FoxO3a represses lymphangiogenesis in gastric cancer

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FoxO3a represses lymphangiogenesis in gastric cancer

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This certifies that the Master's Thesis of

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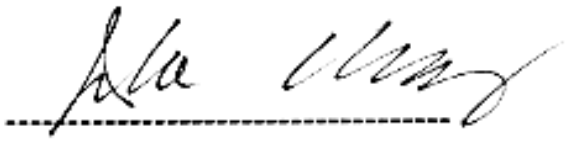

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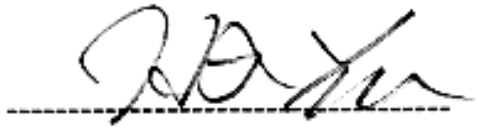

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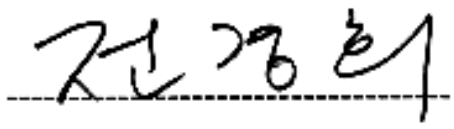

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언제나 항상 저를 믿어주시고 응원해 주셨기에 제가 지금까지 잘 성장할 수 있었던 것 같습니다. 자나깨나 저를 위해 항상 기도하시는 외할머니, 저를 항상 믿어주시고 지원해주시는 엄마, 뒤에서 응원해주시는 아빠, 딸처럼 하나부터 열까지 다 잘 챙겨주시는 외삼촌, 큰이모, 작은이모 그리고 공부하는 누나를 많이 이해해준 동생 문희에게도 고마움과 사랑을 전합니다.

2014년 12월, 논문을 마무리하며 모든 분들께 감사의 마음을 전합니다.

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ABSTRACT

FoxO3a represses lymphangiogenesis in gastric cancer

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Lymph node metastasis is the most important prognostic factor and is associated with about 60% of gastric cancer. For cancer cells to metastasize to lymph nodes, lymphangiogenesis is required. FoxO3a, a tumor suppressive transcription factor, is known to be a negative regulator of angiogenesis. Despite that lymphangiogenesis and angiogenesis in cancer are interrelated processes, the role of Foxo3a in lymphangiogenesis remains unknown. Thus, the goal of the study is to investigate the clinical significance and the role of FoxO3a in lymph node metastasis and lymphangiogenesis in gastric cancer. Foxo3a expression was analyzed across a large number of gastric cancer patient tissue samples with reverse phase protein microarray (RPPA). To study the biological function, FoxO3a was knocked down with RNA interference or overexpressed with an expression vector in gastric cancer cells. Tube formation and migration assays were carried out in human lymphatic endothelial cells (HLECs) with conditioned media of FoxO3a-silenced or overexpressed gastric cancer cells,

respectively. To assess the DNA binding activity of FoxO3a, electrophoretic mobility shift assay (EMSA) was performed. RPPA analysis revealed that FoxO3a expression was inversely correlated with lymph node metastasis and high expression of phosphorylated FoxO3a was a poor prognostic factor in gastric cancer patients. Silencing of FoxO3a in gastric cancer cells profoundly induced VEGF-C expression and secretion, increased tube formation, and migration of HLECs treated with the conditioned media while overexpression of FoxO3a in gastric cancer cells showed the opposite effects. EMSA with supershift assay demonstrated that FoxO3a binds to the FHRE in the promoter region of VEGF-C. Treatment of LY294002, a PI3K pathway inhibitor, reversed the phosphorylation of FoxO3a and increased the nuclear localization of FoxO3a in gastric cancer cells. Collectively, our data clearly illustrate that FoxO3a binds to VEGF-C promoter and transcriptionally represses the expression of VEGF-C thereby inhibiting tumor lymphangiogenesis.

Key words: foxO3a, vegf-c, lymphangiogenesis, gastric cancer

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Ⅰ. INTRODUCTION

Gastric cancer

Gastric cancer is the fourth most frequent malignancy and is the second leading cause of cancer-related death in the world[1-3]. The highest incidences of gastric cancer are reported in China, Japan, Korea, and other Eastern Asian countries. It is also one of the most common malignant tumors in Korea[4-6]. The overall prognosis of gastric cancer is poor with a 5-year survival rate below 30% for most countries[7]. Several potential risk factors include high salt diet, low intake of vegetables and fruits, smoking, chronic gastritis with glandular atrophy and intestinal metaplasia, and *Helicobacter pylori (H. pylori)* infection. The clinical outcomes of *H. pylori* infections have been shown to be

influenced by various genetic factors and induced the expression of pro-inflammatory cyclooxygenase enzyme (COX-2), which shows up-regulated expression in gastric cancer[8]. Some studies have demonstrated the importance of genetic and epigenetic alterations of oncogenes, tumor suppressor genes, and mismatch repair genes in the development of gastric cancer. Compared with other more extensively investigated cancers, such as breast, prostate, and colon carcinomas, the molecular mechanisms involved in the transformation and progression of gastric cancer are poorly characterized. In spite of improvements in early diagnosis and multimodality treatment to this disease, patients at advanced stage frequently have poor prognosis because of the high rate of metastasis[9]. About 60% of gastric cancers have lymph node metastasis, one of the most important prognostic factors[10]. In addition, preoperative staging has low sensitivity for discrimination in clinical characteristics including lymph node metastasis, and controversies remain about the choice of therapeutic regimens[11-13]. Thus, the identification of molecular prognostic markers bearing information on the cancer progression will help to provide a more effective therapeutic approach to the patients with gastric cancer.

Lymph node metastasis

The extent of lymph node (LN) metastasis is a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions. The prognosis of gastric cancer patients is also affected by the presence of LN metastasis[14-16]. Indeed, lymph node metastasis is the most significant prognostic factor for gastric cancer patients[17]. Although the clinical significance of LN involvement is well documented, little has been known about the molecular mechanisms that

promote tumor spread via lymphatic vessels to regional and distant LNs. The early step of lymph node metastasis is considered to be intravasation of the cancer cells into lymphatic vessels which are newly induced by lymphangiogenic factors secreted by cancer cells. In that order cancer cells migrate into the lymph nodes, and proliferate there to form a metastasis[18, 19]. Clinically, a trustworthy indicator is required to predict the presence of LN metastasis. Furthermore, molecular predictors of LN metastasis can be a prognostic marker and be of a potential therapeutic target.

Lymphangiogenesis

Lymphaniogenesis is a dynamic process during embryogenesis while is largely absent under normal physiological postnatal conditions[20]. Indeed, in adults, lymphangiogenesis only takes place during certain pathological conditions such as inflammation, tissue repair, and tumor growth[21]. Under pathological conditions, a major contribution has been established for the proliferation and sprouting of new vessels from preexisting lymphatic vessels. The relative contribution to new vessels from circulating endothelial progenitor cells remains unclear. The identification of several key lymphatic-specific molecular markers and factors that promote lymphatic vessel growth has propelled our understanding of the lymphatic vasculature in both physiological and pathological situations[22]. The first and most comprehensively studied, pro-lymphangiogenic factors identified are vascular endothelial growth factor (VEGF)-C and VEGF-D, which bind to a tyrosine kinase receptor, VEGF receptor 3, expressed on the lymphatic endothelium[23]. Lymphangiogenesis, the growth of new lymphatic vessels, was found to be critically required for solid tumor growth, invasion and distant lymph node metastases[24, 25]. Lymphatic vessels are tubular structures composed of mono-layered

lymphatic endothelial cells (LECs)[26, 27]. The migration and proliferation of LECs contributes to the sprouting of lymphatic vessels[28].

VEGF-C

Vascular endothelial growth factor (VEGF)-C is a member of the VEGF family of growth factors, which are conserved secreted glycoproteins that induce vasculogenesis, angiogenesis, lymphangiogenesis and are implicated in many physiological and pathological processes[23]. The VEGF family is comprised of VEGF-A,-B,-C,-D and –E. Of the three VEGF tyrosine kinase receptors identified thus far (i.e. VEGFR-1,-2 and -3), VEGFR-1 binds VEGF-A and VEGF-B, VEGFR-2 binds VEGF-A,-C,-D and –E, and VEGFR-3 binds VEGF-C and VEGF-D. VEGFRs differ with respect to mechanisms of regulation and patterns of expression. For example, VEGFR-1 and VEGFR-2 are expressed almost exclusively by vascular endothelial cells and hematopoietic precursors, whereas VEGFR-3 is widely expressed in the early embryonic vasculature but becomes restricted to lymphatic endothelium at later stages of development and in postnatal life. VEGF-C displays a high degree of similarity to VEGF-A, including conservation of the eight cysteine residues involved in intra- and intermolecular disulfide bonding. Like VEGF-A, both human and murine VEGF-C are alternatively spiced. In addition, VEGF-C mRNA is first translated into a precursor from which the mature ligand is derived by cell associated proteolytic processing after secretion. The post-secretion processing also allows VEGF-C to bind to VEGFR-2. Processed VEGF-C induces endothelial cell proliferation and migration, as well as increased vascular permeability. However, the respective roles of VEGFR-2 and -3 in mediating the biological effects of VEGF-C are incompletely understood. Unlike VEGF-A,

VEGF-C expression does not appear to be regulated by hypoxia. Based on its expression profile and its binding to VEGFR-3, VEGF-C has been implicated in the development of the lymphatic system[29]. Molecular mechanisms of tumor-associated lymphangiogenesis in cancer have been studied and identified VEGF-C and VEGF-D widely accepted as a key molecules of lymphangiogenesis that also play a critical role via VEGF receptor-3 (VEGFR3) on the surface of lymphatic endothelial cells (LECs)[20, 30-32]. VEGF-C and VEGF-D promote tumor-associated lymphangiogenesis and lymph node metastasis *in vivo* models[33-35]. Besides, VEGF-C and VEGF-D are often expressed in primary human tumors: they are secreted by tumor cells, tumor associated fibroblasts and immune cells[36-38]. It is reported that gastric cancer patients with high expression of VEGF-C protein had a poor prognosis than did those in low VEGF-C expression group[39, 40]. Further, VEGF-C binding to VEGFR-3 induces lymphatic vessel development and lymph node metastasis[41]. A significant correlation between lymph node metastasis and VEGF-C expression has been reported in gastric cancer[39, 42]. Other growth factors reported to be lymphangiogenic are fibroblast growth factor-2 (FGF-2)[43] and platelet-derived growth factor B (PDGFB) ([44]. The lymphangiogenic effect of FGF-2 occurs to be indirect via VEGF-C and D. Gastric cancer cells produce PDGFB that is a regulator of lymphangiogenesis in gastric cancer[44],[45]. In addition, Angiopoietin-2 is essential for establishing the lymphatic vasculature [46, 47]. VEGF-C, D/VEGFR-3 is a key for primary proliferation of lymphatic vessels, whereas angiopoietin-2 is crucial in later remodeling stages[43, 48].

FoxO3a

Forkhead box (FOX) proteins are superfamily of evolutionarily conserved transcriptional factors which play important roles in both normal biological processes and cancer development[49-51]. FOXO transcription factor members include FOXO1, FOXO3, FOXO4 and FOXO6[52-54]. These FOXO factors contribute to the regulation of various processes such as cell cycle regulation, cell size determination, apoptosis, differentiation, resistance to stress, DNA damage repair and energetic metabolism[55-59].

Recently, increasing FOXO subfamilies have been suggested to be involved in carcinogenesis and cancer metastasis[60-62]. Of note, oncogenic growth factor signaling pathways regulate FOXO function by phosphorylation[63]. FOXO transcription factors are directly phosphorylated by PI3K/AKT signaling pathway, resulting in their sequestration in the cytoplasm[64-67]. The FOXO1, FOXO3 and FOXO4 knockdown mouse has been shown to develop lymphomas *in vivo*[68]. Among FOXO transcription factors, FoxO3a was shown to be associated with tumor suppression activity and angiogenesis[69, 70]. Also FoxO3a inhibition promotes cell transformation, angiogenesis and tumor progression[71, 72]. In addition, ERK down regulates FoxO3a expression by directly phosphorylating FoxO3a at Ser294, Ser344 and Ser425, which leads to cell proliferation and tumorigenesis[73-75]. Aforementioned, FOXO functions as a tumor suppressor in cancer[76]. Loss of function of FOXO is a pivotal event in tumorigenesis[57, 77]. FOXO proteins contain a conserved DNA-binding domain (Forkhead domain) which recognizes the consensus sequence, TTGTTTAC[78-80]. An active form of FOXO translocates to the nucleus, binding to cognate DNA sequence and partner proteins, transcriptional co-activators or co-repressors, regulate the transcription of multiple target genes involved in tumor suppression[81-84].

FoxO3a binds to a consensus Forkhead response element (FHRE) in the VEGF promoter and then recruits histone deacetylase2 (HDAC2) to the promoter leading to decreased histones acetylation, and concomitant transcriptional inhibition of VEGF. FoxO3a dependent repression of VEGF in breast cancer cells was recently reported[85]. FoxO3a is a crucial downstream effector of the PI3K/AKT signaling pathway[86]. Phosphorylated by AKT, FoxO3a is mislocalized in cytoplasm and cannot function as a tumor suppressive transcription factor in cancer cells[62, 87].

