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*miR-187** Enhances SiHa Cervical Cancer Cell Oncogenicity *Via* Suppression of WWOX

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Abstract. *Background/Aim: Cervical cancer is one of the most common cancers worldwide and a major cause of cancer-related mortality among women. Previous studies have reported that microRNA-miR-187*, which is one of the non-coding parts of the genome producing small conserved ribonucleic acids, is associated with various cancers. In this study, we explored the function of miR-187* in cervical cancer cells. Materials and Methods: miR-187* mimic, WWOX reporter constructs, siRNA and overexpression constructs were transfected into SiHa cells to investigate the function and regulatory mechanisms of miR-187*. Results: Exogenous miR-187* was found to increase the oncogenic phenotypes of SiHa cells. The tumor suppressor gene WWOX is a novel target of miR-187* in SiHa cells. WWOX siRNA suppressed endogenous WWOX expression and increased the oncogenic phenotypes of SiHa cells. Exogenous WWOX expression was able to suppress the oncogenic phenotypes of SiHa cells and rescue cells from miR-187*-induced migration. Conclusion: miR-187* seems to enhance SiHa cervical cancer cell oncogenicity via suppression of the WWOX pathway.*

Cervical cancer is one of the most common cancers among women worldwide and a major cause of cancer-related mortality. Nearly all cervical cancers are HPV-associated, including cervical squamous cell carcinomas (70%), cervical adenocarcinomas (CA) (25%), and mixed-histology tumors (1). With the increased use of Pap tests for screening, which are able to detect the mild morphological changes present in HPV-infected cervical epithelium at an early stage, the death rate from cervical cancer has decreased appreciably in recent years. However, the median overall survival of individuals with advanced cervical cancer is only 16.8 months and the 5-year overall survival for all stages of cervical cancer remains at 68% (2). The mechanisms involved in cervical cancer pathogenesis remain unclear to a large extent. Therefore, exploring the pathogenesis and molecular mechanisms involved in cervical cancer remain important research objectives.

There is increasing evidence that miRNA expression shows aberrations in human cancers, there being miRNAs that are oncogenic and miRNAs that are tumor suppressors (3-5). miRNAs, through imperfect complementarity, bind with conserved sites in 3'-untranslated regions (3'-UTRs) or in the coding sequences of targeted transcripts; this represses gene activity by blocking translation of the mRNA transcript into protein (6). Previous studies have reported that aberrant miRNA expression contributes to cervical cancer carcinogenesis, either via oncogenic miRNAs or via tumor suppressor miRNAs (7-9).

miR-187 (also called miR-187-3p) and its star-form partner *miR-187** (also called miR-187-5p) are located on chromosome 18q12.2. *miR-187** has been reported to be associated with various cancers and to function as either as an oncomiR or as a tumor suppressor miRNA. In non-small cell lung cancer, the expressions of *miR-187* and *miR-187** has been found to be decreased in primary tumor tissue samples and expression of these miRNAs is able to suppress cancer cell progression (10). In addition, *miR-187** has been

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shown to be up-regulated in oral carcinoma tissue samples and this miRNA seems to enhance the oncogenic phenotype of oral cancer cells. Furthermore, the plasma level of *miR-187** has been identified as a potential biomarker for oral carcinoma (11). *miR-187** has also been shown to enhance the oncogenicity and recurrence rate of bladder cancers (12). Thus, the expression level of *miR-187** seems to vary significantly across a range of different cancer types and therefore the mechanisms whereby they are involved in carcinogenesis would seem to be cancer type specific.

WW domain containing oxidoreductase (WWOX) is located on chromosome 16q23.3–q24.1. A LOH of 16q has been demonstrated to be associated with breast carcinoma and its pre-cancer lesions, with prostate carcinoma and with hepatic carcinomas; therefore, WWOX is considered to act as a suppressor gene in a wide variety of cancers (13-16). Loss of WWOX expression has been reported in breast cancer (14, 16, 17), lung cancer (14, 18), gastric cancer (15, 19) and ovarian cancer (14, 16, 20). Overexpression of WWOX or reactivated of endogenous WWOX has been shown to lead to apoptosis of breast cancer cells *in vitro* and to suppression of breast tumor growth *in vivo* (21). In a similar manner to that which occurs in breast cancer, WWOX overexpression has been shown to induce apoptosis and inhibit cancer cell growth in ovarian cancer (20) and prostate cancer (22). In addition, WWOX is able to directly interacts with RUNX2 to suppresses RUNX2 expression, which then results in an inhibition of osteosarcoma metastasis (23) and reduced lung cancer cells invasion (24). WWOX also is able to suppress the Wnt/β-catenin pathway transcriptional activity through binding to dishevelled (Dvl) proteins, which play a key role in Wnt receptor activation (25). In cervical cancer and invasive cervical squamous cell carcinoma (ICSCC), WWOX is known to be significantly down-regulated and has been associated with tumor angiogenesis (26, 27). WWOX may also be involved in cervical cancer carcinogenesis *via* an induction of apoptosis and inhibition of proliferation (28). Finally, it has been reported that the WWOX SNP, rs11545028, is a genetic variant associated with cervical cancer patient survival, whereby CT/TT would seem to predict better overall survival (29). Nevertheless, notwithstanding the above findings, the regulatory mechanisms associated with WWOC and cervical cancer still remain unclear. In this study, we showed that exogenous *miR-187** expression is able to increase the expression of oncogenic phenotypes by cervical cancer cells *via* suppression of various WWOX pathways.

