Possible role of Pax-6 in promoting breast cancer cell proliferation and tumorigenesis

Xiangyun Zong, Hongjian Yang*, Yang Yu, Dehong Zou, Zhiqiang Ling, Xiangming He & Xuli Meng

Department of Breast Surgery, Zhejiang Provincial Cancer Hospital, Hangzhou 310022, China

Pax 6, a member of the paired box (Pax) family, has been implicated in oncogenesis. However, its therapeutic potential has been never examined in breast cancer. To explore the role of Pax6 in breast cancer development, a lentivirus based short hairpin RNA (shRNA) delivery system was used to knockdown Pax6 expression in estrogen receptor (ER)-positive (MCF-7) and ER-negative (MDA-MB-231) breast cancer cells. Effect of Pax6 silencing on breast cancer cell proliferation and tumorigenesis was analyzed. Pax6-RNAi-lentivirus infection remarkably downregulated the expression levels of Pax6 mRNA and protein in MCF-7 and MDA-MB-231 cells. Accordingly, the cell viability, DNA synthesis, and colony formation were strongly suppressed, and the tumorigenesis in xenograft nude mice was significantly inhibited. Moreover, tumor cells were arrested at G0/G1 phase after Pax6 was knocked down. Pax6 facilitates important regulatory roles in breast cancer cell proliferation and tumor progression, and could serve as a diagnostic marker for clinical investigation. [BMB reports 2011; 44(9): 595-600]

INTRODUCTION

Paired box 6 (Pax6) was first identified as a member of the murine multigene family that shares a conserved Pax sequence motif with developmental control and tissue-specific genes of Drosophila (1). Human Pax6 gene on chromosome 11p13, encodes a 422-amino-acid transcriptional regulator with two DNA-binding domains: a bipartite paired domain (PRD) and a paired-type homeodomain (HD) (2). Pax6 is essential for the development of eyes, central nervous system (CNS), pancreas, endocrine glands of vertebrates and invertebrates (3-8). Besides differentiation, proliferation, apoptosis, and some other important biological processes, Pax6 has been implicated in oncogenesis as well (9-11).

The vertebrate Pax6 locus predominantly produces two alternatively spliced Pax6 isoforms, Pax6 and Pax6-5a, which differs from Pax6 by the presence of 14 additional amino acids encoded by exon 5a (12). In a study of pancreatic cancers, both protein forms of Pax6 can transactivate the expression of Met oncogene that commonly overexpressed in many cancers. And downregulation of Pax6 results in a decline in cancer cell proliferation and Met protein expression (13). In addition, Pax6 was found to be directly linked to glioblastoma, bladder cancer and prostate cancer, suggesting that Pax6 may function as a tumor suppressor and serve as a molecular biomarker for cancer development (14,15). Moreover, it has been reported that Pax6 may interact with androgen receptor (AR) and repress the transcriptional activity and target gene expression of AR to regulate cell growth and regeneration (15).

However, few studies were focused on the role of Pax family members in breast cancers. Human growth hormone (hGH) has been found to contribute to proliferative disorders of the human breast, including breast cancer. Autocrine production of hGH in human breast cancer cells was associated with a strong nuclear accumulation of Pax5 and a significant increase of Pax5-DNA binding activity, suggesting that Pax5 might be involved in breast cancer cell proliferation (16). In a cancer-wide analysis of Pax gene expression in a panel of common cancer cell lines, Pax6 gene was expressed at high levels in breast and other cancer cells (17). In these regards, Pax6 may play a crucial role in regulating breast cancer cell growth by interacting with some important molecules such as Met and AR (13,15).

Yet the functional role of Pax6 in breast cancer carcinogenesis has not been addressed. In this study, we aim to explore the effect of Pax6 inhibition on breast cancer cell growth in vitro and in vivo via lentivirus-based RNAi approach, which might have a major impact on the developing new treatment strategies of breast cancer.

