Huaier aqueous extract inhibits proliferation and metastasis of tuberous sclerosis complex cell models through downregulation of JAK2/STAT3 and MAPK signaling pathways

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Abstract. Tuberous sclerosis complex (TSC) is a genetic disorder with formation of benign tumors in many different organs. It has attracted increasing attention from researchers to search for therapeutic drugs for TSC patients. Traditional Chinese medicine (TCM) has become an important source for finding antitumor drugs. *Trametes robiniophila* Murr. (Huaier) is a kind of officinal fungi in China and has been applied in TCM for approximately 1,600 years. A large number of clinical applications have revealed that Huaier has good antitumor effect. In this study, we have investigated the effects of Huaier aqueous extract on two TSC cell models, including inhibition of proliferation, induction of apoptosis, cell cycle arrest, and anti-metastasis. We demonstrated that Huaier aqueous extract inhibited JAK2/STAT3 and MAPK signaling pathways in a dose-dependent manner. Therefore, based on the low toxicity and the multi-targets of Huaier treatment, Huaier may be a promising therapeutic drug for TSC.

Introduction

Tuberous sclerosis complex (TSC) is a genetic disease characterized by formation of benign tumors in multiple organ systems, including the brain, eyes, heart, kidneys, skin and lungs. There are several typical symptoms in TSC patients, including epilepsy, mental deterioration, facial angiofibroma, pulmonary lymphangiomyomatosis (LAM), kidney angiomyolipoma, and renal cyst. The incidence of TSC is approximately 1:6,000-10,000 (1-3).

Tuberous sclerosis 1 (TSC1) and 2 (TSC2) are two tumor suppressor genes located upstream of mechanistic target of rapamycin (mTOR) (4). TSC is caused by inactivating mutations of either TSC1 or TSC2. Hyperactivation of mTOR caused by deficiency of either TSC1 or TSC2 is thought to be the major cause of TSC development (1,5,6). Therefore, mTOR inhibition is considered to be effective in the treatment of TSC patients. However, rapamycin (mTOR specific inhibitor) mediated disruption of the feedback suppression of phosphatidylinositol 3-kinase (PI3K)/AKT signaling and extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) signaling from mTOR limited the therapeutic effect of rapamycin on TSC patients (7-9). Moreover, the effect of rapamycin treatment is not very satisfactory because of the immunosuppressive property and drug dependence of rapamycin (10,11). Thus, to ameliorate the treatment for TSC, it is extremely important to search for new therapeutic drugs for TSC.

Increasing attention has been paid to antitumor drugs originating from traditional Chinese medicine (12,13). As a kind of officinal fungi, *Trametes robiniophila* Murr. (Huaier) is applied for the treatment of inflammation and cancer in China. There are increasing evidence reporting that Huaier exerts anti-neoplastic activities through inhibition of proliferation, induction of apoptosis, suppression of angiogenesis, and

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inhibition of metastasis of cancer cells (14-18). However, the underlying mechanisms of anticancer effect of Huaier remain poorly understood.

In this study, we investigated the effect of Huaier aqueous extract on Tsc1- or Tsc2-null mouse embryonic fibroblasts (MEFs), two widely used TSC cell models. Huaier aqueous extract inhibited the proliferation and metastasis, and promoted cell cycle arrest and apoptosis of $Tsc1^{-/-}$ or $Tsc2^{-/-}$ MEFs. Interestingly, we have demonstrated that Huaier aqueous extract inhibited JAK2/signal transducer and activator of transcription 3 (STAT3) and MAPK signaling pathways in these two TSC cell models. Thus, this study provide new insight into the treatment of TSC.

Materials and methods

Reagents and antibodies. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and 0.25% trypsin-EDTA were purchased from Corning Life Sciences (Corning, NY, USA). Antibodies against JAK2, p-JAK2 (Y1007/1008), STAT3, p-STAT3 (Y705), ERK, p-ERK, c-Jun N-terminal kinase (JNK), p-JNK, N-cadherin, β-catenin, TCF8/ZEB1, claudin-1, Slug, Snail, and MMP9 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-MMP9 antibody was purchased from Abcam (Cambridge, UK). Anti-β-actin antibody was purchased from Abgent, Inc. (San Diego, CA, USA). Anti-mouse and rabbit IgG-HRP antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Preparation of Huaier aqueous extract. The electuary ointment of Huaier (Gaitianli Medicine Co., Ltd., Jiangsu, China) was dissolved in complete medium and then sterilized by filtration with a 0.22-mm filter to get the 10 mg/ml stock solution, which was stored at 4°C for short-term storage.

Cell culture. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were previously described (7,19). Cells were maintained in DMEM containing 10% FBS and 1% penicillin/streptomycin in 5% CO_2 at 37°C.

