# Effects of forkhead box C2 on carcinogenesis and lymphatic metastasis in endometrial carcinoma

**Y.Y. Fan[1], W.G. Deng[2], Y.N. Liu[3], Y.Y. Li[1], S.L. Deng[4] and Y. Fu[1]**

[1]Department of Obstetrics and Gynecology, First Hospital of Jilin University, Changchun, China
[2]Department of Adolescent and Maternal Health, School of Public Health, Jilin University, Changchun, China
[3]Department of Otorhinolaryngology, Fourth Hospital of Jilin University, Changchun, China
[4]Clinical Medicine Department 2007-grade, Bethune Medical College, Jilin University, Changchun, China

Corresponding author: Y. Fu
E-mail: yanfucn@163.com

**ABSTRACT.** We investigated the distribution of endometrial lymphatic vessels and expression of forkhead box C2 (FOXC2) in normal endometrium during menstrual cycle and in endometrial adenocarcinoma. Full-thickness uterine samples and endometrial adenocarcinoma samples were collected for immunohistochemical analysis using D2-40 and FOXC2 mouse monoclonal antibodies. The lymphatic vessel density (LVD) of the endometrium was significantly reduced compared with the myometrium during the cycle. Intra-tumoral LVD was significantly decreased in both stages of endometrioid adenocarcinoma compared with normal endometrium and myometrium. Intra-tumoral LVD significantly decreased from stage IA to stage IIIC. Peri-tumoral LVD for stage IA and stage IIIC tumors was significantly increased compared with normal endometrial LVD,

but decreased compared with normal myometrial LVD. Stage IIIC showed increased peri-tumoral LVD when compared with stage IA. The positive rate of FOXC2 was 73.3% in proliferative endometrium and 80% in secretory endometrium. Secretory endometrium showed significantly increased FOXC2 expression compared with proliferative endometrium. Endometrioid adenocarcinoma showed significantly increased FOXC2 expression compared with normal endometrium, both in the epithelium and stroma. FOXC2 expression in the stroma significantly increased when pelvic and/or para-aotic lymph nodes were involved. FOXC2 was immunolocalized in low-risk endometrial carcinoma in endometrioid adenocarcinoma, but not in normal endometrium. Endometrial lymphatic vessels were located in normal endometrium and myometrium across the menstrual cycle and in intra- and peri-endometrioid adenocarcinoma, and increased in endometrial adenocarcinoma. Peri-tumoral lymphatics were associated with increased lymphatic metastasis. FOXC2 may be associated with the genesis of endometrial carcinoma and lymphangiogensis in endometrial adenocarcinoma in intra- and peri-tumoral lymphatics.

**Key words:** Endometrial adenocarcinoma; Endometrium; Forkhead Box C2; Lymphangiogenesis; Lymphatic metastasis

## INTRODUCTION

Lymph node metastasis is the most important metastasis route of endometrial cancers; the 5-year survival of patients with positive lymphatic metastasis is significantly lower than that in patients without lymph node metastasis. Lymphangiogenesis in endometrial cancer tissue is a key aspect of the remote metastasis of tumor cells through the lymphatic system. Cancer cells may directly or indirectly induce the formation of new lymphatic vessels through the production of a variety of growth factors, and metastasize through newly generated lymphatic vessels (Cao, 2005; Stacker and Achen, 2008; Holmqvist et al., 2009). Many factors, such as D2-40 (specific antibody for podoplanin), lymphatic vessel endothelial hyaluronan receptor (LYVE)-1, and vascular endothelial growth factor receptor-3, are expressed on the lymphatic endothelial cell membrane (Donoghue et al., 2007), becoming specific markers for lymphatic endothelial cells and useful tools for studying lymphatic vessels. Immunohistochemical staining study using D2-40 as a specific lymphatic endothelial cell (LEC) marker showed that lymphatic vessels are present in the normal myometrium, endometrial functional layer, and basalis layer (Donoghue et al., 2007). In addition, other studies using LYVE-1 as an LEC marker found that although lymphatic vessels could not be detected in the non-pregnant human endometrium (Koukourakis et al., 2005; Red-Horse et al., 2006), pregnancy could rapidly induce lymphangiogenesis in the decidua of the uterus (Red-Horse et al., 2006). Lymphatic vessel density (LVD) at different sites of cancer tissue was shown to be closely correlated with tumor cell invasion and patient survival rate (Holmqvist et al., 2009). Lymphangiogenesis is a necessary stage in the uncontrollable proliferation, invasion, and metastasis processes of cancer. This impacts the treatment effectiveness and prognosis of cancer. As a potential therapy for cancer, "anti-lymphangiogenesis" has been increasingly studied (Stacker and Achen, 2008).

Forkhead box C2 (FOXC2) is a member of the forkhead transcription factor family and has been implicated in lymphangiogensis in the embryo. Deficiency of FOXC2 in human and mouse leads to abnormal lymphatic patterning, failure of lymphatic valve formation, and lymphatic dysfunction (Petrova et al., 2004; Cueni and Detmar, 2008). Because the processes of embryo-associated lymphangiogenesis and carcinoma-associated lymphangiogenesis share some characteristics, we hypothesized that FOXC2 is involved in lymphangiogenesis in endometrial carcinoma. We explored the expression of FOXC2 in the normal endometrium during the menstruation cycle and in endometrial cancer tissue to examine the relationship between FOXC2 mode and LVD. Our aim was to confirm whether the *FOXC2* gene can induce and regulate lymphangiogensis in endometrial cancer. Our results also provide a basis for studies examining the control the growth of lymphatic vessel to treat cancer.

