

Forskolin enhances the antitumor effect of oncolytic measles virus by promoting Rab27a dependent vesicular transport system in the lung cancer A549 cells in vitro

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Research

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Abstract

Background: Measles vaccine strain viruses (MV-Edm) are an ideal platform for developing safe and effective oncolytic vectors. However, despite the promising pre-clinical data, understanding of determinants of efficacy and, thus, the interplay of the oncolytic virus with particular agents remains limited.

Methods: We investigated the potency of forskolin enhancing the antitumor effect of oncolytic measles virus by promoting Rab27a dependent vesicular transport system. Cells were infected with MV-Edm and the vesicles were observed by TEM. The oncolytic effects of MV-Edm/Forskolin were investigated in vitro.

Results: Here we demonstrate that the MV-Edm infection and spread in tumor, which are indispensable processes for the viral oncolysis, depend on the vesicular transport system of tumor cells. On the contrary, the tumor cells display a responsive mechanism to restrain the MV-Edm spread by down-regulating the expression of Rab27a, which is a key member of the vesicle transport system. Over-expression of Rab27a promotes the oncolytic efficacy of MV-Edm towards A549 tumor cells. Finally, we find a Rab27a agonist Forskolin, is capable of promoting the oncolytic effect of MV-Edm in vitro.

Conclusions: Our study reveals the important role of vesicle transporter Rab27a in the whole program of MV-Edm mediated oncolysis. We also provide a combined strategy of Forskolin and MV-Edm, which may exert a synergistic anti-tumor effect, for clinical treatment for patients with tumor.

Introduction

Measles virus (MV-Edm) has been proved to be a kind of safe and effective oncolytic virus because it can selectively kill a variety of tumor cells with no harm to normal cells [1]. At present, it has been applied in a number of clinical trials. The current research about MV-Edm is to improve the tumor lytic effect for developing excellent clinical efficacy [2]. Therefore, it is necessary to target the key molecules to promote its anti-tumor effect.

MV-Edm is an enveloped negative strand RNA virus containing six genes, which encode nucleoprotein, phosphoprotein, membrane protein, fusion protein, virulence factor, hemagglutinin, and large polymerase [3]. The replication of MV-Edm starts with the adsorption of surface H protein to the host cell membrane. After specific binding with the receptor, it fuses with the host cell membrane under the action of F protein, and then the nucleocapsid is released into the cytoplasm and the replication takes place there. MV-Edm kills the receptor cell by inducing multinucleation [4, 5]. Therefore, it is important to promote the assembly, maturation and secretion of MV-Edm to improve its oncolytic effect.

Previous studies have found that enveloped viruses usually achieve assembling and propagation depending on the vesicle transport system of host cells, and formed virus particles were secreted to infect other cells [6, 7]. Cell vesicle transport is the process of transporting biomacromolecules such as proteins, polysaccharide and polynucleotides to specific organelles via different vesicles across membrane, which

is the main form of substance exchange across eukaryotic cells [8]. In the transportation process, biomacromolecules are inside the vesicles or on the membrane, which is called vesicle trafficking [9].

As the largest subfamily of Ras superfamily, Rab regulates most of the intracellular transport and plays an important role in different stages of transportation [10]. It has been studied that Rab can somehow gather specific motor molecules which are related to microtubule and actin to target membrane and regulate the transportation of molecules or vesicles related to organelle [11]. It has also been proved that a few members of Rab family are closely related to the infect process of various viruses [12]. For example, Rab1, Rab5, Rab6, Rab11, Rab27 and Rab43 are involved in the infection of Herpes simplex virus 1 [13–15]. Rab1, Rab5, Rab11, Rab27 and Rab43 are involved in the infection of Influenza A virus [16, 17]. Rab11 is also involved in the infection of Mumps Virus [18].

So far, it has been also reported that Rab proteins such as Rab9 and Rab11 are involved in infection of MV-Edm [19, 20]. Theoretical basis for research on oncolytic mechanism of MV-Edm has been built based on the research findings of combination of vesicle and membrane, across-membrane transport of proteins and cell secretion and endocytosis. New methods are also proposed to improve the oncolytic effect.

Ostrowski M. et al. found that Rab family proteins were involved in the regulation of cell membrane lysis and fusion in tumor cells during vesicle transportation. If the expression of some proteins in Rab family was inhibited, the number of cell membrane lysis was significantly reduced, especially for Rab27a [11, 21]. Further study on the role of Rab27a in tumor cell vesicle transport revealed that Rab27a mainly promoted the fusion of vesicles and cell membrane, and activated lysosome to promote cell membrane lysis [22, 23]. Rab27a has been proved to be closely related to viral assembly and vesicle transport. Recent studies have shown that infection with HCMV increases the expression of Rab27a and gather Rab27a to the membrane structure of assembly site [24].

In this study, in order to solve the key problems of limited local replication and tumor lysis about MV-Edm, we firstly explored the effect of Rab27a-mediated vesicle transport system on the oncolytic effect of MV-Edm, and found a suitable drug to regulate the vesicle transport system of tumor cells to promote the oncolytic effect. We also found that inhibiting Rab27a also reduced the generation of syncytial body induced by MV-Edm, and oncolysis was enhanced when Rab27a was increased. These experimental results could support the new thinking of oncolytic improvement based on targeted strategy. This study would be expected to promote the standardization and industrialization of anti-tumor therapy strategy of oncolytic virus, and find a new method to optimize MV-Edm mediated oncolytic strategy in the future. It may also provide theoretical basis to clinical therapy.

Materials And Methods

Cell lines and cell culture

Human non-small cell lung cancer cell line A549 (CCL-185) and Vero African green monkey kidney cells (CCL-81) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in DMEM supplemented with 0.1 mM nonessential amino acids, 5% fetal bovine serum, and 100 U/mL penicillin-streptomycin (all from Invitrogen, Carlsbad, CA). All cells were maintained in a humidified incubator with 5% CO₂ at 37 °C.

Viruses

Measles virus Edmonston vaccine lineage seed B (MV-Edm), kindly provided by S. Russell, Mayo Clinic, MN, USA, were propagated in Vero cells. The cells were infected with a multiplicity of infection (MOI) of 0.02 in 2 ml OptiMEM (Invitrogen, 31985-062) at 37 °C for 3 h. The medium was replaced with DMEM supplemented with 2% FCS, and the cells were incubated at 37 °C for 1 day before being transferred to 32 °C for another day. When 100% of the cell monolayer was fused into syncytia, the cells were harvested, and the viral particles were released by three cycles of freezing and thawing. The viral titers were determined by 50% end-point dilution assays (TCID₅₀) on the Vero cells. The viral supernatant was centrifuged to remove cell debris and frozen at - 80 °C.

Reagents and siRNAs

The reagents used in this study are listed as follows. Forskolin were purchased from Sigma-Aldrich. All solvents were used directly without further purification. The siRNAs targeting RAB27a (Invitrogen; HSS108985) and negative-control siRNA (Invitrogen; 12935400) were all purchased from the Invitrogen Stealth RNAi collection. All reagents were formulated as recommended by their suppliers.