Cell proliferation assay. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were seeded in 96-well plate at a density of 2.0×10^3 cells/well. Twenty-four hours later, cells were treated with Huaier aqueous extract at the indicated concentrations of ranging from 0 to 8 mg/ml, and incubated for 12, 24 and 48 h, respectively. Ten microliters CCK-8 reagent (Dojindo, Kumamoto, Japan) was added to each well, and then incubated for 2 h at 37°C. The optical density values were measured at 450 nm using a microplate reader (Perkin-Elmer, Waltham, MA, USA).

Colony formation. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were seeded in a 6-well plate at a density of 1,000 cells/well. Twenty-four hours later, cells were treated with Huaier aqueous extract at the indicated concentrations. After 10 days of treatment, the cells were fixed with methanol for 15 min, and then colonies were stained with 0.1% crystal violet. The cell colonies were photographed using a digital camera (Leica Microsystems GmbH, Wetzlar, Germany).

Hoechst staining. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min, and stained with Hoechst 33258 (Biyuntian Biotechnology Co., Ltd., Shanghai, China) for 30 min in the dark before washed with PBS again. Then cells were observed under an inverted fluorescence microscope (Leica Microsystems GmbH).

Apoptosis analysis. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were seeded in 12-well plates at 2.5×10^5 cells/well. The next day, the cells were treated with Huaier aqueous extract at 4 or 8 mg/ml for 48 h. Apoptosis was analyzed with an Annexin V-FITC apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. In brief, cells were collected and washed twice with PBS, then resuspended at a concentration of 1×10^6 cells/ml in binding buffer. Five microliters Annexin V-FITC and 5 µl propidium iodide (PI) were added and incubated for 15 min at room temperature in the dark. Finally, 400 µl the binding buffer was added and then cells were analyzed using a flow cytometer (BD Biosciences).

Cell cycle analysis. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were seeded in 6-well plates at 5×10^5 cells/well. After 12-h starvation in serum-free medium, cells were treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h, and then cells were collected, washed twice with PBS and fixed overnight with 70% ethanol at 4°C. The next day, the fixed cells were centrifuged at 1,000 x g for 5 min and washed twice with PBS, then cells were resuspended with 500 µl binding buffer containing RNaseA and PI (Biyuntian Biotechnology Co., Ltd.). After 30-min incubation at 37°C in the dark, the cells were analyzed by flow cytometry.

In vitro scratch assay. Cells were seeded in 12-well plates. The complete medium was replaced with serum-free medium when the cells grew to a subconfluent state. After 12-h starvation, a straight cell-free wound was created with a 10-µl pipette tip and washed twice with PBS, then the cells were maintained in serum-free medium containing Huaier aqueous extract at the concentrations of 0, 2 and 4 mg/ml. The cells were observed and the scratch width was measured at 0, 12 and 24 h. The migration distances were analyzed quantitatively.

