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***Basic Study***

**Inhibition of SW620 human colon cancer cells by up-regulating microRNA-145**

Li C *et al*. SW620 inhibition and microRNA-145

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**Abstract**

**AIM:** To investigate the targeted inhibition of proliferation and migration of SW620 human colon cancer cells by up-regulating microRNA-145 (miR-145).

**METHODS:** Forty five samples of colon cancer tissues and 45 normal control samples were obtained from the biological database of the First Affiliated Hospital of Liaoning Medical University. Quantitative of miR-145 and N-ras expression in tissues, reverse transcription polymerase chain reaction analysis of miR-145 expression in colon cancer cell SW620 and normal colonic epithelial cells, construction miR-145 lentiviral vector and determination of miR-145 expression in SW620 transduced with miR-145 vector; analysis of miR-145 overexpression on SW620 proliferation. Analysis of miR-145 overexpression on SW620 migration using wound healing test, analysis of miR-145 on N-ras expression using Western blot.

**RESULTS:** MiR-145 expression was significantly down-regulated in colon cancer tissues with its expression in normal colonic tissues being 4-5 fold higher (two sample *t*-test, *P <* 0.05), whereas N-ras expression showed the opposite trend; miR-145 expression in SW620 was down-regulated, which was significantly lower as compared to colonic epithelial cells (two sample *t*-test, *P <* 0.05). miR-145 vector and control were successfully packaged; expression of miR-145 in SW620 transduced with miR-145 was 8.2-fold of that in control (two sample *t*-test, *P <* 0.05), the proliferation of miR-145-transduced SW620 was significantly decreased as compared to control (two sample t-test, *P <* 0.05). At 48 h in wound healing experiment, the migration indexes and control were (97.27% ± 9.25%) and (70.22% ± 6.53%), respectively (two sample *t*-test, *P <* 0.05). N-ras expression in miR-145-tranduced SW620 was significantly lower than others (one-way ANOVA, *P <* 0.05).

**CONCLUSION:** MiR-145 plays a critical role in inhibiting colon cancer cell proliferation and migration. This finding lays a good foundation for development of potential future colon cancer therapy by targeting tumor suppressor miR-145.

**Key words:** MicroRNA-145; Colorectal cancer; N-ras; Targeted therapy; Lentiviral vector

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**Core tip**: MicroRNA-145 (miR-145) may play an important role in inhibiting colon cancer proliferation and invasive migration. This finding lays a good foundation for further investigation on targeting miR-145 as a tumor suppressor in colon cancer treatment, and provides novel evidence for the anti-cancer effects and therapeutic potential of miR-145.

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**INTRODUCTION**

Colorectal cancer is currently one of the most common malignancies worldwide[[1](#_ENREF_1),[2](#_ENREF_2)]. Invasive migration at early stages is a critical factor affecting the prognosis and survival of colorectal cancer patients[[3](#_ENREF_3),[4](#_ENREF_4)]. Despite increasingly improving diagnosis and medical technologies, treatment effects and clinical prognosis are still not ideal. With the development of genetic therapy, targeted therapy has become the top level specific anti-cancer treatment. Blocking invasive migration by modifying the microenvironment on the molecular basis is the frontier of current research in colorectal cancer therapeutics[[5-7](#_ENREF_5)].

Recently, the discovery of a class of small non-coding RNA known as microRNA (miRNA) that regulates gene expression at the level of transcription shed new light on cancer research[[8](#_ENREF_8),[9](#_ENREF_9)]. miRNAs are endogenous, revolutionarily conserved, single chain non-coding RNA consisting of19-22 nucleotides, which inhibit mRNA transcription or induce mRNA degradation by base-paring with the 3’UTR of target mRNA, and thus play an important role in cell proliferation, differentiation, apoptosis, gene regulation, and tumor formation by suppressing the expression of target genes[[10](#_ENREF_10),[11](#_ENREF_11)]. Although current understanding of the biological function of miRNAs is still limited, we know for certain that miRNAs play a critical role in many aspects including modulating development, growth, differentiation, apoptosis, and tumorigenesis. In some malignant tumors, miRNAs function like oncogenes or tumor suppressors by regulating different gene targets. The function of specific miRNA during tumor formation and development is becoming a hot area in cancer research. Some experts predicted that targeting miRNA in biological cancer therapeutics will be more effective than targeting coding genes[[12](#_ENREF_12)].

