JWA deficiency induces malignant transformation of murine embryonic fibroblast cells

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Abstract. The present study aimed to investigate the effects of JWA knockout (JWA$^{-/-}$) on malignant transformation of murine embryonic fibroblast (MEF) cells using a conditional JWA$^{-/-}$ mouse model. Once MEF cells were prepared, the potential role of JWA$^{-/-}$ on proliferation, migration, invasion and colony formation of MEF cells was investigated by cytological examination. The effects of JWA$^{-/-}$ on the regulation and protein expression levels of epithelial-mesenchymal transition (EMT)-related proteins in MEF cells, including poly(ADP-ribose) polymerase-1 (PARP-1), vimentin, β-catenin and E-cadherin, were investigated using western blot analysis. The tumorigenicity of JWA deficiency was explored using nude mouse xenografts and subcutaneous inoculation of MEF cells exhibiting JWA$^{-/-}$. JWA$^{-/-}$ was able to increase cell proliferation, migration, invasion and colony formation in the malignant transformation of MEF cells. The protein expression levels of PARP-1, vimentin and β-catenin were upregulated, whereas E-cadherin was downregulated in JWA$^{-/-}$ MEF cells. The tumor formation was observed in mice following subcutaneous inoculation of MEF with JWA$^{-/-}$, whereas no tumor was formed in the mice treated with functional JWA MEF cells. In conclusion, the present findings suggest that JWA$^{-/-}$ has important roles in cell proliferation, migration, invasion and colony formation and is able to induce the malignant transformation of MEF cells. The expression levels of EMT-related proteins changed and tumorigenicity increased in JWA$^{-/-}$ MEF cells compared with cells with functional JWA. The present findings indicate that JWA may function as an anti-oncogene in tumorigenesis.

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Introduction

As a novel microtubule-binding protein, JWA is essential in regulating cancer cell migration through mitogen-activated protein kinase cascades and cytoskeletal F-actin pathways (1). It has been reported that JWA is essential for promoting cell survival and protection from DNA damage induced by oxidative stress, which may result in cancer cell apoptosis by chemical methods (2,3). Previous studies have associated the JWA gene with reduced cancer risk in various types of cancer, including gastric cancer (4), bladder cancer (5) and hepatocellular carcinoma (6). In addition, previous results have demonstrated the important role of JWA downregulation in promoting cell invasion using Matrigel-coated chambers and accelerating melanoma cell migration and adhesion (7). However, the potential role of JWA in the malignant transformation of cells has not been fully elucidated.

Epithelial-mesenchymal transition (EMT) is a biological process accompanied by mesenchymal gene activation and epithelial gene repression. EMT has been implicated in the loss of function of multiple adhesion proteins, including E-cadherin during tumor progression, and increased cell proliferation, invasion and migration (8-10). Based on tumor xenograft models and cell culture studies, previous results have indicated that the activation of EMT may promote tumor cell dissociation and metastasis to distant organs (9,11).

The present study aimed to explore the role of JWA knockdown (JWA$^{-/-}$) on the malignant transformation of mouse embryonic fibroblast (MEF) cells. In the present study, the effects of JWA$^{-/-}$ on MEF cell proliferation, migration, invasion and colony formation were investigated. The effects of JWA$^{-/-}$ on the regulation of EMT-related proteins and the tumorigenicity of MEF cells were explored. The findings of the present study may provide insightful information into the potential mechanisms of JWA in carcinogenesis.

Materials and methods

Preparation of MEF cells. All experiments were conducted in accordance with the Animal Care and Use Committee of the Model Animal Research Centre and approved by the Nanjing Medical University and the Animal Care Ethics Committee of Nanjing Medical University (Nanjing, China). The conditional JWA$^{-/-}$ murine model used in the present study was

