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ORIGINAL ARTICLE

AKT and JNK Signaling Pathways Increase the Metastatic Potential of Colorectal Cancer Cells by Altering Transgelin Expression

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Abstract

Background Transgelin (SM22) plays a crucial role in colorectal cancer (CRC) progression; nevertheless, its upstream regulatory mechanisms are poorly defined. AKT and JNK signaling pathways are strongly associated with tumor progression and metastasis, and there are some indications that these pathways might be involved in transgelin regulation.

Aims To examine the role of AKT and JNK signaling in transgelin regulation in colorectal cancer progression.

Methods Phospho-AKT (P-AKT), phospho-JNK (P-JNK), and transgelin expression were examined in one normal colon cell line (FHC) and three CRC cell lines (SW620, LoVo, and RKO) as well as in normal colon and CRC tissue samples by Western blot and qRT-PCR. Next, siRNA silencing of AKT or JNK pathways in SW620 cells was performed to examine their role in transgelin regulation. The effects of siRNA silencing on SW620 cell mobility and metastatic properties were examined by cell migration, invasion assays, and actin cytoskeleton.

Results Transgelin, P-AKT, and P-JNK were increased in all examined cell lines. Moreover, transgelin mRNA and protein expression was especially elevated in SW620 cells. Furthermore, inhibition of Akt or JNK signaling resulted in transgelin downregulation. When transgelin, Akt, or JNK signaling was inhibited, SW620 cell migration and invasion were dramatically decreased with inhibition of actin cytoskeleton dynamics.

Conclusion This study demonstrates, for the first time, that activated AKT and JNK signaling pathways promote the overexpression of transgelin, which potentially contributes to CRC progression and metastasis.

Keywords Transgelin · SM22 · AKT · JNK · Metastasis · Colorectal cancer

Introduction

Although colon cancer incidence in the USA has dropped by 30 % over the past decade among patients older than 50 years, epidemiological studies indicate that colorectal cancer (CRC) is the third most common cancer and the third leading cause of cancer death in men and women [1]. Currently, localized CRC is curable by surgical resection. However, many CRC patients will experience recurrent, metastatic disease and the 5-year survival rates are <10 % in these patients [2]. Hence, the search for novel therapeutic biomarkers and better understanding of the underlying mechanisms of CRC progression are highly desired to decrease CRC.

Numerous studies have documented the widespread role of Akt and JNK signaling in cell survival and metastasis in

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CRC, as well as many other types of cancer. These signaling pathways act as key signaling nodes that link oncogenic receptors to many essential pro-survival cellular functions, and are perhaps the most commonly activated signaling pathways in human cancer [3]. However, the mechanisms of Akt and JNK signaling have not yet been completely elucidated. Therefore, examining potential new connections of these signaling pathways with different signaling molecules within a cell could possibly provide the basis for the development of novel strategies for the treatment of metastases.

Transgelin, also known as SM22, is an abundant 22-kDa protein which primarily participates in processes associated with remodeling of the actin cytoskeleton [4]. Although some early studies observed that transgelin is downregulated in early-stage tumor models, more recent studies suggest that transgelin is upregulated in aggressive, late-stage, lymph node-positive cancers and that its expression correlates with tumor stage and prognosis [5–9]. In our previous study in which we performed a proteomic analysis of CRC samples obtained through laser capture microdissection of tumor tissue from lymph node-negative and lymph node-positive patients, we demonstrated that transgelin might be an oncogenic marker for CRC [9]. Regulatory mechanisms that act upstream of transgelin have not yet been identified. Since AKT or JNK signaling pathways participate in modulation of many cancer-related processes in the cell and previous studies have reported that activation of AKT and JNK is closely related to the expression of transgelin [10, 11], we hypothesize that these pathways may contribute to CRC progression possibly by modulating transgelin expression.

In this study, we decided to examine the expression of phospho-AKT (P-AKT), phospho-JNK (P-JNK), and transgelin in CRC lines as well as in normal colon and CRC tissue samples at both the mRNA and protein level. Next, in order to investigate the possible role of AKT or JNK pathways in transgelin regulation, siRNA silencing was performed, and the effects of inhibition on cell mobility and metastatic properties were investigated.