Earlier studies reported that microRNA-145 (miR-145) expression is down-regulated in many cancers including breast cancer, melanoma, ovarian cancer, and liver cancer, suggesting that miR-145 may function as a tumor suppressor[[13-16](#_ENREF_13)]. However, so far there has been no systemic research on the role of miR-145 in colorectal cancer. Therefore, we investigated the expression of miR-145 and its target gene N-ras in colon cancer tissues and normal control tissues, and postulated that miR-145 may play a biological role in colon cancer development and metastasis. Next, we constructed recombinant lentiviral vector expressing miR-145 and examined the inhibitory function of miR-145 after its transduction into SW620 colon cancer cells as an experimental basis for continued research on targeting miR-145 in colon cancer treatment. Lastly, we analyzed the expression of miR-145 target N-ras to clarify the regulatory role of miR-145 in colon cancer.

**MATERIALS AND METHODS**

***Materials and reagents***

Forty five samples of colon cancer tissues and 45 normal control samples were from the biological database of the First Affiliated Hospital of Liaoning Medical University. These samples were obtained from colon cancer patients attending the hospital between January 2013 and December 2013. Patients had no treatments before surgery. All patients gave written informed consent to the study, which was reviewed and approved by the First Affiliated Hospital of Liaoning Medical University Institutional Review Board. pSilencerTM 4.1 lentiviral vector system and Trizol reagent were purchased from Invitrogen. HEK293T cell line, SW620 colon cancer cell line, and normal human colonic epithelial cell line were purchased from ATCC. DMEM, FBS, and trypsin were from Gibco. Ultra-pure plasmid extraction kit was from Promega. Restriction endonucleases, DNA polymerase, plasmid miniprep kit, one-step reverse transcription polymerase chain reaction (RT-PCR) kit, and gel extraction kit were from TaKaRa. Monoclonal rabbit anti-human primary antibodies, secondary antibodies, and internal controls were from Santa Cruz. miR-145 and internal control U6 primer were designed and synthesized by TaKaRa (Table 1). Other common reagents were provided by the Central Laboratory of the First Affiliated Hospital of Liaoning Medical University.

***Determine miR-145 and N-ras expression levels in tissue samples***

One milliliter of Trizol reagent was added to 100 mg or less of frozen samples. After grinding in liquid nitrogen, total RNA was extracted using phenol-chloroform protocol. Quality of RNA was analyzed on 1% agarose gels. Qualified RNA samples were sent to TaKaRa for sequencing and quantitative analysis of miR-145 and N-ras expression in colon cancer and control tissues.

***Determine miR-145 expression levels in SW620 and normal colonic epithelial cells***

SW620 and normal colonic epithelial cells were grown to 3rd passage of exponential growth phase, and total RNA was extracted using phenol-chloroform protocol. Semi-quantitative one-step RT-PCR analysis of miR-145 expression was carried out according to the instruction provided by the manufacturer. PCR products were analyzed on 1% agarose gels and OD values were read on a gel imaging system. Experiments were repeated three times to compare miR-145 expression levels in these two cell lines.

***Construction and packaging of miR-145 lentiviral expression vector***

The complete sequence of miR-145 was PCR-amplified using up-stream and down-stream primers and purified by gel extraction. pSilencerTM 4.1 vector was linearized and purified according to the instruction provided with the lentiviral vector kit, and then ligated with miR-145 sequence. DH5α competent cells were then transformed with the ligation reaction. Positive clone was selected, and the sequence was amplified and linearized. miR-145 lentiviral vector was then packaged in HEK 293T cells using Lipofectamine 2000, and viral titer was determined. The control vector containing a scrambled unrelated sequence was constructed and packaged in the same way.

***Determine miR-145 expression levels in transduced SW620 cells***

SW620 cells that were 90% confluent were passaged at 1:3 at 18-24 h before transduction so that cells were 70%-80% confluent and at exponential growth stage at the time of transduction. SW620 cells were then transduced with successfully packaged miR-145 lentiviral vector or control vector with 50% multiplicity of infection (MOI = 50) and cultured for 48-72 h. When cytopathic effect (CPE) occurred, total RNA was extracted from each of the two groups. PCR products were analyzed on 1% agarose gels and OD values were read on a gel imaging system. Experiments were repeated three times to compare miR-145 expression levels in two groups of cells in order to confirm the successful construction of the expression system.

