

The Putative Tumor Suppressor microRNA-101 Modulates the Cancer Epigenome by Repressing the Polycomb Group Protein EZH2

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Abstract

The Polycomb Repressive Complex 2 (PRC2) mediates epigenetic gene silencing by trimethylating histone H3 lysine 27 (H3K27me3) and is known to aberrantly silence tumor suppressor genes in cancer. EZH2, the catalytic subunit of PRC2, enhances tumorigenesis and is commonly overexpressed in several types of cancer. Our microRNA profiling of bladder transitional cell carcinoma (TCC) patient samples revealed that microRNA-101 (miR-101) is down-regulated in TCC, and we showed that miR-101 inhibits cell proliferation and colony formation in TCC cell lines. Furthermore, our results confirm that miR-101 directly represses EZH2 and stable EZH2 knockdowns in TCC cell lines create a similar growth suppressive phenotype. This suggests that abnormal down-regulation of miR-101 could lead to the overexpression of EZH2 frequently seen in cancer. We conclude that miR-101 may be a potent tumor suppressor by altering global chromatin structure through repression of EZH2. [Cancer Res 2009;69(6):2623–9]

Introduction

Polycomb group proteins are chromatin-modifying enzymes that are important in stem cell maintenance, X-inactivation, imprinting, and development, and many Polycomb group proteins are dysregulated in cancer (1). The Polycomb group protein EZH2 is the catalytic subunit of the Polycomb Repressive Complex 2 (PRC2), which is a critical part of the cellular machinery involved in epigenetically regulating gene transcription (2). PRC2 represses genes by trimethylating the core histone H3 lysine 27 (H3K27me3) at and around the promoter regions of target genes (1).

EZH2 enhances proliferation and neoplastic transformation (3), is overexpressed in many cancers, and is strongly associated with metastatic breast and prostate cancers (3–7). Recent work has shown that overexpression of EZH2 is directly responsible for the *de novo* suppression of multiple genes in human cancer (8, 9). However, the cause of EZH2 overexpression in cancer is not clear. Intriguingly, a significant subset of PRC2 target genes in cancer were also targets of PRC2 in embryonic stem cells (10). This

illustrates a strong association between the function of PRC2 in cancer and stem cells, which represent dedifferentiated and proliferative cell states. Therefore, EZH2 overexpression might cause a normal cell to dedifferentiate back to a stem cell–like state by epigenetically repressing cell fate–regulating genes and tumor-suppressor genes leading to tumor development (1, 9, 11).

MicroRNAs (miRNA) are ~22 nucleotide noncoding RNA molecules that usually function as endogenous repressors of target genes. In animals, miRNAs can bind with imperfect complementarity to the 3' untranslated region (3' UTR) of the target mRNA via the RNA-induced silencing complex. The resulting gene repression occurs by multiple mechanisms including enhanced mRNA degradation and translational repression (12). Developmental timing, cell death, proliferation, hematopoiesis, insulin secretion, and the immune response are just a few examples of critical biological events that depend on faithful miRNA expression (13).

In the past few years, miRNA profiling of various human cancers has revealed many miRNAs that function as tumor suppressors, such as *let-7*, miR-15a/16-1, and the miR-34 family, or oncogenes such as miR-155 and the miR-17-92 cluster (14, 15). In addition, miRNA profiles were better able to predict tumor type than were mRNA profiles (16). However, miRNA profiling of bladder cancer with normal and matched tumor tissues and a functional study of differentially expressed miRNAs in bladder cancer remains to be conducted. Bladder cancers in the United States are almost exclusively transitional cell carcinomas (TCC), and in 2007, TCC was the fifth most common cancer diagnosis according to the National Cancer Institute (NCI).

Therefore, we sought to generate a miRNA expression profile for TCC by comparing primary TCCs to their corresponding normal urothelium. We found many differentially expressed miRNAs, several of which showed putative tumor suppressor functions. The miRNA that most consistently and dramatically suppressed growth was miR-101, which we confirmed can directly target EZH2 and repress H3K27me3. Furthermore, our results indicate that a significant subset of genes is regulated by both miR-101 and EZH2.

Materials and Methods

Detailed protocols and primer sequences are in the Supplementary Data.