Materials and Methods

Reagents. Chemically modified *miR-187** mimic (Cat. No.: C-301040-01) and an appropriate scrambled control (Cat. No.: CN-001000-01-05) were purchased from Dharmacon (GE Healthcare Life Sciences, Pittsburgh, PA, USA). The small interference oligonucleotide, siWWOX (Cat. No.: sc-44193), which can be used to knock down WWOX gene expression, as well as a control oligonucleotide (siControl; Cat. No.: sc-37007), were purchased from Santa Cruz Biotech (Santa Cruz, Dallas, TX, USA). TransFectin™ (Cat. No.: 170-3351, Bio-Rad, Hercules, CA, USA) lipid reagent was used for transfection. Unless specified otherwise, all other reagents were purchased from MilliporeSigma (St. Louis, MO, USA).

Quantitative PCR (Q-PCR). Total RNA was purified using TRI reagent (Sigma-Aldrich). Applied Biosystems® TaqMan MicroRNA Reverse Transcription Kits (ThermoFisher Scientific, Waltham, MA, USA) and TaqMan® MicroRNA Assays (ThermoFisher Scientific) were used to detect the expression of *miR-187** (Cat. No.: 00274) and *RNU6B* (Cat. No.: 001093) was used as a control (10). These reactions were carried out using an iQ5 real-time PCR detection system (Bio-Rad). The mRNA expression levels of WWOX (Cat. No.: Hs03044790_m1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, which was used as an internal control, Cat. No.: Hs02758991_g1) were analyzed using the Applied Biosystems® TaqManq PCR System (Thermo Fisher Scientific). The comparative threshold cycle (Ct) method was used to measure the relative changes in expression. The $2^{-\Delta\Delta Ct}$ values were used to represent the fold change in *miR-187** expression and WWOX expression between the sample groups and between the various experimental setups (30).

Cell culture. The SiHa cell line was grown in PRMI-1640 medium (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Biological Industries, Kibbutz Beit Haemek, Israel) at 37˚C in a humidified atmosphere containing 5% CO_2. The cells were passaged at a density ratio of 1:5 when 80% confluence was reached.

Transwell migration assay. Transwell units (Corning, Acton, MA, USA) were pre-coated with fibronectin (Sigma-Aldrich) as a chemotactic attraction agent in order to induce cell migration. A total of 1×10^5 cells were seeded into each Transwell upper chamber, which contained serum-free medium. Next, 1 μM hydroxyurea was added to arrest cellular proliferation. After 24 h, the migrated cells on the lower surface of membrane were stained with Hoechst 33258 (Thermo Fisher Scientific) and counted by fluorescence microscopy.

Anchorage-independent colony forming assay. Using 6-well plates, a lower agar layer was fabricated using a 1:1 mixture of 1.8% agarose (Sigma-Aldrich) and 30% FBS. Next, an upper agar layer was formed with a 1:1 mixture of methylcellulose (Sigma-Aldrich) and 30% FBS; this was done in such a manner that it covered completely the lower agar layer. Next, 1×10^5 cells were seeded onto the upper layer and the plates cultivated for 7 days. On day 7 the colonies present on the plates were stained with 0.05% crystal violet and colonies with a diameter larger than 50 μm in fifteen randomly selected fields were counted using an inverted microscope.

Reporter plasmid construction and reporter assay. A PCR product of the WWOX 3'UTR, which contains the target sites for *miR-187** (total 871bp), was cloned into the pMIR-REPORT plasmid vector (pMIR-REPORT miRNA Expression Reporter Vector plasmid, ThermoFisher Scientific) using the restriction sites of SpeI and