The vectors of pGEM-T and pcDNA3.1, and RNA extraction kit were purchased from Invitrogen. Restriction endonuclease HindIII, BamH I and DNA ladder were purchased from TaKaRa Corporation. First-strand cDNA Synthesis Kit was purchased from Qiagen Incorporation. T4 DNA ligation enzyme was obtained from Promega. Taq DNA polymerase and marker were purchased from Fermentas. The sequence of Rab27a mRNA was taken from Genbank database (NM_004580 and subjected to restriction enzyme cutting site analysis. On the flanker of gene Rab27a, a pair of primers were searched and the sense primers was 5'- GCCACTGGCAGAGGCCAG - 3', and the anti-sense primers was 5'- GAGTGCTATGGCTTCCTCCT-3'. All the primers were synthesized by Invitrogen Incorporation.

Western blot analysis

Cells were pelleted and lysed using RIPA buffer containing a protease inhibitor cocktail (Roche, Mannheim, Germany, 11873580001). The protein concentration was determined. The samples were migrated on SDS-PAGE and transferred onto PVDF membranes (Roche, 03010040001). After blocking with 5% nonfat milk, the membrane was incubated with primary antibodies followed by incubation with horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence reagent (Millipore, Darmstadt, Germany, WBKLS0500) and subjected to the Alpha Innotech Fluor Chem-FC2 imaging system (Alpha Innotech, San Leandro, CA). Antibodies were as follows: anti-GAPDH (Bioworld, Nanjing, China, 1:5000 diluted), anti-Rab27a (Abcam, ab55667, 1:1000 diluted).

Transfection

100 nM of siRNA or 500 ng/ml expression plasmids coupled with Lipofectamine 2000 (Invitrogen; 11668-019) were used for transfection of A549 cells on a 6- or 12-well plate according to the manufacturer's instructions. For all experiments, MV-Edm infection was performed 24 h after siRNA or plasmids transfection.

Electron microscopy

A549 cells (5×10^4 cells/cm²) were seeded on sapphire discs (Brügger) in a 12-well plate and infected with MV-Edm at an MOI of 0.5 for 3 h. Cells were washed and incubated for an additional 9 h. Samples were frozen under high pressure, dehydrated, and chemically fixed. Ultrathin section was cut and stained with uranyl acetate and lead.

Cell viability assay

To measure viability following MV-Edm or Forskolin/MV-Edm infection. Cells were harvested with trypsin/EDTA and stained with 0.2% trypan blue (C3601-2; Beyotime Inc., Shanghai, China). The cell viability was determined by the trypan blue exclusion assay using a Countstar Automated Cell Counter (Inno-Alliance Biotech Inc., Wilmington, DE, USA).

Quantitative RT-PCR

For quantitative reverse transcription (RT)-PCR (qPCR), total cellular RNA was extracted with TRIzol (Invitrogen, 15596-026), and 1 µg of RNA was reverse-transcribed using the synthesis system (TaKaRa, DRR036A). qPCR was performed using the real-time PCR system (ABI 7300). Gene expression was calculated with the comparative Ct method and normalized to the endogenous levels of GAPDH. Primer sequences used for qPCR are as follows: GAPDH, 5'-CCACCCATGGCAAATTCATGGCA-3' and 5'-TCTAGACGGCAGGTCAGGTCCACC-3'; MV-Edm N-protein, 5'-ACATTAGCATCTGAACTCGGTATCAC-3' and 5'-TTTTTCGCTTTGATCACCGTGTA-3'.

Statistical analysis

All data are expressed as the mean \pm standard error of the mean (SEM). Student's t tests were used for statistical analyses. P-values less than 0.05 was considered to represent statistical significance.

Results

MV-Edm infection can significantly activate the vesicular transport system of tumor cells

The influence of MV-Edm infection to cancer vesicles was explored. A549 tumor cells were infected with MV-Edm and observed by transmission electron microscopy to observe the ultrastructural changes of vesicle inside them. Compared with the untreated group, we observed that the vesicles in the cells increased significantly after the infection (Fig. 1). The results suggested that MV-Edm infection can activate the vesicle transport system of tumor cells.

Vesicle transport molecule Rab27a plays a key role in the generation of syncytia in MV-Edm infected cells

Subsequently, the relationship between vesicle transport molecule Rab27a and MV-Edm infection was investigated. Expression of Rab27a protein was measured after A549 tumor cells were infected with MV-Edm, and it decreased overtime (Fig. 2A). It was indicated that the vesicle transporter Rab27a was closely related to the MV-Edm infection of tumor cells. However, the role of Rab27A in the MV-Edm-infected cells is unknown. It has been reported that tumor cell lysis is induced by forming multinucleated cell bodies after MV-Edm infection [25]. Syncytial size and area were observed by microscope and crystal violet after MV-Edm infection, compared with Control and Rab27a siRNA. The syncytial area was analyzed by ImageJ. It was found that the number and size of syncytia were significantly reduced after Rab27a inhibition with siRNA (Fig. 2B), revealing that Rab27a played a key role in the generation of syncytial bodies in MV-Edm infected cells.

Vesicle transporter Rab27a mainly regulates the secretion of MV-Edm, not the RNA replication of MV-Edm

Then, we explored the relationship between vesicle transporter Rab27a and MV-Edm replication. RNA level of viral structural protein in A549 was detected after infection with MV-Edm in the presence of Rab27a or control siRNA. It was found that viral RNA replication was not significantly affected after inhibition of Rab27a expression (Fig. 2C). Meanwhile, the supernatant of infected cells was collected to measure the RNA replication load of viral structural protein. We found that the exocrine of virus was suppressed significantly when Rab27a expression was inhibited (Fig. 2D). The results showed that vesicle transporter Rab27a mainly regulated the exocrine of MV-Edm, but had no significant effect on the RNA replication of MV-Edm.

Rab27a contributes to the generation of syncytial bodies in MV-Edm infected cells

To further confirm that Rab27a plays a key role in the generation of syncytia in MV-Edm infected cells, we constructed a plasmid for Rab27a overexpression. In A549 cells, after transfection with pcDNA3.1-Rab27a, the expression of Rab27a was significantly higher than that in the control group (Fig. 3A). And then syncytial size and area were observed by microscope and crystal violet after MV-Edm infection. It was found that the number and size of syncytia were significantly upregulated after Rab27a overexpression with pcDNA3.1-Rab27a transfection (Fig. 3B), revealing that Rab27a contributes to the generation of syncytial bodies in MV-Edm infected cells.

Forskolin promotes the oncolytic effect of MV-Edm

It is reported that Forskolin, an activator of adenylate cyclase, can promote the expression of Rab27a [26]. A549 cells were treated with different concentrations of Forskolin, the level of Rab27a indeed concentration dependent increased (Fig. 4A). Then cells were treated with different concentrations of Forskolin and MV-Edm in combination for 48 hours. The formation of syncytial bodies was analyzed. It was shown that as the concentration of Forskolin increased, the size and quantity of syncytial body increased, reflecting that the oncolysis was enhanced (Fig. 4B). Furthermore, the effect of Forskolin on virus spread was also evaluated. Virus expansion was also found to be enhanced, which was indicated by the elevated expression of MV-Edm-N gene (Fig. 4C). These results revealed that Forskolin, an agonist of Rab27a, can promote the syncytial formation of MV-Edm infection and enhance its oncolytic effect.

