miR-145 Contributes to the Progression of Cervical Carcinoma by Directly Regulating FSCN1

Li Ma¹, and Ling-Ling Li¹✉

Abstract
The purpose of our study was to investigate the underlying mechanism and functional role of microRNA-145 (miR-145) in cervical cancer. In this study, quantitative real-time PCR (qRT-PCR) was used to detect miR-145 and FSCN1 expression levels in tissues and HeLa cells. Western blotting was performed to determine the protein level of FSCN1. The luciferase assay was used to verify the direct target of miR-145. The CCK-8 assay and 2D colony formation assays were performed to determine the effects of miR-145 mimics or FSCN1 silencing on cell proliferation. miR-145 expression levels were significantly down-regulated, while FSCN1 expression levels were significantly up-regulated in the cervical carcinoma tissues compared with their matched non-cancerous tissues. In addition, FSCN1 expression levels were negatively correlated to miR-145 in tissues. Next, FSCN1 was verified as the direct target of miR-145 in HeLa cells. Moreover, overexpression of miR-145 dramatically inhibited the proliferation of HeLa cells. The silencing of FSCN1 exhibited the similar patterns on cell proliferation as miR-145 overexpression. The miR-145/ FSCN1 axis contributes to the progression of cervical cancer by inhibition of cervical cancer cell proliferation.

Keywords
microRNA (miRNA), cervical cancer, FSCN1

Introduction
Cervical cancer is one of the leading causes of cancer-related deaths worldwide, responsible for 9% and 8% of the total new cancer cases and cancer deaths, respectively¹. Cervical cancer develops either from cervical intraepithelial neoplasia or squamous intraepithelial lesions². Recently, the incidence of cervical cancer has been greatly reduced by introduction of new screening tests, diagnosis, and treatment. Despite these advances, thousands of women still suffer and die from the disease each year. Therefore, molecular biomarkers are urgently needed for cervical cancer, to open up the potential for molecular therapies.

Among molecular biomarkers, microRNAs (miRNAs) play a critical role in research. In recent years, miRNAs have become the center of interest in oncology. miRNAs, a class of short non-coding RNAs (ncRNAs) of ~22 nucleotides in length, repress the expression of target mRNA by catalyzing mRNA cleavage or by inhibiting mRNA translation³. Numerous studies have demonstrated that the loss or gain of function of specific miRNAs may be key events in the cervical cancer process⁴. miR-145 was first found to be reduced in colorectal neoplasia. Then, miR-145 dysfunction was also showed in breast, ovarian, lung, nasopharyngeal, bladder, gastric, and prostate cancers²,⁴–¹⁰. However, the biological function and molecular mechanism of miR-145 in cervical cancer still remains unclear.

In this study, we demonstrated that miR-145 was significantly down-regulated, while FSCN1 was significantly up-regulated in the cervical carcinoma tissues. In addition, the expression of miR-145 is negatively correlated with FSCN1 levels in cervical cancer tissues. Next, we verified that FSCN1 is directly repressed by miR-145. Subsequently, we observed that overexpression of miR-145 leads to decreased cell proliferation of HeLa cells. Moreover, FSCN1 silencing showed a similar effect on cell proliferation as

¹ Department of Gynecology and Obstetrics, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, Henan, P.R. China

Submitted: April 12, 2019. Revised: May 7, 2019. Accepted: June 4, 2019.

Corresponding Author:
Ling-ling Li, Department of Gynecology and Obstetrics, Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, Henan 471000, P.R. China.
Email: qdqrdm@sina.com
miR-145 overexpression. Our results imply that miR-145 may regulate the process of cervical cancer via targeting FSCN1.

Although miR-145/FSCN1 has been reported to be involved in the progression of cancer in different cancer types, this study provides the first evidence of the regulatory mechanisms of the miR 145/FSCN1 pathway in cervical cancer. Thus, our data indicate that the miR-145/FSCN1 axis may serve as a potential therapeutic candidate target for cervical cancer.