***Effect of miR-145 overexpression on SW620 cell proliferation***

SW620 colon cancer cells were grown to 3rd passage of exponential growth phase, and seeded into 96 well plates. Cells were then transduced with either of the two lentiviral vectors mentioned above. 20 μL of 5 mg/mL MTT solution was added to each well. After incubation for 4 h at 37 ℃, media were removed and 150 μL of DMSO was added. The absorption was then read on a plate reader at 490 nm, and proliferation curves were generated.

***Effect of miR-145 overexpression on SW620 cell migration***

SW620 cells at 3rd passage were plated into two sets of dishes. When cells were confluent, a homogenous scratch wound was created on the monolayer cells using a 1000 μL pipette tip to form a band of open area. MiR-145 lentiviral solution was then added to the experimental group. Media containing no lentivirus was used for the blank control group. Cells were imaged at 0 or 48 h after incubation. The width of the open area was measured at 3 different positions (top, middle, and bottom) and the average width was calculated. The speed of cell migration was described using migration index (MI).

***Effect of miR-145 overexpression on the expression of its target gene N-ras***

Using bioinformatics, we observed that miR-145 may induce colon cancer cell apoptosis *via* N-ras. In other words, N-ras gene might be a target of miR-145. We therefore further tested this at protein level. Total proteins were extracted from miR-145-tranduced SW620 cells and quantified. After separation on 5% stacking gel and 8% separating gel at 60 V-30 min and 100 V-1.5 h, proteins were transferred to a membrane and ponceau stained. After distaining for 3 times, blots were incubated with primary antibody (1:1000, monoclonal rabbit-anti-human) at 4 ℃ overnight. After incubation for 1 h at room temperature with secondary antibody (1:1500, polyclonal goat-anti-rabbit) and BCIP/NBT staining in dark for 3 h, blots were analyzed on a gel imaging system for target bands and internal reference bands. Experiments were repeated 3 times and OD values were calculated.

***Statistical analysis***

SPSS19.0 for Windows was used for statistical analysis. Data were expressed as mean ± SD. Differences among groups were compared using one-way ANOVA. *P <* 0.05 was considered statistically significant.

**RESULTS**

***Expression of miR-145 in colon cancer tissues and normal control tissues***

RNA sequencing and quantitative analysis indicated that miR-145 expression level was markedly decreased in colon cancer tissues as compared to normal colonic tissues. miR-145 level in control tissues was 4-5-fold higher than that in colon cancer tissues (*P <* 0.05). In contrast, N-ras expression level was significantly higher in colon cancer tissues then in control tissues (Figure 1).

***Expression of miR-145 in SW620 and normal human colonic epithelial cell line***

RT-PCR analysis revealed that miR145 expression level in normal colonic epithelial cells was relatively high whereas its expression level in SW620 colon cancer cells was markedly down-regulated. There was an approximately 5-fold difference, which was statistically significant (*P <* 0.05) (Figure 2).

***Results of construction and packaging of miR-145 recombinant lentiviral vector and control vector***

Construction of lentiviral vector and negative control sequence was successful, and sequencing results indicated no mutations. Both sequences were consistent with the expectation, indicating that insertion of miR-145 sequence was successful, and could be used for further experiments. Sequencing was done by TaKaRa. At 72 h post transfection of HEK293T cells with lentiviral vector system, we observed green fluorescence under fluorescence microscope. 80%-90% of cells expressed green fluorescence. Meanwhile we observed CPE phenomenon: shrinking, swelling and rounding of cells; some cells were detached and floating (Figure 3A and B). Viral titers were 2.08 × 109 TU/mL in experimental group and 1.92×109TU/ml in control group. Both met the requirement of the study and could be used for further experiments.

***MiR-145 expression in transduced SW620 cells***

RT-PCR data showed that miR-145 expression in SW620 cells transduced with control lentiviral vector was relatively low, whereas its expression in miR-145-tranduced SW620 cells was 8.2-fold higher. The difference was statistically significant (*P <* 0.05) (Figure 4), indicating the success of generating miR-145 lentiviral transfection system for future experiments.

***Effect of miR-145 overexpression on SW620 cell proliferation***

Two groups of SW620 cells were cultured separately for 24, 48, 72, 96, or 120 h followed by MTT analysis. Plates were read on a plate reader and proliferation curves were generated based on OD values. Results indicated that SW620 cells of the control group proliferated rapidly, whereas cells of the experimental group had significantly slower growth (*P <* 0.05), suggesting that miR-145 lentiviral vector can markedly inhibit the proliferation of SW620 colon cancer cells (Figure 5).

