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Silencing EPB41 Gene Expression Leads to Cell Cycle Arrest, Migration Inhibition, and Upregulation of Cell Surface Antigen in DC2.4 Cells

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Background: Protein 4.1R (EPB41) is the main cytoskeleton component of the erythrocyte membrane and may be involved in cell migration and adhesion. Previous research discovered overexpression of 4.1R in the thymus of patients with myasthenia gravis (MG). The protein 4.1R on dendritic cells may play a pivotal role in MG pathogenesis. This research investigated the effects of small interfering RNA 4.1R-siRNA on cell migration, cell cycle, and surface antigen expression of DC2.4 mouse dendritic cells, thus providing a new direction for the study of MG pathogenesis.

Material/Methods: Three 4.1R-specific siRNAs were designed, and the expression of 4.1R was detected by real-time PCR at the mRNA level and Western blot analysis at the protein level to select out the most efficient siRNAs. Changes in cell morphology were observed and cell migration ability was analyzed by Transwell assay. Cell cycle and surface antigen were both analyzed by flow cytometry.

Results: The cell bodies of DC2.4 diminished, the synapses were increased, and protuberance became more obvious after being transfected with 4.1R-siRNA. After knockdown of 4.1R, cell migration ability decreased and the proportion of cells in S phase significantly increased (both $P<0.05$). The expression levels of MHCII, CD80, and CD86 were all increased in DC2.4 cells (all <0.05).

Conclusions: Silencing the expression of 4.1R in dendritic cells resulted in inhibition of migration ability, cell cycle arrest, and increase in surface antigens, which suggest that 4.1R participates in MG autoimmunity.

MeSH Keywords: Cell Cycle • Dendritic Cells • Myasthenia Gravis

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Background

Myasthenia gravis (MG) is a typical autoimmune disease with antibody-mediated neuromuscular junction disorder [1]. Humoral immunity mediated by acetylcholine receptor antibody (AchRab) and cellular immunity mediated by T cells is the main pathogenesis of MG. The thymus has been reported to be one of the important organs to activate and maintain MG autoimmunity [2], but the detailed pathogenesis of MG has not been fully elucidated. Dendritic cells (DC) are powerful and versatile antigen-presenting cells whose migration ability is the key to trigger a protective inflammatory response and allergenic immune response [3]. Recent studies highlight the importance of DC migration in maintaining immune surveillance and tissue homeostasis, as well as pathogenesis in a range of diseases [4,5]. DCs are the most important antigen-presenting cells in the thymus and play a pivotal role in the pathogenesis of thymic immunity and autoimmune diseases. Since the discovery that DC can induce immune tolerance, studies on the function of DC in the pathogenesis of MG have increased.

A previous study by our team used proteomics analysis to show that cytoskeletal protein 4.1R encoded by the EPB41 gene has an abnormal expression in MG thymus tissues [6]. In the past, protein 4.1R was known only as a membrane-cytoskeleton adaptor. However, recent research shows that 4.1R also regulates the cell cycle, migration, and adhesion [7–9]. Thus, the present study explored the relationship between 4.1R and MG by analyzing the influence of 4.1R on DC biological functions, including migration, cell cycle, and cell surface antigen expression.

Material and Methods

Cell culture

Mouse dendritic cells 2.4 (DC2.4) (Solarbio, Beijing/China) were cultured in fresh MEM medium (Gibco, California/USA) containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C, in an atmosphere of 5% CO_2. All the experiments were approved by the Medical Ethics Committee of the First Affiliated Hospital of Henan University.

Transfection

After being digested with trypsin, cells were transferred 2×10^5 cells/well to a 6-well plate and cultured overnight. Lentiviral vectors (siRNA-NC, 4.1R-siRNA1, 4.1R-siRNA2, and 4.1R-siRNA3) were constructed and then transfected into DC2.4 cells by using ExFect2000 Transfection Reagent (Vazyme, Nanjing/China). The 4.1R target sequences were as follows:
SiRNA-4.1R1: GAAGGAGATAGAACTTGGA
SiRNA-4.1R2: GAAGACTTGACCAAGAACA
SiRNA-4.1R3: GGATCCAAATTCCGATACA
Negative control: ACTACCGTTGTATAGGTGT

Cells were transfected with 5 μl siRNA mixed with 5 μl transfection reagent. After being transfected for 72 h, cells were collected for morphological observation and further experiments.