PI3K/AKT pathway

The phosphatidylinositor-3-kinase (PI3K)/AKT signaling pathway is one of the critical signaling cascades that is estimated to be present in >30% of various types of human cancers, and plays a key role in the extracellular growth factor stimulation to various cellular processes, including cell proliferation, survival, migration, genomic instability, angiogenesis and metastasis[88]. Activation of PI3K is triggered by growth factor binding to receptor tyrosine kinases (RTKs). PI3Ks are lipid kinases that consist of three different classes (class Ⅰ, Ⅱ and Ⅲ) according to their structures and mechanism of activation. Class Ⅰ PI3Ks are composed of a catalytic subunit and a regulatory subunit. PI3K enzyme acts on membrane PI to generate the second messenger lipid PI-3,4,5-triphosphate. PI-3,4,5-triphosphate recruits phosphatidylinositol-dependent kinase 1 and AKT kinase to the membrane. AKT is a serine-threonine kinase that is regulated mainly following activation of the second messenger phospholipid kinase PI3K. The AKT kinase remains a subject of diverse investigations due mainly to its critical roles in a variety of pathways and cellular processes. AKT is activated through phosphorylation of Thr308 and Ser473[67].

In clinical trials, the number of drugs that target proteins involved in this pathway. For example, flavonoid derivative LY294002 is a PI3K inhibitor that acts in the ATP-binding site of PI3K enzyme and targets the PI3K/AKT axis.

Together with the previous knowledge and the notion that lymphangiogenesis and angiogenesis in cancer are interrelated processes, we hypothesize that tumor suppressive transcription factor FoxO3a would suppress lymphangiogenesis in gastric cancer cell line models.

Ⅱ. MATERIALS AND METHODS

1. Cell culture

The human gastric cancer cells SK4 and AGS were cultured in RPMI 1640 medium (Hyclone, South Logan, Utah)) containing 10 % fetal bovine serum, 100 U/ml of penicillin sodium and 100μg/ml of streptomycin sulfate at 37℃ in a humidified incubator containing 5 % CO_2. Cultured human lymphatic endothelial cells (HLECs) were purchased from Promo Cell (Promo Cell, Heidelberg, Germany). This cell line was maintained in complete medium (Endothelial cell Growth medium 1; Promo Cell, Heidelberg, Germany) on gelatin-coated dishes. HLECs were used between passages 5 and 8.

2. Establishment of stable FoxO3a Knockdown cells

For depletion of FoxO3a, short-hairpin RNAs (shRNAs) targeted to FoxO3a were purchased from Origene Technologies (Origene, Suite 200, Rockville, USA). Knockdown vector of FoxO3a was transfected into SK4 cells. SK4 cells were grown at 80% confluence and then transfected with 4μg knockdown vector of FoxO3a or Nontarget vector (Origene, NT-TR30013) for 24h using the TransIT-2020 transfection reagent (Mirus Bio, Madison, WI53711, USA)following the manufacturer's instruction. Stably transfected cell lines were selected using 4μg/ml Puromycin (Merck, Darmstadt, Germany). Stably transfected cells were harvested when plates were 80-90% confluent. Subsequently, stable knockdown cells, designated as SK4-shFoxO3a, were confirmed by western blot used in the

following experiments.

3. Establishment of stable FoxO3a-Overexpression cells

For overexpression of FoxO3a, human green fluorescent protein-FoxO3a construct was obtained from Origene Technologies (Origene, Suite 200, Rockville, USA). Overexpression vector of FoxO3a were transfected into AGS cells, AGS cells at 70-80% confluence and then transfected with 4μg of FoxO3a expression vector or empty vector (pCMV-AC-GFP) for 24h using TransIT-2020 transfection reagent (Mirus Bio, Madison, WI53711, USA) following the manufacturer's instruction. Stably transfected cell lines were selected using 400μg/ml G418 (Geneticin, an analog of neomycin). Stably transfected cells were harvested when plates were 80-90% confluent. Subsequently, stable overexpression cells, designated as AGS-FoxO3a, with successful optimization of gain of expression were determined with Western blotting.

4. siRNA transfection

To silence FoxO3a gene expression, SK4 cells were plated in 6-well plates at $1X10^5$ cells for 16 hour before transfection. siRNA complexes, prepared by incubating 25nM of indicated non-targeted RNA (NT) and FoxO3a –siRNA (Bioneer, Daejeon, Korea) with 8$\mu\ell$ X-tremeGENE siRNA transfection reagents (Roche, Werk Penzberg, Germany) for 20min, were added slowly to the cell plates in a final volume of 2ml. After treatment for 7 hour with the siRNA, the medium contacting siRNA and transfection reagents was removed and cells cultured for 48h with 10%FBS RPMI1640 fresh culture medium. Gene knockdown was assessed by western blotting at 48 hours after siRNA transfection.

FoxO3a small interfering RNA (siRNA) and non-targeted RNA (NT) were purchased from Bioneer (Bioneer, Daejeon, Korea). siRNAs with the following sense and antisense sequences were used:

FoxO3a construct 1, 5'-GACGAUGAUGCGCCUCUCU-3' (sense)

5'-AGAGAGGCGCAUCAUCGUC-3' (antisense);

FoxO3a construct 2, 5'-CAGUUCUAACUUCACUGUU-3' (sense)

5'-AACAGUGAAGUUAGAACUG'3' (antisense)

Non-target construct, 5'-CCUACGCCACCAAUUUCGU-3' (sense)

5'-ACGAAAUUGGUGGCGUAGG-3' (antisense)

5. ELISA

SK4 and AGS cell lines were seeded at $2X10^5$ cells/well of a $10cm^2$ dish and cultured for 24, 48, 72 hour. The amounts of VEGF-C in the cell medium were estimated using VEGF-C human ELISA kit (Abcam, Cambridge, UK) according to the manufacturer's instructions. For each experiment, triplicate samples were measured for statistical significance.

6. Tube formation assay

HLECs ($1X10^5$) were cultured in a 24well plate coated with 150$\mu\ell$ Growth factor reduced Matrigel in MV1 when cell attachment for 1 hour, the MV1 medium was replaced with conditioned medium and continues cell culture for 24 hour. Tube length was quantified after 8 hours by measuring the total cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program Image J. The original magnification used was X100.

7. Migration assay

HLECs (10^6/well) were seeding in MV1 media on 24 well plates. When the cells confluence 100%, we scratch on cells using tips and the media were removed from the cells and replaced with conditioned media from cultured media of gastric cancer cells. Migration of HLECs was determined after 12, 24 hour incubation.

8. Reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from gastric cancer cells using the Trizol reagent according to the manufacturer's protocol, and PCR was performed using the Onestep RT-PCR kit (iNtRON, Gyeonggi-do, Korea). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR amplification was performed using the oligonucleotide primers of FoxO3a sense 5'-AGAGCTGAGACCAGGGTAAA-3', antisense 5'-GACAGGCTTCACTACCAGATTC-3'; VEGF-C sense 5'-AGGCCACGGCTTATGCAA-3', antisense 5'-TAGACATGCATCGGCAGAA-3'; GAPDH sense 5'-GTCAGTGGTGGACCTGACCT-3', antisense 5'-TGTTGAAGTCAGAGGACACC-3', with 35 cycles of 30s at 94℃, 30s at 55℃, and 2min at 72℃. PCR products were resolved on 1.5% agarose gels, stained with ethidium bromide, and photographed.

9. Quantitative Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and real-time PCR reactions were conducted in an illumina Eco Real-time PCR system (Illumina, San Diego, CA). Complementary DNA (cDNA) was synthesized using the M-

MLV Reverse transcriptase (m.biotech, Hanam, Korea). Real-time PCR was performed on 2X QuantiSpeed SYBR No-Rox kit (Philekorea,Deajeon, Korea). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control. Primers used in real-time PCR were as follows: FoxO3a sense 5'-AGAGCTGAGACCAGGGTAAA-3', antisense 5'-GACAGGCTTCACTACCAGATTC-3'; VEGF-C sense 5'-TTCCACCACCAAACATGCAG-3', antisense 5'-GGGACACAACGACACACTTC-3'; GAPDH sense 5'-GTCAGTGGTGGACCTGACCT-3', antisense 5'-TGTTGAAGTCAGAGGACACC-3'.

Relative expression of FoxO3a and VEGF-C mRNAs was given as FoxO3a/GAPDH and VEGF-C/GAPDH. The thermal profile for PCR was 95℃ for 3min, followed by 40 cycles of 95℃ for 10s and 60℃ for 30s. Thermo cycling was carried out in a final volume of 10 $\mu\ell$ containing 1 $\mu\ell$ of a cDNA sample. Each sample was run in triplicate. The melting curve of each tube was examined to confirm a single peak appearance.

10. Western blot analysis

Gastric cancer cells were collected and lysed on ice for 30 min in lysis buffer [1% Triton X-100, 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 25 mmol/L β-glycerophosphate, 25 mmol/L NaF, 5 mmol/L EGTA, 1mmol/L EDTA as a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)]. The lysates were clarified by centrifugation for 13,000 rpm for 30 min. Equal amounts of protein were loaded onto a sodium dodecyl sulfate–polyacrylamide gel (12 % polyacrylamide) followed by electrophoresis at 100V for 3 hour and transferred to polyvinylidene floride membrane at 100V for 1 hour. Subsequently, PVDF membrane was incubated in TBS-T with 5% skim milk (blocking solution)

for 1 h at room temperature. The PVDF membrane was, respectively, incubated 4℃ overnight with the target primary antibody. Anti-FoxO3a (dilution 1:1,000; cell signaling Technology, Massachusetts, USA), Anti-phospho FoxO3a (dilution 1:1,000; cell signaling Technology, Massachusetts, USA), VEGF-C (dilution 1:1,000; cell signaling Technology, Massachusetts, USA) and anti-β-actin (dilution 1:1,000; Sigma-Aldrich, USA) antibodies were diluted in TBS-T (TBS/Tween 20: 2% skim milk). The appropriate secondary antibodies were applied (1:5000, horseradish peroxidase-conjugated anti-rabbit and anti-mouse) at room temperature for 1 hours. Labeled bands were detected by enhanced chemiluminescence (ECL; ThermoScientific, USA).

11. Electrophoretic mobility shift assay (EMSA) and super-shift assay

The DNA binding activity of FoxO3a was confirmed with a ^{32}P-labeled oligonucleotide containing FoxO3a transcription factor binding sites found in the VEGF-C promoter region. The DNA-protein binding detection kit (Promega, Madison, WI, USA) was used with modifications. In brief, DNA-binding reactions were carried out in a final volume of 25μl of buffer containing 10mM Tris (pH 7.5), 100mM NaCl, 1mM DTT, 1mM EDTA, 4% (w/v) glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 15μg of nuclear extract, and oligonucleotides. Oligonucleotides containing consensus VEGF-C (IDT, SanDiego, CA, USA) was end-labelled to a specific activity of 5×10^5 CPM with γ-[^{32}P]-ATP and T4-polynucleotide kinase, followed by purification on a Nick column (GE Healthcare, Piscataway, NJ). Reaction mixtures with radio-labelled oligonucleotides were incubated at room temperature for 20 minutes, and resolved on 6.5% non-denaturing polyacrylamide gels after addition of 3 μl bromophenol blue (0.1%). Gels were dried and subjected to autoradiography. For super-shift assays, 3

μg of antibody (Ab) was added for 20 minutes at room temperature after the initial incubation. Abs specific for were purchased from Cell Signaling Technology.

12. Immunofluorescence assay

Cells were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde for 10 min. Nuclei were premeabilized by treatment with 0.5% Triton X-100/phosphate-buffered saline for 5 min. Cells were blocked in 5% BSA/phosphate-buffered saline for 20 min and incubated with rabbit Foxo3a (Cell Signaling Technology, Danvers, MA, USA) for 1 h at room temperature. Subsequently, cells were incubated with Alexa Fluor 488 anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) for 1 h at 4℃ (1:2000). Images were collected using a confocal microscope (LSM Meta 700, Carl Zeiss, Oberkochen, Germany) and were analyzed using the Zeiss LSM Image Browser software program, version 4.2.0121.The intensity of nuclear staining was determined by using the area measurement

13. Chemicals

PI3K inhibitor, LY294002, was obtained from Invitrogen (Carlsbad, CA, USA) and dissolved in DMSO at a concentration of 50mM and stored at -20℃ until used.

14. Reverse Phase Protein Array (RPPA)

For RPPA assays, cells were lysed in lysis buffer (1% Triton X-100, 50mM HEPES, pH7.4, 150mM NaCl, 1.5mM MgCl2, 1mM EGTA, 100mM NaF, 10Mm Na Pyrophosphate, 1mM Na3VO4, 10%

glycerol, 1mM PMSF, and 10ug/mL aprotinin). Samples were incubated on ice for 30min and centrifuged at 13,000rpm. Protein concentrations in the resulting supernatants were determined by the BCA method (Pierce, Rockford, IL). After dilution to 1mg/mL concentration, lysates were boiled in 1%SDS. Five serial dilutions were prepared for each lysate on 96well plates using additional lysis buffer with 1% SDS, and samples then transferred to 384well plates. Protein was then spotted onto nitrocellulose-coated glass slides (FAST Slides, Schleicher & Solutions Inc.,). Slides were stored at -20℃ after printing.

To detect protein levels, the RPPA slides were first blocked for endogenous peroxidase, avidin, and biotin protein activity and then incubated with primary and secondary antibodies. Antibodies were purchased from Cell Signaling Inc., (Danvers, MA, USA). The antibody signals were amplified using a Dako cytomation catalyzed detection system.

