



# CLIC4 mediates TGF- $\beta$ 1-induced fibroblast-to-myofibroblast transdifferentiation in ovarian cancer

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**Abstract.** Stromal myofibroblasts, activated by crosstalk signaling between the tumour and stroma, play a critical role in tumour development and progression. Chloride intracellular channel 4 (CLIC4) may be functionally important for tumour stromal fibroblast-to-myofibroblast transdifferentiation, but the molecular mechanism of the process has not been addressed. In this study, the expression of CLIC4 in ovarian cancer tissues was analyzed by immunohistochemistry, and we used an indirect co-culture model of ovarian cancer cells and normal fibroblasts to demonstrate the molecular pathway in which CLIC4 participated during the fibroblast-to-myofibroblast transdifferentiation. The results showed that the expression of CLIC4 in 96.7% of ovarian cancer stroma and correlated with the up-regulation of myofibroblast marker  $\alpha$ -SMA. Conditioned medium from ovarian cancer cells (CM) or transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) increased cellular reactive oxygen species (ROS) levels in fibroblasts, which initiated up-regulation of CLIC4 expression, then resulted in myofibroblast conversion. Moreover, inhibition of

CLIC4 significantly reduced the expressions of factors related to the phenotype and functions of myofibroblasts, such as  $\alpha$ -SMA, VEGF and HGF. These results suggest that ROS-initiated CLIC4 up-regulation is required for TGF- $\beta$ 1-induced fibroblast-to-myofibroblast transdifferentiation in ovarian cancer, indicating that inhibiting the CLIC4 might have therapeutic potential targeting tumour stroma.

## Introduction

Epithelial ovarian cancer is the most lethal disease among gynaecologic malignancies (1-3). For years, it has been dubbed as a 'silent killer' - causing no symptom till it is too advanced at the time of diagnosis (2). Five-year survival for patients with advanced ovarian cancer improved little even with the treatment of optimal cytoreductive surgery and systemic combination chemotherapy (3). Increasing evidence has indicated that cancer development is facilitated by interaction between tumour cells and activated stromal cells (4). The tumour stroma (also referred to as 'reactive stroma') is characterized by marked alterations in the phenotype and expression profile of fibroblast-like cells. These cells commonly express  $\alpha$ -SMA and thus are termed as myofibroblasts (5,6). Stromal fibroblasts are located at the tumour border near the invasion front, and play a crucial role in tumour progression (7). Recent studies demonstrated that co-culturing of fibroblasts with tumour cells can promote tumour invasion and angiogenesis (8,9).

Myofibroblasts are recruited from different sources during cancer development and the invasion progression (10,11). Myofibroblasts can differentiate from the local fibroblast population in the epithelial stroma on transforming growth factor- $\beta$ 1 (TGF- $\beta$ 1) secreted by tumour cells stimulation. Tumour-associated myofibroblasts also transdifferentiate from non-malignant or epithelial derived carcinoma cells via epithelial-mesenchymal transition (11). Moreover, myofibroblasts are recruited or derived from distant fibroblasts and bone marrow progenitor cells (12,13). Although it has been shown that the conversion from fibroblasts to myofibroblasts constitutes the major source of myofibroblasts in tumour stroma, the molecular mechanism underlying fibroblast-to-myofibroblast transdifferentiation is still not fully understood.

Chloride intracellular channel 4 (CLIC4), a chloride channel of intracellular organelles, regulates intracellular pH

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**Abbreviations:** CLIC4, chloride intracellular channel 4; CM<sup>SKOV3</sup>, conditioned medium from SKOV3; DCF-DA, 2',7'-dihydro-dichlorofluorescein diacetate; GST, glutathione S-transferase; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; siRNA, small interfering RNA; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1

**Key words:** reactive oxygen species, chloride intracellular channel 4, transforming growth factor- $\beta$ 1, tumour and stroma interaction, myofibroblasts

and cell volume. Besides its presence on the organelle membrane, CLIC4 exists in soluble form in the cytoplasm and nucleus acting as signalling protein or channel regulator (14). Transcription level of CLIC4 is up-regulated when fibroblasts transdifferentiate into myofibroblasts induced by TGF- $\beta$ 1, and more importantly CLIC4 is highly expressed in myofibroblasts of breast cancer (15). The work of Littler and colleagues (16) shows that functional activity of CLIC4 depends on its redox state and oxidative conditions enhance membrane binding and channel activity of CLIC4. Recent findings also reveal that reactive oxygen species (ROS) can alter gene expression level associated with cell differentiation, including fibroblast-to-myofibroblast transdifferentiation and epithelial-mesenchymal transition (10,17,18). Hence, we hypothesized that the factors involved in fibroblast-to-myofibroblast transdifferentiation, such as tumour-cell-derived TGF- $\beta$ 1, promoted the generation of intercellular ROS, which in turn initiated up-regulation of CLIC4 expression, thus triggering fibroblast-to-myofibroblast conversion. Agents that block CLIC4 might inhibit such transdifferentiation and delay myofibroblast-dependent tumour progression.

