MicroRNA-210 Modulates Cell Migration and Invasion by Targeting Vacuole Membrane Protein 1 in U87 Glioma Cells

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Research

Keywords: microRNA-210, glioblastoma, VMP1, proliferation, apoptosis

DOI: https://doi.org/10.21203/rs.3.rs-29861/v1

Abstract

OBJECTIVE: This study was to investigate whether the expression of vacuole membrane protein 1 (VMP1)was correlated with MicoRNA210 (miR-210) in U87 glioma cell line.

MATERIALS AND METHODS: Plasmid was constructed and transfected into U87 glioma cells.The correlation between VMP1 and miR-210 was observed by the double fluorescence sumei experiment. qRT-PCR and Western blotting were used to detect the expression levels of VMP1 at mRNA and protein level, respectively.MTT assay was utilized to examine the effect of miR-210 on cell proliferation and apoptosis.The transwell chamber assay and plate cloning experiments showed the changes in the rate of cell migration and invasion after cells were transfected with miR-210. Computer SPSS 22.0 software was used to perform t-test and analysis of variance on all experimental dat and results were considered statistically significant when $P < 0.05$.

Results: The results of the analysis of double luciferase showed that the relative fluorescence intensity of miR-210 and VMP1 in the co-staining group was significantly lower comparing to other experimental groups($P <0.001$).qRT-PCR and Western blotting experiments showed that the expression level of VMP1 in miR-210 group was downregulated ($P < 0.05$).The MTT assay showed that the cell length curve of the miR-210 group was less comparing to the control group ($P < 0.05$).Transwell experiments showed that the number of cells in the miR-210 group was significantly reduced ($P < 0.01$).Plate cloning experiments showed no significant difference in the number of cell colonies between the experimental group and the control group ($P > 0.05$).

Conclusion: VMP1 is a potential target gene of miR-210 in U87 glioma cell line, and provides a new option for molecular targeted therapy of gliomas in the future.

Introduction

Gliomas are the most common intracranial primary malignant brain tumors.The proportion of all central nervous system tumors is as high as 42-50%.Patients are often found with corresponding clinical symptoms caused by lesion compression.Enlarged tumor tissue can infiltrate and invade the surrounding normal brain tissue,thus increasing the difficulty of surgical resection.The median survival time of patients with malignant tumors is lower than 1 year, and the 5-year survival rate is only 5%[1,2].MicroRNA is an endogenous non-coding RNA that can participate in the growth and development of cells.It has been shown that microRNA is involved in pathogenesis of various diseases including glioma.Studies have been shown that miR-210 is highly expressed in gliomas and is positively correlated with the malignant degree of tumors.Vmp1 is a newly discovered tumor suppressor gene with low expression level in glioma.Ying et al.[3] demonstrated that hypoxia can induce miR-210 expression,thereby the overexpression of miR-210 can promote invasion and metastasis of liver cancer cells.They further confirmed that VMP1 was a direct downstream target of miR-210.The miR-210/VMP1 signaling pathway acted on the

occurrence and development of hepatocellular carcinoma. Therefore,we predicted that VMP1 was also a downstream target gene of miR-210 in glioma,and miR-210 can target VMP1 to modulate its expression.

Studies have been shown that the transfection rate of miR-210 in U87 glioma cell line was considerably higher than in other cell lines[2].In addition, hypoxia can upregulate the expression of miR-210 and promote the proliferation and metastasis of gliomas.In addition,miR-210 is associated with the prognosis of patients[4].Therefore,in this study,we investigated the interactions between miR-210 and VMP1 and its effects on the proliferation and invasiveness of U87 glioma cells.

