Overexpression of the MicroRNA *hsa-miR-200c* Leads to Reduced Expression of Transcription Factor 8 and Increased Expression of E-Cadherin

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Abstract

MicroRNAs are ~22-nucleotide sequences thought to interact with multiple mRNAs resulting in either translational repression or degradation. We previously reported that several microRNAs had variable expression in mammalian cell lines, and we examined one, *miR-200c*, in more detail. A combination of bioinformatics and quantitative reverse transcription-PCR was used to identify potential targets and revealed that the zinc finger transcription factor transcription factor 8 (TCF8; also termed ZEB1, δEF1, Nil-2-α) had inversely proportional expression levels to *miR-200c*. Knockout experiments using anti-microRNA oligonucleotides increased TCF8 levels but with nonspecific effects. Therefore, to investigate target predictions, we overexpressed *miR-200c* in select cells lines. Ordinarily, the expression level of *miR-200c* in non–small-cell lung cancer A549 cells is low in contrast to normal human bronchial epithelial cells. Stable overexpression of *miR-200c* in A549 cells results in a loss of TCF8, an increase in expression of its regulatory target, E-cadherin, and altered cell morphology. In MCF7 (estrogen receptor–positive breast cancer) cells, there is endogenous expression of *miR-200c* and E-cadherin but TCF8 is absent. Conversely, MDA-MB-231 (estrogen receptor–negative) cells lack detectable *miR-200c* and E-cadherin (the latter reportedly due to promoter region methylation) but express TCF8. The ectopic expression of *miR-200c* in this cell line also reduced levels of TCF8, restored E-cadherin expression, and altered cell morphology. Because the down-regulation of E-cadherin is a crucial event in epithelial-to-mesenchymal transition, loss of *miR-200c* expression could play a significant role in the initiation of an invasive phenotype, and, equally, *miR-200c* overexpression holds potential for its reversal. [Cancer Res 2007;67(17):7972–6]

Introduction

The identification of microRNAs and their mechanism of action was originally made in *C. elegans* (1) and, due to their highly conserved nature, subsequently described in many other species including vertebrates. The interpretation of microRNA biogenesis and mode of action has since led to the recognition of a new level of eukaryotic gene regulation (2, 3). The mechanism involves endogenous ~22-nucleotide (nt) microRNAs of which there are thought to be several hundred expressed at varying levels and in a tissue-specific manner. In eukaryotes, initial expression is in the form of nascent pri-microRNA transcripts, generated by polymerase II and capped and polyadenylated (4). These are processed into ~70-nt pre-microRNAs by the Drosha RNase III endonuclease (5) and transported from the nucleus by Ran-GTP/exportin-5 (6). The pre-microRNAs are then processed by Dicer into the final ~22-nt noncoding single-stranded mature microRNAs that regulate gene expression (5). Regulation involves the incorporation of mature microRNAs into the RNA-induced silencing complex and target recognition based on perfect (or almost perfect) complementarity to the mRNA, with the result being either cleavage or transcriptional repression (7, 8). The function of the majority of microRNAs is currently unknown, with identification of many (mRNA) targets being predicted through computational means (9). Such algorithms have to account for the majority of animal microRNAs (unlike plant microRNAs) lacking a perfect pairing to their targets, and predictions may alter following experimental investigation (9). In addition, computational predictions of microRNA/mRNA interactions do not include the outcome (i.e., translational repression or cleavage). Experiments involving transfection of reporter constructs have indicated that, in mammals, the outcome is often translational repression. However, it has recently been reported that transfected microRNAs can also regulate the mRNA levels of a large number of targets (10). To examine these predicted interactions in an endogenous manner, we initially adopted a technique to allow for the quantitation of both microRNA and mRNA from the same sample (11). This experimental approach reasoned that if the interaction between the microRNA and its target mRNA resulted in degradation, then an inverse relationship would be observed in expression levels. The variably expressed microRNA *hsa-miR-200c* was studied further and, by using a combination of bioinformatic and mRNA array data, ~600 predicted targets were reduced to 20. Quantitaive PCR analysis then identified three mRNAs with expression levels inversely related to *miR-200c* (11). The remaining candidate targets had no inverse relationship or were expressed below detection level, and thus may be bona fide targets in other cells, tissues, or stages of development. This study suggested that the vast majority of *miR-200c* targets are translationally repressed; however, the lack of any consensus may also indicate overprediction by *in silico* methods. Further examination of predicted targets using an anti-microRNA oligonucleotide against *miR-200c* did restore expression to transcription factor 8 (TCF8; also termed ZEB1, δEF1, Nil-2-α; ref. 11). However, we observed nonspecific effects using the anti-microRNA oligonucleotide and, in addition, the repression of *miR-200c* expression was transient (11). Therefore, to gain a better understanding of the function of *miR-200c*, we used a retroviral expression system (12) for its overexpression in A549 and MDA-MB-231 cell lines.