Discussion

Although the MV-Edm vaccine strain has been applied in a number of clinical trials for its reliable safety and excellent oncolytic effect, the specific oncolytic mechanism has not been elucidated [27, 28]. It is of great significance to clarify the mechanism of oncolytic virus killing tumor cells for the correct and effective optimization of oncolytic virus treatment strategy. In this study, the mechanism of vesicle transport regulating the oncolytic mechanism of MV-Edm was revealed. The expression of small G protein Rab27a, which can promote the exocrine of MV-Edm is decreased after viral infection, and then oncolytic effect can be suppressed. Up-regulating the Rab27a expression promotes the oncolysis induced by MV-Edm.

Our investigation indicated that MV-Edm infection can activate the vesicular transport system of tumor cells which is closely related to the replication and diffusion of virus in host cells. It was also found that the expression of Rab27a drops significantly as time went on. There may be three possible situations: 1) MV-Edm needs to use Rab27a in host cells to assist its replication resulting in the consuming reduction. 2) The virulence protein of MV-Edm may restrain the Rab27a protein of host cells. 3) The host cell restrained the expression of Rab27a on its own to fight the virus. In general, if the protein expression is required by the viral proteins assembly, it should occur together with the viral infection in early period. If it is the host cell response to the virus infection, the change of protein expression should be in later time after infection reaches a considerable degree. Our results showed that the expression of Rab27a did not change significantly during the first 24 hours after infection, but decreased significantly since 48 and 72 hours, especially at 72 hours. So, the situation 3) might be the fact.

Through siRNA interference experiments, we found that down-regulation of Rab27a expression in A549 cells was not conducive to the expansion of MV-Edm. The amplification of virus in host cells needs to go through three main stages: infection, replication and exocrine [29]. It was indicated in our study that the early infection and replication were not affected by the expression of Rab27a. However, the exocrine was significantly affected after the completion of virus assembly. Therefore, the amount of extracellular virus decreased, the infection to the surrounding cells was delayed, and finally the number of syncytial bodies decreased.

Forskolin is an adenylate cyclase activator, which can promote the function of vesicular transport system by up-regulating the expression of Rab27a [26, 30]. Forskolin can significantly promote the infection and diffusion of MV-Edm virus and the formation of syncytial bodies. These results indicate that the oncolytic effect of MV-Edm virus could be enhanced if it is combined with Forskolin.

Oncolytic virus is an excellent tumor therapy, and one of the main ways is that it infects tumor cells, completes replication and assembly in cells, and eventually causes tumor cells to lyse and die. At the same time, it can activate the host's immune response, effectively recognize the infected cells and kill them immunologically. The combination of these two methods will eventually enable the immune system to recognize tumor specific antigens, and further immune clearance of distant tumors. Meanwhile, it can generate immune memory and prevent tumor metastasis and recurrence. In this complex process, the

release and propagation of virus after assembling are the key prerequisites for the oncolytic effect and immune activation. Therefore, the combination of Forskolin and MV-Edm may significantly improve the therapeutic effect of the oncolytic virus treatment. We will further explore the anti-tumor effect of Forskolin and MV-Edm in vivo.

Conclusions

We found that increasing the expression of Rab27a can enhance vesicular transport and promote the exocrine of MV-Edm, and then enhance the oncolytic effect of MV-Edm significantly. It is revealed that Forskolin can be utilized to enhance the exocrine and propagation of MV-Edm via up-regulating the expression of Rab27a, and enhance the oncolytic effect of MV-Edm. This research provided a new theoretical basis and feasible method for optimizing the strategy of MV-Edm oncolysis, and also provided a reference for antiviral therapy.

List Of Abbreviations

DMEM: Dulbecco's Modified Eagle Medium

MV-Edm: attenuated measles virus of the Edmonston strain

PBS: Phosphate-buffered saline

RT-PCR: Reverse transcription polymerase chain reaction

TCID50: Tissue culture infective dose

Declarations

Ethics approval and consent to participat

Not applicable

Consent for publication

Not applicable

Availability of data and materials

We can share our data if needed.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conception and design: Han Shen, Yan Hong

Development of methodology: Mao Xia, Yongquan Xia

Acquisition of data (provided animals, provided facilities, etc.): Mao Xia, Yongquan Xia, Xuejing Xu

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Xia M, Xuejing Xu

Writing, review, and/or revision of the manuscript: Mao Xia, Yongquan Xia

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Han Shen, Xia M, Yongquan Xia

Study supervision: Han Shen

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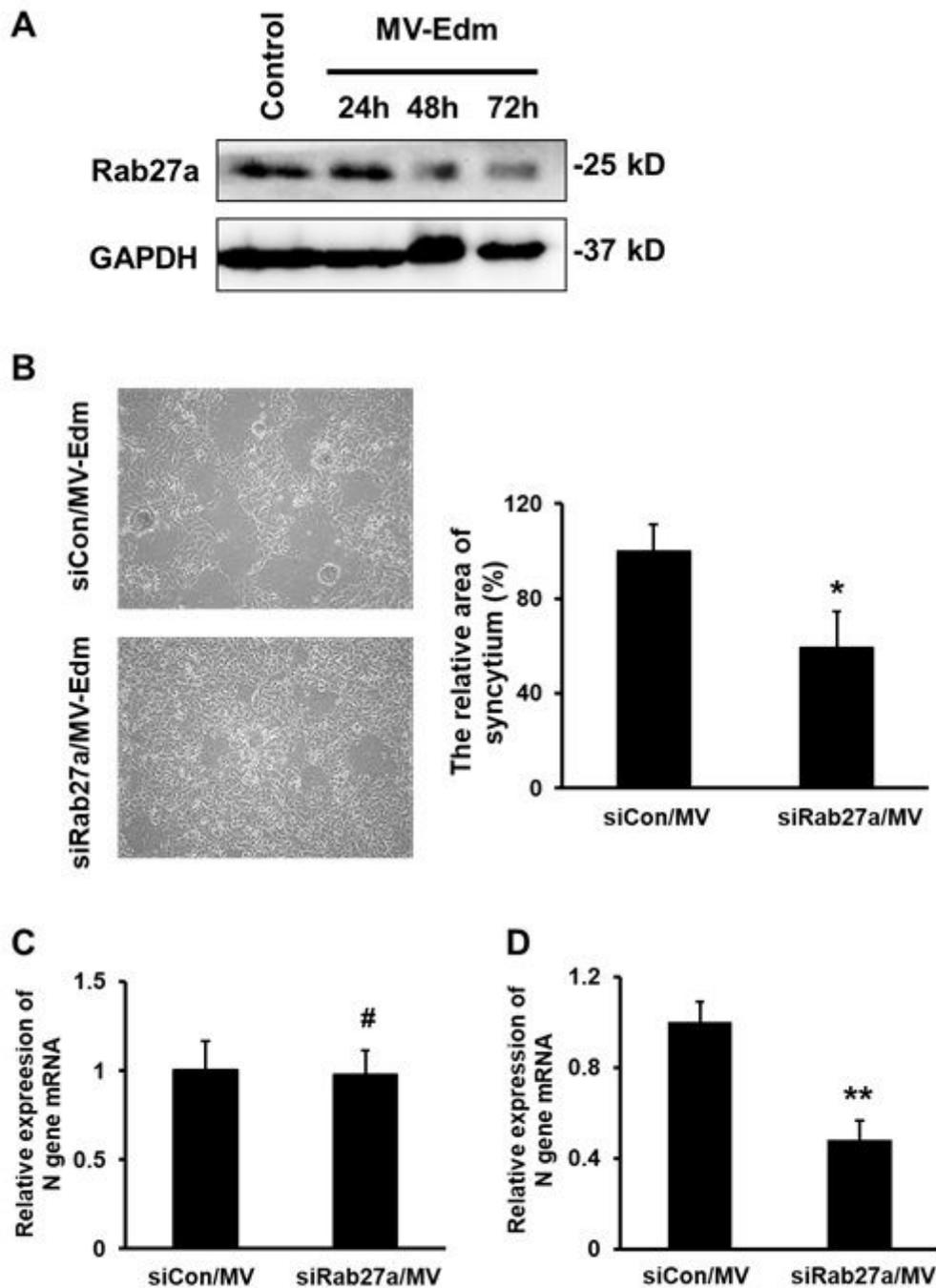