Results

P-AKT and P-JNK Expression in SW620, RKO, and LoVo Colorectal Cancer Cell Lines

In order to examine P-AKT and P-JNK expression in CRC, we have examined their expression in three human CRC cell lines (SW620, RKO, and LoVo) by Western blot analysis. In our study, P-AKT and P-JNK were highly expressed (Fig. 1) which is consistent with findings from previous studies [12, 13].

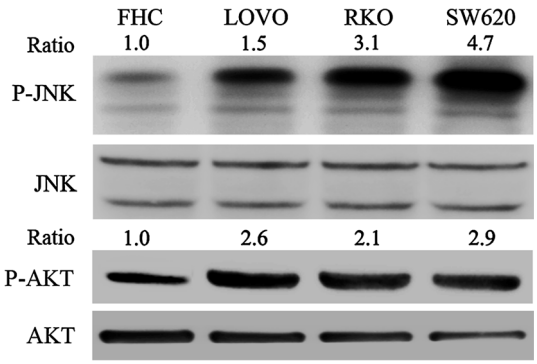

Fig. 1 AKT and JNK are activated in CRC cell lines. Western blot analysis of P-AKT and P-JNK showed that they are highly expressed in human CRC cell lines SW620, LoVo, and RKO compared to FHC ($p < 0.05$)

Transgelin Expression in CRC Cell Lines, Normal Colon, and CRC Tissue Samples

In order to investigate the role of transgelin in tumor genesis, we next examined its expression in human CRC cell lines (SW620, RKO, and LoVo) and one human normal colon cell line (FHC) by Western blot. In our study, transgelin was expressed in all examined CRC cell lines and its expression was highest in the SW620 cell line

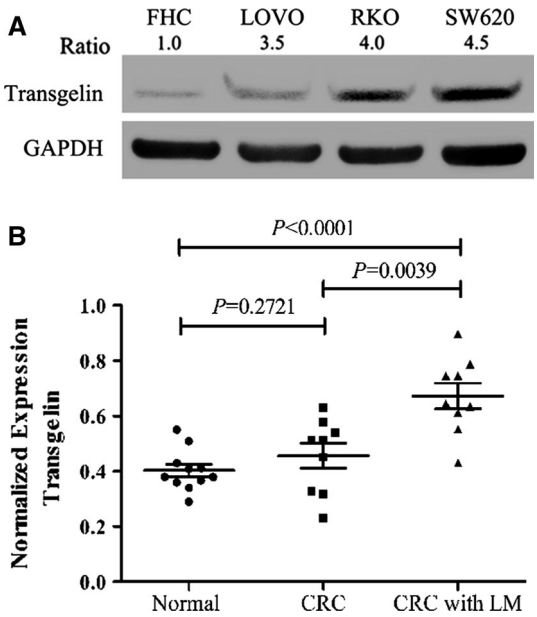

Fig. 2 Transgelin expression is increased in colorectal cancer. **a** Western blot analysis has shown that the highest transgelin expression was observed in SW620 cell line; **b** qRT-PCR analysis of transgelin expression has revealed that transgelin expression was higher in CRC tissues with LM than in normal colonic epithelial tissues ($p < 0.001$) or CRC tissues without metastasis, while no differences were observed between normal colonic epithelial tissues and CRC tissues without metastasis ($p = 0.0039$)

(Fig. 2a). Transgelin expression was further evaluated by qRT-PCR in 10 normal colon epithelial tissues, nine CRC tissues without metastasis, and nine CRC tissues with lymph node metastasis (LM). As shown in Fig. 2b, transgelin expression was higher in CRC tissues with LM than normal colonic epithelial tissues or CRC tissues without metastasis, while no differences were observed between normal colon epithelial tissues and CRC tissues without metastasis, suggesting that transgelin may be closely related to the CRC metastatic processes.