Cell lines and primary tumors. T24, UM-UC-3, and TCCSUP cells were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC protocols. Patient samples were obtained through University of Southern California/Norris Tissue Procurement Core Resource after informed consent and Institutional Review Board approval (IRB #886005 and #926041) at the University of Southern California/Norris Comprehensive Cancer Center.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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miRNA microarray. One microgram of total RNA from each of nine TCCs was pooled and the same was done with nine matched normal tissues. miRNA microarray analysis was done as previously described (17).

Reverse transcription and Taqman qPCR. miRNA Taqman assays (Applied Biosystems) were used according to the manufacturer's protocol. *EZH2*, *U6* primers are provided in the supplemental data.

Expression vectors. Expression vectors were made by cloning ~200 bp surrounding the precursor miRNA into pcDNA3.1(+) (Invitrogen). Expression vectors containing shRNA to *EZH2* were commercially available (Sigma).

Cell proliferation and colony formation assays. Cell proliferation and colony formation assays were conducted as described previously (18, 19).

Western blot. Western blots were performed as previously described (17).

Luciferase assay. Reporter vectors were created by cloning the wild-type or mutated 3'UTR of *EZH2* into the XbaI site of the pGL3-control vector (Promega). Firefly and renilla luciferase activity was analyzed using the Dual Luciferase Reporter assay system (Promega) as previously described (17).

Messenger RNA microarray. UM-UC-3 cells were transfected with pre-miR-101, control precursors, siRNA to *EZH2*, and control siRNA in triplicate. Total RNA was prepared 72 h after transient transfection and the Illumina human 6 v 2 array was used for gene expression analysis. The Norris Cancer Center CORE facility performed the amplification and hybridization according to the manufacturer's protocol (Illumina).

Results and Discussion

Differential miRNA expression in TCCs. We used a miRNA microarray to examine differentially expressed miRNAs in a pool of nine TCCs and a pool of the corresponding normal samples. miR-1, miR-101, miR-143, miR-145, and miR-29c were the most down-regulated transcripts in the tumors, and miR-182, miR-183, miR-203, miR-224, and miR-196a were the most up-regulated (Table 1; Fig. 1A). miR-127 was included in the table because our previous work revealed it is down-regulated in human cancers (17).

We conducted RT-qPCR for 12 differentially expressed miRNAs on 28 additional TCC patients. miR-1, miR-101, miR-143, miR-145, miR-29c, and miR-127 were significantly down-regulated in the tumors, whereas miR-224, miR-182, and miR-183 were significantly up-regulated in the tumors (Fig. 1B). miR-196a, miR-10a, and miR-203 showed no significant differences. Our miRNA microarray analysis and RT-qPCR results showed that there is severe and consistent miRNA misexpression in TCC, and our miRNA panel likely constitutes a TCC miRNA signature. The miRNA that was most down-regulated in TCC, miR-1, and miR-29c were down-regulated in hepatocellular carcinoma and lung cancer, respectively (20, 21). Furthermore, our study supports the link between down-regulation of miR-143 and miR-145 and cancer, which was previously shown in various malignancies (22, 23).

Restored expression of miRNAs in TCC cell lines reveals putative tumor suppressors. We used RT-qPCR to analyze miRNA expression in 10 TCC cell lines. miR-1, miR-101, miR-127, miR-143, and miR-145 were expressed at low levels in all cell lines (data not shown), indicating that these miRNAs are promising tumor suppressor candidates. After creating miRNA expression vectors, T24, TCCSUP, and UM-UC-3 cells were transfected and the enhanced miRNA expression from each vector was confirmed by RT-qPCR (Supplementary Fig. S1). The expression levels for miR-101 were found to be of similar levels to those found in adjacent normal tissues (Supplementary Fig. S2).

Proliferation assays were done by stably transfecting cells with the miRNA expression vectors to determine the functional effects

of miRNA misexpression. The results showed a strong inhibition of cell proliferation by miR-101. In T24 cells, miR-101 suppressed proliferation by 57% (Fig. 1C). Proliferation was significantly inhibited by miR-101, miR-1, and miR-145 in UM-UC-3 and TCCSUP cells (Fig. 1C). These results indicate that miR-101 is the most potent growth suppressor, although miR-1 and miR-145 also significantly inhibited cell proliferation.