***Wound healing study for analysis of the effect of miR-145 on SW620 cell migration***

Our data showed that at 48 h after scratching the monolayer of cells, the wound in the control group was basically healed. miR-145 group showed a small number of cells migrating to the open area, and the wound was still apparent. The MI in controls and experimental groups were 97.27% ± 9.25% and 70.22% ± 6.53%, respectively. The differences between the two groups were statistically significant (*P <* 0.05), suggesting that up-regulating the expression of miR-145 can markedly inhibit the migration of SW620 cells (Figure 6).

***Western blot analysis of miR-145 regulation of N-ras expression***

Western blot analysis revealed that N-ras expression level in miR-145-tranduced SW620 cells was significantly reduced as compared the empty vector control and blank control groups (*P <* 0.05), whereas empty vector control and blank control groups had similar N-ras expression levels (*P <* 0.05), suggesting that miR-145 down-regulates N-ras protein expression by effectively suppressing N-ras mRNA translation (Figure 7).

**DISCUSSION**

Currently, the genes and molecular mechanisms involved in colon cancer development and progression have become a hot research area[[17](#_ENREF_17)]. Studies have shown that many miRNAs are abnormally expressed in colon cancer tissues and cell lines. Among them, miR-143 is down-regulated whereas miR-21 and miR-223 are up-regulated in colon cancer. They each function by regulating different target genes and are tightly related to each other during colon cancer development and progression[[18-20](#_ENREF_18)]. It has been demonstrated that miRNAs down-regulated in cancers often function as tumor suppressors, while miRNAs up-regulated in cancers often function as oncogenes[[21](#_ENREF_21)]. Various miRNAs and their target genes play important roles in colon cancer, but their specific mechanisms remain to be investigated.

MiR-145, like miR-143, is down-regulated in various cancers, but the degree of down-regulation varies in different cancers[[22](#_ENREF_22)]. Research has shown that miR-145 is down-regulated by approximately 2.5-fold in breast cancer, 10-fold in nasal cancer and 20-fold in bladder cancer[[23-26](#_ENREF_23)]. We have observed that miR-145 expression in colon cancer is 4-5-fold lower than normal colonic tissues, and the degree of its down-regulation is positively correlated with colon cancer progression. In the present study, we not only demonstrated miR-145 down-regulation in SW620 colon cancer cells but also found that the expression level of miR-145 in normal human colonic epithelial cells can be 5 times higher than that in SW620 cells. The above evidences support that miR-145 may play an important role in the development and progression of various cancers.

Because miR-145 expression is decreased in SW620 cells, we chose to further examine its function by up-regulating its expression. In this study, we used lentiviral vector to express miR-145. Lentiviral vector is a novel vector type developed in recent years, which has high transfection efficiency and low cellular immune response and exerts its silencing effect *via* gene integration[[27](#_ENREF_27),[28](#_ENREF_28)]. After successful construction of lentiviral vectors, HEK293T cells were transfected for viral packaging. Both transfection efficiency and viral titers met experimental requirement, so the vectors could be used for further experiments.

In terms of inhibiting SW620 proliferation, we found that cells in blank control group proliferated exponentially at 24 h, 48 h, 72 h, 96 h, and 120 h, while the proliferation rate of cells in experimental group (miR-145-tranduced SW620 cells) was significantly lower compared to blank control group, indicating that miR-145 can markedly suppress SW620 cell proliferation. This suggests that miR-145 may play a critical role in inhibiting colon cancer growth.

Cancer cells with metastasis potential have migratory ability, and cells with high metastasis potential often have more active migratory ability[[29](#_ENREF_29),[30](#_ENREF_30)]. We used wound healing assay to examine colon cancer cell migration. Our data showed that overexpression of miR-145 using recombinant lentiviral vector can decrease the MI of SW620 cells by over 20% and markedly suppress wound healing ability, indicating that miR-145 has the ability to suppress colon cancer cell migration and distant invasion. This is an important finding regarding cancer cell migration, which agrees with our expectation.