Cell invasion assay. $Tsc1^{-/-}$ MEFs and $Tsc2^{-/-}$ MEFs were maintained in serum-free medium for 12 h to reduce the interference of serum. Transwell chambers were placed in 24-well plates to constitute the Transwell system. The Matrigel (BD Biosciences, San Jose, CA, USA) was dissolved in serum-free medium at a ratio of 1:12, and then 60 µl mixture was put to the upper chamber gently. Next the Transwell system was incubated at 37°C for 4 h. The cells were collected and resuspended in serum-free medium containing different concentrations of Huaier aqueous extract. In brief, 1×10^5 cells suspended in 200 µl serum-free medium containing drug were added to the upper chamber, and 750 µl complete medium containing 10% FBS was added to the lower chamber. After incubation for 12 or 24 h, the cells on the upper surface of the membrane were wiped off with cotton swabs, and the cells on the lower surface of the membrane were fixed with ethanol for 15 min, then stained with crystal violet for 10 min and washed twice with PBS. The successfully invaded cells were observed and photographed on 5 random fields with an inverted microscope.
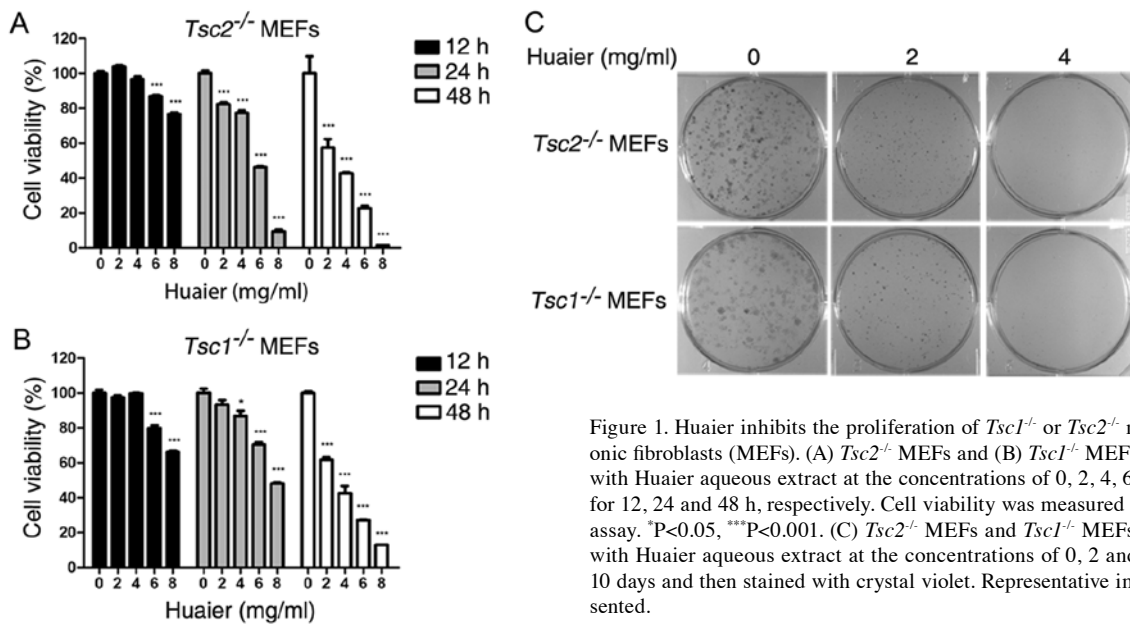
Figure 1. Huaier inhibits the proliferation of *Tsc1*^{-/-} or *Tsc2*^{-/-} mouse embryonic fibroblasts (MEFs). (A) *Tsc2*^{-/-} MEFs and (B) *Tsc1*^{-/-} MEFs were treated with Huaier aqueous extract at the concentrations of 0, 2, 4, 6 and 8 mg/ml for 12, 24 and 48 h, respectively. Cell viability was measured by the CCK-8 assay. *P<0.05, ***P<0.001. (C) *Tsc2*^{-/-} MEFs and *Tsc1*^{-/-} MEFs were treated with Huaier aqueous extract at the concentrations of 0, 2 and 4 mg/ml for 10 days and then stained with crystal violet. Representative images are presented.

Western blotting. Cells were washed twice with PBS and harvested with cell lysis solution as previously described (20). The cell lysate was boiled at 98°C for 10 min. The proteins were separated by 4-12% Bis-Tris Nu-PAGE (Invitrogen, Carlsbad, CA, USA) and then transferred onto PVDF membrane. The membrane was blocked with 3-5% skim milk in TBST at room temperature for 1 h, and then incubated with the primary antibodies at 4°C overnight, followed by the incubation with the HRP-conjugated secondary antibody at room temperature for 2 h. Finally, the membrane was washed three times in TBST and detected by chemiluminescence.

Statistical analysis. The data groups were compared with the two-tailed Student's t-test using GraphPad Prism 5.0 software. The data are presented as mean ± SD. P<0.05 was considered to be statistically significant.

Results

Huaier inhibits proliferation of Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs.* To evaluate the effect of Huaier aqueous extract on *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs, we examined cell viability with the CCK-8 assay. *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs were treated with Huaier at the indicated concentrations (0, 2, 4, 6 and 8 mg/ml) for 12, 24 and 48 h, respectively. As shown in Fig. 1A and B, Huaier significantly suppressed the proliferation of *Tsc2*^{-/-} or *Tsc1*^{-/-} MEFs in a time- and dose-dependent manner. The IC50 values of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs exposed to Huaier aqueous extract of various concentrations for 48 h were 3.89 and 3.56 mg/ml, respectively. Furthermore, we performed colony formation assay to assess the effect of Huaier aqueous extract on cell proliferation. Result indicated that *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs treated with Huaier aqueous extract formed smaller and fewer colonies in a dose-dependent manner (Fig. 1C).

Huaier induced cell cycle arrest of Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs.* To investigate whether the inhibitory effect of Huaier aqueous extract was attributed to cell cycle arrest, we performed flow cytometry assay to evaluate cell cycle distribution of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs exposed to Huaier. *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs were treated with Huaier aqueous extract for 48 h. As shown in Fig. 2A, *Tsc2*^{-/-} MEFs exposed to Huaier showed significantly decreased fraction of the G0/G1 phase and increased fraction of the S phase in a dose-dependent manner. In addition, *Tsc1*^{-/-} MEFs treated with Huaier exhibited significantly decreased fraction of the G2/M phase and increased fraction of the S phase (Fig. 2B). Taken together, these results revealed that Huaier-induced S-phase arrest was partially responsible for the proliferation inhibition of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs.

