Mechanisms involved in biological behavior changes associated with *Angptl4* expression in colon cancer cell lines

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Abstract. Colorectal cancer (CRC) is one of the most common causes of cancer-related deaths throughout the world. Angiopoietin-like-4 (Angptl4), a member of the angiopoietin family of secreted proteins, is frequently expressed in the perinecrotic areas of different human tumors, yet its role is still unclear in colorectal cancer. Angptl4 mRNA expression in primary colorectal cancer tissue and seven colon cancer cell lines was measured by semi-quantitative RT-PCR; the influence of Angptl4 expression on the colon cancer cell lines was investigated by either overexpression or knockdown of Angptl4 in colon cancer cell lines HCT116 and HT29, respectively. The results showed that Angptl4 mRNA is frequently expressed in human colorectal cancer tissues and cell lines. Overexpression of Angptl4 promoted cell migration, F-actin reorganization and formation of pseudopodia. Further investigation showed that high Angptl4 expression was associated with an increase in ezrin/radixin/moesin and vasodilator-stimulated phospho-protein expression and a decrease in E-cadherin expression. These results indicate that overexpression of Angptl4 may promote invasion and metastasis in CRC.

Introduction

Although the incidence of colorectal cancer (CRC) has declined by almost 3% every year in the US (1), it is continually increasing in developing countries, and is still one of the most common causes of cancer-related deaths throughout the world; metastases remains the major cause of mortality. Although a complex multistep process has been recognized in the initiation, promotion and progression of CRC (2), the precise mechanisms involved in cancer metastasis are largely unknown; unraveling its molecular basis is still a challenging issue.

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Human angiopoietin-like 4 (Angptl4), previously known as hepatic fibrinogen/angiopoietin-related protein (HFARP), peroxisome proliferator-activated receptor-γ (PPARγ) angio-poietin-related gene (PGAR), or fasting-induced adipose factor (FIAF), is a circulating plasma protein mainly expressed in the liver, adipose tissue and placenta, as well as in ischemic tissue. It is composed of 406 amino acids and contains an amino-terminal coiled-coil domain and a carboxyl-terminal fibrinogen-like domain (3). The most studied functions of Angptl4 are its role in the regulation of angiogenesis, glucose metabolism and lipid metabolism (4-6).

There is growing evidence that Angptl4 plays an important role in the regulation of cell growth and progression in carci-noma. Li *et al* (7) confirmed that Angptl4 inhibits the growth of liver cancer cells. Galaup *et al* (8) showed that Angptl4, through its action on both vascular and tumor compartments, prevents the metastatic process by inhibiting vascular activity as well as tumor cell motility and invasiveness. Kaneda *et al* (9) reported that the expression of Angptl4 in two gastric cancer cell lines (MKN28 and MKN74) was lost due to methylation, yet it was unmethylated in normal tissues. However, its role in CRC remains unknown.

To determine whether Angptl4 expression influences the biological behavior of colon cancer cell lines, we established a stable Angptl4-overexpressing human colon cancer cell line HCT116 (HCT116/Angptl4) which originally had extremely low gene expression and an Angptl4 shRNA knockdown human colon cancer cell line HT29 (HT29/shRNA-Angptl4) which originally had high gene expression. The results showed that overexpression of Angptl4 markedly promoted the cell migration of HCT116 cells, and Angptl4 knockdown inhibited the cell migration of HT29 cells.

Materials and methods

Patients and tissue samples. A total of 41 frozen tumor and matched normal mucosa blocks retrieved from patients with colorectal adenocarcinoma who underwent surgery from June 2008 to January 2010 at the Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, China) were investigated in this study. There were 24 male and 17 female patients ranging in age from 26 to 85 years. Patients who received preoperative chemotherapy or chemoradiotherapy were excluded from this

study. This study was approved and monitored by the Ethics Committee of Sir Run Run Shaw Hospital.

Cell cultures. Human colorectal cancer cell lines COLO320, DLD1, HT29, SW480 and SW620 were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Grand Island, NY). RKO was cultured in RPMI-1640 medium (Hyclone, USA); HCT116 was cultured in McCoy's 5A medium (Hyclone), containing 10% fetal bovine serum (FBS) (Biochrom, Berlin, Germany), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA) and 100 μg/ml streptomycin (Sigma-Aldrich) under humidified conditions in 95% air and 5% CO_2 at 37˚C.

