ORIGINAL ARTICLE

Combination of Evodiamine with Berberine Reveals a Regulatory Effect on the Phenotypic Transition of Colon Epithelial Cells Induced by CCD-18Co

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Objective Transdifferentiation exists between stromal cells or between stromal cells and cancer cells. Evodiamine and berberine are predominant pharmacological components of *Zuojin* pill, a prescription of Traditional Chinese Medicine, playing crucial functions in remodeling of tumor microenvironment. This study aimed to explore the effect of combination of evodiamine with berberine (cBerEvo) on the phenotypic transition of colon epithelial cells induced by tumor-associated fibroblasts, as well as the involved mechanisms.

Methods Human normal colon epithelial cell line HCoEpiC cells were treated with the prepared conditioned medium of CCD-18Co, a human colon myofibroblast line, to induce epithelial-mesenchymal transition. Phase contrast microscope was used to observe the morphological changes. Epithelial-mesenchymal transition markers including E-cadherin, vimentin and alpha-smooth muscle actin (α -SMA) were observed with immunofluorescence microscopy. Migration was assessed by wound healing assay. Western blotting was used to detect the expressions of E-cadherin, vimentin, α -SMA, Snail, ZEB1 and Smads.

Results In contrast to the control, the tumor-associated fibroblasts-like CCD-18Co cells induced down-regulation of E-cadherin and up-regulation of vimentin, α -SMA, Snail and ZEB1 ($P < 0.05$), and promoted

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migration of HCoEpiCs ($P < 0.05$), with over expression of Smads including Smad2, p-Smad2, Smad3, p-Smad3 and Smad4 ($P < 0.05$), which were abolished by a transforming growth factor- β (TGF- β) receptor inhibitor LY364947 and by cBerEvo in a concentration dependent manner. In addition, cBerEvo-inhibited ratios of p-Smad2/Smad2 and p-Smad3/Smad3 were also dose dependent.

Conclusion The above results suggest that cBerEvo can regulate the differentiation of colon epithelial cells induced by CCD-18Co through suppressing activity of TGF- β /Smads signaling pathway.

BOTH of the cancer cells and the fibroblasts which reside in human solid neoplasm stroma can differentiate into cancer-associated fibroblasts (CAFs) or tumor-associated fibroblasts (TAFs), resulting in modification of the extracellular matrix. These TAFs or CAFs reveal many characteristics of myofibroblasts, such as cell contraction and expression of alpha-smooth muscle actin (α -SMA). Therefore, these cells are also named as myofibroblasts. TAFs or CAFs can produce multiple growth factors and chemokines as the metabolism changes in solid neoplasms to provide an optimal tumor-supporting microenvironment for cancer development.^[1] In this situation, other stromal cells, such as epithelial cells, bone marrow-derived mesenchymal stem cells and perithelial cells, may be likely induced by these growth factors or chemokines and transformed into members of TAFs or CAFs pools through cell transdifferentiation. Substantial studies have revealed that transdifferentiation extensively exists between stromal cells in tumor microenvironment.^[2, 3] Transforming growth factor- β (TGF- β), a key inducer of transdifferentiation of epithelial cells, is the primary family member of secretory factors produced by TAFs or CAFs,^[4] and has been extensively proved the participation in occurrence and progression of tumor through induction of epithelial-mesenchymal transition (EMT).^[5, 6] Orchestration of TGF- β signaling may be a promising strategy to prevent the phenotypic transition of colon epithelium caused by TAFs or CAFs.

Zuojin pill just consists of *Rhizoma Coptidis* and *Evodia rutaecarpa*, two classical Chinese Medicines, in a ratio of 6:1 (w/w). As a classical prescription of Traditional Chinese Medicine, *Zuojin* pill has been reported to have significant clinical efficacy in treatment of gastrointestinal diseases since the 15th century.^[7] Berberine which is an isoquinoline alkaloids derived from *Rhizoma Coptidis* and evodiamine which is an indole alkaloid from *Evodia rutaecarpa*, both exhibit several potent pharmacological activities including anti-inflammatory and anti-tumor effects. Our pre-

vious studies have demonstrated that berberine or evodiamine alone inhibited the EMT of colon epithelial cell HCoEpiCs induced by TAF-like CCD-18Co or colon cancer cell SW480.^[8, 9] However, the effect of combination of berberine and evodiamine (cBerEvo) and the underlying mechanisms are still unclear, thus, our present study aimed to explore this issue by preparing TAF-associated conditioned medium for establishing an *in vitro* EMT model.

