

Construction of a recombinant eukaryotic expression vector containing *DNM3* gene and its expression in colon cancer cells

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Introduction: Dynamin 3 (DNM3) is a large GTPase that possesses mechanochemical properties and has been shown to be involved in malignancies. However, most studies about DNM3 are observational, and knowledge of the precise molecular mechanism of DNM3 remains limited.

Materials and methods: We constructed a PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 recombinant eukaryotic expression vector, which was then transfected into SW620 and LoVo cells. One cell line was divided into three groups. DNM3 mRNA and protein expression was analyzed by quantitative real-time PCR and Western blot assay. To investigate DNM3 biological activity in colon cancer SW620 and LoVo cell line, we performed cell proliferation, transwell migration, and invasion assay. Matrix metalloproteinase (MMP)-2 and MMP-9 protein expressions were detected by Western blot.

Result: We successfully constructed a PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 recombinant eukaryotic expression vector, and stable DNM3 expression was observed in SW620 and LoVo cell lines. The vector overexpressing DNM3 inhibited the proliferation, weak invasion, and migration ability of colon cancer SW620 and LoVo cells relative to those in the control group (all $P < 0.001$). DNM3 downregulated the protein expression of MMP-2 and MMP-9.

Conclusion: DNM3 may weaken the malignant behavior of colon cancer and may have promoted the invasion and migration of colon cancer by regulating the expression of MMP-2 and MMP-9.

Keywords: dynamin 3, colon cancer, proliferation, migration, invasion

Introduction

Dynamin is a large GTPase of the dynamin superfamily¹ that includes classical dynamins (dynamin-1,2,3) encoded by *DNM1*, *DNM2*, and *DNM3* genes in eukaryotic cells,² dynamin-like proteins that are involved in apoptosis,³ OPA1, mitofusins, Mx proteins, and guanylate-binding proteins.² The sequence of dynamin-1,2,3 exhibits ~80% homology. Furthermore, dynamins share a primary structure consisting of an amino-terminal GTPase domain, pleckstrin homology domain, carboxy-terminal proline/arginine-rich domain, middle domain, and GTPase effector domain.⁴ However, dynamins are expressed in different tissues. Dynamin 1 is limited to neuronal cells, dynamin 2 is found ubiquitously, and dynamin 3 is expressed in the lungs, testis, and brain.⁵ Dynamin possesses mechanochemical properties involving membrane trafficking processes, which includes vesicle scission, phagocytosis, cytokinesis, podosome formation, and endocytosis.^{6–11} Recently, DNM3 has been shown to be involved

in malignancies.^{12–17} Inokawa et al and Shen et al found that DNMT3 is hypermethylated in hepatocellular cancer (HCC).^{13,14} Zhang et al have further studied the mechanism of DNMT3 in HCC.¹⁸ DNMT3 weakens the proliferation and induces apoptosis of HCC cells by upregulating P53. Lee et al found that low DNMT2 expression is associated with tumor invasion and metastasis in cervix carcinoma and upregulates matrix metalloproteinase 2 (MMP-2) expression.¹⁹ Inokawa et al found that inhibited expression of DNMT2 or DNMT3 upregulates MMP-2 in HCC.¹³ However, studies about the relationship between DNMT3 and malignancies are still insufficient, and most of them are observational. The precise molecular mechanism is unclear. In this study, we constructed a eukaryotic expression vector that comprises *DNMT3* gene to analyze the effect of *DNMT3* gene on the proliferation, invasion, and migration abilities of MMP-2 and MMP-9 expression in human colon cancer SW620 and LoVo cells.