Cell transfection and stable colony selection. HCT116, with originally very low expression of Angptl4, was used for the gene overexpression assay, and HT29, with originally high Angptl4 expression, was used for the gene knockdown assay. For transfection, HCT116 cells were plated in 6-well plates at a density of $1x10^6$ cells per well and allowed to grow overnight to 70-90% confluency. Transfections were carried out with pCMV6-entry Angptl4-expressing vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as directed by the manufacturer. Empty vector (pCMV6-entry vector; OriGene, Rockville, MD, USA) was added as a control. Stable Angptl4-expressing clones were selected with 600 μg/ml G418 for 14 days; clones were picked and expanded for an additional 2 months for further research. HT29 cells were transfected with Angptl4 shRNA (OriGene) using the same method. To produce stably transfected cells, after transfection with the shRNA expression construct, 3 μg/ml of puromycin (Sigma) was added at 48 h to the medium. The cells were left in selective medium for 2 weeks, clones were picked, trypsinized and recultured in selective medium for propagation and further research.

Semi-quantitative RT-PCR. The extraction of total RNA was carried out using the TRIzol reagent (Invitrogen) according to the manufacturer's specification. First-strand complementary DNA (cDNA) synthesis was performed using a GeneAmp RNA PCR core kit (Perkin-Elmer, Branchburg, NJ). Briefly, mRNA from 2 μg of total RNA was reverse transcribed to cDNA in a 20-μl reaction volume containing 1X PCR buffer, 5 mM $MgCl_2$, 1 mM each of dNTP, 1 U/μl of RNase inhibitor, 2.5 U/μl MuLV enzyme and 2.5 mM Oligo-d(T)16 or random hexamer primer. The RNA was first denatured at 95˚C for 10 min before adding it to the reaction mixture. cDNA was synthesized at 42˚C for 1 h followed by 95˚C for 10 min and stored at -20˚C until use. PCR was performed to amplify Angptl4 with the following primers: forward: 5'-TCCTGGGA CGAGATGAATGT-3' and reverse: 5'-GGTCTAGGTGC TTGTGGTC-3', resulting in a DNA product of 352 bp. PCR conditions were 94˚C for 1 min, 52˚C for 30 sec and 72˚C for 30 sec, for 33 cycles, with initial denaturation at 95˚C for 5 min and a final elongation at 72˚C for 5 min. To normalize the amount of cDNA of each sample analyzed in the semi-quantitative RT-PCR, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene product was also amplified as an endogenous control of the expression. GAPDH forward primer: 5'-GAGTCCACTGGCGTCTTCA-3' and reverse primer: 5'-GGGGTGCTAAGCAGTTGGT-3', resulted in a
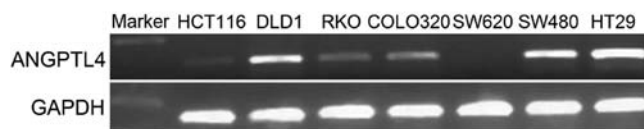

Figure 1. Relative Angptl4 mRNA expression level in seven colon cancer cell lines. Expression of GAPDH served as a control.

DNA product of 190 bp. Each cDNA sample was analyzed in triplicate with the appropriate negative controls. All PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide.

Direct enzyme-linked immunosorbent assay (ELISA). Angptl4 protein levels in the cell supernatants were measured by ELISA. Cells $(1x10^6)$ were cultured in full medium for 48 h, the medium was removed and 3 ml of FBS-free medium was added for another 24 h to the 6-cm dishes; at the end of the incubation the supernatants were harvested, and Angptl4 levels were measured by ELISA. Microwell plates (Costar) were coated with 100 μl cell culture supernatants (4 times dilution for HCT116, 2 times dilution for HT29) at 4˚C overnight. After incubation, the coated plates were washed four times with PBS-Tween and then incubated with the primary antibody (anti-ANGPTL4, BioVendor) for 2 h at room temperature. The primary antibody used in this study is a sheep polyclonal antibody against the human Angptl4 protein (BioVendor). After washing four times with PBS-Tween, the plates were coated with secondary antibodies (anti-sheep HRP) and incubated at room temperature for 1 h. The plates were washed four times with PBS-Tween again, and 100 μl of TMB peroxide-based substrate solution was added to each well. Ten minutes later 50 μl stop solution was added to stop the reaction. The absorbance values were read using a microplate reader at 450 nm.

Cell proliferation assay. Cells $(4x10^3)$ in 200 μl of medium were plated in 96-well plates and grown under normal conditions. Cultures were assayed at 0, 1, 2, 3 and 4 days, and absorbance values were determined on an enzyme-linked immunosorbent assay reader (BioTek, Winooski, VT, USA) at 570 nm. The experiments were performed in triplicate.