Material And Methods

Cell culture

U87 glial cell line was obtained from Shanghai Yubo Biology.U87 glial cells were thawed, passaged,and the culture medium was changed every 3 days.All cells were grown in Dulbecco's Modified Eagle Medium (DMEM, HyClone,USA) supplement with 10% Fatal Bovine Serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin solution (PS, HyClone,USA).Cells were incubated at 37℃ in a 5% CO2 incubator (MCO-18AC Panasonic,Japan).According to the cell density,cells were trypsinized and mixed evenly with the cryopreservation solution that was prepared by 40% Fatal Bovine Serum (FBS) and 10% dimethylsulfoxide(DMSO,Sigma,USA). Cells were then packed into sterilized cryopreservation tubes and stored in a -80 °C refrigerator (MDF-382E, Panasonic, Japan) for overnight.Cells were stored in liquid nitrogen irrigation for long term use.All laboratory equipments were used after sterilization in a high pressure steam sterilizer (Panasonic, japan).

Carrier construction

The binding site of miR-210 to the VMP1 gene was predicted by the targetscan website. Sequences 5’-CGCACAA-3’ in the 3' UTR region of the VMP1 gene was identified.MiR-210 was artificially synthesized and cloned into the pCDNA3.1 vector to generate miR-210-pCDNA3.1.About 200 bp flanking the binding site of VMP1 (-CGCA-) and mutant binding site (Vmp1-MUT:-TATGTGG-) were introduced into pmirGLO vector to generate VMP1-pmirGLO and VMP1-mut-pmirGLO,respectively.These three plasmids along with the empty vector were mixed and divided into 6 groups as follows:1. pCDNA3.1+pmirGLO; 2.miR-210-pCDNA3.1+pmirGLO;3.pCDNA3.1+VMP1-pmirGLO;4.miR-210-pCDNA3.1+VMP1-pmirGLO;5.pCDNA3.1+VMP1-mut-pmirGLO;6.miR-210-pCDNA3.1+VMP1-mut-pmirGLO. miR-210-pCDNA3.1 and pCDNA3.1 were labeled as miR-210 group and NC group, respectively.

Plasmid extraction, cell transfection and dual luciferase assay

Plasmid DNA was extracted by plasmid extraction kit (D6950-01,OMEGA,USA).The concentration and quality of extracted plasmids were determined by nucleic acid protein analyzer (ultramicro spectrophotometer,KAIAO,China).Plasmids were transfected into U87 cells by liposomal (Lipo2000, China) according to the manufacturer's instructions.
The plasmids labeled as group 1-6 were co-transfected for 36 h. Dual luciferase assay was performed to determine the intensity of the ginseng luciferase reaction using Luciferase Reporter Assay Kit.The miR-210 group and the NC group were cultured for 6 hours in an incubator and replaced with anti-complete medium. Cells were then cultured for another 24 hours.

Total RNA isolation, reverse transcription and quantitative RT-PCR

Total RNA was isolated from U87 cells using Trizol reagent (Life technologies,USA) according to the manufacturer's instructions.RNA quality and concentration was determined by a nucleic acid protein analyzer (ultra-micro spectrophotometer,KAIAO,China).RNA was reverse transcribed into cDNA by a reverse transcription system.qRT-PCR was performed using a quantitative polymerase chain reaction kit (TOYOBO, USA) to detect the expression level of VMP1.

Western blotting analysis

Protein samples were separated on by SDS-PAGE gels (Sigma, USA) and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked in a 5% skim milk buffer at room temperature for 2 h on a shaker. Membranes were then incubated with the following primary antibodies (anti-vmp1, Beyotime, USA; anti-GAPDH, Beyotime, USA) for overnight at 4 °C and secondary antibody for 1 h at room temperature. After washing with 1% Tween-20 tris buffer (TBST), membranes were immersed in ECL luminescent solution and developed in darkness for 3 minutes. Membranes were blotted dry with filter paper and placed in a fully automated chemiluminescence image analysis system (Tanon, China) for scanning.

Cell proliferation experiment

MTT assay was used to detect the growth of U87 glial cells. Cells were seeded in a 96-well plate at 1×104 cells/mL. On the next day, 10 μL of MTT reagent was added to each well of the 96-well plate and incubated in an incubator (37℃,5%CO2) for 4 hours.100 μL of dimethylsulfoxide(DMSO,Sigma,USA) was added to the precipitate after discarding the supernatant to fully dissolve the crystal. The OD value was measured using a microplate reader at a wavelength of 490 nm. Each group was tested in triplicate every day for over 7 days.

