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Full Length Research Paper

High mobility group in A1a (HMGA1a) small interfering RNA (siRNA) blockage decreases metastatic potential in cultured osteosarcoma cell lines

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High mobility group A1 (HMGA1) is a non-histone chromosomal nuclear protein that plays important roles on gene transcription, recombination, and chromatin structure stabilization. The aim of this study was to investigate the effect of small interfering RNA (siRNA) on HMGA1 expression and metastatic potential in osteosarcoma cell *in vivo*. We obtained osteosarcoma cell sublines after single cell cloning technique from human osteosarcoma cell line MG-63, and chose the cell subline with high HMGA1 expression. One sequence-specific siRNA targeted to HMGA1 was designed and cloned to generate the pU6mRFP-HMGA1 shRNA vector, then transfected into the osteosarcoma cell subline. The transfection efficiency of the experiment group and control group were 55.68±6.74 and 49.87±4.33%. The specific HMGA1 shRNA effectively down regulated the mRNA and the protein expression of osteosarcoma cell subline. The cells numbers throwing membrane at the experiment group was higher than that of the control group. shRNA of HMGA1 could successfully inhibit the expression of target gene in mRNA and protein levels and inhibit the in *vitro* metastatic potential of osteosarcoma cells.

Key words: RNA interference, osteosarcoma cell, high mobility group A.

INTRODUCTION

The sarcomas constitute a heterogeneous group of rare solid tumors of soft tissues and bones, usually divided into two broad categories: sarcomas of soft tissues and sarcomas of bone (osteosarcoma). Sarcomas affect the pediatric and adult population. Although their etiology is unclear, it is believed that they arise from primitive mesenchymal bone-forming cells, or osteoblasts (Ottaviani and Jaffe, 2009). Nevertheless, recent evidence suggests that genes that give rise to non-histone protein products involved in chromatin stabilization, DNA replication, transcription and recombination such as those of the high mobility group A family (HMGA1a, 1b and 2), may be involved in the etiology and pathogenesis of these tumors, specifically in their metastatic potential. Indeed, the HMGA1 gene has been shown to be highly expressed in sarcoma cells and the magnitude of its expression to be related with tumor cell growth potential (Mellone et al., 2008; Franco et al. 2008; Chiefari et al., 2009). In the paper, we evaluated, the human osteosarcoma cell line MG-63 with highly HMGA1 expression and RNAi technology to knockdown the HMGA1 expression, and investigated the effects on the biological characteristics of the human osteosarcoma by small interfering RNA of HMGA1.

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MATERIALS AND METHODS

The human osteosarcoma cell line MG-63 was purchased from Cellular Biology Institute, Shanghai, China. HMGA1 monoclonal antibody of MEM2G/1 was purchased from Santa Cruz (Santa Cruz Biotechnology, Santa Cruz, CA). Transfection reagent kit was obtained from Gibco Company (USA). Mouse anti-human β-actin, RT-PCR primers and probes were purchased from Applied Biosystern (USA). HRP-labelled sheep anti-mouse IgG, HRP, DNA ladder and low molecular weight protein ladder were obtained from Hongbo Biotechnology (Harbin, China) Co. Mouse anti-human β-actin antibody was purchased from Invitrogen (USA). The pU6mRFP vector was obtained from Ambion (USA).

Table 1. Primers used for siRNA and real-time RT-PCR.

