Expression of Transcription Factor FOXC2 in Cervical Cancer and Effects of Silencing on Cervical Cancer Cell Proliferation

Chun-Hua Zheng¹, Yuan Quan¹, Yi-Yang Li¹, Wei-Guo Deng², Wen-Jing Shao¹, Yan Fu¹*

Abstract

Objective: Forkhead box C2 (FOXC2) is a member of the winged helix/forkhead box (Fox) family of transcription factors. It has been suggested to regulate tumor vasculature, growth, invasion and metastasis, although it has not been studied in cervical cancer. Here, we analyzed FOXC2 expression in cervical tissues corresponding to different stages of cervical cancer development and examined its correlation with clinicopathological characteristics. In addition, we examined the effects of targeting FOXC2 on the biological behavior of human cervical cancer cells.

Methods: The expression of FOXC2 in normal human cervix, CIN I-III and cervical cancer was examined by immunohistochemistry and compared among the three groups and between cervical cancers with different pathological subtypes. Endogenous expression of FOXC2 was transiently knocked down in human Hela and SiHa cervical cells by siRNA, and cell viability and migration were examined by scratch and CCK8 assays, respectively.

Results: In normal cervical tissue the frequency of positive staining was 25% (10/40 cases), with a staining intensity (PI) of 0.297±0.520, in CIN was 65% (26/40 cases), with a PI of 3.00±3.29, and in cancer was 91.8% (68/74 cases), with a PI of 5.568 ±3.449. The frequency was 100% in adenocarcinoma (5/5 cases) and 91.3% in SCCs (63/69 cases). The FOXC2 positive expression rate was 88.5% in patients with cervical SCC stage I and 100% in stage II, showing significant differences compared with normal cervix and CIN. With age, pathologic differentiation degree and tumor size, FOXC2 expression showed no significant variation. On transient transfection of Hela and SiHa cells, FOXC2-siRNA inhibition rates were 76.2% and 75.7%; CCK8 results showed reduced proliferation and relative migration (in Hela cells from 64.5±3.16 to 49.5±9.24 and in SiHa cells from 60.1±3.05 to 44.3±3.98) (P < 0.05).

Conclusion: FOXC2 gene expression increases with malignancy, especially with blood vessel hyperplasia and invasion degree. Targeted silencing was associated with reduced cell proliferation as well as invasion potential.

Keywords: FOXC2 - cervical cancer - RNAi - tumor vessel density - invasion - therapy

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Introduction

Cervical cancer is the common malignant tumor of female reproductive system, each year, there are 100000 new cases of cervical cancer in China, 1/5 of the global cases. Tumor registration report newly increased cases of cervical cancer are about 135000 cases in 2013, whose incidence remains high. According to China’s health and family planning commission issued 2011 China health statistics yearbook of statistics, in 2010 cervical cancer mortality rate is 3.40/10 thousand in big cities, and 4.31/100 thousand in small and medium-sized cities, 2.45/10 thousand in rural area (Forouzanfar et al., 2011). Cervical cancer mortality rate is relatively low in young women, less than 1.0/10 thousand, but after entering the age of 35, the mortality rate doubled, and increased year by year, which threatened middle-aged and old women seriously. Cervical cancer occurred secretly, progress rapidly, therefore, early detection of primary lesions and effective treatment is effective to prolong patient life time and reduce the fatality rate.

Neoadjuvant chemotherapy was used recently, many patient in advanced stage could get the opportunity of surgery, but the majority of patient developed with drug resistance, and end up their lives with progression and reoccurrence. Otherwise, a new effective therapy is essential for cervical cancer. Thetargeting silence of the E6 and E7 oncogene with a technique called RNAi, the HPV positive Hela cells increased sensitivity to cisplatin (Putral et al., 2005). Silencing the expression of HPV16/18 positive cervical cancer cell cyclin B1, increase the sensitivity of cervical cancer cells to camptothecin and other chemotherapeutic drugs (Kreis et al., 2010). For patients who do Preoperative chemotherapy to reduce the lesion and Postoperative chemotherapy to eliminate residual lesions, multidrug resistance is the main obstacle.