Materials and Methods

Cell Culture

HeLa cells were purchased from ATCC (Rockville, MD, USA) and cultured in RPMI 1640 (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, penicillin and streptomycin (100 U/mL, and 100 μg/mL). The cells were incubated at 37°C in a humidified 5% CO2 and 95% air atmosphere.

qRT-PCR

qRT-PCR was used to determine the expression levels of miR-145 and FSCN1 in cells and tissues. Total RNA was extracted from HeLa cells and tissues with TRI Reagent (Molecular Research Center, Cincinnati, OH, USA). Residual DNA was removed using DNA-free DNase (Ambion, Austin, TX, USA). Reverse transcription was performed using the TaqMan™ advanced miRNA cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The analysis of miR-145 levels was performed using the TaqMan Reverse Transcription Kit and TaqMan MicroRNA Assays Kit with U6 as an internal control (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s instructions.

FSCN1 quantitation was determined by SYBR Green I detection with gene-specific primers (Forward primer: 5’-GGAGACCGACCAGGAGAC-3’, reverse: 5’-GATTTG-GAGCCTCAGTG-3’) on an ABI 7500 system (Applied Biosystems). GAPDH (Forward Primer, 5’-CCTGCAC-CACCAACTGCTTA-30. Reverse Primer, 5’-GGCCATC-CACAGTCTTCTGAG-30.) was used as an internal control.

All independent PCR-based reactions were performed in triplicate. The selected internal controls for miRNA and gene were based on previous studies. The amount of each gene and miRNA relative to their internal control was calculated using the comparative Ct method.

3’ UTR Luciferase Assay

The 3’ UTR of FSCN1 identified by Targetscan was cloned into the pmirGLO vector (Promega, Madison, WI, USA). HeLa cells were seeded in a 96-well plate at the density of 2×10^4 per well. The cells were co-transfected with miR-145 mimic or negative control (miR-Ctrl) and 3’ UTR vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) with Opti-MEM (Thermo Fisher) as previously described. Then a mutant 3’UTR plasmid was created by site-directed mutagenesis at the four miR-145 binding sites (Promega). After 48 h incubation, the cells were lysed for measurement of luciferase activities using the Dual Luciferase Reporter Assay System (Promega). The firefly luciferase signal was normalized to the Renilla luciferase activity.

miR-145 Overexpression

HeLa cells were seeded in the 6-well plates (1×10^5 per well) the day before transfection, and then transfected with 100 nM of miR-145 mimic or miR-Ctrl by the RNAiMAX Reagent according to manufacturer protocols (Invitrogen).

FSCN1 Silencing

Small interfering RNA for FSCN1 (si-FSCN1) and the respective control (si-Ctrl) were purchased from Gene-Pharma (San Diego, CA, USA). The cells were transfected with 100nM Si-FSCN1 and si-Ctrl by the Lipofectamine 2000 Reagent according to manufacturer protocols (Invitrogen).

Western Blotting

The whole cells were lysed in lysis buffer (Thermo Fisher), and protein concentrations were quantified with Pierce BCA Protein Assay Kit (Thermo Fisher). Protein samples were separated with 4–15% polyacrylamide SDS gels and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The primary antibodies were used: anti-FSCN1 at 1:5000 dilution (ab49815, Abcam, Cambridge, UK) and anti-GAPDH at 1:5000 (G9545, Sigma, St. Louis, MO, USA) followed by the HRP-conjugated secondary antibodies (Sigma). The signals were determined using an enhanced...
chemiluminescence (Thermo Fisher). All experiments were performed in triplicate. Separate blots were used for each independent experiment to avoid problems related to incomplete membrane stripping.

**Cell Proliferation**

Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Gaithersburg, MD, USA) allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation. It is based on WST-8 reduced by dehydrogenases in cells to give an orange-colored product (formazan). The amount of the formazan dye produced in cells is directly proportional to the number of living cells. HeLa cells were seeded at the density of $5 \times 10^3$ cells per well in the 96-well plates. At the indicated time-point, CCK8 solution was added to each well and incubated for 1 h following the manufacturer’s protocol.

**2D Colony Formation Assay**

HeLa cells were released from clusters by trypsin and cell counts were made. The suspended cells were seeded in the 6-well plate at the density of 1,000 cells per well. After 14 days of culture, the cells were fixed in 80% ethanol and dyed with crystal violet solution (Millipore).

**Statistical Analysis**

SPSS17.0 for Windows (SPSS, Inc., Chicago, IL) was used to execute the statistical analysis. Data were presented as the mean ± standard errors from at least three independent experiments, and the difference between two conditions was analyzed by the two-tailed, unpaired Student’s t-test. The statistical significance was defined as $p < 0.05$. The Spearman’s rank correlation analysis was performed to analyze the correlation. $p$ (probability) $< 0.05$ was considered to indicate a statistically significant difference.

