MicroRNA-661, a c/EBPα Target, Inhibits Metastatic Tumor Antigen 1 and Regulates Its Functions

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Abstract

MicroRNAs (miR) have been identified as posttranscriptional modifiers of target gene regulation and control the expression of gene products important in cancer progression. Here, we show that miR-661 inhibits the expression of metastatic tumor antigen 1 (MTA1), a widely up-regulated gene product in human cancer, by targeting the 3′ untranslated region (UTR) of MTA1 mRNA. We found that endogenous miR-661 expression was positively regulated by the c/EBPα transcription factor, which is down-regulated during cancer progression. c/EBPα directly interacted with the miR-661 chromatin and bound to miR-661 putative promoter that contains a c/EBPα-consensus motif. In addition, we found that the level of MTA1 protein was progressively up-regulated, whereas that of miR-661 and its activator, c/EBPα, were down-regulated in a breast cancer progression model consisting of MCF-10A cell lines whose phenotypes ranged from noninvasive to highly invasive. c/EBPα expression in breast cancer cells resulted in increased miR-661 expression and reduced MTA1 3′UTR-luciferase activity and MTA1 protein level. We also provide evidence that the introduction of miR-661 inhibited the motility, invasiveness, anchorage-independent growth, and tumorigenicity of invasive breast cancer cells. We believe our findings show for the first time that c/EBPα regulates the level of miR-661 and in turn modifies the functions of the miR661-MTA1 pathway in human cancer cells. Based on these findings, we suggest that miR-661 be further investigated for therapeutic use in down-regulating the expression of MTA1 in cancer cells. [Cancer Res 2009;69(14):5639–42]

Introduction

MicroRNAs (miRs) have been identified as posttranscriptional modifiers of target gene regulation (1). miRs are 20 to 22 bases long and are derived from longer noncoding primary transcripts by the actions of the Drosha and Dicer RNA cleaving enzymes (2, 3). Recent spurt of research has revealed that miRs have important roles in development and disease including cancer (4–6). In general, miRs pair up with the 3′ untranslated region (UTR) of target mRNAs in a sequence-specific manner with the help of RNA-induced silencing complex. These pairings lead to the down-regulation of the target mRNA by translational inhibition or degradation of mRNA (7). Recent research suggests that miRs suppress or activate cancer and metastasis. miR-10B, for example, has been shown to promote metastasis in breast cancer (8), whereas miR-7 has been shown to suppress breast and brain cancers (9, 10).

A large body of work (11–13) has linked the up-regulation of metastatic tumor antigen 1 (MTA1) to the maintenance and progression of more invasive phenotypes of many human cancers. MTA1, the founding member of the MTA family, was initially identified as a differentially expressed gene in metastatic rat mammary adenocarcinoma (14). MTA1 is a component of the chromatin remodeling complex and modulates transcription of its target gene chromatin, by recruiting HDACs or RNA PolII. MTA1 is widely up-regulated in many carcinomas, including breast, colorectal, gastric, esophageal, pancreatic, ovarian, non–small cell lung, hepatocellular and renal carcinomas; thymoma; and hematopoietic malignancies (12). Forced overexpression of MTA1 in the mouse mammary gland epithelium leads to hyper proliferation in glands of virgin mice and mammary gland adenocarcinomas (15). Although the role of MTA1 in human cancer has been established, it remains unclear whether MTA1 is targeted by miRs.

Materials and Methods

Cell lines, culture conditions, and transfections. Human cell lines were cultured as described in supplementary methods. Transfections for miRNA mimics and plasmid constructs were done with Oligofectamine or Fugene as described in the Supplementary Methods.

Plasmid constructs, luciferase assay, and Western blotting. MTA1 3′ UTR and miR-661 promoter region were cloned into pGL3 control vector and pGL3 basic promoter less vector, respectively, as described in Supplementary Methods. Luciferase assays and Western blotting were done as described in the Supplementary Methods.

Quantitative real-time PCR analysis of miRs. RNA was isolated using mirVana miRNA isolation kit. For quantitative analysis of miRNAs, two-step TaqMan real-time PCR analysis was performed using primers and probes obtained from Applied Biosystems.

Migration, soft-agar, and confocal studies. Migration, Invasion, Soft agar assays, and Confocal studies were done as described in the Supplementary methods.

Chromatin immunoprecipitation. Chromatin immunoprecipitation and Electrophoretic Mobility Shift Assay was performed as described in the Supplementary methods.