Huaier induces apoptosis in Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs.* Next we performed Hoechst staining assay to determine the effect of Huaier aqueous extract on apoptosis. Apoptotic cells could be stained with Hoechst 33258 dye. As observed in Fig. 3A, the nuclei in the untreated *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs were stained with a less bright blue fluorescence and these two cells appeared to be intact oval shape. Whereas, more condensed or fragmented chromatin were observed in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs exposed to Huaier aqueous extract. To further explore the effect of Huaier on apoptosis in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs, we conducted flow cytometry analysis with Annexin V-FITC/PI staining. As shown in Fig. 3B, the apoptosis rate of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs increased in a dose-dependent manner in response to Huaier. Collectively, Huaier aqueous extract promotes apoptosis of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs.

Effect of Huaier treatment on the morphology of Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs.* Next we determined the effect of Huaier aqueous extract on cell morphology. *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs were treated with Huaier aqueous extract at a concentration of 4 or 8 mg/ml for 24 h. As shown in Fig. 4, the untreated cells showed plump cell body, homogeneous cytoplasm, and good refraction. However, Huaier treatment led to shrinked cell body and weakened refraction. Moreover, more cells became round and died, which was concomitant with an increase in the concentration of Huaier.
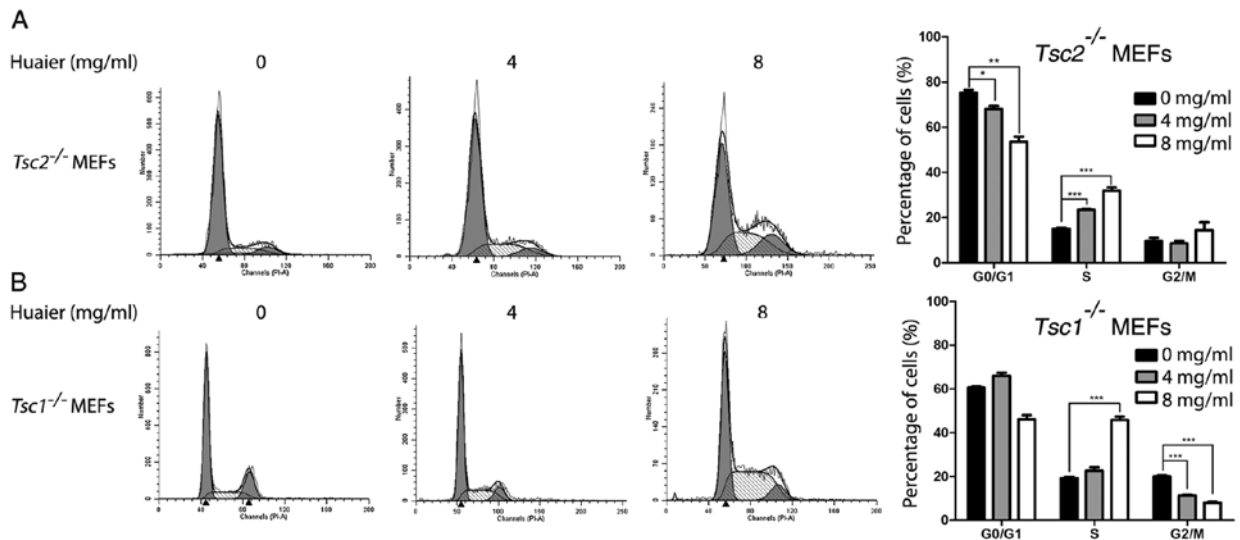
Figure 2. Huaier induces cell cycle arrest of *Tsc1*^{-/-} or *Tsc2*^{-/-} mouse embryonic fibroblasts (MEFs). Cell cycle distribution of (A) *Tsc2*^{-/-} MEFs and (B) *Tsc1*^{-/-} MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were examined by flow cytometry. *P<0.05, **P<0.01, ***P<0.001.
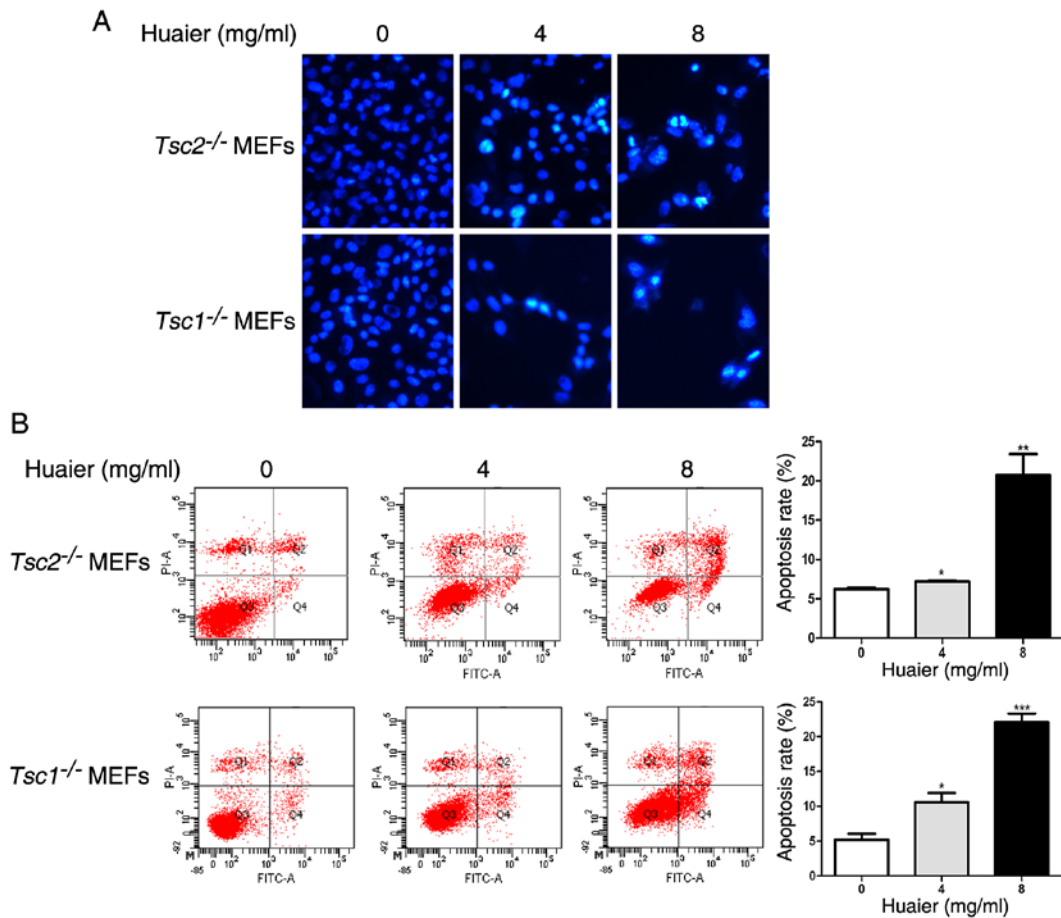