15. Statistical Analysis

Student's t-test was used to evaluate the data. P values of <0.05 were considered statistically significant.

Ⅲ. RESULTS

1. Phosphorylated FoxO3a is prognostic factor in gastric cancer patients

The Forkhead box class O (FoxO) transcription factors are implicated in a broad array of cellular functions including cell differentiation, apoptosis and DNA damage[89]. Also, down regulation of FoxO proteins are associated with cancer progression and tumorigenesis [62]. To find out for prognostic factors in gastric cancer patients, we performed RPPA in tissue of 673 gastric cancer patients. Cox univariate analysis for proteins related to survival revealed an inverse correlation between the phosphorylated FoxO3a, inactive form of FoxO3a, and prognosis of gastric cancer patients. The cluster analysis of RPPA was presented as a heat map (Figure 1).

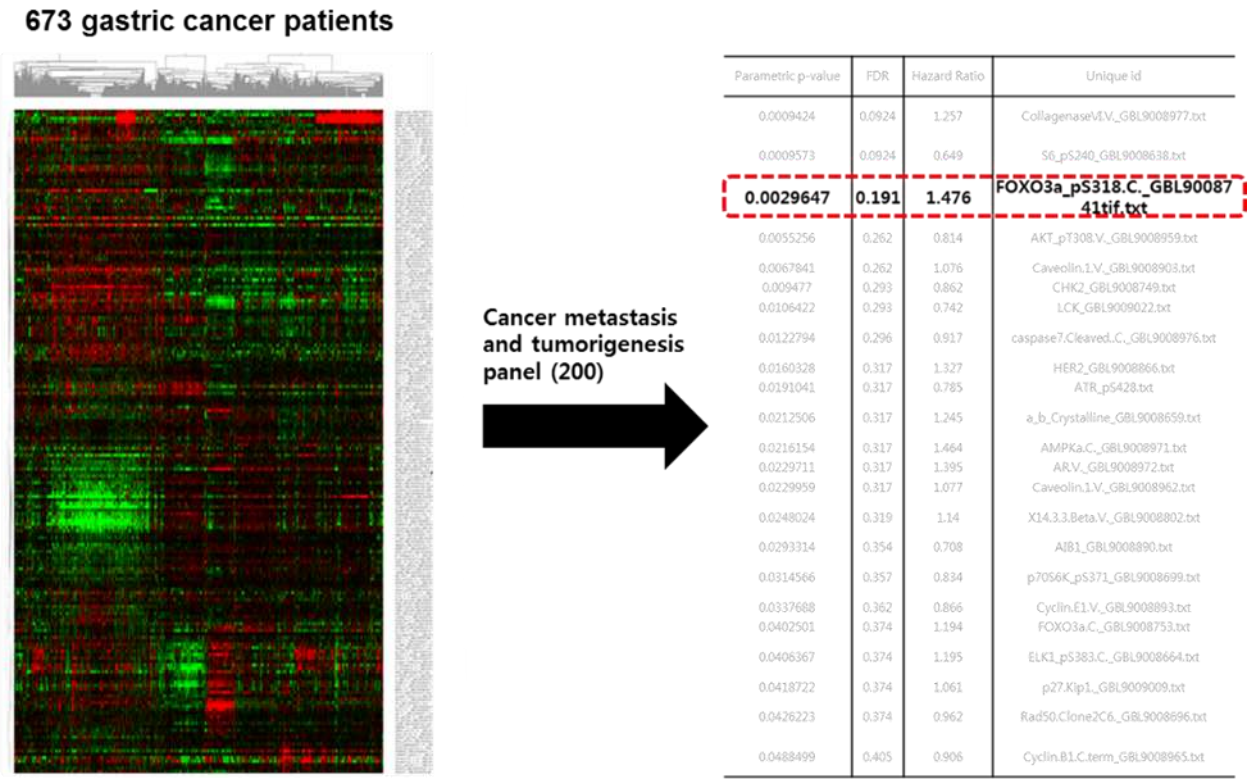


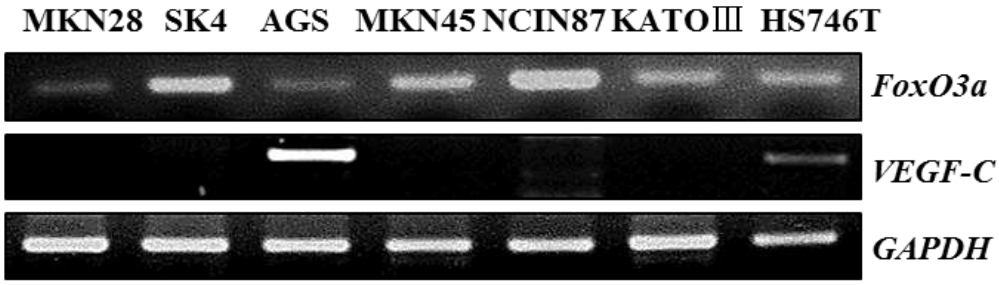
Figure 1. Exploring prognostic proteins using RPPA in gastric cancer patients tumors.

The result of reverse phase protein array (RPPA) was presented as a heat map. The color represents the expression level of the protein. Red represents high expression, while green represents low expression. Cox univariate analysis of protein expression profiling showed that proteins related to critical cancer pathways are prognostic in 673 gastric cancer patients. Among these, p-FoxO3 was a significant poor prognostic factor for gastric cancer.

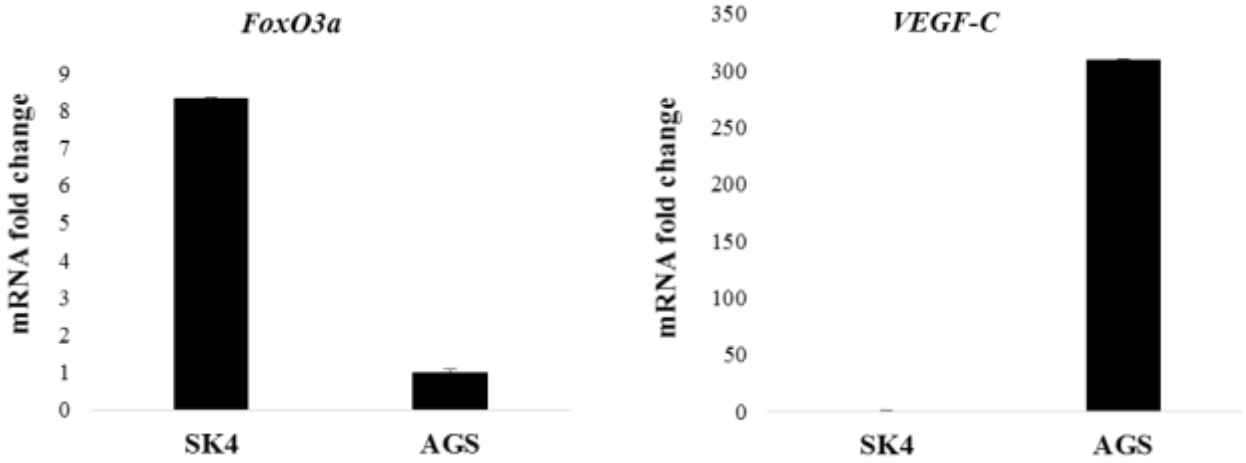
2. FoxO3a and VEGF-C expression showed a negative correlation in SK4 and AGS cell lines

To select model cell lines, we first examined the expressions of FoxO3a and VEGF-C in 7 gastric cancer cell lines (MKN28, SK4, AGS, MKN45, NCIN87, KATOⅢ, HS746T) by RT-PCR and immunoblotting (Figure 1A,C). FoxO3a mRNA was variously expressed in 7 gastric cancer cell lines examined whereas VEGF-C mRNA was expressed significantly only in AGS cells. For quantitative measurement, Real time RT-PCR was performed in SK4 and AGS cells. The expression levels were normalized against the house keeping gene *GAPDH*. There was an inverse relationship between *FoxO3a* and *VEGF-C* in gastric cancer cell lines of SK4 and AGS cells (Figure 1B). Based on these results, we selected SK4 and AGS as model cell lines for downstream analysis in this study.

(A)


MKN28 SK4 AGS MKN45 NCIN87 KATOⅢ HS746T
FoxO3a
VEGF-C
GAPDH

(B)


FoxO3a
mRNA fold change
0
1
2
3
4
5
6
7
8
9
SK4
AGS
VEGF-C
mRNA fold change
0
50
100
150
200
250
300
350
SK4
AGS

(C)

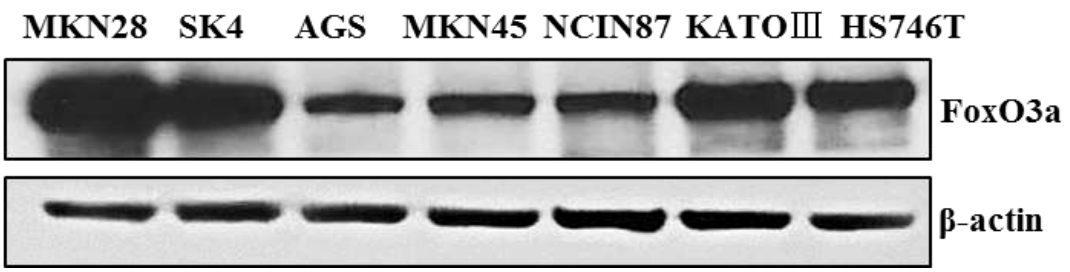

MKN28 SK4 AGS MKN45 NCIN87 KATOⅢ HS746T
FoxO3a
β-actin

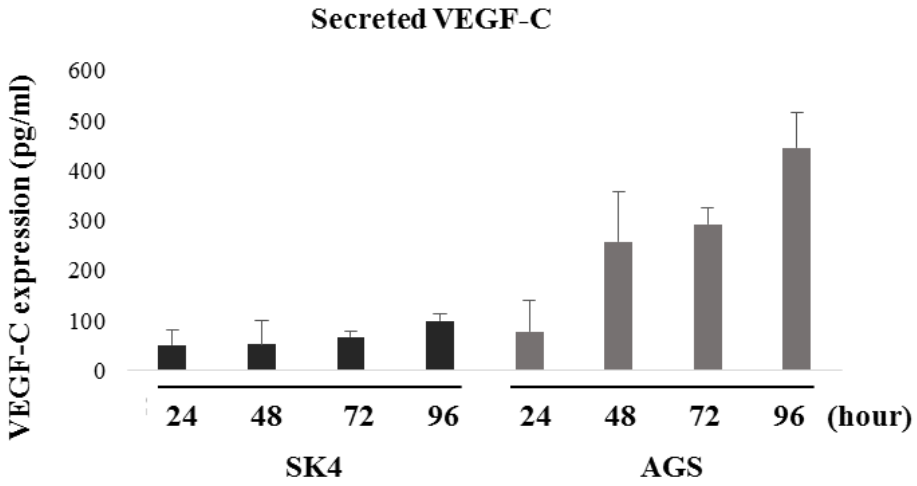
Figure 2. FoxO3a and VEGF-C expressions in gastric cancer cell lines

(A) *FoxO3a* and *VEGF-C* mRNA levels in 7 gastric cancer cell lines were analyzed by RT PCR. Total RNA (1ug) isolated using the Trisol was reverse transcribed using the SuperscriptⅢ reverse transcriptase and random primers, and the resulting first strand cDNA was used as template in the RT-PCR. *GAPDH* was used as loading controls. All experiments were performed in triplicate. (B)Total RNA was extracted from these cells and analyzed for *FoxO3a* and *VEGF-C* mRNA expression using real-time PCR (qRT-PCR). The expression of the *FoxO3a* gene in SK4 cell was high than AGS cell. In contrast, the expression of the *VEGF-C* gene in AGS cell was high than SK4 cell. (C) FoxO3a protein levels were analyzed in 7 gastric cancer cell lines by western blot assay using anti-FoxO3a antibody. β-actin level was used as loading controls. The experiment was repeated three times with reproducible results.

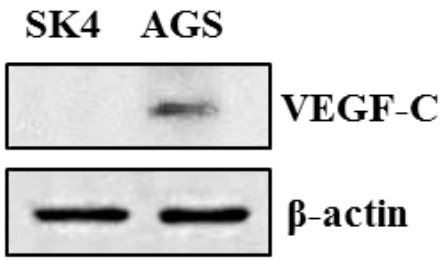
3. Secreted VEGF-C levels and tube formation efficiency of human lymphendothelial cells (HLECs) in conditioned media of AGS were higher than those in SK4 cells

Lymphangiogenesis, the sprouting of new lymphatic vessels from pre-existing ones, and the permeability of lymphatic vessels are regulated by vascular endothelial growth factor (VEGF)-C via its known receptors VEGFR-3 (Flt-4)[90]. Because VEGF-C is a soluble factor that can be released from gastric cancer cells, we next investigated whether the model cell lines, SK4 and AGS cells, secret VEGF-C and thereby tube formation of HLECs being stimulated. To this end, we examined the secreted VEGF-C from the conditioned media of SK4 and AGS cells, using ELISA and immunoblotting. We measured secreted VEGF-C expression in conditioned media of SK4 and AGS cells on day 1, 2 and 3 (Figure 3A). We then performed western blot analysis of secreted VEGF-C protein with conditioned media at 72hour time point in SK4 and AGS cells (Figure 3B). Higher proliferation and tubule formation efficiency were observed when HLEC was cultured in the conditioned media of AGS than of SK4 cells (Figure 3C, D and E). The results suggest that AGS cells with low-level FoxO3a expression secreted high level of VEGF-C thereby promoting HLEC proliferation and tubule formation more efficiently than SK4 cells.

