200c-expressing clones showed a significantly reduced wound-closing efficiency at 48 h (Fig. 4B). The high miR-200c levels expressed by the UMUC3 clones (Fig. 4C) were associated with the appearance of E-cadherin mRNA transcripts and reduced mRNA transcripts for ZEB1 and ZEB2 (Fig. 4D). These differences were also true at the protein levels as shown for ZEB1 and ZEB2 using immunofluorescence microscopy and confocal analysis (Fig. 4E) and for E-cadherin by Western blot analysis (Fig. 4F). These changes were also associated with a reduced ERRFI-1 protein expression (Fig. 4F). Based on morphologic criteria as well as biochemical and biological behavior, we concluded that the UMUC3/200c clones showed a mesenchymal-to-epithelial transition phenotype and reduced ERRFI-1 expression.

miR-200c expression reversed the EGFR-resistant phenotype. Expression of miR-200c in UMUC3 cells constantly but modestly reduced the mRNA transcripts of ERRFI-1 (data not shown), whereas the ERRFI-1 protein levels were significantly reduced, suggesting an additional post-translational mechanism involved in ERRFI-1 down-regulation in mesenchymal-to-epithelial transition clones (Fig. 4F). It is well documented that the EGFR is subject to ligand-induced down-regulation mediated by receptor internalization and subsequent degradation by lysosome and the ubiquitin/proteasome system (27–29). Thus, it has recently been suggested that ERRFI-1 is able to bind EGFR molecules, including the trafficking EGFR molecules and that ERRFI-1 becomes expressed at a time in which cells contain a sizable population of activated EGFRs that are being routed through the endosomal compartment. The blockade of kinase activity imposed by ERRFI-1 to trafficking EGFR molecules is expected to shift the kinase/phosphatase equilibrium in favor of phosphatases, therefore leading to a decline of pEGFR levels and concomitant loss of signaling competence (28).

To interrogate the location of these EGFR/ERRFI-1 complexes, we performed immunofluorescence staining and confocal microscopy studies of UMUC3 cells costained with antibodies specific for EGFR (Fig. 5A, green pixels) and ERRFI-1 (Fig. 5A, red pixels). The results revealed significant colocalized signals (yellow pixels) inside the cytoplasm, corresponding to high levels of EGFR trafficking, as well as on the cell membrane edges, such as ruffles. To our surprise, the distribution of EGFR molecules in the UMUC3/200c clones was very different, with most of the EGFR localized on the cell membrane, suggesting a higher rate of the surface-recycled EGFR. ERRFI-1 expression (red pixels) was also lower in UMUC3/200c cells compared with UMUC3 controls (Fig. 5A, middle and right compared with left), confirming our immunoblot analysis.

