Influence of the Twist gene on the invasion and metastasis of colon cancer

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Abstract. The present study investigated the role of the Twist gene in epithelial-mesenchymal transition (EMT) and its effects on the invasion and metastasis of malignant tumors. *In vitro*, we transfected SW480, HCT116 and HT29 cells with recombinant plasmids, pTracer-CMV/BSD-Twist and pGenesil1.2-Twist-shRNA, to influence expression of Twist. The transfection efficacy of the plasmids in the cell lines was confirmed by flow cytometry. The relative mRNA and protein expression levels of Twist, E-cadherin and vimentin in the transfected cells were detected by RT-PCR and western blotting, respectively. In addition, migration and invasion were assessed by Transwell assays. *In vivo*, we established a xenogenic liver metastasis mouse model by intrasplenic injection with transfected SW480, HCT116 or HT29 human colon cancer cells and used hematoxylin and eosin (H&E) staining to demonstrate the effective establishment of the model. The relative mRNA levels of Twist and vimentin were detected by RT-PCR. *In vitro*, RT-PCR and western blotting showed higher relative mRNA and protein expression levels of Twist and vimentin in cell lines transfected with the recombinant, highly expressed Twist plasmid than in non-transfected cell lines ($P<0.05$), while E-cadherin was inhibited ($P<0.05$). After transfection with the plasmid pGenesil1.2-Twist-shRNA, the relative mRNA and protein levels of Twist and vimentin were markedly inhibited in the HCT116 cells ($P<0.05$), and the levels of E-cadherin were not changed ($P>0.05$), along with inhibition of the migration and invasion abilities of the cell line ($P<0.01$). *In vivo*, relative mRNA levels of Twist and vimentin in both the liver and spleen of the mouse model were higher in the groups that were injected with one of the three cell lines transfected with pTracer-CMV/BSD-Twist than in the groups injected with cells transfected with pGenesil1.2-Twist-shRNA ($P<0.05$). In conclusion, upregulation of Twist gene expression can promote EMT molecular events. Interfering with the Twist gene can effectively silence Twist gene expression in HCT116 cells and consequently inhibit colon cancer cell migration and invasion.

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Introduction

Colorectal cancer (CRC) is the third most common cancer and the fourth most common cause of death worldwide (1). Many countries have experienced an increase in the incidence of CRC during the past few decades, and it is expected to continue increasing in the next decades (1-3). Although there have been improvements in the rate of early diagnosis, the efficacy of comprehensive treatment and the rate of surgical resection along with postoperative survival (4), radical treatment for metastasis of CRC remains limited. The invasion and metastasis of colon cancer cells weaken the treatment effect, leading to a high mortality rate. The key steps involved in metastasis include detachment of the malignant tumor cells from the primary tumor, migration to secondary tissue or organ through a variety of ways and continued growth and proliferation. Metastasis is closely related to the biological transformation process where cells gradually lose an epithelial phenotype and obtain a mesenchymal phenotype, which is known as epithelial-mesenchymal transition (EMT) (5,6). EMT is characterized by the loss of epithelial surface markers, particularly E-cadherin, and the acquisition of mesenchymal markers including vimentin and N-cadherin (7). Recently, many studies have shown that EMT is one of the important steps in the progression of tumors and is a necessary condition for the increased invasive ability of tumor cells, such as in the progression of breast (8-12), prostate (13-15), liver (16) and lung cancer (17,18). Previous studies have also demonstrated that the expression of cell adhesion molecules, including E-cadherin, is reduced in primary tumors, whereas expression levels are elevated in metastatic foci (19). E-cadherin plays an important role in embryonic development and morphogenesis (20), and cell proliferation, survival, invasion and migration (21). The mechanisms by which E-cadherin is inhibited include gene mutations, promoter hypermethylation,

chromatin remodeling, post-translational modification and transcriptional repression (22-25). The major proteins implicated in the transcriptional repression of E-cadherin include ZEB-1 or ZEB-2 (26), Snail and Slug, Smad interacting protein 1 (SIP1), and a basic helix-loop-helix (bHLH) protein called Twist (25,27,28). Twist was originally described as a key factor in early mesodermal development of *Drosophila*, which is also both necessary and sufficient to drive the development of the embryo (29,30). Genetic studies have well illustrated the critical role of Twist in mesodermal development while human studies have shown that Twist gene mutations can lead to a disease called Saethre-Chotzen syndrome (SCS) (31). Recently, more and more research concerning the dysregulation of the Twist gene in the induction of EMT in diseases has been reported (32,33). However, there are few studies on the relationship between the Twist gene and EMT in colon cancer and its molecular mechanism.

In the present study, we mainly focused on the expression of E-cadherin and vimentin after upregulation and downregulation of the Twist gene in SW480, HT29 and HCT116 colon cancer cell lines *in vitro*. In addition, we evaluated the expression of E-cadherin and vimentin genes in primary tumors and metastatic sites by constructing a heterotopic liver metastasis model in nude mice with intrasplenic injection of the colon cancer cell lines (SW480, HT29 or HCT116) transfected with the different plasmids.

Materials and methods

Transformation, purification and extraction of DNA plasmids. The pTracer-CMV/BSD-Twist and pTracer-CMV/BSD plasmids were obtained from our laboratory. The three plasmids pGenesil1.2-Twist-shRNA, pGenesil1.3-Twist-shRNA and pGenesil1.2-shRNA were designed and constructed by Wuhan GenSil Biotechnology Co. Ltd. (Wuhan, China). The plasmids were successfully constructed, transformed with DH5α, and extracted and purified from *E. coli*. The double restriction enzyme digestion of DNA used the enzymes *Nhe*I, *Bsa*I, *Sac*I and *Eco*RI, and we performed agarose gel electrophoresis for confirmation and separation of digested DNA. All the sequences were confirmed by Beijing Liuhe Huada Gene Technology, Co., Ltd. (Beijing, China).

Cell culture and transfection. Human colon cancer cell lines SW480 and HT29 were gifts from the College of Life Science, Nankai University, Tianjin, China, and the HCT116 cell line was purchased from the Tumor Line Database of the Chinese Academy of Medical Sciences, Beijing, China. We used complete medium containing liquid nitrogen and the cryoprotective agent dimethyl sulfoxide (DMSO) to store the cryopreserved cultured cells. Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect the cells according to the manufacturer's protocol.

Flow cytometric analysis and MTT assay. The DNA plasmids coded with green fluorescent protein (GFP) expressed green fluorescence under an inverted fluorescence microscope IX70 (Olympus, Tokyo, Japan) 48 h post-transfection. When cells were in the logarithmic growth phase, we harvested the cells by trypsinization. A FACS flow cytometer (BD Biosciences, Bedford, MA, USA) was used to determine the number of positive cells within a transfected cell population (positive cells %). A MTT colorimetric assay was applied to test the cell proliferation and viability.

Fluorescence quantitative real-time PCR. Total RNA from transfected cells was extracted by TRIzol reagent (Invitrogen). High capacity cDNA reverse transcriptase (Invitrogen) was used for cDNA synthesis. Quantitative real-time PCR was used to determine relative expression levels of Twist, vimentin and E-cadherin. The relative expression of mRNA of the three genes was determined and normalized to the expression of the reference gene GAPDH. The primers used for PCR amplification were as follows: Twist forward, 5'-GGAGTCCGCA GTCTTACGAG-3' and reverse, 5'-TCTGGAGGACCTGGT AGAGG-3'; E-cadherin forward, 5'-GTGTCATCCAACGGG AATGC-3' and reverse, 5'-TGGCGGCATTGTAGGTGTTC-3'; vimentin, forward, 5'-ATGACCGCTTCGCCAACTAC-3' and reverse, 5'-CGGGCTTTGTCGTTGGTTAG-3'; GAPDH forward, 5'-GAAGGTGAAGGTCGGAGTC-3' and reverse, 5'-GAAGATGGTGATGGGATTTC-3'. The qPCR amplification was performed for 40 cycles using the Fluorescence Quantitative Real-Time PCR Machine (MJ, USA); SYBR Select Master Mix (ABI, USA), and it consisted of three steps: 1 min at 94˚C for denaturation, 45 sec at 54˚C for primer annealing and 2 min at 72˚C for extension. The ΔΔCt method was used to calculate the relative amount of mRNA.