MATERIALS AND METHODS

Reagents and antibodies

Berberine (ab142117; purity > 98%; formula: $C_{20}H_{18}ClNO_4$; formula weight: 371.81; structure sees **Figure 1A**), evodiamine (ab142427; purity > 98%; formula: $C_{19}H_{17}N_3O$; formula weight: 303.36; structure sees **Figure 1B**), and a TGF- β receptor inhibitor LY364947 (ab141890; purity > 98%; formula: $C_{17}H_{12}N_4$; formula weight: 272.30; structure sees **Figure 1C**), were purchased from Abcam (Science Park, Cambridge, England), respectively dissolved in DMSO at 5 and 10, 100 mmol/L, and stored at -20°C .

DMEM medium with high glucose (4.5 g/L D-glucose), RPMI-1640, fetal bovine serum (FBS), streptomycin and penicillin were purchased from Thermo Fisher/Gibco (8117280), Life Technologies/Gibco

Figure 1. Chemical structural formula for berberine (A), evodiamine (B), and LY364947 (C).

(1830909), Life Technologies/Gibco (1861242), and Solarbio (20171222), respectively. The anti- α -SMA (ab124964), -vimentin (ab92547), -ZEB1 (ab203829), -Snail (ab216347) antibodies were purchased from Abcam. The anti-E-cadherin antibody was the product of Beyotime Biotechnology (AF0138, China). The antibodies against Smad2 (AF1300), Smad3 (AF1501), p-Smad2 (AF2545), p-Smad3 (AF1759), Smad4 (AF1291) were purchased from Beyotime (China). FITC-labelled goat anti-rabbit IgG (H+L) (A0562), Alexa Fluor 488 (AF488)-labelled goat anti-mouse IgG (H+L) (A0428), HRP-labelled goat anti-mouse IgG (H+L) (A0216), and HRP-labelled goat anti-rabbit IgG (H+L) (A0208) were purchased from Beyotime Biotechnology.

Cell culture and maintenance

The human normal colon epithelial cell line HCoEpiC (American Type Culture Collection, ATCC) and human colon myofibroblast line CCD-18Co (ATCC) were purchased from Guangzhou Jennio Biotech Co., Ltd. (Guangzhou, Guangdong, China) and Guandao Biotech Co., Ltd. (Shanghai, China). The HCoEpiCs were maintained in complete RPMI 1640 supplemented with 10% FBS. The CCD-18Co cells were maintained in DMEM supplemented with 10% FBS. HCoEpiCs and CCD-18Co were cultured at 37°C in a humidified atmosphere with 5% CO₂.

Preparation of CCD-18Co conditioned medium and induction

CCD-18Co conditioned medium (CM) was prepared as previously described.^[9] The prepared CM was diluted to 25% (v/v) with free-serum RPMI 1640. Then, HCoEpiCs were treated by 25% CM with or without LY364947/cBerEvo for 24 h. The different concentrations of cBerEvo, including 25 μ g/ml berberine+5 μ g/ml evodiamine [named as low-cBerEvo (L-cBerEvo)], 50 μ g/ml berberine+50 μ g/ml evodiamine [moderate-cBerEvo (M-cBerEvo)], 100 μ g/ml berberine+100 μ g/ml evodiamine [high-cBerEvo (H-cBerEvo)], were used according to previous results of our study. The HCoEpiCs cells treated with free-serum RPMI 1640 medium without CM were as the Control. The morphological observations of HCoEpiCs were performed by phase contrast microscope (Olympus).