Cell invasion assay and plate cloning experiment

The invasiveness of cells were detected by the transwell chamber.100-200 μl of the trysinized cells were added to the transwell chamber and 500 μl of medium containing 20% FBS were added to the 24-well plate. No bubbles was generated in the middle during the process. 24 hours after incubation, the transwell chamber was washed twice with PBS. The cells were stained with gentian violet for 5-10 min, and allowed to stand at room temperature for 0.5 h. Cells were washed twice with PBS, and cells in the upper surface were wiped off with a cotton ball. Five fields under the microscope were photographed and counted. In parallel, the cell suspension was diluted and cells in each group were inoculated into a dish containing 8 ml of 37℃ pre-heated culture solutions with gradient density of 600, 1000 and 1500 cells per dish, respectively. Cells were evenly dispersed by gently rotating. Cells were incubated in a cell culture incubator for 2 to 3 weeks. Cell culture was terminated when macroscopic clones appeared in the culture dish. The supernatant was then discarded and washed twice with PBS. Appropriate amount of gentian violet staining solution was added and incubated for 10 to 30 minutes. After washing off the staining solution, cells were examined and counted under the microscope. Colony formation rate = (number of clones / number of cells inoculated) × 100%.

Results

1. Double luciferase experiment verifies whether miR-210 and VMP1 have targeted relationship in U87

By literature searching, we found that VMP1 was the direct downstream target of miR-210 in liver cancer cells. We predicted that VMP1 might also be a downstream target gene of miR-210 in U87 brain glial cells. In order to study the expression and the correlation between miR-210 and VMP1 in U87 brain glial cells, miR-210, VMP1 and a VMP1 mutant (-mut) were cloned into pCDNA3.1 vector and pmirGLO vector, respectively. Plasmids were transfected into U87 brain glial cells(Figure 1.A) and double luciferase detection was performed.The relative fluorescence intensity of miR-210 and VMP1 in the co-stained groups were significantly lower comparing to other experimental groups ($P < 0.001$) indicating that there was a interaction between miR-210 and VMP1(Figure 1.B).

In order to investigate the effect of miR-210 on VMP1, we examined the VMP1 expression level in U87 glial cells at mRNA and protein levels by qRT-PCR and Western blotting, respectively.In order to eliminate the influence of empty vector on experimental results,we transfected pCDNA3.1 vector into U87 glial cell line as NC group.The data obtained by QPCR were analyzed by $2^{-\triangle\triangle}$CT.For the convenience of calculation,we standardized the blank group to 1 according to the experimental results.Table 1 and Figure 2.a show the results of QPCR experiment.We found that the expression level of VMP1 mRNA in miR-210 group was significantly lower comparing to other groups（$P < 0.05$）. Figure 2.b and c shows the results of Western blot analysis. VMP1 protein expression level is also lower than other combinations.

	Blank group	NC group	miR-210 group
VMP1	1.00±0.00	1.04±0.14	0.38±0.07
Note: p>0.05: Blank group vs NC group. *p<0.05: miR-210 group vs NC group.			

Table 1
QPCR test results □`x±s□

2. The effect of miR-210 on the proliferation, invasiveness and cloning ability of U87 glioma

In addition,we examined cell proliferation by MTT assay and found that the OD value of cells changed after cell transfer from day 1 to day 7. No differences were found in the first three days of cell culture. From the third day, it was found that the growth trend of the mir-210 group was significantly reduced (Figure 4 A and B).The cell length curve of miR-210 group was lower comparing to the control group indicating that miR-210 had an inhibitory effect on the proliferation of glioma cells. Transwell chamber migration experiment showed that the number of cells in miR-210 group entering the lower culture solution decreased (*P < 0.01*) indicating that miR-210 had inhibitory effect on the migration ability of glioma cells(Figure 3). Moreover, We used a plate cloning experiment to examine the effect of miR-210 on cell aggregation and cloning ability(Figure 5). In order to ensure the accuracy of the experiment, we tested the cloning ability of U87 glioma cells under a 600/1000/1500 cell gradient.Analysis of ABC three groups of experimental results shows that the cloning ability of different cells is not affected by cell gradient.Respectively,within the group analysis,we found that tumor cells that were transfected with miR-210 in the plate clone had no significant difference in the formation of cell colony comparing to the control glioma cells (*P > 0.01*) indicating that miR-210 had no effect on glioma cell clone formation.