To test the hypothesis, we designed this study to confirm whether CLIC4 was highly expressed in epithelial ovarian cancer tissues and to examine the pathway in which CLIC4 participated during the fibroblast-to-myofibroblast transition induced by TGF- $\beta$ 1 from ovarian cancer cells.

## Materials and methods

**Tissue specimens.** Tissue specimens were collected from patients who underwent surgery at the Department of Obstetrics and Gynaecology, Qilu Hospital, Shandong University, following informed consent and approval by the local Human Research Ethics Committee. The specimens consisted of 30 primary epithelial ovarian cancer and 8 normal ovarian tissues. Of 30 patients, 23 were serous carcinomas, 5 mucinous carcinomas and 2 endometrioid carcinomas. Three cases were in stage I, 2 in stage II and 25 in stage III. For paraffin-embedded sections, surgical tissues were fixed in 10% formalin and then embedded in paraffin. For tissue culture, ovaries were placed in DMEM (Gibco) and immediately transported on ice to the laboratory.

**Cell culture.** Both human epithelial ovarian cancer cell line SKOV3 and human fetal lung fibroblast cell line MRC-5 were obtained from Basic Medicine Research Institute, Qilu Hospital, Shandong University, P.R. China. Human ovarian primary fibroblasts were isolated as described previously (19). These cells were cultured in DMEM containing 10% heat-inactivated fetal bovine serum (Gibco) and 100 units/ml penicillin/streptomycin. The cultures were maintained at 37°C in an atmosphere of 5% CO<sub>2</sub>. The purity of primary stromal cells was >98% with keratin and vimentin staining (20). Ovarian fibroblasts between passage 3 and 8 were used for all experiments.

**Treatment of cells with TGF- $\beta$ 1, conditioned medium from SKOV3 (CM<sup>SKOV3</sup>), N-acetyl-L-cysteine (NAC) or IAA-94.** After SKOV3 cells were cultured in serum-free medium for 48 h, the conditioned medium was collected, clarified by centrifugation and stored at -20°C for future use.

When MRC-5 cells and human ovarian primary fibroblasts reached subconfluence, the medium was replaced by serum-free medium, serum-free medium with 10 ng/ml human recombinant TGF- $\beta$ 1 (PeproTech EC Ltd., UK) or CM<sup>SKOV3</sup>, then cultured for another 48 h. In order to study the inhibitory functions of NAC and IAA-94, subconfluent fibroblasts were incubated with 5 mM NAC for 4 h or 10  $\mu$ M IAA-94 for 24 h before addition of TGF- $\beta$ 1 or CM<sup>SKOV3</sup>.

**Immunohistochemical and immunofluorescent staining.** For immunohistochemical staining, the sections were deparaffinized, rehydrated followed by antigen retrieval. The cells were fixed with ice-cold acetone for immunocytochemical analysis. Afterwards, these sections and cells were blocked with 10% normal goat serum, then incubated with rabbit anti-CLIC4 polyclonal antibody (Lifespan Biosciences, USA) and rabbit anti- $\alpha$ -SMA monoclonal antibody (Abcam, UK) at 4°C overnight, respectively. After washing with PBS, the slides were incubated with anti-rabbit IgG secondary antibody for 45 min and biotinylated horseradish peroxidase solution for 45 min. Finally, the slides were stained with diaminobenzidine and observed under light microscope. For immunofluorescent staining, cells were stained with FITC conjugated anti-rabbit IgG secondary antibody, nuclei with 4,6-diamidino-2-phenylindole (DAPI). Images were collected on a Zeiss laser-scanning confocal microscope.

**Western blot analysis.** Cells were lysed in RIPA lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM sodium chloride, 1.0% NP-40, 1% sodium deoxychlorate, and 0.1% SDS) plus protease inhibitors. Cell nuclear and cytoplasmic proteins were extracted using Nuclear/Cytosol Extraction Kit (Pierce Biotechnology, USA). The expressions of CLIC4 and  $\alpha$ -SMA were characterized by Western blots using polyclonal anti-CLIC4 antibody and monoclonal anti- $\alpha$ -SMA antibody as primary antibodies. Reaction products of  $\alpha$ -tubulin were used to control for total or cytoplasmic protein loading, Lamin A+C for normalizing nucleic protein loading. The band intensities were measured by densitometry using the Quantity One software (Bio-Rad, CA, USA).

**Analysis of intracellular ROS.** The intracellular ROS generation was assayed using 2'-7'-dichlorofluorescein diacetate (DCF-DA) (Sigma) dye as previously described (10). DCF-DA can diffuse into cells readily, where it is hydrolyzed to the non-fluorescent polar derivative DCF-DA and trapped within cells. In the presence of the oxidant, DCF-DA turns to highly fluorescent 2'-7'-dichlorofluorescein (DCF).