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doi:10.1158/0008-5472.CAN-07-1058

Materials and Methods

Cell transfection and RNA preparation. The A549 (lung), MDA-MB-231, and MCF7 (breast) cancer cell lines used in this study were obtained from the American Type Culture Collection and cultured according to recommended conditions. To generate stably expressing constructs, the genomic region surrounding the pri-microRNA sequence of *miR-200c* was amplified using primers that generated a ~500-bp product, directionally cloned into pLNCX (Clontech Labs, Inc.) using *Not*I and *Xho*I, and transformed using *E. cloni* 10G Chemically Competent cells (Lucigen). Clones were sequenced to confirm correct orientation of *miR-200c* in relation to the cytomegalovirus (CMV) promoter and Maxipreps (Qiagen) prepared to generate enough DNA (~10 μg) to mix with Phoenix packaging cells (Orbigene, Inc.). After 48 h, the supernatant from the Phoenix cells was used to infect A549 or MDA-MB-231 cells, followed by selection for resistance to G418. Total RNA was extracted with Trizol (Invitrogen) according to the manufacturer's recommended procedures after 3, 7, and 21 days and quality was determined at 260/280 nm with a spectrophotometer (Nanodrop Technologies).

Modification and amplification of mature microRNAs and mRNA. The mRNA and mature microRNAs were amplified as previously described (11) and outlined in Fig. 1*B*. Relative expression was calculated using an ABI HT7900 system with fluorescent Syber Green real-time PCR used to generate C_t values in triplicate. Changes in the expression levels of mRNA and microRNA, relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were calculated as $2^{-\Delta C_t}$ and graphed as shown in Figs. 2 and 3.

Cytospins and immunohistochemistry. Slides and filters were placed into appropriate slots in the cytospin with the cardboard filters facing the center of the cytospin. Aliquots of ~100 μL of each sample (10^5 cells) were placed into the appropriate wells of the cytospin. Lids of the cytospin were placed over the samples and spun at maximum speed for 1 to 3 min. Filters were removed without contacting the smears on the slides. Slides were then examined under the microscope to be sure that the cells had annealed properly. All slides were dried in a desiccation chamber overnight.

Cytospin slides were immunostained with mouse monoclonal antibodies for E-cadherin (DAKO; 1:100) using an automated method (Ventana Medical Systems).

Results and Discussion

To generate stable cell lines that express *miR-200c*, we adopted methods previously used for expressing mature microRNA under the control of a polymerase II promoter (13). The strategy we used for integration of the pri-microRNA sequences regulated by a CMV promoter and subsequent selection is shown in Fig. 1*A*. The two cell lines chosen had previously been shown to have either extremely low (A549) or undetectable (MDA-MB-231) expression of *miR-200c* (11). We established that the integrated construct could generate mature microRNA using the method outlined in Fig. 1*B* to quantitatively measure expression levels, showing that mature *miR-200c* greatly increased in A549 and MDA-MB-231 (Fig. 1*C*). As a control, we generated constructs that had the identical genomic region cloned in the opposite orientation with respect to the CMV promoter. These failed to generate mature microRNA but did integrate as evidenced by cell selection for neomycin resistance using G418. In the correct orientation, the integrated region continued to express *miR-200c* with levels increasing over the 21-day time period shown. It is unclear if this is a result of accumulation of mature microRNAs or due to cell selection (Fig. 1*C*). Having established that the cells could stably express *miR-200c*, we next examined the effect on mRNAs previously predicted to be targets (11); these were bridging integrator 1 (BIN1), lipoma HMGIC fusion partner (LHFP), RAS homologue gene family member E (RND3/ARHE), and TCF8. We also included one mRNA, the RAB11 family–interacting protein 1 (RAB11F1P1), which has no seed match for *miR-200c* in its 3′ untranslated region (UTR) and is a predicted target of *miR-205* (11). The expression levels of these mRNAs were again measured quantitatively relative to GAPDH using the same anchored oligodT–based approach (11).