Transwell assay

After 72-h transfection, cells were digested with trypsin and resuspended with MEM medium containing 2% FBS. Then, the cell concentration was diluted to 2.5×10^5 cells/ml, and then placed in the upper chamber of Transwell plates (5×10^4 cells/hole). Then, we added 500 μL 10% FBS MEM medium to the lower chamber. After being incubated at 37°C and 5% CO_2 for 16 h, the cells in the upper chamber were removed. Then, we fixed the cells in the lower chamber with 4% paraformaldehyde for 15 min. Finally, cells were stained using crystal violet for 15 min and washed 3 times with PBS. The numbers of invaded cells from 3 randomly selected fields were counted using a microscope (Olympus, Tokyo/Japan) and the average number was calculated.

Flow cytometry (FCM)

Cell cycle and surface antigens were analyzed using flow cytometry. To analyze cell cycle distribution, cells were collected after 72-h transfection and then fixed overnight with cold 75% ethanol at 4°C. After being washed with PBS, the cells were stained with 10 μl of RNase-containing propidium iodide (500 μg/ml PI) for 30 min without light. DNA content was detected by flow cytometry (BD, USA). Surface antigens were detected as follows: After 72-h transfection, the cells were centrifuged for 5 min at 1200 rpm, washed twice with PBS, and then resuspended with 100 μl MEM medium. Cells were then incubated for 30 min with FITC-labeled antibody CD80, CD86, and MHCII (Thermo Fisher, America).

The quantitative and real-time PCR analysis

Cell RNA was extracted according to the instructions of the Trizol-spin-column kit (Generay, Shanghai/China). The concentration and purity quotient of RNA was determined by the measurement of 260 nm absorbance and 260/280 nm absorbance by using SMA4000 (Merinton, USA). We used 1000 ng of total RNA to synthesize cDNA by using the HiScript II QRT SuperMix for qPCR kit (Vazyme, Nanjing/China).

The expression pattern of EPB41 mRNA following siRNA transfection was measured by the qRT-PCR using the CFX Connect Real-Time System (BIO-RAD, America). Expression of Mus-GAPDH was measured and used as the internal control to

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Table 1. The sequences of primers.

Name	Sequences
Mus-4.1R-F	5'-ATCCACCCGACCCAGCACA-3'
Mus-4.1R-R	5'-GAGCAGACGCCGAGAATA-3'
Mus-GAPDH-F	5'-CACTGAGCAAGAGAGGCCCTAT-3'
Mus-GAPDH-R	5'-GCAGCGAACTTTATTGATGGTATT-3'

normalize the results of qRT-PCR analysis. qRT-PCR primers are shown in Table 1. qRT-PCR was detected using the ChamQTM SYBR Color qPCR Master Mix kit (Vazyme, China). The program contained 1 cycle of 95°C for 30 s, 1 cycle of 95°C for 10 s, and 45 cycles of 55.7°C for 30 s, and the melting curve was 70°C to 95°C. All samples were analyzed in triplicate. The results were measured using CFX Manager 3.1 software with $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

DC2.4 cells were harvested at 72 h after transfection and lysed by RIPA solution, and then centrifuged for 30 min at 12 000 rpm. The protein concentration was determined using the BCA protein assay kit (Generay, Shanghai/China). The protein was subjected to SDS-PAGE. The primary antibodies (Solarbio, Beijing/China) were used in incubation: anti-EPB41 (1: 1000) and anti-GAPDH (1: 1000). After washing with TBST, the membrane was incubated with goat anti-rabbit antibodies (1: 1000) conjugating with horseradish peroxidase (HRP). The anti-GAPDH was used to confirm equal loading.