RESULTS

Pax6 silencing in MCF-7 and MDA-MB-231 cells

Pax6 expression in human breast cancer cells were first examined by Western blotting assay. It revealed that Pax6 expression levels in MCF-7 and MDA-MB-231 cells are abnormally high in comparison to human mammary epithelial cells MCF10A (Fig. 1A). Then, a lentivirus-RNAi based system was
used to efficiently knockdown Pax6 expression in both breast cancer cell lines. As shown in Fig. 1B, over 80% cells were GFP-positive, indicating that a large proportion of cells were successfully infected. Then we studied the knockdown efficiency of Pax6 by real-time PCR and Western blotting assay. In MCF-7 and MDA-MB-231 cells, the mRNA levels were decreased by 73.3% and 66.3%, respectively (P < 0.01, Fig. 1C). Also, the protein levels were remarkably decreased by

**Fig. 1.** Silencing of Pax6 by RNAi lentivirus. (A) Pax6 expression in human breast cancer cells (MCF-7 and MDA-MB-231) and a human mammary epithelial cell line (MCF10A). (B) Photomicrographs of GFP-positive MCF-7 and MDA-MB-231 cells infected by lentivirus. Bright light (left panel) and fluorescence (right panel) pictures were taken 72 h after infection at a magnification of 200. (C) mRNA levels of Pax6 in MCF-7 and MDA-MB-231 cells were determined by quantitative real-time PCR (b, P < 0.01). (D) Protein levels of Pax6 in MCF-7 and MDA-MB-231 cells were determined by western blotting analysis. GAPDH was used as loading control. Control: control lentivirus; RNAi: Pax6-RNAi-lentivirus.

**Pax6-RNAi-lentivirus** compared to the levels of housekeeping gene GAPDH (Fig. 1D).

**Effect of Pax6 silencing on breast cancer cell growth and in vitro colony formation**

In order to explore the effect of Pax6 on growth of breast cancer cells, MTT cell proliferation assay was performed on MCF10A, MCF-7 and MDA-MB-231 cells for five successive days. The viability of Pax6 knock-down MCF10A cells was only decreased by 12.7% after 5 days of lentivirus infection (P < 0.01, Fig. 2A). However, the proliferation indicates a decrease by 47.9% and 57.9% in MCF-7 and MDA-MB-231 cells compared with controls (P < 0.01, Fig. 2B, C). Then we used BrdU incorporation assay to further confirm the proliferative ability of Pax6 knock-down cancer cells. As shown in Fig. 2D, E, significant decrease in the DNA synthesis ability was observed at 24 h and 72 h in MCF-7 and MDA-MB-231 cells (P < 0.01). To detect the malignant feature of breast cancer cells, colony formation assay was performed. Lentivirus treated cells were allowed to form natural colonies in two weeks. As shown in Fig. 2F, the number of giemsa-stained colonies in Pax6-RNAi-lentivirus treated MCF-7 and MDA-MB-231 cells were 22.3 ± 2.1 and 31.3 ± 5.7, which were dramatically inhibited compared to 83.0 ± 7.0 and 90.0 ± 5.3 in control groups, respectively.

**Effect of Pax6 silencing on cell cycle progression**

To further investigate the potential mechanism regulating the inhibition of growth by Pax6 RNAi, the cell cycle status was determined by flow cytometry analysis. When Pax6 expression was downregulated, there was a significant increase in the cell number at G0/G1 phase in Pax6 knock-down MCF-7 and MDA-MB-231 cells, from 45.0% to 60.0% and 52.6% to 57.6%, respectively (Fig. 3A, B). We speculated that the G0/G1 arrest may contribute to the inhibition of growth in breast cancer cells.

**Fig. 2.** Pax6-RNAi-lentivirus represses breast cancer cell proliferation and in vitro colony formation. The viability of Pax6 RNAi or control lentivirus-infected MCF10A (A), MCF-7 (B) and MDA-MB-231 (C) cells was examined by MTT assay. The proliferative ability in Pax6-RNAi-lentivirus treated MCF-7 (D) and MDA-MB-231 (E) cells were significantly decreased as evidenced by BrdU assay (b, P < 0.01; c, P < 0.05). (F) Giemsa stained colonies (>50 cells in each colony) were counted and statistically analyzed (b, P < 0.01). Control: control lentivirus; RNAi: Pax6-RNAi-lentivirus.
Effect of Pax6 silencing on tumor growth in nude mice
To test whether Pax6 RNAi influenced the growth of human breast cancer cell line in vivo, Pax6-RNAi-lentivirus and control lentivirus infected MCF-7 cells were inoculated in nude mice. After 22 days, all mice developed tumors (Fig. 4A). Tumor size was measured 14, 18 and 22 days after injection. As shown in Fig. 4B, the average volume of tumors in control group was about 6-fold larger than in RNAi group at 14 days; while the difference became more distinctive at 18 and 22 days (P < 0.01, Fig. 4C). Moreover, the weight of tumor from Pax6-RNAi treated mice was markedly smaller than control (P < 0.01, Fig. 4D), indicating Pax6 knockdown inhibited the tumorigenesis of breast cancer cells.