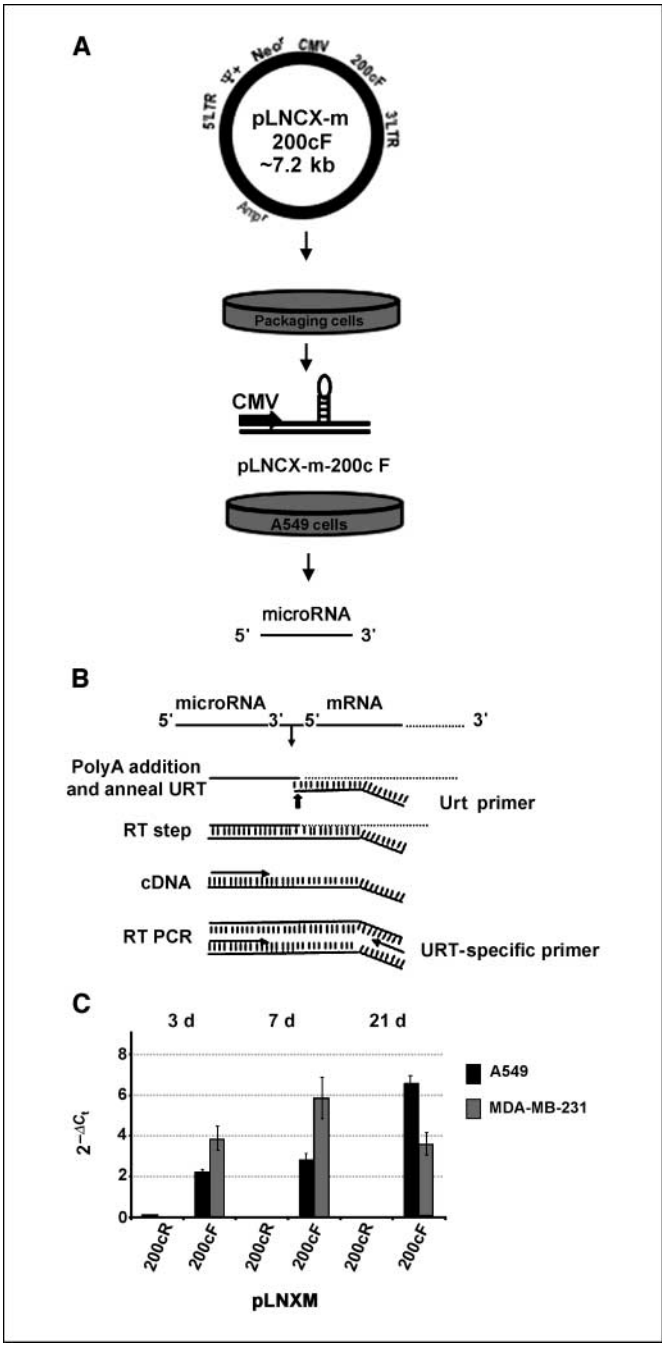

Figure 1. Strategy for the expression of *miR-200c* in A549 and MDA-MB-231 cell lines. *A,* ~500 bp of the flanking region surrounding the pri-miR-200c sequence was cloned into pLNCX-m in either orientation with respect to the CMV promoter. Cell lines were infected and RNA extracted after selection for recombinants using G418 as described in text. *B,* outline of the procedure for the amplification of both mature microRNA and mRNA from total RNA, described in detail in ref. 11. *C,* increase in expression of mature *miR-200c* seen over a 3-wk period following selection in both A549 and MDA-MB-231 cells (*200cF*; *black* and *gray columns*). No increase in *miR-200c* expression is seen with negative control (*200cR*), remains at the endogenous level of limit of detection (A549), or absent (MDA-MB-321).