Figure 2

Rab27a plays a key role in the generation of syncytia in MV-Edm infected cells and mainly regulates the secretion of MV-Edm, not the RNA replication of MV-Edm. (A) A549 lung cancer cells were infected with MV-Edm at a MOI of 0.5 for 24, 48 or 72 h. Cell lysates were then harvested for Rab27a detection. Uninfected cells or cells infected with heat inactivated MV-Edm were used as controls. Representative blots from two independent experiments are shown. (B) A549 cells were transfected with siRNAs against Rab27a, or with non-specific control siRNA for 24 h followed by infection with MV-Edm (MOI of 0.5) for another 48 h. Syncytium formation was observed by phase-contrast microscopy (left panel) and further

analyzed using ImageJ software (right panel) 48 h postinfection. And then A549 cells were transfected with siRNAs against Rab27a, or with non-specific control siRNA for 24 h followed by infection with MV-Edm (MOI of 0.5) for another 48 h. The expression of N viral structural gene was quantified by qRT-PCR in cells (C) and culture supernatant (D). Similar results were obtained in 3 independent experiments. #p > 0.05, * p < 0.05, ** p < 0.01.

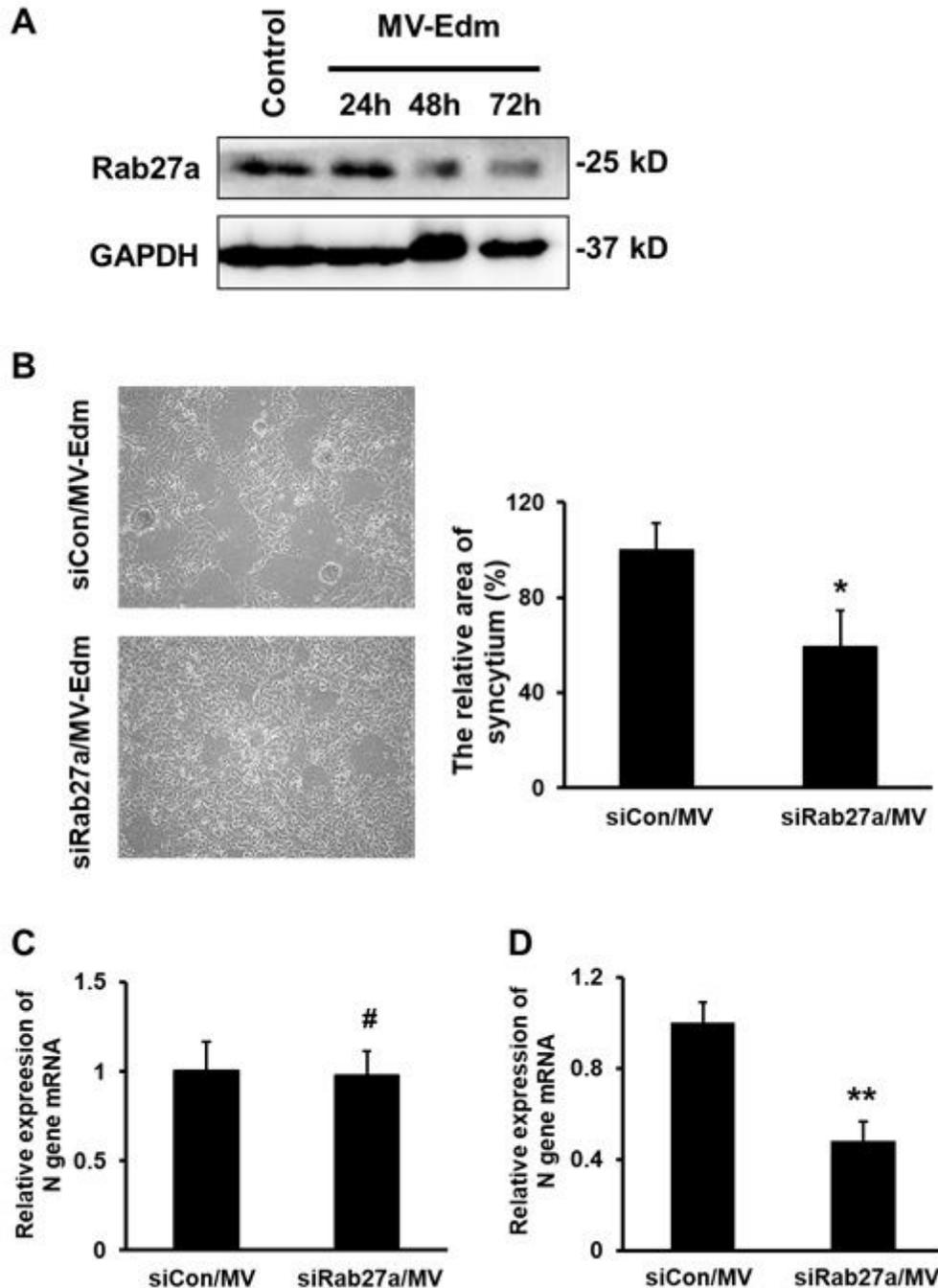


Figure 2

Rab27a plays a key role in the generation of syncytia in MV-Edm infected cells and mainly regulates the secretion of MV-Edm, not the RNA replication of MV-Edm. (A) A549 lung cancer cells were infected with

MV-Edm at a MOI of 0.5 for 24, 48 or 72 h. Cell lysates were then harvested for Rab27a detection. Uninfected cells or cells infected with heat inactivated MV-Edm were used as controls. Representative blots from two independent experiments are shown. (B) A549 cells were transfected with siRNAs against Rab27a, or with non-specific control siRNA for 24 h followed by infection with MV-Edm (MOI of 0.5) for another 48 h. Syncytium formation was observed by phase-contrast microscopy (left panel) and further analyzed using ImageJ software (right panel) 48 h postinfection. And then A549 cells were transfected with siRNAs against Rab27a, or with non-specific control siRNA for 24 h followed by infection with MV-Edm (MOI of 0.5) for another 48 h. The expression of N viral structural gene was quantified by qRT-PCR in cells (C) and culture supernatant (D). Similar results were obtained in 3 independent experiments. #p > 0.05, * p < 0.05, ** p < 0.01.