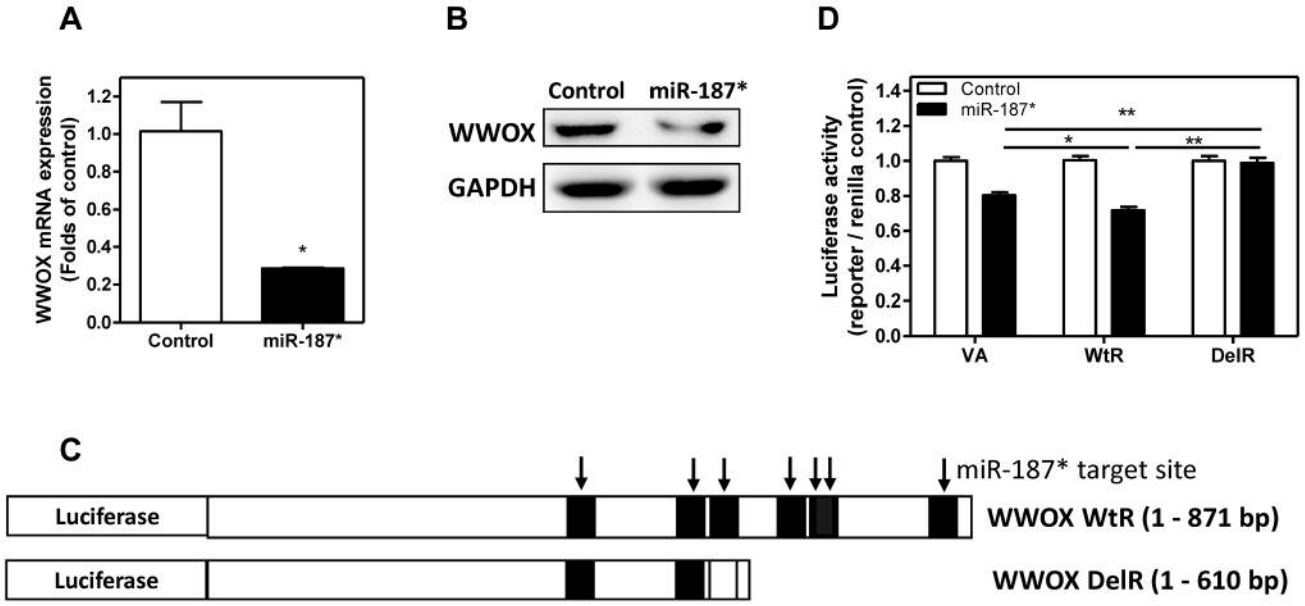

Figure 1. *WWOX is the miR-187* target in SiHa cells. SiHa cells transfected with 20 nM miR-187* mimics for 24-48 h. (A - B) Expression of WWOX mRNA (A) and protein (B). (C) A schematic diagram of the predicted miR-187*/WWOX sequences in the WWOX 3'UTR that seem to be complementary to the seed sequence of miR-187*. (D) WWOX luciferase reporter assay. *p<0.01; **p<0.001; Mann–Whitney test.*

Table I. *Primary and secondary antibodies used in this study.*

Antibody	Supplier	Cat. No.
BRCA1	Santa Cruz Biotech	sc-6954
DKK2	Santa Cruz Biotech	sc-25517
GABAAR1	Bioss Antibodies	bs-1232R
GAPDH	Santa Cruz Biotech	sc-47724
SLC26A3	Abnova	H00001811-M01
α-Tubulin	Santa Cruz Biotech	sc-5286
WWOX	Santa Cruz Biotech	sc-390175
ZEP36 (TTP)	Santa Cruz Biotech	sc-374305
Rabbit anti-goat IgG HRP	Santa Cruz Biotech	sc-2768
Mouse anti-rabbit IgG HRP	Santa Cruz Biotech	sc-2357

MluI. This reporter plasmid was designated WWOX WtR. In addition, a PCR product of the WWOX 3'UTR that had four fewer target sites for *miR-187** (total 610 bp) was cloned into the pMIR-REPORT plasmid vector and this was designated WWOX DelR (Figure 1C). The plasmid pRL-TL, which expresses the renilla luciferase gene, was used as a transfection control. Cells were co-transfected miR-187* mimic with pMIR-REPORTER (vector alone; VA), with WWOX WtR reporter or with WWOX DelR reporter, as well as with pRL-TL, for 24 h. Next the lysate was collected and assayed in accordance with the protocol provided for the Dual-Luciferase® reporter assay system (Promega, WI, USA). Firefly luciferase activity was normalized against the renilla luciferase activity in order to measure the activity levels of the two reporter systems.

Western blot analysis. A total of 30 μg protein from various whole-cell lysates was resolved by electrophoresis on a 10% denaturing polyacrylamide gel by following previously used protocols (30). The primary antibodies and secondary antibodies used in this study are listed in Table I. The protein signals were detected using a Western Lightning Chemiluminescence Reagent Plus Kit (Perkin-Elmer, Wellesley, MA, USA) and a Chemi-Smart 3000 image acquisition system (Viber Lourmat, Collégien, France).

Statistical analysis. Mann–Whitney tests, *t*-tests, and various bioinformatic modules were used for the statistical analysis. The results are considered to be statistically different when $p<0.05$.

Results

miR-187 promotes the oncogenicity of SiHa cells*. The functions of *miR-187** were analyzed by transiently transfecting with mimic. After transfecting with the mimic for 24 h, *miR-187** expression (Figure 2A) was found to be up-regulated in SiHa cells. This exogenous *miR-187** expression was found to significantly increase the migration (Figure 2B) and the anchorage-independent colony formation (Figure 2C) of SiHa cells.

WWOX is a novel miR-187 target*. It has been reported that BRCA1 and DKK2 are *miR-187** targets (31, 32). However, after transfecting with *miR-187** mimic for 48 h, unexpectedly, BRCA1 and DKK2 protein expression levels were found to be up-regulated in SiHa cells (Figure 3A).