Transwell migration and invasion assays. The Transwell migration assay was used to determine the migration and invasion ability of the different tumor cell lines. Transwell filters (Corning Inc., Corning, NY, USA) were coated with EMC Gel (Sigma-Aldrich, St. Louis, MO, USA) [2 mg/l, dilution with serum-free Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 medium] and incubated at 37˚C for 2 h. Fetal bovine serum (FBS) (10%) (as a chemoattractant) was added when EMC became solidified and FBS-free medium suspension including the harvested tumor cells were placed into the bottom and the upper compartment of the chamber (cell concentration, $1x10^5$ cells/ml) respectively. After 24 and 48 h of incubation in a 37˚C atmosphere and 5% CO_2, non-invaded cells were removed from the upper chamber with a cotton swab and the migrated cells were stained using hematoxylin and eosin (H&E) (Sigma-Aldrich). In addition, we counted the number of the cells on the lower side of the filter under a microscope.

Western blot analysis. Cells transfected for 48 h were lysed on ice for 30 min in a lysis buffer (Thermo Fisher Scientific, Waltham, MA USA). Equal amounts of protein from the cell extracts were applied to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were incubated at 4˚C with primary antibodies, including anti-Twist, anti-E-cadherin, anti-vimentin and anti-GAPDH (1:1,000 dilution ratio; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight. The membranes were washed and incubated at room temperature for 2 h with diluted secondary horseradish peroxidase (HRP)-conjugated polyclonal antibodies (1:1,000; dilution ratio; Zhongshan Golden

Bridge, Beijing, China). The blotted proteins were determined using a Bradford protein assay kit (Beyotime Institute of Biotechnology, Beijing, China).

Building spleen-to-liver mouse model of metastatic colon cancer and fluorescence RT-qPCR of xenograft tumors in the spleen and liver of mice. The surgical principle and procedure for xenogenic spontaneous heterotopic liver metastasis were similar to descriptions in the literature (34). Female BALB/c nude mice (10-11 weeks of age) of specific pathogen-free grade were purchased from Beijing Wei Tong Li Hua Experimental Animal Technology Co. Ltd., and raised at the Chinese Academy of Medical Sciences Hematology Hospital, Department of Experimental Animal Center in an aseptic environment. Mice were fed *ad libitum* and maintained in a HEPA filter environment in a cage. All the food and bedding were sterilized by autoclaving. After the nude mice were anesthetized, a left lateral flank incision was made to expose the spleen. Tumors were implanted by intrasplenic injection of $1x10^6$ SW480, HT29 or HCT116 cells using a 27-gauge needle, and the surgical incision was closed using surgical thread. The mice were euthanized on the 30th day after intrasplenic injection with the tumor cell lines (35). Liver and spleen specimens were taken for H&E and immunohistochemical staining and mRNA isolation. The method of fluorescence RT-qPCR of xenograft tumors in the spleen and liver of the mice was the same as described above.

Statistical analysis. Data were analyzed using single-factor analysis of variance (one-way ANOVA) for comparison within groups. Multiple comparisons were carried out among groups using the least significant difference method [least significant difference (LSD)]. For analysis of data with an unknown population, the distribution was carried out by Spearman correlation test. The difference between the mean values for groups was analyzed by t-test for normal distribution. Otherwise, the Mann-Whitney U test was used. Statistical significance was determined at the $P<0.05$ probability level. The software package SPSS 17.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis of all the experimental data. The results are representative of three independent experiments.

Results

mRNA transcription and protein expression levels of Twist in different colon cancer cell lines. The mRNA transcription copies and protein expression levels of Twist in different colon cancer cell lines from high to low were HCT116 → SW480 → HT29 (Fig. 1A and B). The relative mRNA transcription copies of HCT116, SW480 and HT29 were 11.7, 1.03 and 1, respectively. Least significant difference (LSD) showed that the difference in mRNA transcription and protein expression levels of Twist among the groups was significant ($P<0.05$).

Successful transfection of plasmids in colon cancer cell lines. The plasmids pTracer-CMV/BSD-Twist, pTracer-CMV/BSD, pGenesil1.2-Twist-shRNA, pGenesil1.3-Twist-shRNA and pGenesil1.2-shRNA were successfully transformed with DH5α and extracted and purified from *E. coli* (Fig. 2). After

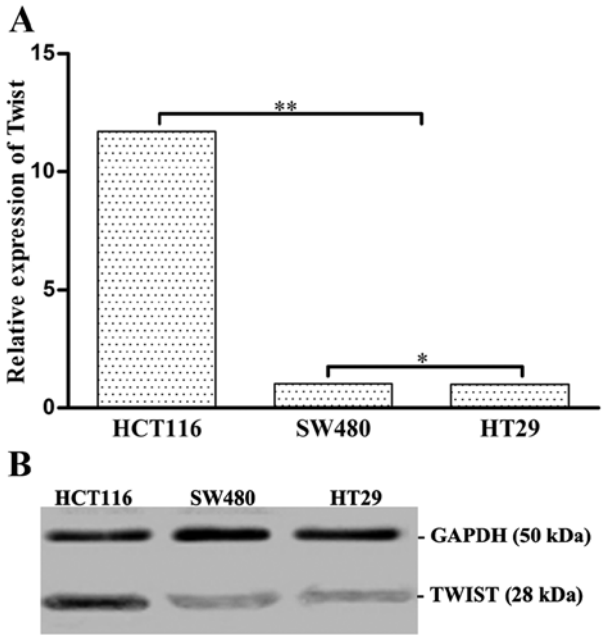

Figure 1. *Twist* has higher expression in colon cancer cell line HCT116 than in SW480 and HT29. (A) Real-time PCR analysis of mRNA transcription levels of *Twist* in three colon cancer cell lines ($P<0.05$). (B) Western blot analysis of protein expression levels of Twist in three colon cancer cell lines.

transfection of the tumor cells using Lipofectamine 2000, 48 h later the DNA plasmids coded with the GFP gene in the colon cancer cell lines expressed green fluorescence under an inverted fluorescence microscope (Fig. 3). We harvested the tumor cells and used FACS flow cytometry to determine the number of GFP-positive cells among the transfected cells (positive cells %). The GFP expression of pGenesil 1.2-Twist-shRNA reached 21.2% while GFP expression of pGenesil1.3-Twist-shRNA reached only 19.8% in the SW480 cells. Thus, we used pGenesil 1.2-Twist-shRNA for further experiments (Fig. 4A). The transfection efficiency of pGenesil 1.2-Twist-shRNA analyzed by CellQuest software in the HCT116, HT29 and SW480 cells was 23.4, 30.3 and 21.2%, respectively (Fig. 4B), and the transfection efficiency of pTracer-CMV/BSD-Twist in the HCT116, HT29 and SW480 cells was 22.3, 22.7 and 21.6%, respectively (Fig. 4C).

MTT proliferation assay results for the transfected cell lines. As shown in Fig. 5A-C, the proliferation and viability of the three cell lines were not affected after the transfection of the recombinant plasmids. The difference in cell proliferation of the plasmid pTracer-CMV/BSD-Twist and pGenesil1.2-Twist-shRNA-transfected groups was statistically insignificant compared with that of the plasmid pTracer-CMV/BSD, pGenesil1.2-shRNA and negative control groups ($P>0.05$).

Transwell migration and invasion assay results. The total number of migrated cells was ~900-1,200 cells, which showed that all three malignant cell lines had elevated spontaneous Transwell migration. The Transwell migration assay results (Figs. 6 and 8A-C) showed that the number of migrated cells in all three cell lines transfected with the

