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Mir-17-5p Regulates Breast Cancer Cell Proliferation by Inhibiting Translation of *AIB1* mRNA▽

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MicroRNAs are an extensive family of ~22-nucleotide-long noncoding RNAs expressed in a wide range of eukaryotes, including humans, and they are important in development and disease. We found that microRNA *Mir-17-5p* has extensive complementarity to the mRNA of *AIB1* (named for "amplified in breast cancer 1"). Cell culture experiments showed that *AIB1* expression was downregulated by *Mir-17-5p*, primarily through translational inhibition. Expression of *Mir-17-5p* was low in breast cancer cell lines. We also found that downregulation of *AIB1* by *Mir-17-5p* resulted in decreased estrogen receptor-mediated, as well as estrogen receptor-independent, gene expression and decreased proliferation of breast cancer cells. *Mir-17-5p* also completely abrogated the insulin-like growth factor 1-mediated, anchorage-independent growth of breast cancer cells. Our results reveal that *Mir-17-5p* has a role as a tumor suppressor in breast cancer cells.

MicroRNAs (miRNAs) are genomically encoded, ~22-nucleotide-long noncoding RNAs found in many organisms. miRNAs are produced from primary RNA polymerase II transcripts by sequential processing in the nucleus and cytoplasm (26, 27). Nuclear precursor RNAs are cleaved by the endonuclease Drosha in a "microprocessor complex" to release pre-miRNAs, which are 60- to 70-nucleotide-long imperfect hairpin structures (10, 20, 25). After being transported to the cytoplasm by exportin-5, pre-miRNAs are processed by the endonuclease DICER, generating ~22-nucleotide duplexes, one strand of which is the mature miRNA (34, 55, 56). miRNAs inhibit the translation of their respective RNA targets through imperfect base-pairing interactions, often with the 3′-untranslated regions (UTRs) of target mRNAs, or degrade their targets through perfect or near-perfect base pairing (1, 9). A single miRNA can regulate a number of genes, as shown by Lim et al. in an experimental model (29), and genetic studies in various organisms suggest that miRNAs have pivotal roles in development, cell death, proliferation, and disease (3, 8, 19, 45).

There is increasing evidence that miRNAs are mutated or differentially expressed in many types of cancer. The miRNAs *Mir-15* and *Mir-16* were found to be deleted in 68% of patients with chronic lymphocytic leukemia (5). Downregulation of *Mir-143* and *Mir-145* has been observed in colorectal cancer (38), and *let-7* expression is often reduced in lung cancers with a poor prognosis (23, 49). In addition, increased expression of the precursor of *Mir-155* has been detected in pediatric Burkitt lymphoma (13). Based on cancer-associated alterations in miRNA expression and the location of miRNAs at genomic regions often involved in cancers, it has been suggested that miRNAs act as tumor suppressors or oncogenes (6, 33). For example, *Mir-17-5p*, also known as *Mir-91*, is located on chromosome 13q31; this gene is amplified in childhood lymphoma (42, 48). The genomic location of *Mir-17-5p* also undergoes loss of heterozygosity in different types of cancer, including breast cancer (12, 30, 47, 51).

The clinical and epidemiological evidence for the necessary role of estrogen in breast cancer is substantial (35). There are two receptors for estrogen: ERα and ERβ (24, 37, 53). Estrogen receptors (ERs) are members of the steroid/thyroid hormone nuclear receptor superfamily, and both of these receptors act as ligand-dependent nuclear transcription factors (36). Transcriptional activation of genes by nuclear receptors is accomplished through the recruitment of coactivators (36). The nuclear receptor coactivator amplified in breast cancer 1 (AIB1) is a member of the p160/SRC family of coactivators (also named ACTR, RAC3, TRAM1, SRC-3, and NCOA3). The *AIB1* gene is amplified in several cancers, including breast, ovarian, pancreatic, and gastric cancers (2, 18, 46), and acts as an oncogene (50). *AIB1* enhances the transcriptional activity of the ER, *E2F1*, and other transcription factors (2, 32). *AIB1* is a rate-limiting factor for the estrogen- and *E2F1*-mediated growth of human breast cancer cells and is involved in growth hormone-signaling pathways (32, 41, 54, 58). We report here on the role of *Mir-17-5p*, a potential translational repressor of the *AIB1* oncogene, in the control of breast cancer cell proliferation.

MATERIALS AND METHODS

Cell cultures, plasmids, stable transfectants, and reporter gene assays. All cell lines were obtained from the American Type Culture Collection and maintained in the recommended media. For the generation of stable MCF-7 cells overexpressing *Mir-17-5p* or *Mir-95*, pSilencer-neo vectors (Ambion) expressing *Mir-17-5p* or *Mir-95* were transfected into MCF-7 cells, and stable cells were selected in medium containing G418.

Reporter genes were constructed by PCR amplification of the *AIB1* 3′-UTR, gel purification, and restriction digestion. The products were inserted into the

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XbaI site immediately downstream of the stop codon in the pGL3 promoter vector (Promega). Site-directed mutagenesis was done by using a site-directed mutagenesis kit (Stratagene).

The AIB1 expression construct pcDNA3AIB1 was generated by PCR amplification of entire AIB1 coding sequences from normal breast tissue and cloned into pcDNA3 vector (Invitrogen). The expression constructs pcAIB3′-UTR and pcAIBm3′-UTR were generated by replacing the original 3′-UTR from pcDNA3AIB1 with wild-type or mutated 3′-UTR of AIB1 cDNA.

Reporter assays were performed by cotransfection of various cell lines with vectors or synthetic 2′-*O*-methyl oligoribonucleotides by using Lipofectamine 2000 (Invitrogen). At 48 h after transfection, cells were collected for use in luciferase assay kits (Tropix). A β-galactosidase expression vector driven by the simian virus 40 promoter was used for transfection control. All reporter gene assays were performed in triplicate, with the entire experiment repeated several times.

Northern blotting and solution hybridization. Northern blots were performed according to standard procedures. Briefly, for conventional Northern blotting, 20 μg of total RNA was separated on formaldehyde-agarose gels, transferred to a nylon membrane, and hybridized with randomly primed, labeled *AIB1*-complementary probes using NorthernMax hybridization buffer (Ambion). The blots were stripped and reanalyzed with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

For miRNA analysis, miRNA was purified from cells by using a mirVANA miRNA isolation kit (Ambion). Total RNA (20 μg) was separated on 15% denaturing polyacrylamide gels, transferred to a nylon membrane, and hybridized with UltraHyb-Oligo buffer (Ambion). An oligonucleotide complementary to *Mir-17-5p* (5′-ACTACCTGCACTGTAAGCACTTTG-3′) was end labeled with T4 polynucleotide kinase (New England Biolabs) and used as a probe. A U6 snRNA probe was used as an internal control. The blots were analyzed with a PhosphorImager.

Breast tumor samples were obtained from the M. D. Anderson Cancer Center tissue repository, and miRNA was isolated as described above. Total RNA (100 ng) was used for the detection of Mir-17-5p and Mir-15a using an mirVANA miRNA detection kit (Ambion, TX) according to the manufacturer's instruction.

Western blotting. Whole-cell extracts were prepared in modified radioimmunoprecipitation assay buffer. Western blots were developed by using SuperSignal West Femto chemiluminescent substrate (Pierce). We used primary antibodies against AIB1 (BD Transduction Laboratories) and β-actin (Santa Cruz Biotechnology) and a peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (Amersham).

Cell proliferation and soft agar colony formation assays. For the estrogen-dependent cell proliferation assay, equal numbers of stable MCF-7 cells expressing *Mir-17-5p*, *Mir-95*, or control vector were plated in a 96-well plate and treated with improved minimum essential medium (Invitrogen) and 1% charcoal-stripped calf serum containing 10 nM E2 or vehicle only. Cell growth was determined by using a standard tetrazolium bromide (MTT) assay and verified by counting with trypan blue and a hemacytometer.