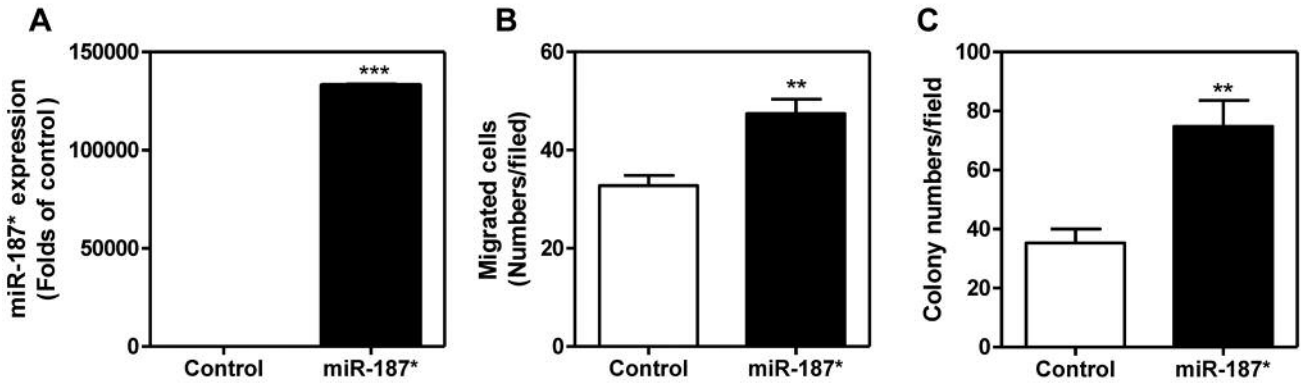

Figure 2. *miR-187* promotes the oncogenicity of SiHa cells. SiHa cells were transfected with 20 nM miR-187* mimics for 24 h. (A) Expression levels of miR-187*. (B-C) Phenotype type assays. (B) Migration. (C) Anchorage-independent growth. **p<0.001; ***p<0.0001; Mann–Whitney test.*

After analysis by the TargetScan (v7.1) *in silico* module, it was predicting that SLC26A3, GABAAR1 and ZFP36 are putative targets of *miR-187**. The predicted *miR-187** binding sequences and total context^{++} scores of BRCA1, DKK2, GABAAR1, SLC26A3 and ZFP36 are listed in Table II. Further analysis using SiHa cells showed that SLC26A3 was obviously up-regulated by *miR-187** mimic, while GABAAR1 was found to show a weak up-regulation by *miR-187** (Figure 3A). By way of contrast, after transfecting with *miR-187** mimic for 48 h, ZFP36 protein was found to be drastically decreased in SiHa cells (Figure 3C). However, the reporter assay studies demonstrated that exogenous *miR-187** expression did not suppress ZFP36 luciferase activity (Figure 3D). It was also noted that transfection with siZFP36 did suppress endogenous ZFP36 expression (Figure 3E) and that migration (Figure 3F) and anchorage-independent growth (Figure 3G) were decreased in these siZFP36 transfected cells. These findings suggest that BRCA1, DKK2, SLC26A3, GABAAR1 and ZFP36 are not *miR-187** targets and neither do they act in SiHa cells as would be expected of tumor suppressors.

WWOX is a tumor suppressor in a wide variety of cancers (14-16) and is known to be significantly down-regulated in cervical cancer (26, 27). Following on from these findings, we discovered that expression of WWOX was decreased in *miR-187** mimic transfected SiHa cells (Figure 1A and B). To explore the correlation between WWOX and *miR-187**, the presence of possible *miR-187** binding sequences was checked by comparing the WWOX 3'UTR with the mature *miR-187** sequence (5'-GGCUACAACACAGGACCCGGGC-3'). In this study, sequences that contain five continuous matched nucleotides in the seed region, and had a total matched nucleotide percentage of more than 60%, were considered as putative *miR-187** target sequences. A schematic diagram of the identified *miR-187** binding sites within the WWOX 3'UTR is presented in Figure 1C. The activity of the WWOX WtR reporter was found to be lower than that of the control reporter in cells transfected with control oligonucleotide. After transfecting with the *miR-187** mimic for 24 h, exogenous *miR-187** was able to suppress further the activity of the WWOX WtR reporter. Furthermore, this change in reporter activity was reversed in WWOX DelR reporter cells, which had had four of the possible *miR-187** binding sites deleted (Figure 1D).

miR-187 suppresses WWOX expression and functions*. The functional implications of WWOX were explored by transfecting siWWOX into SiHa cells. Compared with the scrambled control, siWWOX was able to knock-down endogenous WWOX expression (Figure 4A) and this resulted in an increase in migration (Figure 4B) and a higher level of anchorage-independent colony formation (Figure 4C) by the SiHa cells.