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MDR1 coding P glycoprotein (p-gp), is one of the main genes for chemotherapy drugs. Pickler et compound the shRNA targeting MDR1, inhibit the expression of MDR1 and p-gp protein, which increased the sensitivity to incrusting paclitaxel doxorubicin (Pichler et al., 2005). Survivin gene is new member of the family of apoptosis inhibiting protein gene, has strong resistance to apoptosis role.

High risk human papilloma virus type 16and18 (HPV) infection is the basic factor of cervical cancer and infection rates is as high as 99.7%. HPV E6 / E7 promote tumor suppressor protein P53 and Rb degradation is one of the malignant transformation of cervical epithelial cells. HPV16-E7 shRNA was transfected into HPV16 positive SiHa, CaSkI cells, induced the low phosphorylation of Rb protein, thus lead to massive apoptosis of HPV positive tumor cells selectively. And through the activation of P53, P21 and Rb, leading to apoptosis related HPV16 cancer cells significantly, E7-shRNA may have the potential for gene therapy of HPV16 related tumor (Simia et al., 2008). The inhibition of the HPV16 E7 gene expression vectors by siRNA eukaryotic, promote the apoptosis of CaSkI, leading to the proliferation of CaSkI/P57 cells was significantly decreased (Jiang et al., 2009). The siRNA was transfected into Hela and CaSkI, inhibited the expression of cyclin B1, selective reduced P53 phosphorylation and increased expression of P53, therefore stimulate the function of downstream genes p21, MDM2, Bax, thereby inhibiting the growth of tumor cells (Kreis et al., 2010). These result suggest that E6 E7 protein may become a potential target for HPV positive cervical cancer. HPV pseudotype virus particles may become a effective and convenient RNAi vector for HPV positive cervical cancer.

Blood vessels, lymphatic vessels are important in cervical cancer early metastasis, cervical cancer early metastasis occur in blood vessels and lymphatic vessels mostly, related lymph node has been transferred in early stage of cervical cancer (Yang et al., 2008) Lymphatic vessel formation and its regulation mechanisms and lymphatic generated effect on promoting tumor metastasis have become an important problem in malignant tumor study. Podoplanin antigen epitope (D2-40), lymphatic vessel endothelial hyaluronan receptor (LYVE-1) and vascular endothelial growth factor receptor 3 (VEGFR-3) and other factors are specific to express on the lymphatic endothelial cell membrane, therefore become tumor-specific markers of lymphatic endothelial cells. Vascular endothelial growth factor C (VEGF-C) and cervical lymph node metastases have significantly positive correlation, VEGF-C may act as upstream regulator of cell signal transduction, activate the NF-κB, make the higher expression of BCL-2, boosting the tumor cells of antiapoptotic effect. But knockout of VEGF-C does not fully suppress cervical cancer cell to transfer via the lymphatic and blood vessels. The original tumor lymphatic vessels and newly built lymphatic vessels may act as different roles in promoting cancer metastasis process and tumor cell metastasis, which needs further study (VanTrappen et al., 2002) Therefore, looking for other lymphatic vessel formation, tumor metastasis growth factors and signal pathways to control the distant metastasis of cancer cells play an important role.

Epithelial-mesenchymal transformation is a physiological mechanism during embryonic development of the necessary, but also in the evolution of epithelial tumors played a key role, such as ovarian cancer (Wang et al., 2013), cervical cancer (Lee et al., 2012), breast cancer (Roxanis I. 2013). Snail, Twist, VEGF, COX-2, CDH-1, FOXC2 all take a part in EMT regulation. Tumor cells transfer from the primary tumor to distant organs just like that cell migration in embryonic development.FOXC2 has relation with occurrence morphological remodeling and structure differentiation of lymphatic vessels. FOXC2 expresses differently in the tumor happening which play an important role in early stage of embryonic development. In 2007 someone found that ectopic expression of FOXC2 in breast cancer make breast cancer cells acquire interstitial morphology and more ability to invade an migrate (Sendurai et al., 2007). In 2011 found that FOXC2 is associated with invasion and metastasis of esophageal SCCs, FOXC2 targeted RNA interference can reduce esophageal squamous cancer cells ability to proliferate and migrate (Naohiro et al., 2011). given the EMT in primary tumor cells migrated to the circulatory system or lymphatic system, people can change process by blocking EMT to prevent the occurrence of infiltration and the formation of metastases, provide opportunities for the precious treatment of patients with cervical cancer.