For the estrogen-independent cell proliferation assay, ER-negative BT-20 cells were plated in a six-well plate at 50% confluence 24 h before transfection. The next day, cells were transfected with 2′-*O*-methyl *Mir-17-5p* or 2′-*O*-methyl *Mir-95* by using Oligofectamine (Invitrogen) according to the manufacturer's instructions. After 24 h, transfected cells were treated with trypsin, and equal numbers of viable cells were plated into 96-well plates. The cells were then incubated for another 96 h, with one change of medium after 48 h. Cell growth was determined by using a standard MTT assay and verified by counting and trypan blue exclusion.

For the soft agar assay, stably transformed MCF-7 cells were resuspended in 0.35% soft agar and layered onto 0.6% solidified agar in a 5-cm dish in 10% charcoal-stripped calf serum with or without 100 ng of insulin-like growth factor 1 (IGF-1)/ml. The soft agar colonies were allowed to grow for 12 days at 37°C. Colonies with a diameter of >80 μm were scored as positive. Experiments were carried out in triplicate.

RESULTS

***AIB1* is a target of *Mir-17-5p*.** To identify the mRNA targets of *Mir-17-5p*, we performed a computational screen for genes with *Mir-17-5p* complementary sites in their sequences. One of the high-scoring candidates was the human oncogene *AIB1*. We identified two *Mir-17-5p* complementary sites within the 3′-UTR and coding region of *AIB1* (Fig. 1A). The most important criteria for target recognition is the 5′-eight-nucleotide core sequences of miRNA. However, some mismatches and gaps are allowed between the 3′ end of miRNA and its target mRNA, as this is the case for many proven miRNA targets (14, 15, 23). The degree of similarity at the 3′ end increases the specificity of target recognition by miRNAs (4, 11, 28). The first ten nucleotides of *Mir-17-5p* were perfectly matched with their *AIB1* mRNA targets, and the two-nucleotide gap at positions 11 and 12 decreased the free energy of the duplex and is predicted to favor the formation of a *Mir-17-5p–AIB1* duplex. mFold analysis of the local mRNA secondary structure also revealed no noticeable complex secondary structure surrounding the *Mir-17-5p* binding site in the 3′-UTR of *AIB1*. A duplex between *AIB1* and *Mir-17-5p* was also observed upon using mFold analysis, with a ΔG value of -13.0 kcal mol^{-1}, which is well within the range of a true miRNA target site (11). By fulfilling the above-mentioned criteria *AIB1* became a good candidate target of *Mir-17-5p* and merited further experimental analysis.

To examine whether *AIB1* is repressed by *Mir-17-5p*, segments of the *AIB1* 3′UTR containing wild-type or mutated *Mir-17-5p* complementary sites were inserted into the 3′-UTR of a luciferase reporter gene. The resulting reporter plasmids were transfected into HeLa cells together with a transfection control and 2′-*O*-methyloligoribonucleotides of *Mir-17-5p* (2′-*O*-methyl *Mir-17-5p*) or a noncognate miRNA (2′-*O*-methyl *Mir-95*). As expected, *Mir-17-5p* inhibited luciferase activity from the construct with the wild-type complementary site but not the construct with the mutated complementary site (Fig. 1B). There was no change in luciferase reporter activity when *Mir-95* was cotransfected with either reporter construct, indicating that the observed inhibition is specific to *Mir-17-5p*. The inhibition was essentially the same as that of a reporter with perfect complementarity to *Mir-17-5p* (Fig. 1B). These results suggest that the complementary site in *AIB1* mRNA is a target of *Mir-17-5p*-mediated posttranscriptional silencing of gene expression. To eliminate the possibility that the *Mir-95* used here was nonfunctional, we fused *Mir-95* antisense sequences downstream of the luciferase gene and used these sequences for a *Mir-95* functional assay. *Mir-95* significantly reduced the luciferase activity, but *Mir-17-5p* could not do so (Fig. 1C), indicating that the *Mir-95* used here was indeed functional.

Translation of endogenous *AIB1* is suppressed by *Mir-17-5p*. To gain insight into the mechanism by which *Mir-17-5p* inhibits luciferase expression (Fig. 1B), we transfected 2′-*O*-methyl *Mir-17-5p* or 2′-*O*-methyl *Mir-95* into MCF-7 cells. We chose MCF-7 cells because *AIB1* is highly expressed in these cells, and *AIB1* activity is essential for their growth in vitro and in vivo (31, 41). As expected, *AIB1* mRNA levels were unaffected by *Mir-17-5p* (Fig. 2A), although Northern blotting (Fig. 2A) and real-time PCR (data not shown) revealed that *AIB1* mRNA was sharply reduced by cotransfection of an *AIB1*-specific short interfering RNA (siRNA) molecule. In contrast, AIB1 protein levels decreased substantially after treatment with *Mir-17-5p*, as shown by Western blotting (Fig. 2B) and by pulse-labeling (Fig. 2C); the reduction level was comparable to that using *AIB1*-specific siRNA, which occasionally decreased AIB1 protein to almost undetectable levels (data not shown). In contrast, in the presence of *Mir-95*, the *AIB1* mRNA and protein levels remained unchanged and were similar to those