## MATERIAL AND METHODS

### Patients and tissues

The study was performed on paraffin-embedded full-thickness hysterectomy tissue samples obtained from 30 women (45 ± 1.6 years) undergoing hysterectomy for cervical intraepithelial neoplasia III (CIN III) or prolapse and 40 women (58 ± 1.4 years) undergoing radical hysterectomy and pelvic and para-aortic lymphadenectomy for endometrioid adenocarcinoma in the Gynecology Department of the First and the Second Hospital in Jilin University, China, from 2005 to 2008. Samples were grouped by 2 experienced pathologists as follows: proliferative group (N = 15), secretory group (N = 15), FIGO stage IA endometrioid adenocarcinoma (N = 20, limited to the endometrium), and stage IIIC (N = 20, limited to the endometrium and metastases to the pelvic and/or para-aotic lymph nodes). Informed consent was obtained from each patient at the time of tissue collection.

### Immunohistochemical staining

Formalin-fixed, paraffin-embedded, 5-μm serial tissue sections were stained for the LEC marker D2-40 to detect a fixation-resistant epitope on the podoplanin antigen mouse monoclonal antibody monoclonal antibody (Sigma, St. Louis, MO, USA) and for FOXC2 mouse monoclonal antibody prepared according to Yang et al. (2000). The slides were deparaffinized in xylene twice for 5 min and rehydrated using graded ethanol solutions diluted with distilled water. Antigen retrieval was carried out by microwave pretreatment in tri-sodium citrate buffer, pH 6, for 15 min (cooled for 30 min). Endogenous peroxidase was blocked by incubating the slides in 3% hydrogen peroxide in absolute methanol for 10 min. Non-specific binding was blocked with normal horse serum for 15 min. Both primary antibodies were incubated overnight at 4°C. Biotinylated anti-mouse immunoglobulin G served as a second antibody. Immunohistochemical staining was continued using the Vectastain Elite ABC-Peroxidase kit (Vector Laboratories Inc., Burlingame, CA, USA) according to manufacturer instructions. The slides were washed thoroughly with phosphate-buffered saline after each step. The antibody reaction was visualized using diaminobenzidine peroxidase substrate (Sigma FAST™ 3,3- diaminobenzidine table sets). For negative controls, the primary antibody was replaced with phosphate-buffered saline.

## Immunohistochemical analysis

### *Quantification of LVD*

Tissue sections were initially scanned at low magnification (40X) to identify the so-called 'hotspots', which are areas with the greatest density of D2-40-positive endothelial cells. These areas were then examined at high magnification (200X); microscopy fields with the highest degree of immunoreactivity were chosen for counting of LVD. Counting was performed on 3 separate 200X fields (20X objective and 10X ocular, 0.74 mm-2 per 200X field) within the hotspot using an Olympus GX-51 microscope (Olympus Optical, Tokyo, Japan). Any discrete D2-40-positive structure, regardless of the presence or absence of a lumen, was counted as 1 lymphatic vessel. The microvessel density count was defined as the highest number of lymphatic vessels among three 200X fields (Jaeger et al., 1995). The intratumoral compartment was defined as the area encompassing all malignant cells presented by hematoxylin and eosin staining. The peritumoal compartment was defined as benign uterine tissues that were present in the same 100X-microscopic field as the malignant glands.

### *Staining intensity of FOXC2 expression*

The intensity and distribution patterns of the specific immunohistochemical staining were evaluated using a semi-quantitative method (IRS score) as previously described by Remmele and Stegner (1987). Sections were examined by 2 independent observers. The IRS score was calculated as follows: IRS = SI x PP, where *SI* is the optical staining intensity (graded as 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and *PP* is the percentage of positively stained cells. PP was estimated by counting approximately 200 cells (epithelial cells and stromal cells were counted) and defined as 0, no staining; 1, <10% staining; 2, 11-50% staining; 3, 51-80% staining; and 4, >80% staining. The IRS for the epithelium and stroma were recorded.

## Statistical analysis

The results are reported as means ± standard deviation. All statistical tests were performed using Statistical Package for the Social Sciences for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). LVD and IRS were analyzed using one-way analysis of variance followed by the Student-Newman-Keuls test for individual mean analysis. Significance of differences was assumed at $P < 0.05$.

## RESULTS

## LVD of normal uterine tissue

Lymphatic endothelium was identified in normal endometrium and myometrium across the menstrual cycle with monoclonal antibody D2-40 (Figure 1). The lumen or line appearance the lymph vessel wall was formed by a continuous monolayer of pavement cells, and the nucleus of pavement cells was round or fusiform-shaped in the middle of the cell. Almost all lymphatic vessels of the endometrium were located in the basalis layer, while myometrial