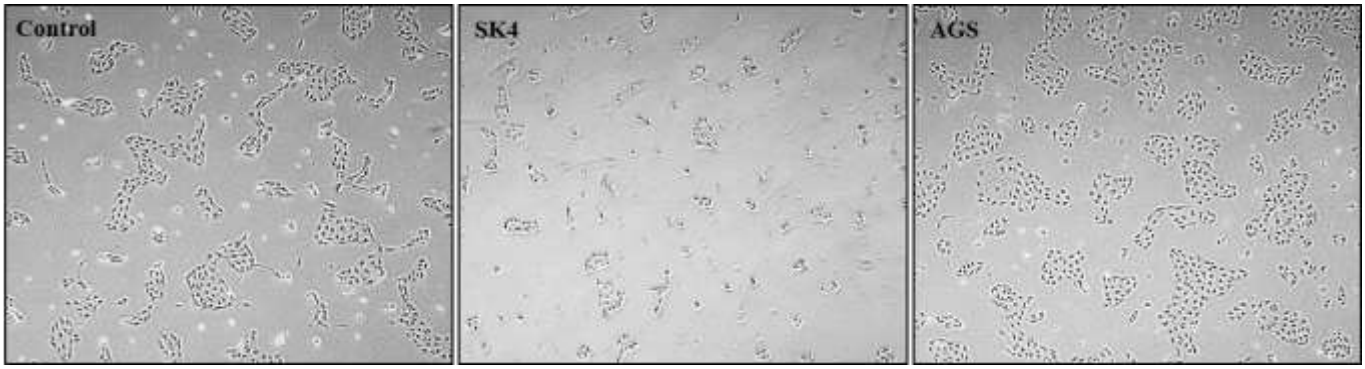
(A)


Secreted VEGF-C
VEGF-C expression (pg/ml)
600
500
400
300
200
100
0
24
48
72
96
24
48
72
96
(hour)
SK4
AGS

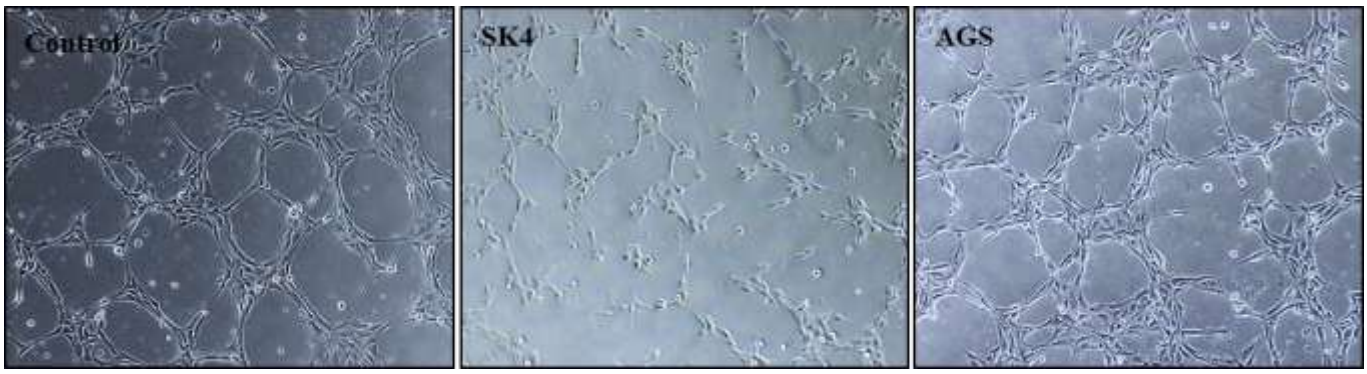
(B)


SK4
AGS
VEGF-C
β-actin

(C)


Control
SK4
AGS

(D)



(E)

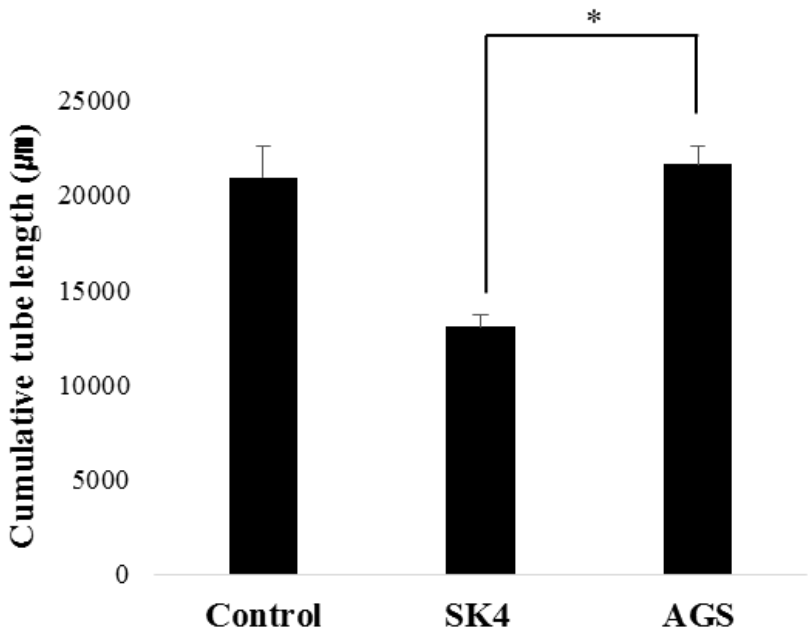


Figure 3. Secreted VEGF-C expression and tube formation of HLECs in conditioned media of gastric cancer cell lines

(A)The time course of the secreted VEGF-C expression in cultured media SK4 and AGS gastric

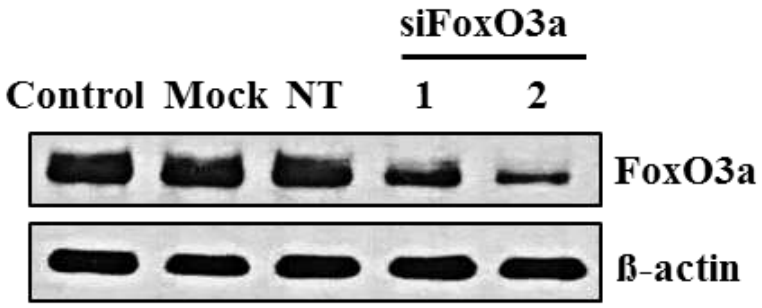
cancer cells. Conditioned media extracts were analyzed by VEGF-C ELISA kit. (B) Secreted VEGF-C expression in media of cultured gastric cancer cells were taken at 72h after seeding by western blot with media concentration method using a methanol. (C) The proliferation of HLECs with media of 72h cultured gastric cancer cells was observed in a 10 cm^2 dish. Cultured media of AGS cells induces HLECs proliferation more than SK4. (D) The tube formation of HLECs with media of 72h cultured gastric cancer cells was observed in a 24-well plate coated with growth factor reduced matrigel. (E)Tube length of tube formation quantified after 7hours by measuring the total cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program Image J. The original magnification used was X100. Data were expressed as mean +SD, n=3. Significant differences (* $P<0.05$) were determined by t-test using Graphpad prism 5.0 software.

4. Silencing FoxO3a in gastric cancer cells enhances the lymphangiogenic activity of HLECs

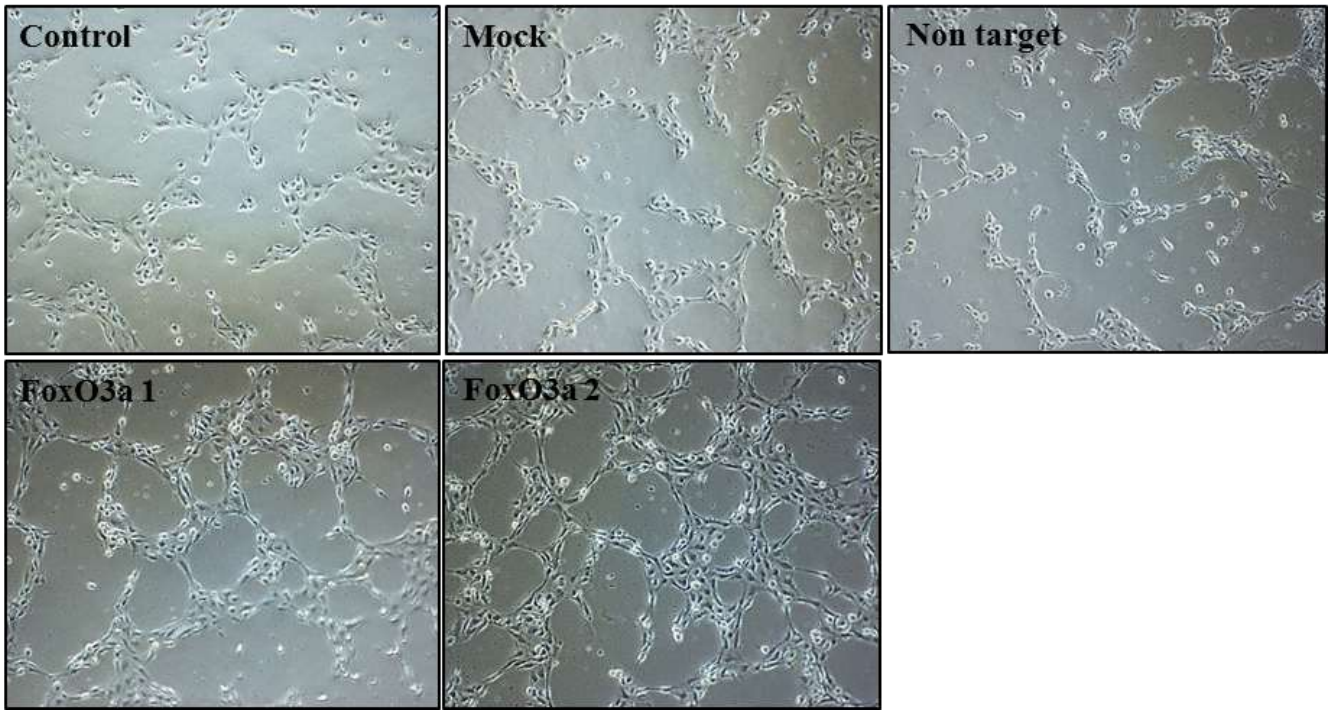
Based on the above results, we next investigated the effect of FoxO3a in gastric cancer cells on tube formation and migration of HLECs *in vitro*, which are crucial for tumor lymphangiogenesis. First, we examined the effect of FoxO3a suppression using siRNA. SK4 cells, highly expressing FoxO3a, were transfected with 2 different constructs of FoxO3a siRNAs.

After transfection, media changed with MV1 media and after 72hours the cells and conditioned media were collected. FoxO3a protein levels were determined by western bot. (Figure 4A). Human lymphatic endothelial cells (HLEC) were seeded on a growth factor reduced matrigel and examined the tube formation capacity Compared with control, vector only or non-target groups, tube formation of HLECs were significantly promoted by the conditioned media of FoxO3a knock-down SK4 cells (Figure 4B, C). Capillary-like structures were measured under light microscopy after 8 hours. In addition, the conditioned media of FoxO3a knockdown SK4 cells induced migration of HLECs (Figure 4D, E). Taken together, these results suggest that knockdown of FoxO3a in SK4 cells promote lymphangiogenesis.