EGFR downstream mitogenic signaling is affected by miR-200c expression. Feedback inhibition is an important mechanism of signaling pathway regulation and is crucial in modulating the intensity and duration of signals generated by receptors. Thus, sustained activation of the signal-regulated kinase group of MAPKs is necessary for agonist-induced cell proliferation (30). EGF-stimulated extracellular signal-regulated kinase activation is often relatively transient, and in fibroblasts, EGF is a less effective mitogen than platelet-derived growth factor. A whole-genome expression analysis on two of the UMUC3/200c clones revealed that they express moderate levels of mRNA/TGF-α, which may be responsible for autocrine loop-driven baseline EGFR activation, although the overall levels of ERRFI-1 protein are lower than the empty vector–transfected UMUC3 cells as described previously. Given that TGF-α uncouples internalized EGFR molecules from degradation by driving their rapid recycling to the cell surface (31, 32), ERRFI-1 may represent the major, if not the only, means of restraining the recursive positive feedback loop generated by autocrine stimulation of the EGFR via TGF-α. Thus, a reduction of the ERRFI-1 baseline levels in the stable UMUC3/200c clones would result in higher baseline EGFR activation. Western blot analysis showed that UMUC3/200c clones have indeed higher baseline EGFR autophosphorylation associated with reduced levels of ERRFI-1 compared with UMUC3 controls (Fig. 5B). Furthermore, concentrations of cetuximab (C225) as low as 10 nmol/L were sufficient to block the baseline MAPK activity in UMUC3/200c clones, whereas 1 μmol/L cetuximab (C225) had no effect on MAPK activity in UMUC3/E (Fig. 5C). To determine whether morphologic and biochemical changes were relevant for the mitogenic response to anti-EGFR therapy, we treated UMUC3 controls and miR-200c clones with increased concentrations of C225 for 36 h and measured their proliferation index (Fig. 5D). We found that expression of miR-200c in the resistant UMUC3 cells resulted in a significantly higher cell growth inhibition to anti-EGFR therapy using cetuximab (C225; Fig. 5D) as well as other anti-EGFR agents, such as erlotinib/Tarceva (data not shown). Thus, cetuximab (C225) concentrations of up to 1 μmol/L produced cell cycle inhibition ranging from 40% to 60% translated into similar or even higher efficiency in cell growth inhibition than the rest of epithelial bladder cancer cells tested in baseline conditions. The importance of ERRFI-1 in modulating the cell cycle response to EGFR-targeted therapy was further shown by specific ERRFI-1 silencing in UMUC3 and in T24 cells, another E-cadherin-negative urothelial cell (Fig. 6A), where a significant reduction of ERRFI-1 levels was associated with a 30% cell cycle inhibition of cells treated with 10 nmol/L cetuximab (Fig. 6B). In addition, transfection of the full-length myc-tagged ERRFI-1 into sensitive, E-cadherin-positive UMUC5 cells resulted in the loss...
of EGFR sensitivity to relevant EGFR inhibitor concentrations (Fig. 6C). As we have mentioned previously, and according to the computer-based prediction programs, the 3′-UTR of the ERRFI-1 gene contains two putative miR-200-binding sites (Fig. 6D, red rectangles). To show that ERRFI-1 is indeed a direct target of miR-200 family members, we constructed four constructs, encompassing the 650-bp region of the 3′-UTR shown in Fig. 6D. We also modified the two binding sites one by one as single mutants or both sites as double mutants. We engineered a 2-bp site-directed mutagenesis, which allowed us to identify binding differences of miR-200b/c/429 translated to modulation of the activity of the luciferase reporter (Fig. 6E). Because cotransfection with either miR-200c (Fig. 6E), miR-200b, or miR-429 (data not shown) and wild-type 3′-UTR/ERRFI-1 significantly reduced the luciferase activity in UMUC3 and T24 and because the double-mutant constructs were able to rescue this activity, we concluded that ERRFI-1 is a direct target of the miR-200 in these cells (Fig. 6E). Because we used M1 and M2 point mutations and not deletions of the miRNA-binding sites and because the sequence complementarities between the miR-200 and the M2 site is expanding beyond the "seed" region, we cannot conclude that the M2 site is less important than the M1 site, as it may also be the result of a residual binding despite the point mutations that we introduced at this site. To finally show that expression of miR-200 into T24 results in modulation of cell proliferation response using EGFR inhibitors, we transfected a lentiviral construct with or without the miR-200b sequence into T24, an EGFR-resistant cells (Fig. 6F). Treatment of these cells with various concentrations of the EGFR inhibitor Iressa resulted in an increase in the response of the cell to this inhibitor as measured by DNA replication (Fig. 6G). We have included in this experiment the highly sensitive UMUC9 cells for comparison (Fig. 6G). In addition, miR-200b expression resulted into expression of E-cadherin, a 18-fold reduction of ERRFI-1 mRNA (data not shown), suggesting a similar phenotype to the UMUC3/200c clones above.