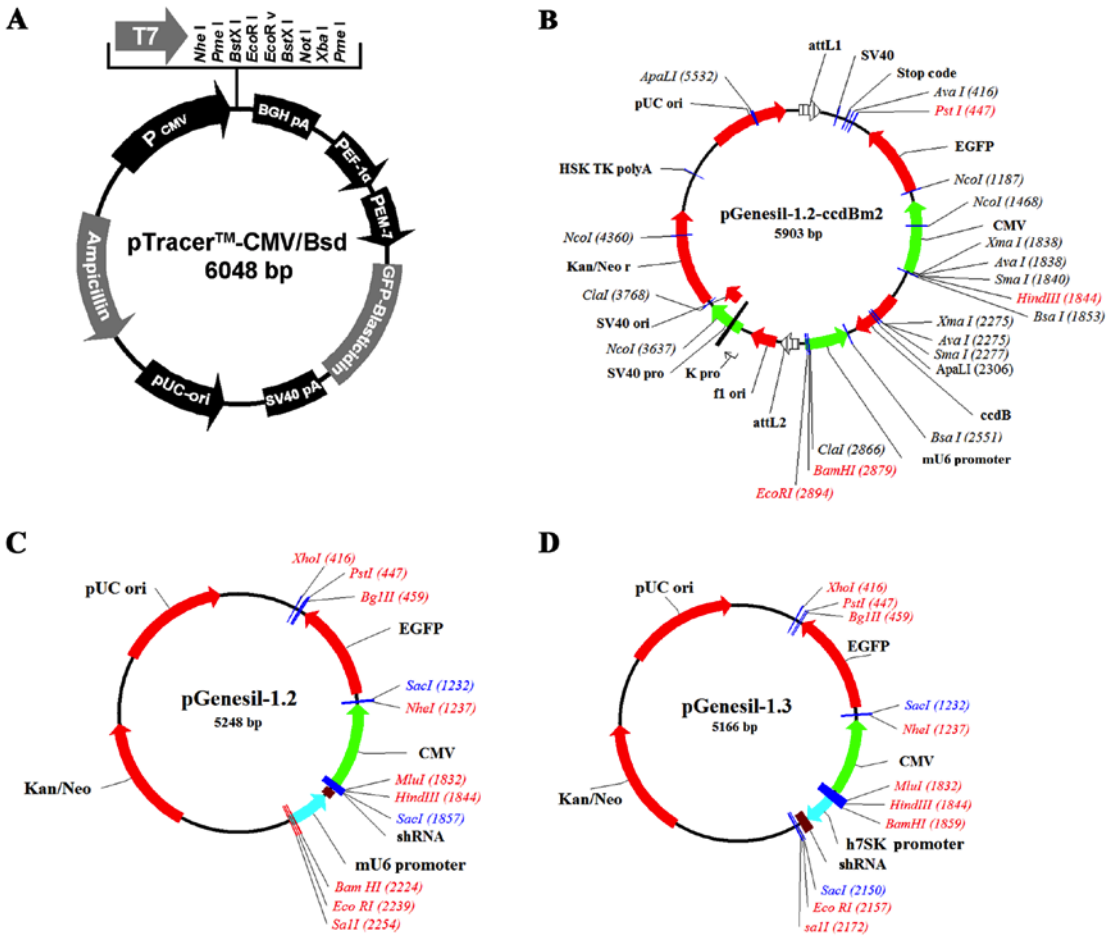

Figure 2. The plasmids were successfully transformed, extracted and purified. (A) pTracer-CMV/BSD-Twist, pTracer-CMV/BSD. (B) pGenesil1.2-shRNA. (C) pGenesil1.2-Twist-shRNA. (D) pGenesil1.3-Twist-shRNA.

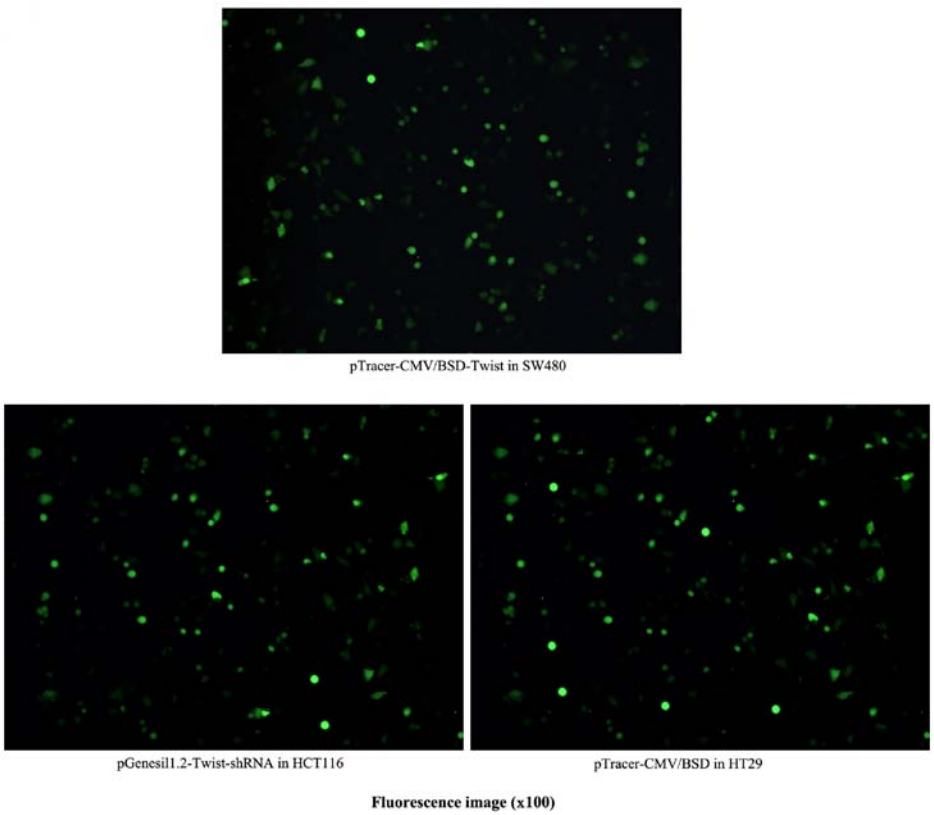

Figure 3. Colon cancer cell lines were successfully transfected. Immunofluorescence images of the transfected plasmids

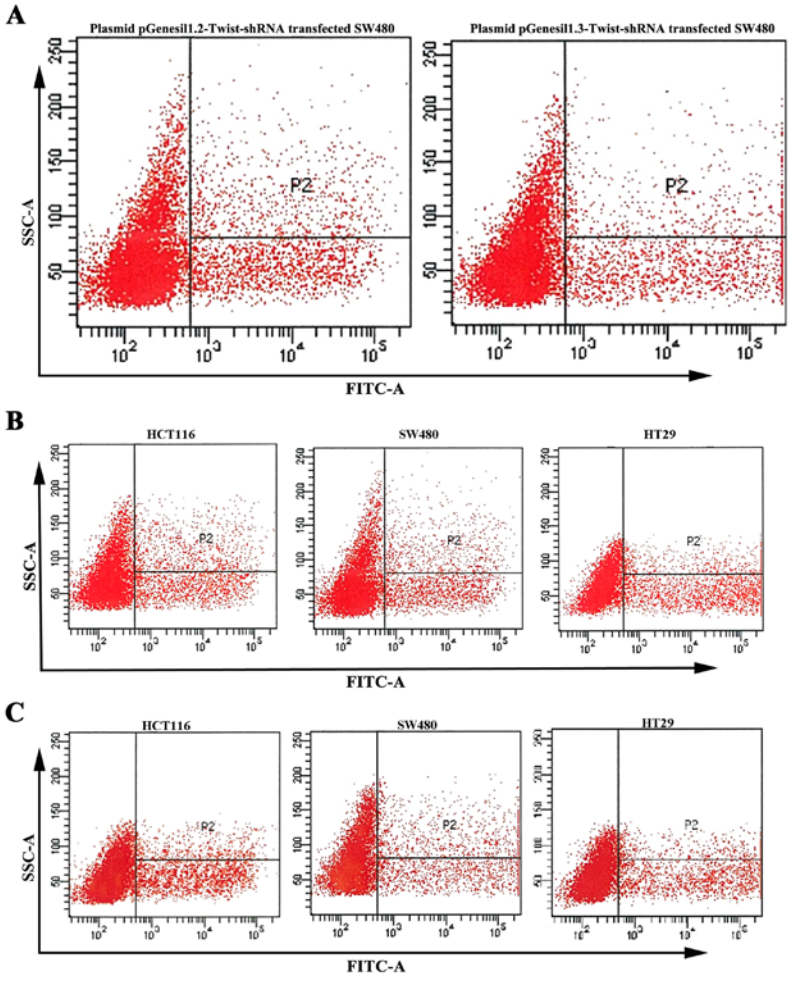

Figure 4. Transfection efficacy of the different plasmids in each cell line. (A) The transfection efficacy of plasmid pGenesil1.2-Twist-shRNA and pGenesil1.3-Twist-shRNA by FCM in SW480 cells was 21.2 and 19.8%, respectively. (B) The transfection efficacy of pGenesil1.2-Twist-shRNA in HCT116, HT29 and SW480 cells was 23.4, 30.3 and 21.2%, respectively. (C) The transfection efficacy of pTracer-CMV/BSD-Twist in HCT116, HT29 and SW480 cells was 22.3, 22.7 and 21.6% respectively.