To further clarify the relationship between *miR-187** and WWOX, *miR-187** mimic and the WWOX overexpression construct (pEGFP-WWOX) were co-transfected into SiHa cells. After 24 and 48 h, it was found that the expression of both *miR-187** and WWOX had been increased successfully, respectively (Figure 4D). In addition, WWOX overexpression was found to suppress both migration (Figure 4E) and anchorage-independent growth (Figure 4F) of SiHa cells. In the co-transfected cells, the levels of *miR-187** and WWOX expression were slightly lower than in the cells transfected with *miR-187** mimic or pEGFP-WWOX alone (Figure 4D, Group 4). Although WWOX overexpression had no effect on *miR-187**-induced anchorage-independent growth (Figure 4F), WWOX overexpression was able to rescue *miR-187**-induced migration (Figure 4E).

miR-187 is up-regulated and WWOX is down-regulated in cervical cancer tissues*. The GEO datasets were used to analyze the expression of *miR-187** and WWOX in cervical

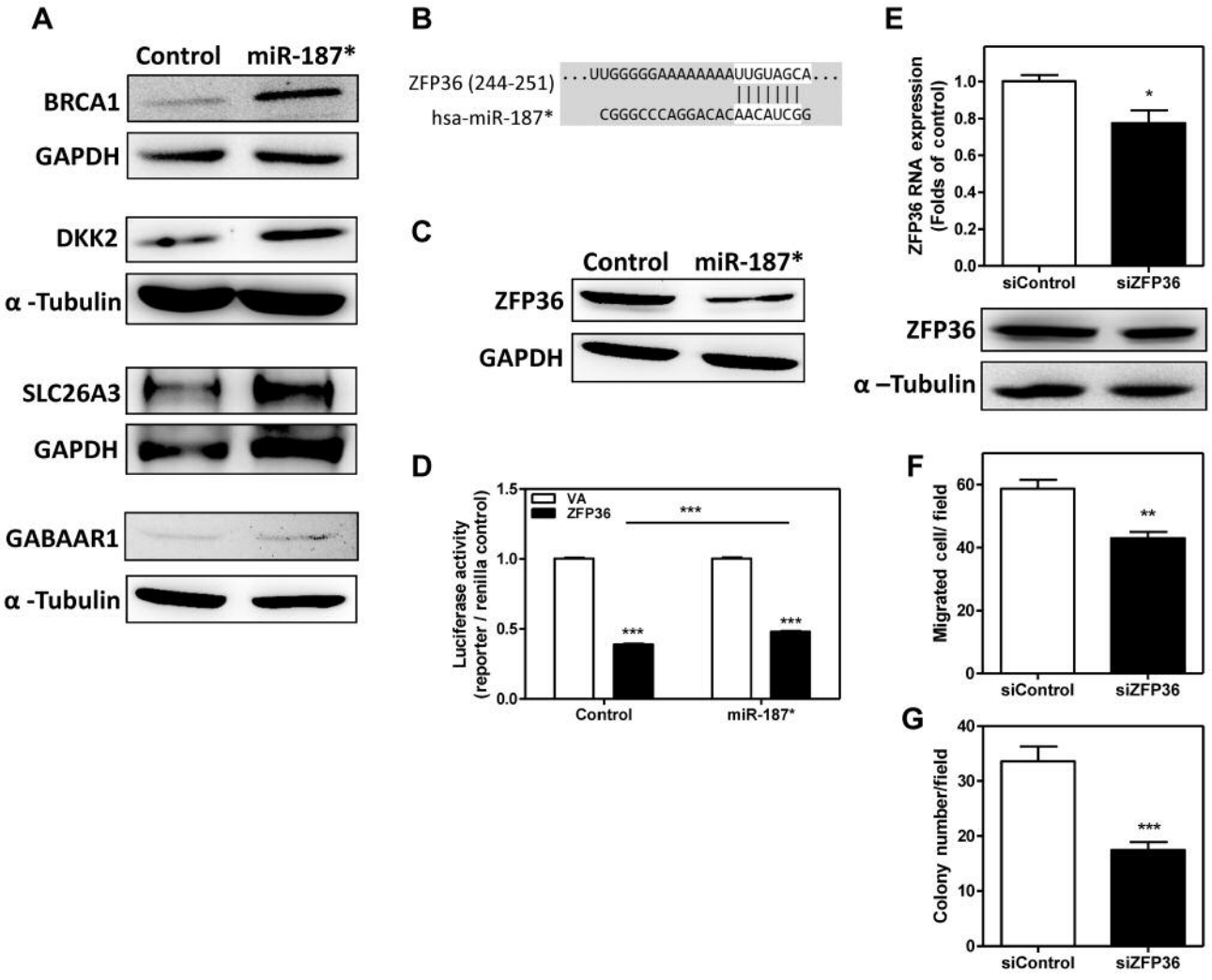

Figure 3. *Exploring potential miR-187* targets in SiHa cells. (A-D) SiHa cells were transfected with 20 nM miR-187* mimics for 24-48 h. (A) Protein expression of various potential miR-187* target genes, namely BRCA1, DKK2, SLC26A3 and GABAAR1. (B) A diagram generated by TargetScan to show the complementarity between miR-187* and the targeted sequence in the 3'UTR of the ZFP36 gene. (C) ZFP36 protein expression. (D) ZFP36 luciferase reporter assay. (E-G) SiHa cells transfected with 10 nM ZFP36 siRNA oligonucleotide for 24-48 h. (E) ZFP36 mRNA (upper) and protein (lower) expression. (F) Migration. (G) Anchorage-independent growth. *p<0.01; **p<0.001; ***p<0.0001; Mann–Whitney test.*