**Small interfering RNA (siRNA) transfection.** The siRNAs used in this study were designed by and purchased from Qiagen. The sequences of siRNA were as follows: CLIC4 validated siRNA, 5'-GCAGUACAAUGAUUAGUAAAdTdT-3' (sense) and 5'-UUACUAAUCAUUGUACUGCdTdT-3' (antisense); negative control siRNA, 5'-UUCUCCGAACGUGUCACGUGdTdT-3' (sense) and 5'-ACGUGACACGUUCGGAGAAdTdT-3' (antisense); MAPK1 positive control siRNA, 5'-UGCUGACUCCAAAGCUCUGdTdT-3' (sense) and 5'-CAGAGCUUUGGAGUCAGCAdTdT-3' (antisense). Lipofectamine 2000 reagent was used with a final concentration of 30 nM of each siRNA. After 16 h of transfection,

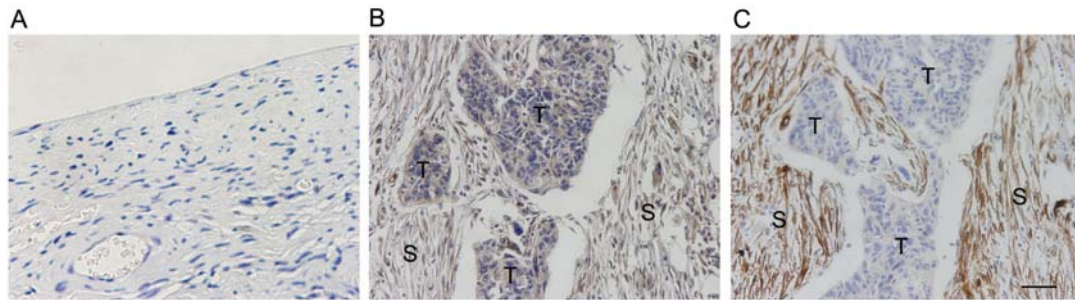


Figure 1. Immunohistochemical stainings of CLIC4 and  $\alpha$ -SMA in normal ovarian tissue and cancer tissue. (A) Normal ovarian tissue was negative for CLIC4 expression. (B) CLIC4 expression was detected in the stroma and epithelium of tumour. (C)  $\alpha$ -SMA expression is shown in the same tumour stroma. S indicates tumour stroma, T indicates tumour epithelium. Scale bar, 50  $\mu$ m.

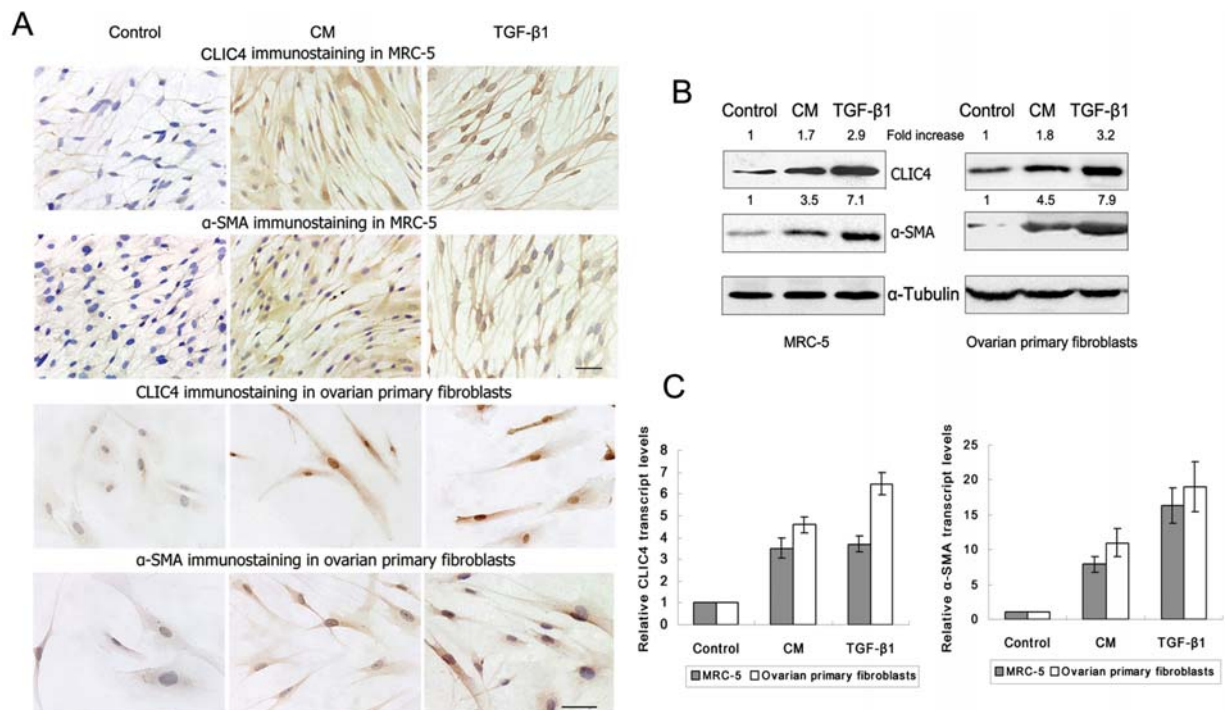


Figure 2. Expression levels of CLIC4 and  $\alpha$ -SMA in fibroblasts up-regulate during transdifferentiation of fibroblasts to myofibroblasts. Subconfluent MRC-5 cells or primary ovarian fibroblasts were cultured in serum-free medium (control), in CM<sup>SKOV3</sup> or treated with 10 ng/ml TGF- $\beta$ 1 for 48 h. CLIC4 and  $\alpha$ -SMA expressions were observed using immunocytochemistry (A), Western blotting (B) and real-time RT-PCR (C). Scale bar, 50  $\mu$ m.

cells were incubated in serum-free medium with or without 10 ng/ml TGF- $\beta$ 1 or CM<sup>SKOV3</sup> for another 48 h.