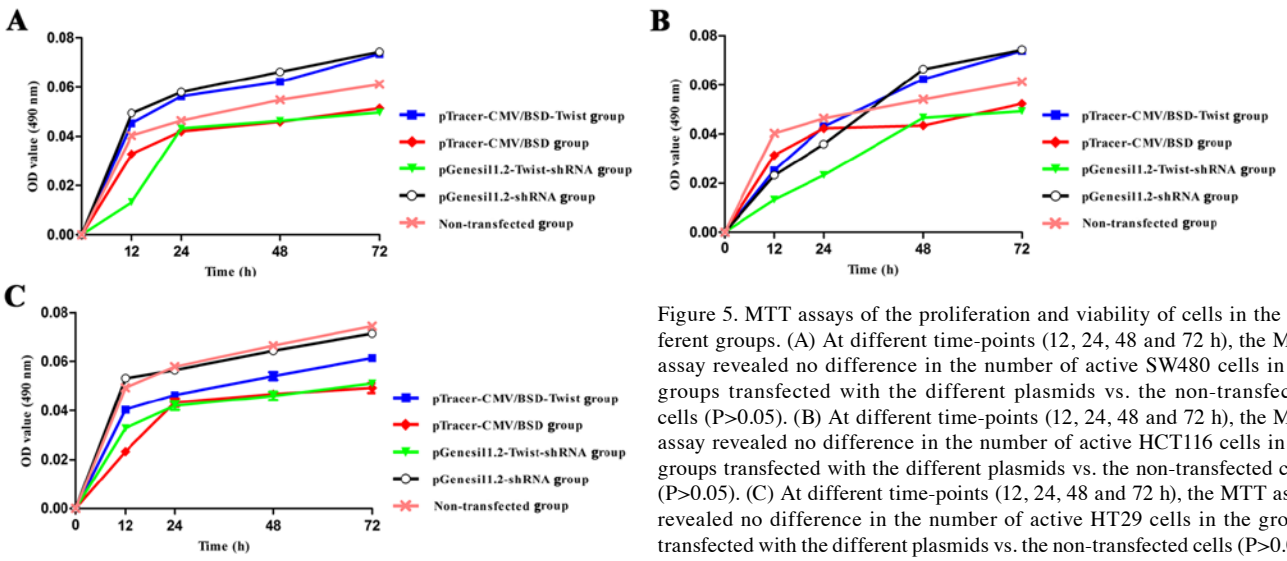

Figure 5. MTT assays of the proliferation and viability of cells in the different groups. (A) At different time-points (12, 24, 48 and 72 h), the MTT assay revealed no difference in the number of active SW480 cells in the groups transfected with the different plasmids vs. the non-transfected cells (P>0.05). (B) At different time-points (12, 24, 48 and 72 h), the MTT assay revealed no difference in the number of active HCT116 cells in the groups transfected with the different plasmids vs. the non-transfected cells (P>0.05). (C) At different time-points (12, 24, 48 and 72 h), the MTT assay revealed no difference in the number of active HT29 cells in the groups transfected with the different plasmids vs. the non-transfected cells (P>0.05).

pTracer-CMV/BSD-Twist was increased compared with that in the control groups, and the difference was statistically significant (P<0.01). The number of migrated cells in the HCT116 cell line transfected with pGenesil1.2-Twist-shRNA was decreased compared with that in the control group, and the difference was statistically significant (P<0.01), but there was no statistically significant difference between the plasmid pGenesil1.2-Twist-shRNA-transfected group and the

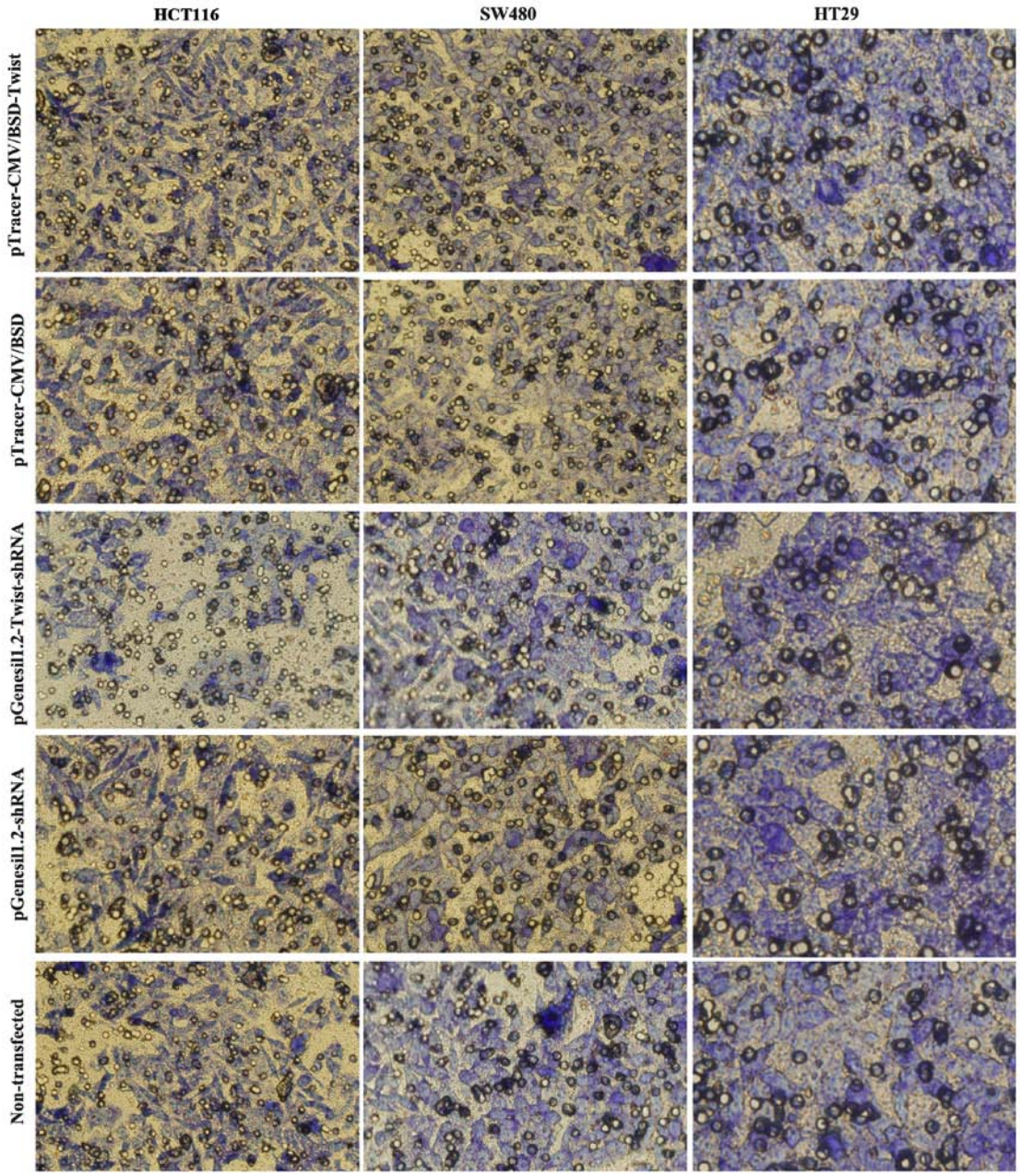

Figure 6. Transwell migration assay showing the migrated colon cancer cells in the different groups (magnification, x200).

control groups in the SW480 and HT29 cell lines (P>0.05). The Transwell invasion assay results (Figs. 7 and 8D-F) showed that cell invasion in all three cell lines in the plasmid pTracer-CMV/BSD-Twist-transfected groups was higher than that in all other groups (P<0.01), whereas cell invasion only in HCT116 in the pGenesil1.2-Twist-shRNA-transfected group was comparatively lower than that in all other groups (P<0.01). The results above indicated that after transfection with pGenesil1.2-Twist-shRNA plasmid, the migration and invasion ability of the HCT116 cell line was significantly inhibited (P<0.01).

Twist promotes EMT in tumor cells in vitro. After transfection of the tumor cells for 48 h, fluorescence qRT-PCR results (Fig. 9A-C) and western blot results (Fig. 9D-F) showed higher mRNA transcription and protein expression of Twist and vimentin in the pTracer-CMV/BSD-Twist-transfected groups compared with those in the other groups (P<0.01).In addition, E-cadherin mRNA and protein expression levels were decreased (P<0.01) in all three colon cancer cell lines after Twist gene transfection. Correlation analysis showed that Twist and vimentin mRNA transcription and protein levels were positively correlated (P<0.01), while negatively correlated (P<0.01) with E-cadherin.