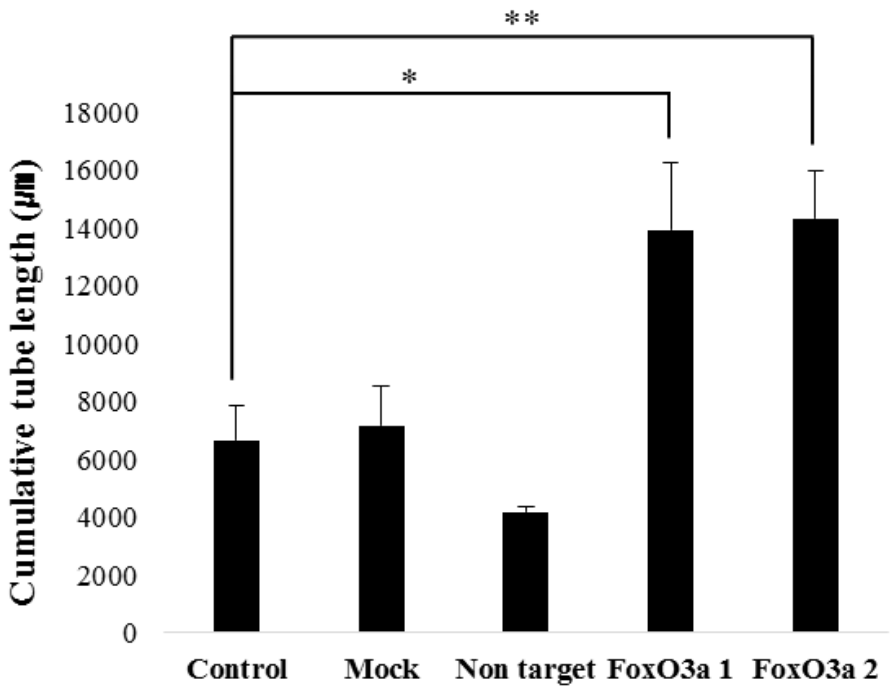
(A)


siFoxO3a
Control
Mock
NT
1
2
FoxO3a
ß-actin

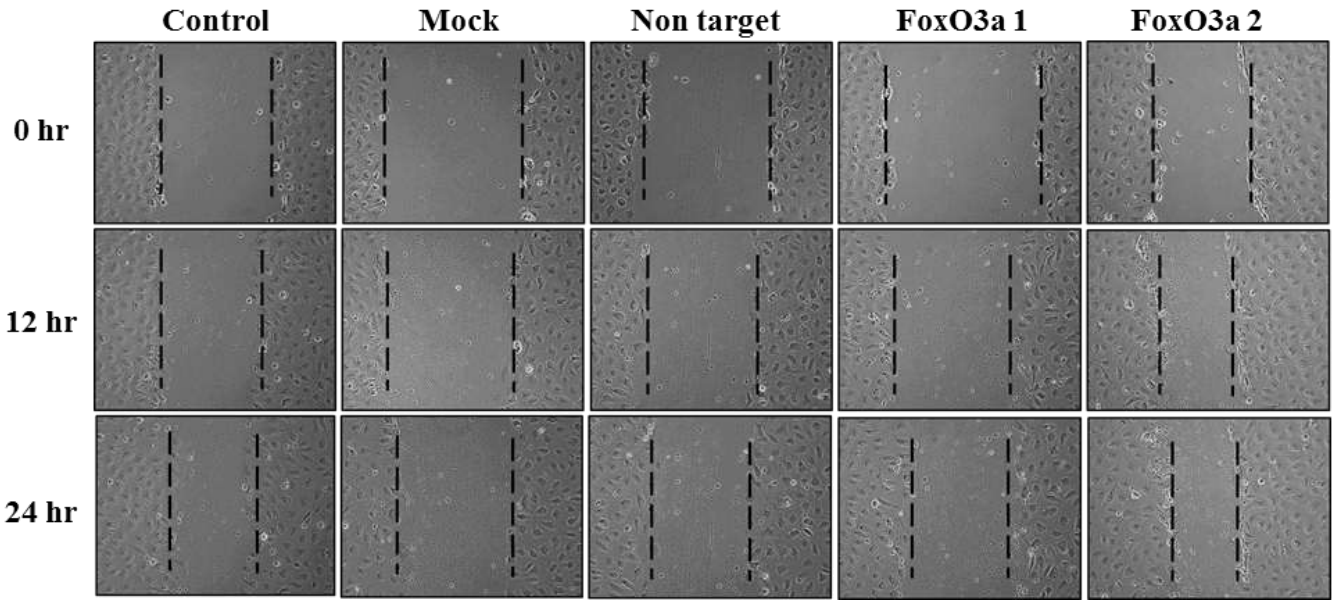
(B)


Control
Mock
Non target
FoxO3a 1
FoxO3a 2

(C)


**
*
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Cumulative tube length (μm)
Control
Mock
Non target
FoxO3a 1
FoxO3a 2

(D)


Control
Mock
Non target
FoxO3a 1
FoxO3a 2
0 hr
12 hr
24 hr

(E)

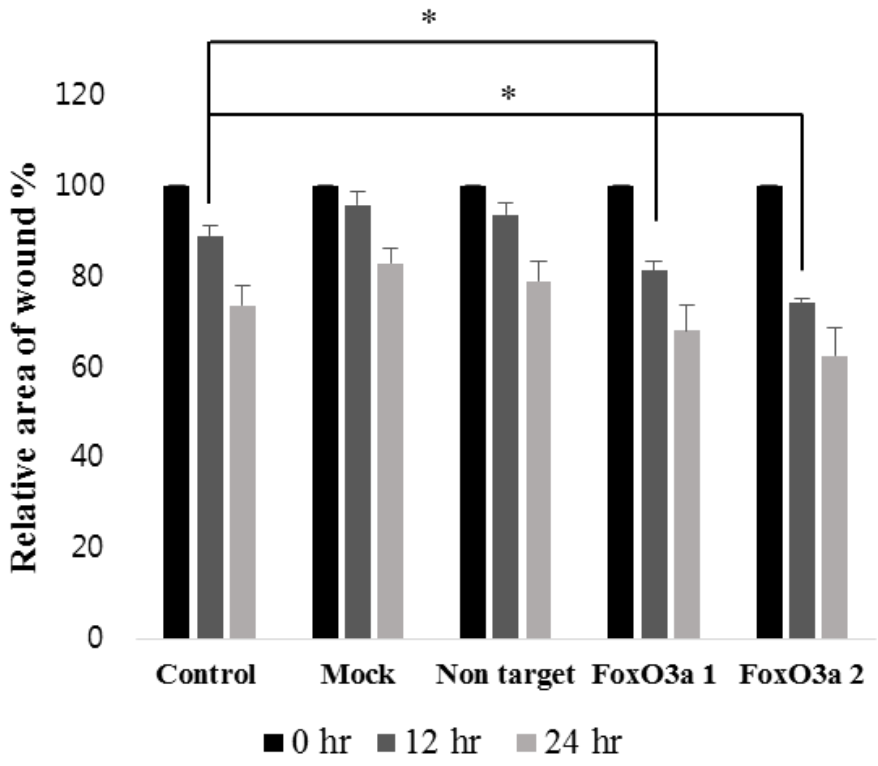


Figure 4. Knockdown of FoxO3a in SK4 cells enhances lymphangiogenesis

(A) FoxO3a was specifically down-regulated by FoxO3a siRNA. SK4 cells were trypsinized and seeded on 6well plates at $2X10^5$ cells per well. Cells were transfected with 25nM non-targeted siRNA and 2 different constructs of FoxO3a siRNAs for 7hour with 2% FBS RPMI1640 media. After 72h, the cells were collected and FoxO3a protein levels were determined by western blot. β-actin expression was monitored for normalization. (B) After transfection, media changed with MV1 media and after 72h the cells and cultured media were collected. The silencing of FoxO3a in SK4 cells profoundly induced tube formation of HLECs treated with corresponding conditioned media than in

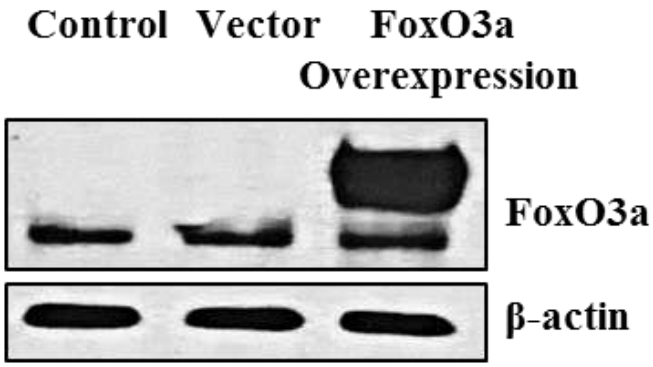
non-targeted control siRNA transfected cells. Images show tube formation 7 h after HLECs seeding on matrigel. The data represent the average from there independent experiments (error bars = SD). (C) The tube formation of HLECs quantified after 7hours by measuring the cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program Image J. The original magnification used was X100. Data were expressed as mean +SD, n=3. Significant differences (* $P<0.05$) were determined by t-test using Graphpad prism 5.0 software. (D) Migration of HLECs with cultured media of knocked down FoxO3a SK4 cells or with the vector only control transfected cells. (E)Images of approximate same field were taken at 12 and 24 hour after seeding, respectively. Representative images of three fields were shown (n=3, 100x).

5. Overexpression of FoxO3a in gastric cancer cells inhibits the lymphangiogenic activity of HLECs

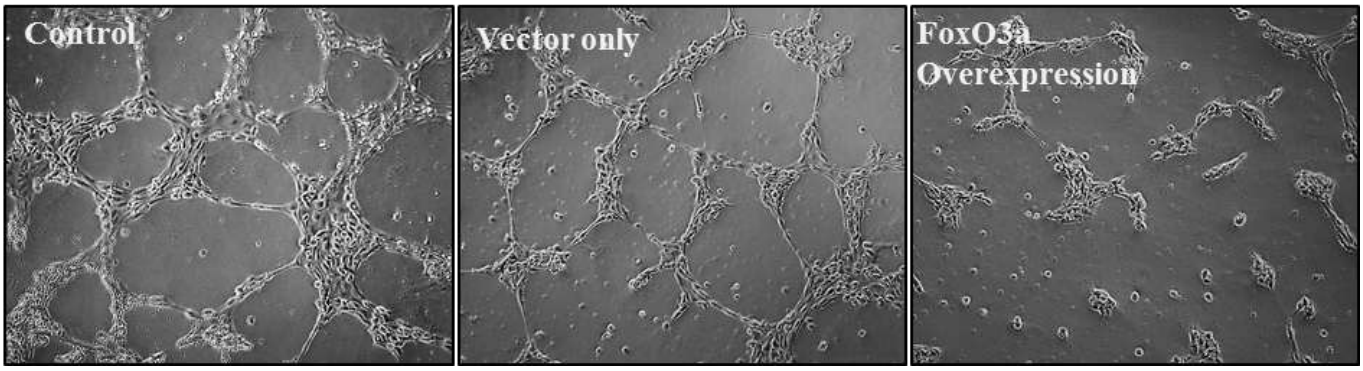
Next, we investigated the effect of FoxO3a overexpression in gastric cancer cells on lymphangiogenesis activity of HLECs. AGS cells, which expresses very low level FoxO3a, were transfected with the FoxO3a expression vector. After transfection, media changed with MV1 media after 72hour the cells and conditioned media were collected. FoxO3a protein levels were determined by western blot. (Figure 5A). To investigate the effect of FoxO3a expression in cancer cells on lymphangiogenic activity, HLECs were seeded on a growth factor reduced matrigel. Compared with control, vector only or non-target groups, tube formation of HLECs were significantly repressed by the conditioned media of FoxO3a overexpressed AGS cells. Capillary-like structures were measured under light microscopy after 8 hours. To quantify lymphangiogenesis, the parameters analyzed were cumulative tube length using an image J (Figure 5B, C). In addition, the conditioned media culture of FoxO3a overexpressed AGS cells suppress migration of HLECs (Figure 5D, E). These results suggest that overexpression of FoxO3a in AGS cells repress lymphangiogenesis.

Collectively, we found that FoxO3a expression in cancer cells has a negative effect on lymphangiogenesis in our model systems.

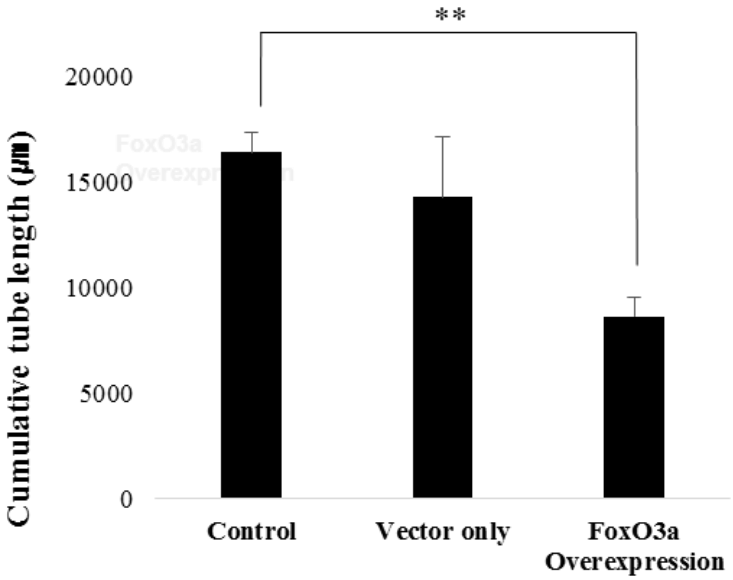
(A)