Materials and methods

Materials

High Fidelity Prime Script™ RT-PCR Kit and DNA Ligation Kit Ver.2.1 were purchased from Takara (Shiga, Japan). Ampicillin was purchased from Generay (Shanghai, China). Universal DNA Purification Kit was purchased from Tiangen (Beijing, China). Endo-free Plasmid Mini Kit II was purchased from Omega (Norcross, GA, USA). Competent *Escherichia coli* DH5α was purchased from Transgen Biotech (Beijing, China). Luria Bertani broth, agarose, and agar were purchased from Generay. Colon cancer cell lines, SW620 and LoVo, were obtained from Genesee (Guangzhou, China). FBS and Roswell Park Memorial Institute 1640 (RPMI-1640) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Lipofectamine™ 2000, Opti-MEM, and Trizol were purchased from Thermo Fisher Scientific. Puromycin was purchased from MP Biomedicals (Solon, OH, USA). PrimeScript™ RT reagent kit with gDNA Erase and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara. BCA protein assay kit was purchased from Beyotime (Shanghai, China). Mini GEL tank, NUPAGE 10% BT GEL 1.0 MM 12W Western, NuPAGE™ LDS sample buffer (4×), and NuPAGE™ MES SDS running buffer (20×) were purchased from Thermo Fisher Scientific. DNMT3 antibody was purchased from GeneTex (Irvine, CA, USA), and anti-MMP-2, anti-MMP-9, and GAPDH antibody were purchased from Abcam (Cambridge, MA, USA). Pierce™ ECL Western blot substrate was purchased from Thermo Fisher Scientific. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Tokyo, Japan). Matrigel was purchased from BD Biosciences (San Jose, CA, USA).

Construction of the PCDH-CMV-MCS-EF1a-GFP-Puro-DNMT3 vector

Obtaining and identification of DNMT3

The DNMT3 RNA was obtained from the gene bank of Genesee Biotech Co., Ltd (China). A pair of specific primers containing EcoRI and NotI restriction enzyme cutting sites was designed as follows: DNMT3-EcoRI-F: 5'-CCGGAA TTTCATGGGGAACCGGGAGATGGAGGAGC-3' and DNMT3-NotI-R: 5'-ATTTGCGGCCGCCTAAATGTTCT CATCATCTTTATAC-3'. The primers were designed and synthesized by Sangon Biotech Co., Ltd (China). DNMT3 complementary DNA (cDNA) was synthesized by High Fidelity Prime Script™ RT-PCR Kit according to the manufacturer's instructions. The PCR products were analyzed by 1% agarose gel electrophoresis.

Purification of DNMT3 cDNA and vector

The PCR products and PCDH-CMV-MCS-EF1a-GFP-Puro plasmids were digested by EcoRI and NotI restriction enzymes. The target fragments and the vector were then purified with a Universal DNA purification kit (according to the manufacturer's instructions).

Construction and identification of the recombinant eukaryotic expression vector

For the formation of the recombinant vector PCDH-CMV-MCS-EF1a-GFP-Puro-DNMT3, the digested and purified PCR products and plasmids were ligated with DNA Ligation Kit Ver.2.1. The recombinant vector was transformed into competent *E. coli* DH5α. The recombinant vector was amplified in positive transformants, which were selected by ampicillin. The positive clones with DNMT3 were identified by PCR and then sequenced. Approximately 5 μL of PCR products were analyzed by 2% agarose gel electrophoresis, and the positive vector was sequenced by Genesee Biotech Co., Ltd.

Expression of the recombinant PCDH-CMV-MCS-EF1a-GFP-Puro-DNMT3 eukaryotic expression vector in colon cancer SW620 and LoVo cells

Plasmid extraction

The recombinant vector PCDH-CMV-MCS-EF1a-GFP-Puro-DNMT3 and the empty vector plasmid PCDH-CMV-MCS-EF1a-GFP-Puro were transformed to competent *E. coli* DH5α. The bacterial strains were amplified in 1× LB supplemented with ampicillin, and the vector and plasmid were extracted with an Endo-free Plasmid Mini Kit II.