DISCUSSION
Based on the levels of ER and drug sensitivity status, breast cancers falls into two broad categories, ER-positive or ER-negative types (18). ER is expressed in about 60% of all breast
cancers, which generally possess better prognosis and is often responsive to anti-estrogen therapy. On the other hand, ER-negative breast cancers are unresponsive to antiestrogens, thus being more aggressive (19). Therefore, it is of great importance to understand the cellular regulatory mechanisms governing both kinds of breast cancers. In this scenario, we chose two breast cancer cell lines, MDA-MB-231 (ER-negative) and MCF-7 (ER-positive) as cell models to study the effect of Pax6 silencing on breast cancer cell growth.

Pax6 is a transcription factor that regulates the expression of a broad range of molecules, hormones and structural proteins (10). In our study, we first constructed an efficient lentivirus-RNAi based system to downregulate Pax6 expression (Fig. 1). It should be noted that Pax6 has three different protein isoforms: normal Pax6, Pax6 (5α), and paired domain-less Pax6 (ΔPD) (20). Thereinto, Pax6 (5α) is expressed in pancreatic carcinoma cell lines at higher levels than the canonical Pax6 protein (13). Yet it is still unknown if there is any difference in expression levels or functional roles in breast cancer for individual variants. Therefore, the short hairpin RNA (shRNA) was designed against all three transcript variants of Pax6 in this experiment, thus could decrease the expression of total Pax6 at protein level.

RNAi and control lentiviruses were applied in both breast cancer cell models and a nude mouse model to explore the function of Pax6 in breast cancer carcinogenesis. These experiments showed that decreased Pax6 expression resulted in remarkable suppression of in vitro cell growth and in vivo tumorigenesis. Our observations were in consistency with that in previous report about pancreatic cancer (13).

Disturbed regulation of the cell cycle is a hallmark of cancers. We then investigated the cell cycle distribution of Pax6 knock-down breast cancer cells. Analysis on both cells showed arresting cells in the G0/G1-phase of the cell cycle. It suggests that Pax6 controls the growth of breast cancer cells by controlling the progression of cells through the cycle. Further investigations of cell cycle regulators may help us better understand the mechanism(s) of the oncogene effect of Pax6.

However reports on other tumors such as glioblastoma, bladder cancer and prostate cancer suggested that Pax6 may function as a tumor repressor (14,15). For example, methylated Pax6 may represent as a biomarker for bladder cancer, and Pax6 may inhibit prostate cancer by transcriptionally regulate AR. Thus, Pax6 may play different roles in different microenvironment of cancers. Interestingly, most estrogen receptor-positive breast tumors also express AR. And AR could exert growth inhibitory effects on ER and progesterone receptor (PR)-negative breast cancer cell lines that show AR expression (21). Thus, it is possible that Pax6 RNAi may repress the growth of breast cancer cell growth by upregulating the expression of AR. However, this assertion needs to be confirmed in future experiments.

In conclusion, our data suggests that Pax6 is a novel regulator in both ER-positive and -negative breast cancer cell growth. And it could serve as a diagnostic marker for breast cancer clinical investigation.

MATERIALS AND METHODS

Construction and production of lentivirus

Lentivirus system from Shanghai Genechem, Co. Ltd consists of three packing vectors: pGCL-GFP, pHelper 1.0 (gag/pol) and pHelper 2.0 (VSVG). Short hairpin RNA (shRNA) targeting Pax6 (5'-ATGGGCGGAGTTATGATACCTAC-3') and scrambled control (5'-AATTCTCGAAGCTGCTACGGG-3') were cloned into pGCL-GFP vector, respectively, which were then co-transfected with pHelper 1.0 and 2.0 vectors into 293T cells to generate individual lentiviruses. After 48 h, Pax6-RNAi-lentivirus and control lentivirus were harvested and purified.