Kinds	Sequence
Target sequence	Target sequence Nucleotides 2760 -2780 (Genebank no:BC071864) GGCAUCCGAGCCGCAUUUGCU
HGMA1 siRNA	Forward 5'-GAAGGCAUCCGCAUUUGCU dTdT-3' Reverse 3'-dTdTCUUCCGUAGGCGUAAACGA-5'
non-specific	Target sequence Nucleotides 2134 -2154 of (Genebank no:BC071864) AATTCACTCCAAGTCTCTTCC
non-specific	Forward 5'-UUCACUCCAAGUCUCUUCCdTdT-3' Reverse 3'-GGAAGAGACUUGGAGUGAAdTdT-5'
Real-time RT-PCR	Forward 5'-ATGAACTCCGAAGGCCAGCC-3' Reverse 5'-CCTTCCTAGGTCTGCCTCTTGG-3'
β-actin (450 bp)	Forward 5'-TGCGCAAAACAAGATGAGATT-3' Reverse 5'-TGGGGACAAAAAGGGGGAAGG-3’
Probe of HGMA1	5'-TCTGCGACGTGAAAGCCTCT- 3'

Vector construction

HMGA1 specific siRNA and their negative control were designed *in silico* based on the annoted sequences deposited in the GeneBank data base (no.BC071864). Primers were synthesized following the protocols provided by Qiagen (USA) and the corresponding sequences are shown in Table 1. The pU6mRFP vector was digested with *Apa* I /*EcoR* I and ligated with oligonucleotide fragments of the synthesized and annealed human HMGA1 siRNA to generate the pU6mRFP-HMGA1 RNAi vector. The recombinant vector was transferred into *E. coli* DH5α. The positive clones were screened through ampicillin and sequenced.

Cell culture and cell colonies selection

Human osteosarcoma MG-63 cells (MG-63) were seeded in poly-lysine embedded 24 well culture plates (company) and cultured with in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C temperature (95% O_2/ 5% CO_2) until achieving 70% of the logarithmic growth phase. MG-63 cells (5×10^3) were then detached from the culture plate by incubating them with 0.25% trypsin in PBS buffer during 5 min at 37°C and prepared into 50, 10 and 5 cells/ml suspension. Individual cells were then seeded in 96 well culture plates (0.1 ml/well) in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) during 5 min at 37°C temperature. Only one cell each well was picked out under the inverted microscope after 24 h; it was labeled and cultured in 0.1 ml medium. When the larger cell colonies formed and reached 1/3 to 1/2 of the well bottom area, they were digested and further seeded into 6 wells 24-wells, and 100 ml culture flasks.

Detection of cell clones with HMGA1 highly expression and experimental group

RT-PCR was performed to determine the HMGA1 mRNA expression level of cultured osteosarcoma cells. Total RNA was extracted by Trizol reagent, 1 μg RNA was subjected to a reverse transcriptase and cDNA was synthesized by reverse transcription (RT). The PCR program consisted of an initial denaturing step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 2 min. Incubation for 10 min at 72°C followed to complete extension. β-actin was used as the internal control. PCR products were detected by agarose gel electrophoresis and gel image analysis system. Cells with the highly expressed HMGA1 mRNA level were chosen as research object.

Human osteosarcoma cells with high HMGA1 expression were cultured into the logarithmic growth phase before transfection, then digested with 0.25% trypsin and cell numbers were counted. MG-63 cells were subcultured into 6 wells culture plates (0.2×10^6 cells/well) in Iscoves Modified Dubecco’s Media (IMDM)/F12 medium for 24 h. Three groups were divided: the experimental group transfected with HMGA1 siRNA**,** the negative control group transfected with HMGA1 no-specific sequence siRNA and untransfected MG-63 cells served as the blank control group.

Cell transfection

MG-63 cells were transfected according to the manufacturer’s protocol after the plasmid was purified. Briefly, one day before transfection, the attached cells in logarithmic growth phase were respectively implated into 12-well plates at density of 1×10^5 /well. Transfection mixes containing Solution A (5 μg plasmid + 100 μl OptiMEM medium) and Solution B (10 μl Lipofectamine™ 2000 +100 μl OptiMEM medium) were prepared in 96-well plates. When cells reached 70% confluence, the plasmid DNA was transfected into the cells. The transfection efficiency was observed by fluorescence microscopy after 24 h. Cells were transfected for 6 h per day, lasted for 3 days and were collected in the 4th day. The transfection concentration of the experimental group HMGA1 shRNA was 1.0, 2.5 and 5.0 μg/L, respectively. The negative group transfected with HMGA1 no-specific sequence and the control group transfected with IMBM medium were all used at the same dose.