lymphatic vessels were located within the connective tissue matrix between smooth muscle bundles. Lymphatics with irregular lumens and thin walls were larger and sometimes closely associated with spiral arterioles. There was no significant difference between proliferative and secretory LVD within the endometrium (proliferative $4.96 \pm 0.70$/mm$^2$; secretory $5.01 \pm 0.98$/mm$^2$), and myometrium (proliferative $10.63 \pm 1.13$/mm$^2$; secretory $10.78 \pm 1.22$/mm$^2$). The LVD of the endometrium was significantly reduced when compared with the myometrium across the cycle (endometrium $4.99 \pm 0.84$/mm$^2$; myometrium $10.70 \pm 1.16$/mm$^2$, P < 0.001).
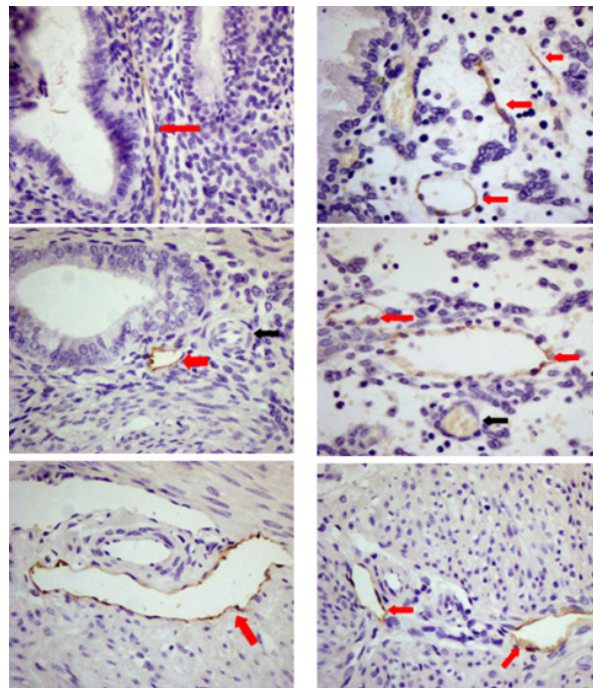


**Figure 1.** D2-40 expression in normal uterine tissues. Immunohistochemical demonstration of endometrial lymphatics in the normal uteri. Lymphatic vessels (red arrows) identified by D2-40 are shown in the proliferative (**A**, **D**) and secretory (**B**, **E**) endometrium and the myometrium (**C**, **F**), Some lymphatic vessels were intimately associated with the spiral arterioles (black arrows) in the endometrium (**B**). **A**, **B**, **C** were 200X, and **D**, **E**, **F** were 400X.

## Analysis of LVD in endometrial adenocarcinoma tissue

The D2-40 positively stained lymph vessels were found at the inside and at the edge of endometrial adenocarcinoma tissue (Figure 2). The lymph vessels on the inside of endometrial adenocarcinoma appeared as smaller and applanatus lumens filled with tumor emboli, but the lymph vessel at the edge of endometrial adenocarcinoma tissue exhibited larger lumens with an irregular shape. Intra-tumoral LVD was significantly reduced in grade IA and IIIC adenocarcinomas compared with normal endometrial and myometrial LVD (stage IA: $3.49 \pm 0.94$/mm$^2$, stage IIIC: $2.43 \pm 1.08$/mm$^2$, normal endometrium: $4.98 \pm 0.84$/mm$^2$, normal myometrium: $10.71 \pm 1.16$/mm$^2$, P < 0.001). Peri-tumoral LVD for grade IA and grade IIIC tumors was significantly increased compared with normal endometrial LVD and was significantly reduced compared with myometrial LVD (stage IA: $7.09 \pm 1.22$/mm$^2$, stage IIIC: 8.63

± 2.88/mm$^2$, normal endometrium: 4.98 ± 0.84/mm$^2$, normal myometrium: 10.71 ± 1.16/mm$^2$, P < 0.005). Peri-tumoral LVD significantly increased in grade IIIC tumors compared with in grade IA tumors (stage IA: 7.09 ± 1.22/mm$^2$, stage IIIC: 8.63 ± 2.88/mm$^2$, P = 0.012) (Table 1).
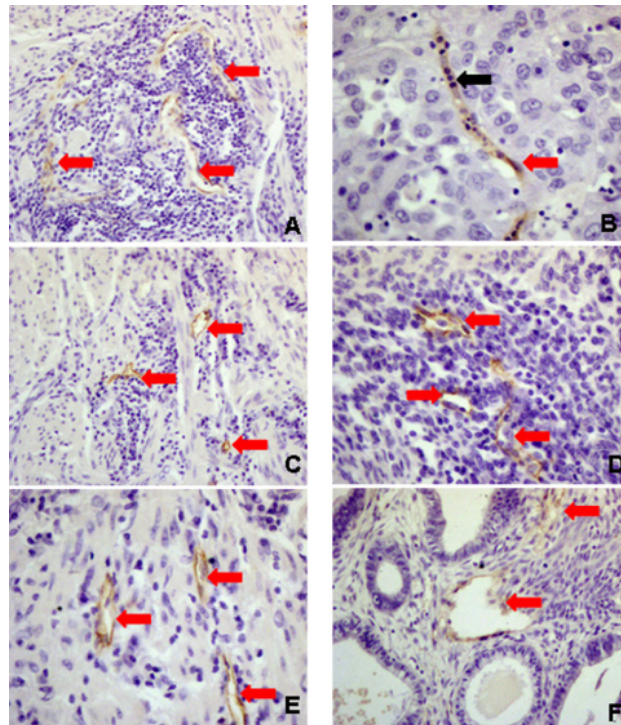


**Figure 2.** D2-40 expression in intra- and peri- endometrial adenocarcinoma tissue immunohistochemical staining of endometrial adenocarcinoma for lymphatics using antibody to D2-40. The lymphatic vessels (red arrows) were located within intra-tumor (**A**, **B**, **C**, **D**, **E**) and peri-tumor (**F**). Tumor embolus was found within lymphatic vessels (black arrows in **B**).