**Results**

miR-145 is Down-regulated in Cervical Cancer Tissues

miR-145 has been found to be tumor-suppressive in various cancer types; however, data are still limited regarding miR145 and cervical cancer. In this study, we first assessed whether or not miR-145 was dysregulated in cervical cancer tissues. miR-145 expression levels from 13 pairs of cervical cancer tissues and their adjacent non-cancerous tissues were
analyzed by qRT-PCR. We found significantly lower levels of miR-145 in cervical cancer tissues compared with their matched non-cancerous tissues (Fig. 1). These results suggested that miR-145 down-regulation could contribute to the tumor progression.

FSCN1 is Up-regulated and Negatively Correlated with miR-145 in Cervical Cancer Tissues

FSCN1 overexpression has been indicated in patients with breast cancer\(^{16}\). FSCN1 has also been verified as a direct target of miR-145 in other cancer types\(^{17,18}\). We determined FSCN1 expression levels in the 13 pairs of cervical cancer tissues and their adjacent non-cancerous tissues using qRT-PCR. As shown in Fig. 2A, FSCN1 mRNA expression levels are significantly up-regulated 2.5 fold in tumor tissues compared with non-cancerous tissues. The protein levels of FSCN1 were also higher in tumor tissues than in non-cancerous tissues (Fig. 2B). In cervical cancer tissues, miR-145 was down-regulated while FSCN1 was up-regulated. To elucidate whether FCSN1 interacts with miR-145 in cervical cancer, we executed a correlation analysis between the expression of FSCN1 and miR-145. The protein expression of FSCN1 levels correlated inversely with miR-145 (Fig. 2C).

miR-145 Directly Suppresses FSCN1

To elucidate whether FSCN1 is directly repressed by miR-145, HeLa cells were co-transfected with the FSCN1 3’-UTR and miR-145 mimic or miR-Ctrl. Luciferase reporter assay results showed that co-transfection with miR-145 obviously reduced the luciferase activity of the FSCN1 3’-UTR wild-type (WT) reporter gene, but not the mutant reporter gene (Fig. 3A), but miR-Ctrl had no effect on the luciferase activity of both WT and mutant (Fig. 3A). Next, we detected the expression level of FSCN1 after miR-145 overexpression (Fig. 3B) by qRT-PCR and Western blotting in HeLa cells. We noticed that the FSCN1 mRNA and protein levels were significantly decreased by miR-145 mimic compared with the miR-Ctrl (Fig. 3C, D).

miR-145 Inhibits Cell Proliferation Through Regulating FSCN1

To decipher the biological role of miR-145/FSCN1 in the progression of cervical cancer, we monitored the cell proliferation rate after miR-145 overexpression. As shown in Fig. 4A, CCK-8 cell proliferation assays indicated that proliferation was inhibited when HeLa cells were transfected with miR-145 mimics compared with miR-Ctrl and mock
cells. Furthermore, the 2D colony formation assay also revealed that miR-145 overexpression dramatically inhibited HeLa cell growth (Fig. 4B). Next, we silenced FSCN1 to assess whether FSCN1 also involved in the regulation network. We observed similar patterns of decreased cell proliferation after FSCN1 silencing in HeLa cells (Fig. 4C, D). Our data showed that miR-145/FSCN1 signaling could significantly inhibit the proliferation of cervical cancer cells.

Discussion

A growing body of studies have suggested that miRNAs are important regulators in various cellular processes, such as cell cycle control, growth, apoptosis, and migration. Aberrant expression of miRNAs has been reported to be associated with a broad spectrum of human diseases, including cancer. miR-145 has been frequently reported as a tumor suppressor in various kinds of cancers. miR-145 up-regulation has been found to inhibit SW620 human colon cancer cells. miR-145 has also been observed to inhibit invasion of bladder cancer cells by targeting PAK1. In addition, miR-145 has been shown to suppress human rhabdomyosarcoma cell proliferation and invasion by targeting ADAM19. However, elucidating the features of expression and roles of miR-145 in cervical cancer remains an ongoing process. In this study, miR-145 was found to be downregulated in cervical carcinoma tissues compared with the matched non-cancerous tissues. Moreover, the overexpression of miR-145 can inhibit HeLa cell proliferation.