Although many observations support FOXC2 as a potential target for cancer therapy, minimal knowledge is available on the association between FOXC2 expression and cervical cancer development, or on the molecular mechanisms by which FOXC2 may modulate cervical cancer progression. To address these questions, we profiled the expression of FOXC2 in normal cervical tissues, as well as those with different pathological alterations. In addition, we examined the significance of FOXC2 in regulating the biological behaviors of cervical cancer cells.

Materials and Methods

Cervical tissue specimens

From 2008 April to 2013 October were collected, in The First Hospital of Jilin University Department of gynaecology accept operation treatment of cervical tissue samples were 154 cases, including 40 cases of normal cervical tissues, 40 cases of CINI-III and 74 cases of cervical cancer.

Immunohistochemical staining and analysis

The expressions of FOXC2, CD34 or D2-40 in cervical tissues were detected by immunohistochemical staining using the Streptavidin-Peroxidase (SP) staining kit (Fuzhou Maixin Biotech., Fuzhou, China) following the manufacturer’s instructions. Briefly, paraffin sections were deparaffinized and hydrated. After heat-induced antigen retrieval, endogenous peroxidase activity was blocked using 0.3% H2O2 at room temperature for 20 min, and nonspecific binding sites were blocked with goat serum for 20 min. After incubation with the specific primary antibody diluted in PBS at 4 °C overnight followed by secondary antibody, the sections were developed in diaminobenzidine solution and counterstained with
hematoxylin. Negative control slides were treated the same as above except that the primary antibody was replaced with PBS only (vehicle). The following primary antibodies were used in this study: anti-human FOXC2 polyclonal antibody (1:100, Abcam), anti-human D2-40 monoclonal (1:100) and anti-human CD34 monoclonal antibody (1:200) (ZSGB-Bio, Beijing, China).

Paraffin sections were deparaffinized, hydration, antigen hot repair; Peroxidase blocking agent blocking 20 min at room temperature; Goat serum blocking 20 min; Dropping the Rabbit anti human FOXC2 antibody (1:100), 4°C Overnight; Add two anti 10 min was incubated at room temperature; DAB color, hematoxylin staining; Sliced dehydrated and transparent and neutral gum mounting. D2-40 antibody (1:100) staining, to observe of lymphatics. CD34antibody (1:200) staining, to observe the vascular. The intensity of FOXC2 staining staining intensity PI = SI ×PP, 1-4; were negative to weak positive; 5-8 is divided into moderate positive; 9-12 was strongly positive. The SI result: no staining of cells for 0, cells were stained yellow for 1, cells were stained brown for 2, cells were stained brown for 3; PP result: each section to find expression in densely populated areas 100× magnification, 200× of counting, number 3 horizons, each field were counted 200 cells, averaging, the positive rate was 0 recorded as 0 points, < 10% was 1, 11-50% was 2, 51-80% was 3, 80% was 4.

Cell culture

The human cervical carcinoma Hela cells were generously provided by Dr. Ping Wang (Department of Otolaryngology, the First Hospital of Jilin University) and SiHa cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Both cells were cultured in DMEM medium (Gibco, CA, USA) containing 10% newborn calf serum (Gibco) and penicillin/streptomycin in a 37°C, 5% CO₂ incubator. The human cervical carcinoma Hela cells were 4.

siRNA transfection

The siRNA specifically targeting human FOXC2 (siFOXC2, 5’-GACCCAAACCAGACAATTAAGG-3’) and a control siRNA sequence targeting no known human genes (siCtrl, 5’-GTTCCTCGAAGATGTGCTGT-3’) was designed and cloned into the siRNA-expressing vector pGPU6/GFP/Neo by GenePharma (Shanghai, China). The transfection of these plasmids into cells was performed using Lipofectamine 2000 (Invitrogen, CA, USA) following the manufacturer’s instruction.