We determined the effect of restored miRNA expression on colony formation, and again, the results varied depending on the cell line and miR-101 was the most potent suppressor of colony formation. In T24 cells, miR-101 suppressed colony formation by 45% (Fig. 1D). miR-101, miR-127, miR-143, and miR-145 significantly reduced colonies in UM-UC-3 cells, whereas in TCCSUP cells, miR-101, miR-1, and miR-143 showed significant suppression (Fig. 1D). Clearly, restored expression of miR-101 potently suppresses colony formation in these cell lines, whereas other miRNAs also suppress colony formation, although less substantially and consistently. The cell proliferation and colony formation assays indicate that miR-101 is a putative tumor suppressor and may be a therapeutic target for cancer.

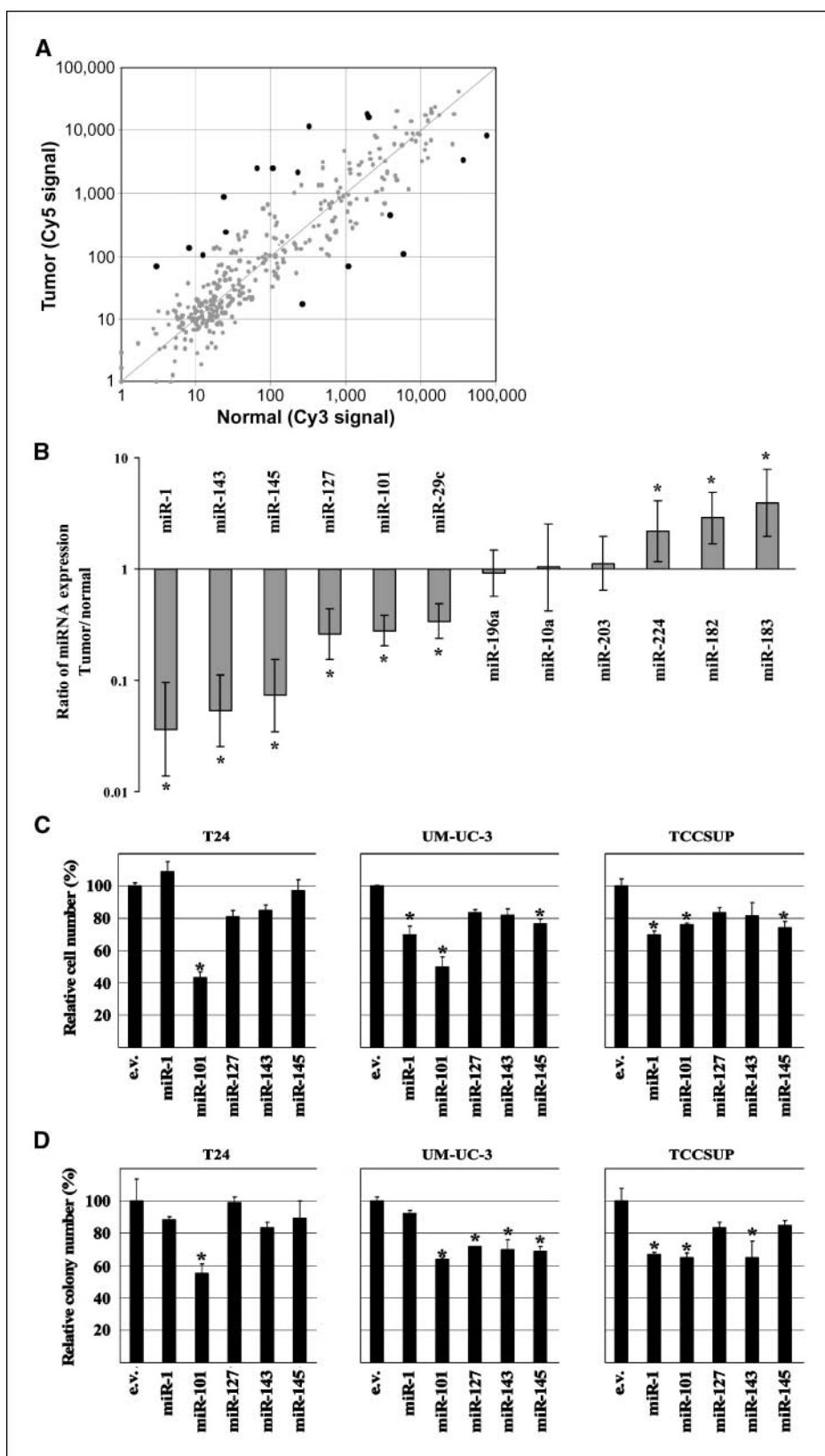
There are a plethora of mechanisms that could lead to decreased miRNA expression in cancer including copy number alterations (16), epigenetic silencing (17), and *trans*-acting factors (15). There are two copies of miR-101, with miR-101-1 located at chromosome 1p31 and miR-101-2 located at chromosome 9p24. The two precursors have different sequences, but the mature forms are identical. We found that miR-101 silencing in TCC cell lines was probably not due to epigenetic phenomena because treatment with the DNA demethylating agent 5-aza-2'-deoxycytidine and the histone deacetylase inhibitor 4-phenylbutyric acid did not induce expression of miR-101 (data not shown). Intriguingly, loss of heterozygosity (LOH) at chromosome 1p occurs in many different solid tumors and is negatively associated with survival (24), whereas LOH at chromosome 9p also commonly occurs in cancer, particularly TCC (25). While this article was being revised, genomic