constructed by the Model Animal Research Center of Nanjing University (Nanjing, China) and generated according to a previous study (12). Embryos from JWA$^{+/-}$ x JWA$^{+/-}$ crossed female mice were obtained on day 13.5 of gestation (12). Once the heads and all visible organs of the embryos, such as the heart and spleen were removed, the embryos were placed in a 50-ml centrifuge tube and minced with scissors. A total of 5 ml 0.25% trypsin, which was inactivated using 5 ml of Dulbecco's modified Eagle medium (DMEM) (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) was added to the tube and incubated at 37°C for 20 min. Subsequently, the cells of embryo were centrifuged at 1,500 x g for 5 min at room temperature and resuspended in 15 ml fresh medium. After standing for 10 min, the top layer of cell suspension (10 ml) was collected and plated in a 100-mm dish. The cells were cultured at 37°C in humidified atmosphere containing 5% CO_2. All experiments were conducted in accordance with the Animal Care and Use Committee of the Model Animal Research Centre and approved by Nanjing Medical University and the Animal Care Ethics Committee of Nanjing Medical University (Nanjing, China).

Identification of the JWA gene. Genomic DNA from MEF cells was extracted using standard protocols to detect the JWA gene. The sequences of primers for detecting wild type and null JWA alleles were as follows: Wild-type and null JWA forward primer P1: 5'-CCACTGTTTCCTCTGTTG-3'; wild-type reverse primer P2: 5'-GTGAAAACCACTGAG AACC-3'; and null JWA reverse primer P3, 5'-CAGATGTTC CTCGTGTATC-3'. The JWA gene structure is presented in Fig. 1. The extracted genomic DNA was amplified by polymerase chain reaction. Taq DNA polymerase and PCR kit were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The PCR procedures were as follows: Initial denaturation step at 94°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min and elongation at 72°C for 45 sec, and a final extension at 72°C for 10 min. The products were analyzed by 1.5% agarose electrophoresis.

MEF cell proliferation. MEF cell proliferation was analyzed using the Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. A total of 1,000 cells for each of MEF with wild-type JWA and JWA$^{-/-}$ MEF cells were collected and inoculated into 96-well plates, which were routinely cultured. A total of 10 μl CCK-8 reagent was added into the wells and the absorbance was measured at 450 nm using an ELISA spectrophotometer (Hyphen Biomed, Neuville-sur-oise, France), following incubation for 1 h.

MEF cell migration and invasion assays. Cell migration and invasion assays were performed using the Transwell invasion assay. In the upper chamber of the Transwell unit (Corning Incorporated, Corning, NY, USA), 6.5-mm diameter polycarbonate filters with 8-μm pore size (EMD Millipore, Billerica, MA, USA) were inserted. For the migration assay, 200 μl cell suspension with a density of 2x10^5 cells/ml were seeded in
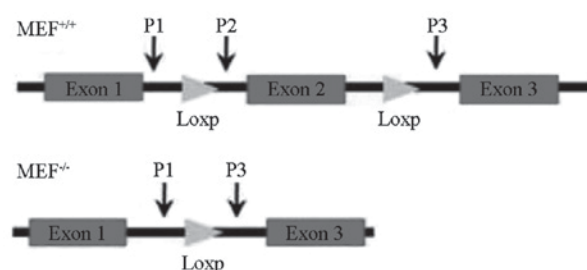

Figure 1. Diagram of JWA gene structure. JWA gene structure was determined in MEF cells. Wild type JWA in MEF (JWA$^{+/+}$) and JWA knockout (JWA$^{-/-}$). P1 and P2 amplified a fragment between exon 1 and 2. P1 and P3 amplified a fragment between exon 1 and 3. Following JWA knockout, exon 2 was deleted in MEF cells. Loxp is the mark of gene knockout in mice. MEF, murine embryonic fibroblast; P1, primer 1; P2, primer 2; P3, primer 3.

serum-free DMEM in the upper chamber and incubated for 8 h at 37°C. MEF cells were fixed in methanol, stained with 1% crystal violet solution for 15 min at room temperature and counted. A total of 9 random fields were counted using a light microscope at x200 magnification (Olympus BX41; Olympus Corporation, Tokyo, Japan). For invasion assay, MEF cells were suspended in serum-free DMEM at a density of 5x10^5 cells/ml. Subsequently, 200 μl of cell suspension were added into the upper chamber and 500 μl of culture medium supernatant were added into the lower chamber. Following incubation at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h, the cells on the surface layer of cells in the upper chamber were swabbed with cotton-swappers. Cells in the lower chamber were fixed with 4% paraformaldehyde, stained with crystal violet, washed with distilled water, dried at room temperature and counted following the same steps as those in the migration assay.