Soft agar assay. For the soft agar colony formation assay, ~500 cells were suspended in medium containing 0.3% low melt agarose, seeded into a 6-well plate that was overlaid with 0.5% low melt agarose, and allowed to grow for 2 weeks at 37˚C in 5% CO_2. Colonies containing >50 cells were counted under a microscope. Three to five wells were analyzed for each experiment.

Cell migration assay. Cells were trypsinized and resuspended in medium containing 1% FBS at a density of $1.5x10^6$ cells/ ml. In all, 100 μl of the cell suspension was added to the upper chamber of a Transwell (Corning, USA) consisting of inserts containing 8-μm pore-size PET membranes. Medium (600 μl) containing 10% FBS was placed in the lower chamber. After incubation for 24 h, cells remaining in the upper chamber were removed carefully using a cotton swab, and the membrane
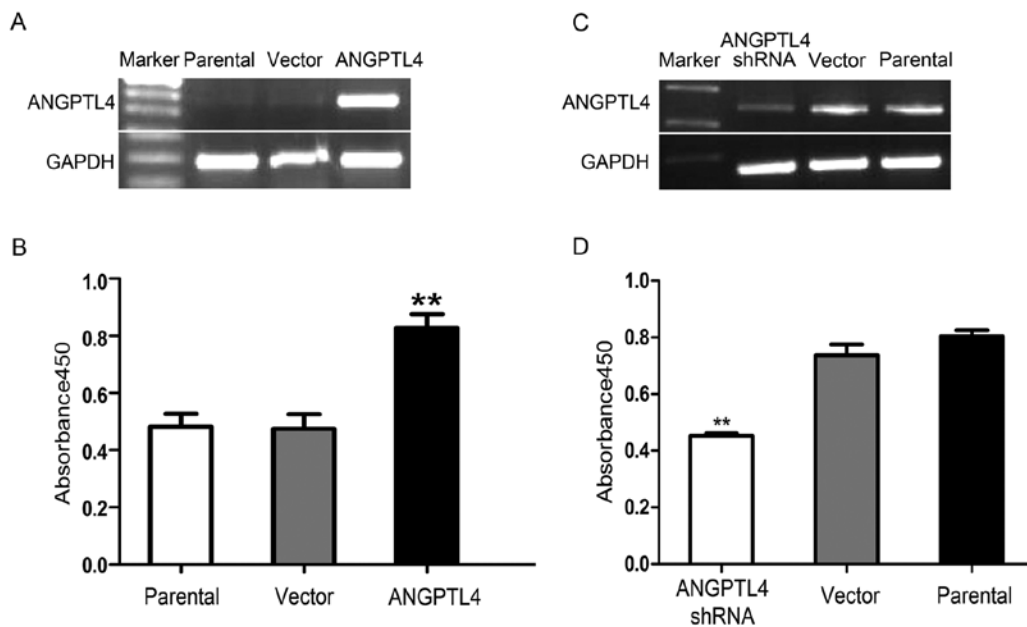
Figure 2. Stable transfection of Angptl4 in HCT116 cells. (A and B) Angptl4 mRNA and medium protein levels in the HCT116 cells increased significantly (**P<0.05) according to RT-PCR and ELISA assay; parent and vector-transfected HCT116 cells served as the control. (C and D) After HT29 cells were transfected with Angptl4 shRNA, Angptl4 mRNA and medium protein levels decreased significantly (**P<0.05) according to RT-PCR and ELISA assay; parent and vector transfected HT29 cells served as the control.

was cut off with a surgical knife. The side facing the lower chamber was stained with 0.05% crystal violet and attached cells were counted under a light microscope. The experiment was performed 3 times.

Fluorescence microscopy. Cells were cultured on glass coverslips and fixed in 3.7% paraformaldehyde in phosphate-buffered saline (pH 7.4) for 20 min, permeabilized in 0.1% Triton X-100 in phosphate-buffered saline for 5 min, blocked with 1% bovine serum albumin/phosphate-buffered saline for 1 h, and then incubated at room temperature for 1 h with rhodamine-conjugated phalloidin (Invitrogen) at 1:100 in blocking solution. Nuclei were counterstained with DAPI. Images were acquired with an Olympus BX51 microscope.