Figure 3. Huaier induces apoptosis in *Tsc1*^{-/-} or *Tsc2*^{-/-} mouse embryonic fibroblasts (MEFs). (A) *Tsc2*^{-/-} MEFs and *Tsc1*^{-/-} MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were stained with Hoechst 33258 and were photographed under a fluorescence microscope. Representative images are presented (x200). (B) *Tsc2*^{-/-} MEFs and *Tsc1*^{-/-} MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were subjected to analysis of apoptosis by flow cytometry. *P<0.05, **P<0.01, ***P<0.001.

Huaier inhibits cell motility of Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs.* Migration and invasion play a critical role in metastasis of cancer cells (21). To evaluate the migration ability of cells *in vitro*, we performed *in vitro* scratch assay. We treated *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs with Huaier aqueous extract at the concentrations of 0, 2 and 4 mg/ml, and then measured the scratch width at time-points of 0, 12 and 24 h. As shown in Fig. 5A, the migration distance was decreased in response to Huaier in a dose-dependent manner.
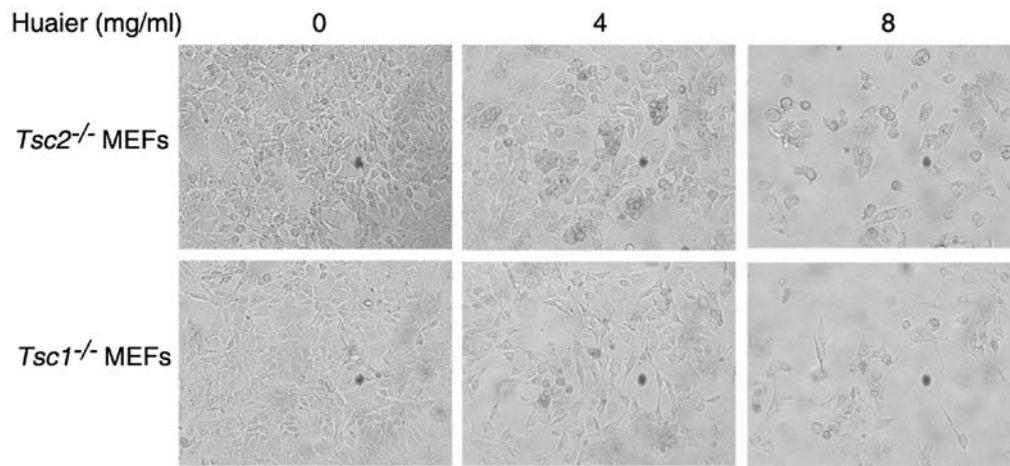
Figure 4. Effect of Huaier treatment on the morphology of *Tsc1*⁻ᐟ⁻ or *Tsc2*⁻ᐟ⁻ mouse embryonic fibroblasts (MEFs). The morphology of *Tsc2*⁻ᐟ⁻ MEFs and *Tsc1*⁻ᐟ⁻ MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were observed. Representative images are presented (x200).
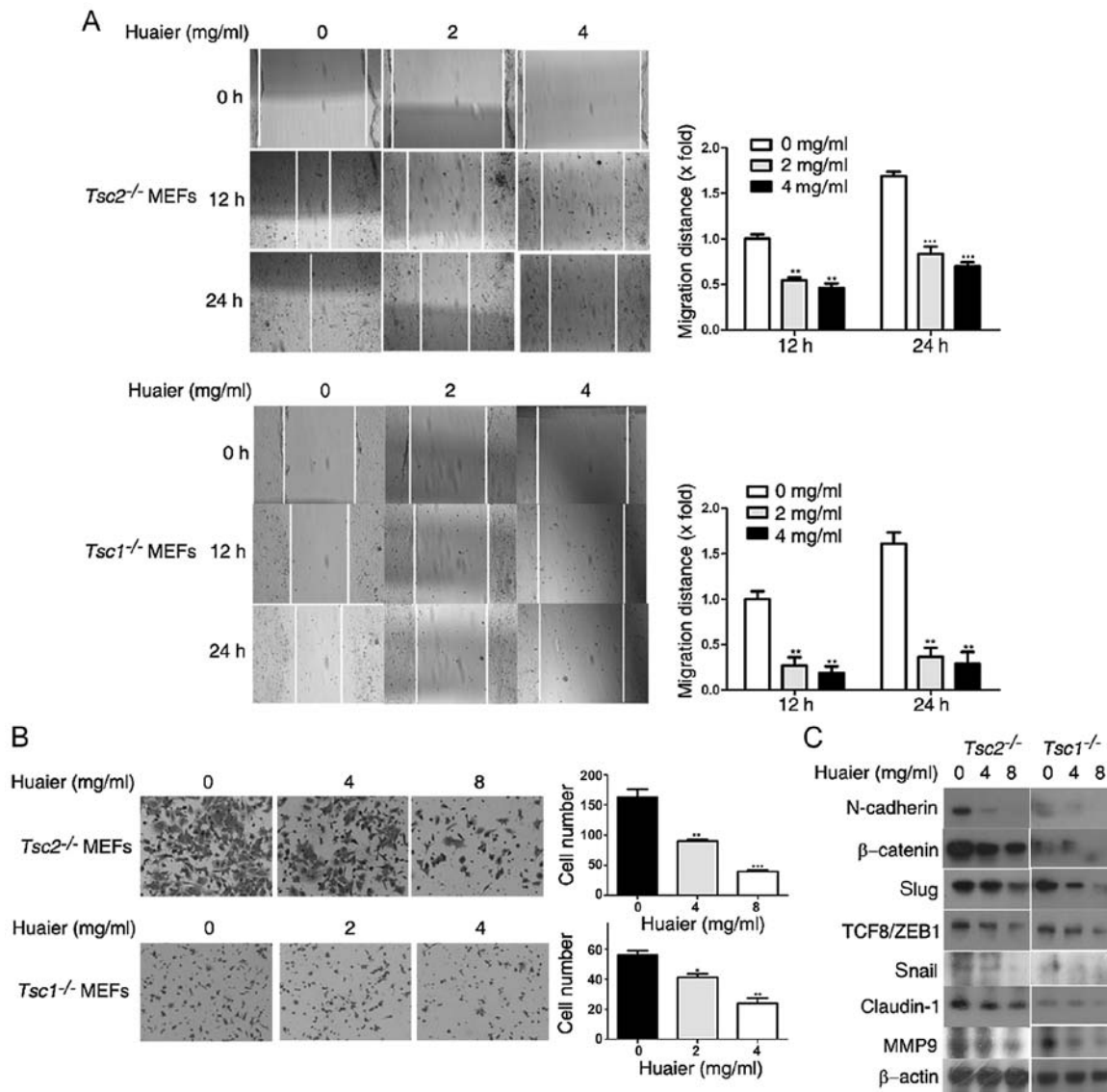