Taken together,our results showed that miR-210 had an inhibitory effect on the proliferation and invasion of U87 brain glial cells under normal oxygen environment. MiR-210 did not promote the colony formation of tumor cell clone. VMP1 was a miR-210 targeting gene in glioma. MiR-210 can act on VMP1 to inhibit its expression and participate in the occurrence and development of the tumor.

Discussion

MicroRNA (miRNA) is an endogenous non-coding small RNA composed of 17-27 nucleotides, which exists in the human body in various forms, and can participate in the regulation process of almost all organisms, such as cell proliferation, differentiation and development[5]. Multiple studies have shown that miRNA plays a key role in the development of tumors and can be secreted into the systemic circulation outside cells. Different tissues have different miRNA expression pattern, and miRNAs are widely studied as candidate markers for the diagnosis and prognosis of tumors.

Recently,the computer-based method in predicting miRNAs target genes has become a research hotspot. One can predict the targeting genes of a miRNA by analyzing the sequence complementarity between miRNA and target gene's 3'UTR. Glioma is a rapidly developing malignant tumor. Surgical resection and postoperative chemotherapy are still the gold standard for the treatment of malignant tumors, such as glioma. The biological function of miR-210 and its role in pathological state has attracted more and more attention. Molecular biological markers are part of the basic items in modern diagnostic pathology. Therapy targeting genes at molecular level is expected to play an important role in the treatment of malignant tumors[21,23]. Signal transduction pathways related to tumor genes have become a hotspot of cancer research. Studies from many research groups have confirmed that miR-210 is highly expressed in various malignant tumors, such as glioma, whereas VMP1 is poorly expressed. However, whether there is a correlation between miR-210 and VMP1 in glioma is still not fully understand.

In this study,we predicted the binding site of miR-210 to vmp1 by using the targetscan website, and investigated the relationship between miR-210 and vmp1 in U87 glial cells at the cellular level. We found that VMP1 was the target gene of miR-210 in glioma. MiR-210 can target VMP1 to inhibit its expression indicating the downregulation of VMP1 was partially due to the upregulation of miR-210 in U87 glial cells. MiR-210 is a recently discovered oncogene which plays an important role in cell survival, endothelial cell migration and cell regeneration. MiR-210 can be induced in renal cancer, breast cancer, pancreatic cancer, lung cancer and Glioblastoma[12,6,7,8,9,10]. Moreover, overexpression of miR-210 are associated with patient survival rate in multiple cancers[11]. VMP1 was originally identified a protein that was related to acute pancreatitis in mice in 2002 by Dusetti et al.. Recently, VMP1 is shown to be a tumor suppressor gene[15,16]. A large number of studies have found that the expression level of VMP1 is low in most malignant tumors, such as liver cancer, kidney cancer, breast cancer, colorectal cancer and pancreatic cancer[3,19,15,20]. There are also differences in the expression levels of VMP1 in different tissues in the human body.For instance,VMP1 is found to have high expression in liver tissue or normal brain astrocytes.In contrast,there are limited VMP1 expression in some self-organizations or even not expressed[21].

In this study,we found that miR-210 can act on VMP1 and inhibit its expression in U87 glial cells. Hypoxia-inducible factor (HIF) is a key regulator in the hypoxia signaling pathway, which has the dual properties of activating gene expression and suppressing gene expression. Studies have been shown that HIF-1a is stably expressed under hypoxia conditions. HIF-1a can regulate expression of downstream target genes by combining with hypoxia response elements. Some target genes are highly expressed, such as miR-210, miR-107, which are involved in cell proliferation and differentiation. On the other hand, HIF-1a can be activated and degraded under normal oxygen environment reducing the expression of some regulated target genes[13,14]. MiR-210 is currently the most widely studied miRNA that is regulated by HIF.The longer miR-210 is exposed to hypoxia, the stronger miR-210 is induced. Therefore, miR-210 is considered as a landmark miRNA for hypoxia. MiR-210 and VMP1 are closely related in terms of the occurrence of tumors. VMP1 can inhibit cell proliferation and induce apoptosis by acting on the S phase of cell proliferation.In addition,VMP1 is involved in autophagy of cells as an active autophagy marker and

can pass through the zonal-1 (ZO-1) between cells[17,18].Therefore, the proliferation and migration of tumor cells can be promoted by the mechanisms mentioned above imparting the value-added and invasive properties of tumor cells when the expression level of VMP1 is lowered.