Table 1. Summary of the most differentially expressed miRNAs in TCC compared with normal bladder tissue from Fig. 1A

miRNA	Fold change (tumor/normal)
hsa-miR-183	38
hsa-miR-182	37
hsa-miR-203	36
hsa-miR-224	23
hsa-miR-196a	23
hsa-miR-10a	10
hsa-miR-127	-3
hsa-miR-29c	-9
hsa-miR-145	-9
hsa-miR-143	-11
hsa-miR-101	-16
hsa-miR-1	-54

NOTE: Data are presented as the fold change of miRNA expression from the tumor/normal for the most differentially expressed transcripts (including miR-127) that were validated with RT-qPCR in additional patient samples.

Figure 1. miRNAs are dysregulated in TCC. **A**, plot for miRNA microarray of the Tumor signal versus Normal signal shows all transcripts with those showing fold change of >8 in black. **B**, miRNA RT-qPCR of 28 clinical TCC samples and matched normal urothelium. All reactions were done in duplicate and *U6* was the internal control. The graph shows the ratio of miRNA expression of Tumor/Normal on a logarithmic scale. *, statistical significance; error bars, 95% confidence interval. **C**, cell proliferation assays were conducted by transferring equal cell numbers to 10-cm dishes 48 h posttransfection with miRNA expression vectors or empty vector (*e.v.*) control. After 13 d under G418 selection, total cells were counted and normalized to the empty vector control. *, $P < 0.02$ according to Dunnet's method (except UM-UC-3 miR-145, P value = 0.044; *C* and *D*); columns, mean; bars, SE. **D**, colony formation assays were conducted by seeding equal cell numbers 48 h posttransfection into 6-well plates. Colonies were stained and counted after 13 d under G418 selection and normalized to the empty vector control. *, $P < 0.02$ according to Dunnet's method (except UM-UC-3 miR-145, P value = 0.044; *C* and *D*); columns, mean; bars, SE.



loss was found to be a mechanism for miR-101 down-regulation in prostate cancer (26). This suggests that DNA copy number may regulate miR-101 expression, although a *trans*-acting mechanism should be investigated in the future.

miR-101 represses the Polycomb group protein EZH2. We used the prediction algorithm TargetScan³ to identify targets of

³ <http://www.targetscan.org>

miR-101 and found the histone methyltransferase *EZH2* had a very high score, a highly conserved sequence, and two predicted sequence matches to the miR-101 seed. To make a clear comparison, we plotted miR-101 RT-qPCR data from 20 individual patient samples from Fig. 1B above the mRNA levels of *EZH2* that were obtained by RT-qPCR (Fig. 2A). Our results showed that *EZH2* was up-regulated in the tumor samples relative to the adjacent normal specimens, whereas miR-101 is down-regulated in these same samples (Fig. 2A).