Colony formation assay. The MEF cells in logarithmic phase were harvested and resuspended in DMEM with 10% heat-inactivated FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). The molten 0.3% agarose was placed in a 60 mm culture dish. The cell suspension was applied onto the base layer and cultured in the incubator at 37°C in a humidified environment containing 5% CO_2 for 2 weeks. The cells were counted using a hemocytometer under a light microscope. Clusters containing >50 cells were identified as a colony. The colony forming rate was calculated as: Colony forming rate = number of colonies/number of total cells seeded x 100.

Expression of EMT-related proteins and western blotting. The effects of JWA knockout on PARP-1 and EMT-related proteins were observed. MEF cells (1x10^6) were seeded in a 100 mm culture dish. NU1025 (Sigma-Aldrich; Merck KGaA Darmstadt, Germany), a specific inhibitor of PARP-1, was used to inhibit the function of PARP-1 in MEF cells. NU1025 was dissolved in dimethylsulfoxide (DMSO) and added into the wells to give a final concentration of 50 μmol/l. DMSO of same volume was used as a blank control. The small interfering (si)RNA of PARP-1 (PARP-1 siRNA: sc-29438; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was also used to inhibit the function of PARP-1 simultaneously. The MEF cells were transfected with 60 μl of 10 μmol/l PARP-1 siRNA according to
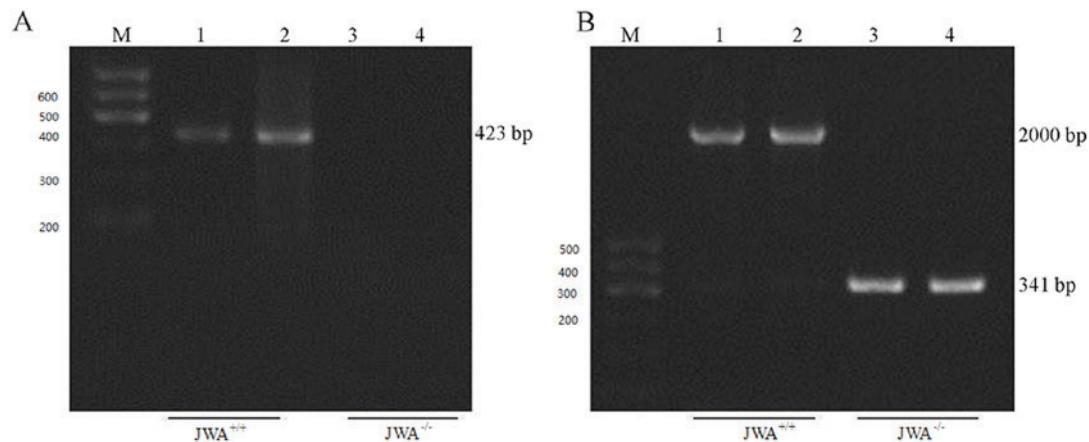
Figure 2. Genotype verification of MEF cells in agarose electrophoresis. (A) The results of amplification by primers 1 and 2. The 423-bp fragment between exon 1 and 2 was wild JWA (JWA^{+/+}). No bands were observed in the JWA^{-/-} MEF cells, which indicates that JWA knockout induced the deletion of exon 2 in genomic DNA of MEF cells. (B) The results of amplification by primers 1 and 3. A fragment of ~2,000 bp was observed between exons 1 and 3 in the MEF cells with wild-type JWA, whereas a 341 bp band was the remaining fragment between exons 1 and 3 in JWA^{-/-} MEF cells M, DNA marker. MEF, murine embryonic fibroblast; MEF^{+/+}, wild-type JWA in MEF cells; MEF^{-/-}, JWA knockout in MEF cells.

the manufacturer's protocol (Lipofectamine® 3000, Invitrogen; Thermo Fisher Scientific, Inc.). A mimical nonsense siRNA was used as blank control. A 24 h interval was left before subsequent experimentation.