Discussion

We found that expression of the miRNA members of the miR200 RNA family, particularly miR-200c and miR-200b, is sufficient to reverse the biology of a mesenchymal cell line not only by its morphologic appearance but also by its EGFR signaling. Patterns of miRNA expression are thought to coordinately regulate a specific biological function, not just a gene involved in a specific pathway, so we examined whether the miRNA expression patterns found in bladder cancer cell lines might reflect potential markers of an epithelial phenotype in tight connection with EGFR signaling, which might actually represent a signature of an EGFR-sensitive phenotype in bladder cancer. ZEB1, ZEB2, and ERRFI-1 are three examples of miR-200 direct targets, their down-regulation by miR-200 being associated with an EGFR-sensitive phenotype. What would be the ERRFI-1-specific role to this change? We have recently reported that an "uninterrupted flow" from an active EGFR and its downstream phosphoinositide 3-kinase and MAPK signaling pathways toward glycogen synthase kinase-3β inactivation seems to play central roles in defining the EGFR sensitivity or the EGFR dependency in bladder cancer cells (25). Thus, we conclude that a higher baseline expression of ERRFI-1 (as found in many mesenchymal cell lines) that is not the resultant of an autocrine loop, might impair the EGFR-MAPK signaling flow and switch the dependence for cell proliferation from EGFR-driven to another signaling or growth factor receptor signaling pathway.

ERRFI-1, also known as RALT, gene 33, or Mig6, is a widely expressed adaptor protein that can be transcriptionally induced by a variety of external stimuli, including various growth factors, cytokines, and stress factors (33–36). Because several studies have reported that loss of ERRFI-1 signaling mediated by RNA interference enhances the mitogenic potency of suboptimal doses of EGF (37, 38), with paralleled extracellular signal-regulated kinase/MAPK and phosphoinositide 3-kinase/AKT activation and accumulation of cyclins, crucial in the G1-S transition, we reasoned that low baseline expression of ERRFI-1 might define the "addiction" of a cell line for ligand/EGFR signaling loop, promoting cell proliferation and finally defining an "EGFR-dependent" phenotype. An intriguing property of ERRFI-1 is its ability to bind more tightly to activated EGFR than to the unliganded receptor (38, 39) by using a double-headed mechanism for inhibiting EGFR to the cyclin-like face of the kinase domain of EGFR, a region that is conserved between all four EGFR family members (40). Based on these observations, we reasoned that the higher the ratio between TGF-α expression and ERRFI-1 expression in a cell line, the higher would be the chances that that cell line would be dependent on EGFR-driven mitogenic signals and would be mostly sensitive to anti-EGFR therapy in vitro. Two of the cell lines expressing miR-200 displayed very low levels of ERRFI-1 protein, whereas other three lines expressing miR-200 and TGF-α displayed slightly higher levels of ERRFI-1 protein, although all five were defined as EGFR sensitive. These cell lines were indeed some of the most sensitive to EGFR inhibitors, including cetuximab (C225) treatment (12). Of particular interest are two recent studies that show that secretion of EGFR agonists facilitate disease control in patients with metastatic colorectal cancer treated with cetuximab (C225) as well as in experimental metastatic lung model in mice (41, 42). Based on these reports and the present work, we conclude that high expression of miR-200 would facilitate the best EGFR functionality, hence most efficiently targeted by anti-EGFR therapy, and that additional TGF-α expression through autocrine or paracrine secretion would probably serve to fine-tune the EGFR dependency in vitro.