Ying et al. found that miR-210 inhibited cell proliferation and colony formation in Huh-7 cells under normoxic conditions[3].Hirofumi Yoshino et a. found that deletion of miR-210-3p promoted tumor cell proliferation and invasiveness[5].Huang et al. found that HIF was a key regulatory gene of miR-210, and miR-210 can inhibit the proliferation of tumor cells both in vitro and in vivo[26,27].In this study, we found that miR-210 inhibited proliferation and invasiveness using U87 cell line tumor cells under normoxic conditions.However, some studies from other groups have found that miR-210 promotes the proliferation and invasion of tumor cells[25]which is contrary to our results.The combination of HIF and hypoxia response elements in a hypoxic environment induced high expression of the miR-210.We think this may due to the difference in the experimental environment.

There are limitations in our study.For instance,the occurrence of tumor is determined by the joint action of multiple mechanisms,we have only studied one of them.Our experiment is limited to the cell level and does not involve relevant animal experiments and corresponding clinical case studies to further confirm the relationship between VMP1 and miR-210 in glioma. The selected cell line is single and not representative.In addition,in vivo study is needed to verify our in vitro results.Last but not least,the identified interactions between VMP1 and miR-210 in in U87 glioma cells may provide a new approach for the treatment of glioma.

Conclusions

We confirmed the presence of the miR-210/VMP1 pathway in gliomas.MiR-210 can target VMP1 to suppress its expression in gliomas,which contributes to tumor development. It provides new options for molecular targeted therapy of gliomas.

List Of Abbreviations

English abbreviations	English name
miR-210	MicoRNA210
VMP1	Vacuole membrane protein 1
OD	Optical Density
DMEM	Dulbecco's Modified Eagle Medium
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
HIF1	hypoxia inducible factor-1
cDNA	Complementary Deoxyribonucleic acid
PCR	Polymerase Chain Reaction
qPCR	Quantitative real-time PCR

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and/or analysed during the current study are not publicly available due [REASON WHY DATA ARE NOT PUBLIC] but are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests

Funding

This research was funded by the Heilongjiang Provincial Health and Family Planning Research Project(Project No.2018378); the Mudanjiang Medical College Graduate Innovation Research Project(Project No.2018YJSCX-03MY) and the Foundation of HongQi (Project No. 2018HQ-17).

Authors' contributions

We are very grateful to Professor Guangyao Liu and Professor Yanhai Ren for providing experimental reagents and microscopes and other experimental equipment. Thanks to Guangdong Liu and Chuang Wang for their assistance in cell culture and other experiments. Thanks to Liqiu Ma for collecting relevant data in the early stage of the experiment. Thanks to Jianxin Li and Lijiao Jin for analyzing the experimental data. Thanks to Haidong Gong for the application of this topic and the overall grasp and revision of the article.

Acknowledgements

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Thanks to the Heilongjiang Provincial Health and Family Planning Research Project (Project No. 2018378); the Mudanjiang Medical College Graduate Innovation Research Project (Project No. 2018YJSCX-03MY) and the Foundation of HongQi (Project No. 2018HQ-17) for providing us with the project Financial support.