Transgelin May Be a Downstream Target of AKT or JNK Signaling

Further, we examined the relationship between transgelin and AKT and JNK signaling pathways. For this purpose, SW620 cells were treated with 50 μM LY294002 (AKT inhibitor) or 25 μM SP600125 (JNK inhibitor) for 16 h and the transgelin mRNA and protein levels were quantified by Western blot and qRT-PCR. The results of these analyses show that in SW620 cells treated with inhibitors, transgelin expression was markedly decreased when compared with the mock group or control group as shown in Fig. 3a, b. In addition, transgelin expression was inhibited by transgelin siRNA as a positive control in these experiments ($p < 0.05$). To additionally examine the influence of AKT and JNK signaling pathways on transgelin expression in SW620 cells, cells were incubated in the presence of SP600125 (25 μM) or LY294002 (50 μM) for 0, 8, 16, or 24 h, and transgelin protein expression was examined. The results of this time response analysis showed that transgelin protein expression was decreased at 8 h after the addition of AKT and JNK inhibitors by 59 and 63 %, respectively, and remained decreased by 80 % (AKT inhibitor) and 86 % for up to 24 h (Fig. 3c, d).

AKT or JNK Signaling Pathways May Promote Cell Migration and Invasion in CRC Partly by Activation of Transgelin

Cell motility and invasiveness closely correlate with cancer metastasis. In order to evaluate the metastatic properties of CRC cells, we carried out cell migration and invasion assays in SW620 cells treated with AKT or JNK inhibitor, as well as siRNA for transgelin. The results of these analyses have shown that both the treatment of SW620 cells with AKT and JNK inhibitors and silencing of transgelin by siRNA were able to suppress the migration and invasion capacity of these cells when compared with the control or mock group ($p < 0.05$, Fig. 4a, b).

AKT or JNK Signaling Pathways May Regulate Actin Cytoskeleton by Activation of Transgelin in Human CRC Cells

To test the hypothesis that the regulatory effect of AKT or JNK signaling on CRC cell migration may be mediated by regulation of actin cytoskeleton via transgelin, changes in the action cytoskeleton in human SW620 cells were determined by actin staining. After stimulation with