Cell stable transfection

Colon cancer SW620 and LoVo cells were cultured in RPMI-1640 containing 10% FBS at 37°C in a humidified atmosphere of 5% CO₂. Puromycin dose used for the selection of positive clones was determined by challenging SW620 and LoVo cells with gradient concentration of puromycin. The optimal dose (2 µg/mL) was determined by titration. Vector PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 and plasmid PCDH-CMV-MCS-EF1a-GFP-Puro were transfected into SW620 and LoVo cells by Opti-MEM and Lipofectamine™ 2000 reagent according to the manufacturer's instructions. Approximately 48 hours after transfection, the cells were passaged (1:10) and cultured in RPMI-1640 medium containing 10% FBS and 2 µg/mL of puromycin. Puromycin-resistant clones were visible ~10 days after the selection. The puromycin-resistant clones were then selected and cultured. After 2 weeks of cloning, four cell lines with stable expression of PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 and PCDH-CMV-MCS-EF1a-GFP-Puro were obtained. The cells were divided into six groups, namely no treatment SW620, empty plasmid PCDH-CMV-MCS-EF1a-GFP-Puro SW620-P, PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 SW620-DNM3, no treatment LoVo, empty plasmid PCDH-CMV-MCS-EF1a-GFP-Puro LoVo-P, and PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 LoVo-DNM3.

Detection of DNM3 mRNA by quantitative real-time PCR

Total RNA was extracted from the transfected cells by Trizol, and cDNA was synthesized with a PrimeScript™ RT reagent kit with gDNA Erase according to the manufacturer's instructions. Real-time PCR was performed with a SYBR® Premix Ex Taq™ II (Tli RNaseH Plus). The gene-specific primer pairs were as follows: β-actin-F: 5'-GCATGGGTCAGAAGGATTCCT-3', β-actin-R: 5'-TCGTCCAGTTGGTGACGAT-3', and DNM3-F: 5'-GCTGGCAGAGAGGAAGTTTTTC-3', DNM3-R: 5'-TTTGGTAGGGTATCCCGAATG-3'. The data were analyzed through the 2^{-ΔΔCT} method.

Detection of DNM3, MMP-9, and MMP-2 by Western blot assay

The cells were collected and lysed, and the total proteins were extracted. The concentration of protein was determined with a BCA protein assay kit. Approximately 15 µg of protein samples mixed with the NuPAGE™ LDS sample buffer (4×) were loaded on a NUPAGE 10% BT GEL 1.0 MM 12W Western and processed in the Mini GEL Tank with

NuPAGE™ MES SDS running buffer according to the manufacturer's instructions. The gel was wet-transferred onto polyvinylidene fluoride membranes. After blocking the membranes with 5% milk (western blocking buffer) at room temperature for 2 hours, the membranes were incubated with the corresponding primary antibodies: DNM3, 1:300; MMP-2, 1:1,000; and GAPDH, 1:1,000 at 4°C overnight and secondary antibodies (1:2,000) at room temperature for 2 hours simultaneously. The bands were detected by Pierce™ ECL Western blot substrate. Finally, the blot was photographed with a VersaDoc 5000 Imager (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The image was scanned for the relative value of protein expression in gray scale by ImageJ software 1.45.

Cell proliferation assay

To analyze the effects of DNM3 on proliferation of colon cancer SW620 and LoVo cells, we performed CCK-8 assay according to the manufacturer's instructions. The cells were plated onto 96-well plates at a density of 3,000 cells per well. Each group was seeded to 16 well cells, and every four succeeding wells were cultured for 12, 24, 48, and 72 hours. Approximately 10 µL of CCK-8 was added to the wells and incubated for 2 hours. The absorbance value (OD) of the cells was measured using Multiskan Mk3 (Thermo Fisher Scientific) with a 450 nm filter.

Transwell migration and invasion assay

Transwell chambers were covered with 50 µL of Matrigel diluted with serum-free RPMI-1640 (1:7) and incubated at 37°C for 2 hours (this step was omitted in the transwell migration assay). Approximately 5×10⁵ cells were suspended in 100 µL of serum-free RPMI-1640 and 600 µL of RPMI-1640 with 10% FBS and were seeded in the upper and lower chambers, respectively. After incubation at 37°C in 5% CO₂ for 48 hours (36 hours for the migration assay), the cells were fixed and stained for 15 minutes in a crystal violet solution in PBS. The cells were viewed under the microscope.