We transiently transfected miR-101 precursors (pre-miR-101) into T24, TCCSUP, and UM-UC-3 cells and visualized *EZH2* levels by Western blot. In TCCSUP and UM-UC-3 cells, *EZH2* levels decreased by 86% and 91%, respectively, whereas H3K27me3 decreased by 58% in both cell lines (Fig. 2B). In T24 cells, there was a 52% decrease in *EZH2* levels and the H3K27me3 levels remained the same, which is likely due to lower transfection efficiencies that yielded 50% less mature miR-101 levels when compared with UM-UC-3 cells (data not shown). We determined the effect of physiologic levels of miR-101 on *EZH2* by transfecting 5 nmol/L pre-miR-101 in UM-UC-3 cells. The results showed a 39% down-regulation of *EZH2* (Fig. 2B) and mature miR-101 levels comparable with the average normal patient sample (Supplementary Fig. S2). These results indicate that miR-101 can repress *EZH2* in TCC cell lines even at levels that are similar to those found in normal urothelium (Fig. 2B).

***EZH2* is a direct target of miR-101.** To confirm that *EZH2* is a direct target of miR-101, we created luciferase reporter vectors by

cloning either the wild-type or a mutated portion of the 3'UTR of *EZH2* into the 3'UTR of the pGL3-control vector. The mutated 3'UTR had three bases changed, from GUACUGU to CUAGUCU, at each of the two putative miR-101 binding sites (Fig. 2C). We transfected these vectors with pre-miR-101 into UM-UC-3 cells and the lysates were analyzed 24 hours later. The results showed a 42% decrease in luciferase activity for the wild-type 3'UTR of *EZH2*, whereas the mutated 3'UTR showed no repression when compared with the empty reporter as was also found previously (Fig. 2D; ref. 27). Therefore, miR-101 represses *EZH2* by binding to the 3'UTR of *EZH2* in a direct and sequence specific manner.

Knockdown of *EZH2* shows phenotypic overlap with the restoration of miR-101 expression in TCC cells. After confirming that miR-101 leads to a decrease in H3K27me3 levels by targeting *EZH2*, we examined if there was a phenotypic overlap between stable miR-101 transfection and knockdown of *EZH2* by shRNA in TCC cells. We conducted cell proliferation assays and colony formation assays in three TCC cell lines using four expression vectors (clones 74, 75, 76, and 77) containing distinct shRNAs targeting *EZH2*. The results showed that two of four, one of four, and four of four shRNAs suppressed cell proliferation in T24, TCCSUP, and UM-UC-3 cells, respectively (Fig. 3A). The colony formation results were more striking as all four shRNAs suppressed colony formation in T24 and TCCSUP cells, whereas three of four shRNAs suppressed colony formation in UM-UC-3 cells (Fig. 3B and C).

To examine additional phenotypic overlap, expression microarrays were conducted on UM-UC-3 cells transfected with