Statistical analysis

All data were analyzed using SPSS 20.0 (IBM, Chicago/USA) and $P<0.05$ was considered as significant. The data are shown as mean±SD. Comparisons among multiple groups were conducted by one-way ANOVA and comparisons between 2 groups were performed with a post hoc test (the LSD t test).

Results

The expression of EPB41 in DC2.4 after 4.1R-siRNA transfection

To knock down EPB41 expression, 3 targeting sequences specific to EPB41 (4.1R-siRNA1, 4.1R-siRNA2, and 4.1R-siRNA3) were designed and transfected to DC2.4. Then, real-time PCR for mRNA level detection was conducted to validate the silencing efficiency. The results showed that all of the 3 different sequences of 4.1R-siRNA could significantly downregulate the expression of EPB41 compared to the blank group and negative control group ($P<0.05$, Figure 1A), and 4.1R-siRNA1 had the most significant silencing efficiency. The gene silencing effect on EPB41 was verified at the protein level by Western blot (Figure 1B), which was consistent with the results of the mRNA-level verification. We selected 4.1R-siRNA1 for subsequent experiments.

Effect of 4.1R-siRNA on morphology of DC2.4

After transfection with 4.1R-siRNA on DC2.4 cells, cell morphology was changed. As shown in Figure 2, cell bodies were smaller than in the control group after transfection with 4.1R-siRNA. The DC2.4 synapses were increased and the protuberance became more obvious compared with the blank group. The increased numbers of DC synapses indicate that the maturity of DC is enhanced, since the function of mature DC is quite different from that of immature DC, suggesting that silencing of 4.1R causes functional changes in DC.

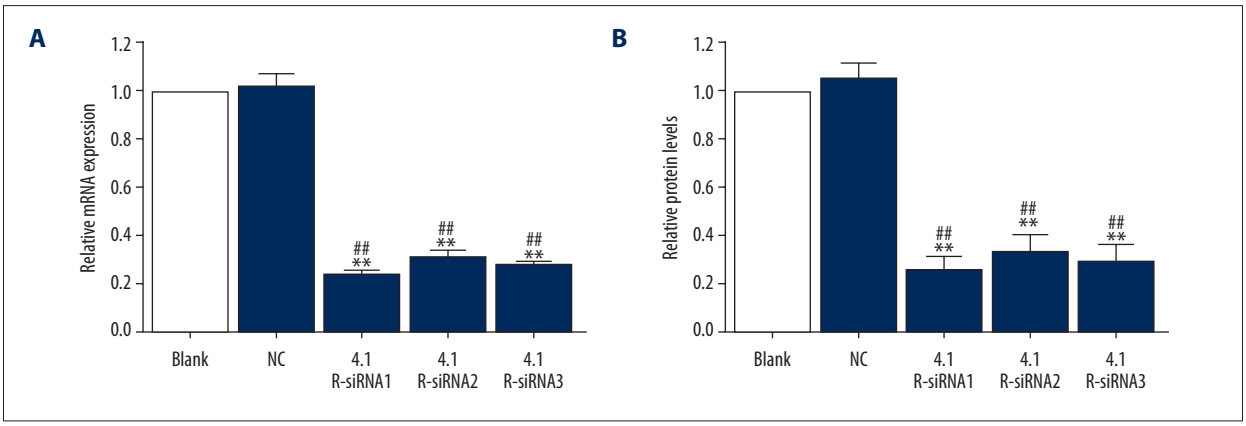

Figure 1. Validation of the silencing efficiency at mRNA and protein level. (**A**) EPB41 mRNA expression level determined by qRT-PCR. (**B**) EPB41 protein level was analyzed by Western blot. Values are shown as means±SD. NC – negative control. * $P<0.05$, ** $P<0.01$ compared with blank control group; # $P<0.05$, ## $P<0.01$ compared with NC group.