RNA extraction, synthesis of cDNA and RT-qPCR

RNA was extracted from manual calculation according to TRLzol (Takara, Dalian, China), the purity and concentration, 5 μg RNA in 1% agarose gel electrophoresis, UV detection of integrity of RNA, all qualified RNA reverse transcription, in accordance with the first strand cDNA synthesis kit (TAKARA) that synthesis of cDNA.

FOXC2 primer sense 5’-GCCTAAGGACCTGTGAAAGC-3’ antisense 5’-TGTACGAAACGTCTGTTGAG-3’; GAPDH primer sense 5’-GCACCGTCAAGGCTGAGAA-3’ antisense 5’-TGTTGAAGACGCCAGTGGGA-3’.

cDNA 2 μL, 2 pairs of primer 1 μL, 2×SYBR MIX 10 μL, ddH₂O added to 20 μL. The reaction conditions: 94°C, 1 min, 94°C, 10 s, 60°C, 20 s. A total of 50 cycles. Each sample underwent 3 complex hole detection, the cycle threshold (CT) value. Taking GAPDH as the reference gene, 2^ΔΔCT method to calculate the relative expression amount.

Cell viability assay

Cell viability was examined using Cell Counting Kit-8 (CCK8, Beyotime Biotech., Jiangsu, China) following the manufacturer’s instructions. Briefly, cells were seeded into 96-well plate at 2000 cells/well. At 24 to 72 h after siRNA transfection, 10 μL of CCK8 solution was added into each well and the cells were incubated for 1 h. The optical density (OD) was then read at 450 nm. DMEM medium alone was used as control. The relative cell viability at a specific time point was calculated ad OD (cells)/OD (control) and plotted to generate the viability curve over time after siRNA transfection.

The scratch migration assay

24 hours after transfection, using pipette gun head is straight scratch on the culture plate at the bottom, on the migration of 0 h and 24 h after the scratch cells were observed under inverted microscope and photographed, distance measurement of 0h scratches (A) and 24 h scratch area distance (B), relative movement distance = (A-B)/A×100%.

Statistical analysis

Continuous variables were presented as mean±SD. Quantitative data were expressed as percentage. Data comparison was performed using a Student’s t test and a P value of < 0.05 was considered statistically significant.

Results

The expression of FOXC2 and staining intensity in cervical tissue

Immunohistochemical staining of FOXC2 positive expression, mainly in the cytoplasm of the tumor cells showed brown yellow or brown staining. The positive expression of FOXC2 protein in 68 cases of cervical carcinoma tissues accounting for 74 of all cases of cervical carcinoma tissues of 91.89%, the normal cervical tissue FOXC2 staining intensity of PI was 5.568 ± 3.449, cervical χ² = 12.92, P< 0.01. The positive expression rate of FOXC2 in CINI-III was 65% (26/40 cases), compared with the cervical cancer χ² = 12.98, P< 0.01; compared with normal cervical χ² = 12.92, P< 0.01 (Figure 1a). Cervical cancer tissue FOXC2 staining intensity of PI was 5.568 ± 3.449, Significantly higher than that in normal cervical PI 0.297 ± 0.520, t=9.59, P< 0.05; cervical CIN I-III PI 3 ± 3.29, compared with normal cervix, PI t = 5.15, P< 0.05 (Figure 1b). Expression of FOXC2 in adenocarcinoma was 100%, 5 cases of adenocarcinoma were weak positive expression, PI was 3.2 ± 0.837 (Figure 1c). Expression of FOXC2 in SCC was 91.30% (63/69 cases), PI was 5.74 ± 3.51; weak.
positive accounted for 31.70% (20 cases), PI was 2.70 ± 0.92; moderate positive accounted for 39.70% (25 cases), PI was 6.24 ± 0.66; the strong positive rate was 28.6% (18 cases), PI was 10.33 ± 1.53 (Figure 1c and Figure 2).