cancer tissue samples. In one pooled samples study (GSE105409), *miR-187** expression was found to be increased in pre-cancer lesion – cervical intraepithelial neoplasia (CIN) samples and in cervical cancer tissue samples (Figure 5A). In addition, the expressions of *miR-187** in sixteen cervical cancer tissue samples were higher than in two cervical cancer cell lines, namely HeLa and Me180 (Figure 5B), which seems to suggest that there is up-regulation of *miR-187** in cervical cancers. In contrast to the above, WWOX was significantly down-regulated in cervical cancer tissue samples (GSE30760, Figure 5C). ROC analyses showed that the expression level of WWOX in cervical cancer tissue had a predictive power of 0.73 when separating cervical cancer from normal tissue (Figure 5D). Furthermore, although patients who had a low level of WWOX expression showed a trend towards having a worse 10-year overall survival and 10-year recurrence-free survival compared to the remaining patient group, the differences were not statistically significant (detailed analysis not shown).

Discussion

In this study, we demonstrated that *miR-187** expression enhances the migration and anchorage-independent growth of

Table II. *miR-187* potential targets, prediction by TargetScan7.2.*

Target Gene	Gene name	Link to sites in UTRs	Total context++ score
BRCA1	Breast cancer 1, early onset	Position 37-43 of BRCA1 3' UTR 5' ...GUGGGUGUUGGACAGUGUAGCAC... / hsa-miR-187-5p 3' CGGGCCCAGGACACA--ACAUCGG Position 1131-1137 of BRCA1 3' UTR 5' ...GUAAAAUGUUUAUUGUUGUAGCU... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG	-0.17
DKK2	Dickkopf WNT signaling pathway inhibitor 2	Position 2057-2064 of DKK2 3' UTR 5' ...UUCUUACAGAAAGCUUUGUAGCA... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG	-0.37
GABAAR1	Gamma-aminobutyric acid (GABA) A receptor, alpha 1	Position 1402-1408 of GABRA1 3' UTR 5' ...AAGUGUUGUACCAUAUGUAGCAU... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG Position 1480-1486 of GABRA1 3' UTR 5' ...ACAUUUCAUUUAUACUGUAGCAA... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG	-0.59
SLC26A3	Solute carrier family 26 (anion exchanger), member 3	Position 203-209 of SLC26A3 3' UTR 5' ...UCUAAAUUUUAUAUCUUGUAGCU... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG Position 249-256 of SLC26A3 3' UTR 5' ...UGUUCAUACAUAUUUUUGUAGCA... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG	-0.71
ZFP36	ZFP36 ring finger protein	Position 244-251 of ZFP36 3' UTR 5' ...UUGGGGGAAAAAAAAUUGUAGCA... / hsa-miR-187-5p 3' CGGGCCCAGGACACAACAUCGG	-0.45

SiHa cells. Previous studies have shown that *miR-187* is a tumor suppressor miRNA in cervical cancers (33, 34). However, no previous study has investigated the involvement of its star-form partner, *miR-187*,* in cervical cancer. Preliminary analyses were able to exclude several genes that had been predicted to be *miR-187** targets. Notwithstanding the above, we found that *miR-187** seems to act through suppression of WWOX expression and that this suppression enhances the expression of a range of oncogenic phenotypes, in particular migration. It had been previously reported that WWOX is able to induce the apoptosis of and inhibit the proliferation of cervical cancer cells (28). WWOX protein expression has also been found to be reduced or absent in various cervical cancer tissue samples and cell lines (26). In the GEO datasets that were analyzed in this study, we found that *miR-187** was up-regulated in cervical cancer. The above studies and our results agree with the hypothesis that *miR-187** has a role in oncogenesis. This study provides novel evidence demonstrating that miR-187* is an oncomiRNA in cervical cancers that silences WWOX by epigenetic targeting.