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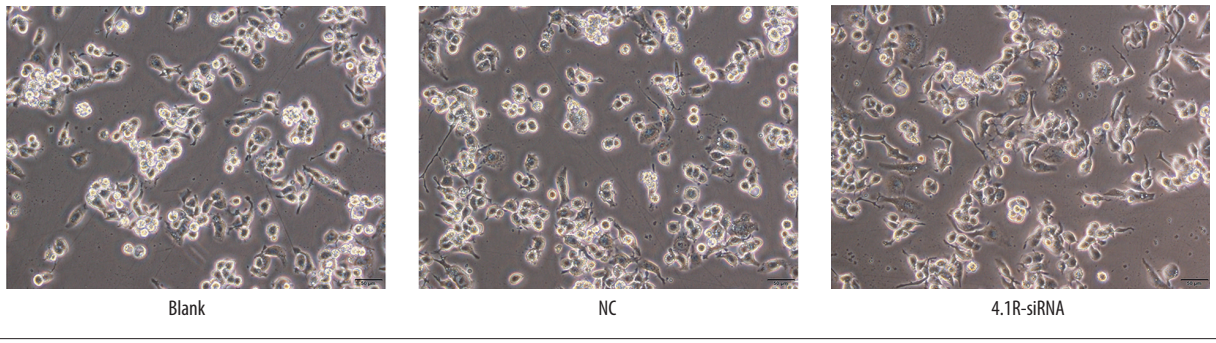

Figure 2. Morphology observation of DC2.4 (200×, Olympus). NC – negative control.