Results and Discussion

To search for miRs that might regulate MTA1 expression, we screened the 3′UTR region of MTA1 mRNA using the public database for possible complementation of a minimum of 8-bp to the seed region of miRs (16). This exercise resulted in identification of human miR-661 and miR-559 as candidate miRs that might regulate MTA1 (Fig. 1A). To assess the effects of these miRs on

Figure 1. Prediction and target validation of MTA1-targeting miRs. *A,* prediction of MTA1-targeting miRs by mirBase (version 4). *B,* ZR75, MCF-7, MDA-231, and HeLa cells were transfected with 100 nmol/L miR-559 or miR-661 or a negative control mimic using Oligofectamine. Protein lysates were prepared by radioimmunoprecipitation assay lysis buffer and Western blot analysis was used to determine the levels MTA1, MTA2, and vinculin. *C,* MiR-559 or mir-661 targeted the mRNA 3′UTR of MTA1. Target regions of miR-559 or miR-661 in the mRNA 3′UTR were cloned downstream of a pGL3-luciferase reporter. Reporter constructs were transfected along with miR-559 or miR-661 or negative control mimics. After 48 h, cells were harvested and luciferase activity was measured. Luciferase reporter assay was performed in triplicate.

MTA1 expression, we transfected human cancer cell lines with miR-559 or miR-661 or a negative control (designated con-miR) and MTA1 protein levels were determined by Western blot analysis. Both miRs inhibited the expression level of MTA1 but not MTA2 or vinculin. (Fig. 1*B*). To investigate whether the 3′UTR region of MTA1 was directly targeted by the miRs, we cloned the 3′UTR region of MTA1 that is complementary to miR-559 or miR-661 into the pGL3-luciferase reporter (MTA1 3′UTR-luc). Transfection of the miRs and MTA1 3′UTR-luc into cells inhibited luciferase reporter activity, whereas no such inhibitory effect was observed in the control, con-miR (Fig. 1*C*). These findings suggested that miR-559 or miR-661 targeted the 3′UTR region of MTA1 and inhibited MTA1 expression. Because the expression of only miR-661 was observed in human cells, we focused on the regulation of MTA1 expression and functions by miR-661 in subsequent experiments.

We analyzed the 2-kb region directly upstream of the miR-661 to identify the mechanisms by which miR-661 might be regulated in physiologic settings. Using transcriptional factor prediction program Alibaba2 (17), we determined whether binding motifs for various transcription factors were present. This analysis revealed the presence of consensus motifs for c/EBPα within the −1426 to −1417, −1322 to −1311, −885 to −875, −740 to −730, and −331 to −320 in the upstream of the miR-661 (Fig. 2*A*). Because

expression of c/EBPα has been shown to reduce as cancer progresses (18, 19) and MTA1 expression is up-regulated during the progression of cancer cells to more invasive phenotypes (12), we reasoned that c/EBPα has a role in miR-661 expression and in turn MTA1 regulation. To evaluate this hypothesis, we used chromatin immunoprecipitation analysis to determine that c/EBPα is recruited at its binding sites, −331 to −320 (R4), on the *miR-661* promoter region but not to the other three regions of the *miR-661* chromatin (Fig. 2*B*). To establish a direct binding of c/EBPα to the *miR-661* promoter DNA, we performed an electrophoretic mobility shift assay analysis using a *miR-661* DNA fragment containing the consensus site for c/EBPα. We found that incubating the labeled DNA fragment with nuclear extract from MDA-MB-231 cells transiently transfected with c/EBPα resulted in the formation of discrete protein-DNA complex. These complexes could be effectively supershifted by inclusion of c/EBPα antibodies and their formation blocked by the unlabeled DNA fragment (Fig. 2*C*). We next cloned the putative *miR-661* promoter into a TATA-less basic pGL3-luc reporter (pGLmiR-661) to study the functionality of the interaction of c/EBPα with *miR-661* DNA. We found that transient expression of c/EBPα efficiently stimulated the transcription of miR-661 from the pGLmiR-661 reporter in HeLa and MDA-231 cells (Fig. 2*D*). Similarly, transient expression of c/EBPα led to the