**Table 1.** LVD in normal endometrium and endometrial adenocarcinoma.

| Group | N | LVD | |
|---|---|---|---|
| Normal endometrium | 15 | 4.98 ± 0.84 | |
| Normal myometrium | 15 | 10.71 ± 1.16 | |
| | | Intra foci LVD | Peri-foci LVD |
| Endomet. Adenocar. IA | 20 | 3.49 ± 0.94**†† | 7.09 ± 1.22*† |
| Endomet. Adenocar. IIIC | 20 | 2.43 ± 1.08**†† | 8.63 ± 2.88*† |

*Means compared with normal endometrium, P < 0.05; †means compared with normal myometrium, P < 0.05; **means compared with normal endometrium, P < 0.01; ††means compared with normal myometrium, P < 0.01.

## Expression of FOXC2 in epithelium of normal endometrium

A total of 73.3% (11/15) of normal endometrial glandular epithelial cells in proliferation phase were FOXC2-positive. However, in contrast to embryonic tissues, the brown FOXC2-positive particles in tissues were located on the lumen side of the cytoplasm of glan-

dular epithelial cells. In addition, 80% (12/15) of endometrial glands in the secretory phase showed positive expression of FOXC2, but brown FOXC2-positive particles located in the cytoplasm of the glandular epithelial cells were observed to be away from the luminal side. IRS scores of FOXC2 expression in the proliferative and secretory phases showed no significant difference (proliferative IRS = $1.53 \pm 1.30$, secretory IRS = $2.1 \pm 1.68$, P = 0.284). The brown particles were also located in the nuclei of glandular epithelial cells (Figure 3).
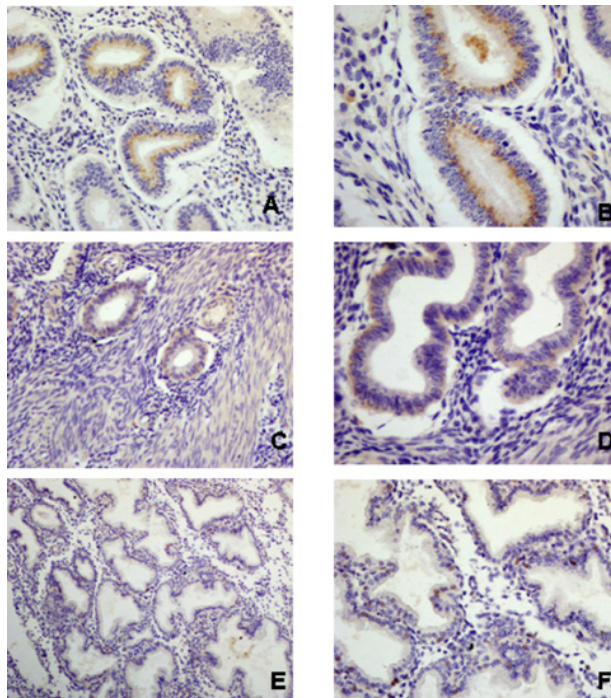


**Figure 3.** Expresssion of FOXC2 in normal endometrium. **A. B.** Positive FOXC2 in normal endometrial glandular epithelial cells in proliferation phase with the brown FOXC2-positive particles located in the lumen side cytoplasm of the glandular epithelial cells. **C. D.** Positive FOXC2 in normal endometrial glandular epithelial cells in secretory phase with the brown FOXC2-positive particles located away from the luminal side. **E. F.** FOXC2-positive in the nucleus of glandular epithelial cells. **A**, **C**, **E** were 200X; **B**, **D**, **F** were 400X.

## Expression of FOXC2 in endometrial adenocarcinoma tissue

### Expression of FOXC2 in epithelium of endometrial adenocarcinoma tissue

The positive staining of FOXC2 in endometrial adenocarcinoma cells was mainly located in the nuclei. As shown in Figure 4, the expression of FOXC2 in endometrial adenocarcinoma cells was increased compared to that in normal endometrium (normal endometrium IRS = $1.83 \pm 1.51$, endometrioid adenocarcinoma IRS = $5.18 \pm 2.82$, P < 0.01). The IRS score of FOXC2 expression in endometrial adenocarcinoma with lymph metastasis was slightly higher than that without lymph metastasis; however, as shown in Table 2, the difference was not significant (stage IA IRS = $4.45 \pm 2.48$, stage IIIC IRS = $5.9 \pm 3.01$, P = 0.104).
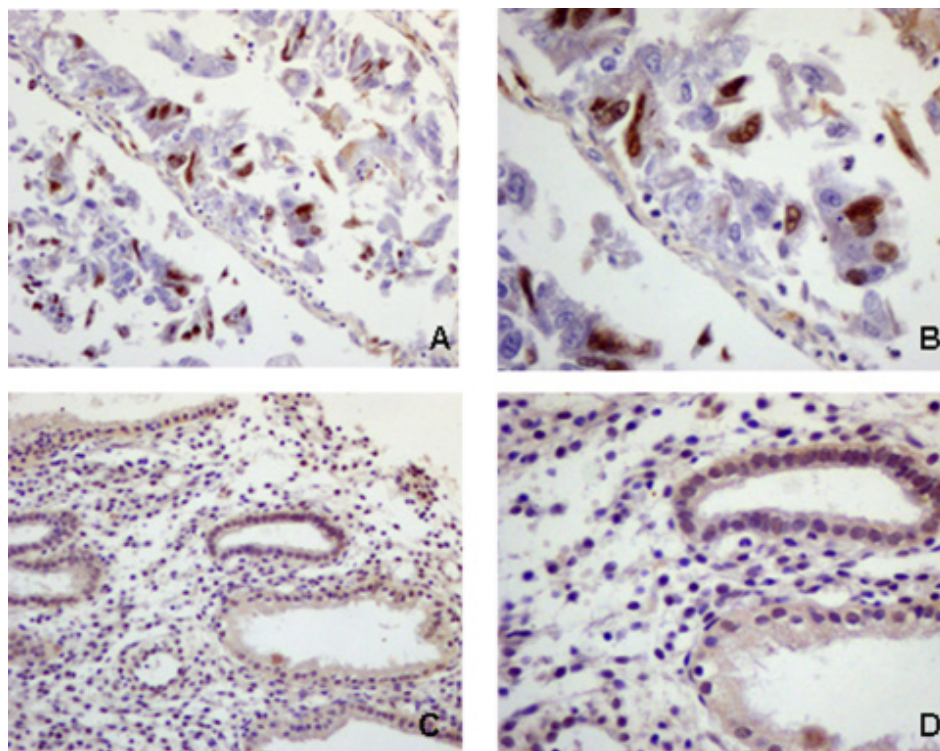
**Figure 4.** Expression of FOXC2 in endometrial adenocarcinoma epithelial tissue. By positive FOXC2-stain slice in endometrial adenocarcinoma epithelial tissue, the bigger cell nucleus with irregular shape was seen.