Ras oncogene is involved in human cancer development and progression. Although Ras mutation in colon cancer mainly involves K-ras, and N-ras mutation accounts for only 5%, the role of N-ras in colon cancer cannot be replaced by K-ras. K-ras expression promotes colon cancer cell proliferation, but it also makes these cancer cells prone to apoptosis. In contrast, high expression of N-ras does not promote cancer cell proliferation, but markedly inhibits cancer cell apoptosis. This function of N-ras cannot be replaced by K-ras[[31](#_ENREF_31),[32](#_ENREF_32)]. By determining N-ras expression level in colon cancer tissues, we found that N-ras expression in cancer tissues is increased as compared to normal tissues, and this trend is the opposite from miR-145. This is consistent with our finding using bioinformatics that N-ras is a target gene of miR-145. miR-145 may induce apoptosis of SW620 colon cancer cells by regulating N-ras. We postulate that down-regulation of miR-145 caused by a certain reason during colon cancer development may lead to decreased suppression of N-ras and therefore increased N-ras expression in cancer cells. N-ras signaling then leads to resistance to pro-apoptotic signals and promotes colon cancer development.

In conclusion, miR-145 may play an important role in inhibiting colon cancer proliferation and invasive migration. This finding lays a good foundation for further investigation on targeting miR-145 as a tumor suppressor in colon cancer treatment, and provides novel evidence for the anti-cancer effects and therapeutic potential of miR-145.

**comments**

***Background***

Invasive migration at early stages is a critical factor affecting the prognosis and survival of colorectal cancer patients. With the development of genetic therapy, targeted therapy has become the top level specific anti-cancer treatment. Blocking invasive migration by modifying the microenvironment on the molecular basis is the frontier of current research in colorectal cancer therapeutics. Some experts predicted that targeting miRNA in biological cancer therapeutics will be more effective than targeting coding genes.

***Research frontiers***

MicroRNA-145 (miR-145) expression is down-regulated in many cancers including breast cancer, melanoma, ovarian cancer, and liver cancer, suggesting that miR-145 may function as a tumor suppressor. However, so far there has been no systemic research on the role of miR-145 in colorectal cancer.

***Innovations and breakthroughs***

MiR-145 may induce apoptosis of SW620 colon cancer cells by regulating N-ras. The authors postulate that down-regulation of miR-145 during colon cancer development may lead to decreased suppression of N-ras and therefore increased N-ras expression in cancer cells. N-ras signaling then leads to resistance to pro-apoptotic signals and promotes colon cancer development.

***Applications***

MiR-145 may play an important role in inhibiting colon cancer proliferation and invasive migration. This finding lays a good foundation for further investigation on targeting miR-145 as a tumor suppressor in colon cancer treatment, and provides novel evidence for the anti-cancer effects and therapeutic potential of miR-145.

***Terminology***

MicroRNA: microRNA (miRNA) are endogenous, revolutionarily conserved, single chain non-coding RNA consisting of19-22 nucleotides, which inhibit mRNA transcription or induce mRNA degradation by base-paring with the 3’UTR of target mRNA, and thus play an important role in cell proliferation, differentiation, apoptosis, gene regulation, and tumor formation by suppressing the expression of target genes.

***Peer-review***

Authors presented a study in which they evaluated the role of microRNA-145 on colon cancer. They showed evidences of the tumor suppressor activity of microRNA-145 in colon cancer using several approaches.