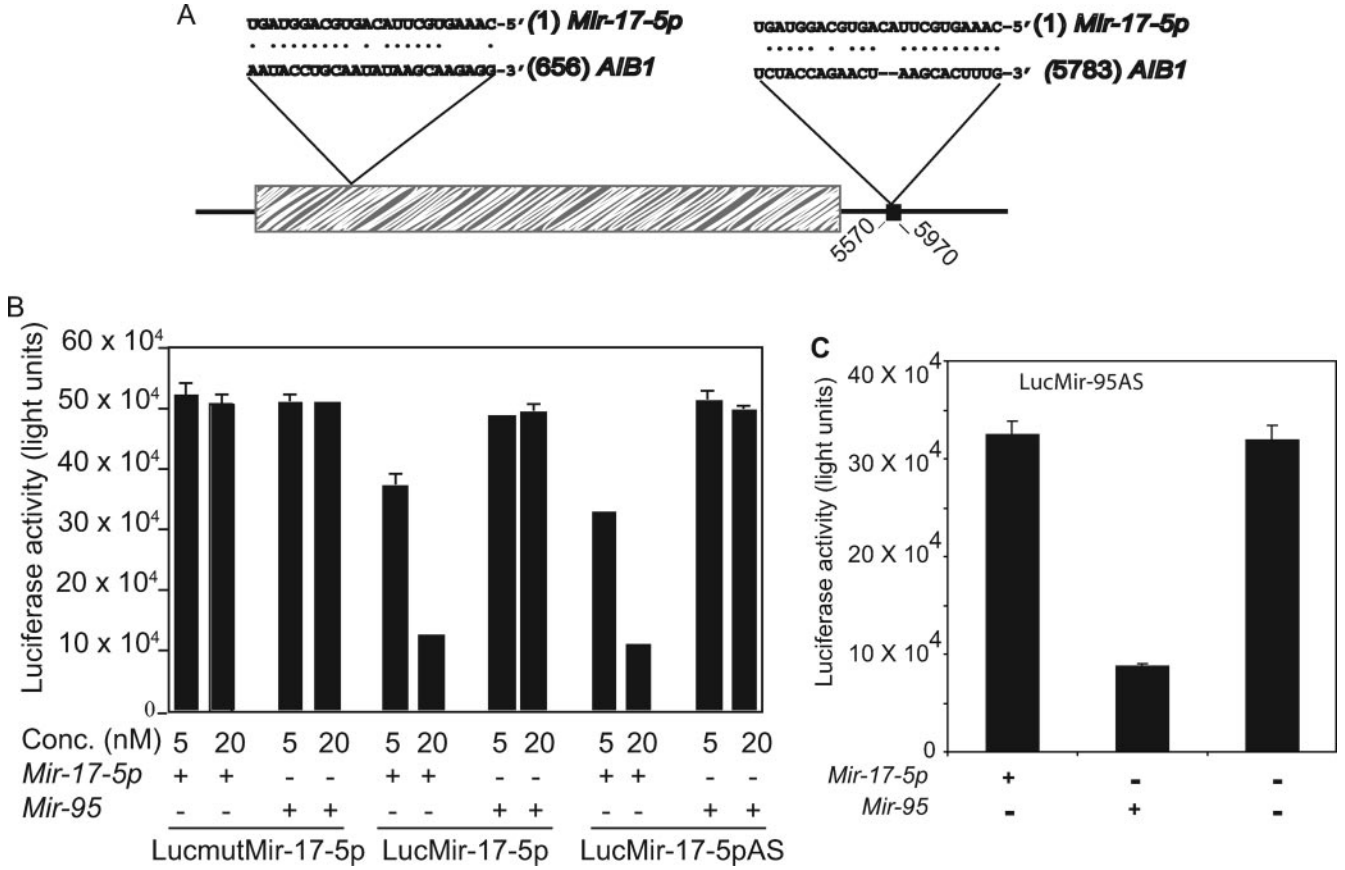

FIG. 1. AIB1 is a target of *Mir-17-5p*. (A) Sequence alignment and diagram of *Mir-17-5p* and its complementary sites in the *AIB1* cDNA. The hatched area indicates the coding region; the black square indicates the region cloned at the end of the luciferase reporter gene. (B) Activity of luciferase reporters containing the *Mir-17-5p* complementary site from human *AIB1* (LucMir-17-5p), a mutant *Mir-17-5p* complementary site from human *AIB1* (mutLucMir-17-5p), or the perfect antisense sequence of *Mir-17-5p* (LucMir-17-5pAS). The reporters were cotransfected with the indicated amounts of 2′-*O*-methyl *Mir-17-5p* or *Mir-95*. Firefly luciferase activity was normalized to that of β-galactosidase. The data shown are the mean of three independent experiments, and error bars indicate the standard deviations. (C) Activity of luciferase reporters containing the perfect antisense sequence of *Mir-95* (LucMir-95AS). The reporters were cotransfected with the indicated amounts of 2′-*O*-methyl *Mir-17-5p* or *Mir-95*. Firefly luciferase activity was normalized to that of β-galactosidase. The data shown are means of three independent experiments, and error bars indicate standard deviations.

in untreated MCF-7 cells, indicating that *Mir-17-5p* regulation is specific to *AIB1* (Fig. 2A and B).

To further examine the function of *Mir-17-5p*, we tested whether the levels of endogenous *Mir-17-5p* in HeLa cells could be reduced by using synthetic siRNA targeted to the *Mir-17-5p* precursor. We selected HeLa cells for their high expression of endogenous mature *Mir-17-5p*. The intracellular levels of *Mir-17-5p* decreased significantly after treatment with *Mir-17-5p*-specific siRNA (Fig. 2D). As expected, the levels of endogenous AIB1 protein increased in the presence of *Mir-17-5p*-specific siRNA, whereas mutant siRNA did not affect the expression of *Mir-17-5p* or AIB1 protein (Fig. 2E). These results suggest that synthetic *Mir-17-5p* suppresses the expression of *AIB1*, primarily at the level of translation.

***Mir-17-5p* modulates ER-mediated signaling.** Because AIB1 enhances the transcriptional activity of ER (2), we tested the effects of *Mir-17-5p* by using ER-mediated transactivation or estrogen-responsive elements (EREs) containing a luciferase reporter gene (ERE-Luc). Transfection of ERE-Luc with *Mir-17-5p* in CHO-K1 cells reduced ER-dependent transcriptional activity by ca. 50% in the presence of estrogen (Fig. 3A). As expected, noncognate *Mir-95* had no effect on ER-dependent transcriptional activity (Fig. 3A).

To confirm the negative effect of *Mir-17-5p* on estrogen signaling, we fused wild-type (pcAIB3′-UTR) and mutated (pcAIBm3′-UTR) *AIB1* 3′-UTR to the *AIB1* expression vector, pcDNA3AIB1, by replacing the original 3′-UTR sequences present in the pcDNA3 vector. These constructs were used for transcription assays by transient transfection into CHO-K1 cells, along with reporter plasmids containing the ERE-Luc and ER expression vectors. Cotransfection of the pcAIB3′-UTR and ERE-Luc in the presence of 2′-*O*-methyl *Mir-17-5p* substantially reduced ER-mediated transactivation (Fig. 3B). In contrast, cotransfection of pcAIBm3′-UTR and ERE-Luc resulted in potent transcriptional activity in the presence of *Mir-17-5p* (Fig. 3B). No significant differences were observed upon treatment with 2′-*O*-methyl *Mir-95*. These results clearly demonstrate that *Mir-17-5p*-mediated reduction of AIB1 protein requires the presence of *AIB1* 3′-UTR target sites and can effectively block ER-mediated signaling.