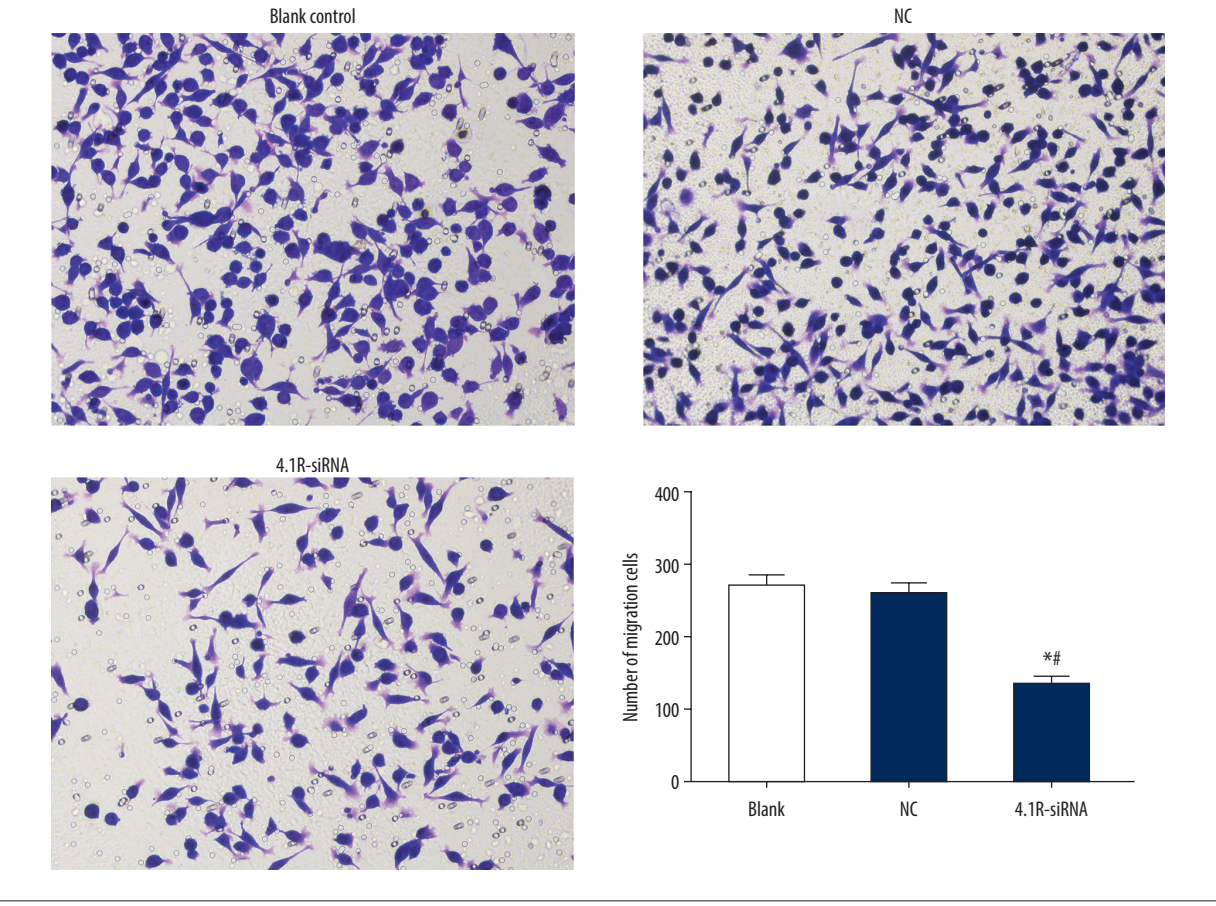

Figure 3. Cell migration ability of DC2.4 was analyzed by Transwell assay. NC – negative control. * $P<0.05$, compared with blank control; # $P<0.05$, compared with NC group.

Effect of 4.1R-siRNA on migration of DC2.4

Transwell analysis was used to examine the effect of EPB41 silencing on the migration of DC2.4 cells. As shown in Figure 3, the migration ability of DC2.4 cells in the 4.1R-siRNA transfected group was significantly decreased ($P<0.05$). These results suggest that downregulation of 4.1R suppresses the migration ability of DC2.4 cells.

Effect of 4.1R-siRNA on cell cycle of DC2.4 cells

FCM was carried out to examine the effect of silencing 4.1R on the cell cycle of DC2.4 cells. The results of analysis indicated that downregulation of 4.1R induced significant differences of cell cycle distribution in DC2.4 cells. The percentage of cells at G1 phase in the 4.1R-siRNA group decreased and the percentage of cells at S phase increased significantly compared