In order to understand the effects of miR-145 on the progression of cervical cancer, we studied the target gene regulated by miR-145. Fascin actin-bundling protein 1 (FSCN1) was verified as the direct target of miR-145 in esophageal cancer. FSCN1 has also been shown to contribute to tumor suppression in gastric cancer cells. FSCN1 has also been found as a biomarker for the development and progression of breast cancer. In this study, FSCN1 was found to be upregulated in cervical carcinoma tissues. In addition, FSCN1 was also identified as a direct target of miR-145 by 3’ UTR luciferase assay and Western blotting. Furthermore, FSCN1 silencing exhibited similar patterns of decreased cell proliferation as miR-145 overexpression. Future study will need to verify the in vitro results in an in vivo animal model.

In summary, this study suggests that miR-145 can inhibit cell growth in HeLa cells in vitro through directly regulating
FSCN1. This newly identified miR 145/FSCN1 pathway implicates that tight regulation of the miR-145/FSCN1 pathway may be a basis for therapeutic applications in cervical cancer in the future.

**Ethical Approval**
The study was approved by the Institutional Ethics Committee of Luoyang Central Hospital Affiliated to Zhengzhou University, Luoyang, Henan, China.

**Statement of Human and Animal Rights**
This article does not contain any studies with human or animal subjects.

**Statement of Informed Consent**
There are no human subjects in this article and informed consent is not applicable.

**Declaration of Conflicting Interests**
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**
The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the 2018 Henan Province medical science and technology research plan joint construction project (No.B2018108), Luoyang Central Hospital Incubation project, and the 2018 Henan Province medical science and technology research plan joint construction project (No.2018—FY17).

**ORCID iD**
Ling-Ling Li https://orcid.org/0000-0001-9194-2527

**References**
1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jamal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin. 2018;68(6):394–424.
2. Park TW, Fujiwara H, Wright TC. Molecular biology of cervical cancer and its precursors. Cancer. 1995;76(10 Suppl):1902–1913.
3. Farazi TA, Hoell JI, Morozov P, Tuschl T. MicroRNAs in human cancer. Adv Exp Med Biol. 2013;774(2):1–20.
4. Pardini B, De Maria D, Francavilla A, Di Gaetano C, Ronco G, Naccarati A. MicroRNAs as markers of progression in cervical cancer: a systematic review. BMC Cancer. 2018;18(1):696.
5. Iorio MV, Ferracin M, Liu CG, Veronese A, Spizzo R, Sabbioni S, Magri E, Pedriali M, Fabbri M, Campiglio M, Menard S, et al. MicroRNA gene expression deregulation in human breast cancer. Cancer Res. 2005;65(16):7065–7070.
6. Iorio MV, Visone R, Di Leva G, Donati V, Petrocca F, Casalini P, Taccioli C, Volland S, Liu CG, Alder H, Calin GA, et al. MicroRNA signatures in human ovarian cancer. Cancer Res. 2007;67(18):8699–8707.
7. Michael MZ, Young GP, James RJ. Reduced accumulation of specific microRNAs in colorectal neoplasia. Mol Cancer Res. 2003;1(12):882–891.
8. Takagi T, Iio AY. Decreased expression of microRNA-143 and -145 in human gastric cancers. Oncology. 2009;77(1):12–21.
9. Ichimi T, Enokida H, Okuno Y, Kunimoto R, Chiyoumaru T, Kawamoto K, Kawahara K, Toki K, Kawakami K, Nishiyama K, Tsujimoto G, et al. Identification of novel microRNA targets based on microRNA signatures in bladder cancer. Int J Cancer. 2009;125(2):345–352.
10. Liu X, Sempere LF, Galimberti F, Freeman MJ, Black C, Dragnev KH, Ma Y, Fiering S, Memoli V, Li H, DiRenzo J, et al. Uncovering growth-suppressive MicroRNAs in lung cancer. Clin Cancer Res. 2009;15(4):1177–1183.
11. Luo M, Gao Z, Li Q, Zhang C, Xu W, Song S, Ma C, Wang S. Selection of reference genes for miRNA qRT-PCR under abiotic stress in grapevine. Sci Rep. 2018;8(1):4444.
12. Su J, Zhang R, Dong J, Yang C. Evaluation of internal control genes for qRT-PCR normalization in tissues and cell culture for antiviral studies of grass carp (Ctenopharyngodon idella). Fish Shellfish Immunol. 2011;30(8):830–835.
13. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 2001;25(4):402–408.
14. Liu R, Liao J, Yang M, Sheng J, Yang H, Wang Y, Pan E, Guo W, Pu Y, Kim SJ, Yin L. The cluster of miR-143 and miR-145 affects the risk for esophageal squamous cell carcinoma through co-regulating fascin homolog 1. PLoS One. 2012;7(3):e33987.
15. Skjefstad K, Johannessen C, Grindstad T, Kilvaer T, Paulsen EE, Pedersen M, Donnem T, Andersen S, Brennes R, Richardsen E, Al-Saad S, et al. A gender specific improved survival related to stromal miR-143 and miR-145 expression in non-small cell lung cancer. Sci Rep. 2018;8(1):8549.
16. Wang CQ, Tang CH, Wang Y, Jin L, Wang Q, Li X, Hu GN, Huang BF, Zhao YM, Su CM. FSCN1 gene polymorphisms: biomarkers for the development and progression of breast cancer. Sci Rep. 2017;7(1):15887.
17. Li Y-Q, He Y-M, Ren X-Y, Tang X-R, Xu Y-F, Wen X, Yang X-J, Ma J, Liu N. MiR-143 inhibits metastasis by targeting fascin actin-bundling protein 1 in nasopharyngeal carcinoma. Plos One. 2015;10(3):e0122228.
18. Zhang Y, Lin Q. MicroRNA-145 inhibits migration and invasion by down-regulating FSCN1 in lung cancer. Int J Clin Exp Med. 2015;8(6):8794–8802.
19. Adams BD, Kasinski AL, Slack FJ. Aberrant regulation and function of microRNAs in cancer. Curr Biol. 2014;24(16):R762–R776.
20. James CD. Aberrant miRNA expression in brain tumors: a subject attracting an increasing amount of attention. Neuro Oncol. 2013;15(4):405.
21. Montagnana M, Benati M, Danese E, Giudici S, Perfranceschi M, Ruzzeneñete O, Salvagno GL, Bassi A, Gelati M, Paviati E,
Guidi GC, et al. Aberrant microRNA expression in patients with endometrial cancer. Int J Gynecol Cancer. 2017;27(3):459–466.