Statistical analysis

All the experiments were carried out in triplicates, and the data were expressed as mean ± SD of six independent experiments. Data were analyzed with SPSS21.0 software. A *P*-value of <0.001 indicated significant difference. Statistical comparisons among three groups and between two groups were conducted with Virance and LSD test, respectively. The experimental charts were produced by GraphPad Prism5.

Results

mRNA expression levels of DNM3 in colon cancer SW620 and LoVo cells

After transfection, the DNM3 expression was analyzed by quantitative real-time PCR, which showed that the DNM3 transfected group was significantly overexpressed compared to the control groups (all $P < 0.001$, Figure 1).

DNM3 protein expression and its effect on MMP-9 and MMP-2 protein expression levels

The expression levels of DNM3, MMP-9, and MMP-2 proteins were analyzed by Western blot. The result showed that the DNM3 transfected group was significantly overexpressed compared with each of the control group (all $P < 0.001$), and DNM3 overexpression group downregulated the MMP-9 (all $P < 0.001$) and MMP-2 (all $P < 0.001$) protein expression levels compared with the control groups (Figure 2).

Effect of DNM3 on the proliferation of colon cancer SW620 and LoVo cells

The OD values of the six groups were measured every 12 hours after transfection. The mean value was calculated. As shown in Figure 3, the survival number of either SW620 or SW620-P group was higher than that of SW620-DNM3 group ($P < 0.001$, Figure 3A). The survival number of either the LoVo or LoVo-P group was higher than that the LoVo-DNM3 group ($P < 0.001$, Figure 3B). DNM3 overexpression was associated with decreased proliferation in SW620 and LoVo cells.

Effect of DNM3 on migration and invasion of colon cancer SW620 and LoVo cells

As shown in Figure 4, DNM3 overexpression weakened the ability of cell invasion and migration. SW620-DNM3 group migration ability was weaker than that of either the SW620 or SW620-P group (all $P < 0.001$, Figure 4A). The migration ability of the LoVo-DNM3 group was weaker than that of either the LoVo or LoVo-P group (all $P < 0.001$, Figure 4A). The invasion ability of the SW620-DNM3 group was weaker than that of either the SW620 or SW620-P group (all $P < 0.001$, Figure 4B). The invasion ability of the LoVo-DNM3 group was weaker than that of either the LoVo or LoVo-P group (all $P < 0.001$, Figure 4B).

Discussion

DNM3 gene can be a predictor for prognosis and is a molecular marker for diagnosis and gene therapy of malignant disease.¹⁷ Booken et al¹⁶ indicated that DNM3 is overexpressed in Sezary syndrome (SS) in a screen for differential gene expression in peripheral blood mononuclear cells of ten healthy controls and ten SS patients by Affymetrix genechip. Shen et al¹⁴ analyzed the genome-wide DNA methylation profiles in tumor and adjacent nontumor specimens of 62 HCC patients and demonstrated that DNM3 is hypermethylated in HCC. Inokawa et al¹³ showed that DNM3 is hypermethylated in HCC. Methylation downregulates DNM3 expression and is associated with poor prognosis, which was determined by analyzing the disease-specific survival of 48 HCC patients. Teicher et al¹⁵