Real-time RT-PCR

The expression of HMGA1 mRNA in human osteosarcoma cells was detected by real time RT-PCR. Total RNA of each group transfected cells was extracted by Trizol and cDNA was synthesized by reverse transcription (RT) at 37°C for 60 min. Amplification reactions (25 μl) contained 1 μl (50 ng) of cDNA, 2 μl (10 μmol/L) of each primer, 0.5 μl (10 μmol/L) of probe, 12.5 μl of PCR mix and 7 μl of deionized water. Mixed them gently and amplified by real time RT-PCR, cycling parameters were 55°C for 2 min, then 35 cycles of 93°C for 10 min, 93°C for 10 min and 60°C

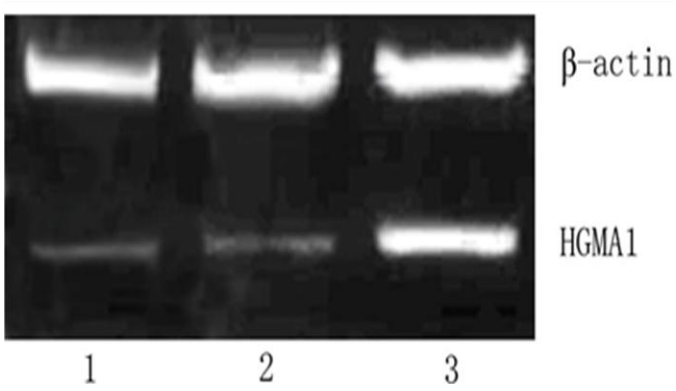

Figure 1. HMGA1 mRNA expression detected by RT-PCR in human ostesarcoma cells. Lane 1: Uncloned cells; lane 2: Cloned cell A; lane 3: Cloned cell B; β- actin was used as internal control of RT-PCR.

for 55 min. β-actin which served as the internal control was also amplified by PCR. Cycle threshold (Ct) of PCR represents the expression level of HMGA1 mRNA. The Ct value means cycles of each tube fluorescent signals reach predetermined threshold values, Ct value is negative correlated with the expression level of HMGA1 mRNA, Ct value increased to represent the expression level of HMGA1 mRNA decreased. Based on the determination of the standard curve, when 1 fold decreased of the transfection concentration and 1 cycle increased of Ct value, indicated the expression level of HMGA1 mRNA decreased by 50%. The differences of HMGA1 mRNA expression of each group were compared**.** Each experiment was repeated triplicates.

Western blot analysis

The expression level of HMGA1 protein in human osteosarcoma cells was detected by Western blot. Total cell lysates collected from each group were run on 12% SDS-polyacrylamide gels and then transferred onto nitro-cellulose membranes. The membranes were subsequently incubated at room temperature for 1 h with the mouse anti-human HMGA1 mAb MEM2G/1 antibody and mouse anti-humanβ-actin polyclonal antibody respectively. Secondary antibody was HRP-labelled sheep anti-mouse IgG. Reactive proteins were detected by enhanced chemiluminescence plus reagent.

Transfection concentration in the three groups of vectors was 2.5 μg/L. Cells of each group were collected at 6, 12, 24, 48, 72 h, respectively, after transfection for 3 days. The changes of HMGA1 protein expression levels of three groups transfected cells at the different time point were detected and compared. The ratio of HGMA1/β-actin was used to present the expression level of HMGA1 protein. Each experiment was repeated triplicates.