Western blot assay. Cell extracts were prepared with lysis buffer. Total protein concentration was measured by using a BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Forty micrograms of sample per well was loaded onto a 10% SDS-PAGE and transferred electrophoretically to a nitrocellulose membrane. After blocking in TBS-T buffer with 5% dry milk for 1 h at room temperature, the membranes were incubated sequentially with the primary antibody overnight at 4°C and HRP-conjugated secondary antibody (1:1000; Cell Signaling, Beverly, MA) for 2 h at room temperature in TBS-T milk. Primary antibodies were used as follows: anti-VASP rabbit antibody (1:1000; Cell Signaling); anti-phospho-VASP (Ser157) rabbit antibody (1:1000; Cell Signaling); anti-phospho-VASP (Ser239) rabbit antibody (1:1000; Cell Signaling); anti-ezrin/radixin/moesin rabbit antibody (1:1000; Cell Signaling); anti-phospho-ezrin/radixin/moesin rabbit antibody (1:1000; Cell Signaling); anti-E-cadherin antibody (1:1000; Cell Signaling); anti-CD44 antibody (1:250; BD) and anti-GAPDH monoclonal antibody (1:5000; Santa Cruz

Biotechnology, Santa Cruz, CA). Detection was carried out using the ECL kit (Amersham Biosciences, Piscataway, NJ, USA) and the blots were developed using a Fujifilm Las-4000 imaging system.

Animal experiments. Male BALB/c nu mice were housed in laminar flow cabinets under specific pathogen-free conditions. All of the *in vivo* experimental protocols were approved by the Animal Care Committee of Sir Run Run Shaw Hospital, Zhejiang University. Tumors were established by subcutaneous injection of $5x10^6$ viable HCT116/Angptl4 or HCT116/empty vector cells suspended in 100 μl PBS into the right flank of 5-week-old nude mice (n=5 for each cell type), respectively. Tumor size was monitored by measurement of the longest (L) and shortest (W) diameters, and the tumor volume was calculated using the formula: $V = 0.5 \times L \times W^2$.

Statistical analysis. Statistical analysis was performed using SPSS11.0 for Windows (SPSS Inc., Chicago, IL, USA). Data are presented as the means ± SD and were compared by the Student's t-test. P<0.05 was considered to indicate statistical significance.

Results

Analysis of Angptl4 expression in the colorectal cancer cell lines. The relative Angptl4 expression level in seven different colorectal cancer cell lines, COLO320, DLD1, HT29, HCT116, RKO, SW480 and SW620 was measured. Semi-quantitative RT-PCR showed that Angptl4 gene expression was high in the COLO320, DLD1, HT29, RKO and SW480 cell lines, but extemely low in the HCT116 and SW620 cells (Fig. 1). After stable transfection of Angptl4 in the HCT116 cell line, Angptl4 mRNA and medium protein levels in the HCT116
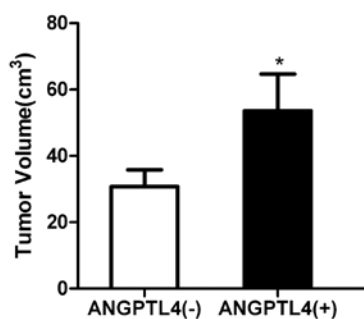
Figure 3. The tumor size of a total of 35 of 41 colorectal cancer tumors was measured and the volume was calculated by: $V = 0.5 \times L \times W^2$ (L is the longest and W the shortest diameters). The tumor volume of the Angptl4 expression group and the Angptl4-negative group was compared. The Angptl4 expression group showed a larger tumor volume (*P=0.04).

cells increased significantly as compared with that in the HCT116/vector cells according to RT-PCR and ELISA assay (Fig. 2A and B). In contrast, in the HT29 cells, after being transfected with Angptl4 shRNA, the Angptl4 mRNA and medium protein levels decreased significantly as compared with the values in the control group according to RT-PCR and ELISA assay (Fig. 2C and D).

Expression of Angptl4 in colorectal cancer and paired normal tissues. We analyzed Angptl4 mRNA expression status in 41 primary colorectal cancer cases. Angptl4 mRNA expression was detected in 41.5% (17/41) of the tumors, whereas Angptl4 mRNA expression was detected in 19.5% (8/41) of the normal colorectal mucosal tissues.

Correlation of Angptl4 expression with clinicopathological features of the colorectal cancer patients. We investigated whether there is any correlation between Angptl4 expression and the clinicopathological features of the colorectal cancer patients. The expression of Angptl4 in the tumor tissues was correlation with tumor volume (P=0.04) (Fig. 3). Positive Angptl4 expression was linked to larger tumor volume. The expression of Angptl4 mRNA expression was not correlated with patient gender, age, tumor grade, lymph node metastasis, TNM stage (data not shown).

Angptl4 inhibits colony formation of colorectal cancer cells in vitro, but no difference in cell proliferation was noted by the MTT assay. To investigate the effects of Angptl4 expression on the growth of colorectal cancer cells, the colony formation efficiency of the transfected cell line was evaluated by soft agar assays. Overexpression of Angptl4 significantly suppressed the colony-formation efficiency in HCT116 cells, while knockdown of Angptl4 promoted colony formation in HT29 cells (Fig. 4). There was no difference in cell proliferation noted in either the HCT116 or HT29 cells after Angptl4 overexpression or knockdown, respectively, according to the MTT assay (Fig. 5).