Silencing of the Twist gene by Twist-shRNA in the HCT116 cell line. After transfection of the tumor cells for 48 h, the qRT-PCR results (Fig. 9A-C) and western blot analysis (Fig. 9D-F) showed that in the SW480 and HT29 cell lines, the mRNA transcription and protein expression levels of Twist, vimentin and E-cadherin in the pGenesil1.2-Twist-shRNA groups were the same as the control groups (P>0.05). However, the mRNA transcription and

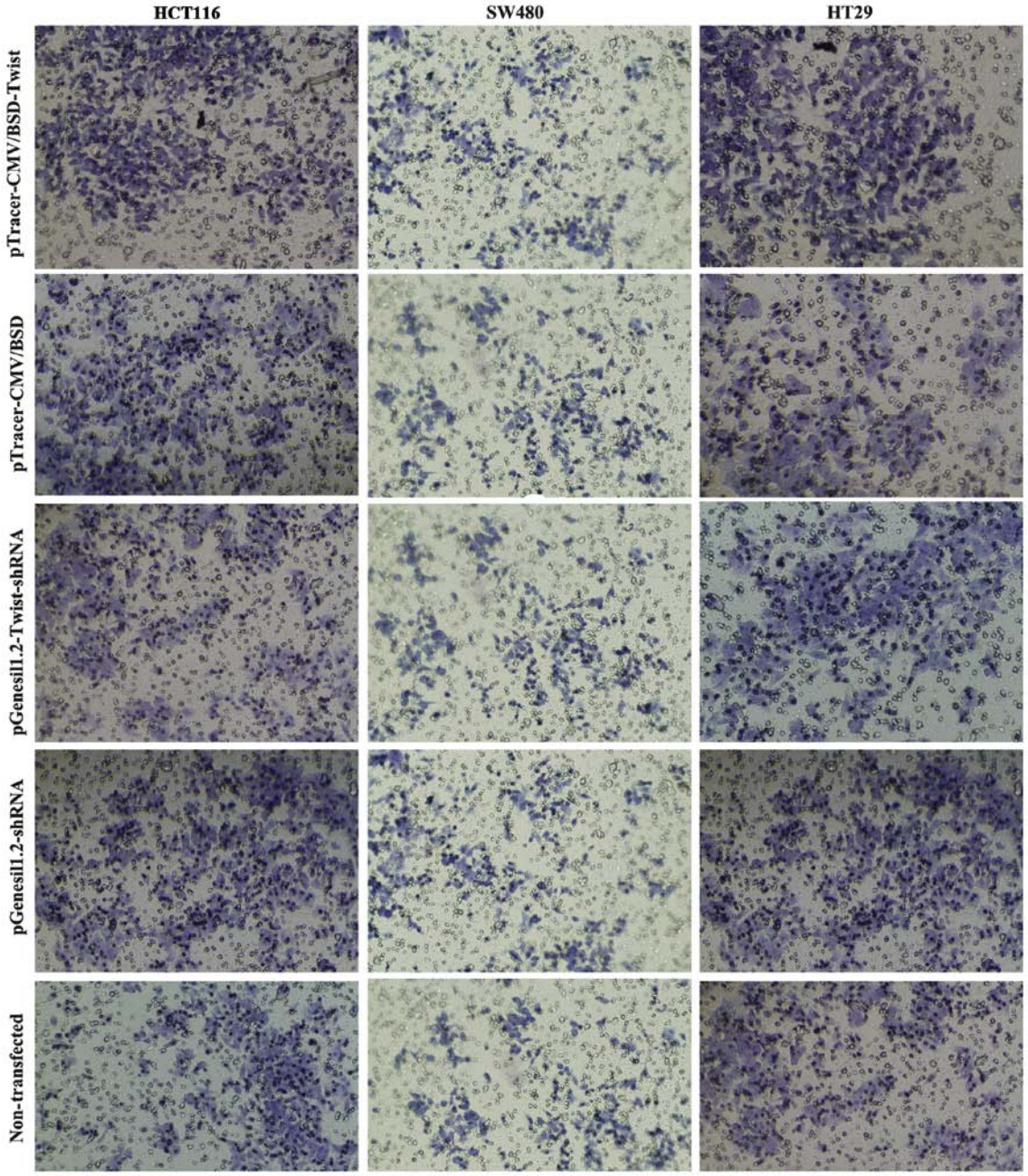

Figure 7. Transwell invasion assay showing the invaded colon cancer cells in the different groups (magnification, x100).

protein expression levels of Twist and vimentin were decreased only in the HCT116 cell line compared with those in the control groups ($P<0.01$); the difference in E-cadherin mRNA transcription and protein expression level was statistically insignificant ($P>0.05$) between the pGenesil1.2-Twist-shRNA transfected and control groups.

Successful construction of the xenogenic spontaneous heterotopic liver metastasis model. A spleen-to-liver colorectal cancer metastasis model was successfully constructed. On the 30th day of intrasplenic injection with SW480, HT29 and HCT116 cell lines, the animals were euthanized and examined for tumor growth (only the animals with primary tumor and secondary metastatic lesions were used for the experiment) (Fig. 10). H&E and immunohistochemical staining results of spleen and liver tumor tissue demonstrated the presence and location of colon cancer cells in the liver and spleen tissue sections (Figs. 11 and 12), which demonstrated the successful construction of the xenogenic spontaneous heterotopic liver metastasis model.

Experimental groups of the intrasplenic injection model. The experimental groups were characterized as follows: Tw, liver or spleen tissues in the model injected with pTracer-CMV/BSD-Twist plasmid-transfected cell lines (HCT116, SW480 and HT29); ShTw, liver or spleen tissues in the model injected with pGenesil1.2-Twist-shRNA plasmid-transfected cell lines; control, liver or spleen tissues in the model injected with non-transfected cancer cells; normal, normal tissue (liver and spleen).