Figure 2. Regulation of miR-661. *A,* the 2,000 bp upstream region of miR-661 was analyzed for putative transcriptional factors using Alibaba2. Recruitment of c/EBPα to region 4 (*R4*) of the miR-661 putative promoter region. *B,* chromatin immunoprecipitation analysis was performed with transfected c/EBPα cDNA, and immunoprecipitation was performed using a c/EBPα antibody. *C,* c/EBPα binding to the consensus site in the putative promoter of miR-661. *D,* c/EBPα positively regulates miR-661. The 2,000 bp upstream region of mir-661 was cloned in to the pGL3 basic vector, with luciferase as a reporter. Luciferase reporter assay was performed in triplicate. *E,* ectopic expression of c/EBPα led to the up-regulation of miR-661 and down-regulation of MTA1. Real-time PCR was performed to measure miR-661 levels using miR-specific probes (*top*). U6 RNA was used as an internal control. Western blot analysis was performed to determine the levels of MTA1 using a polyclonal MTA1 antibody (*bottom*).
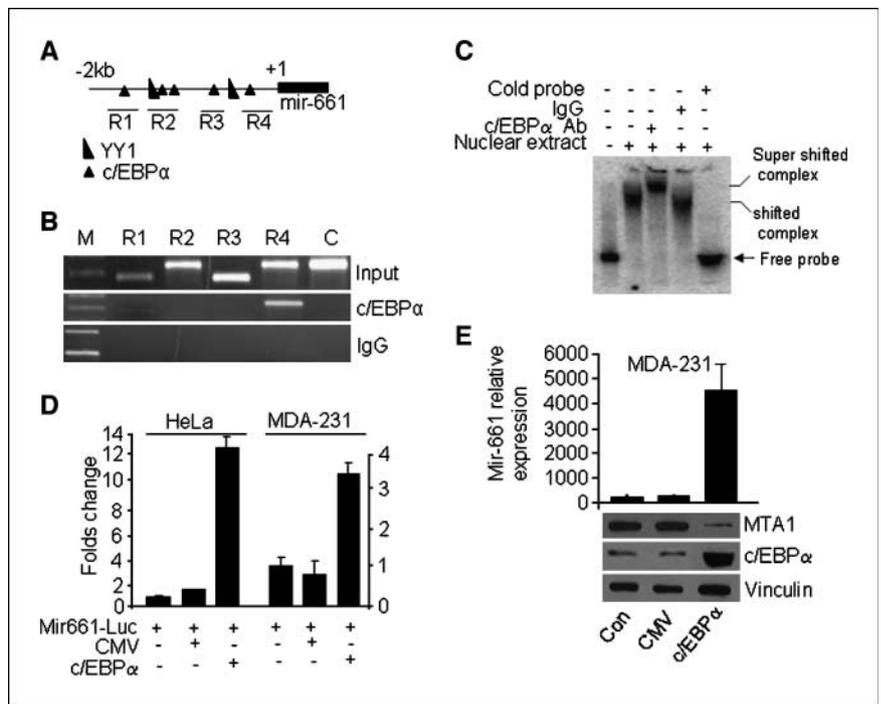
decreased expression of MTA1 and elevated expression of miR-661 in MDA-231 cells (Fig. 2*E*). However, the same was not observed for the control vector. Together, these findings suggest that c/EBPα directly interacted with the putative *miR-661* promoter and positively regulated miR-661 expression.

Because miR-661 targeted MTA1, because c/EBPα positively regulated the expression of miR-661 (this study), and because loss of c/EBPα is commonly observed during cancer progression, we hypothesized that a dynamic relationship exists between MTA1, miR-661, and c/EBPα and specifically that c/EBPα is involved in the action of miR-661 in cancer cells. We tested these hypotheses by analyzing the levels of MTA1, miR-661, and c/EBPα in exponentially breast cancer progressive isogenic model MCF-10A cells (nonmalignant), MCF-10AT cells (weakly tumorigenic cells), MCF-10CA1D cells (undifferentiated metastatic cells), and MCF-10DCIS cells (highly proliferative, aggressive, and invasive cells; ref. 20). The levels of MTA1 protein were progressively up-regulated, whereas those of miR-661 and c/EBPα were down-regulated

from non invasive MCF-10A to highly invasive MCF-10DCIS cells (Fig. 3*A*). To confirm these findings, we showed that c/EBPα expression led to up-regulation of miR-661 expression and down-regulation of MTA1 (Fig. 3*A*). To independently validate these findings, we showed that c/EBPα down-regulated the activity of MTA1 3′UTR-luc in MDA-231, ZR-75 breast cancer cells, and PC-3 prostate cancer cells (Fig. 3*B*). These results suggested that c/EBPα up-regulates the level of miR-661 and consequently affects the functions of the miR-661-MTA1 pathway in human cancer cells.