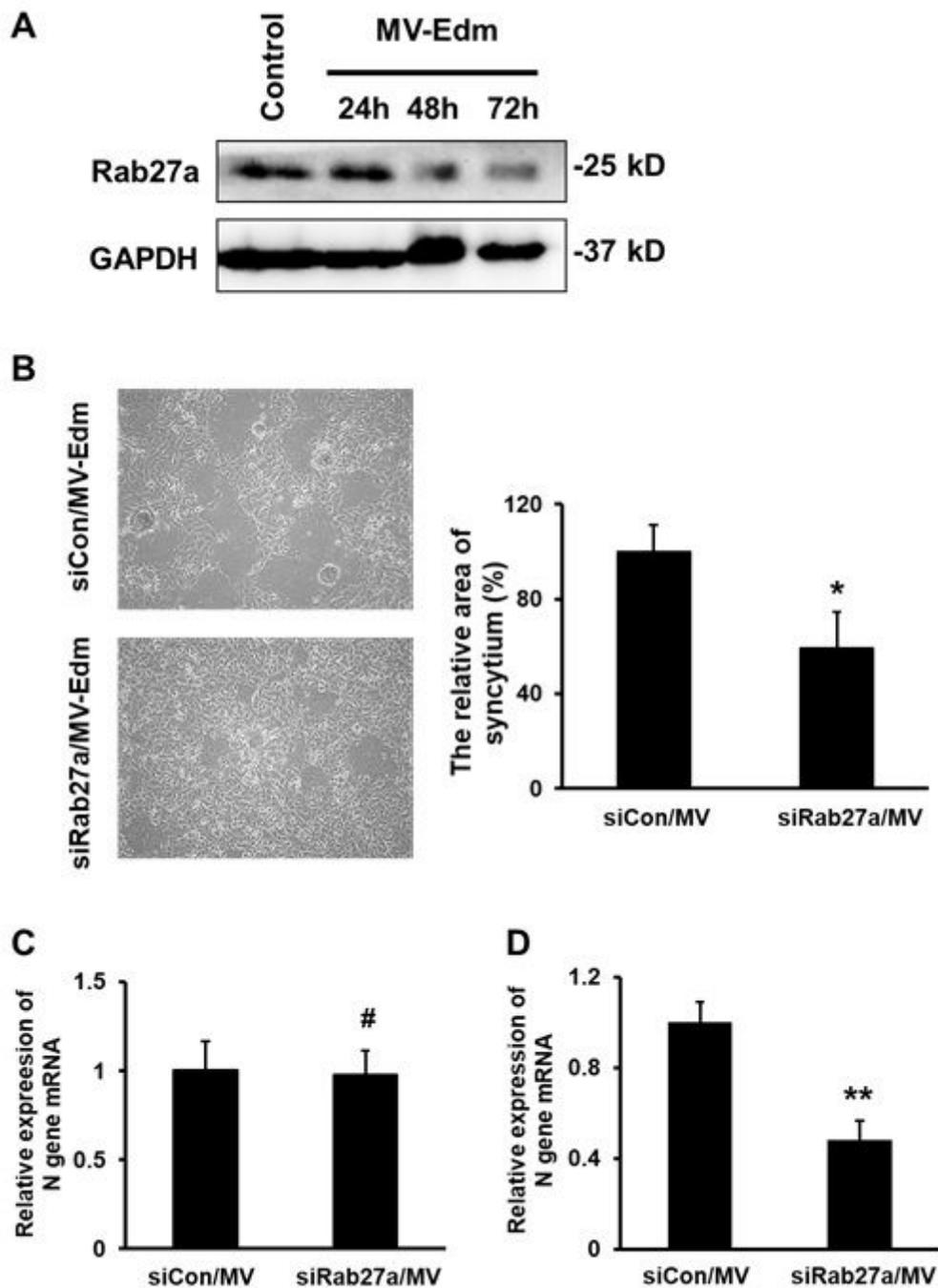


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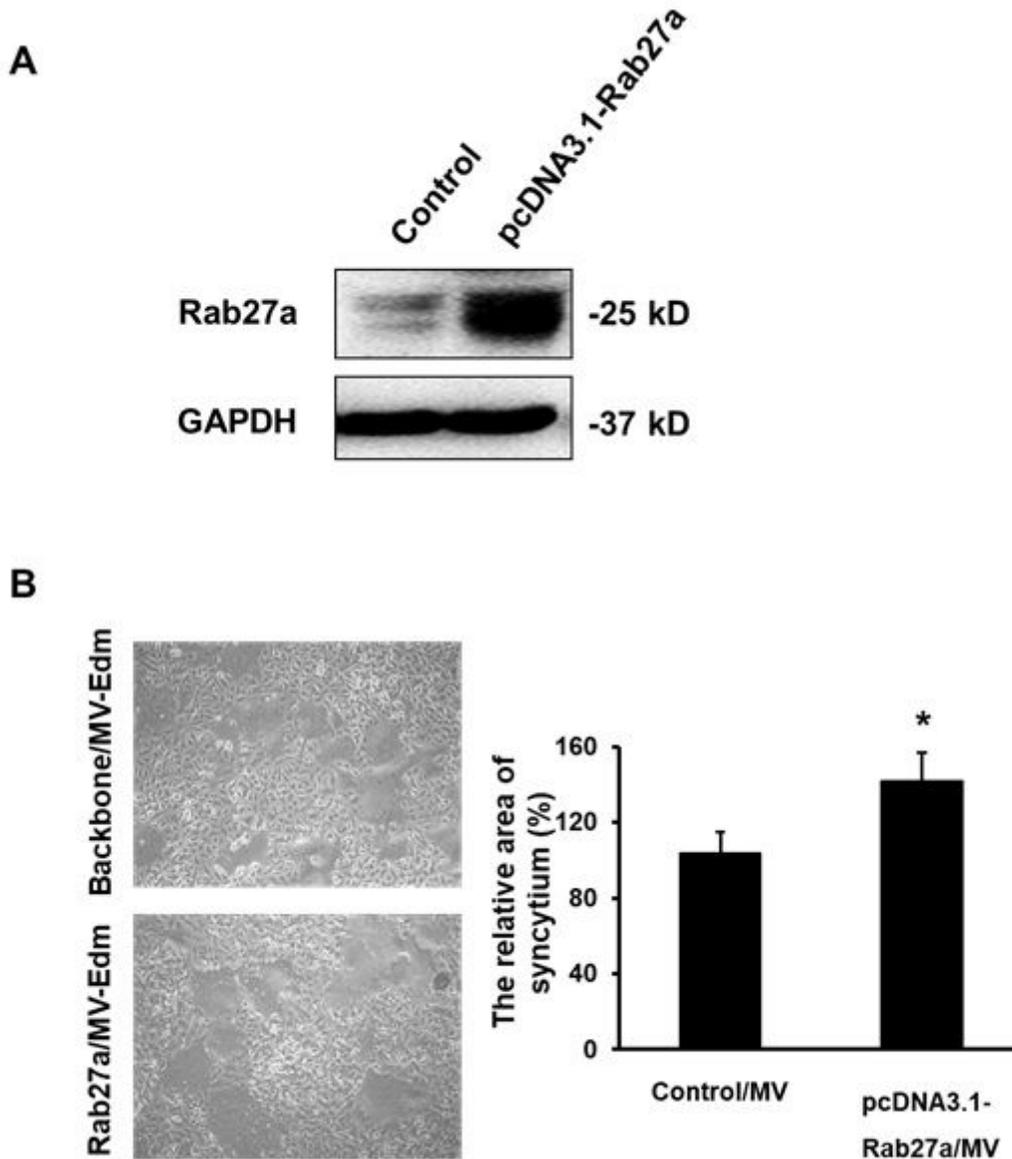