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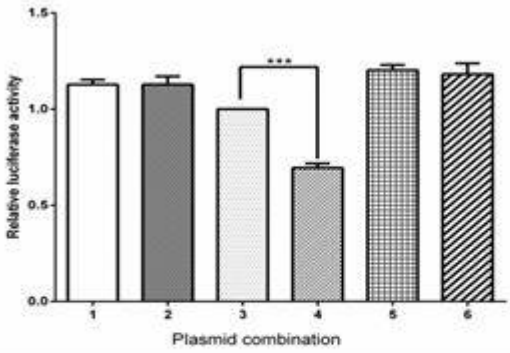
Figures

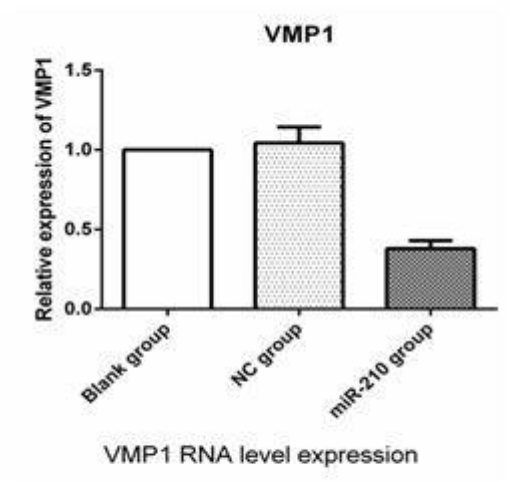
A U-87 cells (100x)



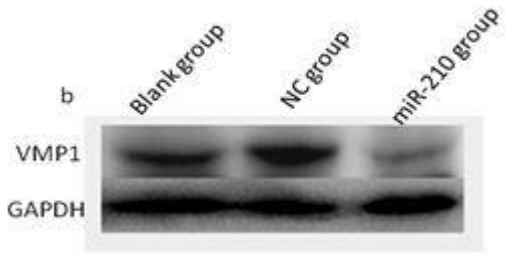
B Dual luciferase assay results
Note:***p<0.001: Third group vs fourth group.

Figure 1

A: A denotes U87 glioma cells under (100X);B: Immunofluorescence experiment results:The experimental results were compared between group 1 and group 2,group 3 and group 4,and group 5 and group 6.Note:***p<0.001:Third group vs fourth group.



a PCR experiments



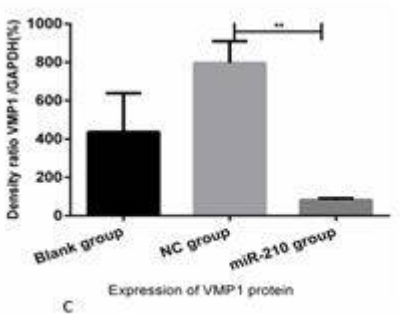


Figure 2

a PCR experiments,The results show that the expression level of VMP1 in miR-210 group is lower than that in other groups (p<0.05).b and c are the westernblot experiment.The expression level of VMP1 protein in miR-210 group is lower than that in other groups (p<0.05).

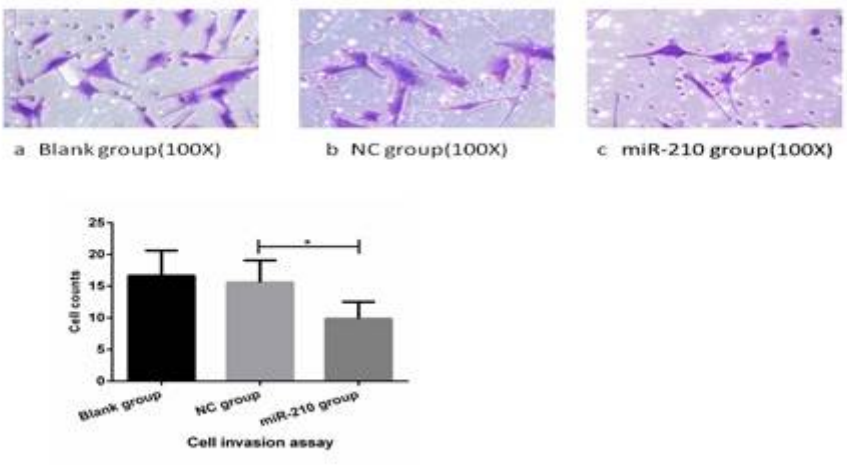


Figure 3

Cell invasion assay: a, b and c are cells observed under a microscope (100X) We analyzed by data analysis that the cell count of miR-210 group was significantly lower than that of other groups (P<0.01)and the difference was statistically significant.