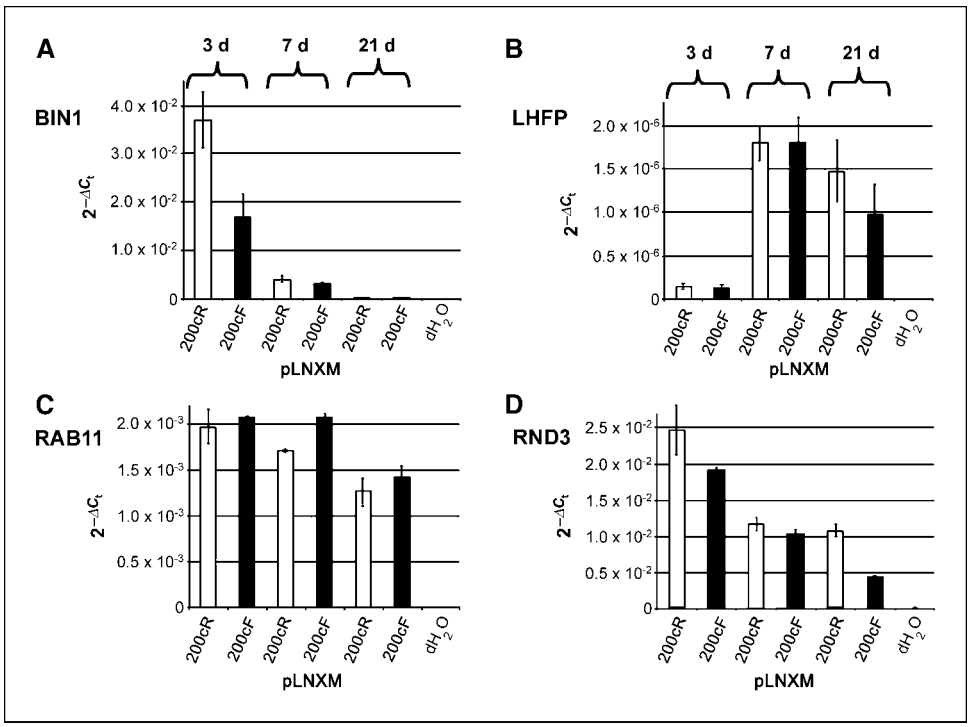

Figure 2. Expression levels of BIN1, LHFP, RAB11, and RND3 in A549 cells. Expression of putative mRNA targets measured by relative quantitative reverse transcription-PCR (RT-PCR) at 3, 7, and 21 d. Cell lines are either overexpressing (+*200cF*; *black columns*) or have endogenous expression (+*200cR*; *white columns*) of *miR-200c. A* and *B,* expression of BIN1 and LHFP (predicted targets of *miR-200c*) are altered but levels are not significantly correlated to overexpression of *miR-200c* at each of the time points shown. *C,* expression of RAB11 (not a predicted target) is essentially unaffected in both cell lines. *D,* RND3 levels are slightly reduced 21 d after G418 selection in cell lines overexpressing *miR-200c*.

In the A549 cells that overexpress *miR-200c*, the level of BIN1 mRNA expression was initially reduced but levels then decreased equally in both the overexpressing and control cells (Fig. 2*A*). The levels of LHFP expression were similarly affected but then increased in both cell lines over the 3-week period (Fig. 2*B*). The expression levels of RAB11F1P1, which was not predicted to be a target, were virtually unaffected in both cell lines over the 3-week time period examined (Fig. 2*C*). However, examination of RND3 expression indicates that although it is initially unaffected, a reduction can be seen in those lines expressing *miR-200c* after