**Real-time PCR.** Total RNA was extracted from cells using TRIzol reagent (Invitrogen). RNA was reverse transcribed with oligo(dT) and M-MLV reverse transcriptase (Promega). The primers were designed as follows:  $\alpha$ -SMA (accession no. NM\_001613), 5'-AGGTAACGAGTCAGAGCTTTGGC-3' (sense) and 5'-CTCTCTGTCCACCTTCCAGCAG-3' (antisense); CLIC4 (accession no. NM\_013943), 5'-CACGTAAA TTTCTGGATGGCAATG-3' (sense) and 5'-ATCACTGGG ACAGGTATTGGTGAAC-3' (antisense); VEGF (accession no. NM\_001025366), 5'-CCTGGTGGACATCTTCCAGGA GTACC-3' (sense) and 5'-GAAGCTCATCTCTCCTATGTG CTGGC-3' (antisense); HGF (accession no. NM\_000601), 5'-GTAAATGGGATTCCAACACGAACAA-3' (sense) and 5'-TGTCGTGCAGTAACAACCACTC-3' (antisense);  $\beta$ -actin (accession no. NM\_001101), 5'-AACTCCATCATG

AAGTGTGA-3' (sense) and 5'-ACTCCTGCTTGCTGATC CAC-3' (antisense).  $\beta$ -actin was chosen as house-keeping gene. The quantification was performed with SYBR Green (Takara Biotechnology, Inc., Japan). Samples were analysed using LightCycler<sup>®</sup> 2.0 Instrument (Roche Applied Science, Switzerland).

**Statistical analysis.** The data represent means  $\pm$  SD of three independent experiments. Statistical differences were analysed with the Student's t-test. Differences were considered significant at P-value <0.05.

## Results

*The expression of CLIC4 is up-regulated in epithelial ovarian cancer tissues.* CLIC4 may play an important functional role in stromal myofibroblasts, which are critical in tumour development and progression. As shown in Fig. 1B, CLIC4



was detected in epithelium and stroma of patients with primary epithelial ovarian tumours. Positive CLIC4 staining in stroma was shown in 96.7% (29/30) of patients. Moreover, the expression of CLIC4 in cancer stroma was correlated with up-regulation of a well-accepted myofibroblast marker,  $\alpha$ -SMA (Fig. 1C), which was detected in 93.3% (28/30) of the same stroma. Co-expression of CLIC4 and  $\alpha$ -SMA were seen in 93.3% (28/30) of cases, while both negative in 1 case and CLIC4(+)/ $\alpha$ -SMA(-) expression in only one. In normal tissues, previous studies have reported that CLIC4 is detected in multiple epithelial cells, such as epidermis, esophagus, colon, and vascular endothelial cells (21-23). CLIC4 expression level in the stroma is very low (21). However, ovarian surface epithelium is in fact a single layer of mesothelial cells covering the ovarian surface and contiguous with coelomic mesothelium. Our result showed (Fig. 1A) that CLIC4 was not observed in normal ovarian surface epithelium and stroma. In addition, CLIC4 staining in primary tumours had no significant correlation with the clinicopathologic features, including tumour grade, histological type and clinical stage (data not shown).

*The expression of CLIC4 is up-regulated during fibroblast-to-myofibroblast transdifferentiation in vitro.* TGF- $\beta$ 1 is the major mediator in the fibroblast-to-myofibroblast conversion process. Tumour cells release large amounts of active TGF- $\beta$ 1 (24). In order to define the alteration of CLIC4 expression in myofibroblast transdifferentiation, we used a myofibroblast conversion cell model where ovarian primary fibroblasts or human fibroblast MRC-5 cells were cultured with CM<sup>SKOV3</sup> or TGF- $\beta$ 1. Fig. 2A showed that the expression of  $\alpha$ -SMA in the cytoplasm of the activated fibroblasts increased expectedly in the presence of CM<sup>SKOV3</sup> or 10 ng/ml TGF- $\beta$ 1 for 48 h compared with that in serum-free medium control. Coincidentally with up-regulation of  $\alpha$ -SMA expression, the activated fibroblasts displayed evident increase of CLIC4 expression both in the cytoplasm and nucleus. Furthermore, the immunoblot and real-time RT-PCR data revealed that the relative amounts of  $\alpha$ -SMA and CLIC4 expression in CM<sup>SKOV3</sup>- or TGF- $\beta$ 1-treated fibroblasts were up-regulated both at the transcription and protein levels compared with the untreated control (Fig. 2B, C).