Immunofluorescence microscopy

To perform an immunofluorescence analysis, the

HCoEpiCs plated in dishes (2 $\times$ 10⁵ cells/dish) were treated with the above conditions and cultured for 24 h at 37°C. The cells were washed with phosphate buffer solution (PBS, pH 7.2-7.4) for three times, followed by fixation with 4% paraformaldehyde, permeabilization with 0.5% Triton X-100 and blocking with 10% sheep serum. The cells were incubated with primary antibodies of E-cadherin (1:50), vimentin (1:100) and α -SMA (1:100) (EMT markers) for 2 h at 37°C, followed by staining with AF488-conjugated goat-anti-mouse IgG (for E-cadherin) and FITC-conjugated goat-anti-rabbit IgG (for vimentin and α -SMA) for 1 h at 37°C. Counterstaining with 4', 6-diamidino-2-phenylindole (DAPI) solution was carried out to show the nuclei. Immunofluorescence observations of E-cadherin, vimentin and α -SMA were performed using a fluorescence microscope (Olympus) equipped with a 960S-Fluor oil immersion lens. Capture of fluorescence images and calculation of the mean fluorescence intensity of each image were performed by Image-Pro®Plus v6.0 (Media Cybernetics, USA).

Western blot analysis

Our western blotting was performed as previously described.^[10] Briefly, protein sample preparation was established using RIPA lysate, followed by separation of total proteins with SDS-PAGE and electro-transfer of the separated proteins to PVDF membranes. The membranes were blocked with Tris-buffered saline Tween (TBST) buffer (0.1% Tween-20) containing 10% non-fat milk at 25°C for 2 h, and incubated with the antibodies against E-cadherin (1:500), vimentin (1:500) and α -SMA (1:1000), ZEB1 (1:500), Snail (1:500), Smads (1:500) overnight at 4°C. Then, incubation of HRP-conjugated secondary antibodies were performed at 25°C for 2 h. All protein bands were acquired and the intensities were analyzed using Molecular Imager® Chemi Doc™ XR+ system with Image Lab™ Software (Bio-Rad).

Wound healing assay

When the HCoEpiC cells which were assigned to "+" type, grown in 35-mm dishes, were converged to 80%-90%, a wound of the cell layer was performed by using a sterile 200- μ l pipette tip, followed by PBS washing. The cells were then exposed to CM or CM combined with 5 μ g/ml LY364947 or with CM+cBerEvo of the indicated concentrations. The changes of wound area were observed by a phase contrast microscope

at 0, 6, 12, 18, and 24 h, and the wound images were captured. To measure and quantify the degree of healing, an image analysis software Image J (National Institutes of Health, USA) was adopted.

Statistical analysis

All the data were analyzed with statistical software SPSS 20.0, and expressed as mean±standard deviation (SD). One-way analysis of variance (ANOVA) was used to analyze the difference among multiple groups. Multiple comparisons between groups were performed by LSD. *P*-values < 0.05 were considered to be statistically significant.

RESULTS

cBerEvo inhibited the CCD-18Co-induced EMT-like morphological changes in colon epithelial cell HCoEpiCs

In the present study, we found that TAF-like CCD-18Co-derived CM induced the spindle-like morphological changes of HCoEpiCs, which was abolished by a TGF- β receptor inhibitor LY364947 (**Figure 2**). Moreover, the CCD-18Co-stimulated EMT changes were also repressed by cBerEvo in a concentration-dependent

manner (**Figure 2**).

cBerEvo mediated the expression of CCD-18Co-induced EMT biomarkers and relative transcription factors in HCoEpiCs

One of the remarkable hallmarks of EMT occurrence are loss of epithelial markers including adhesion molecule E-cadherin and tight junction protein ZO-1, and acquisition of mesenchymal markers including cytoskeletal protein α -SMA, vimentin or N-cadherin.^[11] As shown in **Figure 3**, the fluorescence intensity of E-cadherin (0.0986 ± 0.0084 vs. 0.2258 ± 0.0231 , $P=0.001$) was significantly weakened and that of vimentin (0.5462 ± 0.0248 vs. 0.0246 ± 0.0065 , $P=0.001$) and α -SMA (0.5634 ± 0.0247 vs. 0.0984 ± 0.0081 , $P=0.001$) was significantly enhanced in HCoEpiCs with the stimulation of CCD-18Co CM, comparing with the control. These phenomena were reversed by LY364947 and cBerEvo. After the treatment of cBerEvo, up-regulation of E-cadherin was significant in the M- and H-cBerEvo group (0.2195 ± 0.0487 and 0.2243 ± 0.0715) compared with the CCD-18Co CM group ($P=0.001$), while L-cBerEvo showed no significantly stimulating effect (0.0897 ± 0.0077 , $P=0.845$, **Figure 3**). As for the vimentin and α -SMA expression, the down-reg-

Figure 2. Morphological observation of HCoEpiCs with phase contrast microscope.