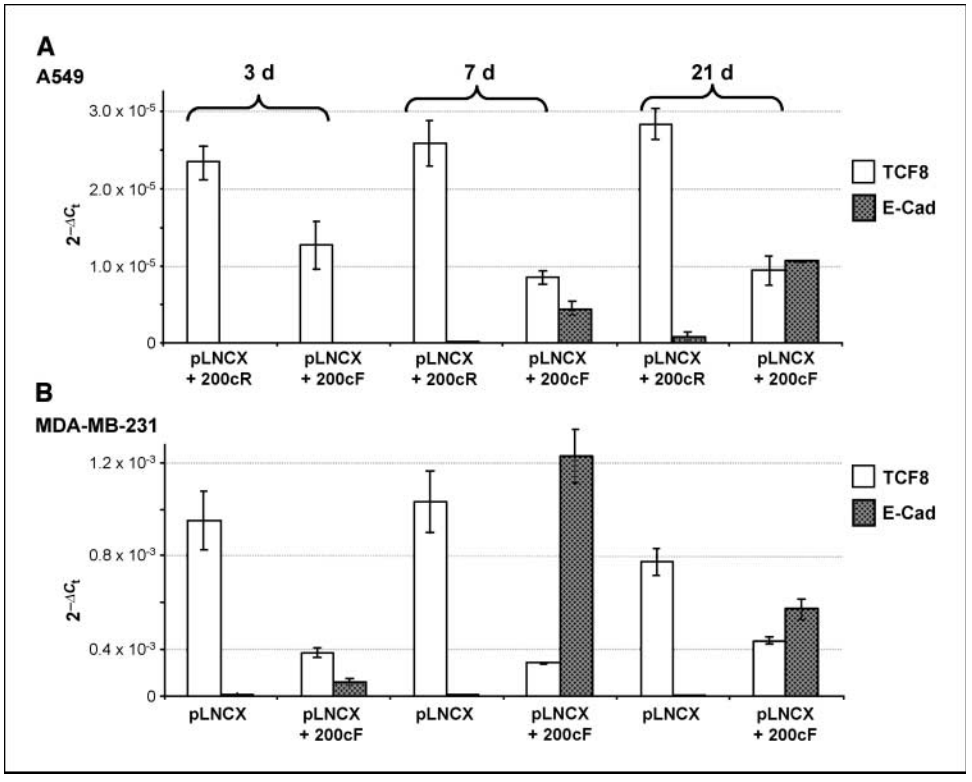

Figure 3. Relative quantitation by RT-PCR of TCF8 and E-cadherin expression. A549 or MDA-MB-231 cells expressing mature *miR-200c* (pLNCX + 200cF) or a nonexpressing control (pLNCX + 200cR) at 3, 7, and 21 d. A reduction in TCF8 is shown in (*A*) and (*B*); compare white columns at each time period for cells positive for *miR-200c* versus nonexpressing control. In A549 cells, the loss of TCF8 coincides with increased expression of E-cadherin (*E-Cad*; *gray columns*) after 7 and 21 d, respectively. In MDA-MB-321 cells, an increase in E-cadherin is observed after only 3 d in lines expressing *miR-200c*.