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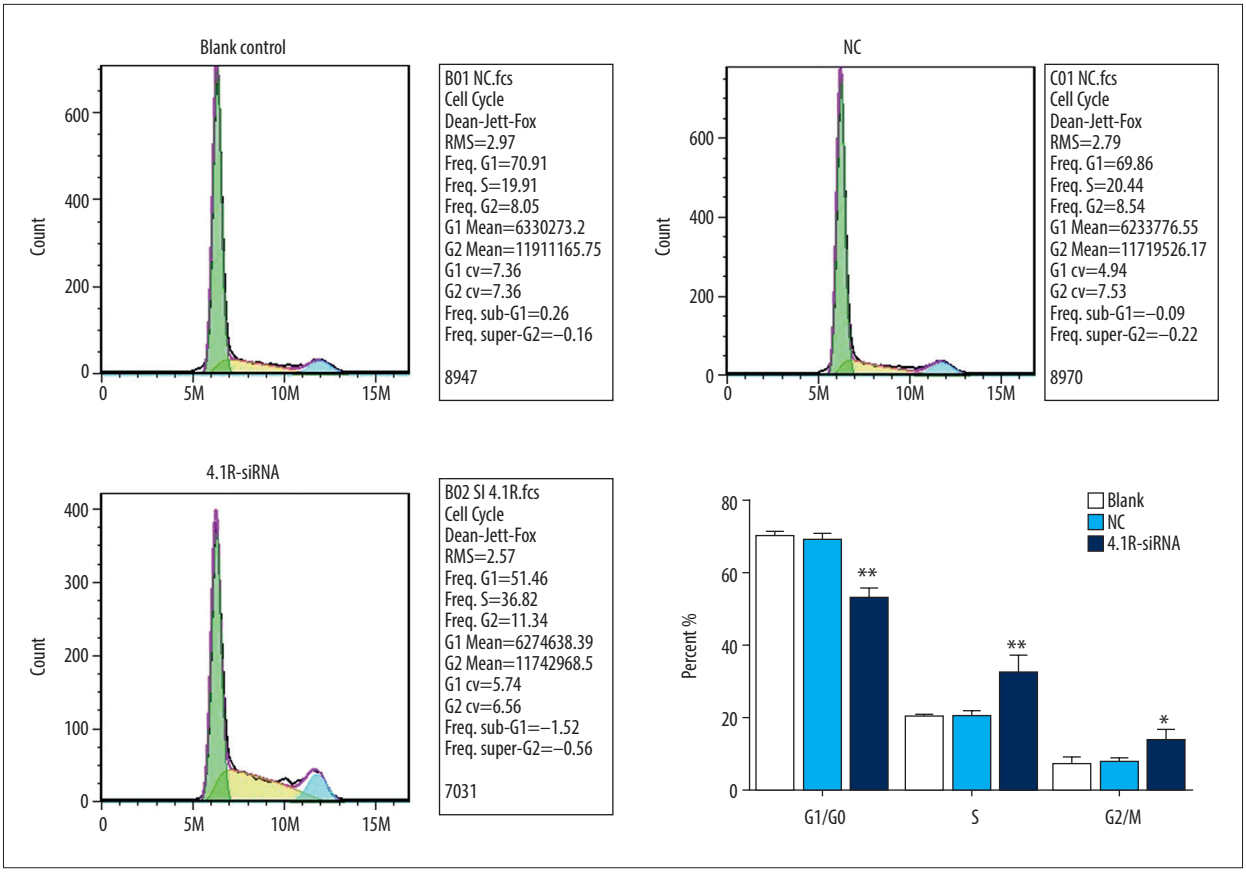

Figure 4. Cell cycle analysis was determined by FCM. The percentage of cell cycle phase are shown as means±SD. Asterisks indicate significant differences, * $P<0.05$, ** $P<0.01$ compared with blank control group; # $P<0.05$, ## $P<0.01$ compared with NC group.

with the blank group and NC group (both $P<0.05$), which indicates that silencing of 4.1R of DC2.4 can induce S phase arrest (Figure 4).

Effect of 4.1R-siRNA on the expression of surface antigens in DC2.4 cells

The expression of surface antigen is a sign of DC2.4 cell maturation, which determines the antigen-presenting ability. Therefore, the effect of 4.1R-siRNA on the expression of DC2.4 surface antigens MHCII, CD80, and CD86 were detected by FCM. As shown in Figure 5 and Table 2, the expression of MHCII, CD80, and CD86 were all upregulated in the 4.1R-siRNA-transfected group compared with the blank control group and NC group (all P<0.05), while there was no difference between the blank group and the NC group. These results indicate that 4.1R can change the capacity of antigen presentation of DC2.4 cells.

Discussion

In this research, we investigated the function of 4.1R in dendritic cells by downregulating 4.1R in DC2.4 cells and then explored its possible relationship with the pathogenesis of MG. The silencing of 4.1R in DC2.4 cells affects cell morphology, cell cycle, cell migration, and surface antigen expression. Protein 4.1 (EPB41) was initially considered to be the main cytoskeleton component of erythrocyte membranes and the core of action in skeleton tissue and transmembrane protein assembly. In recent years, multiple functions of 4.1R in non-erythroid cells have also been reported. For example, Yang et al. reported that, compared with normal tissues, the expression of 4.1R mRNA and protein was downregulated, and found that 4.1R has an anti-cancer effect in the development of HCC (hepatocellular carcinoma) [10]. MG is a chronic autoimmune neuromuscular disease [11]. Researchers reported thymus abnormality was closely related the development of MG [12]. Our previous study found that 4.1R was overexpressed in the thymuses of MG patients. Dendritic cells are the core antigen presentation cells in the thymus and play pivotal roles in innate and adaptive immunity [13]. Dendritic cells activate naive T cells to initiate the immune response and maintain the tolerance of T cells, thereby regulating homeostasis and preventing autoimmunity [14], suggesting that 4.1R can affect the biological characteristics of dendritic cells. siRNAs of 4.1R were designed in the present study and the results showed that