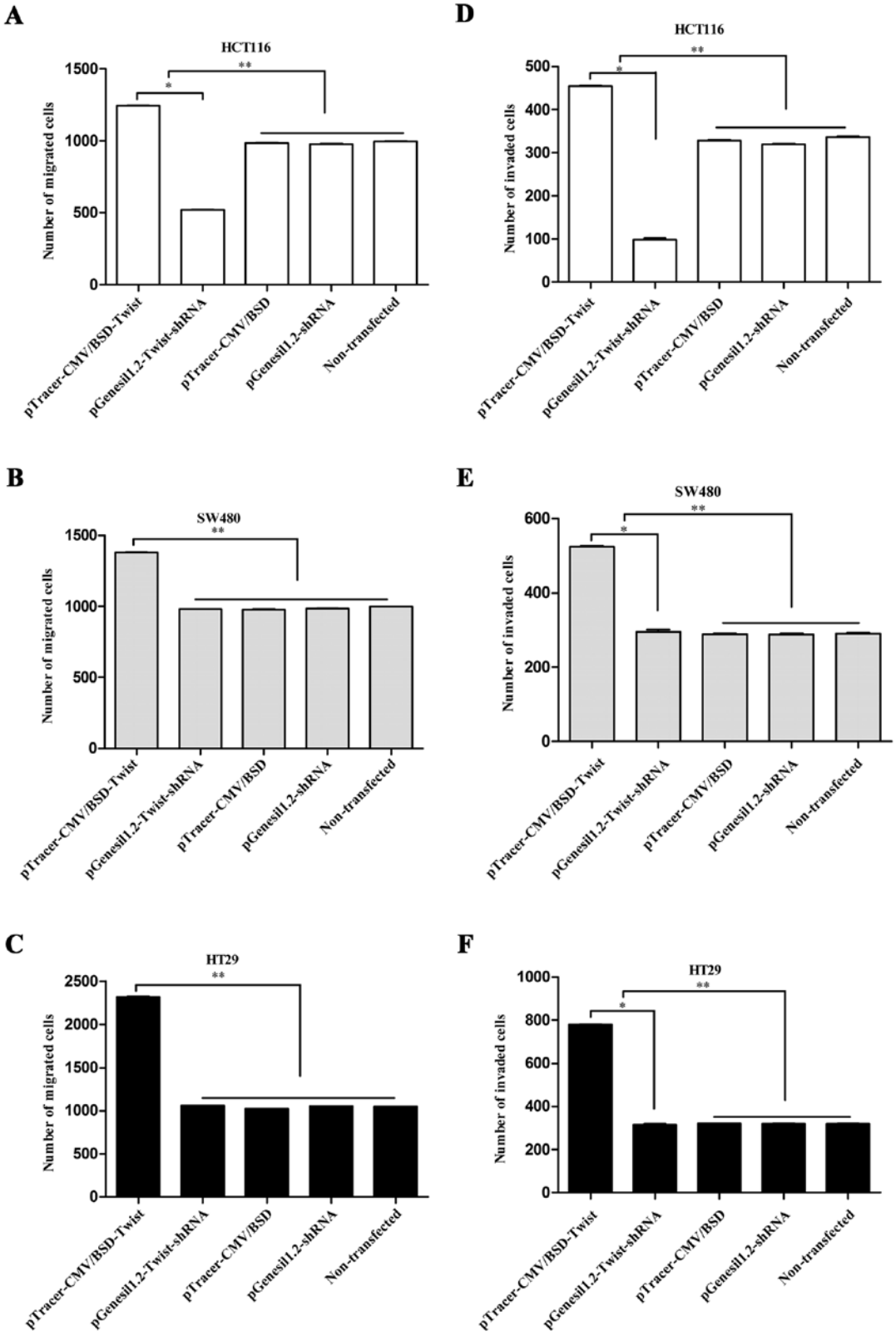

Figure 8. (A) The Transwell migrated number of HCT116 cells through the insert in the plasmid pTracer-CMV/BSD-Twist-transfected, plasmid pGenesil1.2-Twist-shRNA-transfected and non-transfected group was 1,245.45±2.32, 521.76±2.85 and 996.21±1.32. (B) The Transwell migrated number of SW480 cells through the insert in the plasmid pTracer-CMV/BSD-Twist-transfected, plasmid pGenesil1.2-Twist-shRNA-transfected and non-transfected group was 1,380.21±1.13, 979.43±1.42 and 998.54±1.32. (C) The Transwell migrated number of HT29 cells through the insert in the plasmid pTracer-CMV/BSD-Twist-transfected, plasmid pGenesil1.2-Twist-shRNA-transfected and non-transfected group was 2,320.32±1.10, 1,058.54±1.54 and 1,047.76±1.65. (D) The Transwell invaded number of HCT116 cells through the insert in the plasmid pTracer-CMV/BSD-Twist-transfected, plasmid pGenesil1.2-Twist-shRNA-transfected and non-transfected group was 454.24±1.55, 98.23±3.74 and 335.98±2.47. (E) The Transwell invaded number of SW480 cells through the insert in the plasmid pTracer-CMV/BSD-Twist-transfected, plasmid pGenesil1.2-Twist-shRNA-transfected and non-transfected group was 524.12±2.3, 295.12±6.3 and 290.76±2.4. (F) The Transwell invaded number of HT29 through the insert in plasmid pTracer-CMV/BSD-Twist transfected, plasmid pGenesil1.2-Twist-shRNA transfected and non-transfected group was 780.12±1.6, 315.32±4.5 and 319.89±1.5, respectively. The data obtained by counting the average numbers of cells from four different fields are represented as the means ± SEM (*P<0.05 and **P<0.01).

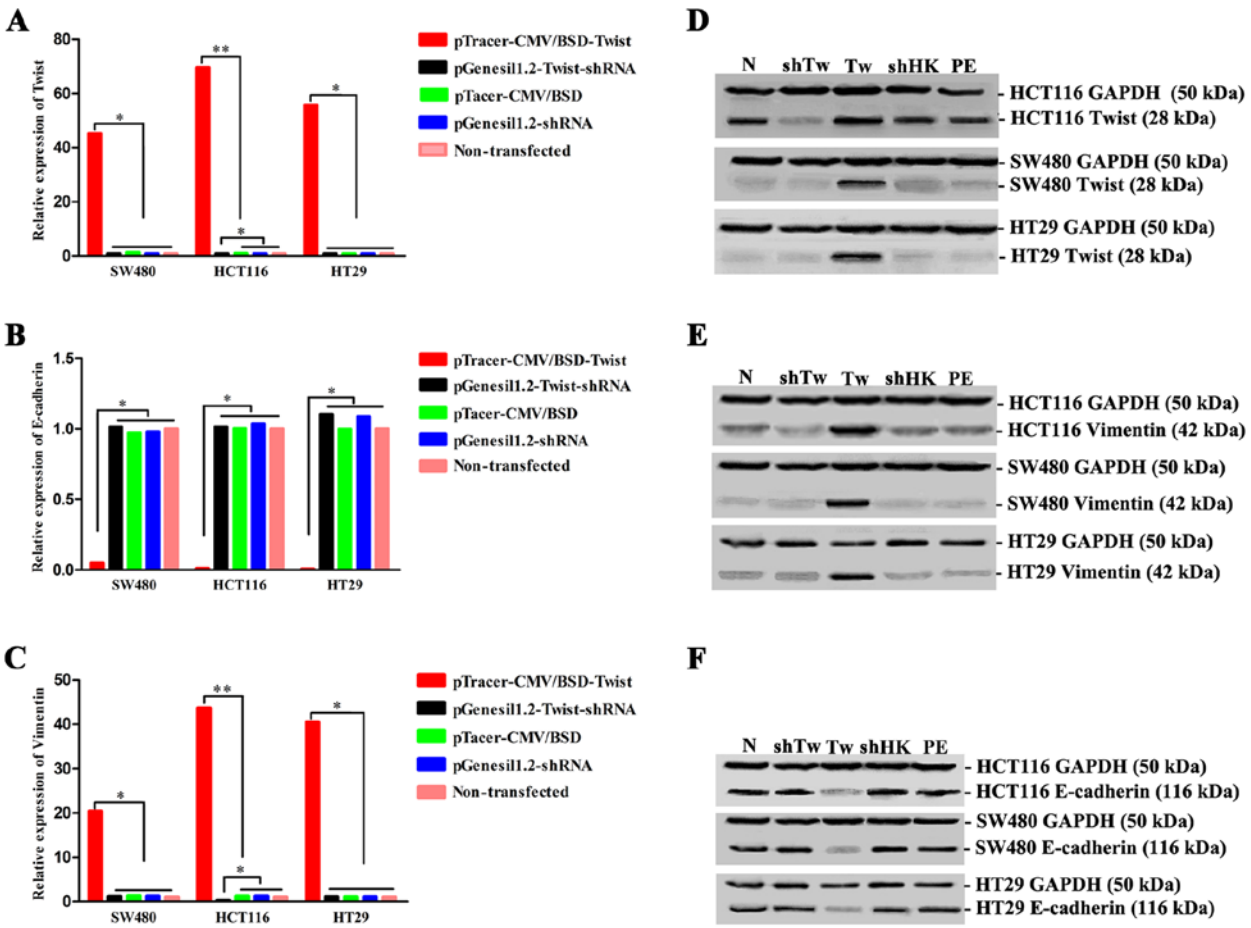

Figure 9. Twist promotes the process of EMT in tumor cells and the Twist gene was successfully silenced by Twist-shRNA in the HCT116 cell line *in vitro*. The fluorescence real-time PCR results detecting mRNA transcription level of (A) Twist, (B) E-cadherin and (C) vimentin in pTracer-CMV/BSD-Twist-, pGenesil1.2-Twist-shRNA-, pTracer-CMV/BSD-, pGenesil1.2-shRNA-transfected and non-transfected groups in three colon cancer cell lines. Protein expression of (D) Twist, (E) vimentin and (F) E-cadherin by western blot analysis in each transfection group in the three colon cancer cell lines (N, non-transfection group; ShTw, pGenesil1.2-Twist-shRNA-transfected group; Tw, pTracer-CMV/BSD-Twist-transfected group; ShHK, pGenesil1.2-shRNA-transfected group; and PE, pTracer-CMV/BSD-transfected group) (*P<0.05 and **P<0.01).