Cell invasion assay

Matrigels were prepared on the polycarbonate filter film of the transwell chamber (50 μg/well), incubated it at 37°C for 3 h and dried it overnight at the room temperature. 100 μl of cell suspension after transfection for 6 h was seeded to the upper transwell and RPMI1640 medium of 500 μl containing 10% FBS and 100 ng/ml SDF-1a were seeded to the lower transwell. Tanswell plate was incubated at 37°C with 5% CO_2, pulled out the polycarbonate filter film and wiped out the cells with the cotton swab. Cells were fixed with 4% paraformalde for 30 min and subjected to hematoxylin-eosin staining, and then total cell numbers, which migrated to the back of the polycarbonate filter film, was counted under the inverted microscope. Five visual eyeshots under the microscope were randomly chosen, and the cells that penetrated to the lower chamber were counted in each visual field, respectively, and the average value was taken. Each experiment was repeated triplicates.

Statistical analysis

The data were subjected to SPSS10.0 Software and the categorical data were presented as mean ± SD. Different transfection concentrations of HMGA1 protein and mRNA expression were compared with single factor analysis of variance and the comparison among groups was performed with SNK-q test. Total cell numbers with the invasion ability before and after transfection were analyzed by *t* test. $p<0.05$ were considered statistically significant.

RESULTS

HMGA1 mRNA expression

RT-PCR was performed to determine the HMGA1 mRNA level of the human osteosarcoma cell strains. Two cell strains (single cloned cell A and B) which showed significant differences in the expression of HMGA1 mRNA were chosen from the five cloned human osteosarcoma cell strains. The ratio of target PCR fragment in cloned cells A and B to internal control was 2.06±0.47 and 0.71±0.36, respectively, while the ratio in the uncloned MG-63 cells was 0.37±0.05. Significant difference was found among groups ($p<0.05$) (Figure 1).

Transfection efficiency assay

The sequence of HMGA1 PCR product was identical to that of the published sequence in the GenBank, suggesting that the HMGA1 targeted recombinant shRNA plasmid pU6mRFP-HMGA1 was successfully constructed. Cells were observed with a fluorescence inverted microscope after 24 h transfection. Cytoplasm of human osteosarcoma cell clones with high HMGA1 expression levels shrink partly, round or ellipse and the proportion of cells expressing green fluorescence protein in the experimental group and the negative group were 55.68±6.74 and 49.87±4.33%, respectively, while no cells expressing green fluorescent protein were observed in the blank control group (Figure 2).

Down-regulation of HMGA1 expression

To observe the HMGA1 mRNA change in human osteosarcoma cells by RNA interference, RT-PCR was preformed. Different concentrations of shRNA (1.0, 2.5

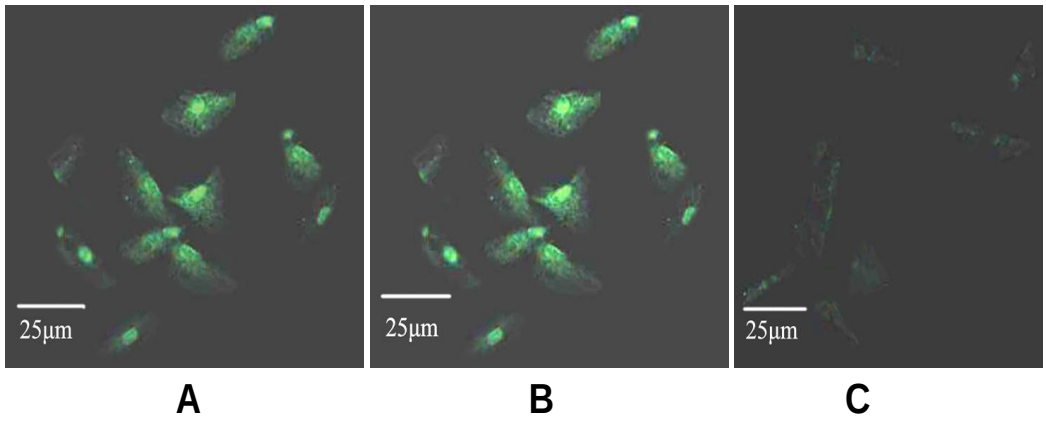

Figure 2. Green fluorescence protein expression of different group after transfection observed under fluorescence microscopy (×20 magnification) (A) Experimental group; (B) Negative control group; (C) Blank control group.