3 weeks (Fig. 2*D*). Similar results for these four mRNAs were observed in MDA-MB-231 cell lines that ectopically expressed *miR-200c* (data not shown). The most significant change in expression was observed in the expression level of TCF8 in A549 (Fig. 3*A*) with an even greater loss seen in the altered MDA-MB-231 cells (Fig. 3*B*) at each of the time points shown over 21 days. However, we noted a disparity between the endogenous *miR-200c* action seen in MCF7 and that observed in the altered A549 and MDA-MB-231 cells. For example, we had previously shown that TCF8 is completely absent in MCF7 (11), but this mRNA can still be detected in MDA-MB-231 and A549 cells where expression of *miR-200c* is now at least equivalent relative to GAPDH (Fig. 3*A*). One explanation is that there is mixed cell population in both A549 and MDA-MB-231 with variety of integration sites. Consequently, some cells may either only weakly express miR-200c or may have inactivated the CMV promoter. It is also possible that another microRNA with a recognition site in the 3′-UTR of TCF8 is required for its effective removal. Alternatively, *miR-200c* represses translation of TCF8 (followed by gradual degradation) and, consequently, the mRNA can still be detected. This may also be the case for RND3, although the delay seen before message is reduced is longer (Fig. 2*D*) and may indicate an alternative pathway initially regulated by *miR-200c*. We are currently investigating another possibility: that translational repression is followed by degradation using an antibody for TCF8 to determine protein levels during overexpression of *miR-200c*. Finally, the possibility that TCF8, which has a large CpG island, is regulated in MCF7 by epigenetic mechanisms is currently under investigation. Such an epigenetic change could prevent initiation of TCF8 transcription with the high level of *miR-200c* degrading any residual or "leaky" transcripts. However, our initial experiments with anti-microRNA oligonucleotides to knock down *miR-200c* did partially restore TCF8 expression (11), suggesting that in these cell lines, TCF8 expression could be primarily regulated by the microRNA. Nevertheless, the loss of TCF8 mRNA in these cells is notable, in particular MDA-MB-231 in which *miR-200c* was undetectable before transformation. It has previously been reported that an almost perfect match between a microRNA and its target in the 3′-UTR is required for degradation (14). However, a recent report by Garzon et al. (15) indicated that *miR-130a* could degrade its target MAFB without a perfect match being present in the 3′-UTR. A third possibility presented here is the observation that the 3′-UTR of TCF8 has multiple perfect matches to the seed region of *miR-200c*. It is possible that either the multiple matches or the spatial differences (two sites are within 50 bp of each other) are prerequisites for degradation. Further investigation either through expression of engineered reporter constructs or using prediction programs may provide an answer to this. Having established stable cell lines in which overexpression of *miR-200c* resulted in a reduction of TCF8 mRNA levels, we then decided to examine the effect on targets of TCF8. A literature search revealed two targets that are both reported to be regulated by TCF8 but in a different manner. The zinc finger protein encoded by the *TCF8* gene is also known as Nil-2-α. This is reported to inhibit expression of the T lymphocyte–specific interleukin-2 (*IL-2*) gene by binding to a negative regulatory domain 100 nt 5′ of transcription start site (16). A second study reported that TCF8 or δEF1 is a transcriptional repressor of E-cadherin and regulates epithelial plasticity in breast cancer cells (17). Finally, it has also been shown that the zinc finger transcriptional repressor ZEB1 (TCF8) inhibits E-cadherin expression by recruiting histone deacetylases (18). We therefore examined both E-cadherin and IL-2 levels in MCF7, A549, and MDA-MB-231 cells and found strong expression of both in MCF7 (data not shown) with little expression in A549 and none detectable in MDA-MB-231. The overexpression of *miR-200c* in both A549 and MDA-MB-231 initially has little or no effect on E-cadherin or IL-2 expression. However, after 1 week and following the reduction in TCF8, increased E-cadherin expression is evident in A549 and pronounced in MDA-MB-231 cells (Fig. 3*A* and *B*). The level of IL-2 does increase in both altered cell lines after 3-week overexpression of *miR-200c*, but the increase is not significant (data not shown). The expression levels of E-cadherin continue to increase in an inverse relationship to TCF8 in both cell lines (Fig. 3). After 3 weeks of expression of miR-200c in MDA-MB-231 and A549, the cells begin to detach from the plates and die. However, there is no evidence that cell death is directly caused by overexpression of *miR-200c*. Therefore, at the mRNA level, E-cadherin expression is directly related to miR-200c and inversely related to TCF8. At the protein level, examples of E-cadherin expression in A549 and MDA-MB-231 cells expressing *miR-200c* are shown in Fig. 4. The cytospin of A549 cells shows a distinct staining for E-cadherin at the cell boundaries (Fig. 4*A*). In Fig. 4*B*, cell morphology was preserved and those cells expressing E-cadherin appear rounder and seem to form more cell-to-cell junctions in comparison with the controls. Thus, at both the mRNA and protein levels, expression of E-cadherin has