The EMT-related proteins in MEF cells, including poly (ADP-ribose) polymerase-1 (PARP-1), vimentin, β-catenin and E-cadherin, were extracted for western blotting as described previously (12). In brief, the cells were lysed in radioimmunoprecipitation buffer (50 mmol/l Tris-HCl pH 7.2, 150 mmol/l NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid sodium, 1 mmol/l PMSF, 25 mmol/l $MgCl_2$, and supplemented with phosphatase inhibitor cocktail). The protein concentrations were determined using the bicinchoninic acid assay method (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins (20 µg per lane) were separated by 12.5% SDS-PAGE and electroblotted onto polyvinyl difluoride membranes. Membranes were blocked with 5% milk at 37°C for 1 h and subsequently incubated with specific primary antibodies for 1 h at room temperature. Monoclonal rabbit anti-PARP-1 (1:1,000; EMD Millipore; MABE365), mouse anti-vimentin (1:500; EMD Millipore; MABT121), mouse anti-β-catenin monoclonal (1:200; BD Biosciences, San Jose, CA, USA; 610153), mouse anti-E-cadherin (1:200; BD Biosciences; 610404) and mouse anti-β-actin (1:2,000; Beyotime Institute of Biotechnology, Nantong, China; AA128) were used as the primary antibodies as described above. The relevant proteins were stained with the secondary antibody (goat anti-mouse IgG-HRP, 1:2,000; Santa Cruz Biotechnology, Inc.; sc-2005,) for 1 h at room temperature. Immunoreactive bands were detected using Beyond electrochemiluminescence (BeyoECL) Plus kit (P0018; Beyotime Institute of Biotechnology, Haimen, China). Protein bands were visualized and measured using ImageJ software (version 1.44; National Institutes of Health, Bethesda, MA, USA; data not shown), following normalization to the corresponding β-actin level.

Nude mouse xenograft assay. To determine the effects of JWA^{-/-} on the tumorigenicity of MEF cells *in vivo*, nude murine xenograft assays were performed. A total of 10 female nude mice (8-9 weeks old, weight 16-20 g) were obtained from the Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China). Mice were maintained in a temperature-controlled room (23°C), relative humidity of 50% with a 12-h light/dark cycle and free access to food and water. A xenograft assay was performed. The mice were randomly divided into two groups (n=5 per group): MEF cells with wild type JWA (JWA^{+/+}) and JWA^{-/-} MEF cell groups. MEF cells were suspended in DMEM and adjusted to a density of $2x10^6$ cells/ml. Subsequently, 200 µl cell suspension of JWA^{+/+} or JWA^{-/-} MEF cells was injected subcutaneously into nude mice backs. The volume and weight of the formed tumors were measured in 4 weeks after inoculation.