22. Eades G, Wolfson B, Zhang Y, Li Q, Yao Y, Zhou Q. lncRNA-RoR and miR-145 regulate invasion in triple-negative breast cancer via targeting ARF6. Mol Cancer Res. 2015;13(2):330–338.

23. Yin Y, Yan ZP, Lu NN, Xu Q, He J, Qian X, Yu J, Guan X, Jiang BH, Liu LZ. Downregulation of miR-145 associated with cancer progression and VEGF transcriptional activation by targeting N-RAS and IRS1. Biochim Biophys Acta. 2013;1829(2):239–247.

24. Li C, Xu N, Li YQ, Wang Y, Zhu ZT. Inhibition of SW620 human colon cancer cells by upregulating miRNA-145. World J Gastroenterol. 2016;22(9):2771–2778.

25. Kou B, Gao Y, Du C, Shi Q, Xu S, Wang CQ, Wang X, He D, Guo P. miR-145 inhibits invasion of bladder cancer cells by targeting PAK1. Urol Oncol. 2014;32(6):846–854.

26. Sun Z, Zhang A, Jiang T, Du Z, Che C, Wang F. miR-145 suppressed human retinoblastoma cell proliferation and invasion by targeting ADAM19. Int J Clin Exp Pathol. 2015;8(11):14521–14527.

27. Li J, Lu J, Ye Z, Han X, Zheng X, Hou H, Chen W, Li X, Zhao L. 20(S)-Rg3 blocked epithelial-mesenchymal transition through DNMT3A/miR-145/FSCN1 in ovarian cancer. Onco-target. 2017;8(32):53375–53386.

28. Zhang M, Dong BB, Lu M, Zheng MJ, Chen H, Ding IZ, Xu AM, Xu YH. miR-429 functions as a tumor suppressor by targeting FSCN1 in gastric cancer cells. Onco Targets Ther. 2016;9(Issue 1):1123–1133.