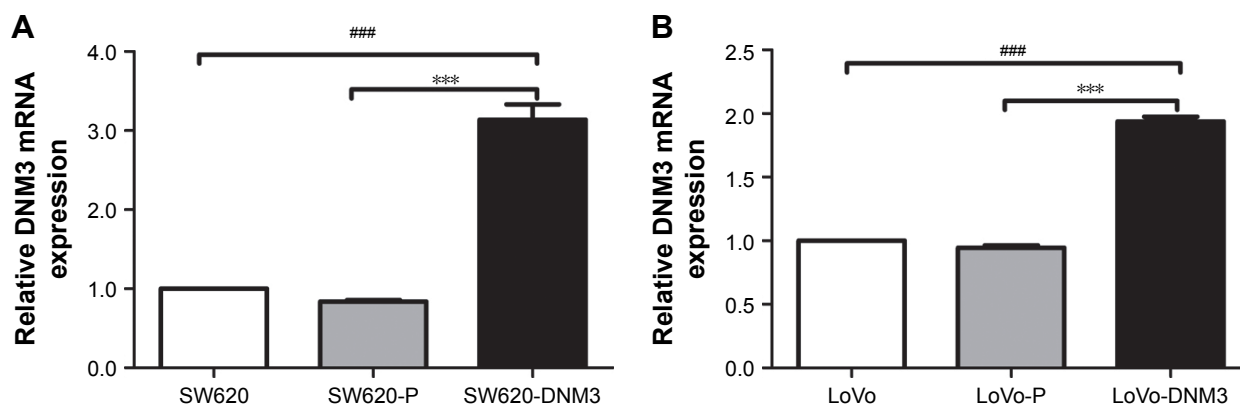


Figure 1 DNM3 mRNA was analyzed by quantitative real-time PCR.

Notes: (A) SW620-DNM3 groups significantly overexpressed DNM3 compared with the SW620 and SW620-P groups. (B) LoVo-DNM3 groups significantly overexpressed DNM3 relative to the LoVo and LoVo-P groups. SW620: no treatment, SW620-P: empty plasmid PCDH-CMV-MCS-EF1a-GFP-Puro, SW620-DNM3: PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3, LoVo: no treatment, LoVo-P: empty plasmid PCDH-CMV-MCS-EF1a-GFP-Puro, LoVo-DNM3: PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3, *** $P < 0.001$, ### $P < 0.001$.

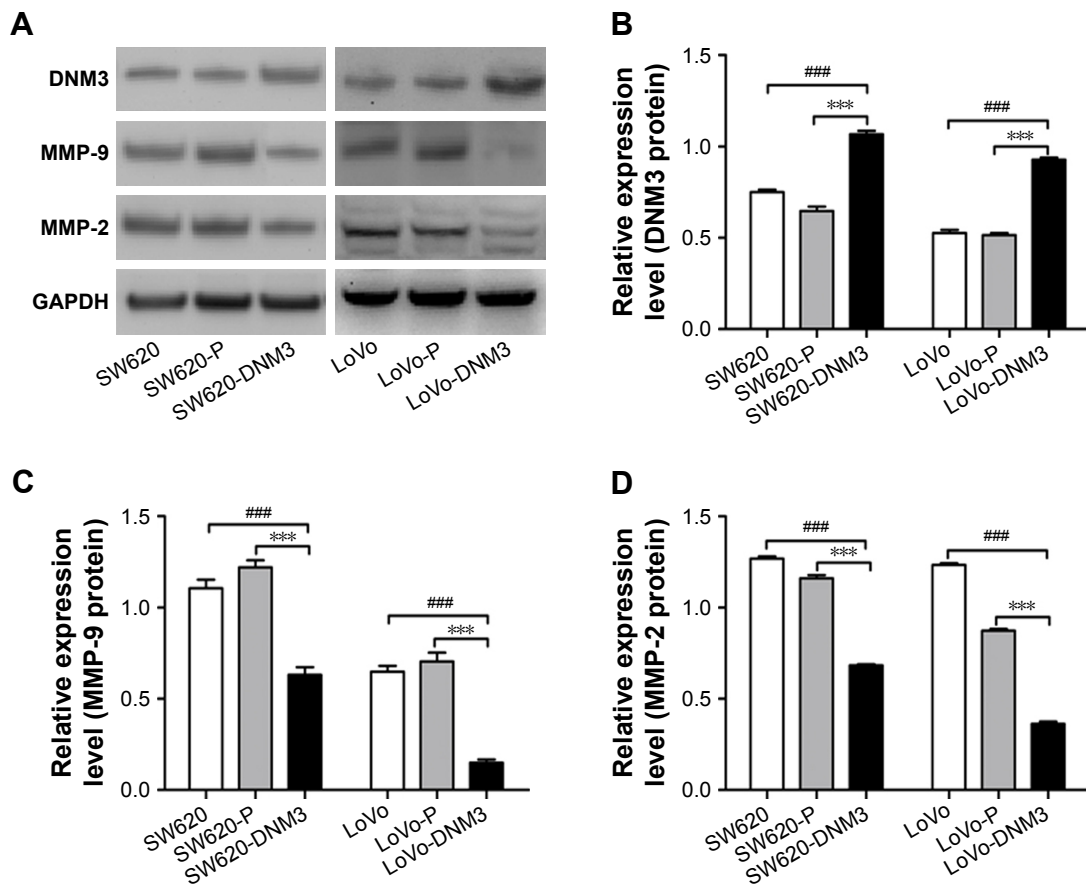


Figure 2 DNM3, MMP-9, and MMP-2 proteins were analyzed by Western blot.