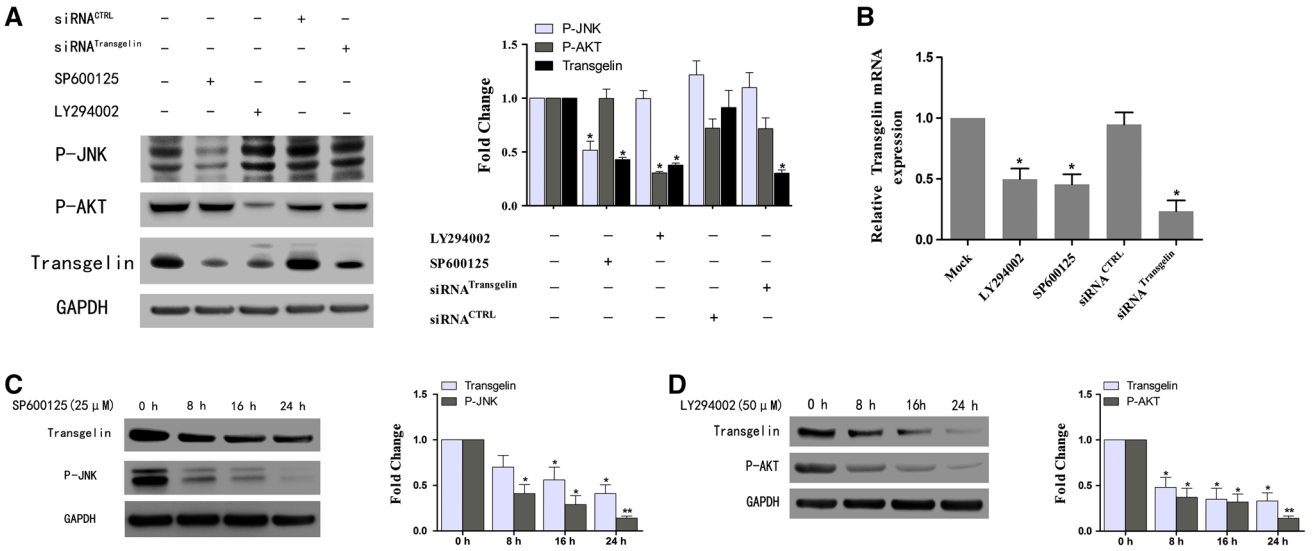

Fig. 3 Transgelin expression is downregulated by inhibition of AKT or JNK pathway in CRC cells. **a** Western blot and **b** qRT-PCR validation of transgelin showed that the protein and mRNA expression of transgelin was significantly decreased after inhibition of AKT or JNK signaling pathways as well as after the transfection of SW620 cells with siRNA^{transgelin} (protein: $p < 0.05$; mRNA: $p < 0.05$; $n = 3$). **c**, **d** Western blot and qRT-PCR analysis of transgelin expression in SW620 cells treated with SP600125 (25 μM) or LY294002 (50 μM) at 0, 8, 16, and 24 h after the treatment with the inhibitors