Expression of FOXC2 in Adenocarcinoma and SCC

FOXC2 expression was mainly located in the interstitial and interstitial vascular, Expression is clearly visible in the cell membrane of small vascular endothelial cell wall thickness of FOXC2, and found positive cells around blood vessels, no expression in glandular cells of FOXC2 (Figure 2). In SCC, CD34 staining showed a large amount of new blood vessels in the basal layer beneath the mesenchymal region. In the same region, FOXC2 staining also showed that FOXC2 positively expressed not only in the new vessels, but also did in squamous cell carcinoma. There was a coincidence between positive expression of CD34 and positive expression of FOXC2. D2-40 staining revealed that there are a lot of lymphangiogenesis in the region which accumulated FOXC2 staining positive cells. FOXC2 immuno staining color degree in lymphangiogenesis was weakened by that in mesenchymal cells. This suggests that micro lymphatic also expressed FOXC2 (Figure 3).

Table 1. Correlation Between FOXC2 Expression and Clinicopathological Characteristics of Patients with Cervical SCC

<table>
<thead>
<tr>
<th>Clinical characteristics</th>
<th>Number of cases</th>
<th>Expression of FOXC2</th>
<th>X²</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td></td>
<td>Positive (%)</td>
<td>Negative (%)</td>
<td></td>
</tr>
<tr>
<td>≥50</td>
<td>22</td>
<td>22 (100)</td>
<td>0 (0)</td>
<td>3.08</td>
</tr>
<tr>
<td>&lt;50</td>
<td>47</td>
<td>41 (87.3)</td>
<td>6 (12.7)</td>
<td></td>
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<tr>
<td>Differentiation degree</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>11</td>
<td>10 (90.9)</td>
<td>1 (9.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Medium - low</td>
<td>58</td>
<td>53 (91.4)</td>
<td>5 (8.6)</td>
<td></td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥4</td>
<td>34</td>
<td>31 (91.2)</td>
<td>3 (8.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>&lt;4</td>
<td>35</td>
<td>32 (91.4)</td>
<td>3 (8.6)</td>
<td></td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>52</td>
<td>46 (88.5)</td>
<td>6 (11.5)</td>
<td>2.15</td>
</tr>
<tr>
<td>II</td>
<td>17</td>
<td>17 (100)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Vascular invasion</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>27</td>
<td>24 (88.9)</td>
<td>3 (11.1)</td>
<td>0.33</td>
</tr>
<tr>
<td>Positive</td>
<td>42</td>
<td>39 (92.9)</td>
<td>3 (7.1)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>62</td>
<td>56 (90.3)</td>
<td>6 (9.7)</td>
<td>0.74</td>
</tr>
<tr>
<td>Positive</td>
<td>7</td>
<td>7 (100)</td>
<td>0 (0)</td>
<td></td>
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</table>

Relationship between SCC and clinical characteristics

The positive expression of FOXC2 in cervical SCC in stage I was 88.5%, stage II was 100%. There are significant statistical difference compared with the normal cervix or CIN. The positive rate was obvious increasing tendency, but there was no significant difference between the two groups. P>0.05.

On the staining intensity of the cervical SCC stage II FOXC2’s PI was 6.59 ± 3.64, higher than that of stage I PI 5.404 ± 3.414, both of which were significantly higher than in normal cervix, P < 0.05, but there was no significant difference between the two groups. P>0.05. The expression of FOXC2 in the blood vessels interstitial, in cervical SCC in stage I. Secondary, thin pipe wall, While the II expression of FOXC2 was detected in blood vessels lumen is larger, the wall thickness. Blood vessels lumen were observed in the tumor thrombus, expression of FOXC2 around positive blood vessels visible multiple vacuolar organization, Thes staining positive of A single nuclear cells were seen around the blood vessels. In the
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**Figure 4. Expression of Foxc2 in SCC.** (4a) Expression of Foxc2 in SCC of Clinical Stage; (4b) SCC Clinical Stage II interstitial bureaucratic large, visible tumor throbous wall thicker vascular lumen, around which there are single nuclear staining positive cells (black the arrow); (4c) in clinical stage I SCC keratosis; (4d) in clinical stage II SCC keratosis (200x).

visible II stage of squamous keratosis with I dyeing range. No significant difference between FOXC2 expression with cervical cancer patient’s age and the tumor size and the tumor differentiation, P > 0.05.