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****

**Figure 1 Expression of miR-145 and N-ras in colon cancer tissues and normal colonic tissues.**



**Figure 2 MiR-145 expression in SW620 colon cancer cells and normal colonic epithelial cells.**

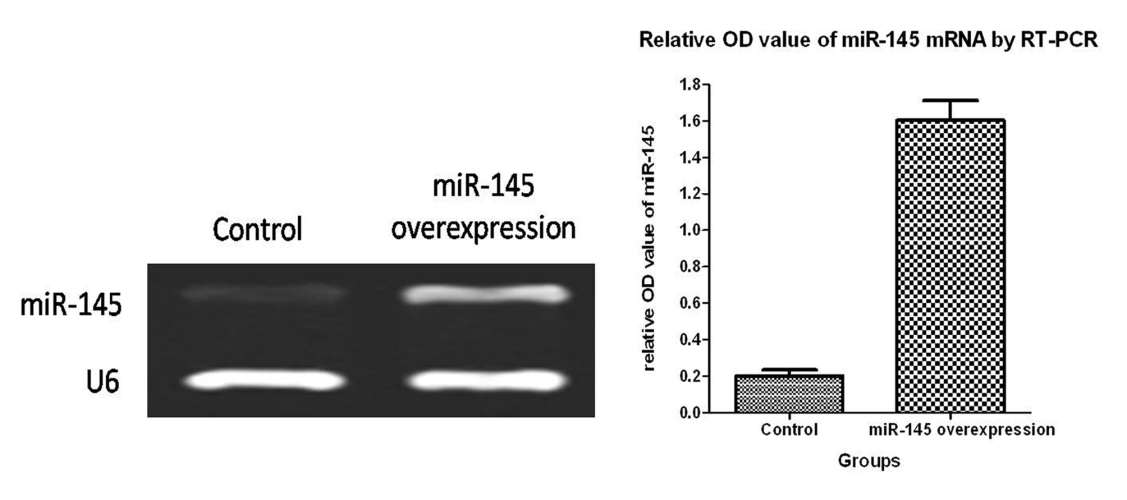
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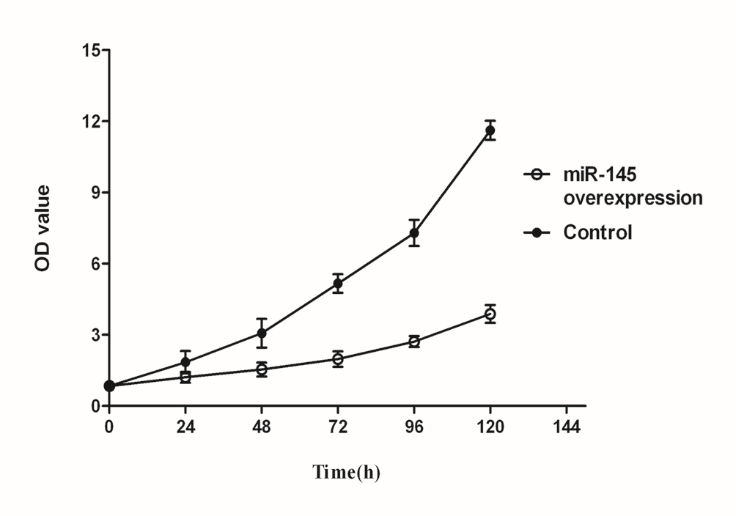
B

**

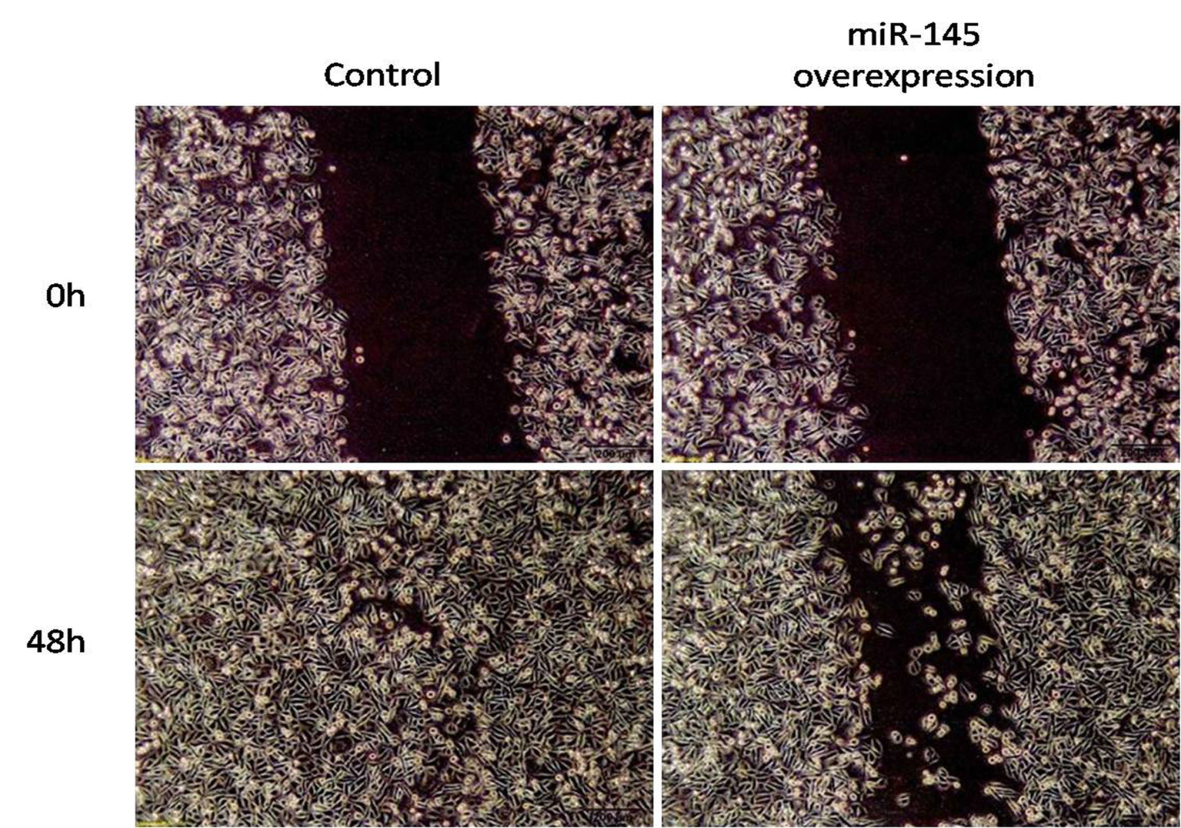
**Figure 3 HEK239T cells under regular (A) and fluorescence microscope (B) (****magnification × 40) at 72 h after transfection with lentiviral vector.**