*ROS mediate up-regulation of CLIC4 expression during myofibroblast transdifferentiation.* ROS act as signalling molecules of TGF- $\beta$ 1 in the regulation of myofibroblast differentiation (10,17). Therefore, we attempted to investigate whether ROS regulated expression of CLIC4 and  $\alpha$ -SMA during fibroblast activation. We examined the intracellular ROS levels in fibroblasts using DCF-DA dye. MRC-5 cells treated with 10 ng/ml TGF- $\beta$ 1 or CM<sup>SKOV3</sup> for 1 h showed a significant increase in the fluorescence intensity, suggesting increased intracellular ROS (Fig. 3A). When cells were pre-treated with the antioxidant NAC, the up-regulation of  $\alpha$ -SMA and CLIC4 expression stimulated by TGF- $\beta$ 1 or CM<sup>SKOV3</sup> was suppressed (Fig. 3B, C).

CLIC4 subcellular location is critical in its physiological functions (25). Therefore, we further assessed the expression of CLIC4 in the nucleus and cytoplasm during myofibroblast differentiation using Western blotting. As shown in Fig. 3D,

the expression of CLIC4 increased not only in the cytoplasm but also in the nucleus, particularly in the nucleus when fibroblasts were treated with TGF- $\beta$ 1 or CM<sup>SKOV3</sup>. Pre-treatment with antioxidant NAC repressed both cytoplasmic and nuclear CLIC4 expressions.

*CLIC4 is required for TGF- $\beta$ 1 induced myofibroblast transdifferentiation.* The above data suggested CLIC4 was implicated in the process of myofibroblast transdifferentiation. However, it is not clear whether CLIC4 serves as a late differentiation marker like  $\alpha$ -SMA or promotes myofibroblast conversion as an early differentiation marker. We thus reduced CLIC4 level by transfection with validated specific CLIC4 siRNA in MRC-5 fibroblasts. CLIC4 siRNA efficiently down-regulated CLIC4 transcript levels by an average of 90% (Fig. 4A). The induction of  $\alpha$ -SMA transcription induced by TGF- $\beta$ 1 or CM<sup>SKOV3</sup> was significantly blocked in CLIC4 siRNA-transfected fibroblasts (Fig. 4B). At the protein level, immunoblot and immunofluorescent data showed that silencing of CLIC4 prevented the expression of  $\alpha$ -SMA (Fig. 4C, D).

*Blocking CLIC4 inhibits the expression of myofibroblast-mediated up-regulation of pro-angiogenic factors.* Angiogenesis plays a key role in tumour development by supplying the tumour with oxygen and nutrients. Recent data show that myofibroblast is the major cell type expressing pro-angiogenic factors in cancer stroma. Meanwhile, tumour stromal fibroblasts secrete abundant angiogenic factor VEGF only after activated by tumour cells (12). Since CLIC4 is required for TGF- $\beta$ 1 induced myofibroblast transdifferentiation, we next tested whether blockade of CLIC4 could prevent the generation of pro-angiogenesis factors. As expected and shown in Fig. 5, when fibroblasts were stimulated by CM<sup>SKOV3</sup> or TGF- $\beta$ 1, the expressions of VEGF and HGF increased significantly compared with that in the unstimulated fibroblasts. Whereas, pre-treatment with CLIC4 siRNA to silence CLIC4 expression or chloride channel inhibitor IAA-94 to block function of CLIC4 lowered VEGF and HGF expression levels in the activated fibroblasts by 29-80%. This suggested that inhibiting CLIC4 could reduce the pro-angiogenesis production related to prevention of myofibroblast conversion. Furthermore, after fibroblasts were relieved from these treatments, CM<sup>SKOV3</sup> or TGF- $\beta$ 1 still could induce expressions of the factors related to the phenotype and functions of myofibroblasts (data not shown), indicating these repressing activities were not due to cytotoxic effect.

## Discussion

In the present study, we showed CLIC4 was highly expressed in ovarian cancer stroma and correlated with up-regulation of a well-accepted myofibroblast marker,  $\alpha$ -SMA. *In vitro*, the expression of CLIC4 was up-regulated during fibroblast to myofibroblast conversion induced by TGF- $\beta$ 1 or CM from ovarian cancer cells. In addition, we showed for the first time that TGF- $\beta$ 1 or CM stimulated the production of cellular ROS, which caused up-regulation of CLIC4, then in turn led to myofibroblast transdifferentiation. Moreover, the agents which inhibit CLIC4 alleviated the expression of