The positive rate of infiltration pipe midvein cervical SCC patients was 60.9%, the positive rate of invasion in patients with clinical stage I tube midrib is 50%, and clinical stage II was 94.1%. The two groups had statistical significance, $\chi^2$=10.47, P<0.01. Patients with cervical SCC in patients with vascular invasion positive FOXC2 staining intensity of PI was 6 ± 3.47, and negative PI was 5.33 ± 3.49, no significant difference between two groups, P > 0.05.

In the patients with lymph node metastasis in cervical SCC tissue FOXC2 staining intensity PI was 8.29 ± 4.49, and the patients without lymph node metastasis in cervical SCC tissue FOXC2 staining intensity PI was 5.40±3.27. Although, lymph node metastasis groupthe staining intensity were significantly higher than those without lymph metastasis group, but no significant differences between the two groups, P>0.05. (Table1).

The experimental results of RT-qPCR differential degree Expression of FOXC2 mRNA in Hela cells was 1.089 ± 0.054, expression of FOXC2 mRNA in SiHa cells was 1.155 ± 0.053 (with Hela cell as a reference for relative quantitative), t=0.427, P>0.05 (Figure 5a). Group pGP-siRNA was interference, and group pGP-siNC was negative vector control group, set a blank control, for transient transfection. Group pGP-siRNA decreased expression of FOXC2 75.7% in SiHa cells, in Hela cells down 76.2%.

**The scratch test results**

Transient transfection of 24h after scratch, image acquisition in 0h, 24h respectively, the detection of Hela cell migration, the control group 64.47 ± 3.16 relative mobility, interference group relative migration 49.5 ± 9.24, two group differences (P<0.05). The invasion and migration of SiHa cells in control group detection, the relative migration of 60.12 ± 3.05, 44.3 ± 3.98 relative migration interference group, the two group differences (P<0.05) (Figure 5b).

The experimental results of CCK8

pGP-siRNA and pGP-siNC in Hela and SiHa cell proliferation activity of the change is not very obvious, Hela and SiHa cell proliferation in 48h-72h see more obviously slowed down, but no statistical significance compared with the control group (Figure 5c, 5d).

**Discussion**

FOXC2 belongs to a highly evolutionally reserved winged-helix transcription factor family with DNA binding site. FOXC2 is highly expressed in mammal embryonic vascular endothelium as a regulatory factor in Notch pathway modulating the specific differentiation from endothelial cells to arterial endothelial cells. The function of FOXC2 in directly inducing transcription and expression of CXCR4 and integrin β3 by binding fox-binding element in their promoters (Kume et al., 2008), hence inducing the expression of VEGF and stimulates angiogenesis. VEGF may also participate in the regulation of FOXC2 transcription activity in the endothelial cells. Both in vivo and in vitro evidences have proved that FOXC2 enhances migration of vascular endothelial cells to VEGF and SDF-1 (Hayashi et al., 2008). DI4 is a ligand of Notch signaling. Kuhnert and Jessica suggested that mutation of notch signaling gene such as notch and its downstream target Hey1/Hey2 might lead to the deficiency of arterial marker expression such as ephrinB2 and manifest as arteriovenous malformations, AVMs (Kuhnert et al., 2011). In FOXC2 mutated cells, DI4 is down regulated or deficient, whereas in cells with FOXC2 highly expressed, DI4 expression is also up-regulated. This suggests potential role of FOXC2 in angiogenesis and vascular...
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References