**Table 2.** Expression of FOXC2 in normal and abnormal endometrium.

| Group | Classification | N | Positive rate | IRS | P |
|---|---|---|---|---|---|
| Epithelium | Proliferative phase | 15 | 73.3 | $1.53 \pm 1.30$ | >0.05 |
| | Secretary phase | 15 | 80 | $2.1 \pm 1.68$ | |
| Stroma | Stage IA endometrioid adenocarcinoma | 20 | 90 | $4.45 \pm 2.48$ | >0.05 |
| | Stage IIIC | 20 | 90 | $5.9 \pm 3.01$ | |
| Epithelium | Proliferative phase | 15 | 100 | $1.93 \pm 1.03$ | <0.05 |
| | Secretory phase | 15 | 100 | $3.93 \pm 1.53$ | |
| Stroma | Stage IA endometrioid adenocarcinoma | 20 | 100 | $5.9 \pm 3.01$ | <0.05 |
| | Stage IIIC | 20 | 100 | $7.85 \pm 2.08$ | |

## *Expression of FOXC2 in the stroma of endometrial adenocarcinoma tissue*

Positive expression of FOXC2 was observed in the stroma of nearly all endometrial adenocarcinoma tissues, with positive staining mainly located in the cytoplasm and nucleus. FOXC2 expression in the stroma of the secretory endometrium was higher than that in the proliferative stage (proliferative IRS = $1.93 \pm 1.03$, secretory IRS = $3.93 \pm 1.53$, P < 0.01). Expression of FOXC2 in the stroma of endometrial adenocarcinoma tissue was significantly higher than that in the normal endometrium (normal endometrium IRS = $2.93 \pm 1.64$, endometrioid adenocarcinoma IRS = $6.78 \pm 2.15$, P < 0.01). As shown

in Table 2 and Figure 5, the stroma IRS of FOXC2 in endometrial adenocarinoma was increased when lymph metastasis was observed (stage IA IRS = $5.70 \pm 1.66$, stage IIIC IRS = $7.85 \pm 2.08$, P = 0.01).
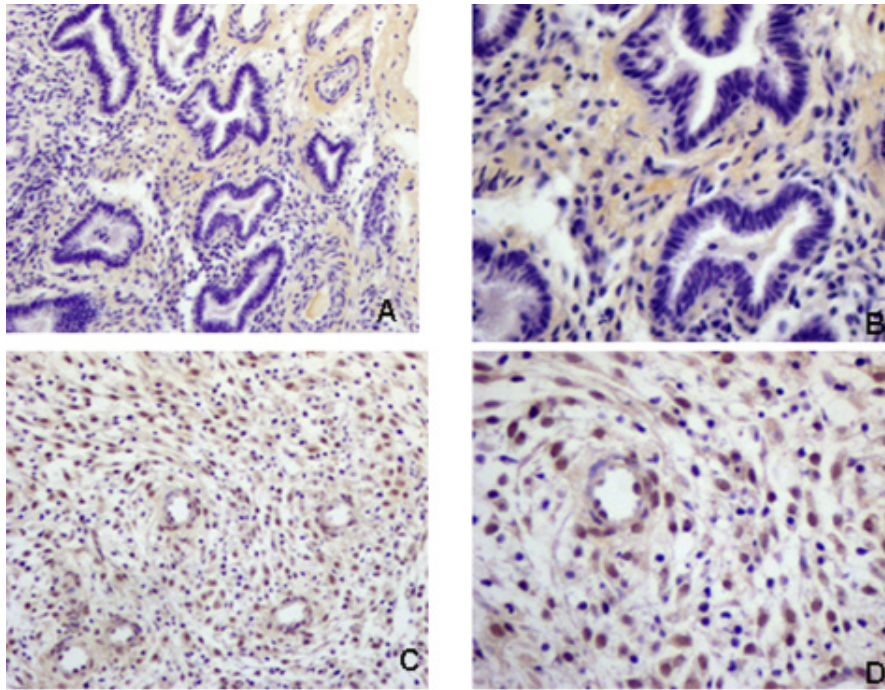


**Figure 5.** Expression of FOXC2 in normal endometrium and endometrial adenocarcinoma stroma. FOXC2 expression was positive in stroma of normal endometrium (**A**, **B**) and endometrial adenocarcinoma (**C**, **D**). The endometrial adenocarcinoma cell has embryonization feature with FOXC2-positive stain located in normal endometrium cytoplasm but not in endometrial adenocarcinoma cell nucleus. **A** and **C** were 200X; **B** and **D** were 400X.