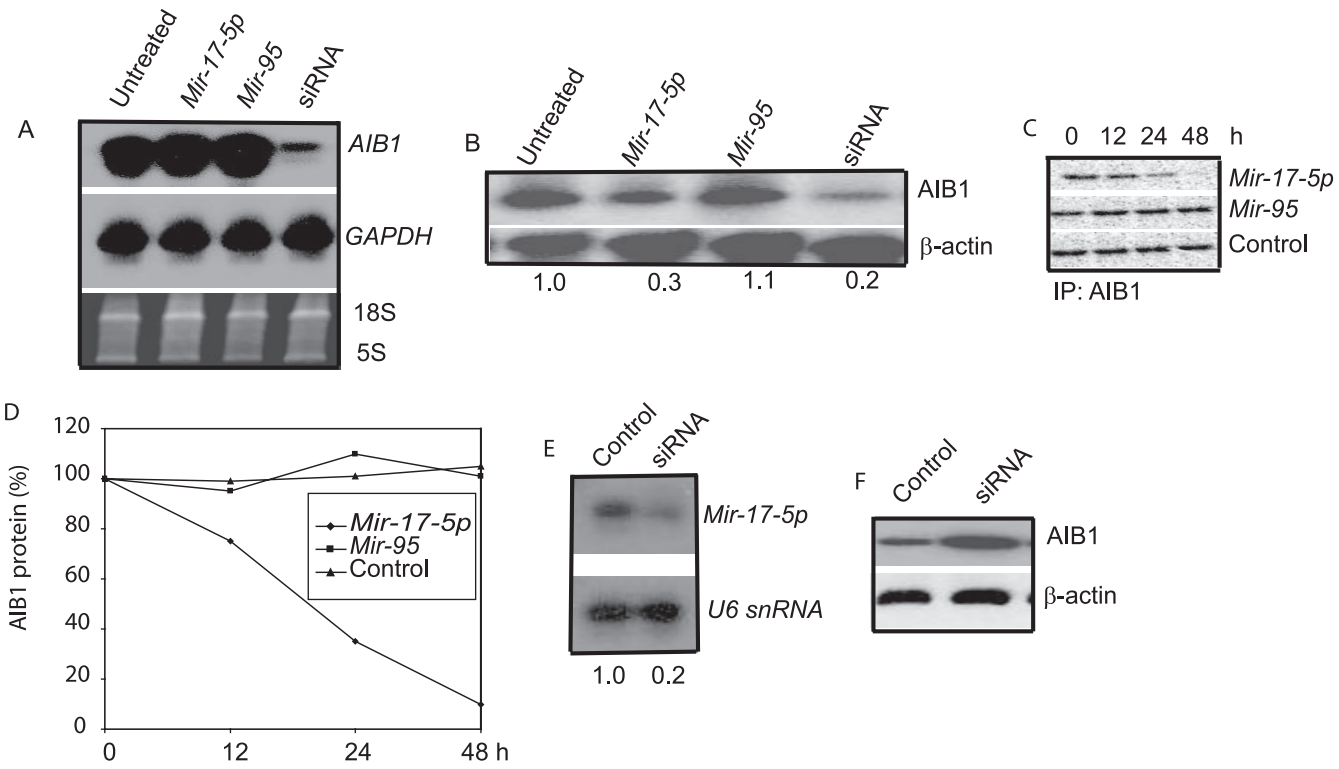

FIG. 2. *Mir-17-5p* regulates translation of endogenous *AIB1*. (A) Northern blot of RNA from untreated MCF-7 cells and cells treated with *Mir-17-5p*, *Mir-95*, or anti-*AIB1* siRNA. The RNA transcript levels were equal in all experimental samples, as shown by GAPDH and ethidium bromide staining (bottom two panels). (B) Western blot of AIB1 protein in untreated MCF-7 cells and cells treated with *Mir-17-5p*, *Mir-95*, anti-*AIB1* siRNA, or scrambled siRNA. Anti-β-actin antibody was used as a loading control (bottom panel). (C and D) MCF-7 cells were transfected with *Mir-17-5p*, *Mir-95*, or vehicle only. Cells were starved in methionine-free medium and then pulse-labeled with [^{35}S]methionine. The cells were then harvested and immunoprecipitated with anti-AIB1 antibody. Precipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by using a phosphorimager. (E and F) Level of *Mir-17-5p* mature transcript (E) and AIB1 protein (F) in HeLa cells treated with siRNA targeted to the loop region of pre-*Mir-17p* transcript or a scrambled siRNA (control). U6 snRNA (snU6) and β-actin were used as internal controls.

Expression of *Mir-17-5p* in cancer cell lines and breast tumors. Upregulation of *AIB1* by gene amplification or other means is a key event in breast cancer cell proliferation (32, 41, 54, 58). Because *Mir-17-5p* clearly affects estrogen signaling and because *AIB1* is a rate-limiting factor for estrogen-mediated growth of breast cancer cells (31), it is possible that loss of *Mir-17-5p* control over *AIB1* translational regulation can lead to unregulated breast cancer cell growth. To test this, we used Northern blotting to examine the expression of *Mir-17-5p* in several breast cancer cell lines: ZR-75-1, MCF-7, BT-20, MDA-MB-361, BT-474, T-47D, MDA-MB-468, MDA-MB-453, Hs 748.T, BT-549, SW527 and in non-breast cancer cell lines: BG-1, EB-3, HeLa, OV90, HEL60, and K562. *Mir-17-5p* expression was low to barely detectable in the breast cancer cell lines compared to the non-breast-tumor cell lines (Fig. 4A). To detect any existing correlation between *Mir-17-5p* and AIB1 protein expression, we used Western blotting with cell extracts from selected cell lines. We found that AIB1 was highly expressed when *Mir-17-5p* expression was low (Fig. 4B). These data indicate that the downregulation of *Mir-17-5p* may be important for breast cancer cell growth.

Translation of endogenous *E2F1* is regulated by *Mir-17-5p*. A recent report suggests that *E2F1* is a target of *Mir-17-5p* at the translational level (40). By careful examination of *E2F1* coding and noncoding 3′-UTR, we found only one *Mir-17-5p* recognition site. It is also known that *AIB1* is involved in *E2F1* transcriptional regulation (32). Therefore, there is a possibility that *Mir-17-5p* may not directly regulate the expression of *E2F1* but rather have an indirect effect on *E2F1* activity. To resolve at which level *Mir-17-5p* may affect *E2F1*, we treated MCF-7 cells with *Mir-17-5p* and *Mir-95*, and cell extracts were analyzed by Western blotting with E2F1 specific antibody. E2F1 protein level was significantly downregulated by the treatment of *Mir-17-5p* (Fig. 5A and B). However, this does not mean that *E2F1* is a direct target of *Mir-17-5p*, since AIB1 expression was also completely abrogated by *Mir-17-5p*. We therefore tested the effects of *Mir-17-5p* in ZR-75-1 cells, which express a very low level of AIB1 (Fig. 4B). Residual expression of AIB1 was completely abrogated by the treatment of *Mir-17-5p* (Fig. 5C). In contrast, we did not observe a large reduction of E2F1 levels by the treatment of *Mir-17-5p* compared to its effects on E2F1 protein levels in MCF-7 cells that express high levels of AIB1. However, we also observed a measurable reduction of E2F1 protein level by the treatment of *Mir-17-5p* in this cell line. This observation further established that *Mir-17-5p* could directly inhibit the translation of *E2F1*, albeit less effectively than the reduction of *AIB1*. We included *E2F1*-specific siRNA to avoid any doubt concerning

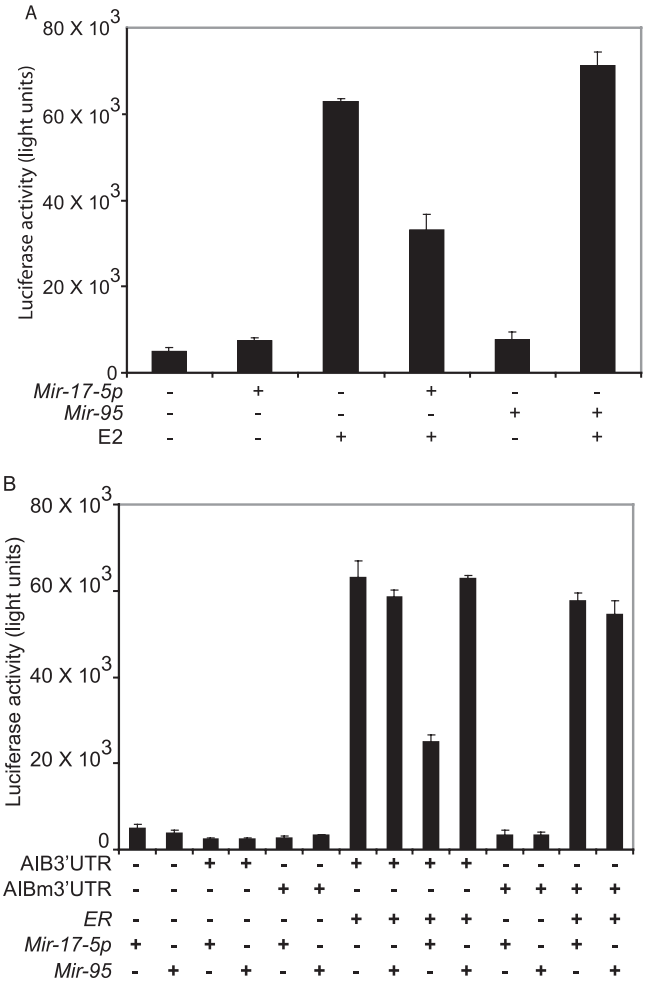

FIG. 3. Mir-17-5p suppresses ER-mediated signaling. (A) Luciferase activity in CHO-K1 cells transiently transfected with an ER expression vector, a luciferase reporter plasmid, and 2′-*O*-methyl *Mir17p* or *Mir-95*, in the absence or presence of 10 nM E2. The data shown are means of triplicate experiments, and error bars indicate the standard deviations. (B) Luciferase activity in cells cotransfected with *AIB1*-expressing reporter plasmids containing wild-type (AIB3′UTR) or mutant (AIBm3′UTR) *AIB1* 3′UTRs, ER, ERE-Luc, and 2′-*O*-methyl *Mir-17-5p* or 2′-*O*-methyl *Mir-95*. The data shown are means of triplicate experiments, and error bars indicate the standard deviations.