Notes: (A) Gray scale image of protein expression. (B) Cells transfected with DNM3 significantly overexpressed DNM3 protein compared with the control groups. (C) Cells transfected with DNM3 significantly downregulated MMP-9 protein compared with the control groups. (D) Cells transfected with DNM3 significantly downregulated MMP-2 protein compared with the control groups, *** $P < 0.001$, #### $P < 0.001$.

Abbreviation: MMP, Matrix metalloproteinase.

reported that liposarcoma had 1q24.3 amplifications involving DNM3.

With an increased attention of DNM3, the precise molecular mechanism should be clarified. Marino et al¹²

performed the MTT and migration assays in breast cancer cells and found that the migration ability of breast cancer cells is weakened after stable silencing of DNM3. However, cell proliferation ability exhibits no difference. In our study, we

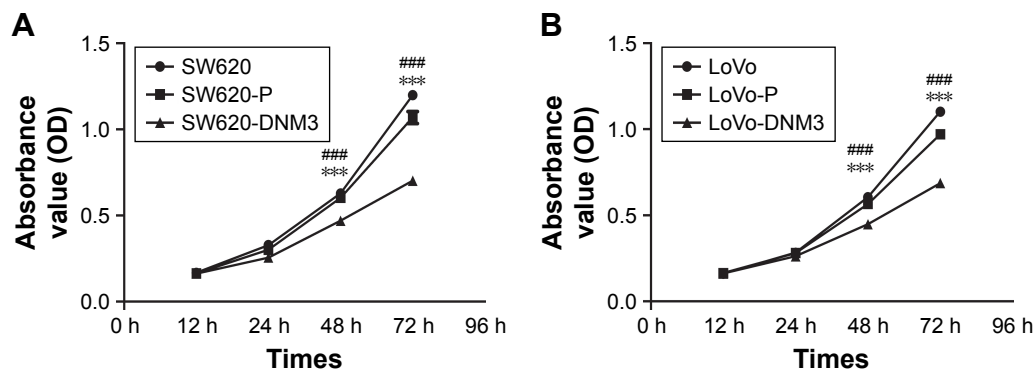


Figure 3 SW620 and LoVo cell growth curves. Compared with the control groups, DNM3 overexpression significantly inhibited cell proliferation.

Notes: (A) SW620 cell growth curves. Compared with the SW620 and SW620-P groups, SW620-DNM3 group significantly inhibited cell proliferation. (B) LoVo cell growth curves. Compared with the LoVo and LoVo-P groups, LoVo-DNM3 group significantly inhibited cell proliferation, *** $P < 0.001$, #### $P < 0.001$.

research methods were different. Marino et al¹² silenced the *DNM3* gene, but we used the overexpressed *DNM3* gene. *DNM3* is worthy of further study. To further explore the molecular mechanisms of motility inhibition induced by *DNM3*, we examined the expression of two major members of the MMP family, MMP-2 and MMP-9. We analyzed the relative expression level histogram and showed that overexpression of *DNM3* can downregulate MMP-2 and MMP-9 protein expression.

Conclusion

In conclusion, we constructed a PCDH-CMV-MCS-EF1a-GFP-Puro-DNM3 recombinant eukaryotic expression vector, which indicated that *DNM3* overexpression can inhibit proliferation, weak invasion, and migration ability in colon cancer SW620 and LoVo cells. *DNM3* might be involved in the invasion and migration of colon cancer by regulating the expression of MMP-2 and MMP-9. *DNM3* might weaken the malignant behavior of colon cancer.

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Disclosure

The authors report no conflicts of interest in this work.

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