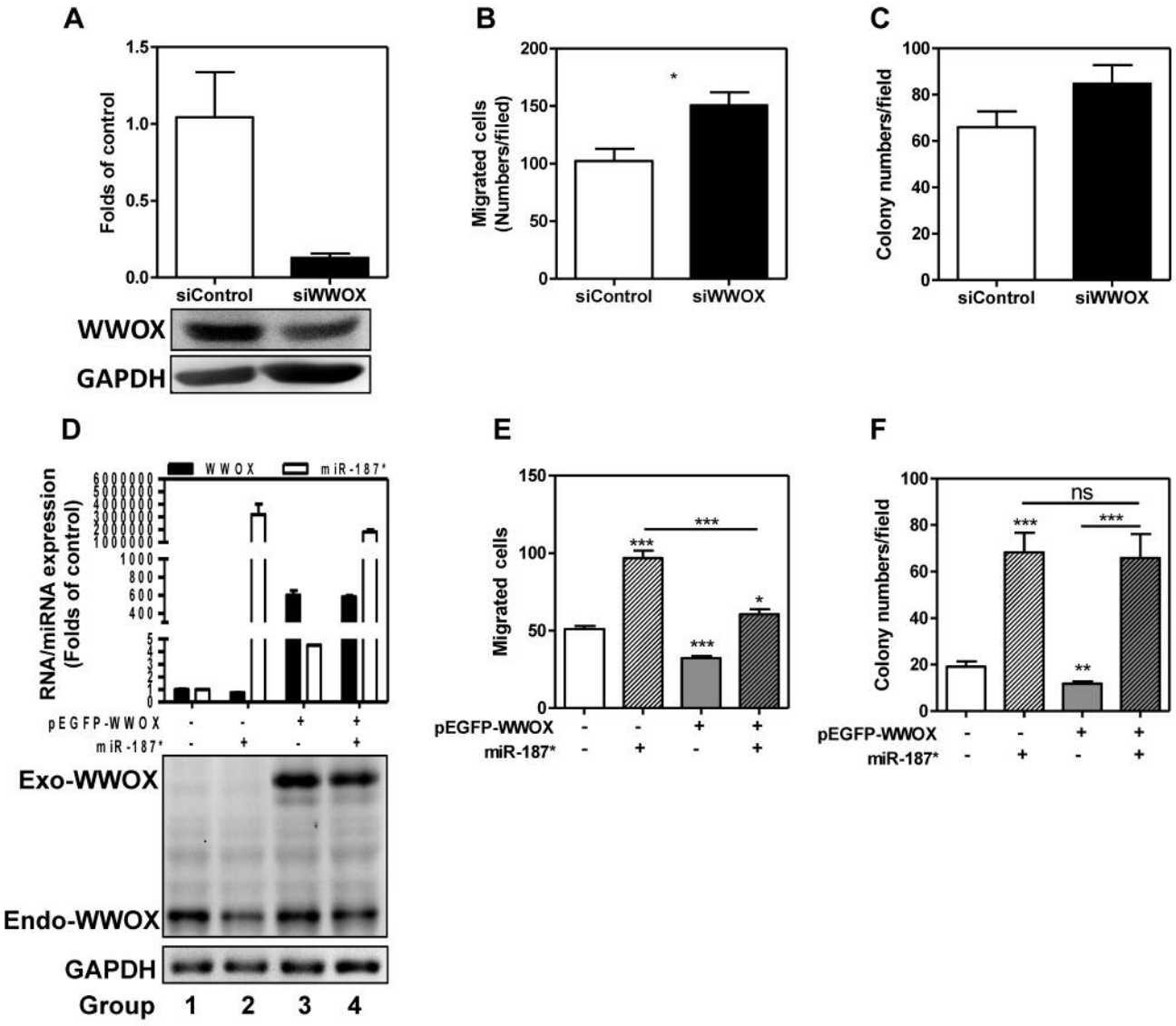

Figure 4. *WWOX participates in the oncogenic functioning of miR-187* in SiHa cells. (A-C) SiHa cells were transfected with 10 nM WWOX siRNA for 24-48 h. (A) WWOX mRNA (upper) and protein (lower) expression. (B) Migration. (C) Anchorage-independent growth (C). (D-F) pEGFP-WWOX plasmid was transfected into cells after transfecting with miR-187* mimic for 1 h. Total RNA was collected 24 h later and cell lysate was isolated 48 h later. (D) miR-187* (upper) and WWOX protein (lower) expression. (E) Migration. (F) Anchorage-independent growth. *p<0.01; **p<0.001; ***p<0.0001; Mann–Whitney test.*

Previous studies and our GEO dataset analysis show that WWOX expression loss occurs in a range of different cancers, including cervical cancer (14-17, 19, 26, 27). Epigenetic modification of CpG islands by methylation that leads to alteration in WWOX expression has been shown to occur in breast cancer (35). The chromosome region 16q23.1, which harbors FRA16D/WWOX, is a hot spot for specific deletions in many human cancers (16, 35, 36). The allelic loss associated with such deletions will result in loss of the WWOX gene in breast cancer and gastric cancer (14-16, 36). Other studies have shown that changes in the expression of various miRNAs are able to increase oncogenicity in various cancers via WWOX targeting. For example, *miR-134* has been shown to suppress WWOX expression and promoted tumorigenesis in head and neck carcinoma (37) and in lung cancer (18). Both *in vivo* and *in vitro* studies have demonstrated that *miR-24* is able to suppress the malignancy of human non-small cell lung cancer cells by targeting WWOX (38). In hepatocellular carcinoma, *miR-153* is also known to suppress WWOX expression, which then promotes