Figure 3

Rab27a contributes to the generation of syncytial bodies in MV-Edm infected cells. (A) The expression of Rab27a after transfected with pcDNA3.1-Rab27a in A549 cells versus the control group. (B) A549 cells were transfected with pcDNA3.1-Rab27a, or with control plasmid for 24 h followed by infection with MV-Edm (MOI of 0.5) for another 48 h. Syncytium formation was observed by phase-contrast microscopy

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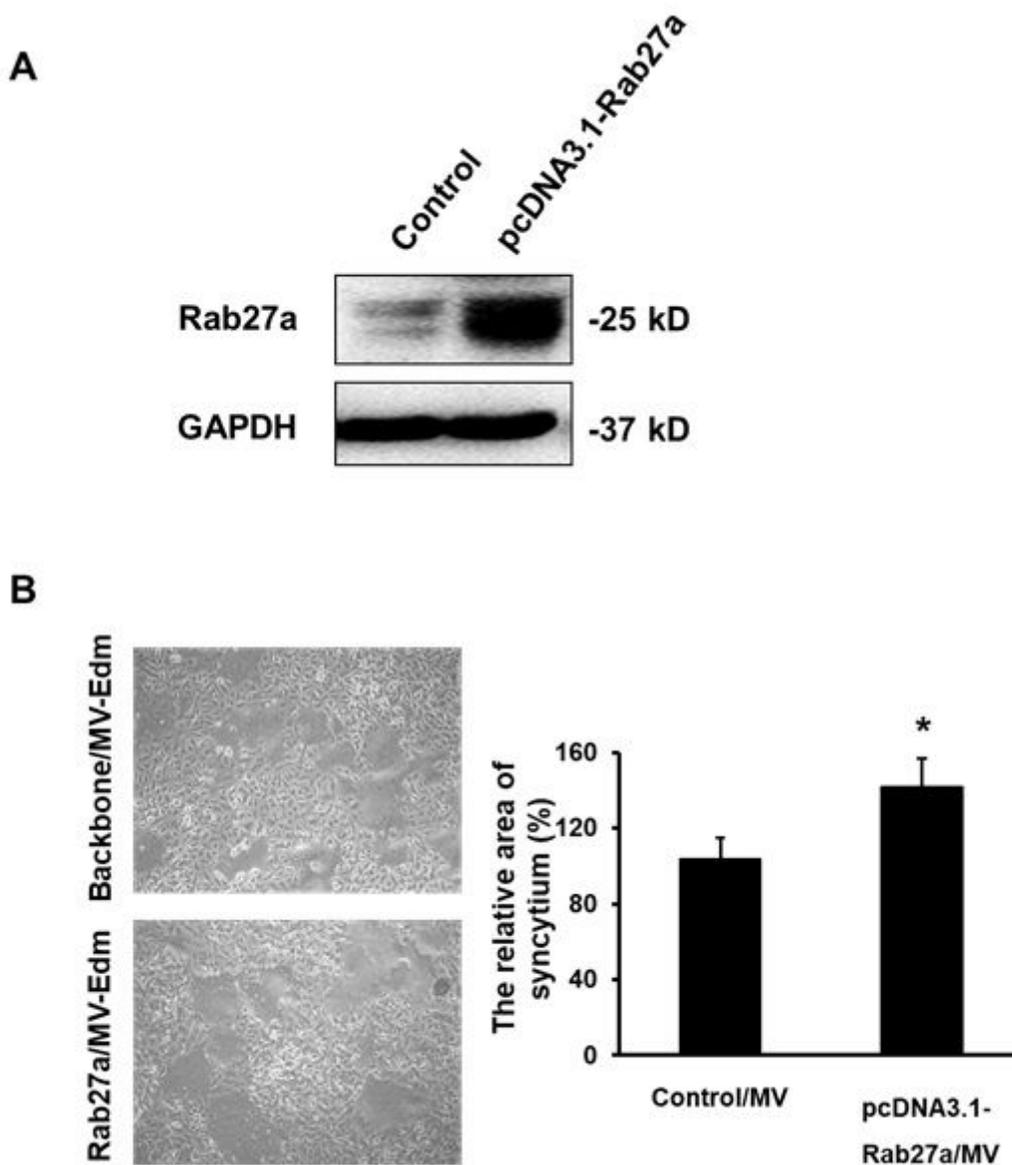