Angptl4 promotes the migration of colorectal cancer cells. To investigate whether Angptl4 plays an important role in cell migration, we compared the migratory ability of the cancer
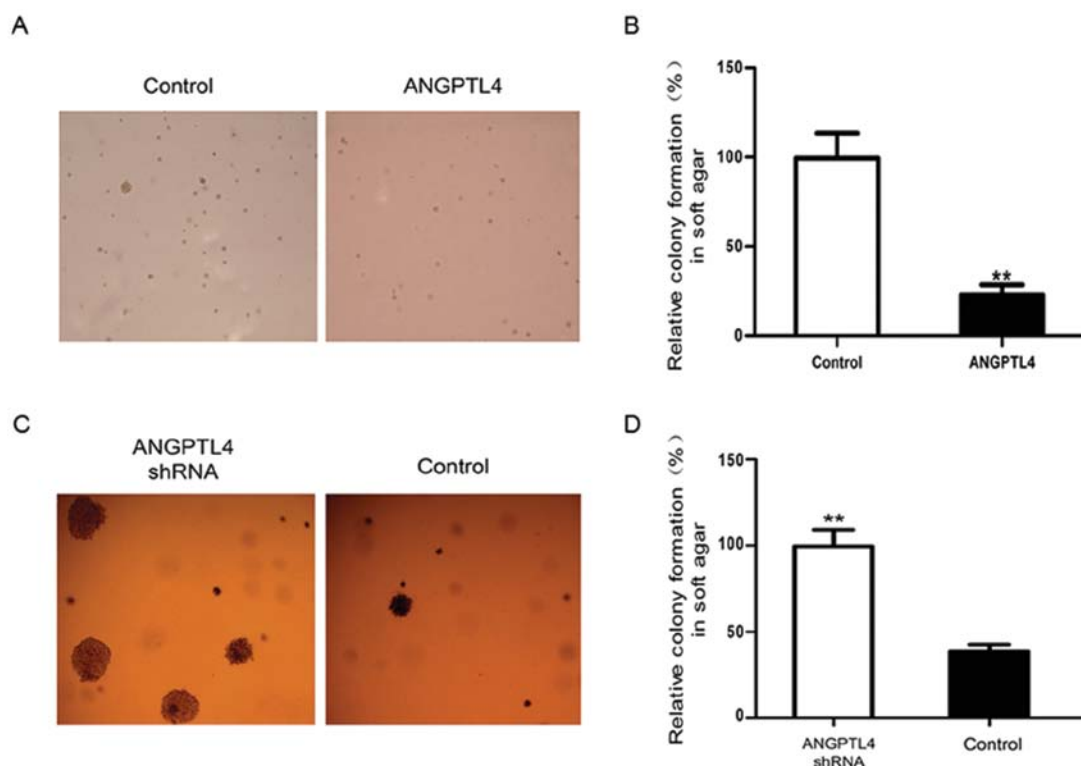