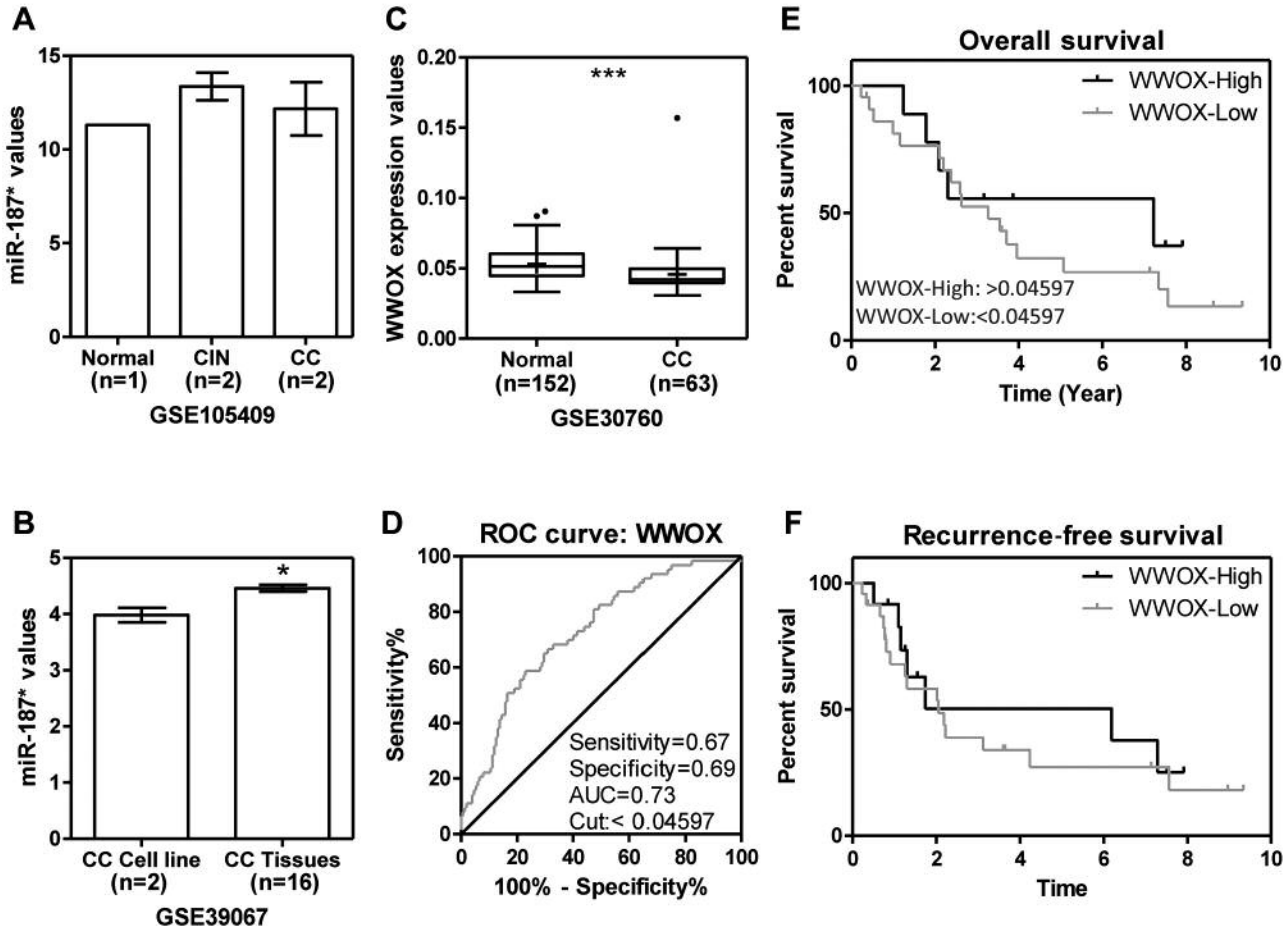

Figure 5. *The expression of miR-187* and WWOX in cervical cancer. The results of the GEO dataset analysis. (A-B) miR-187* expression. (A) GSE105409 dataset. Normal, normal cervix tissues; CIN, cervical intraepithelial neoplasia tissues; CC, cervical cancer tissues. (B) GSE39067 dataset. CC cell line – cervical cancer cell lines, HeLa and Me180. (C-F) WWOX expression levels in the GSE30760 dataset. (C) Whisker plot. Normal, normal cervix tissues; CC, cervical cancer tissues. +, mean value. (D) ROC analysis of WWOX. AUC, area under curve.*

Wnt/β-catenin activation (39). In the present study, we have shown that *miR-187** is able to suppress WWOX expression and that WWOX is involved in a novel regulation mechanism for cervical cancer.

In this study, *miR-187** has be shown to enhance the oncogenic phenotypes of SiHa cells. By way of contrast, *miR-187* has been reported to act as a tumor suppressor miRNA in SiHa cells (33, 34) and CaSki cells (33). Many studies have shown that *miR-187* and *miR-187** can have opposite expression and regulatory effects during the development of various cancers (10-12, 34, 40, 41). In addition, in another study, two miRNAs from the same precursor, miR-574-5p and miR-574-3p, have been to have reverse functionality during the same series of gastric cancer progression events (42). The above studies suggest that it is perfectly possible for *miR-187* and *miR-187** to have different, and even opposite roles, in cervical cancer.

Conflicts of Interest

The Authors declare that they have no conflicts of interest.

Authors' Contributions

All Authors contributed to the study conception and design. The experimental conception, design and manuscript preparation were performed by Su-Shun Lo and Fu-Jie Chuang. Experiments and analyses were carried out by Pei-Shih Hung, Chin-Yau Chen, Chung-Hsien Chou, and Hsi-Feng Tu. The first draft of the manuscript was written by Pei-Shih Hung and all authors commented on previous versions of the manuscript. All Authors read and approved the final manuscript.

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