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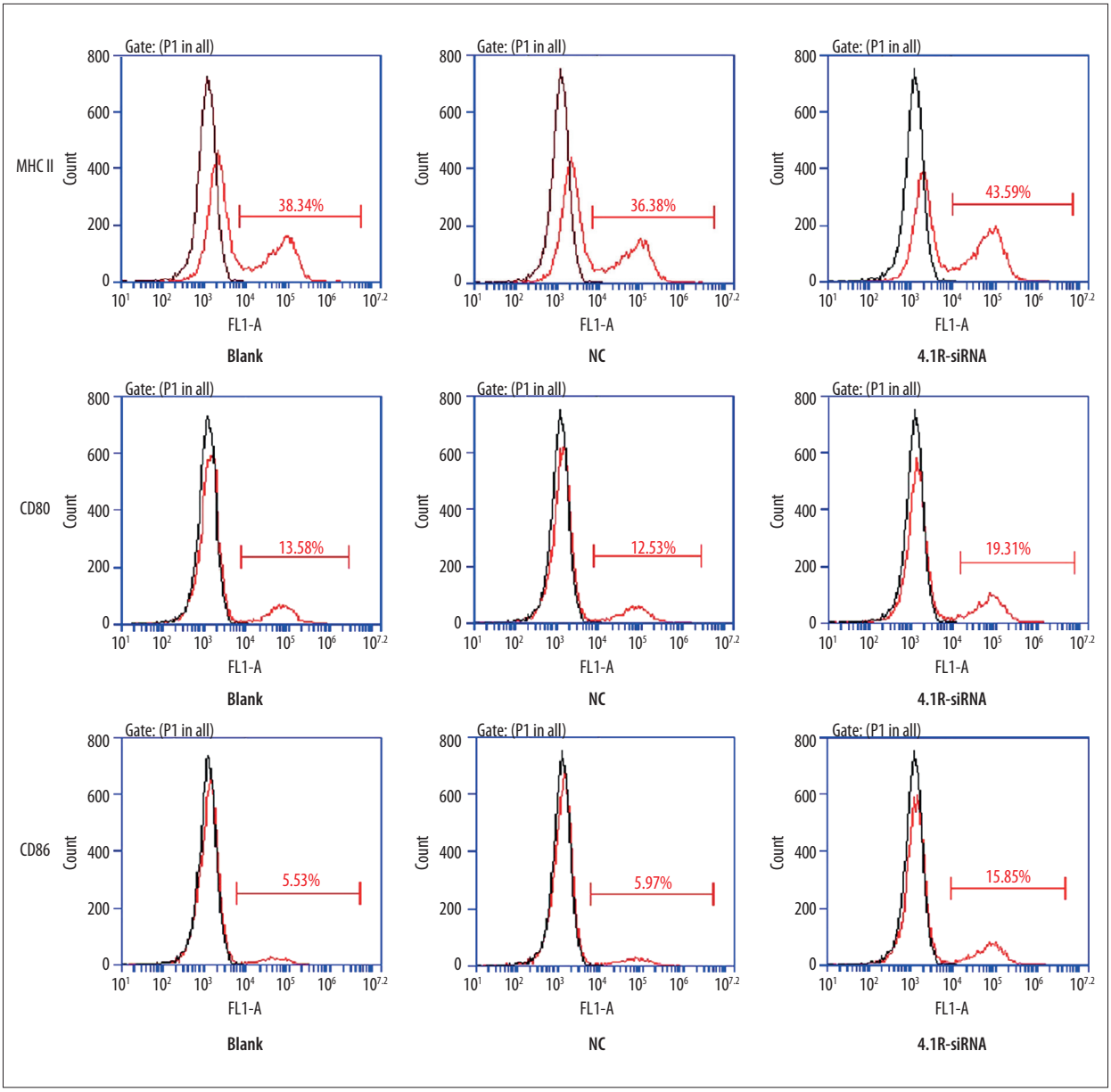

Figure 5. The expression levels of surface antigens MHCII/CD80/CD86 were determined by FCM.