Figure 4. (A) Stable Angptl4-expressing HCT116 (HCT116/Angptl4) cells were assayed for colony formation (500 cells in 6-well plate) in soft agar. Colonies were counted and photographed after 2 weeks of growth (original magnification, x400). These results are representative of three independent experiments. A separate cluster with >50 cells was regarded as a clone. (B) Quantitative analysis of the colony formation efficiency. Error bars represent the standard error of the mean. **P<0.05 compared to the control cells. (C and D) Stable Angptl4 shRNA-transfected and scrambled shRNA-transfected HT29 cells were assayed for colony formation in a similar manner.
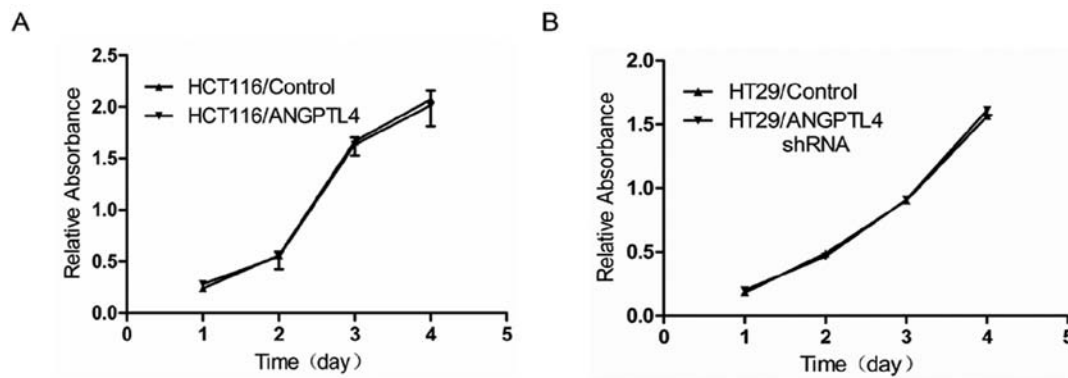
Figure 5. Cells ($4x10^3$) in 200 μl of medium were plated in 96-well plates for MTT assay. There was no difference in cell proliferation in the (A) HCT116 or (B) HT29 cell lines with Angptl4 overexpression or knockdown, respectively, compared with the control group. The experiments were performed in triplicate.
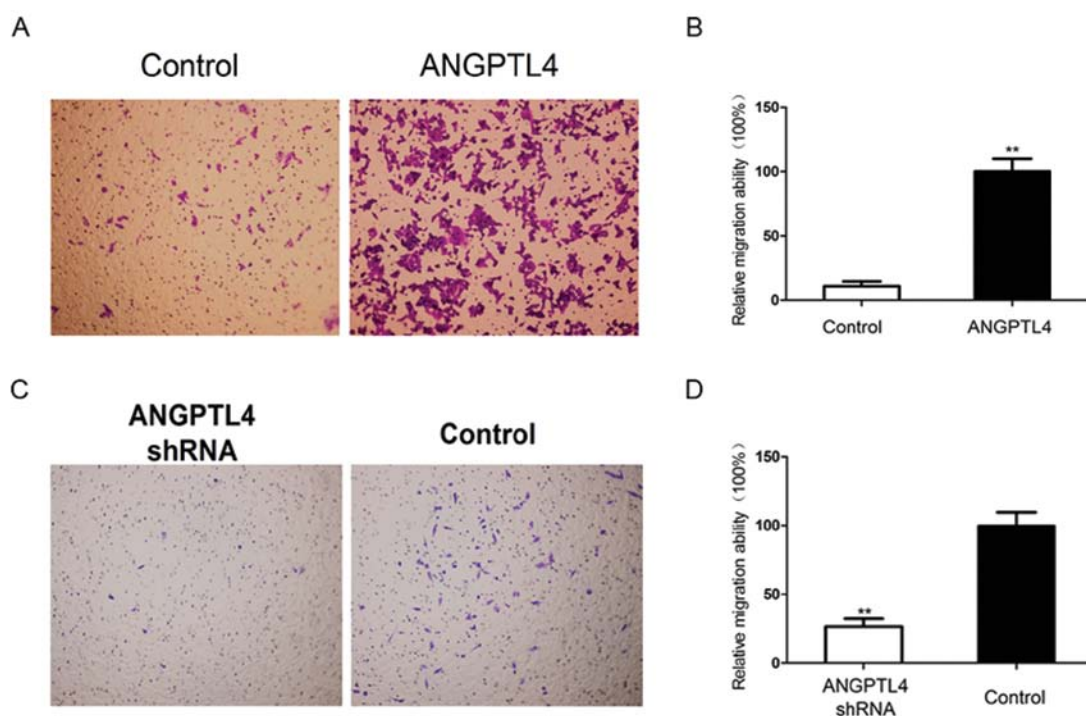

Figure 6. Migration and invasion of the HCT116/Angptl4 and control group was assayed by 24-Transwell system. (A) Images of migrated cells were captured 24 h after seeding (original magnification, x100). (B) Quantitative analysis of the cell migration and invasion. The number of cells that had migrated or invaded was counted in five representative high power fields per Transwell insert. Errors bars represented the standard error of the mean. **P<0.05 compared to the control cells. (C and D) Migration and invasion of the HT29/shRNA-Angptl4 and control group were assayed in a similar manner.

cells by a 24-Transwell system. As shown in Fig. 6A and B, Angptl4 expression led to a significant promotion of HCT116 cell migration after 24 h of incubation, while knockdown of Angptl4 expression resulted in significant inhibition of HT29 cell migration (Fig. 6C and D).

Angptl4 overexpression promotes actin reorganization and formation of pseudopodia. Reorganization of actin cytoskeleton is an early event of cell migration. Rhodamine-phalloidin staining revealed that HCT116 produced F-actin reorganization and formation of pseudopodia upon overexpression of the Angptl4 gene. This was consistent with a similar but opposite change in HT29 cells, which showed inhibition of the formation of pseudopodia in the Angptl4-knockdown clone (Fig. 7).