Control
Vector
FoxO3a Overexpression
FoxO3a
β-actin

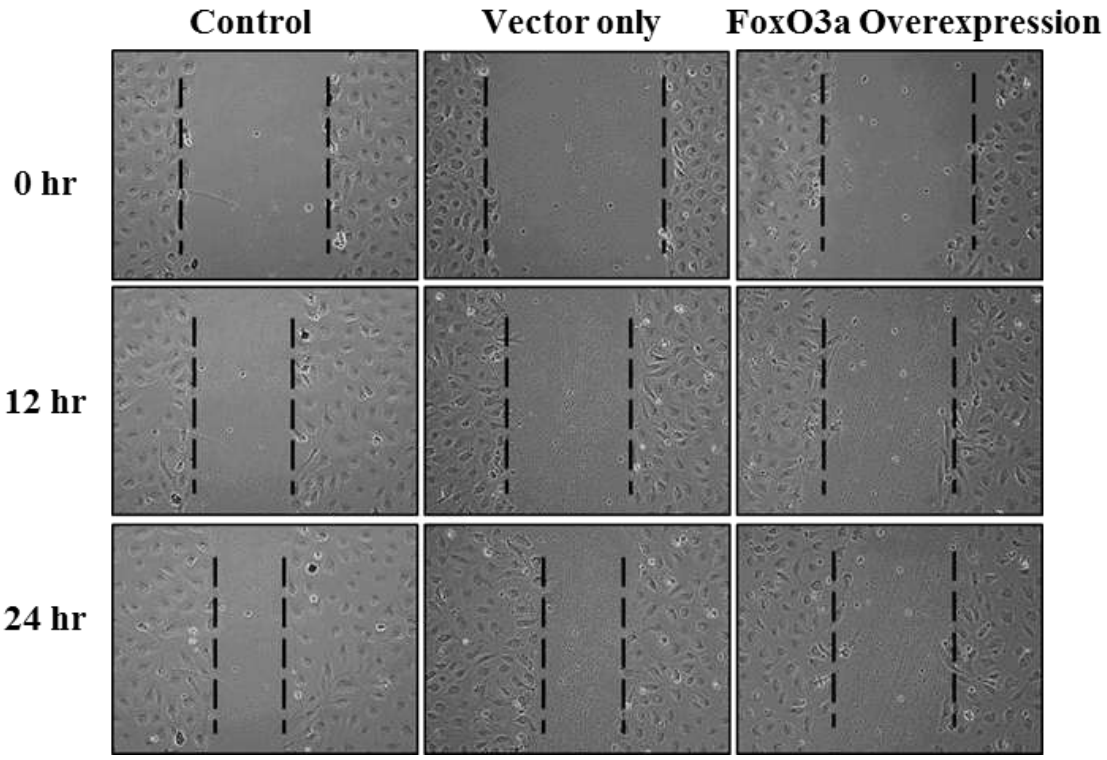
(B)


Control
Vector only
FoxO3a Overexpression

(C)


**
20000
15000
10000
5000
0
Cumulative tube length (μm)
Control
Vector only
FoxO3a Overexpression

(D)


Control
Vector only
FoxO3a Overexpression
0 hr
12 hr
24 hr

(E)

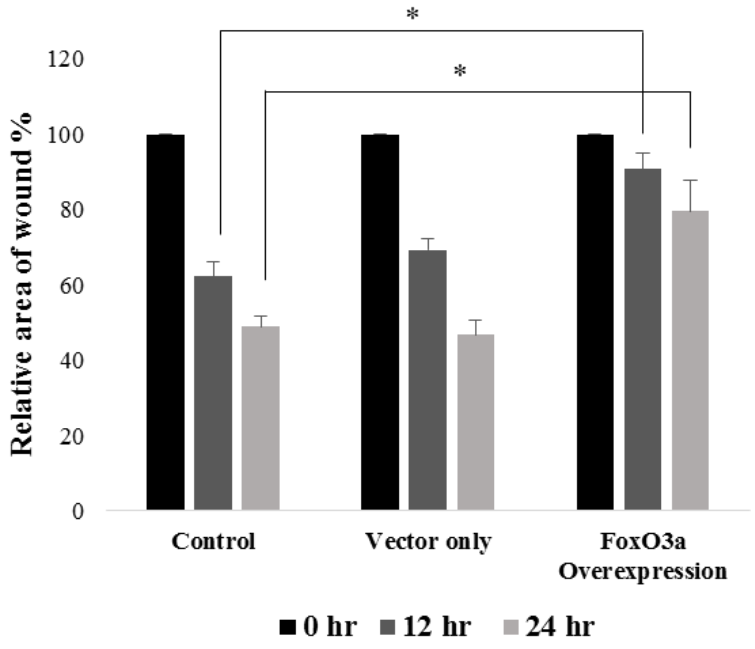

*
*
120
100
80
60
40
20
0
Relative area of wound %
Control
Vector only
FoxO3a Overexpression
0 hr
12 hr
24 hr

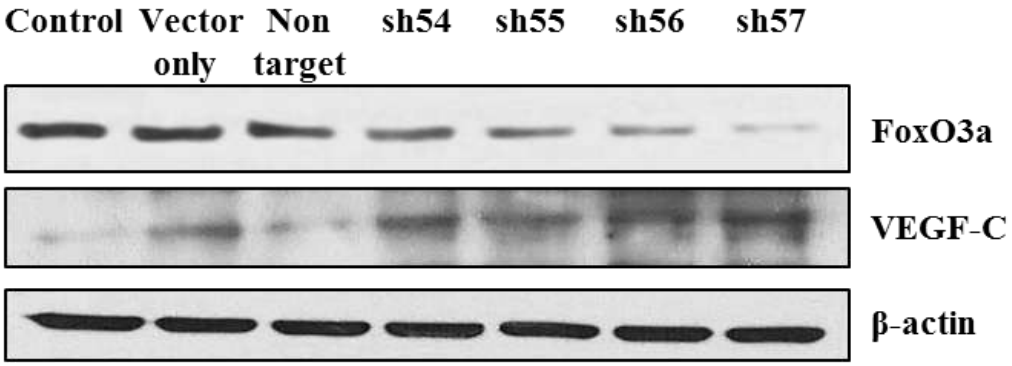
Figure 5. FoxO3a overexpression in AGS cells represses lymphangiogenesis

(A)Foxo3a was overexpressed in AGS cells by FoxO3a overexpression plasmid vector. AGS cells were trypsinized and seeded on 6well plates at $2X10^5$ cells per well. Cells were transfected with 1 μg plasmid vector for 7hour with 2% FBS RPMI1640 media. After 72h, the cells were lysed and subjected to western blot analysis with FoxO3a antibody. β-actin expression was monitored for normalization. (B) After transfection, media changed with MV1 media and after 72h the cells and cultured media were collected. The FoxO3a overexpression in AGS cells profoundly reduced tube formation of HLECs treated with corresponding conditioned media than in vector only control transfected cells. Images show tube formation 7 h after HLECs were seeded on matrigel. The data represent the average from there independent experiments (error bars = SD). (C) The tube formation of HLECs quantified after 7hours by measuring the cumulative tube length in 3 random microscopic fields with a computer-assisted microscope using the program Image J. The original magnification used was X100. Date were expressed as mean +SD, n=3. Significant differences (*$P<0.05$) were determined by t-test using Graphpad prism 5.0 software. (D) The migration of HLECs with cultured media of FoxO3a overexpressed AGS cells or with the vector only control transfected cells. (E) Images of approximate same field were taken at 12 and 24hour after seeding, respectively. Representative images of three fields were shown (n=3, 100X).

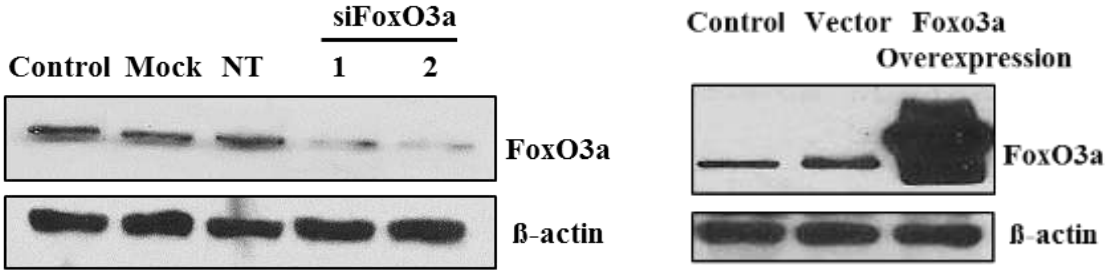
6. FoxO3a represses VEGF-C expression in gastric cancer cells

Together with the observation that knockdown of transcription factor FoxO3a in gastric cancer cells promote lymphangiogenesis in HLECs, we next investigated whether the FoxO3a in gastric cancer cells would transcriptionally regulate the expression of lymphangiogenic factors. A recent study showed that FoxO3a could bind to the VEGF-A gene promoter and inactivate transcription of VEGF-A[85]. Interestingly, we found that the FoxO3a binding site, FHRE, is also present in VEGF-C gene promoter region. VEGF-C is the most representative molecule inducing lymphangiogenesis in cancer. Therefore, we set out to investigate whether FoxO3a regulate VEGF-C expression and thereby influencing tube formation efficiency of HLECs. To do this, secreted VEGF-C in the conditioned media of gastric cancer cells were detected using western blot with media concentration method and ELISA. We used FoxO3a shRNAs to inhibit FoxO3a signaling in FoxO3a high expressing cells, SK4 cell. As a result, when FoxO3a was knocked down in SK4 cells, secreted VEGF-C was significantly increased (Figure. 6A). Also, when FoxO3a was knocked down or overexpressed using either siRNA or plasmid vector respectively, FoxO3a and secreted VEGF-C expression showed a negative correlation in SK4 and AGS cells. Secreted VEGF-C was measured by specific VEGF-C ELISA (Figure 6B, C). These results indicate that FoxO3a repressed lymphangiogenesis of HLECs by transcriptionally downregulating VEGF-C.

(A)


Control
Vector only
Non target
sh54
sh55
sh56
sh57
FoxO3a
VEGF-C
β-actin

(B)


siFoxO3a
Control
Mock
NT
1
2
FoxO3a
ß-actin
Control
Vector
Foxo3a Overexpression
FoxO3a
ß-actin

(C)

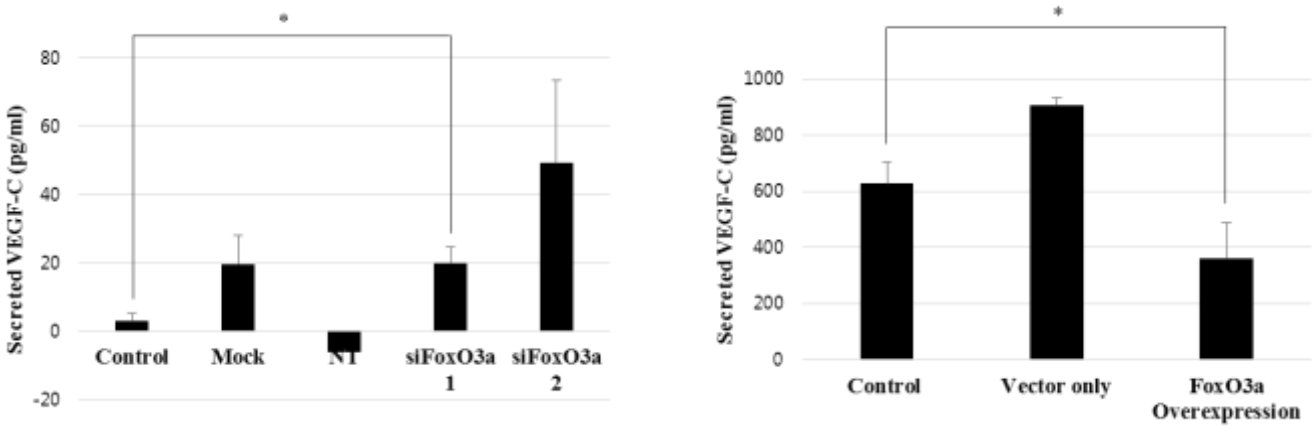

*
Secreted VEGF-C (pg/ml)
80
60
40
20
0
-20
Control
Mock
NT
siFoxO3a 1
siFoxO3a 2
*
Secreted VEGF-C (pg/ml)
1000
800
600
400
200
0
Control
Vector only
FoxO3a Overexpression

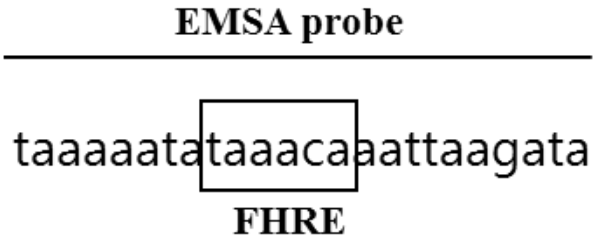
Figure 6. Silencing of FoxO3a in SK4 enhances the VEGF-C expression

(A)Western blot analysis showing inducible shRNA knockdown of endogenous FoxO3a in SK4 cells. shFoxO3a line was seed at 10^5 cells per well in 6-well plates and cultured for 72 hour. At this time point, using a conditioned media, secreted VEGF-C expression was estimated by western blot with media concentration method using a methanol. (B) FoxO3a was specifically down-regulated by FoxO3a siRNA in SK4 cells. Foxo3a was overexpressed in AGS cells by FoxO3a overexpression plasmid vector. SK4 and AGS cells were trypsinized and seeded on 6well plates at $2X10^5$ cells per well. Cells were transfected with 2% FBS RPMI1640 media for 7hours. After 72h, the cells were collected and FoxO3a protein levels were determined by western blot. β-actin expression was monitored for normalization. (C) Secreted VEGF-C was measured in conditioned media by specific VEGF-C ELISA.

7. FoxO3a binds to the FHRE of VEGF-C promoter region

To investigate whether FoxO3a directly binds to VEGF-C promoter region thereby repressing transcriptional activity, we next performed electro mobility shift assay (EMSA) to directly show that FoxO3a binds to the FHRE in promoter region of VEGF-C (Figure 7A). The probe corresponding to the VEGF-C promoter region was designed as described under "Materials and Method". Our results showed that there was a shifted band when the probe was employed. When the five-fold nuclear protein was used in the test, the shifted band was induced significantly. The results suggest that FoxO3a binds to the FHRE of VEGF-C gene promoter (Figure 7B). Taken together, these results demonstrated that FoxO3a can repress the VEGF-C through binding to VEGF-C promoter region.