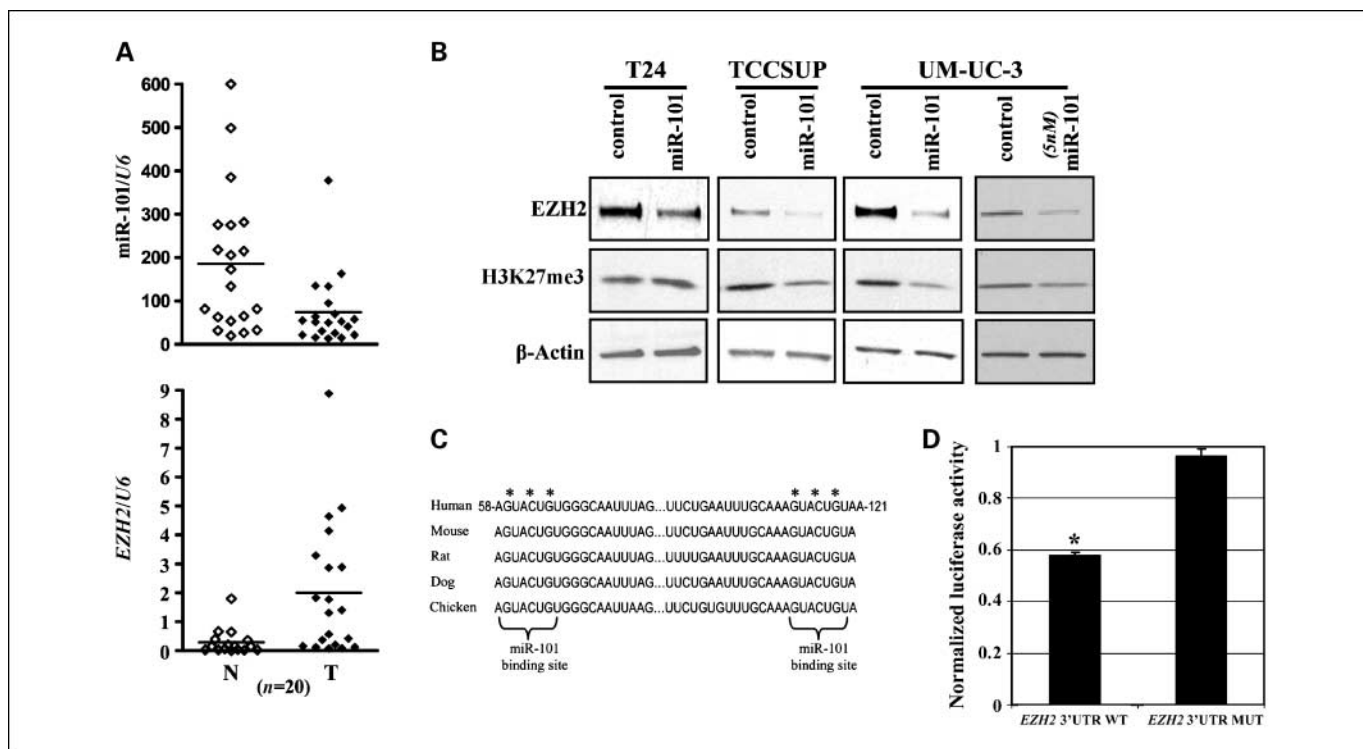


Figure 2. miR-101 directly targets *EZH2*. **A**, RT-qPCR analysis of 20 TCC samples shows that *EZH2* is up-regulated in tumors compared with adjacent normal tissue. The data from Fig. 1B showing miR-101 levels was modified to show individual patient samples and this was compared with *EZH2* levels in these same samples. Each experiment was duplicated and the value for miR-101 or *EZH2* was normalized to *U6*. Data are presented as individual samples with the line indicating the mean (for *EZH2* paired *t* test, $P = 0.004$). **B**, Western blot analysis of TCC cell lines after transient transfection with pre-miR-101 or control precursors at a final concentration of 50 or 5 nmol/L where indicated. Lysates were prepared 48 h after transfection and membranes were probed with antibodies to *EZH2*, H3K27me3, and β -actin as a loading control. Bands were quantitated by Quantity One software (Bio-Rad). **C**, the highly conserved sequence of the 3'UTR of *EZH2* for human, mouse, rat, dog, and chicken are shown. *, nucleotides that were mutated for the luciferase insert. **D**, luciferase assay conducted in UM-UC-3 cells 24 h after cotransfection with pre-miR-101, renilla luciferase vector pRL-SV40, and either the firefly luciferase reporter pGL3-control containing wild-type *EZH2* 3'UTR insert (GUACUGU) or mutated *EZH2* 3'UTR insert (CUAGUCU). Relative luciferase activity was normalized to the no insert control (*, $P < 0.01$). Columns, mean; bars, SE.