Statistical analysis. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± or + standard deviation. Student's t-test was used to determine the differences between the two groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Knockout of JWA in MEF cells. A MEF cell line with JWA^{-/-} was constructed and the wild-type MEF cells (JWA^{+/+}) were used as the negative control. Genotyping of JWA^{-/-} mice was performed at the genomic DNA level (Fig. 2). Using P1 and P2 primers, a 423-bp band was observed in wild-type MEF cells, which indicated that primer 1 and 2 amplified a 423-bp fragment between exon 1 and 2. No bands were present in the JWA^{-/-} MEF cells, which suggested that the deletion of exon 2 lead to JWA^{-/-} (Fig. 2A). In addition, P1 and 3 primers amplified a fragment of ~2,000 bp between exons 1 and 3 in the wild-type MEF cells, whereas a 341 bp band was observed in JWA^{-/-} MEF cells, which suggested that P1 and 3 primers amplified the remaining 341-bp fragment between exons 1 and 3 (Fig. 2B).
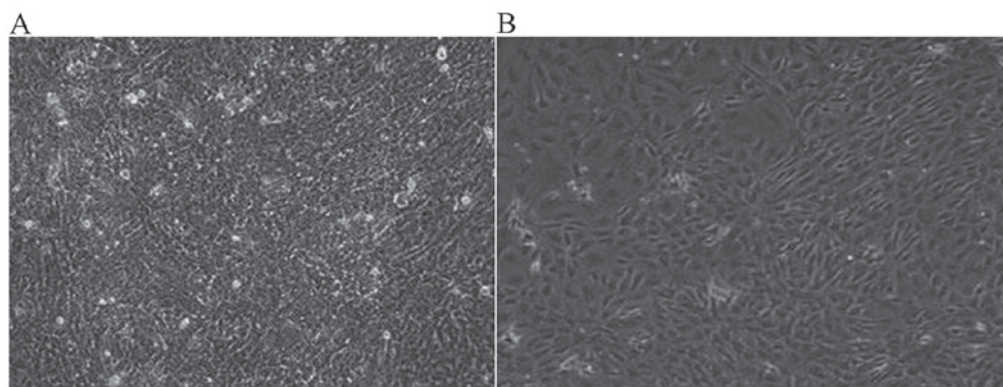
Figure 3. Effect of JWA knockout on MEF cell proliferation. (A) JWA$^{-/-}$ MEF cells covered the full bottom of the culture dish and (B) MEF cells with wild-type JWA underwent apoptosis. MEF, murine embryonic fibroblast; MEF$^{+/+}$, wild-type JWA in MEF cells; MEF$^{-/-}$, JWA knockout in MEF cells.
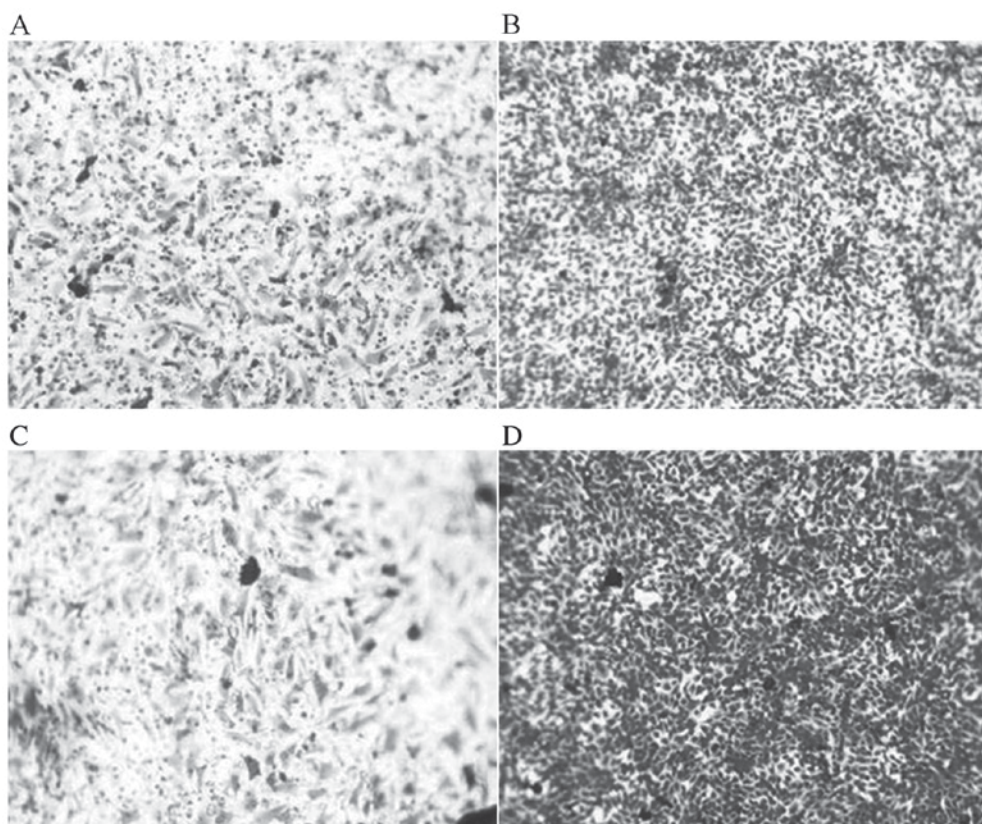

Figure 4. Effects of JWA knockout on migration in (A) JWA$^{+/+}$ MEF cells and (B) JWA knockout MEF cells, and on invasion in (C) JWA$^{+/+}$ MEF cells and (D) JWA knockout MEF cells. MEF, murine embryonic fibroblast; MEF$^{+/+}$, wild-type JWA in MEF cells; MEF$^{-/-}$, JWA knockout in MEF cells.