(A)


EMSA probe
taaaaatataaacaaattaagata
FHRE

(B)

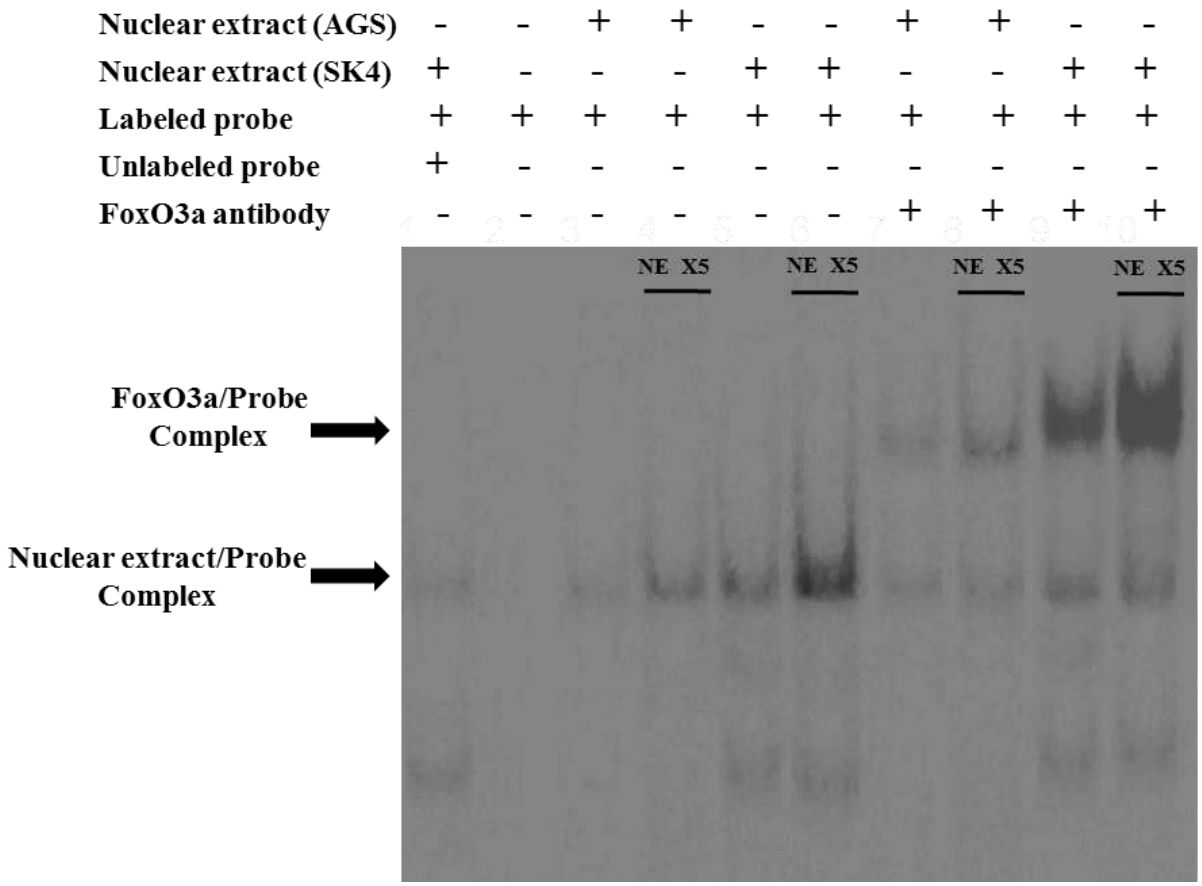

Nuclear extract (AGS) - - + + - - + + - -
Nuclear extract (SK4) + - - - + + - - + +
Labeled probe + + + + + + + + + +
Unlabeled probe + - - - - - - - - -
FoxO3a antibody - - - - - - + + + +
1 2 3 4 5 6 7 8 9 10
NE X5 NE X5 NE X5 NE X5
FoxO3a/Probe Complex
Nuclear extract/Probe Complex

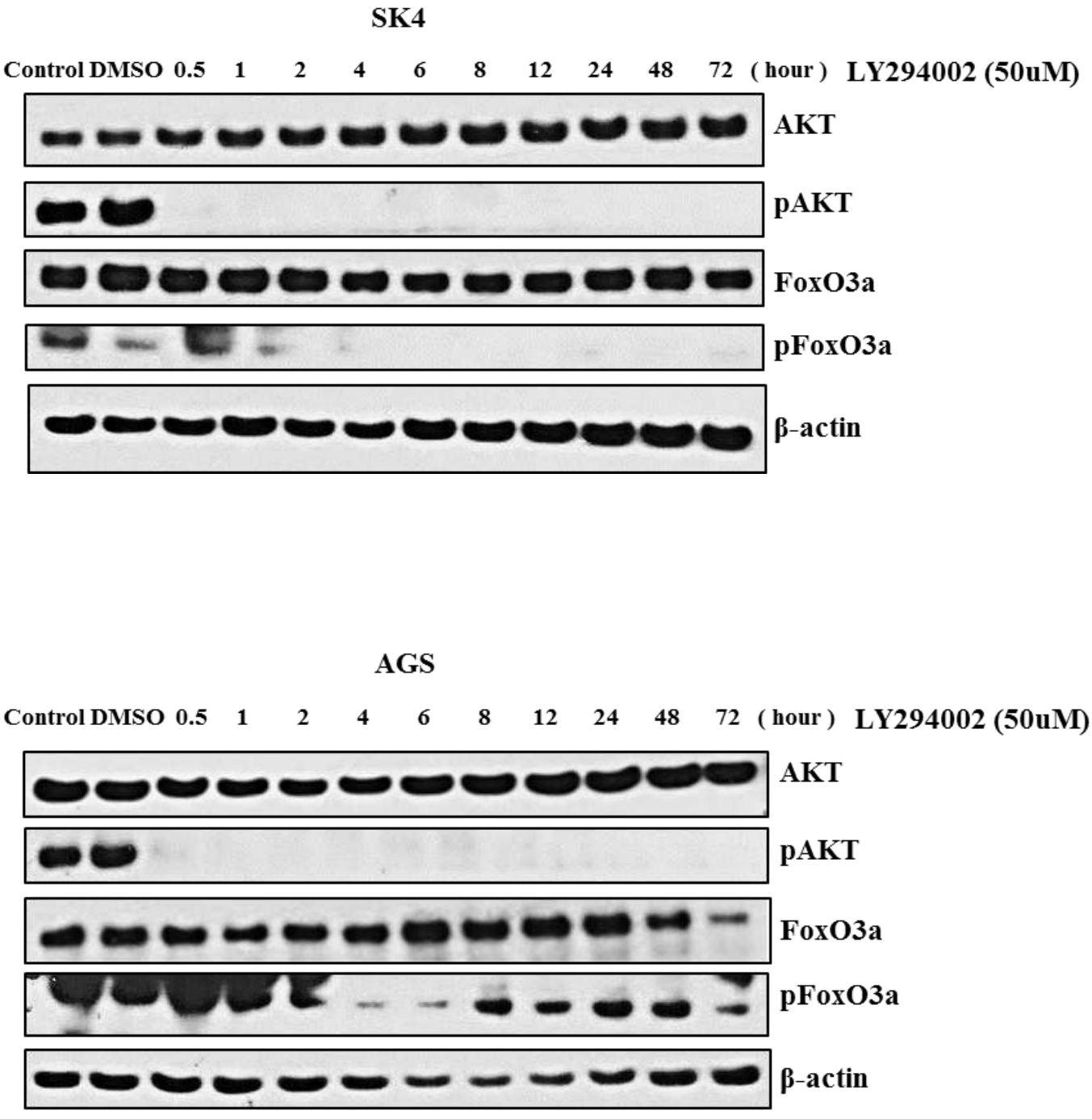
Figure 7. FoxO3a binds to the FHRE of VEGF-C promoter region

(A) Design of the probe including forkhead response element (FHRE) of *VEGF-C* promoter region. (B) Incubation of nuclear extract in SK4 and AGS with ^{32}P-labeled FoxO3a-*VEGF-C* sequence produced a DNA-protein band shift. To confirm the binding of FoxO3a to the FoxO3a-*VEGF-C* sequence, these EMSA reactions were further incubated with anti-FoxO3a antibody. The addition of this antibody resulted in a super shifted complex in addition to the DNA-protein band. These data confirmed the presence of FoxO3a in the nuclear protein complex that binds the FoxO3a binding site of the *VEGF-C* promoter.

8. FoxO3a is inactivated by PI3K/AKT pathway

The PI3K/AKT signaling pathway is activated in many human cancers, and plays a key role in the extracellular growth factor stimulation to various cellular processes, including cell proliferation, survival, and angiogenesis[88]. PI3K downstream is the serine/threonine kinase AKT. AKT is activated through phosphorylation of Thr308 and Ser473[67]. FoxO3a is a crucial downstream target of the PI3K/AKT signaling pathway[86]. FoxO3a function is suppressed by PI3K/AKT pathway. When phosphorylated by AKT, FoxO3a is inactivated and cannot function as a transcription factor in cancer cells[62, 87]. Specific small molecule inhibitors of PI3K (LY294002) were used to suppress endogenous PI3K pathway. To identify the FoxO3a regulation by PI3K/AKT pathway in gastric cancer cells, we examined the time course of phosphorylated AKT (p-S473) and p-FoxO3a (p-S253) expressions in SK4 and AGS cells with LY294002 (50uM) by western blot. As a result, we observed that LY294002 treatment decreased phosphorylation levels of AKT and FoxO3a in SK4 and AGS cells. (Figure 8A). After 2 hours of treatment, we observed FoxO3a localization using immunocytochemistry (ICC) method. Of note, SK4 cells which express high level of FoxO3a at baseline showed minimal change in nuclear localization of FoxO3a after LY294002 treatment compared to AGS cells which showed dramatic increase in nuclear localization of FoxO3a upon LY294002 treatment (Figure 8B). These results suggest that FoxO3a nuclear localization that is dependent on phosphorylation is regulated by the PI3K/AKT pathway.

(A)


SK4
Control DMSO 0.5 1 2 4 6 8 12 24 48 72 (hour) LY294002 (50uM)
AKT
pAKT
FoxO3a
pFoxO3a
β-actin
AGS
Control DMSO 0.5 1 2 4 6 8 12 24 48 72 (hour) LY294002 (50uM)
AKT
pAKT
FoxO3a
pFoxO3a
β-actin

(B)

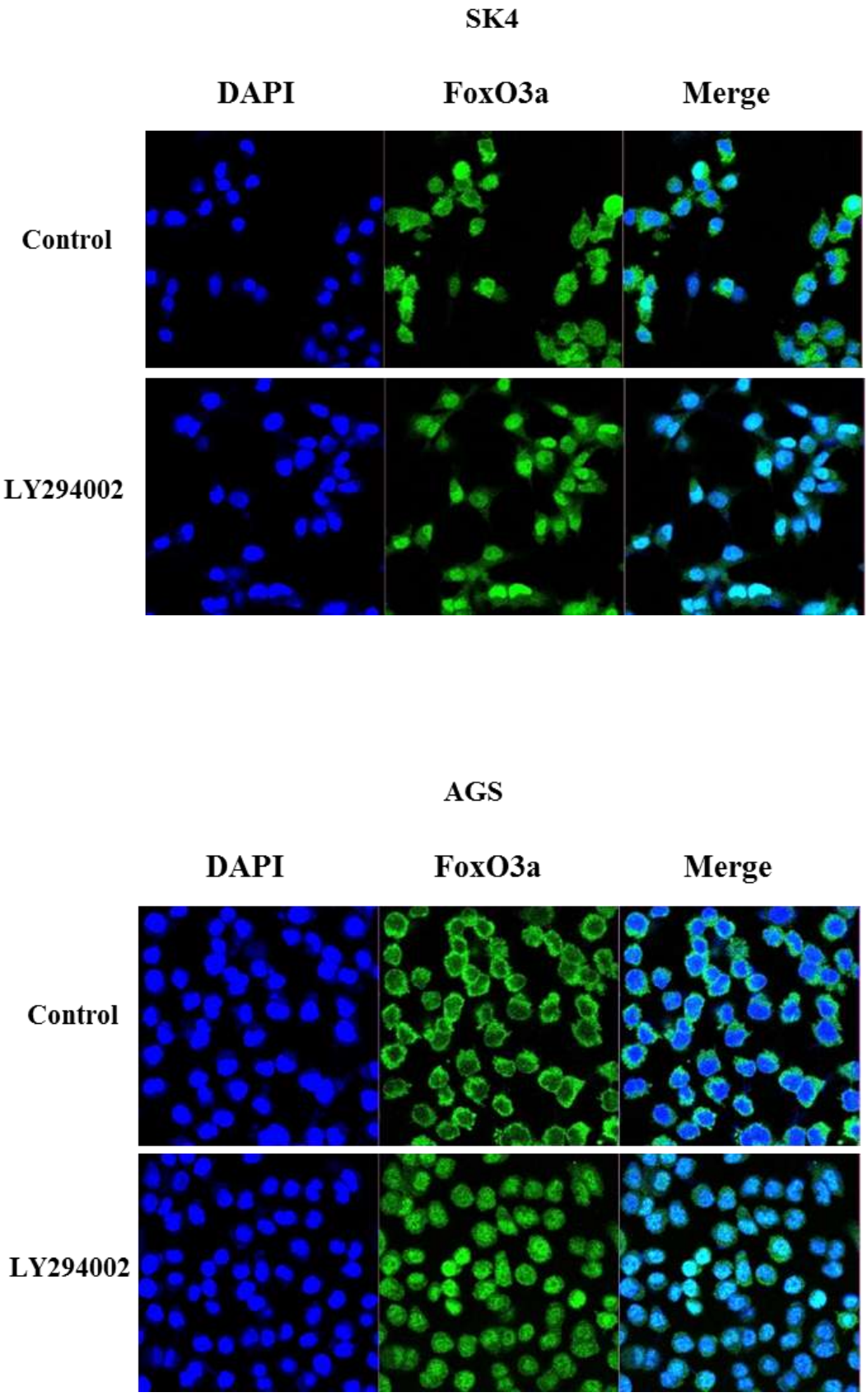

SK4
DAPI
FoxO3a
Merge
Control
LY294002
AGS
DAPI
FoxO3a
Merge
Control
LY294002

Figure 8. FoxO3a localization and PI3K/AKT activity in gastric cancer cells

(A) SK4 and AGS cells were treated the PI3K-specific inhibitor LY294002 (50uM) for the indicated times. Expression of AKT and FoxO3a phosphorylated proteins were examined by western blotting of the total cell lysates and β-actin was used as the loading control. (B) SK4 and AGS cells were seeded in glass coated plates at 80% confluence and treated with LY294002 (50uM). After 2 hours of treatment, ICC shows nuclear localization of FoxO3a in SK4 and AGS cells. Hoechst was used to stain the nuclei.