Particularly intriguing was our previous finding that bladder cancer cells displaying an EMT are also less responsive to anti-EGFR-targeted therapy (12), suggesting that alterations in the
ERRFI-1 is a direct target of miR-200 and implicated in response to EGFR inhibitors. A, Western blot measurement of ERRFI-1 protein in UMUC3 and T24 cells after transfection with a nontargeting short hairpin RNA construct, a glyceraldehyde-3-phosphate dehydrogenase, or an ERRFI-1sh construct. Vinculin served as internal control. B, cell proliferation measurement of the UMUC3 and T24 series using radioactive thymidine incorporation after transfections described in the previous panel. Each experiment was done in at least two triplicates. C, cell proliferation measurement of the UMUC5 series using radioactive thymidine incorporation after ERRFI-1 transfection. Right, level of ERRFI-1 protein. Vinculin served as internal control. D, schematic representation of the 3′-UTR of ERRFI-1 displaying the miRNA potential binding sites as predicted by TargetScan. Red boxes, miR-200b/c/429 potential binding sites. E, measurement of miR-200-repressive activity on wild-type and mutant 3′-UTR/ERRFI-1 reporters. F, real-time RT-PCR of T24 series after miR-200b transduction using a lentiviral system. PEV, empty vector–transduced cells served as negative controls. G, cell proliferation measurement of the T24 series using radioactive thymidine incorporation after miR-200b lentiviral transduction. Each experiment was done in at least two different triplicates. UMUC9 served as positive controls being highly sensitive responders to EGFR blockers, in this case, Iressa.
EGFR signaling are associated with such a phenotype. We reasoned that the association between E-cadherin expression and high anti-EGFR sensitivity could be the result of two situations: (a) EMT-regulating miRNAs might coregulate additional targets involved in EGFR signaling or (b) there are additional miRNAs that primarily target components of the EGFR pathway and showed similar expression patterns to those that regulate an EMT phenotype. In that study, we showed the importance of E-cadherin expression in an epithelial background where the silencing of E-cadherin substantially modulated EGFR response to therapy in 253J BV cell lines from a sensitive to a resistant phenotype (12). In the present study, we were able to completely reverse the EGFR responsiveness to therapy using a mesenchymal background (e.g., UMUC3 and T24) by the introduction of miR-200 and modulation of its direct or indirect target genes, including E-cadherin and ERRFI-1. The two studies support each other by placing emphasis on the importance of an epithelial phenotype and its relation to EGFR responsiveness. Although the exact mechanistic explanation of how E-cadherin expression affects EGFR responsiveness in an epithelial background is still hypothetical at this point, based on previous reports and our findings, we can assume a scenario in which EGFR spatial distribution may play a role in the EGFR responsiveness. Thus, in an epithelial background with low expression of ERRFI-1, EGFR is rapidly recycled to the cell membrane and E-cadherin may serve as a scaffold for EGFR, which would result in increased stability and exposure on the cell surface for optimal accessibility of EGFR-targeted therapy. This was also illustrated by findings in our current study that, in mesenchymal-to-epithelial transition UMUC3/200 clones in which E-cadherin expression was induced by miR-200c expression and ERRFI-1 was down regulated, the EGFR was mostly exposed on the cell membrane and the responsiveness to its inhibitors was optimal. In this case, either down regulation of ERRFI-1 or E-cadherin might affect the EGFR spatial distribution that would affect its signaling. It would be important to know whether E-cadherin is required for EGFR to be accessible by ERRFI-1 or whether E-cadherin down-regulation triggers feed-forward upregulation of ERRFI-1 (43). In contrast, in a mesenchymal background, there is higher baseline expression of ERRFI-1, which binds EGFR to its kinase domain, maintaining the EGFR/agonist loop in a rather inhibited state. Again, this finding was supported by our confocal microscopy analysis, which showed that the cell-surface pool and most of the internalized, trafficking EGFR were bound to ERRFI-1, and by Western blot, which showed that baseline EGFR autophosphorylation was lower in the mesenchymal background compared with the epithelial background of UMUC3 cells and the expression of miR-200c facilitates the EGFR downstream signaling pathways toward MAPK modulation. Importantly, the “switch” in EGFR responsiveness was shown for two different mesenchymal cell lines and using two members of the miR-200 family.

Conclusions. Recent therapeutic advances have identify targeted therapy, such as growth factor inhibition, as being more effective in the context of a documented patient selection and using algorithms that take into account targeted expression and molecular pathway validation. Growth factor receptors, such EGFR, fibroblast growth factor receptor, and platelet-derived growth factor receptor, are differentially expressed by bladder cancer cell lines or tumors, making the use of inhibitors to these molecules an attractive therapeutic strategy. By exploring the mechanistic relationship between miRNAs-driven gene reprogramming and the implications to the therapeutic response, our studies open a new avenue to the understanding the mechanism of acquired resistance to the EGFR-targeted therapy in the context of bladder cancer oncology and unravel additional markers to evaluate for a future informed therapeutic decision.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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