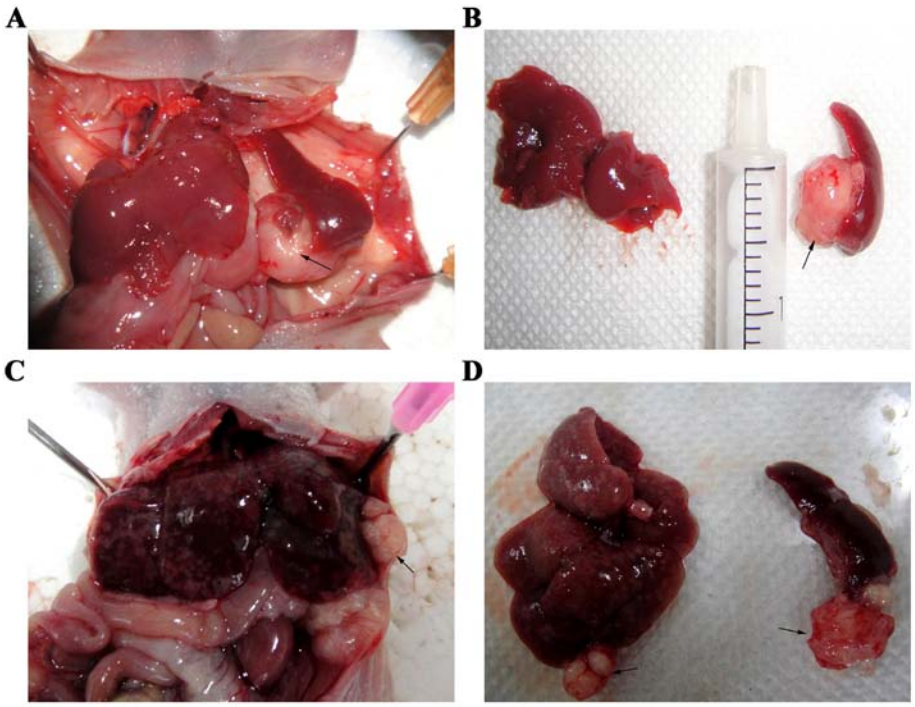

Figure 10. Tumor xenografts in nude mice. Tumor lesion on the (A and B) 20th and (C and D) 30th day after intrasplenic injection of colon cancer cells.

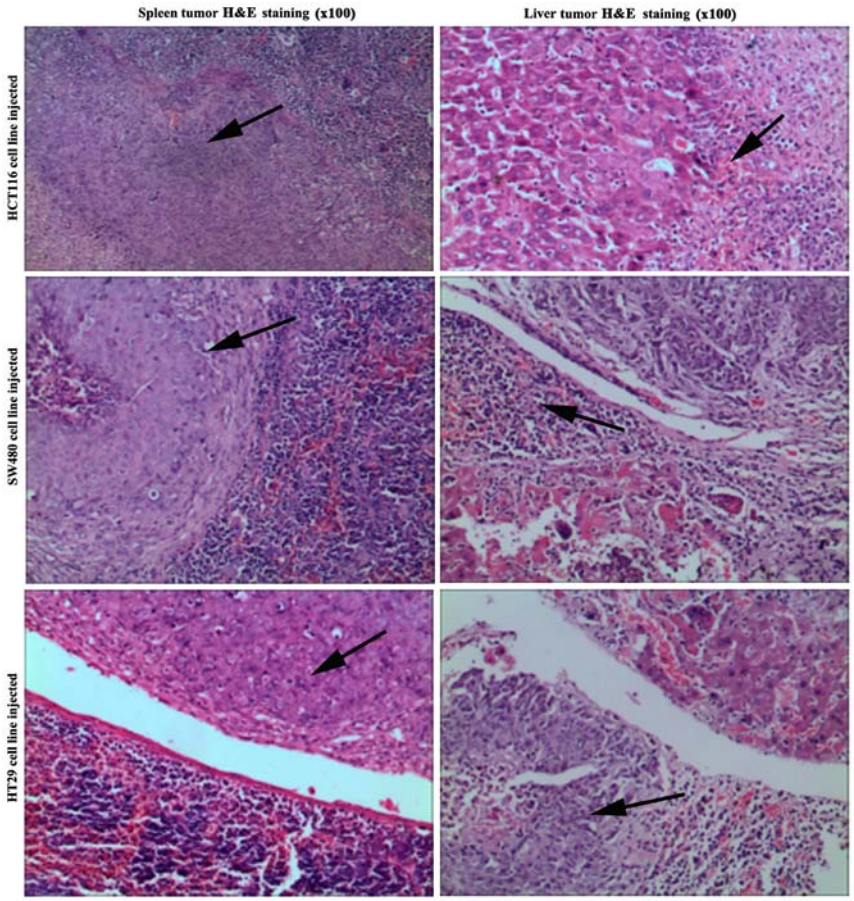

Figure 11. H&E staining results of spleen and liver tumor tissues (magnification, x100).

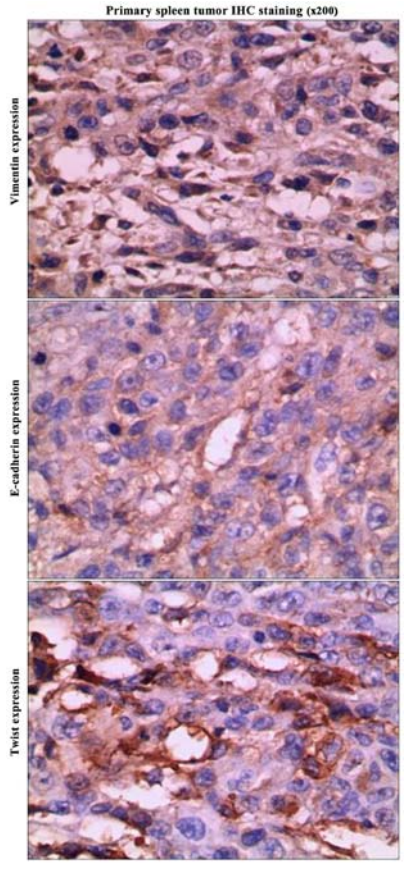

Figure 12. Immunohistochemical staining results of spleen tumor tissues (magnification, x200).

mRNA transcription level of Twist and vimentin in the liver and spleen tissues. Fluorescence qRT-PCR analysis (Fig. 13A and B) showed that Twist and vimentin mRNA transcription levels were higher in the liver than in the spleen of the model after injection of non-transfected cells ($P<0.05$). Twist and vimentin mRNA transcription levels in both the liver and spleen were higher in the model injected with the three cell lines transfected with pTracer-CMV/BSD-Twist (Tw) compared with the levels in the cells transfected with pGenesil1.2-Twist-shRNA (shTw) ($P<0.05$). However, compared with the liver and spleen tissues of the non-transfected cell groups, only the liver and spleen tissues of the model injected with plasmid pGenesil1.2-Twist-shRNA-transfected (shTw) HCT116 cells appeared to have a prominently inhibited mRNA transcription level of Twist (Fig. 13C and D) and vimentin (Fig. 13E and F).

Discussion

Colon cancer is one of the most common diseases worldwide, and its incidence is increasing (36,37). There are two biological processes that are essential for the progression of this disease, despite the diversity of etiology and pathophysiology. One of these processes is epithelial-mesenchymal transition (EMT), which has a significant role in the development of the human body, is involved in the onset and progression of colon cancer, and has an important role in tissue fibrosis (38-42). Cells that experience EMT show many morphologic and characteristic changes, such as the loss of cell adhesion, repression of