JWA$^{-/-}$ induces malignant transformation in MEF cells. Once JWA$^{-/-}$ MEF cells were cultured for 6 months, malignant transformation was observed in the JWA$^{-/-}$ MEF cells. Under the light microscope (1:40), the proliferation activity of JWA$^{-/-}$ MEF cells was notably increased compared with wild-type MEF cells. The number of cells was markedly increased and once the saturation density was reached, cells began to grow overlapping one another. Compared with wild-type MEF cells, the difference was marked. In Fig. 3A, JWA$^{-/-}$ MEF cells covered the bottom of the culture dish. The cell nuclei of JWA$^{-/-}$ MEF with malignant transformation became large and the contact inhibition of growing cells disappeared. However, the MEF cells with wild-type JWA began to die and only a few scattered cell colonies were observed (Fig. 3B).

Effects of JWA$^{-/-}$ on cell proliferation, migration, invasion and colony formation. The migration and invasion abilities of MEF cells with JWA$^{-/-}$ were markedly increased compared with those of the MEF cells with wild type JWA (JWA$^{+/+}$). The results of migration ability of MEF cells indicated that JWA$^{-/-}$ promoted cell migration and invasion of MEF cells with wild type JWA. The number of MEF cells with JWA$^{-/-}$ was markedly greater than MEF cells with wild type JWA (Fig. 4). The results of the invasion assay indicated that the number of MEF cells with JWA$^{-/-}$ was increased (Fig. 4). As
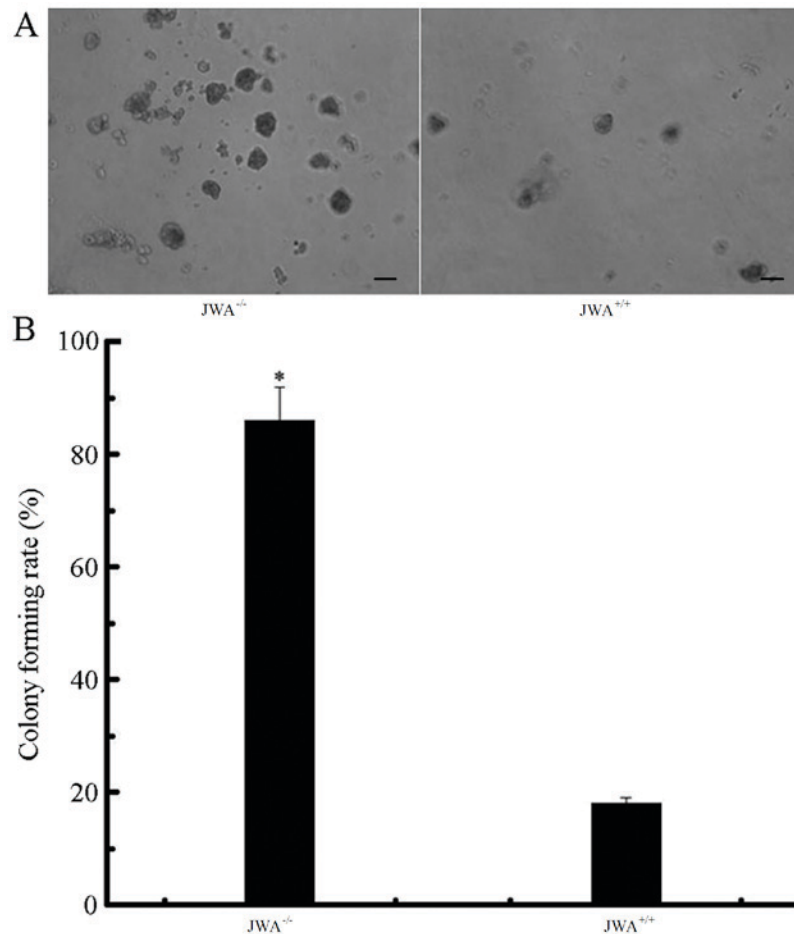
Figure 5. Effects of JWA knockout on (A) colony formation and (B) colony forming rate in MEF cells. Scale bar=250 μm. *P<0.05 vs. MEF+/+ group. MEF, murine embryonic fibroblast; MEF+/+, wild-type JWA in MEF cells; MEF-/-, JWA knockout in MEF cells.

presented in Fig. 4D, a large number of JWA-/- MEF cells penetrated into the lower chamber. Colony formation assay results suggested that the number of colonies of JWA-/- MEF cells was notably increased when compared with the wild-type MEF cells (Fig. 5A). Quantitative analysis demonstrated that the rate of colony formation of JWA-/- MEF cells was 83.4±5.2%, and the JWA+/+ MEF cells was 19.6±1.3%. The colony-forming rate in JWA-/- MEF cells was significantly greater than that of the MEF cells with wild type JWA (P<0.05, Fig. 5B).