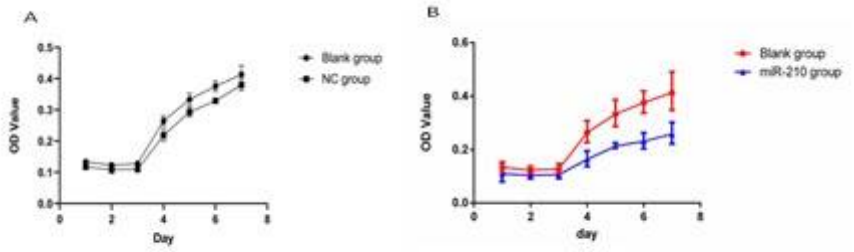


Figure 4

Cell proliferation experiment A: cell proliferation level in NC group and Blank group (P>0.05); B: cell proliferation level in miR-210 group and Blank group showed difference from the 3rd day (P<0.05). The difference is more significant with the extension culture time.

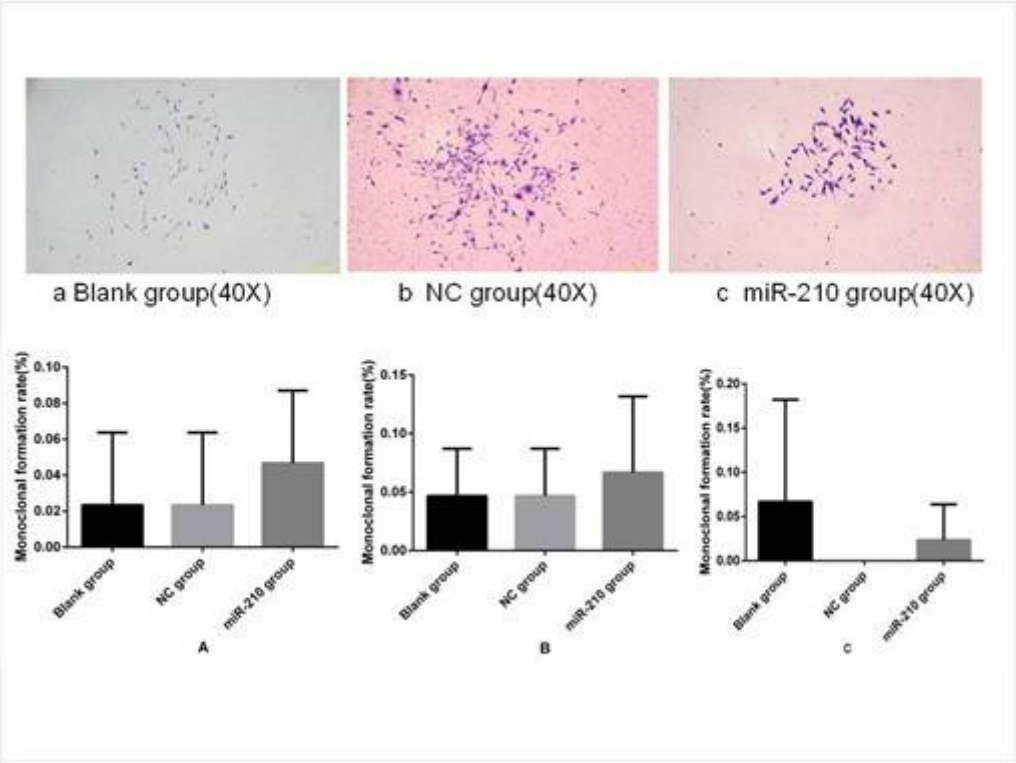


Figure 5

Plate cloning experiment:a,b and c are cell colonies observed under microscope (40X). A,B and C was inoculated into culture dish according to gradient density of 1500,1000 and 600 cells respectively to detect cell clone formation.Analysis of experimental data shows that miR-210 has no statistical difference in cell colony forming ability in each gradient compared with other groups ($p>0.05$).