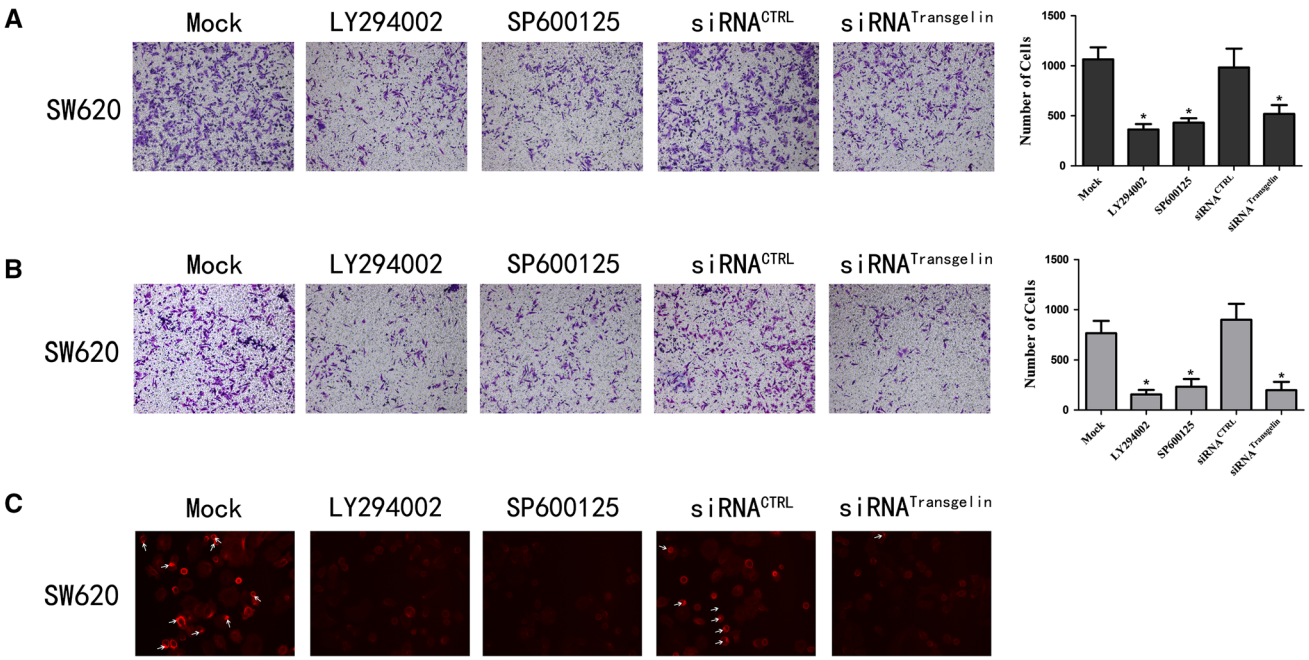

Fig. 4 AKT or JNK signaling pathways may induce CRC metastasis partly by upregulating the expression of transgelin. **a** The migration activity of LY294002- and SP600125-treated SW620 cells was significantly reduced by approximately 66 and 60 % compared with mock group, respectively ($p < 0.05$). Cells treated with LY294002 or SP600125 or incubated with siRNA for transgelin showed less motility when compared to untreated control cells; however, no significant difference was observed between these three treated groups ($p > 0.05$). The results are presented as the mean results ± SD of a representative experiment performed in triplicate ($n = 3$). **b** The cell invasion assay showed that adding LY294002 or SP600125 or knockdown transgelin expression by siRNA resulted in decreased cells invasion capability when compared with the control untreated cells or mock-treated cells ($p < 0.05$). The results are presented as the mean ± SD ($n = 3$). **c** Red fluorescence represents the actin cytoskeleton of SW620 cells (×200). After the stimulation of SW620 cells with CXCL12, mock cells or cells transfected with siRNACTRL showed a normal pattern of actin polymerization and polarization, in which the actin aggregated in the region where cells were in contact with chemokines, and then a projection of cells was formed (*white arrow*). Cells treated with LY294002 or SP600125 or in which siRNA-mediated transgelin knockdown was performed, resulted in an abnormal response, and no obvious actin polymerization and polarization were seen ($n = 3$)

CXCL12, mock cells or cells transfected with siRNACTRL showed a normal pattern of actin polymerization and polarization, while an inhibitory result was shown in SW620 cells treated with AKT and JNK inhibitors as well as with transgelin siRNA silencing (Fig. 4c). These results demonstrated that AKT or JNK signaling pathways might promote the actin polymerization and polarization through transgelin in CRC cells.

Discussion

Colorectal cancer is one of the most common malignancies with high incidence rates globally, and in recent years, it has been the leading cause of cancer-related death in China too [1]. Although CRC progresses slowly to the invasive stage, the progression from invasive carcinoma to metastatic phase occurs rapidly [14]. However, useful markers and complete understanding of their mechanisms of action are still not available and their delineation might be vital in improving the clinical outcome of CRC patients with metastasis. Transgelin has been primarily studied as an abundant protein of smooth muscle cells and an important factor in the determination of actin cytoskeleton dynamics. Structurally, it belongs to a calponin protein family characterized by the presence of a single N-terminal calponin homology (CH) domain and by one C-terminal calponin-like repeat [4]. There is growing evidence that transgelin is both directly and indirectly involved in many cancer-related processes. However, its role in tumorigenesis is complex and often contradictory and therefore requires further examination [4].

The PI3K/AKT pathway acts as a key signaling node that provides the link between oncogenic receptors and downstream pro-survival molecules and is one of the most frequently activated signaling pathways in human cancers [12]. Multiple small GTPases are known to activate PI3K/AKT signaling, and Rho GTPases are downstream activators of PI3K [13]. Genomic analyses have also revealed that many components of the PI3K/Akt pathway are frequently mutated or altered in human cancers [15]. Numerous studies have documented the widespread role of

Akt in cell metastasis in CRC, as well as many other types of cancer [3].

The JNK protein kinases are encoded by MAPK8, MAPK9, and MAPK10 and alternatively spliced to form the JNK isoforms. Multiple growth factors and extracellular signals can lead to activation of the JNK signaling pathway which is involved in the regulation of cell proliferation and inducing apoptosis [16]. In addition, persistent activation of JNK has been found in human tumors and it has been shown that it influences cancer development and progression [17]. Overexpression of signaling molecules involved in this pathway as well as its constitutive activation is commonly detected in CRC, and several lines of evidence indicate that activation of JNK plays an important role in progression and metastasis of this type cancer [18].

In our previous study, we showed that transgelin can promote invasion, survival, and resistance to anoikis and correlates with CRC metastasis [9]. The aim of this study was to investigate whether there was a correlation between AKT or JNK signaling and transgelin and to examine the transgelin function in CRC cells. For this purpose, we examined the expression of active phosphorylated forms of AKT and JNK as well as transgelin expression in three human CRC cell lines, SW620, LoVo, and RKO compared with normal colon cell line FHC. We confirmed their pronounced expression. Next, we have examined transgelin mRNA expression in colorectal tumors of patients with and without LM as well as of normal colonic epithelial tissue. Interestingly, elevated transgelin mRNA expression was observed in the CRC tissues with LM compared with normal colonic epithelial tissues or CRC tissues without metastasis, but no difference was found between normal colon epithelial tissues and CRC tissues without metastasis. These findings suggest that transgelin may play a critical role in CRC metastasis. Moreover, Western blot analysis has shown that the levels of transgelin were downregulated when SW620 cells were incubated with AKT and JNK signaling pathway inhibitors, LY294002 or SP600125. These results confirm transgelin as one of the downstream targets of AKT and JNK cascade in CRC. In addition, the results of cell migration and cell invasion assays as well as actin cytoskeleton demonstrated that AKT or JNK pathway may promote CRC metastasis via upregulation of transgelin.