Figure 5. Huaier inhibits cell motility of *Tsc1*⁻ᐟ⁻ or *Tsc2*⁻ᐟ⁻ mouse embryonic fibroblasts (MEFs). (A) *Tsc2*⁻ᐟ⁻ MEFs and *Tsc1*⁻ᐟ⁻ MEFs treated with Huaier aqueous extract at the concentrations of 0, 2 and 4 mg/ml were subjected to *in vitro* scratch assay and observed at 0, 12 and 24 h, respectively. Left panel, representative images (x100); right panel, quantitative data. **P<0.01, ***P<0.001. (B) *Tsc2*⁻ᐟ⁻ MEFs or *Tsc1*⁻ᐟ⁻ MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 or 0, 2 and 4 mg/ml for 12 or 24 h, respectively, were subjected to cell invasion assay. Representative images are presented (x200). (C) *Tsc2*⁻ᐟ⁻ MEFs and *Tsc1*⁻ᐟ⁻ MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were subjected to immunoblotting. *P<0.05, **P<0.01, ***P<0.001.
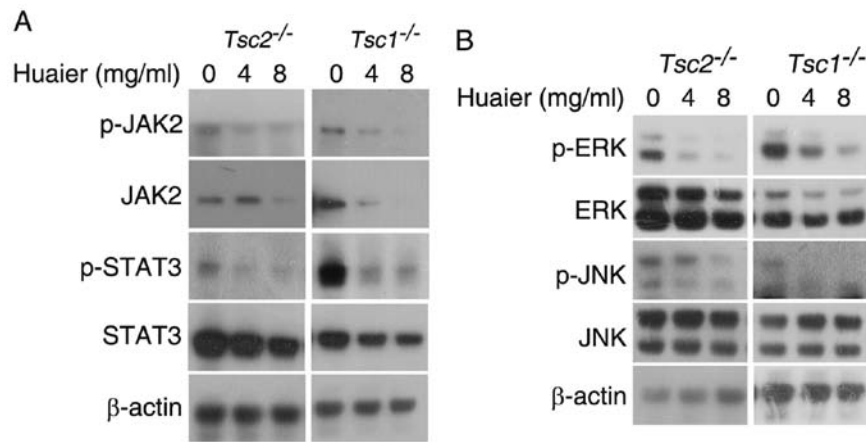
Figure 6. Huaier inhibits JAK2/signal transducer and activator of transcription 3 (STAT3) and mitogen-activated protein kinase (MAPK) signaling pathways in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs. *Tsc2*^{-/-} mouse embryonic fibroblasts (MEFs) and *Tsc1*^{-/-} MEFs treated with Huaier aqueous extract at the concentrations of 0, 4 and 8 mg/ml for 48 h were subjected to immunoblotting for indicated proteins of (A) JAK2/STAT3 signaling pathway or (B) MAPK signaling pathway.

The scratch healing inhibition rates of *Tsc2*^{-/-} MEFs and *Tsc1*^{-/-} MEFs were 58.57±4.52 and 82.01±13.97% after treatment with Huaier aqueous extract at a concentration of 4 mg/ml for 24 h. Thus, Huaier significantly inhibits the migration of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs. Furthermore, we performed Transwell assay to assess the invasive capacity of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs *in vitro*. *Tsc2*^{-/-} MEFs were treated with Huaier aqueous extract at the concentrations of 4 and 8 mg/ml for 12 h. As observed in Fig. 5B, the number of *Tsc2*^{-/-} MEFs invading through the Matrigel-coated membrane was drastically decreased. In addition, the invasive capacity of *Tsc1*^{-/-} MEFs was also reduced by treatment with Huaier (Fig. 5B).

The epithelial-mesenchymal transition (EMT) is a critical process in cancer development, which enhances the metastatic capacity of cancer cells (22,23). We therefore investigated the effects of Huaier on EMT of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs. We examined the expression levels of EMT markers by western blotting. As shown in Fig. 5C, Huaier treatment downregulated the protein levels of N-cadherin, β-catenin, Slug, TCF8/ZEB1, Snail, and claudin-1 (24-26) in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs, indicating that Huaier inhibited EMT of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs. MMP-9 has been reported to be important in cancer metastasis (27,28). Here we found that MMP-9 expression was reduced by Huaier treatment in a dose-dependent manner (Fig. 5C). Taken together, Huaier suppressed the metastatic ability of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs *in vitro*.