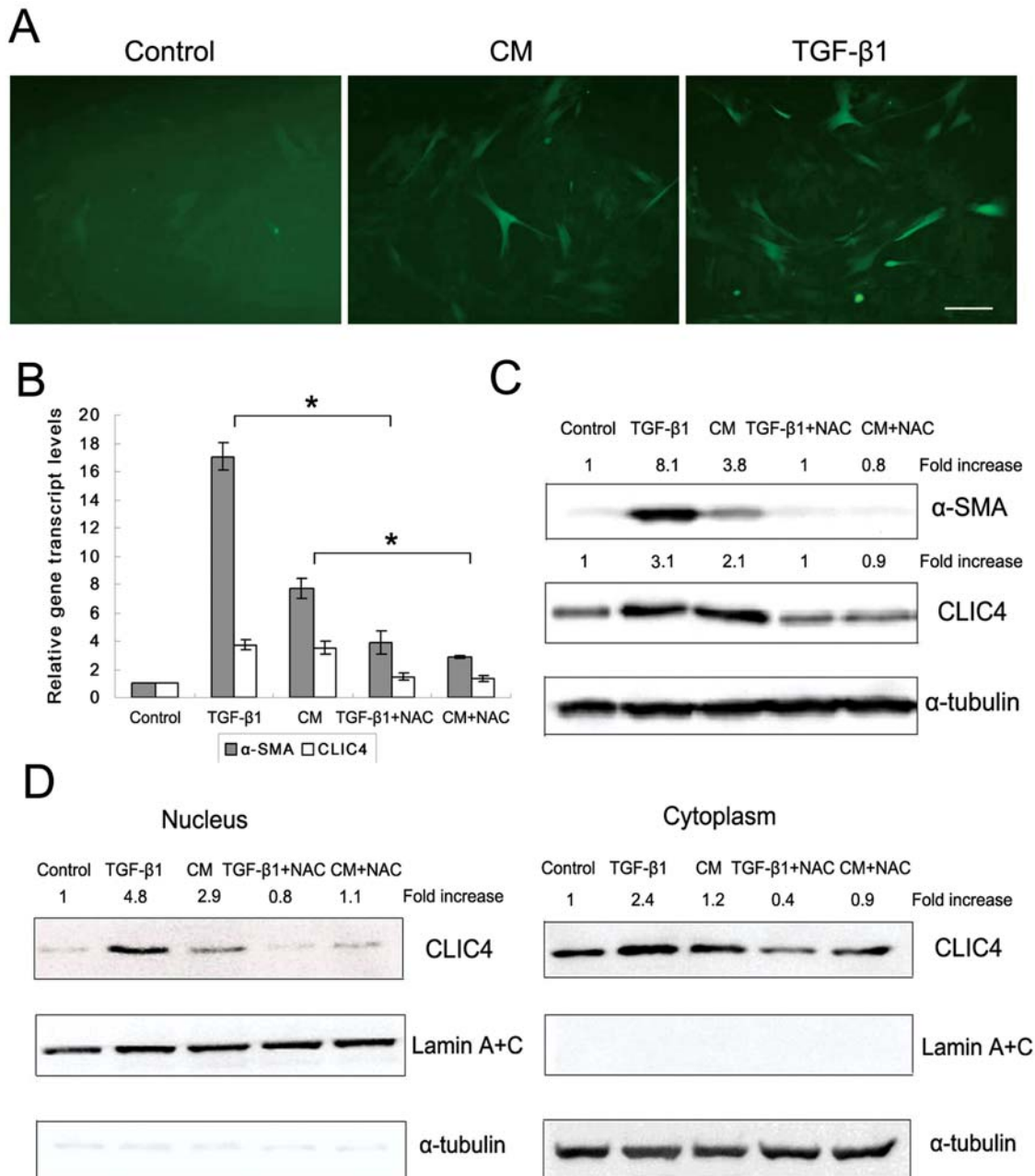


Figure 3. ROS mediate up-regulation of CLIC4 expression during myofibroblast transdifferentiation. (A) CM<sup>SKOV3</sup> and TGF-β1 induced oxidative stress in fibroblasts. MRC-5 cells were loaded with DCF-DA, and then treated with CM<sup>SKOV3</sup> or 10 ng/ml TGF-β1 for another hour, cells were viewed using fluorescent microscope. Scale bar, 50 μm. (B and C) Myofibroblast transdifferentiation was dependent on ROS. The α-SMA and CLIC4 expressions were determined by real-time RT-PCR and Western blotting, when MRC-5 cells were treated with or without antioxidant NAC for 4 h before addition of CM<sup>SKOV3</sup> and TGF-β1 for further 48 h. \*P<0.05. (D) ROS mediated up-regulation of CLIC4 expressions in both cytoplasm and nucleus during myofibroblast transdifferentiation. CLIC4 protein expressions in cytoplasm and nucleus were determined by Western blot analysis. MRC-5 cells cultured in serum-free medium served as control for all the experiments.

pro-angiogenesis factors related to myofibroblast differentiation.

Ion transportation through the membrane sets an environment that promotes initiation of cell differentiation programming. The CLIC family, a major class of intracellular chloride channels, comprises so far seven highly homologous members. Serum- or TGF-β1-stimulated fibroblasts in breast cancer have a gene expression profile of myofibroblasts including a marked up-regulation of CLIC4 (15,26). *In vivo*, CLIC4 expression markedly increased in stroma of breast, kidney, esophagus and colon cancers (21). We not only found that

CLIC4 expression was up-regulated in epithelial cancer stroma correlated with α-SMA expression, but also used an indirect co-culture model to reveal that human primary ovarian fibroblasts and human fibroblast cell line MRC-5 exposed to CM from ovarian cancer cells enhanced CLIC4 and α-SMA expressions, which similarly occurred during TGF-β1-induced fibroblast-to-myofibroblast transdifferentiation. We thus conclude that CLIC4 directly participates in stromal fibroblast activation in ovarian cancer.