Table 2. The expression levels of surface antigens MHCII/CD80/CD86 were determined by FCM (mean±SD).

Groups	MHCII	CD80	CD86
Blank	38.2±2.1	13.6±1.8	5.6±0.8
NC	37.5±2.2	13.0±2.0	5.9±0.7
siRNA-4.1R	44.6±3.1*#	19.6±2.4*#	15.8±1.6*#
F	9.623	10.213	25.334
P	0.000	0.000	0.000

NC – negative control. * P<0.05, compared with blank control; # P<0.05, compared with NC group.

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4.1R-siRNAs could effectively downregulate the level of 4.1R mRNA and protein expression.

Some studies reported that 4.1R and its family members are involved in cell proliferation, apoptosis, and migration [15]. Robb et al. [16] demonstrated that overexpression of 4.1R induced IOOM-Lee and CH157-MN cell proliferation in support of a meningioma tumor suppressor function. Ruiz-Sáenz et al. [17] showed that 4.1R plays a crucial role in migration of non-erythroid cells, and the recruitment of the scaffold protein IQGAP1. Yang et al. [10] also showed that overexpression of 4.1R could inhibit cell proliferation and cell migration and induce cell apoptosis in hepatocellular carcinoma, but has no effect on cell cycle progression, and acts as an anti-cancer factor. In tumor cells, 4.1R was proved to inhibit cell migration. However, in the present research, we found that the silencing of 4.1R in DC2.4 cells inhibited their migration ability. The cause of this difference between study results may be that the main characteristics of dendritic cells are different from those of cancer cells. Krauss et al. [8] demonstrated that downregulation of 4.1R disrupts centrosomes, alters cell cycle progression, and perturbs the mitotic spindle and anaphase. In cells with impaired p53 pathway, such as HeLa cells, 4.1R deletion significantly delays S-stage progression [18]. These results are consistent with our study. Knocking down 4.1R leads to DC2.4 cell cycle arrest. The migration of dendritic cells is very important for activation of T cells, which is an important pathogenesis of autoimmune diseases. Thus, it can be speculated that 4.1R is involved in MG by regulating the migration and cell cycle of dendritic cells.

Dendritic cells are considered to be the most powerful antigen-presenting cells [19] and can present antigens to T lymphocytes to activate immune response [20]. The antigen presentation ability of DCs is related to their stage of maturity. CD80 and CD86 are the later molecules called co-stimulatory molecules that interact with CD28 and cytotoxic T lymphocytes [21]. MHCII has been proved to be involved in most immune diseases [22]; they present exogenous antigens and mainly are phagocytosed and digested into antigenic polypeptides by antigen-presenting cells (APCs) such as DCs, macrophages, and B cells [23]. CD80, CD86, and MHCII are the markers of maturity of dendritic cells, and their increased expression means that the capacity of antigen presentation is higher.

In the present study, silencing 4.1R induced the upregulation of DC2.4 cell surface antigens. Thus, we speculated that the upregulation of 4.1R in MG patients may reduce the ability of DC to present antigens, inhibit its activation, and even induce the inactivation of T cells, leading to immunologic tolerance and the occurrence of MG. However, more research is needed to elucidate the role of 4.1R in MG.

Conclusions

We found that 4.1R is associated with dendritic cell characteristics. Knockdown of 4.1R can affect DC2.4 cell morphology, cell cycle, cell migration, and surface antigen expression. The identification of the role of 4.1R in dendritic cells provides a new direction for studying the pathogenesis of myasthenia gravis.

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