## Positive expression of FOXC2 in endometrial adenocarcinoma neogenesis lymphatic vessels

As shown in Figure 6, D2-40-positive staining of micro-lymph vessel in the normal endometrial stroma was irregular, and the expression of FOXC2 in LECs of adjoining serial sections was negative. The neogenesis lymph vessel grew as a clump or arcuation in endometrial adenocarcinoma, and positive expression of FOXC2 in the micro-lymph vessel cell cytoplasm was observed in continuous slices. FOXC2 was not expressed in the lymphatic vessels of the normal endometrium, but was strongly expressed in the new lymphatic vessels formed in endometrial cancer tissues.
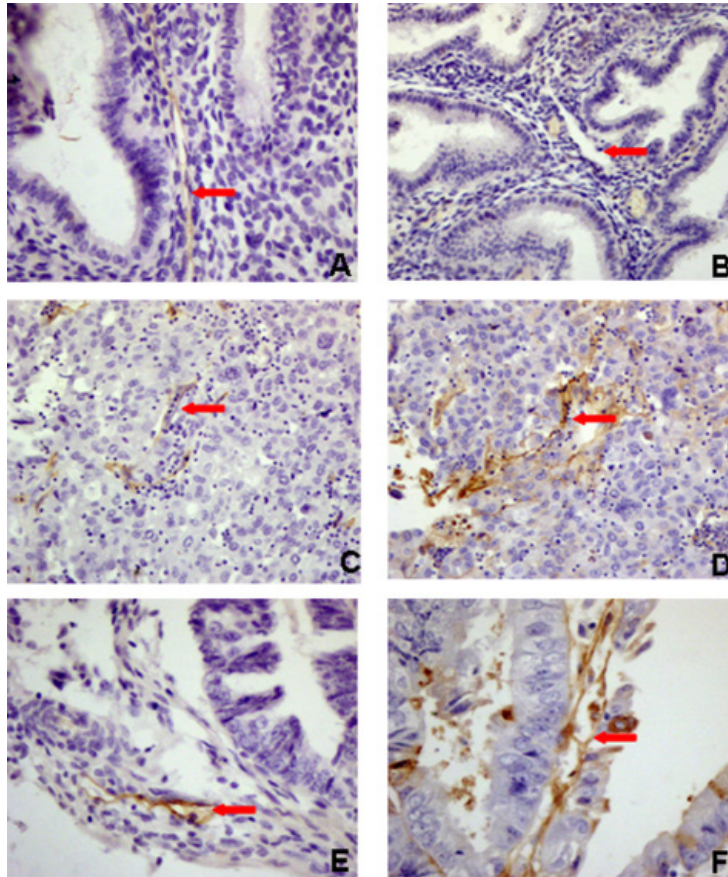
**Figure 6.** Concordance expression of D2-40 and FOXC2 in normal endometrium and endometrial adenocarcinoma. **A. C. E.** D2-40 stain; **B. D. F.** FOXC2 stain in continuous slice of **A**, **C**, **E**. In normal endometrium, lymph vessels (red arrows) presented positive D2-40 stain and negative FOXC2 stain (**A**, **B**), but in endometrial adenocarcinoma, lymph vessel presented both positive (**C**, **D**, **E**, **F**). Magnification400X.

## DISCUSSION

Endometrial cancer is one of the most common malignant tumors of the female reproductive system. Because most endometrial cancers can be detected in early stages when the foci are confined to the uterus (Jemal et al., 2008), the prognosis of endometrial cancer is relatively good. However, each year, approximately 50,000 people die of endometrial cancer (Parkin et al., 2005), among which recurrence and distant metastasis are the leading causes. The mechanism of endometrial cancer recurrence remains unclear, but lymphangiogenesis in cancer foci may activate latent cancer cells in lymphatic vessels; metastases may also occur.

The human endometrium is a highly dynamic tissue, which undergoes approximately 400 cycles of growth, differentiation, shedding, and regeneration during the lifetime of a woman. Our results showed that during the proliferative and secretory phases of a normal menstrual cycle, lymphatic vessels were present in the endometrial layer of the uterus and

there were no significant differences in the endometrial layer LVD between the proliferative and secretory phases. Endometrial lymphatic vessels were mostly concentrated in the basal layer, parallel to the spiral arteries. This indicates that the small lymphatic vessels in the endometrium also underwent regeneration during the menstrual cycle.

Lymphangiogenesis in tumor tissue and the number of lymphatic vessels are correlated with distant lymphatic metastasis of the tumor (van Netten et al., 1998; Clarijs et al., 2001; Pepper, 2001; Van Trappen and Pepper, 2005), as tumor tissue lymphangiogenesis increases the probability of tumor metastasis via lymphatic vessels (Da et al., 2008) and the lymphatic system provides nutritional support for the survival of cancer tissue. Beasley et al. (2002) found that LYVE-1-positive intratumoral lymphatic vessels expressed a cell proliferation marker, pKi67, confirming the presence of newly generated lymphatic vessels in the tumor. Lymphangiogenesis has been observed in ovarian cancer, cervical cancer, and many other tumors (Gombos et al., 2005; Yang et al., 2009). In this study, we detected lymphangiogenesis in endometrial adenocarcinoma foci and surrounding tissues, while the LVD in tumor foci was significantly lower than that in the normal endometrium and myometrim. In addition, the LVD in para-cancerous tissues was significantly higher than in the normal endometrium, but still lower than in the normal myometrium. This may be because solid tumor tissue is hard and exhibits high pressure, making it difficult for lymphatic vessels to form. Previously, it was thought that although solid tumors lacked functional lymphatics (Leu et al., 2000), these functional lymphatic vessels surrounding the tumor are still sufficient to support lymphatic metastasis of cancer cells (Padera et al., 2002). Our study found that the para-cancerous tissue LVD in patients with lymph node metastasis was significantly higher than that in those without lymph node metastasis, suggesting that lymphangiogenesis in cancer tissue may provide the metabolic needs and a pathway for the metastasis of cancer cells.