It has been verified that tumour stromal myofibroblasts favour tumour invasion and metastasis both in experimental

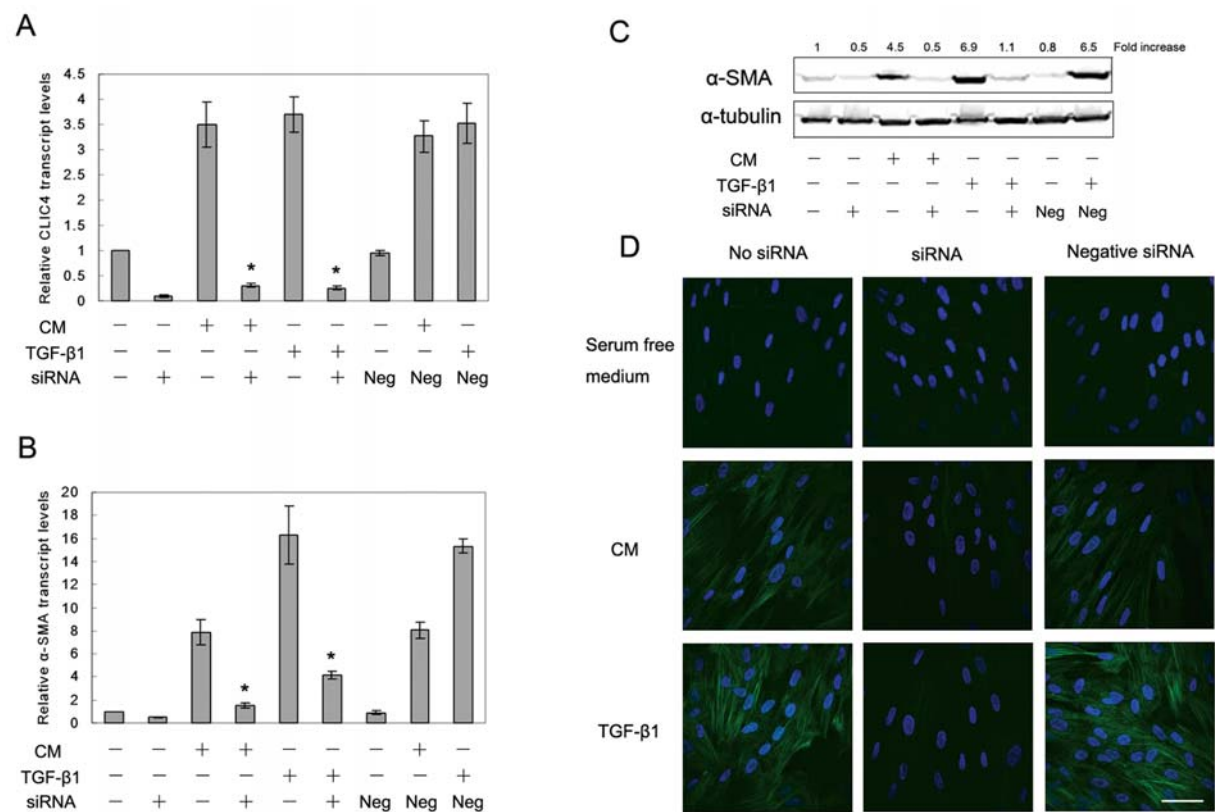


Figure 4. CLIC4 is required for myofibroblast transdifferentiation. MRC-5 cells were transfected with specific CLIC4 siRNA and then treated with or without CM<sup>SKOV3</sup> and TGF- $\beta$ 1 for 48 h. Mock transfection with transfection reagent alone and negative control siRNA (neg siRNA) served as negative control. (A) The relative level of CLIC4 transcript was assessed by real-time RT-PCR. (B-D) The relative levels of  $\alpha$ -SMA transcript and protein expression were assessed by real-time RT-PCR, Western blotting and immunofluorescent staining. \* $P < 0.05$ . Scale bar, 50  $\mu$ m.