The HCoEpiCs were treated by 25% CCD-18Co-derived conditioned medium (CM) for 24 h, and the LY364947 was 5 mg/ml. L-cBerEvo: low concentration-cBerEvo; M-cBerEvo: moderate concentration-cBerEvo; H-cBerEvo: high concentration-cBerEvo.

ulated effect of cBerEvo was dose-dependent, which were consistent with the detection of western blotting (**Figure 4**).

Another characteristic of EMT is induction of EMT transcription factors. Consistent with the changes of the above EMT markers, CCD-18Co CM promoted over-expression of ZEB1 (0.8161 ± 0.0628 vs. 0.4483 ± 0.0502 , $P=0.001$) and Snail (0.5209 ± 0.0421 vs. 0.0599 ± 0.0106 , $P=0.001$), comparing to the control (**Figure 4**), which was abolished by LY364947 (0.6568 ± 0.0905 and 0.1829 ± 0.0724 , $P=0.001$). The treatment of cBerEvo inhibited their expressions and this effect was dose-concentration dependent (**Figure 4**).

cBerEvo repressed the CCD-18Co-induced migration of HCoEpiCs

Enhanced migration is the result of the cells undergoing EMT. The above results revealed that cBerEvo regulated the EMT of the HCoEpiCs induced by CCD-18Co. We hypothesized that cBerEvo could also mediate the migration. Previous study has demonstrated that the berberine and evodiamine-loaded mesoporous silica nanoparticles showed excellent synergistic therapy effects *in vitro* including cytotoxicity, cell migration and invasion, angiogenesis, and *in vivo* including growth of tumor grafts in mice.^[12] In the present study, we observed that CCD-18Co CM obviously promoted the migration of HCoEpiCs at 18 h (4652 ± 1485 pixel/cm²), in contrast to the control (14386 ± 2011 pixel/cm², $P=0.001$) and LY364947 treated cells (9964 ± 252 pixel/cm², $P=0.001$, **Figure 5**). Besides, cBerEvo significantly repressed the migration induced by the CM, and this effect was time- and dose-dependent (**Figure 5**).

cBerEvo inactivated the CCD-18Co-induced TGF- β /Smads signaling in HCoEpiCs

As proved in substantial evidence, TGF- β signaling plays key functions in the induction and maintenance of EMT. Therefore, activated TGF- β signaling stimulated the EMT induction of colon epithelial cells treated with CCD-18Co CM. The above results have suggested that TGF- β receptor inhibitor LY364947 abolished the EMT changes and migration of HCoEpiCs induced by CCD-18Co CM. However, we wondered whether or not the TGF- β signaling was activated in the process. As shown in **Figure 6**, over-expression of Smad proteins that involve in TGF- β signaling was observed during the EMT induction. CCD-18Co CM promoted the expres-

sions of Smad2 (0.8914 ± 0.0542 vs. 0.5350 ± 0.0321 , $P=0.001$) and p-Smad2 (0.4938 ± 0.0258 vs. 0.1649 ± 0.0089 , $P=0.001$), Smad3 (0.3916 ± 0.0212 vs. 0.1514 ± 0.0185 , $P=0.001$) and p-Smad3 (0.5446 ± 0.0526 vs. 0.2313 ± 0.0475 , $P=0.001$) and Smad4 (0.4974 ± 0.0521 vs. 0.1094 ± 0.0151 , $P=0.001$), versus the control cells, which was reversed by LY364947 (0.7339 ± 0.0324 , 0.1777 ± 0.0215 , 0.1728 ± 0.0582 , 0.2633 ± 0.0482 , 0.1514 ± 0.0185 , respectively, $P<0.05$, **Figure 6A**). In addition, CCD-18Co-derived CM improved the ratio of p-Smad2/Smad2 (0.9539 ± 0.0642 vs. 0.3082 ± 0.0148 , $P=0.001$) or p-Smad3/Smad3 (1.3907 ± 0.0554 vs. 0.6277 ± 0.1014 , $P=0.001$, **Figure 6B**) compared with the control. These effects of LY364947 were similar to those of cBerEvo. However, the influence of treatment of cBerEvo was different in concentration. Although inhibitory effect of L-cBerEvo on Smad2 expression was the most obvious (0.2472 ± 0.0421 , $P=0.001$), followed by M-cBerEvo (0.4116 ± 0.0213 , $P=0.001$, **Figure 6A**), the effect on Smad3 was the most significant in the M-cBerEvo group (0.1887 ± 0.0168 , $P=0.001$). cBerEvo-inhibited p-Smad2 expression was not associated with the concentration, but the repressive influence on the p-Smad3 and Smad4 expression was dose-dependent, in contrast to the CM group ($P<0.05$). Interestingly, cBerEvo decreased the ratios of p-Smad2/Smad2 and p-Smad3/Smad3 in a dose-dependent manner, comparing with the CM group ($P<0.05$, **Figure 6B**). These results suggest that cBerEvo inhibits the EMT of HCoEpiCs through mediating the CCD-18Co CM-stimulated hyperactivity of TGF- β /Smads signaling.