To date, many studies have proposed that transgelin is a multifunctional protein and it affects CRC cells and it has now been associated with prognosis, metastasis, and invasiveness of CRC [5, 8, 19, 20]. A recent study showed that apigenin upregulates transgelin and inhibits invasion and migration of colorectal cancer through decreased phosphorylation of AKT [21]. However, the final consensus on the role of transgelin in colorectal tumorigenesis has still not been reached. In this study, we found that the expression level of transgelin was upregulated in colorectal cancer with lymph node metastasis, while there was no significant difference between the normal and tumor tissue of colorectal patients (without metastasis). Furthermore, the expression of transgelin was significantly increased in SW620 (CRC cell line derivated from lymph node metastasis) based on the results of our previous study in which we have shown that transgelin may be an oncogenic marker in CRC by using the proteomic analysis of samples obtained through laser capture micro-dissection of tumor tissue from node-negative and node-positive patients, and we hypothesized that transgelin may be closely related to the CRC metastasis [9]. In addition, transgelin is also known to play a critical role in actin cytoskeleton which is closely related to cancer metastasis [4]. Indeed, the results of this study showed that transgelin promoted migration and invasion of CRC cells and actin cytoskeleton dynamics, confirming our hypothesis. On the other hand, our results highlight the possible strategy of transgelin targeting for CRC therapy, especially for stage III or IVCRC which are characterized by metastasis. Indeed, future studies are warranted in order to increase understanding of the details of cross talk between AKT and/or JNK pathways and transgelin and to clarify what components downstream of transgelin are activated by AKT and/or JNK signaling pathways in tumorigenesis.

Materials and Methods

Cell Lines and Cell Culture

The CRC cell lines (SW620 and LoVo) were obtained from ATCC and maintained in RPMI-1640 cell culturing medium supplemented with 10 % fetal bovine serum (HyClone, USA), whereas the RKO cell line was cultured in MEM cell culturing medium supplemented with 10 % fetal bovine serum. Additionally, FHC cell line was also purchased from ATCC and was cultured in DMEM/F12 supplemented with 10 % fetal bovine serum (HyClone, USA), 10 ng/ml cholera toxin (Sigma, USA), 0.005 mg/ml insulin (Roche, USA), 0.005 mg/ml transferrin (Sigma, USA), and 100 ng/ml hydrocortisone (Sigma, USA). Cells were grown in a humidified incubator at 37 °C with 5 % CO_2. LY294002 (Akt inhibitor) and SP600125 (JNK inhibitor) were purchased from CST manufacturer (USA) and were diluted to the final concentration 50 and 25 μM by adding the normal medium, respectively.

Clinical Samples

For validation of transgelin expression in 11 normal human colon and 18 CRC tissue samples, primary postoperative

CRCs and normal epithelium specimens were obtained from Sun Yat-sen Memorial Hospital. All tissues were histologically verified with hematoxylin and eosin staining by a pathologist. Informed consent was obtained from all participants of the study according to the Internal Review and Ethics Boards of Sun Yat-sen University Cancer Center, Guangzhou, China.

Small Interfering RNAs (siRNAs) Transfection

Effective siRNA targeting of human transgelin was performed according to the protocol which was established in the previous study [14]. Small interfering RNAs were synthesized by RiboBio (Guangzhou, China), and SW620 cells (1×10^6) were seeded in six-well plates and transfected using RNAiMAX reagent (Invitrogen Life Technologies, USA) for 48 h. Subsequently, quantitative real-time PCR analysis was performed to verify the changes in transgelin mRNA expression.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, USA) and reverse transcribed with the RT kit from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The primers used in this analysis for transgelin (target gene) were as follows: forward: GTTCCAGACTGTTGACCTCT TT, reverse: CTGCGCTTTCTTCATAAACC; for GAPDH (endogenous control), forward: TGGTGAAGACGCCAGT GGA, reverse: GCACCGTAAGGCTGAGAAC. The qRT-PCR was performed using a Light Cycler® 480 SYBR Green I Master mix (Roche, USA) on a Light Cycler® 480 System (Roche, USA) according to the manufacturer's instructions. The PCR conditions were as follows: 95 °C for 30 s, 35 cycles at 95 °C for 5 s, then 60 °C for 30 s. The relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ comparative CT method.