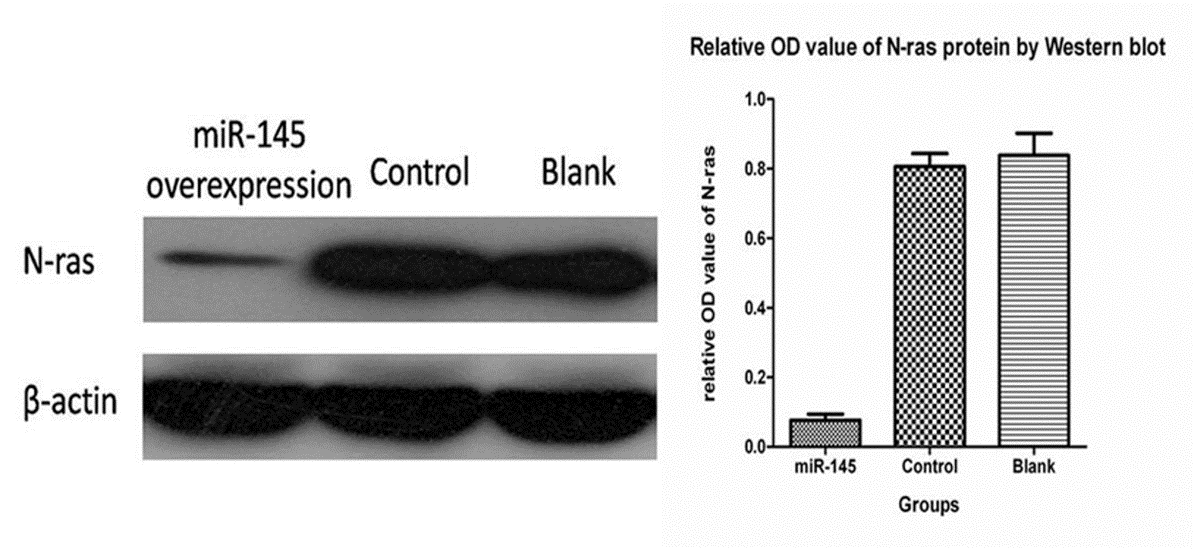
**Figure 4 Reverse transcription-polymerase chain reaction analysis of mature miR-145 expression in two groups of cells.**



**Figure 5 Growth curves of SW620 cells at indicated time points after transduction with lentiviral vectors.**



**Figure 6 Effect of miR-145 on SW620 cell migration (magnification × 100).** MI = 100% (g0-gt)/g0; g0 represents the width of the open area immediately after scratching the monolayer (0 h); gt represents the width of the open area at t h post scratching the monolayer.



**Figure 8 Effects of miR-145 on N-ras protein expression.**

**Table 1 Primer sequences**

|  |  |
| --- | --- |
| **Primers** | **Sequences (5’-3’)** |
| miR-145-F | ACACTCCAGCTGGGGTCCAGTTTTCCCAGGA |
| miR-145-R | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGAGGGATTC |
| U6-F | CTCGCTTCGGCAGCACA |
| U6-R  URP | AACGCTTCACGAATTTGCGT  TGGTGTCGTGGAGTCG |

F: Forward; R: Reverse.