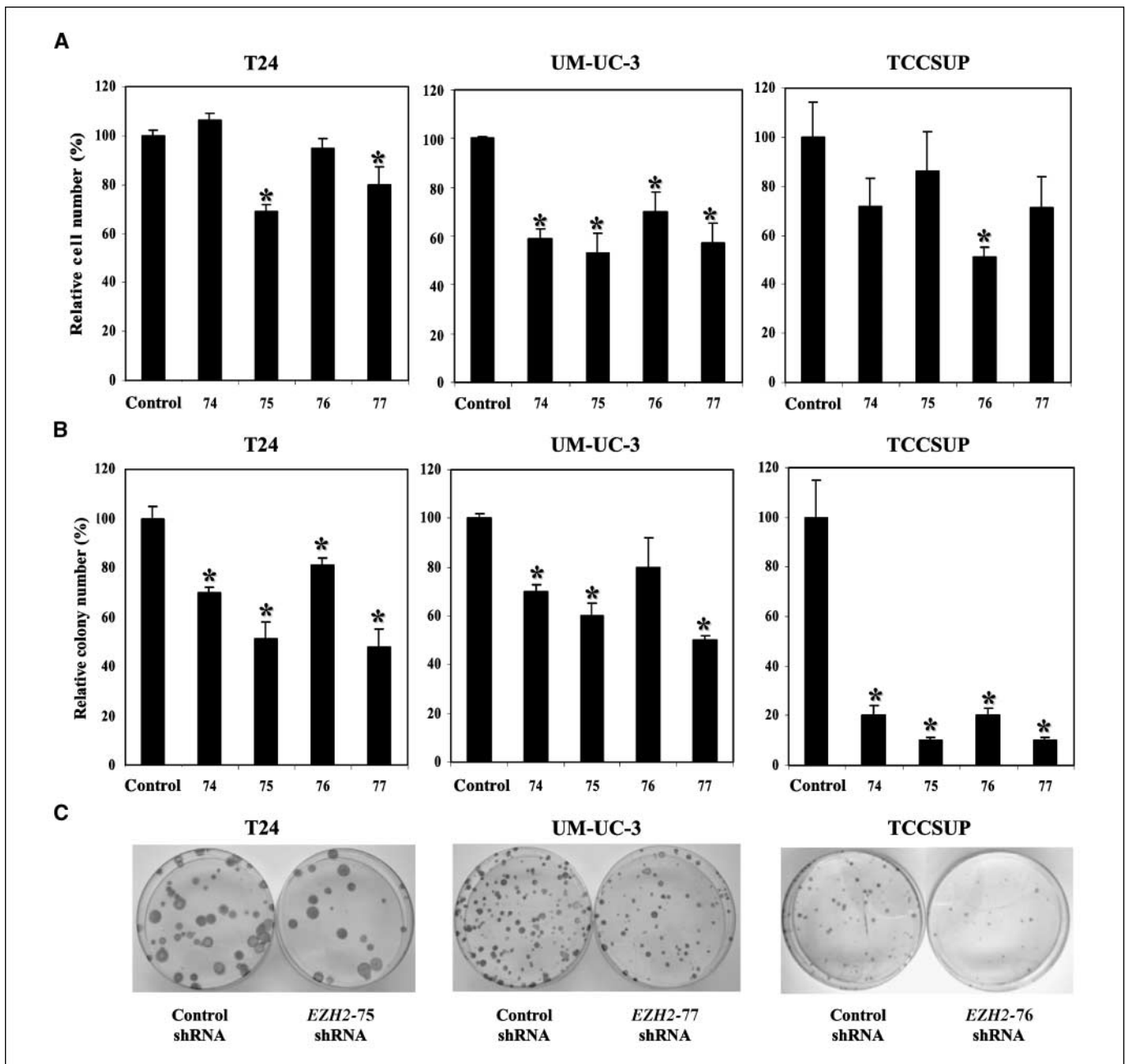


Figure 3. Knockdown of EZH2 decreases cell proliferation and colony formation in TCC cell lines. *A*, cell proliferation assays were conducted by transferring equal cell numbers to 10-cm dishes 48 h posttransfection with 4 different expression vectors (clones 74, 75, 76, and 77) containing distinct shRNAs to *EZH2* or control shRNA vector. After 13 d under puromycin selection, total cells were counted and normalized to the empty vector. *B*, colony formation assays were conducted by seeding equal cell numbers 48 h posttransfection into 10-cm dishes. Colonies were stained and counted after 13 d under puromycin selection and normalized to the control shRNA vector. *, $P < 0.05$ according to *t* test; columns, mean; bars, SE. *C*, photographs of representative colony formation assays.

pre-miR-101 or siRNA to *EZH2*. We identified a significant overlap of 43 up-regulated genes ($P < 10^{-11}$ by hypergeometric distribution), which suggests that restoring miR-101 expression in cancer cells re-expresses a subset of genes that are repressed by *EZH2* (Fig. 4*B*). The extra decrease in *EZH2* and H3K27me3 from the siRNA to *EZH2* may explain why there were 62 up-regulated genes that did not overlap with the genes up-regulated by pre-miR-101 transfection (Fig. 4*A* and *B*). In contrast, we expect the pre-miR-101 transfection to have much wider effects on gene expression than the siRNA to *EZH2* because miRNAs are more promiscuous

and likely repress several targets that influence the transcriptome. We conducted chromatin immunoprecipitation analysis on the promoters for the Polycomb target genes *FAM84* and *DDIT4* after treating UM-UC-3 cells with siRNA to *EZH2* and pre-miR-101 for 72 hours. The results showed that both treatments depleted the promoters of H3K27me3 (Supplementary Fig. S3), indicating that miR-101 regulates histone methylation through *EZH2*.