DISCUSSION

Recently, researches about the interaction between tumor cells and surrounding microenvironment are hot fields. Targeting tumor microenvironment has become a promising strategy for prevention and treatment of cancer.^[13] Most traditional cancer therapies mainly focus on single component in microenvironment. Although newly developed drugs maybe have multiple targets, more adverse reactions are also followed, leading to dissatisfied effects. Surprisingly, Traditional Chinese Medicine (TCM) plays close attention to the overall efficacy. TCM not only kills or inhibits the cancer cells, but also mediates the stromal components, thus relieving clinical symptoms, improving survival rates, reducing toxic effects of chemotherapy

Figure 3. Fluorescent detection of epithelial-mesenchymal transition (EMT) markers including E-cadherin, alpha-smooth muscle actin (α -SMA) and vimentin using a fluorescence microscope (Olympus) equipped with a 960S-Fluor oil immersion lens (1000 $\times$). The cell nuclei was counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (A). Fluorescence intensity comparison of the above EMT markers (B). The error bar represents the SD ($n = 3$). * $P < 0.05$ versus the control, ** $P < 0.05$ versus the CCD-18Co-CM, # $P < 0.05$ versus the CM+L-cBerEvo (ANOVA).

Figure 4. Detection of EMT markers and transcription factors including ZEB1 and Snail using western blotting.

The error bar represents the SD ($n=3$). * $P < 0.05$ versus the control, ** $P < 0.05$ versus the CCD-18Co CM, # $P < 0.05$ versus the CM+L-cBerEvo (ANOVA).

or radiotherapy, and further delaying or blocking tumor progression and recurrence.^[14-18] However, detailed mechanisms of the most TCM are still obscure. Some herb medicines have been reported to inhibit many origination and metastasis of cancer stem cells through abolishing EMT.^[19]

Zuojin pill, a famous prescription of TCM, consists of *Coptis chinensis* and *Evodia rutaecarpa*. *Zuojin* pill has been reported to treat digestive system conditions including gastritis, gastric ulcer and hepatic diseases since the 15th century.^[7] A recent report has demonstrated that *Zuojin* pill and its constituents suppressed

AP-1 and NF- κ B activities, resulting in inhibition of anchorage-independent growth in HepG2 cells, which are consistent with that of the major alkaloidal ingredients in *Zuojin* pill including berberine and evodiamine.^[20] The findings suggest that berberine and evodiamine may be the primary pharmacological basis for the functions of *Zuojin* pill. Our previous study has revealed that *Zuojin* pill-containing serum of rats inhibited the *in vitro* EMT changes of colon epithelial cells HCoEpiCs induced by TAF-like CCD-18Co-derived CM in a dose-dependent manner (Data has been published in monograph "Herbal Medicine"^[21]). In addition, our

Figure 5. Analysis of effects of CM and cBerEvo and LY364947 (LY) on HCoEpiC cells motility by wound-healing assay.