and 5.0 μg/L, respectively) were transfected in the experimental group, Ct values of osteosarcoma cell were 17.17±0.04, 18.23±0.02 and (19.34±0.02) cycles, respectively (F=571.22, p<0.01). In the negative group, shRNA were transfected using the same concentration, Ct values of osteosarcoma cell were 16.62±0.02, 16.84±0.04 and 18.30±0.03 cycles, respectively (F=385.09, p<0.01). Compared with the corresponding experimental concentration, Ct values of the blank control group were 16.65±0.05, 16.73±0.03 and 16.92±0.03, respectively. The comparison of the same concentration in each group showed that there was a significant difference in various concentrations (p<0.05).

Different concentrations of shRNA (1.0, 2.5 and 5.0 μg/L, respectively) were transfected in the experimental group. The expression level of HMGA1 protein of the experimental group cells was 0.83±0.04, 0.45±0.02 and 0.23±0.03, respectively (F=126.87, p<0.01). The expression level of HMGA1 protein of the negative control group was 1.18±0.13, 1.24±0.25 and 1.06±0.36, respectively (F=154.29, p<0.01), while the blank control group was 0.98±0.09, 0.93±0.12 and 0.88±0.16), respectively (F=237.57, p<0.01). The comparison of the same concentration in each group showed that there was a significant difference in different concentrations (p<0.01). The HMGA1 protein expression level of the experimental group (1.0, 2.5 and 5.0 μg/L, respectively) was reduced by 29.7, 63.7 and 78.3%, respectively. After transfection with 2.5 μg/L HMGA1 shRNA at the different time point (6, 12, 24, 48 and 72 h), we found that the expression of HMGA1 protein in the experimental group decreased slightly at the 6 h, then the reduction range increased gradually, and the expression level was lowest at 72 h after transfection (Figure 3).

The expression level of the negative control group and the blank control group had no obvious changes with time increased.

Cell invasion assay

After knocking down the expression of HMGA1 by RNA interference, the cell numbers through the membrane at the experiment group, the negative control group and the blank control group were 36.80±6.33, 68.76±10.28 and 71.25±6.53%, respectively (p<0.01) (Figures 4 and 5).

DISCUSSION

High mobility protein group A1 (HMGA1) is the structural transcriptional factor of the high mobility group (HMG) family and belongs to a group of non-histone highly charged nuclear chromosomal proteins. Recent evidence has shown that HMGA1 regulate the transcription of several genes that are important in the control of tumor development and metastastic potential (Frasca et al., 2006; Grad et al., 2007). HMGA1 plays an important role in oncogenesi and tumor development (Eilebrecht et al., 2011). Accordingly, abnormal expression of HMGA1 exists in many malignant tumors, including osteosarcoma (Matta and Panagiotidis, 2008; Beuing et al., 2008; Rahman et al., 2009). In the present study we then evaluated whether inhibition of HMGA1 expression in human osteosarcoma cells by using small interfering RNA,decreases the metastatic potential of human osteosarcoma cells.

RNAi technology is a newly discovered double-stranded RNA now frequently used to induce gene silencing in oncologic grounds (Sun et al., 2005, 2008; Fu et al., 2009).