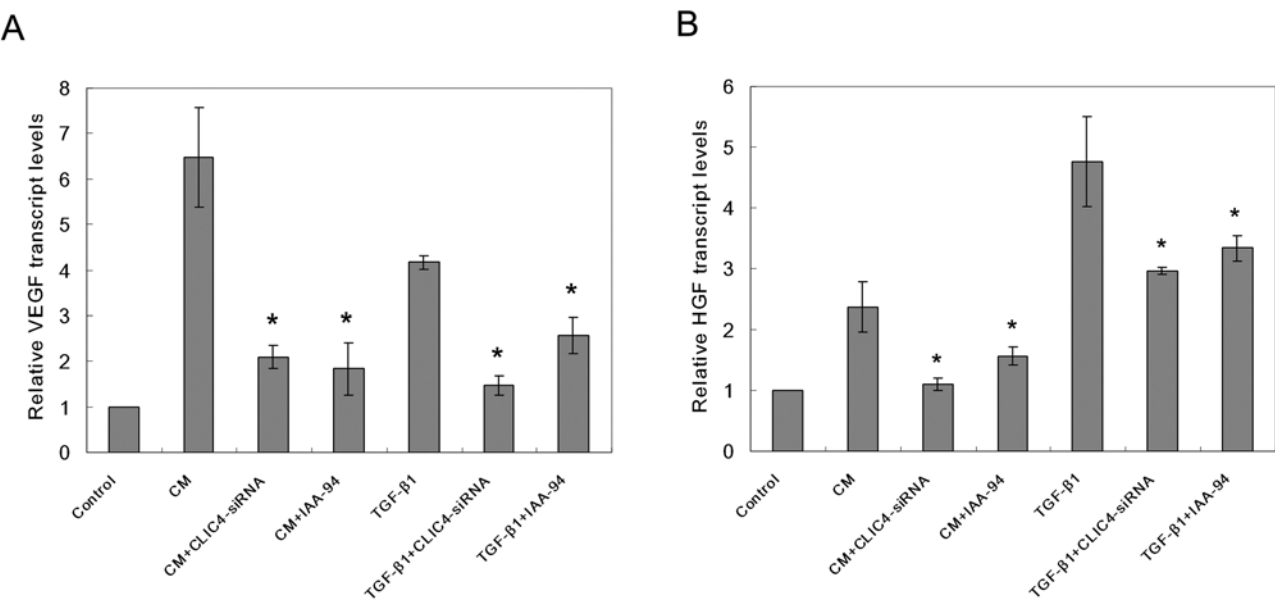


Figure 5. Blocking CLIC4 inhibits the expression of myofibroblast-mediated pro-angiogenic factors. Quantitative PCR was used to analyse transcript levels of VEGF (A) and HGF (B) in MRC-5 cells, when they were treated with or without CLIC4 siRNA or chloride channel inhibitor IAA-94 before addition of CM<sup>SKOV3</sup> and TGF- $\beta$ 1. \* $P < 0.05$ .

settings and in clinical specimens (12,27,28). The over-expression of  $\alpha$ -SMA in tumour stroma is currently used for tumour clinical grading including breast, gastric, colorectal

and pancreatic cancer (27,28). CLIC4 co-localized with  $\alpha$ -SMA in cancer stroma, and the extent of CLIC4 up-regulation correlated with tumour stage and histological grade in kidney,





breast cancer (21). In this study, we did not observe significant relationship between CLIC4 expression in stroma and the progression of epithelial ovarian cancer. Further investigations are required to clarify the clinical significance of CLIC4 in ovarian cancer.

ROS can alter gene expression levels, and ROS signalling is involved in TGF- $\beta$ 1-induced myofibroblast conversion by regulating the expression of multiple phenotype markers, such as  $\alpha$ -SMA (10). Our study showed that not only  $\alpha$ -SMA but also CLIC4 transcription and translation levels were significantly up-regulated following the generation of ROS during TGF- $\beta$ 1 induced fibroblast-to-myofibroblast differentiation. This effect could be blocked by pre-treatment with the antioxidant NAC. Furthermore, the crystal structure of CLIC4 is homologous to that of the glutathione S-transferase (GST) superfamily, suggesting its function may depend on the cellular redox state. A cysteine at position 35 preceding the N-terminal transmembrane domain is redox sensitive and is modified by glutathione (16). Thus, ROS may exert an effect on function of CLIC4 through reducing both its expression and activity. It has been reported that CLIC4 can promote cytoskeleton function by interaction with dynamin, tubulin, actin and 14-3-3 proteins (29), and recombinant CLIC4 in fibroblasts reduces cell motility (15). Thus, the relationship between CLIC4 and  $\alpha$ -SMA seems to be of consequence in the physiological function. To investigate whether myofibroblast conversion was a consequential outcome of CLIC4 up-regulation, fibroblasts were transfected with specific CLIC4 siRNA prior to TGF- $\beta$ 1 or CM<sup>SKOV3</sup>, which could significantly depress the expression of myofibroblast marker  $\alpha$ -SMA. Taken together, these data suggest that TGF- $\beta$ 1 or CM<sup>SKOV3</sup> increases cellular ROS, which induces up-regulation of CLIC4, then leads to myofibroblast trans-differentiation.

Recent studies have emphasized the advantage of therapeutic targeting of the tumour stroma, due to the fact that stromal cells reveal genomic stability in contrast to cancer cells, which acquire adaptive mutations during therapy process and these mutations are critical to promote tumour growth. Fibroblasts and myofibroblasts are the major cell types present in various types of human tumour stroma. Therefore, the stromal fibroblast is an attractive target for therapeutic intervention. CLIC4 promotes proliferation and vascular tubulogenesis of endothelial cells in the formation of new blood vessels (23,30). Here, we found that treatment of fibroblasts with the agents blocking CLIC4 had a marked repressive effect on the production of angiogenic factors associated with myofibroblast transdifferentiation, indicating that inhibiting the CLIC4 might have therapeutic potential targeting tumour stroma.

In conclusion, our results not only demonstrate the up-regulation of CLIC4 expression during conversion of fibroblasts to myofibroblasts in ovarian cancer, but reveal that tumour-stroma interaction promotes stromal fibroblast activation through ROS-initiated CLIC4 expression. Therefore, inhibiting CLIC4 might be a useful strategy for the prevention of myofibroblast differentiation and myofibroblast-dependent tumour progression. Further investigations of the processes governing myofibroblast transdifferentiation will likely suggest additional approaches to interfere with tumour growth.

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