To evaluate the effect of miR-661mediated MTA1 down-regulation on the biology of breast cancer cells, we next examined the effects of miR-661 on of MDA-231 cells. We found that miR-661 expression in MDA-231 cells was accompanied by a substantial inhibition of cell motility, reduced cell invasiveness and suppression of anchorage-independent growth in soft agar, and a reduced ability of the cells to form tumors in a xenograft model (Fig. 4*A–D*). As expected from the data presented here,

Figure 3. miR-661 and c/EBPα are less expressed in MCF-10A tumor progression model cells. *A,* RNA and cell lysates were prepared from the indicated cells by Trizol method. MiR-661 and c/EBPα levels were determined by real-time PCR, and MTA1 protein levels were determined by Western blot analysis. *B,* c/EBPα down-regulated the MTA1 3′UTR reporter. A c/EBPα expression plasmid and MTA1 3′UTR reporter construct were transfected to the indicated cells, and a luciferase reporter assay was performed after 48 h of transfection. All experiments were repeated thrice; *columns,* mean; *bars,* SD.
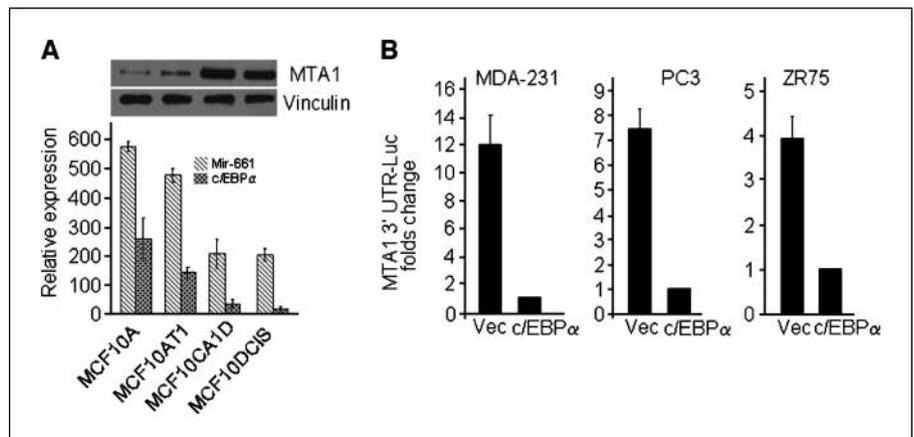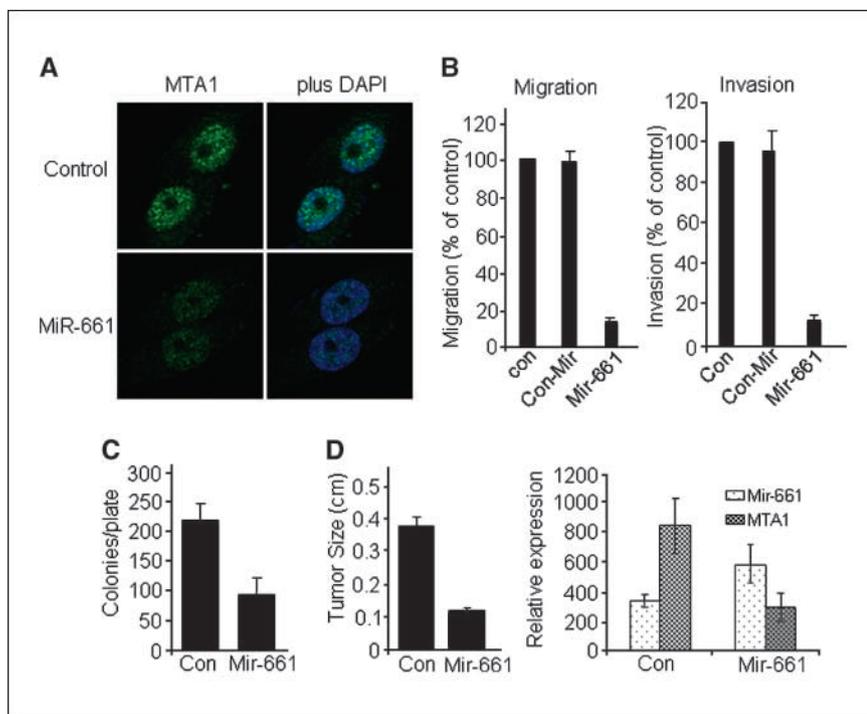
Figure 4. miR-661 compromises the MTA1 functions. *A,* confocal analysis of MTA1 expression in miR-661 transfected MDA-231 cells. *B* and *C,* miR-661 compromised the motility, invasiveness, and anchorage-independence of MDA-231 cells. All experiments were repeated thrice; *columns,* mean; *bars,* SD. *D,* miR-661 reduced the ability of MDA-231 cells to form tumors in nude mice. miR-661 and MTA1 levels were determined in the tumor samples isolated from control and mir-661 treated mice by real-time PCR.