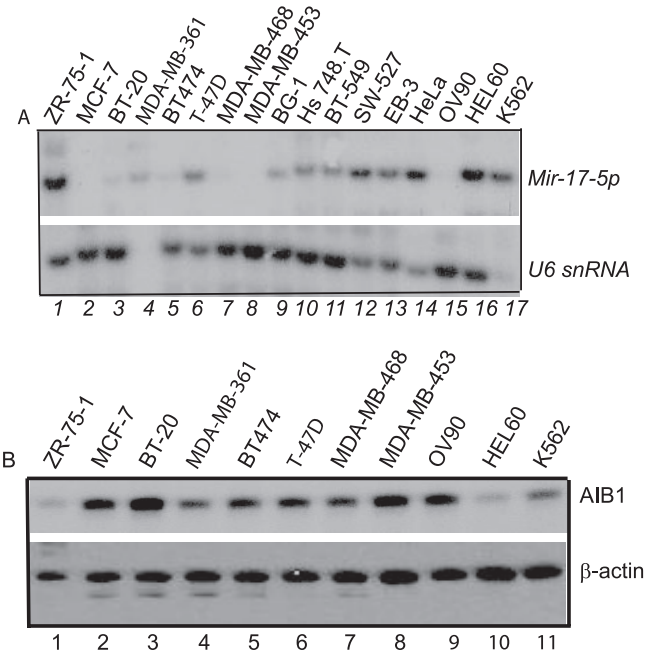

FIG. 4. Expression of *Mir-17-5p* is reduced or silent in some cancer cell lines. (A) Northern blot sequentially assayed with radiolabeled probes specific for *Mir-17-5p*, with U6 snRNA used as a control. Lanes 1 to 17 show RNA from the cell lines ZR-75-1, MCF-7, BT-20, MDA-MB-361, BT-474, T-47D, MDA-MB-468, MDA-MB-453, BG-1, Hs 748.T, BT-549, SW527, EB-3, HeLa, OV90, HEL60, and K562. Lanes 1 to 12 show breast cancer cell lines, and lanes 13 to 17 show other types of cancer cells. (B) Western blot of AIB1 protein in different cell lines. Cell lysates (50 μg) of the different cell lines were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-AIB1 antibody. The same blot was stripped and reprobed with anti-β-actin antibody and was used as a loading control (bottom panel).

the transfection efficiency in this cell line. These results suggest that the negative influence of *Mir-17-5p* on *E2F1* was both directly and indirectly due to AIB1 downregulation at least in breast cancer cells.

***Mir-17-5p* represses *ER* and *E2F1* target genes.** To understand better the role of *Mir-17-5p* on estrogen-mediated signal transduction, we tested the effects of *Mir-17-5p* on ER-regulated genes. The cyclin D1 gene is a known target of ER (43, 44). Transfection of *Mir-17-5p* in MCF-7 cells reduced the ER-dependent upregulation of cyclin D1 significantly in the presence of estrogen (Fig. 6). As expected, noncognate *Mir-95* had no effect on the ER-dependent upregulation of cyclin D1 (Fig. 6).

We also tested the regulation of known E2F1 target gene *CDC2* by the treatment of *Mir-17-5p* (17). The Cdc2 protein level was reduced significantly by the treatment of *Mir-17-5p* in MCF-7 cells (Fig. 6). These results suggest that *Mir-17-5p* has an important role on both ER- and E2F1-mediated signal transduction pathways.

***Mir-17-5p* suppresses estrogen-stimulated breast cancer cell proliferation.** Our findings that *Mir-17-5p* is involved in regulating *AIB1* translation and that *Mir-17-5p* expression is low in breast cancer cells prompted us to examine the role of *Mir-17-5p* in the proliferation of estrogen-responsive breast cancer cells. To this end, we established stable transformants containing vectors overexpressing *Mir-17-5p* or *Mir-95*. We used pooled transformants instead of selective clones to avoid clonal bias. AIB1 protein levels were markedly reduced in MCF7 cells expressing *Mir-17-5p*, whereas no significant effect was observed in cells expressing *Mir-95* (Fig. 7A).

To examine the effect of *Mir-17-5p*-mediated AIB1 depletion on estrogen-stimulated cell proliferation, we treated *AIB1*-depleted MCF-7 cells with the estrogen 17β-estradiol (E2). Depletion of *AIB1* by *Mir-17-5p* markedly inhibited E2-stimulated cell proliferation (Fig. 7B). Reintroduction of *AIB1*-containing mutated 3′-UTRs, which are capable of escaping *Mir-17-5p*-mediated translational control, rescued the cells from the proliferation defects caused by the overexpression of *Mir-17-5p* in MCF-7 cells (Fig. 5B). These results were confirmed in another ER-positive breast cancer cell line. Depletion of AIB1 by *Mir-17-5p* by transient transfection also reduced E2-stimulated growth of BT-474 cell line (Fig. 7C).

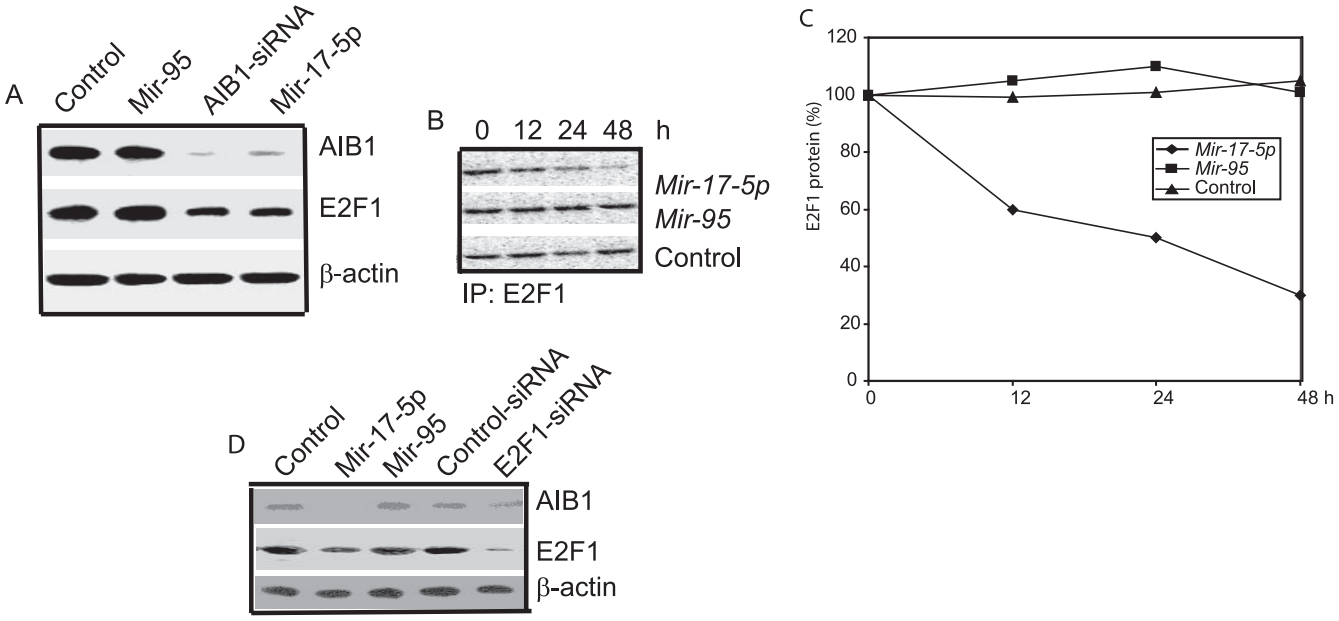

FIG. 5. *Mir-17-5p* regulates translation of endogenous *E2F1*. Cell lysates (50 μg) from MCF-7 (A) and ZR-75-1 (D) were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-AIB1 and anti-E2F1 antibody. The same blot was stripped and reprobed with anti-β-actin antibody and used as a loading control (bottom panel). (B and C) MCF-7 cells were transfected with *Mir-17-5p*, *Mir-95*, or vehicle only. Cells were starved in methionine-free medium and then pulse-labeled with [^{35}S]methionine. The cells were then harvested and immunoprecipitated with anti-E2F1 antibody. Precipitated proteins were separated by SDS-PAGE and visualized by using a phosphorimager.