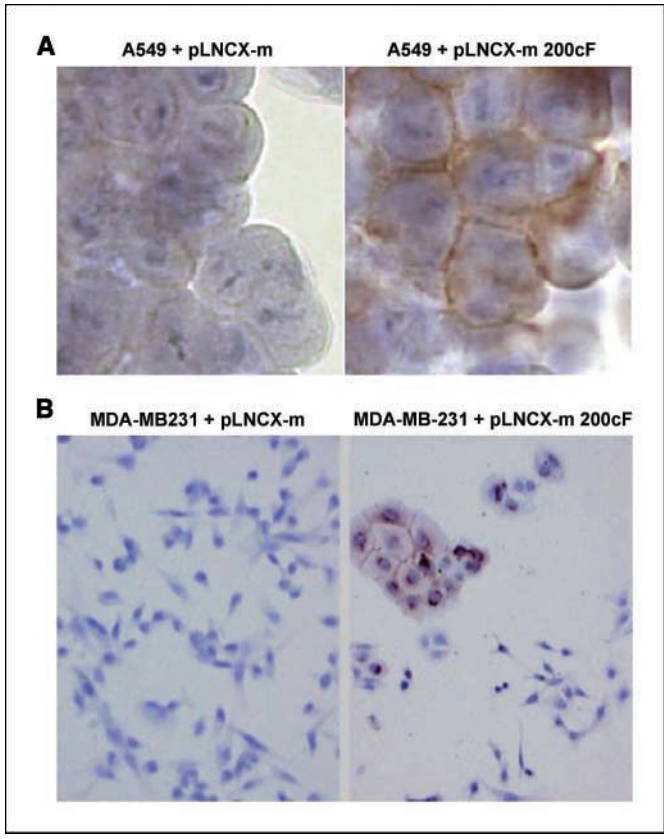

Figure 4. Cell morphology and E-cadherin staining in cells expressing *miR-200c* in comparison with controls. *A,* cytospins of A549 cell lines at 7 d show little or no staining for E-cadherin at the boundaries of cells that do not overexpress *miR-200c*, but strong staining is seen in overexpressing cells (+ *200cF*). *B,* MDA-MB-231 cells grown on coverslips for 7 d and fixed to preserve morphology. Cells that do not express *miR-200c* have normal morphology and do not stain for E-cadherin. Cells expressing *miR-200c* show mixed cell population, with those positive for E-cadherin having a different morphology (+ *200cF*).

apparently been restored. The restoration of expression proceeds several days after reduction in TCF8 levels (Figs. 3 and 4) although the promoter region (at least in MDA-MB-231) is reportedly methylated. One potential explanation is in previous reports that TCF8 recruits a histone deacetylase (18), and its loss over several cycles of cell division could give rise to the specific demethylation or loss of heterochromatin in the promoter region of E-cadherin. Further investigation of epigenetic changes and a detailed investigation of the effects on the proteome in these cell lines are warranted. However, these results do indicate that the mRNA level of the transcription factor TCF8 can be regulated by *miR-200c*, which in turn will alter levels of both E-cadherin and, to a lesser extent, IL-2. Endogenously, the expression levels of miR-200c are highly variable across several cell lines (11) but a preliminary examination of the genomic region flanking pri-miR-200c revealed no large-scale deletions or single-nucleotide polymorphisms (data not shown). The promoter region may lie outside the examined area or the regulation of these microRNA could be mediated by *trans*-acting factor(s). The potential therefore exists for the specific restoration of E-cadherin (and IL-2) by the identification of specific molecules that could up-regulate the expression of *miR-200c*. Whereas the examination of microRNA expression remains a technically challenging problem, its potential effect is underscored by recent reports showing that changes in microRNA expression might be used to classify cancers (19). Our study indicates that a single microRNA can indirectly regulate at least one important cellular pathway that includes alteration of E-cadherin expression levels. Whereas this work represents preliminary findings *in vitro*, further studies are required to assess the implications of such findings *in vivo*. The potential effect of the reported large-scale microRNA changes, and by extension, of the alteration to mRNA and protein targets, presents a daunting prospect from a therapeutic viewpoint. However, this study and previous work indicate that the *miR-200c* degrades only a small number of its predicted targets, the question remains how many of the remaining target predictions are translationally regulated by *miR-200c*. Cell lines generated using this method present a model system for a proteomic analysis and will enable unaffected targets to be identified, which may improve the ability of algorithms to predict targets.

Acknowledgments

Received 3/20/2007; revised 6/12/2007; accepted 7/3/2007.

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