introduction of miR-661 in tumors was accompanied by a reduced MTA1 expression. Collectively, these findings allow us to propose a model wherein the expression of miR-661 and its activator, c/EBPα, are progressively reduced during cancer progression and the loss of miR-661, in turn allows MTA1 levels to be sustained. These results raise the possibility that miR-661 may be developed as a therapeutic agent for down-regulating the expression of MTA1, which is widely up-regulated in human cancers (12).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

References

1. Jackson RJ, Standart N. How do microRNAs regulate gene expression? SciSTKE 2007;2007:re1.
2. Kim YK, Kim VN. Processing of intronic microRNAs. EMBO J 2007;26:775–83.
3. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature 2003;425:415–9.
4. Aboobaker AA, Tomancak P, Patel N, Rubin GM, Lai EC. Drosophila microRNAs exhibit diverse spatial expression patterns during embryonic development. Proc Natl Acad Sci U S A 2005;102:18017–22.
5. Iorio MV, Ferracin M, Liu CG, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res 2005;65:7065–70.
6. Makeyev EV, Maniatis T. Multilevel regulation of gene expression by microRNAs. Science 2008;319:1789–90.
7. Behm-Ansmant I, Rehwinkel J, Izaurralde E. Micro-RNAs silence gene expression by repressing protein expression and/or by promoting mRNA decay. Cold Spring HarbSympQuantBiol 2006;71:523–30.

8. Ma L, Teruya-Feldstein J, Weinberg RA. Tumour invasion and metastasis initiated by microRNA-10b in breast cancer. Nature 2007;449:682–8.
9. Reddy SD, Ohshiro K, Rayala SK, Kumar R. MicroRNA-7, a homeobox D10 target, inhibits p21-activated kinase 1 and regulates its functions. Cancer Res 2008;68:8195–200.
10. Kefas B, Godlewski J, Comeau L, et al. microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma. Cancer Res 2008;68:3566–72.
11. Kumar R, Wang RA, Bagheri-Yarmand R. Emerging roles of MTA family members in human cancers. Semin Oncol 2003;30:30–7.
12. Manavathi B, Kumar R. Metastasis tumor antigens, an emerging family of multifaceted master coregulators. J Biol Chem 2007;282:1529–33.
13. Manavathi B, Singh K, Kumar R. MTA family of coregulators in nuclear receptor biology and pathology. Nucl Recept Signal 2007;5:e010.
14. Toh Y, Pencil SD, Nicolson GL. A novel candidate metastasis-associated gene, mta1, differentially expressed in highly metastatic mammary adenocarci-

noma cell lines. cDNA cloning, expression, and protein analyses. J Biol Chem 1994;269:22958–63.
15. Bagheri-Yarmand R, Talukder AH, Wang RA, Vadlamudi RK, Kumar R. Metastasis-associated protein 1 deregulation causes inappropriate mammary gland development and tumorigenesis. Development 2004;131:3469–79.
16. Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. miRBase:microRNA sequences, targets and gene nomenclature. Nucleic Acids Res 2006;34:D140–4.
17. Grabe N. AliBaba2: context specific identification of transcription factor binding sites. In Silico Biol 2002;2:S1–15.
18. Gery S, Tanosaki S, Bose S, Bose N, Vadgama J, Koeffler HP. Down-regulation and growth inhibitory role of C/EBPα in breast cancer. Clin Cancer Res 2005;11:3184–90.
19. Halmos B, Huettner CS, Kocher O, Ferenczi K, Karp DD, Tenen DG. Down-regulation and antiproliferative role of C/EBPα in lung cancer. Cancer Res 2002;62:528–34.
20. Heppner GH, Miller FR, Shekhar PM. Nontransgenic models of breast cancer. Breast Cancer Res 2000;2:331–4.

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