A. Area of wound in the HCoEpiC cells is observed by phase contrast microscopy (10×10). B. Analysis of wound area by Image J, and the area of wound is presented by pixels/cm². The error bar represents the SD (n=3). **P*<0.05 versus the control, ***P*<0.05 versus the CCD-18Co CM, #*P*<0.05 versus the CM+L-cBerEvo.

previous research has demonstrated that the alone effects of berberine and evodiamine existed in the TAF-induced EMT-like changes, and these were concen-

tration-associated.^[9, 22] However, the effect of cBerEvo is unclear, and whether this effect is associated with their concentration is also obscure. Therefore, we per-

Figure 6. Detection of main components in Smad signaling by western blotting.

A. Protein bands analysis of western blot in the HCoEpiC cells treated by different conditions. B. Analysis of the ratios of p-Smad2/Smad2 and p-Smad3/Smad3 in these groups. The error bar represents the SD ($n=3$). * $P<0.05$ versus the Control, ** $P<0.05$ versus the CCD-18Co CM, # $P<0.05$ versus the CM+L-cBerEvo (ANOVA).

formed the present study. We speculated that cBerEvo plays an important role in the suppressive effects of *Zuojin* pill.

As a matter of fact, berberine and evodiamine have been reported to have the synergistic effects on multiple human cancer cells including breast and liver by induction of cell cycle arrest and apoptosis.^[23-25] Our previous study has demonstrated that berberine and evodiamine down-regulated oncogene miRNA-429 expression in human colorectal cancer.^[26] Recently, we found that berberine inhibited the *in vitro* EMT changes induced by colon cancer cell SW480,^[8] and that evodiamine suppressed the TAF-like CCD-18Co cells-stimulated EMT of colon epithelial cells.^[9] However, the previous studies only explored the single effect of berberine or evodiamine. In this scenario, we performed a combination of berberine with evodiamine (cBerEvo), which is more consistent with the components of *Zuojin* pill. Our observations suggest that cBerEvo can inhibit the EMT transformation and the migration of HCoEpiCs stimulated by CCD-18Co, and these effects are dose-dependent. cBerEvo increases the expression of epithelial marker E-cadherin and decreases the mesenchymal markers vimentin and α -SMA expression promoted by CCD-18Co CM. In addition, expression of EMT transcription factors including Snail and ZEB1 is also down-regulated by the treatment of cBerEvo. These effects are similar to that of TGF- β receptor inhibitor LY364947, implying that cBerEvo likely functions through TGF- β signaling.

TGF- β , a crucial inducer of EMT, takes part in extensive physiological and pathological processes. TGF- β signaling is activated in multiple epithelial or tumoral cells to induce EMT occurrence, resulting in enhanced migration and invasion, which has been proved in several types of cancer including breast, lung, liver, colon and pancreatic cancer.^[27-29] Smads play crucial roles in the activation of TGF- β signaling. The TGF- β /Smads signaling controls the expression or activities of multiple important transcription factors including Snail/Slug, ZEB1/2, Twist1/2 that induce transdifferentiation of epithelium into mesenchyma. Therefore, targeting Smad-dependent pathway has been considered to be an important tactics in prevention of multiple fibrotic or tumorous diseases.^[30] Some researches have revealed that berberine abrogated the high glucose-induced EMT in NRK 52E cells or in rats through repressing the activation of TGF- β /Smad signaling.^[31, 32] Evodiamine is also reported to inhibit TGF- β 1-induced EMT in NRK52E

cells *via* Smad pathway.^[33] Similarly, evodiamine improved the tetrachloride-induced liver fibrosis in rats and HSCs *in vitro via* the TGF- β 1/Smad pathway.^[34] However, the effect of cBerEvo on the Smad signaling pathway in TAF-induced EMT of colon epithelial cells was still not explored.

In our study, we found that, although cBerEvo decreased the expression of Smad2 in a negatively correlated manner and Smad3 expression in a dose-independent manner, it repressed the ratios of p-Smad2/Smad2, p-Smad3/Smad3, as well as expression of p-Smad3 and Smad4 in a concentration-dependent manner. Altogether, cBerEvo reduces the activity of Smads signaling in HCoEpiC cells. Our results suggest that hyper-activities of Smads signaling stimulated by CCD-18Co CM induce the transdifferentiation of colon epithelial cells, and treatment of cBerEvo reverses the transition, which is likely related to the repression of Smads pathway.

Conflict of Interests Statement

The authors declare no conflict of interests.

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