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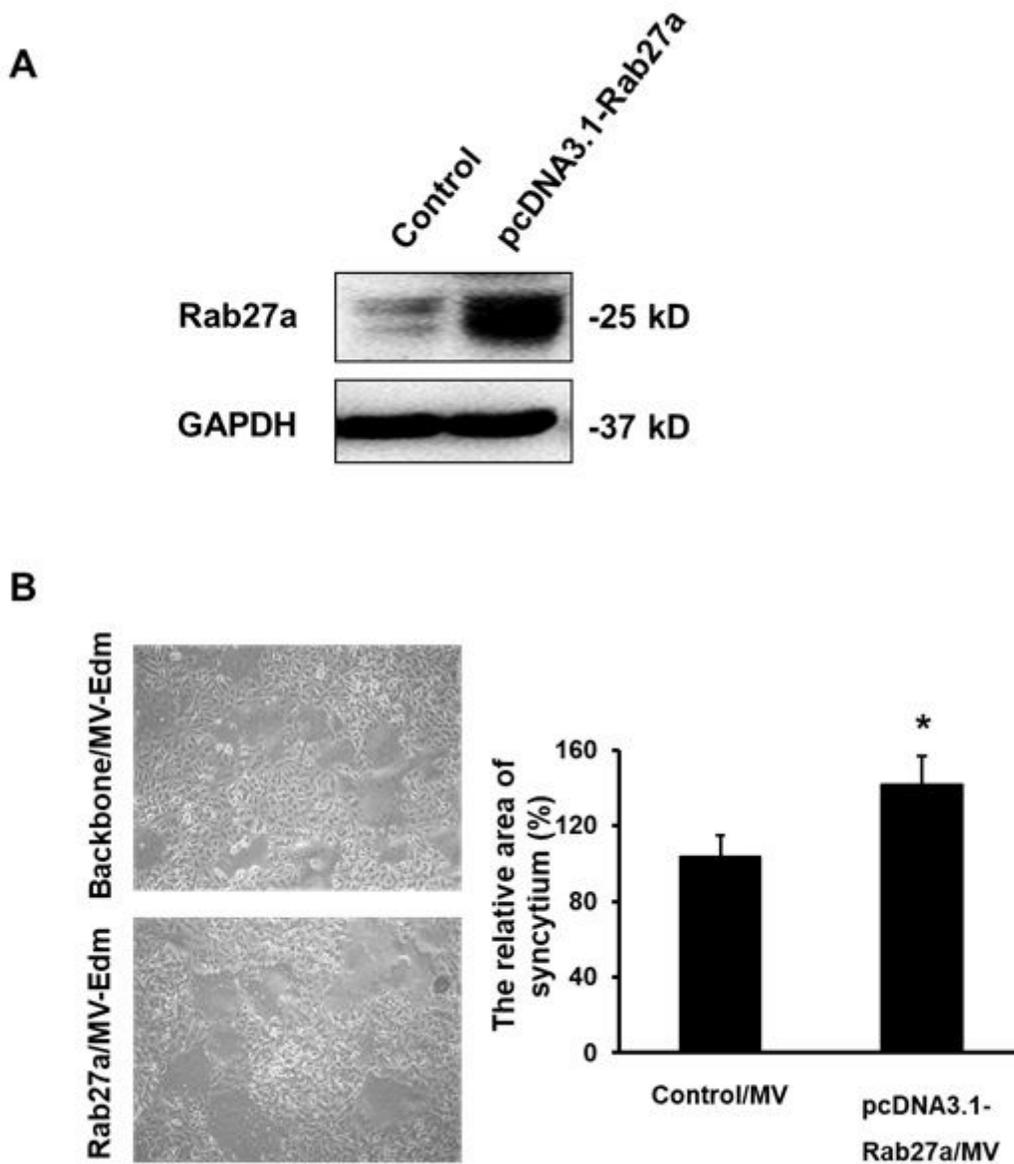


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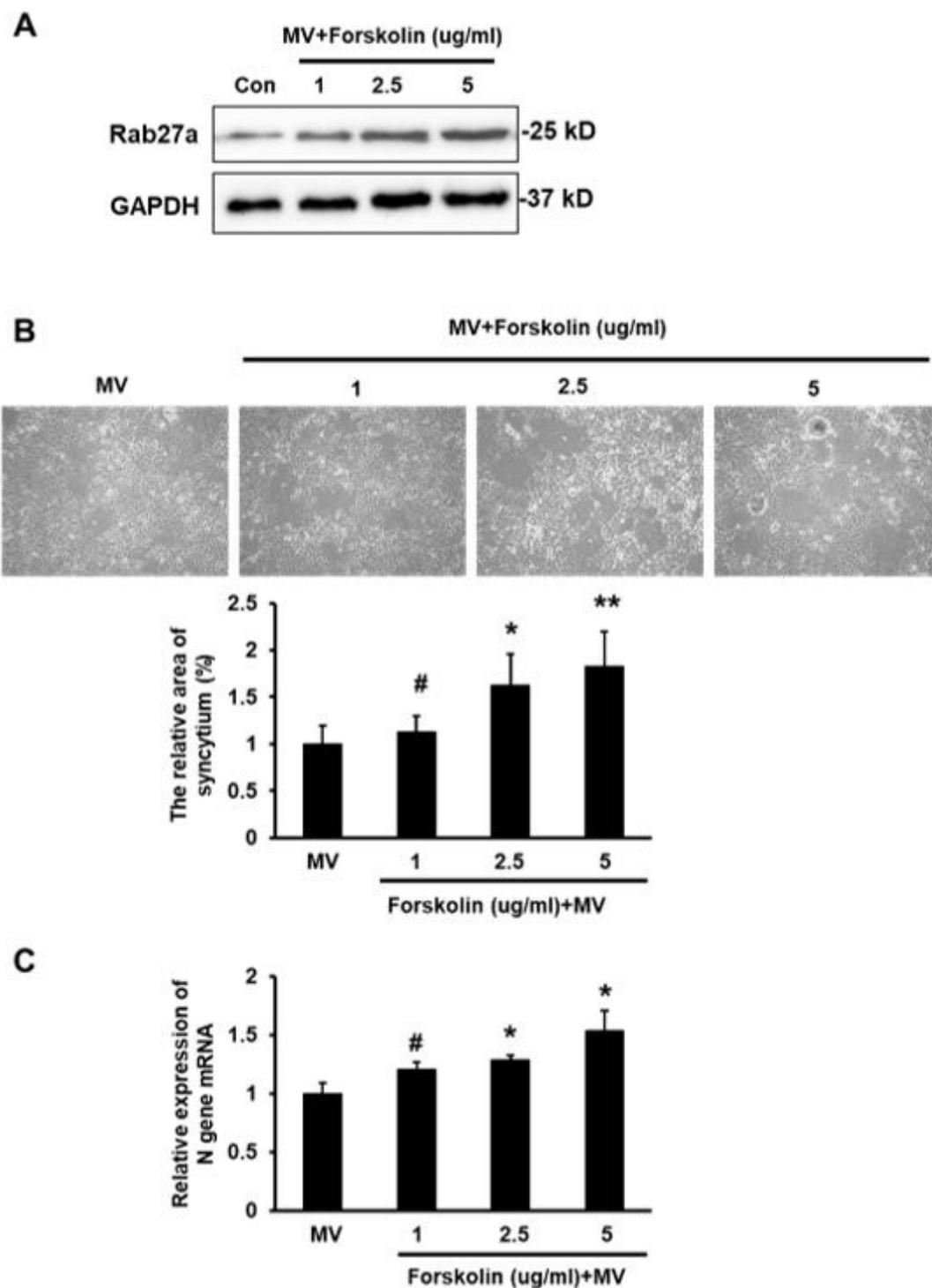


Figure 4

Forskolin promotes the oncolytic effect of MV-Edm. (A) A549 cells were treated with different concentrations of Forskolin for 12 h, cell lysates were then harvested to determine the level of Rab27a by western blot. Blots are representative of two independent experiments. (B) A549 cells were pretreated with different concentrations of Forskolin for 12 h followed by MV-Edm infection with a MOI of 0.5 for another 48 h. Syncytium formation was observed by phase-contrast microscopy (upper panel) and further analyzed using ImageJ software (lower panel). (C) The expression of N viral structural gene was