Effects of JWA-/- on the regulation of EMT-related proteins. JWA-/- markedly altered the expression levels of EMT-related proteins. Western blot analysis revealed that JWA-/- upregulated the protein expression levels of PARP-1, vimentin and β-catenin, and downregulated the protein expression levels of E-cadherin (Fig. 6A). Following the application of PARP-1 inhibitor NU1025 or relevant siRNA, the protein expression levels of PARP-1, vimentin and β-catenin were reduced compared with blank DMSO group or mock group as mimical control (Fig. 6B).

Effects of JWA-/- on the tumorigenicity of MEF cells. In 8 weeks following subcutaneous injections of MEF cells with wild type JWA, no control mice exhibited tumor formation. However, the group injected JWA-/- MEF exhibited obvious tumor formation

(Fig. 7). The tumor volume was 2.56±0.36 cm³ and the tumor weight was 1.74±0.43 g in the JWA-/- MEF group.

Discussion

JWA has been previously reported as a novel regulator in inhibiting melanoma cell adhesion, invasion and metastasis (7) and has been demonstrated to have prognostic and predictive roles in gastric cancer (13). In the present study, JWA-/- was able to increase MEF cell proliferation, and stimulate cell migration, cell invasion and colony formation, ultimately promoting malignant transformation in MEF cells. Additionally, JWA-/- was able to upregulate the expression levels of EMT-related proteins (PARP-1, vimentin and β-catenin) and downregulate the expression levels of E-cadherin.

JWA has been recognized as a typical tumor suppressor and stress response gene (3,7). It has been demonstrated that JWA is an essential signaling gene in the regulation of tumor cell migration and differentiation (14). Additionally, JWA protein expression levels are closely correlated with the occurrence, invasion and metastasis of malignant tumors (15). Using liver cells with different metastatic potential, a previous study revealed that reduced expression levels of JWA protein resulted in increased metastasis potential (6). Previous results have indicated that downregulation of JWA expression may affect cell function, such as proliferation, apoptosis, migration
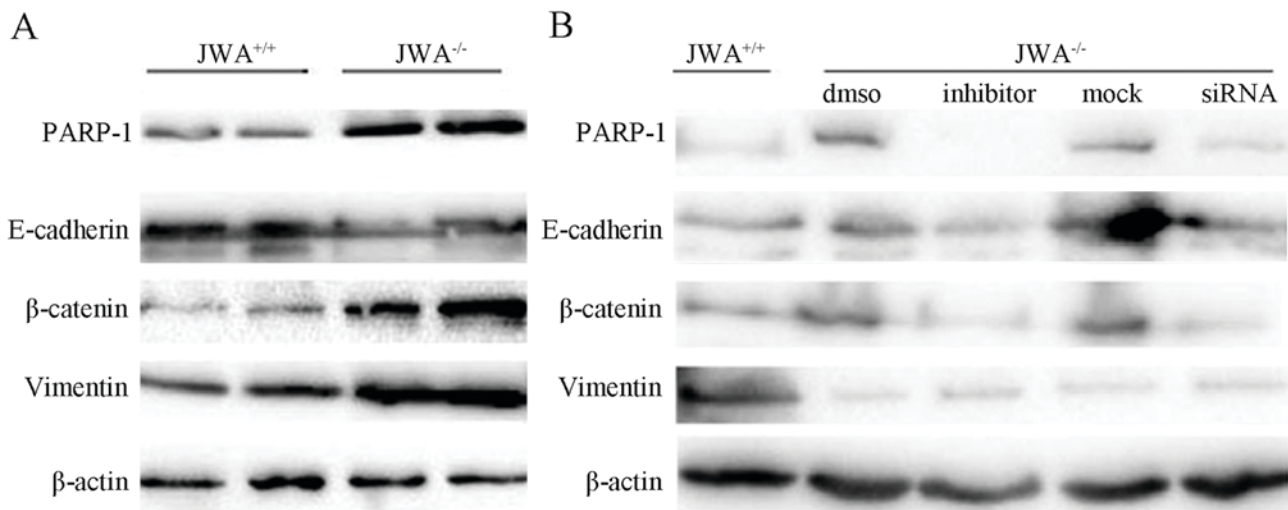
Figure 6. Western blotting results of JWA knockout on PARP-1 inhibition and the regulation of EMT-related protein expression. (A) The effects of JWA knockout. JWA$^{-/-}$ upregulated the expression levels of PARP-1, vimentin and β-catenin, and downregulated the expression of E-cadherin. (B) The effects of inhibitor NU1025 and siRNA. The application of PARP-1 inhibitor NU1025 reduced the expression of PARP-1, vimentin and β-catenin compared with the DMSO group. DMSO was blank organic solvent without NU1025. The application of relevant siRNA reduced the expression of PARP-1, vimentin and β-catenin compared with the mock group in a similar manner. JWA$^{+/+}$, wild-type JWA; JWA$^{-/-}$, JWA knockout; PARP-1, poly(ADP-ribose) polymerase-1; NU1025, PARP-1 inhibitor; DMSO, pure organic solvent without NU1025; siRNA, small interfering RNA for PARP-1, mock: Nonsense RNA.