Cell culture and infection

Human breast cancer cell lines MCF-7 and MDA-MB-231, human mammary epithelial cells MCF10A were obtained from the Institute of Biochemistry and Cell Biology, Shanghai, China. Cells were cultured in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2. For lentivirus infection, cells were cultured in 6-well plates to reach 80% confluence and then treated with lentivirus at m.o.i. (multiplicity of infection) of 20 and 10, respectively. GFP expression was determined 72 h after infection by fluorescence microscopy.

Quantitative real-time PCR

Total RNA from Pax6-RNAi-lentivirus and control lentivirus treated cells was prepared with Trizol reagent (Invitrogen) 72 h after infection. cDNA was then synthesized with random primers following the manufacturer’s protocol (TAKARA). In quantitative real-time PCR, two sets of primers were used as follows. Actin-F, 5'-GGGCGCAGGACTTCATACCT-3', Actin-R, 5'-AGGGGCCGGACTCGTCATACT-3'; Pax6-F, 5'-ATGGGCGGAGTTATGATACCTAC-3'; Pax6-R, 5'-AATTCTCGAAGCTGCTACGGG-3'. Real-time PCR was performed using a standard SYBR Green PCR kit protocol on a Rotor-Gene RG-3000A (Corbetter Research) thermocycler.

Western blotting analysis

Total cell extracts from Pax6-RNAi-lentivirus and control lentivirus treated cells were isolated 72 h after infection. The concentration of total protein was determined by BCA protein assay (Pierce). Cell extracts from all samples were separated on 10% SDS-polyacrylamide gels and transferred electrophoretically to a polyvinylidene fluoride membrane, which were then blocked with 5% skim milk. Membranes were incubated with specific primary antibodies for Pax6 (Santa Cruz) or GAPDH (Santa Cruz), followed by incubation in goat anti-rabbit or anti-mouse secondary antibody (Santa Cruz) and visualized by enhanced chemiluminescence.
Measurement of cell proliferation
The proliferative ability of Pax6-RNAi-lentivirus and control lentivirus treated cells was measured by methylthiazolete-tetrazolium (MTT) and bromodeoxyuridine (BrdU) assay. In detail, MCF-7 and MDA-MB-231 cells were infected with lentivirus in 6-well plates and re-seeded in 96-well plates at a density of 1,500 cells per well. For MTT assay, cells were treated with MTT solution (5 mg/ml) after 1, 2, 3, 4, and 5 days. The crystals were then dissolved in dimethyl sulfoxide (DMSO) and the absorbance was recorded at 570 nm. For BrdU incorporation assay, cells were treated with 20 μl BrdU (1 : 500 in dilution) after 24 h and 72 h of culture. After incubated with anti-BrdU monoclonal (1 : 200 in dilution) and goat anti-mouse IgG (peroxidase conjugate, 1 : 2,000 in dilution), the absorbance was recorded by a spectrophotometer at dual wavelength of 450/550 nm.

Colony formation assay
MCF-7 and MDA-MB-231 cells infected with Pax6-RNAi-lentivirus and control lentivirus were cultured in 6-well plates (200 cells per well) as described previously (22), where cells were allowed to form colonies. After 14 days, cells were washed, fixed with 3.7% formaldehyde and stained with giemsa. All stained cultures were photographed with a digital camera and colonies of greater than 50 cells were counted.

Flow cytometry assay
The cell cycle distribution of Pax6 knock-down cancer cells were analyzed by FACScan flow cytometry. Briefly, 5 × 10⁵ cells were seeded in 6-cm dishes and infected with lentiviruses for 5 days. Cells were collected, washed with PBS, and fixed with 70% cold ethanol. The fixed cells were then dissolved in dimethyl sulfoxide (DMSO) and the absorbance was recorded at 570 nm. For BrdU incorporation assay, cells were treated with 20 μl BrdU (1 : 500 in dilution) after 24 h and 72 h of culture. After incubated with anti-BrdU monoclonal (1 : 200 in dilution) and goat anti-mouse IgG (peroxidase conjugate, 1 : 2,000 in dilution), the absorbance was recorded by a spectrophotometer at dual wavelength of 450/550 nm.

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Statistical analysis
All of the data is expressed as mean ± standard deviation of three experiments. Statistical significance of differences was determined by Student’s t-test. A P value of less than 0.05 was considered significant.

REFERENCES