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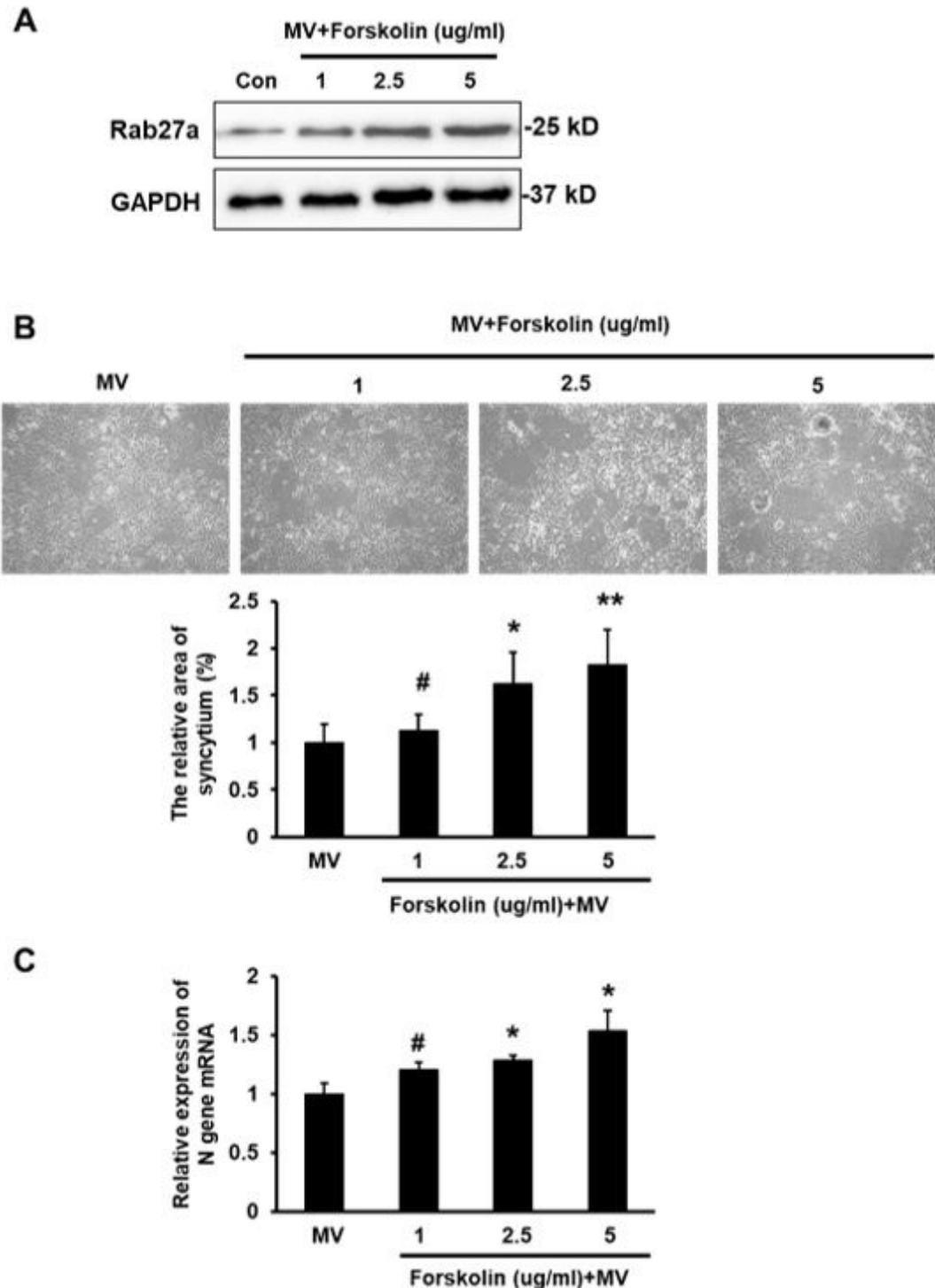


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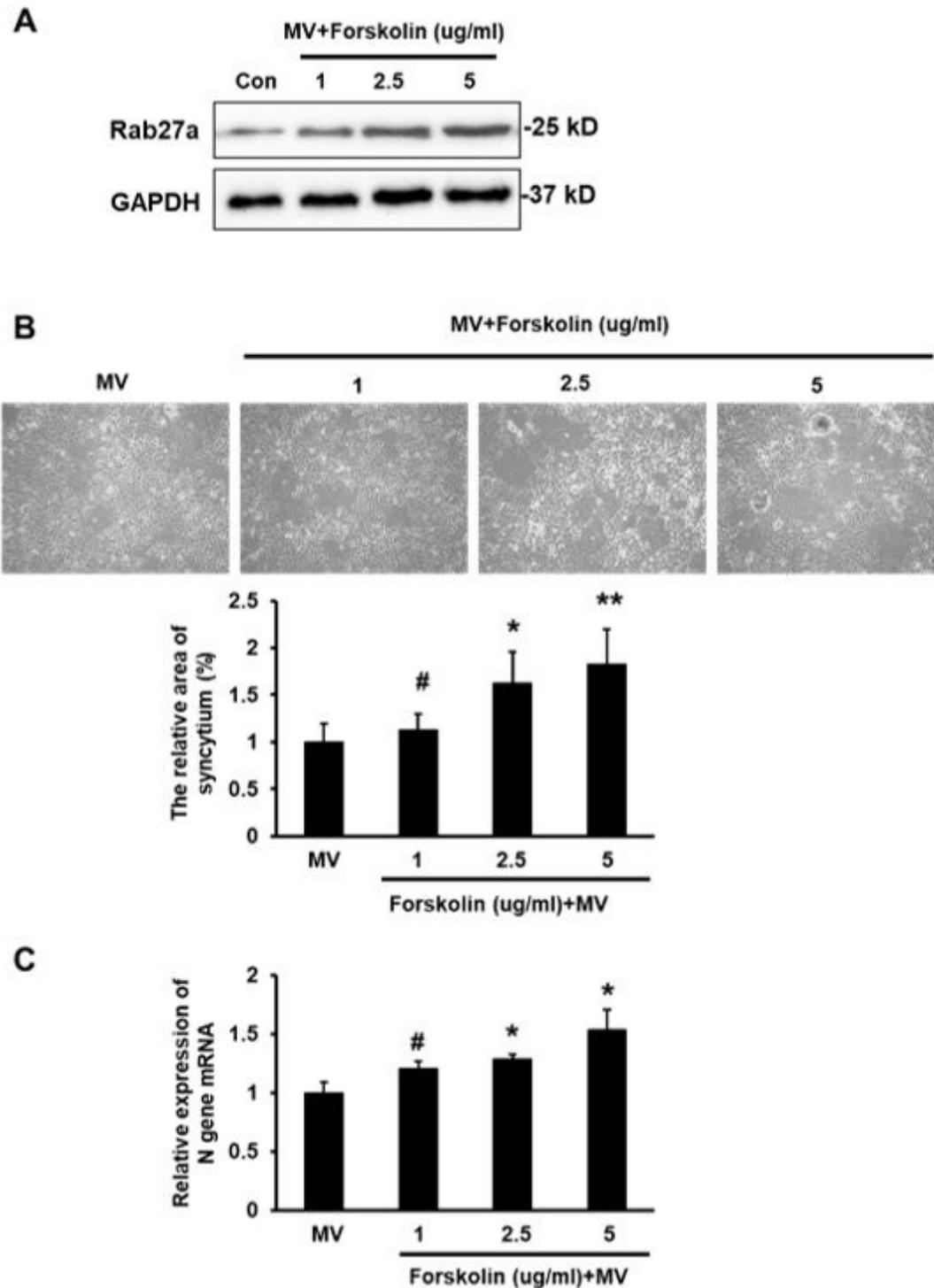


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