FOXC2 is an important transcription factor in embryonic development and is mainly expressed in actively proliferating tissues. Previous studies found that *Foxc2* haploid gene knockout mice developed lymphedema symptoms similar to those of lymphedema-distichiasis patients (Kriederman et al., 2003), indicating that FOXC2 is involved in the generation and development of lymphatic vessels and plays an important role in lymphangiogenesis. We found that FOXC2 was not expressed in lymphatic vessels of the normal endometrium, but strongly expressed in newly formed lymphatic vessels in endometrial cancer tissues, suggesting that FOXC2 is an important factor in embryonic and tumor tissue lymphangiogenesis. Newly generated lymphatic vessels are necessary structures for lymph node metastasis of endometrial cancer. The results of this study showed that there were tumor thrombi within the new lymphatic vessels in endometrial carcinoma, indicating that endometrial cancer cells can migrate to distant tissues or organs via intratumoral lymphangiogenesis. Cancer cells may enter lymphatic vessels through the open connection among endothelial cells of the lymphatic vessels, or through the destructed lymphatic wall following secretion of type IV collagenase or other active substances.

The cyclical proliferation of the endometrium shows similar characteristics to embryonic cell proliferation, suggesting that some embryonic proteins may also be expressed in the endometrium. Side-population cells isolated from the human endometrium not only have long-term regeneration capacity but also can differentiate into endometrial gland stem cells and stromal-like stem cells, as well as show some characteristics of embryonic cells such as distance migration and differentiation across tissue boundaries. This process is similar to the mesenchyme-to-epithelium transition. Therefore, side-population cells are considered to be

cancer stem-like cell subpopulations (Götte et al., 2008; Kato et al., 2010), which have similar biological properties to early embryonic cells. Our results showed that 73.3% of normal endometrial glandular epithelial cells in the proliferation phase were FOXC2-positive. However, in contrast to embryonic tissues, brown FOXC2-positive particles in tissues located in the lumen side cytoplasm of the glandular epithelial cells. A total of 80% of endometrial glands in the secretory phase also showed positive expression of FOXC2, but the brown FOXC2-positive particles located in the cytoplasm of the glandular epithelial cells were away from the luminal side. The IRS scores of FOXC2 expression in the proliferative and secretory phases showed no significant difference. In addition, the abundance of FOXC2 in the cytoplasm suggests that FOXC2 protein is an important component of the proliferation and secretion processes in the endometrium. FOXC2 is a transcription factor involved in the embryogenesis of multiple tissues and organs and is present in high amounts in actively proliferating embryonic cells. When cells enter the synthesis phase, FOXC2 accumulates in the nucleus to regulate gene transcription and function in tissue and organ formation and remodeling. However, unlike in the embryonic stage, FOXC2 in the normal endometrium did not appear in the nucleus, and FOXC2 localization in the secretory phase and proliferative phase endometrium also differed. In addition, nuclear expression of FOXC2 was observed in a few endometrial stromal cells and glandular epithelial cells, showing similar expression as human fetal tissue FOXC2. These cells may be side-population cells, which also suggests that FOXC2 is involved in the transition of side-population cells to endometrial stem cells.

The process of squamous cell carcinoma metastasis from the primary site to distant organs is very similar to the process of embryonic cell migration. By activating the endothelial-mesenchymal transition, cancer cells gain the ability to fulfill the multi-stage invasion-metastasis cascade. Mani et al. (2007) found that metastasis of mice breast cancer cells to the lung required expression of FOXC2, while FOXC2 overexpression enhanced the metastatic ability of cancer cells, suggesting that FOXC2 plays an essential role in tumor invasion and metastasis. The high invasion ability of human basal cell-like breast cancer is also closely related to FOXC2. Our results detected FOXC2-positive expression in the cytoplasm and nucleus of endometrial cancer cells. The FOXC2 expression level in the endometrial epithelial cells was significantly higher than in normal endometrial glands, but FOXC2-positive staining intensity in cancer epithelial cells showed no correlation with lymph node metastasis. All cancer samples contained FOXC2 in the tumor stroma. In endometrial tumor stroma, FOXC2 expression was significantly higher than in the normal endometrium, and the interstitial FOXC2 staining intensity in patients with lymph node metastasis was significantly higher than in those without lymph node metastasis. Our results support that the invasion and metastasis of cancer cells require FOXC2 expression. We hypothesize that FOXC2 is also involved in the carcinogenesis and metastasis of endometrial carcinoma. We found that FOXC2 expression levels in endometrial stromal cells and epithelial cells were not significantly correlated with the LVD in para-cancerous tissues, suggesting that FOXC2 acts on cancer cells and lymphatic epithelial cells in different stages.

Currently, little is known regarding the role of FOXC2 in intratumoral lymphangiogenesis. FOXC2 may play an important role in lymphangiogenesis of the embryo and tumor, as well as lymph node metastasis of endometrial cancers. This provides a theoretical foundation to explore the prevention of congenital defects in vascular development and lymphatic metastasis of tumors, as well as the discovery of new diagnostic methods for treating cancer. In addition, our results may open a new avenue for the treatment of lymphatic diseases or anti-lymphangiogenesis therapy in tumors.