Western Blot

Cells were lysed in RIPA buffer (Beyotime, China) containing protease and phosphatase inhibitors (complete Mini, EDTA-free Protease Inhibitor Cocktail and PhosSTOP Phosphatase Inhibitor Cocktail, Roche, USA). Protein lysates were centrifuged, and the supernatants were collected for protein quantification using the bicinchoninic acid assay kit (Pierce, USA). Protein lysates were resolved through 8–10 % SDS-PAGE (Beyotime, China) and electrophoretically transferred to nitrocellulose membranes (Millipore, USA). Membranes were then probed with antibodies against transgelin (1:1000, ab155272 Abcam, USA), P-AKT (1:1000, cat. No. 13038, CST, USA) and P-JNK (1:1000, cat. No. 4668, CST, USA), and Akt (1:1000, cat. No. 4685, CST, USA) and JNK (1:1000, cat. No. 9252, CST, USA). GAPDH (1:800, cat. No. 5174, CST, USA) was used as protein loading control. Followed by HRP-conjugated goat anti-rabbit IgG (1:1000, CST, USA) as the secondary antibody, the intensity of the proteins was visualized with Syngene G:BOX Chemi XT4 fluorescence and chemiluminescence gel imaging system (Cambridge, UK).

Cell Migration Assay

Five groups of SW620 cells (1×10^5) were suspended in serum-free RMPI-1640 medium and seeded into the upper chambers of 24-well transwell plates (Corning, USA). The lower chamber of each well was incubated with 500 µl of RPMI-1640 with 40 % FBS at 37 °C for 18 h. Cells were fixed with 4 % paraformaldehyde and stained with crystal violet, and non-migratory cells on the upper chamber were removed with a cotton swab. Migrated cells on the other side of the filter were counted in 10 random high-power fields. All experiments were performed in triplicate.

Cell Invasion Assay

The invasion assay was performed using the Bio-Coat Matrigel invasion assay system (BD, USA) following the manufacturer's protocol. Five groups of SW620 cells were suspended in serum-free RPMI-1640 medium and seeded into the upper chambers of 24-well transwell plates. FBS (10 %) was added to the bottom chambers. After 24 h, the cells on the upper side were removed with a cotton swab, while the cells on the bottom side of the filter were fixed with 4 % paraformaldehyde, stained with crystal violet, and counted. The invasive rate was expressed as a percentage of control. All experiments were performed in triplicate.

Actin Cytoskeleton Staining

For the analysis of actin cytoskeleton, SW620 cells were stimulated by CXCL12 (100 ng/ml; peprotech) for 30 min after incubated with LY294002 or SP600125 as well as siRNA-targeting transgelin. After fixing with 4 % paraformaldehyde (YongJin, China) and permeabilization with 0.1 % Triton-X 100 (Sigma, USA), cells were stained with rhodamine-conjugated phalloidin (Invitrogen, USA) for 30 min. Then, the fluorescent images were captured by fluorescent microscopy (Olympus DP72, Japan).

Statistical Analysis

Statistical analyses were performed using SPSS 20.0 software (SPSS Inc., USA). The values were expressed as

mean ± standard deviation (SD) of three independent experiments, and the significance of the differences between two groups was calculated using a two-tailed Student's *t* test. Transgelin expression was compared between CRC tissues with lymphatic metastasis (LM) and CRC tissues or normal tissues using the Wilcoxon signed-rank test. p values <0.05 were considered to be statistically significant.

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Author contributions Huimin Zhou and Qikui Chen conceived and designed the experiments; Huimin Zhou and Yiming Zhang performed the experiments; Ying Lin analyzed the data; and Huimin Zhou wrote the paper.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

Ethical standard All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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