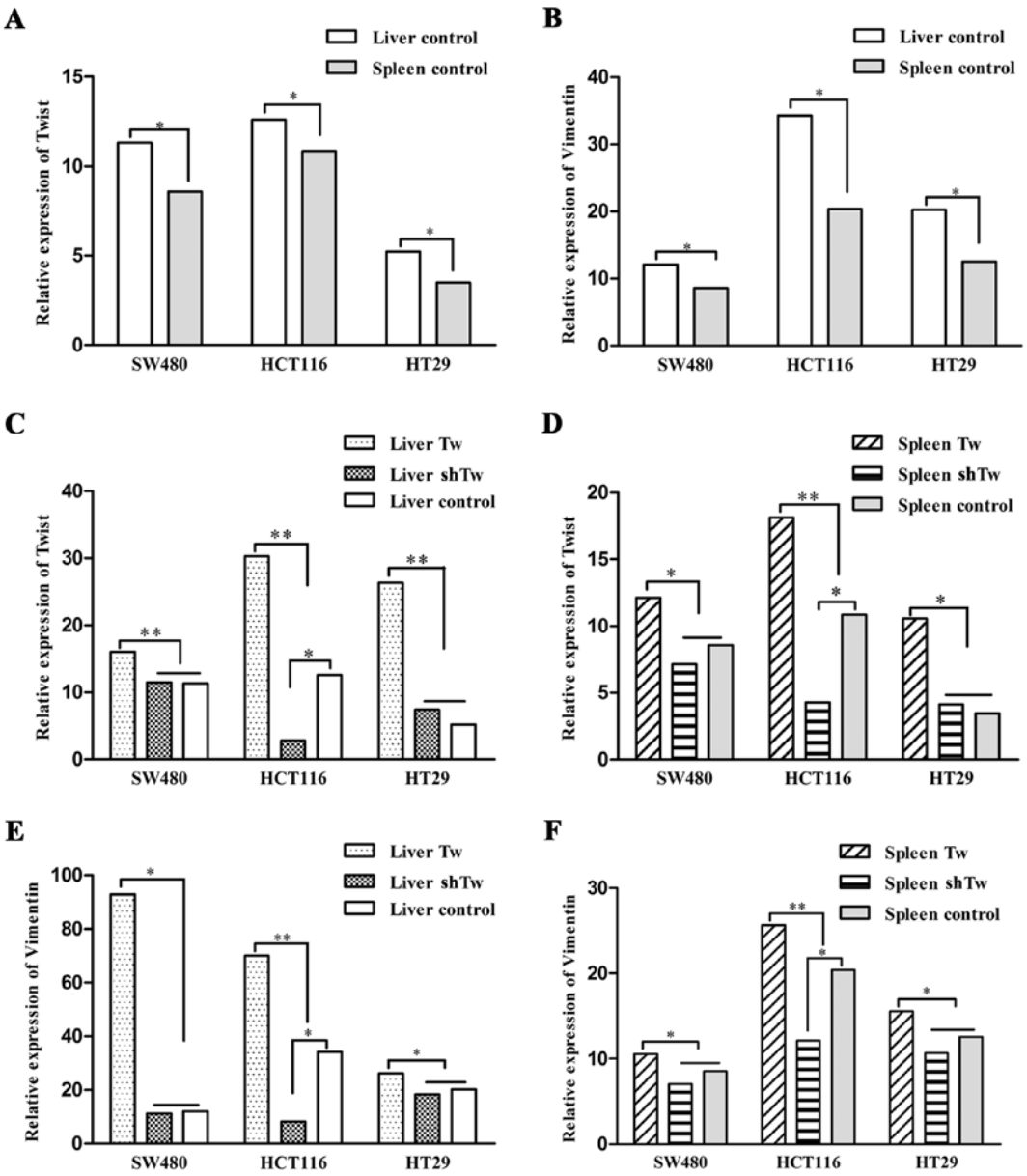

Figure 13. RT-qPCR results of Twist and vimentin gene in the five experimental groups *in vivo*. The mRNA expression levels of (A) Twist and (B) vimentin in the liver or spleen tissues inthe model injected with non-transfected cancer cells. The mRNA expression levels of (C) Twist and (E) vimentin in liver tissue in the model injected with the cell lines transfected with the different plasmids. The mRNA expression levels of (D) Twist and (F) vimentin in spleen tissue in the model injected with the cell lines transfected with the different plasmids. Tw, model injected with pTracer-CMV/BSD-Twist plasmid-transfected cell lines (HCT116, SW480 and HT29); ShTw, model injected with pGenesil1.2-Twist-shRNA plasmid-transfected cell lines; control, model injected with non-transfected cancer cells ($^{*}P<0.05$ and $^{**}P<0.01$).

E-cadherin and increased cell motility (43,44). At the same time, activation or elevated expression of tyrosine kinases, upregulation of N-cadherin, vimentin, fibronectin, zinc-finger domain proteins and basic helix-loop helix domain protein Twist1 expression are all related to a mesenchymal-like phenotype (45-48). In basic research, these proteins are often used as markers for EMT, indicating strong motility and invasiveness of the cells. *In vitro* and *in vivo* evidence suggests that EMT in primary invasive breast, lung, prostate, liver and pancreatic cancer, and other cancers and secondary transfer plays an important role. The other process closely related to normal tissue and organ development is a reverse phenomenon, called the mesenchymal-epithelial transition (MET). MET is also involved in the initial step of colorectal carcinogenesis. Research shows that it may play an important role in the regeneration of colonic mucosa (49,50). Currently, the induction and regulation of these complex, reversible biological processes is not fully understood. The significant influence of EMT and MET in treatment is widely recognized; however, the exact description of these biological phenomena is still unclear. Although the understanding of the mechanisms and developmental processes of EMT and MET have great clinical importance, data on their role in the induction and regulation, as well as their role in the pathogenesis of colonic diseases, are scarce in the scientific literature. Therefore, the present study selected the Twist gene, which may have a close relationship to tumor progression and metastasis. We evaluated Twist expression and EMT markers (E-cadherin, vimentin) after

induction and inhibition (knockdown) of the Twist gene in colon cancer cell lines by transfection of the plasmids pTracer-CMV/BSD-Twist and pGenesil-Twist-shRNA, respectively. In the *in vitro* study, after we transfected human colon cancer cell lines SW480, HCT116 and HT29 with pTracer-CMV/BSD-Twist and pGenesil1.2-Twist-shRNA, we found differences in Twist expression in all the cell lines transfected with the Twist over-expression and silencing plasmids. Expression of Twist was higher in the HCT116 cells in comparison to the SW480 and HT29 cells. Upregulation of Twist gene expression in the three cell lines induced high expression of vimentin molecules and low expression of E-cadherin molecules, hence promoting the EMT-enhanced metastatic ability of the tumor cells (48,51,52). Twist-shRNA effectively silenced Twist gene expression in the HCT116 cell line, inhibiting vimentin expression, reversing epithelial-mesenchymal transition, promoting mesenchymal-epithelial transition, and effectively inhibiting colon cancer cell migration and invasion. The mRNA and protein expression analyses showed that Twist expression was negatively correlated ($P<0.01$) with E-cadherin expression where it was positively correlated ($P<0.01$) with vimentin expression. The Transwell migration and invasion assays showed an increased capability for invasion in the three cell lines transfected with the Twist over-expression plasmid. However, only HCT116 showed decreased invasion capability after transfection with Twist-shRNA. Thus, the results confirmed that the Twist gene plays a vital role during the processes of cell EMT, epithelial invasion and metastasis.

One of the most typical features of cancer is metastatic progression. The process of metastasis is not only related to the characteristics of the primary tumor cells but is also related to the tumor microenvironment, which usually presents as extreme conditions, such as hypoxia and acidosis, elevated tumor interstitial fluid pressure, extracellular matrix and isolation from the host organ by stromal barriers (53). Moreover, the progression of metastases also depends on the ability of target tissues and stromal cells to cause metastasis (54). Injecting cancer cells directly into the liver parenchyma (53) or portal vein (55) has been used to induce tumors in the liver. However, neither approach allows for selection of metastatic cells from injected cells, which may be the potential reason for the variation in the number of mice that develop an intrahepatic tumor in this type of experimental model (56). Previous studies have described the implantation of a primary tumor into the cecum (55) or spleen (56) subsequent release into the portal system. The main advantage of such a model is that only some animals develop disseminated tumors and/or at different locations of primary or metastatic tumors, which leads to an increase in the number of experimental animals needed. There is evidence to support that when tumor cells are implanted in the spleen, more uniform tumor development is achieved compared with direct injection of the same cell line into the portal vein, which demonstrates the importance of metastatic selection of the cells (57). Some metastatic animal models are available for research and therapeutic development. Metastatic cells can easily spread from splenic tumors to the liver through the portal system, which however may also directly mediate implantation of the cells in hepatic tissue after intrasplenic injection. Thus, it is important to distinguish these events in the course of study. Nude mice present with hepatic tumors in 100% of the mice used with regard to liver metastases originating from primary spleen tumors (58). The spleen is a suitable injection site with low-mortality surgery, while the portal system is the most similar to the clinical situation, which may be the most relevant route for the dissemination of tumor cells into the liver. In addition, the progression of liver metastases cannot be tracked by external observation, so a model that can be used to assess the spread of the disease at its early stage is what we needed. Comprehensively considering the above, the spleen-to-liver colorectal cancer model established in nude mice was used in our research to study the expression of Twist in primary tumors (in spleen) and metastatic tumors (in liver), to reveal the relationship between the Twist gene and colon cancer progression and metastasis. We found that Twist expression was much higher in the metastatic lesions than in the primary tumor. The high expression of Twist in the liver may indicate that the invasiveness and metastatic potential of the cancer cells remained high. RNA interference of the Twist gene effectively silenced Twist gene expression in the spleen and liver in mice injected with the HCT116 cell line, which suggests that the expression status of the EMT-related transcription factor Twist may play an important role in the implantation of metastatic foci.

In summary, from the experimental findings of the present study, we conclude that upregulation of the Twist gene promotes EMT molecular events and enhances the metastatic ability of tumor cells, while Twist-shRNA effectively silences Twist gene expression in the HCT116 cell line, promoting mesenchymal-epithelial transition and effectively inhibiting colon cancer cell migration and invasion. Activation and deactivation of Twist may be used as a prognostic factor and as a cancer therapeutic target.

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