Overexpression or knockdown of Angptl4 is associated with a change in E-cadherin, vasodilator-stimulated phosphoprotein (VASP), CD44 and ezrin/radixin/moesin (ERM) expression. To address the possible molecular mechanism contributing to the effects of Angptl4 expression, we examined the effect of Angptl4 on the expression of cytoskeleton signaling, including ERM, VASP, cell adhesion molecule E-cadherin, and CD44 by western blot analysis. The HCT116 cells with overexpression of Angptl4 showed an increased expression of ERM, phospho-ERM, VASP and a decline in E-cadherin expression compared with the control group. In HT29 cells with Angptl4 shRNA knockdown a decline in CD44, ERM and VASP was observed compared with the control group (Fig. 8).
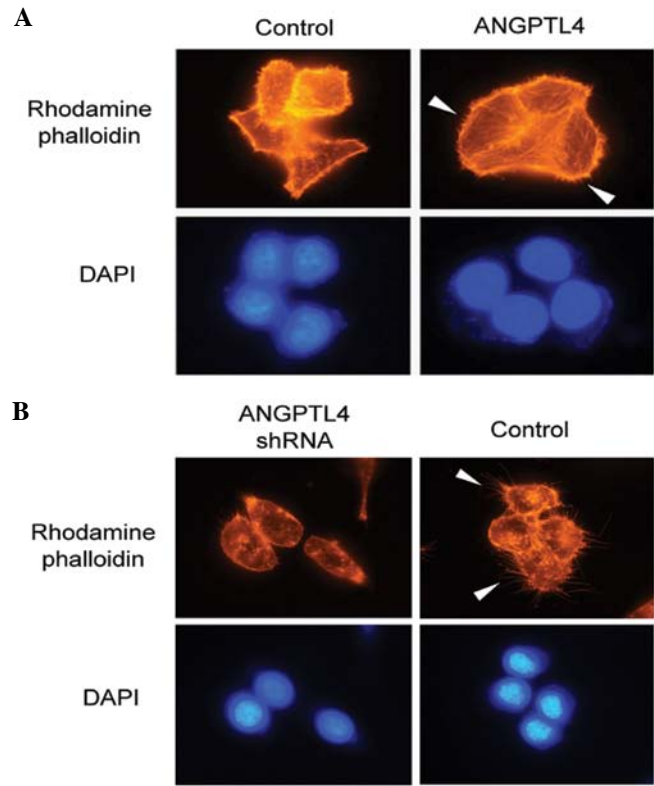
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Figure 7. Angptl4 overexpression promotes actin reorganization and formation of pseudopodia. (A) Rhodamine-phalloidin staining revealed that HCT116 cells with overexpression of the Angptl4 gene exhibited F-actin reorganization and pseudopodia formation, and (B) inhibition of the formation of pseudopodia in the Angptl4 knockdown clone of HT29 cells was noted. Arrowheads indicate formation of pseudopodia. Images were captured under magnification, x400.

Angptl4 inhibits tumor growth in nude mice. We aimed to ascertain whether Angptl4 suppresses the growth of colorectal cancer cells in nude mice *in vivo*. The tumor growth curve of stably transfected HCT116/Angptl4 expression vector and HCT116/control cells in nude mice is shown in Fig. 9. The tumor volume was significantly smaller in the nude mice bearing Angptl4-transfected cell tumors as compared with the control group.

Discussion

Angptl4 is a secreted protein involved in angiogenesis, glucose metabolism and lipid metabolism. It is consistently expressed in ischemic tissues and in the perinecrotic areas of different human tumors as a direct or indirect target gene of hypoxia (10), and may be involved in tumor progression. There have been several reports concerning Angptl4 protein or gene expression in cancer tissues (11-13), and in breast cancer, Angptl4 induction by TGF-β via the Smad signaling pathway primes lung metastases (14). Yet, the role of Angptl4 in CRC remains unclear. In the present study, we performed a semi-quantitative RT-PCR to detect mRNA expression of Angptl4 in 41 colorectal cancer and matched normal colorectal tissues. We also analyzed the correlation between Angptl4 mRNA expression and the clinicopathological features in colorectal cancer patients. Our results demonstrated that Angptl4 expression in colorectal cancer tissue was more frequent than in normal colorectal tissues, and the expression of Angptl4 was associated with tumor volume. The possible reason contributing to this result is hypoxia when tumors become larger in size.