Huaier inhibits JAK2/STAT3 and MAPK signaling pathways in Tsc1^{-/-} *or Tsc2*^{-/-} *MEFs*. JAK2/STAT3 pathway plays an important role in tumorigenesis and metastasis (29,30). Inhibition of JAK2/STAT3 signaling pathway constrains cancer cell growth and induces apoptosis (31,32). As shown in Fig. 6A, Huaier treatment downregulated the protein levels of JAK2, p-JAK2, STAT3, and p-STAT3 in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs, suggesting that Huaier markedly inhibits JAK2/STAT3 signaling pathway in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs. The MAPK pathway plays a crucial role in multiple cellular processes, such as cell proliferation, apoptosis, migration and invasion (33,34). The MAPK superfamily consists of three mammalian MAP kinases, ERK, JNK, and p38 MAPK (35). As shown in Fig. 6B, we found that the phosphorylation of ERK and JNK was inhibited by

Huaier treatment in these two cell lines, indicating that Huaier restrains the MAPK signaling pathway in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs.

Discussion

Tumorigenesis is a multiple-step process involving aberrant genetic alterations. These complicated mechanisms may confer resistance to drugs which were used for targeted therapy of cancers. Therefore, it plays an important role in the treatment of cancers to target multiple pro-survival signaling pathways. Traditional Chinese medicine (TCM) has become an important source for developing new antitumor drugs based on some advantages including low toxicity, low cost, and the multi-targets (13). Huaier has been applied in TCM for approximately 1,600 years in China and has good clinical effects. As a single drug or adjuvant drug in the treatment of cancers, Huaier has attracted increasing attention in recent years.

The dysregulation of cell growth and cell death signals are main characteristics of tumors, so the strategy of inhibition of cell proliferation and induction of apoptosis could be exploited to treat tumors. *Tsc1*^{-/-} MEFs and *Tsc2*^{-/-} MEFs, two widely used TSC cell models (7,36-38), were applied in this study. We have demonstrated that Huaier could inhibit cell proliferation of *Tsc1*^{-/-} MEFs and *Tsc2*^{-/-} MEFs *in vitro* by CCK-8 assay, colony formation, and cell cycle analysis (Figs. 1 and 2). Moreover, we also showed that Huaier induced apoptosis in *Tsc1*^{-/-} MEFs and *Tsc2*^{-/-} MEFs by flow cytometry and Hoechst staining (Fig. 3). These data provide potent *in vitro* evidence for treatment of TSC with Huaier.

Tumor metastasis is an extremely complicated multi-step process and play a critical role in tumorigenesis. Migration and invasion are two important cellular processes in tumor metastasis. In this study, we performed *in vitro* scratch assay and Transwell assay to evaluate the effects of Huaier on migratory and invasive ability *in vitro* of *Tsc1*^{-/-} MEFs and *Tsc2*^{-/-} MEFs. We showed that migration and invasion of *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs were markedly impaired by Huaier treatment in a dose-dependent manner (Fig. 5A and B). In addition, EMT is a process characterized with the conversion of epithelial cells into motile mesenchymal cells and the increase in

cellular migration and invasion. EMT has been reported to occur in tumor metastasis (23,33). Our study revealed that Huaier compromised the process of EMT in *Tsc1*^{-/-} or *Tsc2*^{-/-} MEFs (Fig. 5C). As LAM is considered to be initiated by cells with mutations of TSC1 or TSC2, which metastasized from renal angiomyolipomas (39,40), Huaier may be exploited as a potential drug targeting metastasis in the treatment of LAM and TSC.

JAK2/STAT3 signaling pathway has a critical role in cell proliferation, migration, apoptosis, and differentiation (41,42). JAK2/STAT3 signaling pathway has been considered as a novel target for drugs against cancers (43,44). STAT3 is an oncoprotein highly overexpressed in many cancers (45). STAT3 is required for aberrant proliferation and survival of TSC2-null cells (46). In addition, the role of MAPK signaling pathway in cell proliferation, apoptosis, motility, and morphogenesis has been extensively investigated (33). Suppression of MAPK signaling inhibited *Tsc2*^{-/-} cell proliferation (47). In this study, we reported that Huaier inhibited JAK2/STAT3 signaling through down-regulation of the total expression and phosphorylation of JAK2 and STAT3. Additionally, Huaier inhibited the phosphorylation/ activation of ERK and JNK in a dose-dependent manner in both *Tsc1*^{-/-} MEFs and *Tsc2*^{-/-} MEFs (Fig. 6). Collectively, we propose that Huaier inhibits the proliferation and metastasis of Tsc1- or Tsc2-null cells partially via downregulation of JAK2/STAT3 and MAPK signaling pathways.

In conclusion, we have clarified that the attenuation of JAK2/STAT3 and MAPK signaling are partially responsible for the inhibition of proliferation and metastasis of two TSC cell models induced by Huaier. Therefore, based on the low toxicity and multi-targets of Huaier treatment, Huaier may be a candidate drug for TSC treatment.

Acknowledgements

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