In this study, Ct value of osteosarcoma cells was determined by real-time RT-PCR, Ct value of 5.0 μg/L was the highest among the 1.0, 2.5 and 5.0 μg/L concentration, the concentration of 2.5 and 5.0 μg/L Increased one cycles respectively, namely the

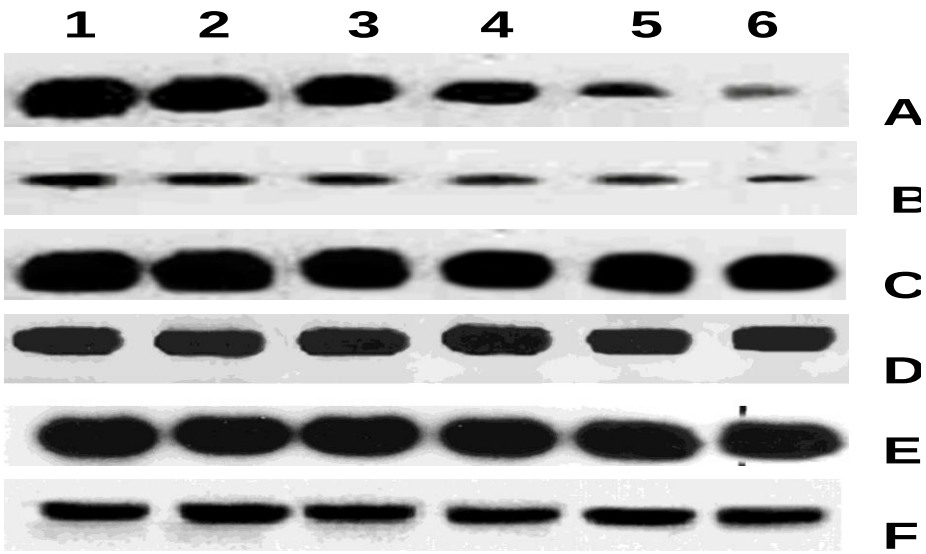

Figure 3. The expression of HMGA1 protein in three groups after HMGA1 shRNA transfected cells at different time points as monitored by Western blotting. Lanes 1 to 6: different time point at the 0, 6, 12, 24, 48, 72 h after transfection. (A) HMGA1 in the experimental group; (B) β-actin; (C) HMGA1 in the negative control group; (D) β-actin; (E) HMGA1 in the blank control group; (F) β-actin.

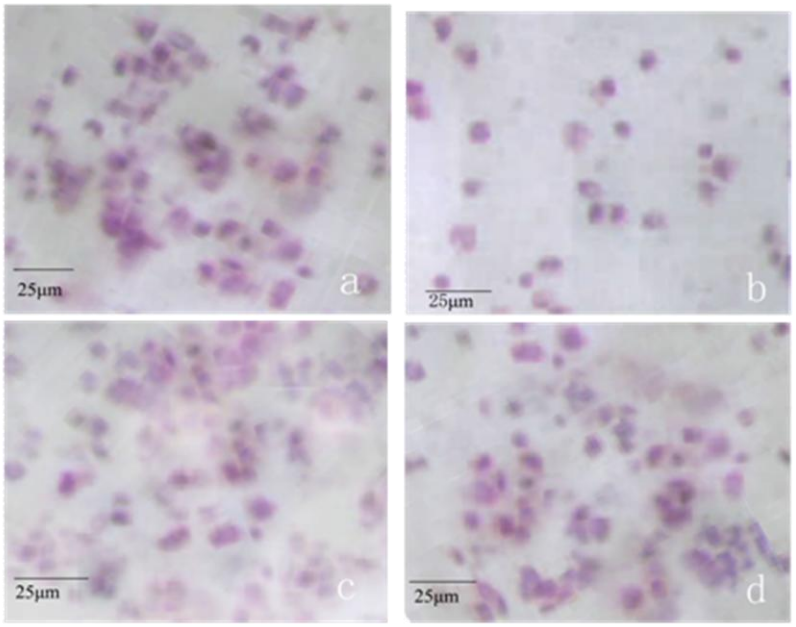

Figure 4. Cells number invading passing through the membrane determined by transwall *vitro* invasion assay after transfection observed under inverted microscope. (a) non-transfected cells; (b) transfected cells in the experimental group; (c) transfected cells in the negative control group; (d) transfected cells in the blank control group.

expression level of HMGA1 mRNA decreased by 50%, the down-regulated level was the most obvious at the concentration of 5.0 μg/L, which showed that the approach was effective, feasible and the intensity was related to the transfection concentration.