## REFERENCES

Beasley NJ, Prevo R, Banerji S, Leek RD, et al. (2002). Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res*. 62: 1315-1320.

Cao Y (2005). Opinion: emerging mechanisms of tumor lymphangiogenesis and lymphatic metastasis. *Nat. Rev. Cancer* 5: 735-743.

Clarijs R, Ruiter DJ and de Waal RM (2001). Lymphangiogenesis in malignant tumors: does it occur? *J. Pathol.* 193: 143-146.

Cueni LN and Detmar M (2008). The lymphatic system in health and disease. *Lymphat. Res. Biol.* 6: 109-122.

Da MX, Wu XT, Wang J, Guo TK, et al. (2008). Expression of cyclooxygenase-2 and vascular endothelial growth factor-C correlates with lymphangiogenesis and lymphatic invasion in human gastric cancer. *Arch. Med. Res*. 39: 92-99.

Donoghue JF, Lederman FL, Susil BJ and Rogers PA (2007). Lymphangiogenesis of normal endometrium and endometrial adenocarcinoma. *Hum. Reprod.* 22: 1705-1713.

Gombos Z, Xu X, Chu CS, Zhang PJ, et al. (2005). Peritumoral lymphatic vessel density and vascular endothelial growth factor C expression in early-stage squamous cell carcinoma of the uterine cervix. *Clin. Cancer Res.* 11: 8364-8371.

Götte M, Wolf M, Staebler A, Buchweitz O, et al. (2008). Increased expression of the adult stem cell marker Musashi-1 in endometriosis and endometrial carcinoma. *J. Pathol.* 215: 317-329.

Holmqvist A, Gao J, Adell G, Carstensen J, et al. (2009). The location of lymphangiogenesis is an independent prognostic factor in rectal cancers with or without preoperative radiotherapy. *Ann. Oncol*. 21: 512-517.

Jaeger TM, Weidner N, Chew K, Moore DH, et al. (1995). Tumor angiogenesis correlates with lymph node metastases in invasive bladder cancer. *J. Urol*. 154: 69-71.

Jemal A, Siegel R, Ward E, Hao Y, et al. (2008). Cancer statistics, 2008. *CA Cancer J. Clin*. 58: 71-96.

Kato K, Takao T, Kuboyama A, Tanaka Y, et al. (2010). Endometrial cancer side-population cells show prominent migration and have a potential to differentiate into the mesenchymal cell lineage. *Am. J. Pathol*. 176: 381-392.

Koukourakis M, Giatromanolaki A, Sivridis E, Simopoulos C, et al. (2005). LYVE-1 immunohistochemical assessment of lymphangiogenesis in endometrial and lung cancer. *J. Clin. Pathol*. 58: 202-206.

Kriederman BM, Myloyde TL, Witte MH, Dagenais SL, et al. (2003). FOXC2 haploinsufficient mice are a model for human autosomal dominant lymphedema-distichiasis syndrome. *Hum. Mol. Genet*. 12: 1179-1185.

Leu AJ, Berk DA, Lymboussaki A, Alitalo K, et al. (2000). Absence of functional lymphatics within a murine sarcoma: a molecular and functional evaluation. *Cancer Res.* 60: 4324-4327.

Mani SA, Yang J, Brooks M, Schwaninger G, et al. (2007). Mesenchyme Forkhead 1 (FOXC2) plays a key role in metastasis and is associated with aggressive basal-like breast cancers. *Proc. Natl. Acad. Sci. U. S. A.* 104: 10069-10074.

Padera TP, Kadambi A, di Tomaso E, Carreira CM, et al. (2002). Lymphatic metastasis in the absence of functional intratumor lymphatics. *Science* 296: 1883-1886.

Parkin DM, Bray F, Ferlay J and Pisani P (2005). Global cancer statistics, 2002. *CA Cancer J. Clin*. 55: 74-108.

Pepper MS (2001). Lymphangiogenesis and tumor metastasis: myth or reality? *Clin. Cancer Res*. 7: 462-468.

Petrova TV, Karpanen T, Norrmén C, Mellor R, et al. (2004). Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasia. *Nat. Med*. 10: 974-981.

Red-Horse K, Rivera J, Schanz A, Zhou Y, et al. (2006). Cytotrophoblast induction of arterial apoptosis and lymphangiogenesis in an *in vivo* model of human placentation. *J. Clin. Invest*. 116: 2643-2652.

Remmele W and Stegner HE (1987). Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. *Pathologe* 8: 138-140.

Stacker SA and Achen MG (2008). From anti-angiogenesis to anti-lymphangiogenesis: emerging trends in cancer therapy. *Lymphat. Res. Biol*. 6: 165-172.

van Netten JP, Cann SA, Maxwell CA and Finegan RP (1998). Lymphagenesis and cancer metastasis. *Br. J. Cancer* 78: 277-278.

Van Trappen PO and Pepper MS (2005). Lymphangiogenesis in human gynaecological cancers. *Angiogenesis* 8: 137-145.

Yang S, Cheng H, Cai J, Cai L, et al. (2009). PlGF expression in pre-invasive and invasive lesions of uterine cervix is associated with angiogenesis and lymphangiogenesis. *APMIS* 117: 831-838.

Yang XL, Matsuura H, Fu Y, Sugiyama T, et al. (2000). MFH-1 is required for bone morphogenetic protein-2-induced osteoblastic differentiation of C2C12 myoblasts. *FEBS Lett.* 470: 29-34.