To further address the role of *Mir-17-5p* in E2-stimulated growth, we introduced *Mir-17-5p*-specific siRNA into another ER-positive cancer cell line, ZR-75-1, that expresses high levels of endogenous *Mir-17-5p* (Fig. 5A). Depletion of endogenous *Mir-17-5p* by anti-*Mir-17-5p* siRNA increased E2-stimulated growth of ZR-75-1 cells (Fig. 7D). Taken together, these results suggested that *Mir-17-5p* plays an important role in estrogen-dependent breast cancer cell proliferation by regulating *AIB1* translation.

***Mir-17-5p* represses estrogen/ER-independent breast cancer cell proliferation by targeting *AIB1*.** *AIB1* is also involved in the estrogen-independent proliferation of breast cancer cells (32). To examine whether *Mir-17-5p* can inhibit estrogen-independent breast cancer cell proliferation, we transiently transfected ER-negative BT-20 cells with 2′-*O*-methyl *Mir17-5p* or *Mir-95*. *Mir-17-5p* repressed the hormone-independent proliferation of BT-20 cells (Fig. 8A and B), and the reintroduction of pcAIBm3′-UTR partially restored *Mir-17-5p*-mediated growth of the BT-20 cells (Fig. 8B). Thus, in addition to regulating breast cancer cell proliferation, *Mir-17-5p* has an important role in breast cancer cell proliferation that is independent of estrogen and ER.

***Mir-17-5p*-mediated *AIB1* depletion impairs the anchorage-independent growth of MCF-7 cells.** To further establish the role of *Mir-17-5p* in breast cancer cell proliferation, we investigated the anchorage-independent growth of breast cancer cells in the presence or absence of *Mir-17-5p*. *AIB1* has an important role in the anchorage-independent growth of MCF-7 cells caused by IGF-1 by regulating E2F1 function (41). Since E2F1 is a direct target of *Mir-17-5p*, we used a *Mir-17-5p*-insensitive *E2F1* expression construct, i.e., *E2F1* coding sequences without their own 3′-UTRs. First, we checked the expression of E2F1 and AIB1 after the transient transfection of *Mir-17-5p* and control *Mir-95*. As expected, the levels of both AIB1 and E2F1 expression were reduced by the treatment of *Mir-17-5p*. We were able to rescue the expression of AIB1 and E2F1 by overexpressing the *Mir-17-5p*-insensitive forms of *AIB1* and *E2F1* (Fig. 9A) in these cells. We then checked the effect of *Mir-17-5p* on the IGF-1-induced anchorage-independent growth of MCF-7 cells. IGF-1 induced severalfold increases in the numbers of colonies larger than 80 μm formed by control MCF-7 cells; this increase was completely abrogated in cells treated with *Mir-17-5p* (Fig. 9B and C). We attempted

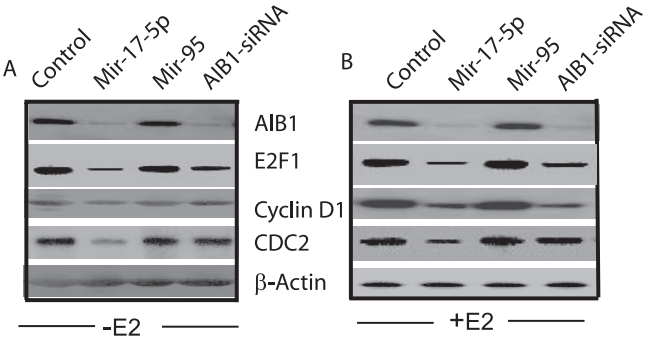

FIG. 6. *Mir-17-5p* represses the expression of some *ER* and *E2F1* target genes. Cells were treated with the indicated 2′-*O*-methyl RNA, and cell lysates (50 μg) were prepared and separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the indicated antibodies. Cells were treated with (B) or without (A) E2 to check target gene expression. The control reflects cells treated with phosphate-buffered saline only. The same blot was stripped and reprobed with anti-β-actin antibody and used as a loading control (bottom panel).