Figure 7. A formed tumor on the back of a JWA$^{-/-}$ mouse. Subcutaneous injection with JWA$^{-/-}$ MEF cells promoted tumor formation in the mice.

and invasion via the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway of mitogen-activated protein kinase signaling cascades (16).

In the present study, the proliferation of MEF cells was increased in JWA$^{-/-}$ MEF cells, and cell migration and invasion were promoted. Previous studies have indicated that loss of JWA was able to increase cell migration and metastasis (1,7,17). The loss of JWA combined with p53 mutation has been demonstrated to promote aggressiveness and metastasis of gastric cancer cells, which may be attributed to the fact that JWA is a member of the DNA repair pathway and may have inhibitory roles in gastric carcinogenesis. The expression of JWA gene may contribute to better chemotherapy outcome in gastric carcinogenesis (4). As a multi-functional microtubule-associated protein, JWA is associated with DNA damage repair and apoptosis in various physiological contexts and inhibits multiple steps of metas-

tasis, including cell invasion, cell adhesion and angiogenesis in diverse carcinoma (18).

Overall, the results of the present study suggest that JWA$^{-/-}$ may induce MEF cell malignant transformation by having important roles in promotion of cell proliferation, migration, invasion and colony formation. These results were consistent with the findings of previous numerous researches on JWA gene (19).

The present results indicated that the protein expression levels of PARP-1, vimentin and β-catenin were upregulated and E-cadherin was downregulated. PARP-1 is considered an important molecule in regulating lung cell proliferation, as a previous study revealed that mice lacking PARP-1 exhibited excessive lung cell proliferation and hyperplasia (20). Furthermore, microglial activation is associated with cell proliferation and the increased release of pro-inflammatory cytokines (21). A previous study reported that PARP-1 promoted microglial activation and proliferation (22). Additionally, vimentin has been indicated to have a crucial role in cell division and proliferation (23). Previous results have suggested that the association of vimentin and epigallocatechin gallate regulates cell proliferation (24). β-catenin expression and vascular endothelial cadherin binding requires the inhibition of vascular endothelial growth factor-induced cell proliferation (25). E-cadherin-associated β-catenin is implicated in inhibiting cell proliferation through regulating the Hippo signaling pathway (26). The findings of the present study suggest that JWA may mediate cell proliferation by regulating the expression of EMT-related proteins. However, further studies are required to investigate this hypothesis.

In conclusion, the present study demonstrated that JWA$^{-/-}$ was able to induce malignant transformation in MEF cells by altering cell proliferation, migration, invasion and colony formation. The role of JWA in mediating cell proliferation may involve regulating the expression of EMT-related proteins.

The present findings suggest that JWA may function as an anti-oncogene in cancer.

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