9. FoxO3a expression is negatively correlated with lymph node metastasis in gastric cancer patients

Lastly, we examined whether the expression of FoxO3a and phospho-FoxO3a in gastric cancer patient tissues is correlated with lymph node metastasis. In RPPA data, we evaluated the quantified expression levels of both FoxO3a and phospho-FoxO3a in relation to lymph node status. In accordance with the projected role of FoxO3a in lymphangiogenesis we explored in this study, there was an inverse correlation between FoxO3a expression and lymph node metastasis in gastric cancer patients (Table 1).

Table 1. FOXO3a expression in anti-correlated with lymph node metastasis in human gastric cancer tissue

	Lymph node(-) A.U. (n=124)	Lymph node(+) A.U. (n=506)	p value
FOXO3a	0.12	-0.04	0.03
pFOXO3a	-0.13	-0.02	0.031

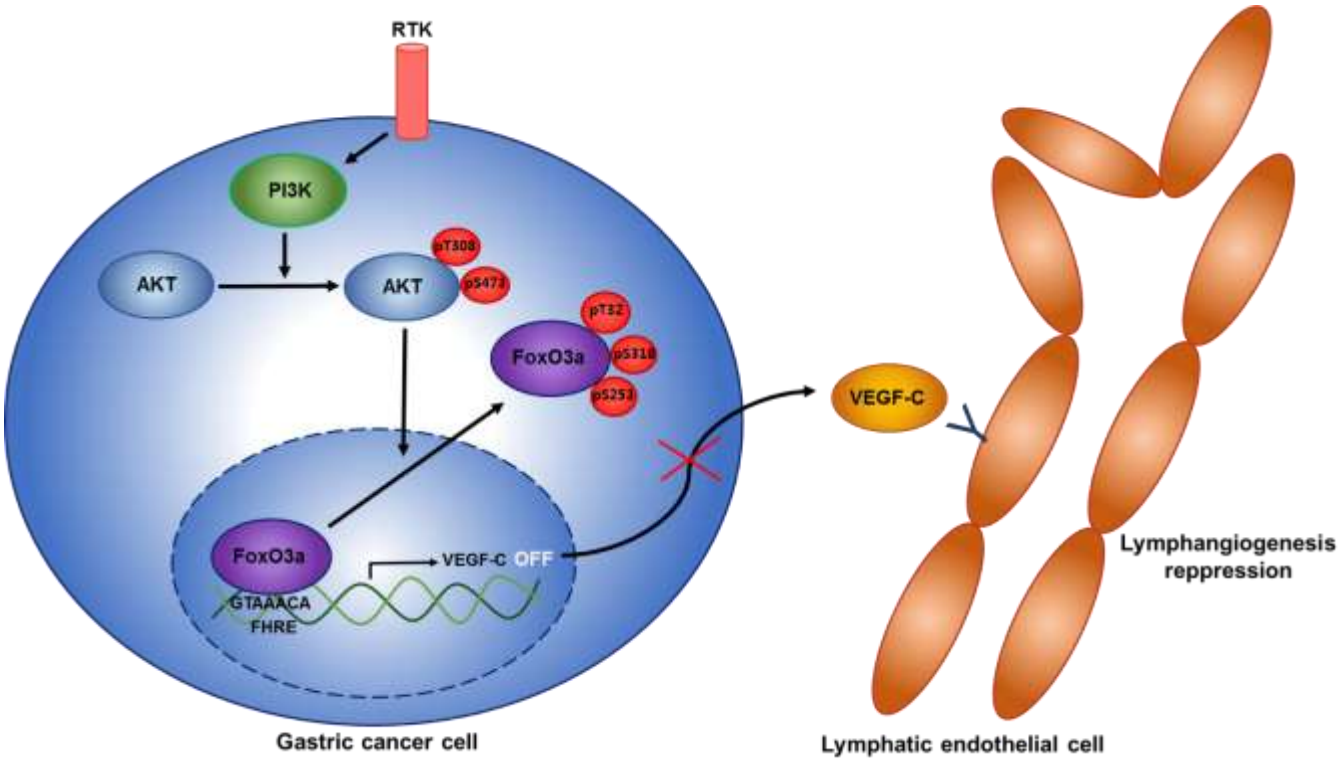


Figure 9. Diagram illustrating the relationship between FoxO3a and lymphangiogenesis in gastric cancer

Stimulation with growth factors leads to activation of the phosphatidylinositol-3 kinase (PI3K)/AKT signaling cascade. Phosphorylation of Foxo3a by AKT leads to their exclusion from the nucleus, and a reduction in the expression of key genes involved in protein degradation. Our data of gastric cancer cells revealed that Foxo3a nuclear localization is significantly but inversely associated with VEGF-C expression, suggesting FoxO3a negatively regulates VEGF-C expression. Signals mediated through VEGF-C and their receptors have been shown to be essential for gastric cancer carcinogenesis, cell migration, lymphangiogenesis and lymph node metastasis.

IV. DISCUSSION

Lymph node metastasis is the major cause of relapse and death in gastric cancer patients. Thus, it is critical to identify reliable predictive markers and molecular targets for lymph node metastasis to improve clinical outcome of gastric cancer. The primary aim of this study was to determine the clinical significance and biological role of FoxO3a in gastric cancer progression, in particular lymphangiogenesis. We found that phosphorylated FoxO3a, indicative of inactivation of FoxO3a, increased in gastric cancer tissues and was correlated with poor prognosis. Moreover, high level of FoxO3a in patient tumor was significantly negatively correlated with lymph node metastasis.

Since angiogenesis and lymphangiogenesis are interrelated processes and FoxO3a is already known to suppress VEGF-A, we speculated that FoxO3a might be involved in regulation of lymphangiogenesis as well. Together with the clinical tumor protein assay data indicating FoxO3a is inversely correlated with lymph node metastasis, we prompted to test the hypothesis that FoxO3a would suppress lymphangiogenesis. Functional studies revealed that overexpression of FoxO3a inhibited tube formation and migration of HLECs, while silencing of FoxO3a caused opposite effects. These data indicate that decreased FoxO3a in gastric cancer promotes lymphangiogenesis. Further, we found that VEGF-C, a major regulator of lymphangiogenesis, has a FHRE in the promoter region that consolidated the hypothesis. We revealed that FoxO3a binds to the FHRE in the promoter region of VEGF-C through EMSA. Further, gene silencing of FoxO3 using RNA interference increased the expression of VEGF-C in gastric cancer cells. Together with EMSA results, these data indicate that FoxO3a could transcriptionally repress VEGF-C expression in gastric cancer cells to decrease the tube formation and migration of HLECs. According to the recent study, FoxO3a suppresses VEGF-A

expression through competing off the transcriptional activator FoxM1. [85] Also, FoxO3a recruits HDAC2 to the proximal region of the VEGF-A promoter thereby repressing VEGF-A transcription independent of FOXM1.[85]

Obviously, the phosphorylation status and subcellular location of FoxO3a seems important in mediating anti-lymphangiogenic effects since phosphorylation by upstream oncogenic signals such as PI3K-AKT can inactivate FoxO3a thereby impeding nuclear localization[91]. Indeed, treatment of LY294002, a PI3K pathway inhibitor, reversed the phosphorylation of FoxO3a and increased the nuclear localization of FoxO3a in gastric cancer cells. Collectively, our data clearly illustrate that FoxO3a binds to VEGF-C promoter and represses the expression of VEGF-C thereby inhibiting tumor lymphangiogenesis. In summary, the present study demonstrates that the decreased expression of FoxO3a is a critical factor in the progression and poor clinical outcome of gastric cancer. Functional study elucidated the role of FoxO3a in tumor induced lymphangiogenesis in gastric cancer cell models. These findings may contribute to identifying FoxO3a as an attractive biomarker for lymph node metastasis and a potential therapeutic target to suppress lymph node metastasis in patients with gastric cancer.

Ⅴ. Conclusion

We identify that FoxO3a demotes tumor induced lymphangiogenesis and thereby lymph node metastasis in gastric cancer. Clinical gastric tumor tissue protein analysis and *in vitro* cancer cell model assays suggest that Foxo3a functions as a negative regulator of tumor lymphangiogenesis by transcriptionally repressing VEGF-C. For this reason, FoxO3a might be a potential clinical biomarker for lymphatic metastasis and poor prognosis in gastric cancer.

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ABSTRACT (IN KOREAN)

위암에서 Fox03a에 의한 신생림프관생성 억제 효과

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위암은 우리나라에서 두 번째로 많이 발생하는 암종으로 진행성 위암의 60%에서 림프절전이가 발생하며 림프절 전이가 동반된 환자의 예후는 매우 좋지 않다. 그러나 위암에서 림프절 전이의 분자적 기전에 관한 연구는 많이 되어있지 않다. 다양한 생물학적 기능을 갖는 전사인자인 Fox03a는 기존 연구에서 신생혈관 생성을 억제하는 것으로 알려져 있다. 신생혈관 생성과 신생림프관 생성이 분자적으로 밀접한 생물학적 공정임에도 불구하고 Foxo3a의 신생림프관 형성에서의 기능은 규명되지 않았다. 본 연구의 목적은 위암세포모델에서 림프절 전이에 필요한 신생림프관생성에서 Fox03a 의 분자생물학적 기능을 규명하고자 함이다. 먼저 저자는 위암조직의 종양신호전달 관련 단백체 분석(RPPA)을 통해 인산화 형태의 Fox03a 발현이 위암 환자의 림프절 전이 및 예후에 좋지 않은 인자임을 확인하였

다. FoxO3a 의 림프관생성에서의 기능 연구를 위한 실험모델로 FoxO3a 발현을 억제한 위암세포를 배양한 조건화 배지를 림프내피세포에 처리 했을 때 림프내피세포의 튜브형성이 증가하고 세포이동이 증가하는 것을 발견하였다. 반대로 FoxO3a를 과발현시킨 위암세포 조건화 배지에서는 튜브형성이 감소하고 림프내피세포 이동 또한 감소한다는 것을 확인하였다.

또한 위암세포 배양 조건화 배지에서 림프관 생성에 중요한 인자인 VEGF-C의 발현이 FoxO3a 발현과 역 상관관계에 있음에 착안하여 전사인자인 FoxO3가 VEGF-C 유전자 발현을 조절하는지 확인하기 위해 electrophoretic mobility shift assay (EMSA)를 시행한 결과 FoxO3a가 VEGF-C 프로모터에 결합하는 것을 확인하였다. 마지막으로 위암세포주 모델에서 LY294002처리 실험을 통해 PI3K 경로는 하위에 있는 FoxO3a를 인산화 시켜 핵 내로 위치하지 못하게 함으로서 FoxO3a의 전사조절을 통한 종양억제 기능이 비활성됨을 확인하였다.

결론적으로 위암세포주 모델에서 FoxO3a가 신생림프관생성을 유도하는 가장 대표적인 물질인 VEGF-C의 전사를 저해하여 신생림프관생성을 억제하는 것을 확인하였으며 실제 위암환자 종양조직에서 단백질 발현 분석을 통해 FoxO3a 발현과 림프절 전이가 역 상관관계에 있음을 제시하였다. 이러한 항신생림프관생성 효과에 기반하여 FoxO3a가 위암의 임상적 예후인자 및 림프절 전이의 바이오마커로서 활용될 수 있을 것이다.

핵심되는 말: FoxO3a, VEGF-C, 신생림프관생성, 위암세포