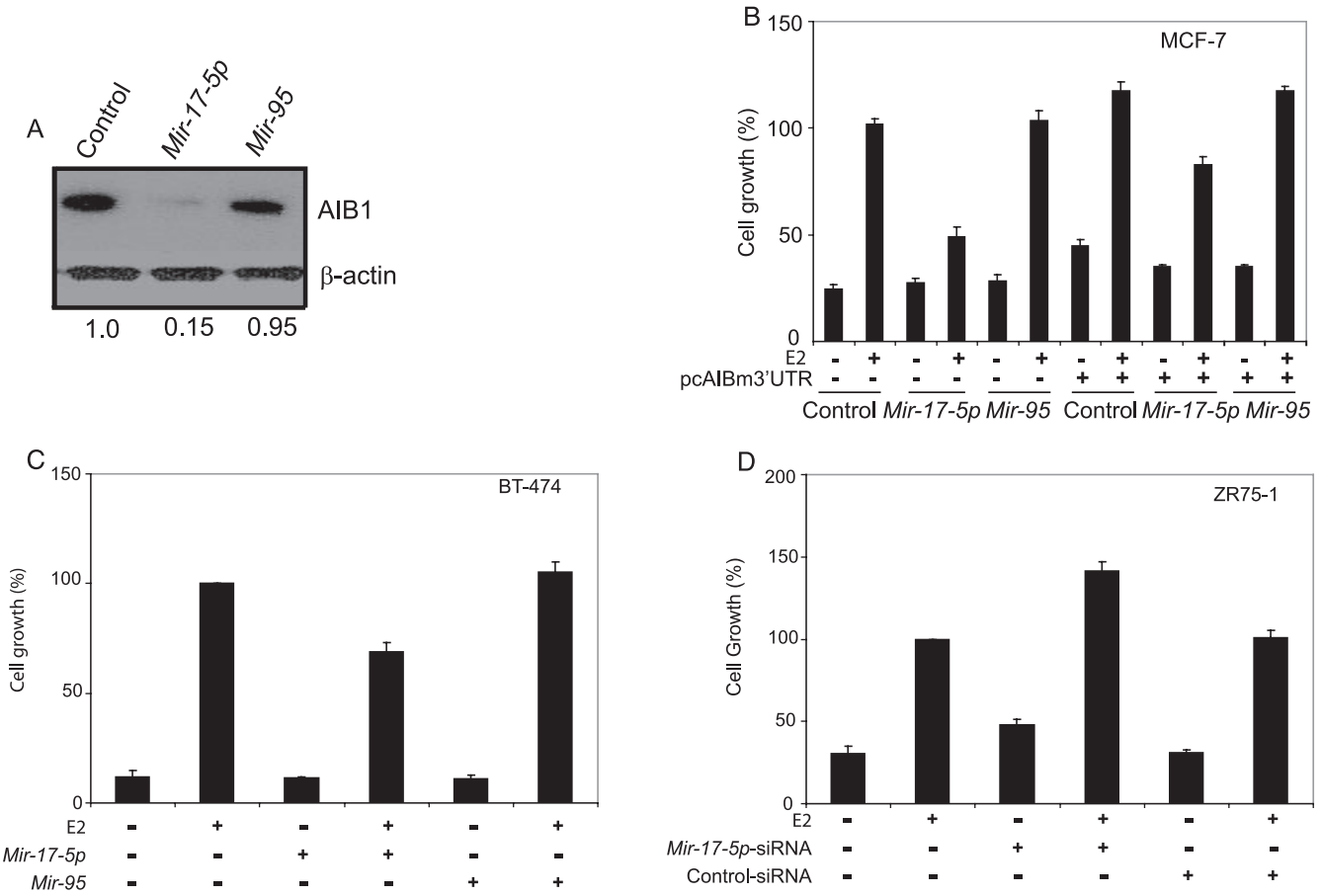

FIG. 7. *Mir-17-5p* suppresses both estrogen-stimulated and estrogen/ER-independent breast cancer cell proliferation. (A) Reduction of AIB1 protein in stably transfected MCF-7 cells expressing *Mir-17-5p* but not *Mir-95* compared to pSilencer vector only (control). Anti-β-actin antibody was used as a loading control. (B) Estrogen (E2)-stimulated cell proliferation assay. MCF-7 cells were stably transformed with pSilencer empty vector (control), pSilencer*Mir-17-5p* (Mir-17-5p), or pSilencer*Mir-95* (Mir-95) and treated with 10 nM E2 or vehicle only. pcAIBm3′UTR was reintroduced by transient transfection in the stably transformed MCF-7 cells described above, which were treated with 10 nM E2. Cell proliferation was measured by the MTT assay. The data shown are means of triplicate experiments, and error bars indicate the standard deviations. (C) 2′-*O*-methyl *Mir-17-5p* or 2′-*O*-methyl *Mir-95* was transiently transfected into BT-474 cells and treated with 10 nM E2 or vehicle only. Cell growth was measured by the MTT assay after 96 h. The data shown are means of triplicate experiments, and error bars indicate the standard deviations. (D) ZR75-1cells were treated with siRNA targeted to the loop region of pre-*Mir-17p* transcript or a scrambled siRNA (control) and treated with 10 nM E2 or vehicle only. Cell growth was measured by MTT assay after 96 h. The data shown are means of triplicate experiments, and error bars indicate the standard deviations.

to rescue the growth inhibition of MCF-7 cells by *Mir-17-5p* using previously characterized *Mir-17-5p* insensitive forms of *AIB1* and *E2F1*. Overexpression of either alone did not significantly rescue the anchorage-independent growth of these cells. However, the coexpression of both *AIB1* and *E2F1* significantly improved the anchorage-independent growth of these cells in the presence of IGF-1 and *Mir-17-5p* (Fig. 9B and C). Therefore, the inhibitory effects of *Mir-17-5p* on estrogen-independent breast cancer cell growth appears to be due to translational regulation of two important players in this pathway: *AIB1* and *E2F1*.

DISCUSSION

Increasing evidence has implicated several miRNAs in tumorigenesis (7). Many miRNA genes are located within regions associated with amplification, deletion, and translocation in cancer (6, 33). The lack of knowledge about genuine miRNA gene targets impedes a full understanding of the true role of miRNAs in tuomorigenesis.

In the present study, we identified *Mir-17-5p* as a genuine regulator of oncogene *AIB1* translation. *AIB1* is overexpressed in primary breast cancer and is involved in hormone-dependent and hormone-independent breast cancer cell proliferation. We also observed that Mir-17-5p represses E2F1-mediated pathways. We clearly demonstrated an important role of *Mir-17-5p* in breast cancer cell proliferation and anchorage-independent growth by both overexpression and knockdown experiments. We also showed that *Mir-17-5p* expression was reduced in breast cancer lines.

AIB1 is a coactivator for nuclear receptors, including ER, and AIB1 accelerates breast cancer cell proliferation by amplifying the mitogenic effect of estrogen through an increase in the transactivation functions of ER (2, 16, 58). The growth of MCF-7 cells in response to estrogen depends on ER and AIB1

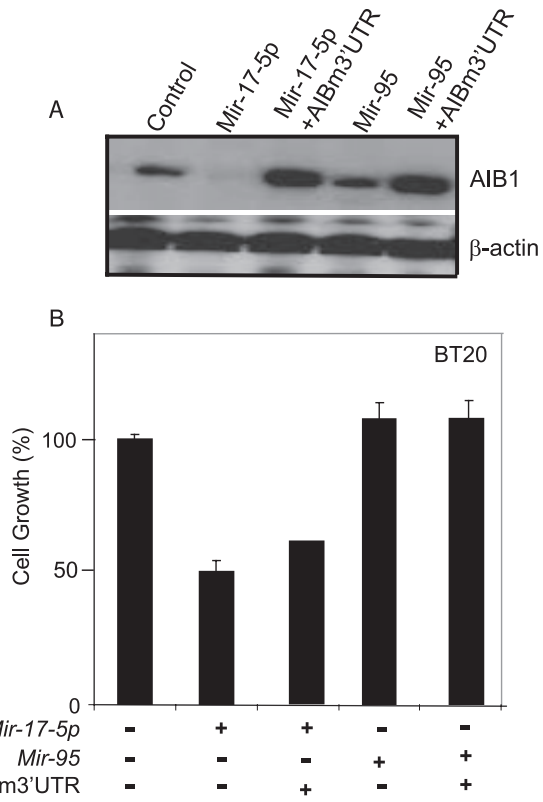

FIG. 8. *Mir-17-5p* suppresses estrogen/ER-independent breast cancer cell proliferation. 2′-*O*-Methyl *Mir-17-5p* or 2′-*O*-methyl *Mir-95* with or without pcAIBm3′UTR was transiently transfected into BT-20 cells, and cells were maintained in hormone-deprived medium. (A) Western blot of AIB1 protein. Cell lysates (50 μg) of transfected cells were separated by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with anti-AIB1 antibody. β-Actin was used as a loading control. (B) Cell proliferation assay. Cell growth was measured by the MTT assay after 96 h. The data shown are means of triplicate experiments, and error bars indicate the standard deviations.

(31). The treatment of MCF-7 cells with *Mir-17-5p* substantially reduced their ability to proliferate in response to estrogen. However, reintroduction of *Mir-17-5p* recognition-defective *AIB1* in these cells restored their growth. This finding indicates that *AIB1* is the target of *Mir-17-5p* and that *Mir-17-5p*-mediated growth reduction of estrogen-sensitive breast cancer cells is caused by the regulation of *AIB1* translation by *Mir-17-5p*. Hormone-independent growth of BT-20 cells was also inhibited by *Mir-17-5p*. However, reintroduction of the *Mir-17-5p* recognition-defective *AIB1* in these cells only partially restored cell proliferation. A recent report suggests that *E2F1* is also a target of *Mir-17-5p*-mediated translational regulation (40). Louie et al. reported that *AIB1* and *E2F1* cooperate in the proliferation of ER-negative BT-20 breast cancer cells (32). This is most likely the reason why we could not restore maximum proliferation activity of *Mir-17-5p*-treated BT-20 cells by reintroducing *AIB1* only. *AIB1* amplification and/or overexpression are commonly observed in primary breast cancer (2). Analogously, the loss of heterozygosity at chromosome region 13q31 has been documented in breast carcinomas (12). A genetically amplified *AIB1* gene is likely to bypass the *Mir-17-5p*-mediated translational regulation of growth hormone signaling, resulting in uncontrolled growth of tumor cells. Our results suggest a scenario in which *Mir-17-5p* functions as a key regulator of hormone-mediated enhancement of breast cancer cell growth.