Our study showed that HMGA1 shRNA had obviously

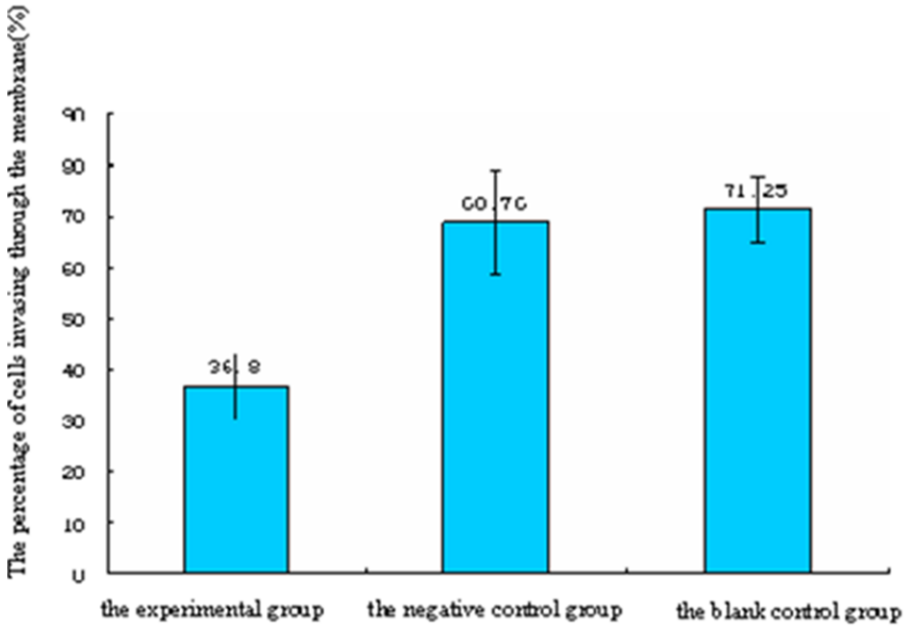

Figure 5. Number of cells invading the lower face of the membrane following treatment.

down-regulated effect to HMGA1 protein expression of osteosarcoma cells; the concentration of 2.5 μg/L was higher than 1.0 μg/L and the down-regulated effect was the most significantly at the concentration of 0. 5 μg/L, which was consistent with some literatures reported on the down-regulated effect of siRNA been related to the transfection concentration (Pierantoni et al., 2007; Koehler et al., 2009). The concentration of 2.5 μg/L HMGA1 siRNA at different transfection time (was observed; we found that the expression level of the experimental group HMGA1 protein decreased slightly at 6 h after transfection, then the reduction range increased gradually and the expression level became the lowest at 72 h after transfection. Although only transient transfection was performed in the experiment, the earliest occurred down-regulated effect of HMGA1 protein was found at 6 h after transfection; the expression level of HMGA1 protein decreased obviously with time increase, while the expression level of the negative control group and the blank control group had no obvious changes. This was further in favor of the important characteristic of shRNA such as efficient, specific, time-and dose-dependent and has its incomparable advantages compared with the traditional gene therapy. In addition, shRNA HMGA1 could down-regulate the expression level of HMGA1 mRNA and protein in osteosarcoma cells; the invasive ability was also decreased.

In conclusion, the specific shRNA HMGA1 could effectively inhibit HMGA1 expression. The inhibition effect was characterized by time-and dose-dependent transfection. After HMGA1 knocking down, metastasis of osteosarcoma cells was effectively reduced.

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