The molecular similarities between cancer and stem cells are becoming ever more apparent. For example, *EZH2* is critical for the maintenance of proliferation and pluripotency in stem cells (1).

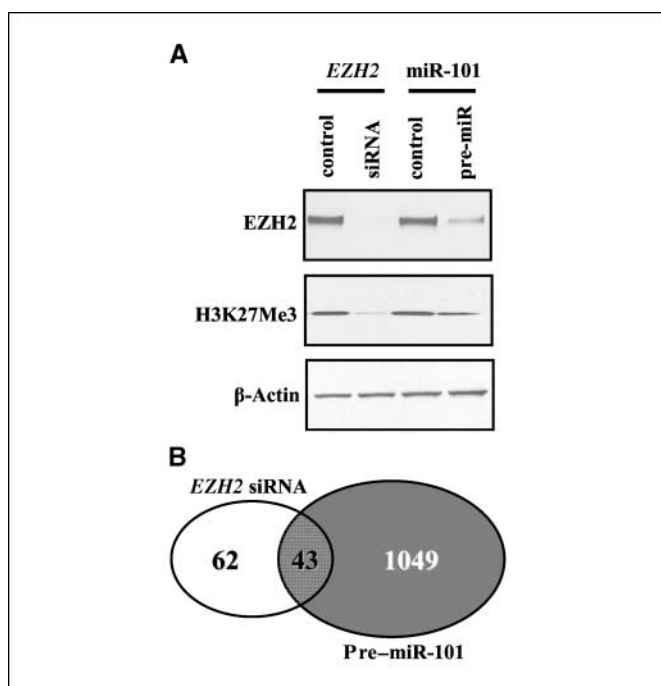


Figure 4. Pre-miR-101 transfection and siRNA to *EZH2* lead to the up-regulation of overlapping genes. **A**, Western blot analysis of UM-UC-3 cells transfected with siRNA to *EZH2*, control siRNA, pre-miR-101, or control precursors to a final concentration of 50 nmol/L. Total protein was extracted 72 h after transfection and membranes were probed with antibodies to *EZH2*, H3K27me3, and β -actin as a loading control. **B**, Illumina Human 6 v 2 chips were used to interrogate the mRNA levels from UM-UC-3 cells 72 h after transfection with pre-miR-101, control precursors, siRNA to *EZH2*, and control siRNA. Based on the criteria fold change of >1.5 and *t* test *P* value of <0.05 , 1,092 genes were up-regulated after pre-miR-101 transfection, whereas 105 genes were up-regulated after treatment with siRNA to *EZH2*. There was an overlap of 43 genes ($P < 10^{-11}$ based on hypergeometric distribution).

Global levels of *Ezh2* and H3K27me3 decrease when mouse embryonic stem cells differentiate and PRC2 target genes specifically lose *Ezh2* binding and H3K27me3 enrichment (28). In addition, two groups found that miR-101 was up-regulated after differentiation of human embryonic stem cell lines (29, 30). These

reports present a strong association between miR-101 activation and *EZH2* repression upon differentiation of stem cells, suggesting that miR-101 might be part of the complex network, which includes Polycomb group proteins, that determines developmental outcome. Therefore, in addition to its potential role in cancer, miR-101 might be involved in normal differentiation by directly repressing *EZH2* and re-expressing cell fate regulating genes.

The role of miRNAs in controlling epigenetics is just emerging. Embryonic stem cell-specific miRNAs control *de novo* DNA methylation during differentiation by targeting *Rbl2* (31, 32). A recent report suggests that the down-regulation of the miR-29 family in lung cancer could lead to the overexpression of DNA methyltransferases 3A and 3B and subsequent DNA hypermethylation and gene silencing (21). This study shows another instance in which miRNA-mediated epigenetic mechanisms can be dysregulated in cancer with global consequences.

Our results and previously published data examining miR-101 in lung cancer, breast cancer, colorectal cancer, and prostate cancer (22, 23, 33, 34) indicate that miR-101 is down-regulated in the five most frequently diagnosed cancers in the United States (NCI), demonstrating that miR-101 might be part of a solid tumor signature. This report suggests that aberrant silencing of miR-101 may be a cause of the overexpression of *EZH2* seen in cancer and that restoring miR-101 expression could hinder *EZH2*-mediated neoplastic progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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