IGF-1-mediated and estrogen-independent growth of breast cancer cells are dependent on functional *AIB1* and *E2F1* (32). We observed that the anchorage-independent growth of MCF-7 cells in response to IGF-1 was abrogated by *Mir-17-5p*. Growth retardation of these cells was directly related to the downregulation of *AIB1* and *E2F1* translation, since we were able to rescue substantial growth of MCF-7 cells by the reintroduction of *Mir-17-5p*-insensitive forms of AIB1 and E2F1. These results further established the role of *Mir-17-5p* in the hormone-dependent and hormone-independent growth of breast cancer cells.

During the preparation of the present report, two independent groups described the regulation and possible function of the *c13orf25* locus that contains the *Mir-17*92* cluster of seven miRNAs (*Mir-17-5p*, *Mir-17-3p*, *Mir-18*, *Mir-19a*, *Mir-20*, *Mir-19b-1*, and *Mir-92-1*) (22, 40). The *c13orf25* locus is overexpressed in human B-cell lymphomas, and forced expression of *c13orf25* and *c-myc* accelerates tumor development in a mouse B-cell lymphoma model (22, 42); however, it was not clear from these studies (22, 42) which individual miRNA is crucial for B-cell lymphoma development. It is also important to mention that in lung cancer, all members of *Mir-17*92* are overexpressed, except for *Mir-17-5p* (21). This indicates that although miRNAs are expressed as a cluster in many cases, individual miRNA biogenesis is under differential control by a still unknown mechanism. In agreement with the present study, O'Donnell et al. showed that translation of the oncogene *E2F1* is negatively regulated by *Mir-17-5p* and suggested that *Mir-17-5p* acts as a tumor suppressor (40).

Because expression of *Mir-17-5p* is very low in some breast tumors and cell lines (32), it is plausible that the transcriptional regulation of *Mir17-5p* is somewhat different in breast tissue than in B cells. For example, expression of the *Mir-17*92* cluster in breast cancer cells may be downregulated by an epigenetic mechanism, since a loss of heterozygosity at the 13q31 region has been reported in some cases (12). During the preparation of the present report, Volinia et al. reported that *Mir-17-5p* is upregulated in breast cancer (52). Their observation directly contradicts an earlier report by Lu et al. (33). These two groups used different techniques, and this may explain the discrepancy between these two reports. It is important to mention that *Mir-106a* is a homolog of *Mir-17-5p* and was downregulated in the study by Volina et al. (39, 52). Structural differences between these two miRNAs (*Mir-17-5p* and *Mir-106a*) are insignificant and should give similar results with the solid base hybridization technique. The small size of miRNAs made it complicated to detect by conventional hybridization techniques. A comprehensive assay with large number of samples using both solid- and liquid-based hybridization techniques are required to uncover the true status of *Mir-17-5p* in breast cancer. During the final preparation of the present study, Zhang et al. (57) reported that the region containing this *Mir-17-92* polycistron was deleted in

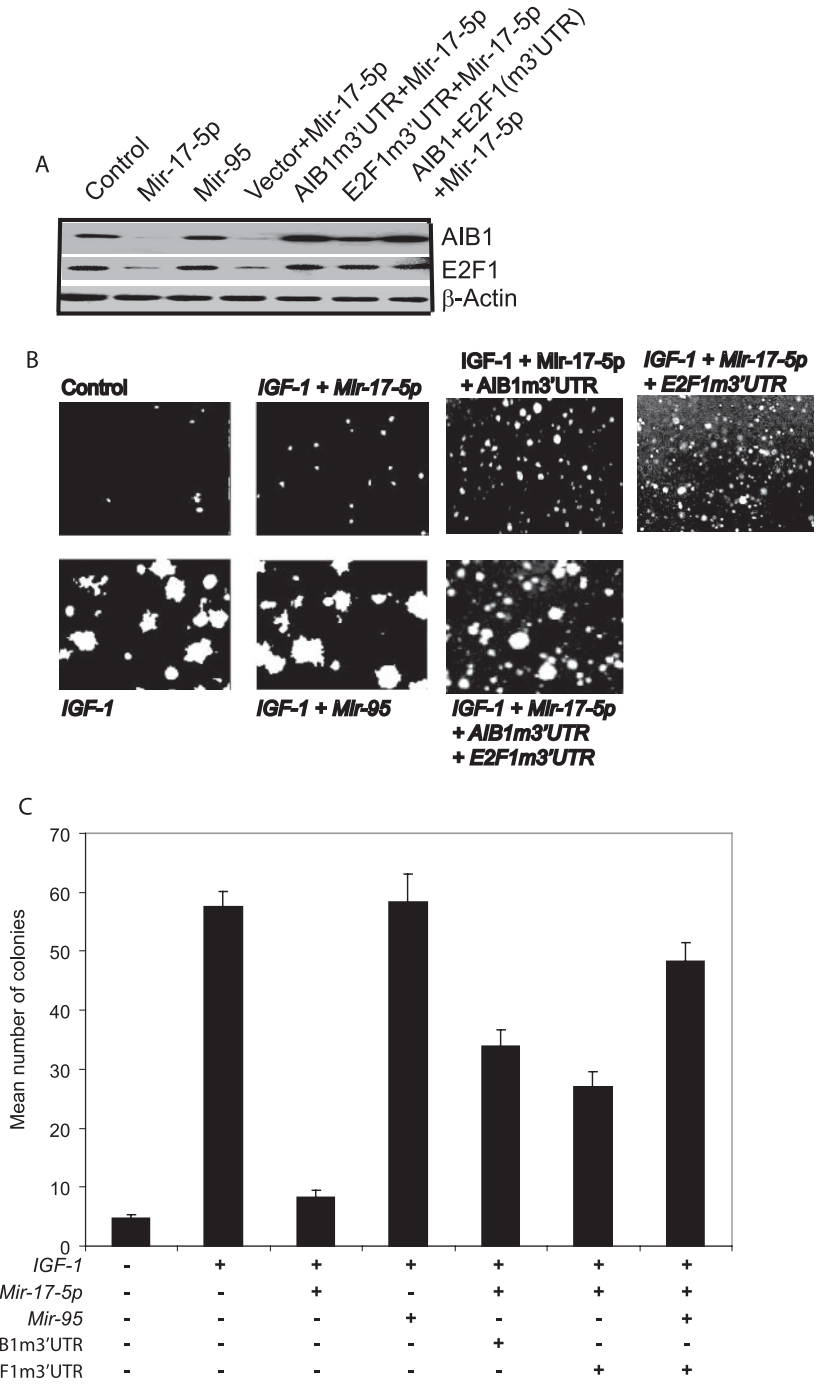

FIG. 9. Reduction of endogenous *AIB1* by *Mir-17-5p* inhibits the anchorage-independent growth of MCF-7 Cells. (A) *Mir-17-5p* and *Mir-95* overexpressing stably transfected MCF-7 cells were grown in improved minimal essential medium and 1% charcoal-stripped calf serum in 0.35% soft agar dishes in the absence or presence of 100 ng of IGF-1/ml. (B) Mean numbers of colonies with a diameter >80 μm. The data shown are means of triplicate experiments, and error bars indicate the standard deviations.

16.5% of ovarian cancers, 21.9% of breast cancers, and 20.0% of melanomas.

Deregulation of *Mir-17-5p* function could represent an important alternative mechanism to derail the control of cell proliferation in breast cancer. Our observations in the present study and from others suggest that *Mir-17-5p* acts as both as a tumor suppressor and as an oncogene by influencing cell proliferation in a cell-type-specific manner, depending on the cellular environment and target mRNAs that are expressed.

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