The function of Angptl4 in colon cancer cell lines was then investigated by either overexpression or knockdown of its expression *in vitro*. Overexpression of Angptl4 promoted a marked increase in anchorage-independent colony formation as well as promotion of cell migratory ability in the HCT116 colon cancer cell line. In contrast, in the HT29 cells, knockdown of Angptl4 expression resulted in inhibition of cell migration. Notably, Rhodamine-phalloidin staining revealed significant formation of pseudopodia in HCT116/Angptl4 cells in contrast to the inhibiton of pseudopodia in the HT29/shRNA-Angptl4 cells, suggesting that Angptl4 promotes cell migration through the cytoskeleton pathway.

Reorganization of actin cytoskeleton is an early event in cell migration. Our further investigation revealed that in the HCT116 colon cancer cell line with high expression of Angptl4, the expression of cytoskeleton-related molecules such as ERM, phospho-ERM and VASP was increased, while phospho-VASP157 was reduced. These results indicate that overexpression of Angptl4 led to the activation of the downstream cytoskeleton signal pathway and cell migration. In addition, there was a decrease in E-cadherin expression upon
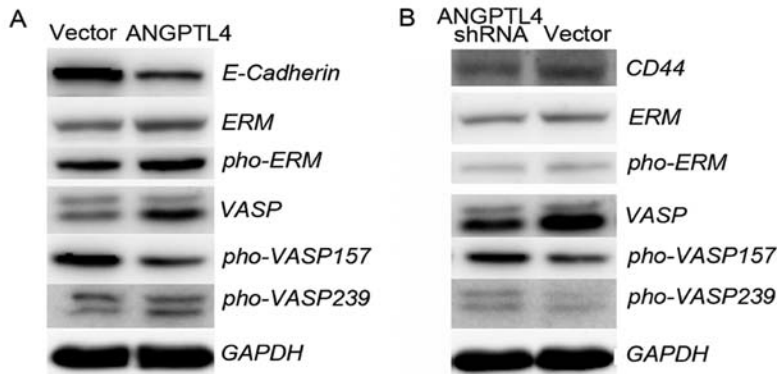

Figure 8. (A) Expression of several key cytoskeleton reorganization regulators and E-cadherin was compared between HCT116/Angptl4 cells and control group. (B) Expression of several key cytoskeleton reorganization regulators and CD44 was compared between HT29/shRNA-Angptl4 cells and control group.
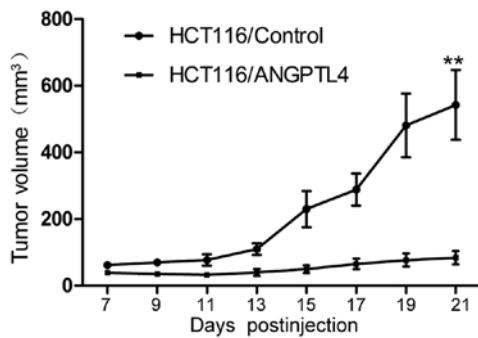
Figure 9. Tumor growth curves of stable Angptl4-overexpressing HCT116 cells and the control in nude mice in the tumor xenograft experiment. [**]P<0.05 compared to the control group.

Angptl4 overexpression. As known, diminished E-cadherin expression is a hallmark observed in many malignant cancers with invasive and metastatic potential (15,16). At present, it is unknown how Angptl4 expression results in the reduction of E-cadherin expression. After the knockdown of Angptl4 expression in the HT29 colon cancer cell line, a decrease in ERM, phospho-ERM and VASP was observed, consistent, but opposing, the changes in HCT116 cells overexpressing Angptl4. Notably, there was a reduction in CD44 expression after knockdown of Angptl4 expression in the HT29 colon cancer cell line. CD44 is a transmembrane cell-surface adhesion molecule that is synthesized in multiple isoforms because of alternative splicing of its pre-mRNA. CD44 has been found to support anchorage-independent growth *in vitro* and tumor growth and metastasis in experimental models of solid cancers (17-19). CD44 may also be involved in the directional motility of human colon cancer cells (20). CD44 may partial contribute to the cell migration inhibition in HT29 cells after knockdown of Angptl4 expression with shRNA in our experiments.

Notably, no cell proliferation effect was noted with over-expression of Angptl4 in the HCT116 colon cancer cell line *in vitro*, and overexpression led to the tumor growth inhibition *in vivo*, suggesting that the function of Angptl4 to promote cancer cell invasion is a late event during the multistep process of CRC initiation, promotion and progression.

In conclusion, our results revealed that Angptl4 is more frequently expressed in CRC cancer tissues than that in normal tissues, and overexpression promotes colon cancer cell migration through the cytoskeleton signal pathway. From our gathered data, it is possible to hypothesize that the development of a hypoxic microenvironment drives Angptl4 expression, and further promotes cancer invasion and metastases in CRC.

Acknowledgements

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