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ORIGINAL PAPER

HoxA10 Induces Proliferation in Human Prostate Carcinoma PC-3 Cell Line

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Abstract The objectives of this study were to examine the expression levels of Homeobox A10 (HoxA10) in prostate cancer cells and to study the molecular mechanism of HoxA10-mediated regulation of prostate cancer cell growth and development. We investigated the effect of HoxA10 on cell proliferation by stably overexpressing or silencing HoxA10 in prostate cancer PC-3 cell line using lentiviral vectors. Quantitative real-time PCR and western blotting analysis were used to compare the expressions of HoxA10 in prostate cancer cell lines and normal prostate epithelium. Cancer cell proliferation was examined by MTT assay and colony formation assay. The levels of HoxA10 expression were significantly increased in prostate cancer cell lines and tissues compared to those in normal prostate epithelium. Overexpression of HoxA10 in PC-3 cells induced significant cancer cell proliferation, whereas silencing of HoxA10 expression by RNAi resulted in decreased proliferation rates. HoxA10 was highly expressed in prostate cancer cells and tissues, suggesting its functional involvement in cancer cell proliferation. We successfully overexpressed or silenced HoxA10 in prostate cancer PC-3 cell line and discovered that the levels of HoxA10 directly correlate with cancer cell proliferation. These findings contribute to a better understanding of the regulatory mechanism of HoxA10 in prostate cancer.

Keywords HoxA10 · Prostate cancer · Lentivirus · Cancer cell line · Cell proliferation

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Introduction

Homeobox (HOX) genes are a family of evolutionarily highly conserved regulatory genes, encoding transcription factors that are essential for regulating cell development, maturation, differentiation, and proliferation. There are numerous reports of pro-proliferative and anti-apoptotic roles of some HOX genes and their overexpression in a range of malignancies [1].

Homeobox A10 (HoxA10) is an important member of the HOX family [2], which is involved in modulating cancer cell differentiation and development in leukemia, lung cancer, or endometrial cancer [3–7]. HoxA10 belongs to cluster A of the class I HOX gene, located at 7p15–p14, and its overexpression has been shown to promote excessive proliferation of early hematopoietic cells, thus leading to the development of myeloid leukemia, breast cancer, glioma, and other forms of cancer [7–11].

Prostate cancer is the most prevalent male malignancy with just under one million new cases worldwide each year [12]. Recent report showed that overexpression of HOX genes is associated with the loss of tumor differentiation in human prostate cancer [13]. In the present study, we found that HoxA10 was highly expressed in prostate cancer cells. By stably overexpressing or, alternatively, silencing HoxA10 expression, we evaluated the effect of HoxA10 on the development of the prostate cancer cells and its role in the pathogenesis of the prostate cancer.

Methods

Materials

Cell Lines and Plasmid

Prostate cancer cell lines, DU145, PC-3, 22RV1, and LNcap, were purchased from the ATCC (Manassas, VA, USA). Lentiviral packaging cells, 293T cells, were purchased from the Shanghai Academy of Sciences Library (China). pMSCV-puro and pSuper-puro plasmids were made in our laboratory.

Reagents

FBS, RPMI-1640, and K-SFM were purchased from Life Technologies (Carlsbad, CA, USA), and pDH5α competent cells were purchased from Guangzhou Bion Company (Beijing, China). T4 DNA ligase, AMV reverse transcriptase, and random 9-mer primers were purchased from TaKaRa Shuzo Co. (Seoul, Korea), and XhoI and EcoRI restriction endonucleases were purchased from NEB (Ipswich, MA, USA). PCR reagents were purchased from Tiangen Biotech (Beijing, China), and qPCR reagents were purchased from FulenGene (Beijing, China). Anti-HoxA10, anti-β-actin primary antibodies, and horseradish peroxidase (HRP)-conjugated rabbit and mouse secondary antibodies were purchased from Sigma (St. Louis, CA, USA), and HRP-ECL was purchased from Perkin Elmer (Shelton, CT, USA).

Human Tissue

The human samples of prostate cancer tissue and cancer adjacent tissues were provided by the Department of Urology, Guangzhou First People's Hospital. The protocol was approved by the Ethics Committee of Guangzhou First People's Hospital, Guangzhou Medical University. The written informed consent was provided by the patients.

Cell Culture

Human prostate cancer cell lines, DU145, PC-3, 22RV1, and LNcap, were grown in RPMI-1640 medium supplemented with 10 % FBS.

Analysis of HoxA10 Expression in Prostate Cancer Cells

RNAs were isolated from frozen tissue, using TRIzol reagent (Life technologies, Carlsbad, CA, USA), and 2 μg of total RNA was used to synthesize cDNAs using AMV reverse transcriptase and random 9-mer primers (Takara Shuzo Co., Seoul, Korea), according to the manufacturer's protocols. HoxA10 gene expression was evaluated in various cell lines by SYBR qPCR using the following oligonucleotides: HoxA10 F: 5′-CTCCCACACTCGCCATC TC-3′; HoxA10 R: 5′-CAAACCCAGCCCAGTCAGG-3′. Expression levels were normalized to β-actin.

Generation of pMSCV-HoxA10 and pSuper-puro-HoxA10/RNAi Constructs

HoxA10 gene (NM_018951.3) was amplified by PCR, digested with EcoRI and XhoI restriction exonucleases, and cloned into the Xho I–EcoRI restriction site of the pMSCV-puro and pSuper-puro expression vectors using T4 DNA ligase (Fig. 1a). The constructs were subsequently transformed into pDH5α competent cells; plasmid DNA from the ampicillin-resistant colonies was extracted using QIAprep Spin miniprep kit (Quiagen, Limburg, Netherlands) and digested with Xho I and EcoR I restriction endonucleases to verify the presence of 1233-bp HoxA10 insertion (Fig. 1b). Positive colonies were further verified by sequencing at the DNA Sequencing Facility, Guangzhou YingJun Company (China).

Stable Expression of HoxA10 in Prostate Cancer PC-3 Cells

HEK 293T cells were plated in 10-cm tissue culture dishes at the density of 3×10^6 cells/well, and maintained at 37 °C, 5 % CO2 for 24 h. Calcium phosphate transfection was used for viral packaging. Transfection was done in two groups: HEK 293T cells in the experimental group were transfected with pMSCV-puro-HoxA10 (PC-3/HoxA10 group) or pSuper-puro-HoxA10/RNAi (PC-3-HoxA10/RNAi group), and cells in the control group were transfected with pMSCV-puro (PC-3/Vector group) or pSuper-puro-scramble (PC-3-scramble group) (Fig. 1). 24 h after transfection, virus solution was filtered using 0.45 μm filter and stored at −80 °C.

PC-3 cells were seeded in 10 cm cell culture dish at the density of 2×10^6 cells/well. Upon reaching 70 % confluence, the cells were treated with 8 μg/ml viral vector. The infection was repeated every 12 h for 2 days. Cells were passaged 24 h after the last infection and incubated in the presence of 0.5 μg/ml puromycin (Sigma, St. Louis, CA, USA).

Quantitative PCR of HoxA10 in PC-3 Cells

After 10 generations, puromycin-resistant PC-3/Vector, PC-3/HoxA10, PC-3-scramble, and PC-3-HoxA10/RNAi cells were collected, cellular DNA was extracted, and HoxA10 gene expression was evaluated using qPCR as

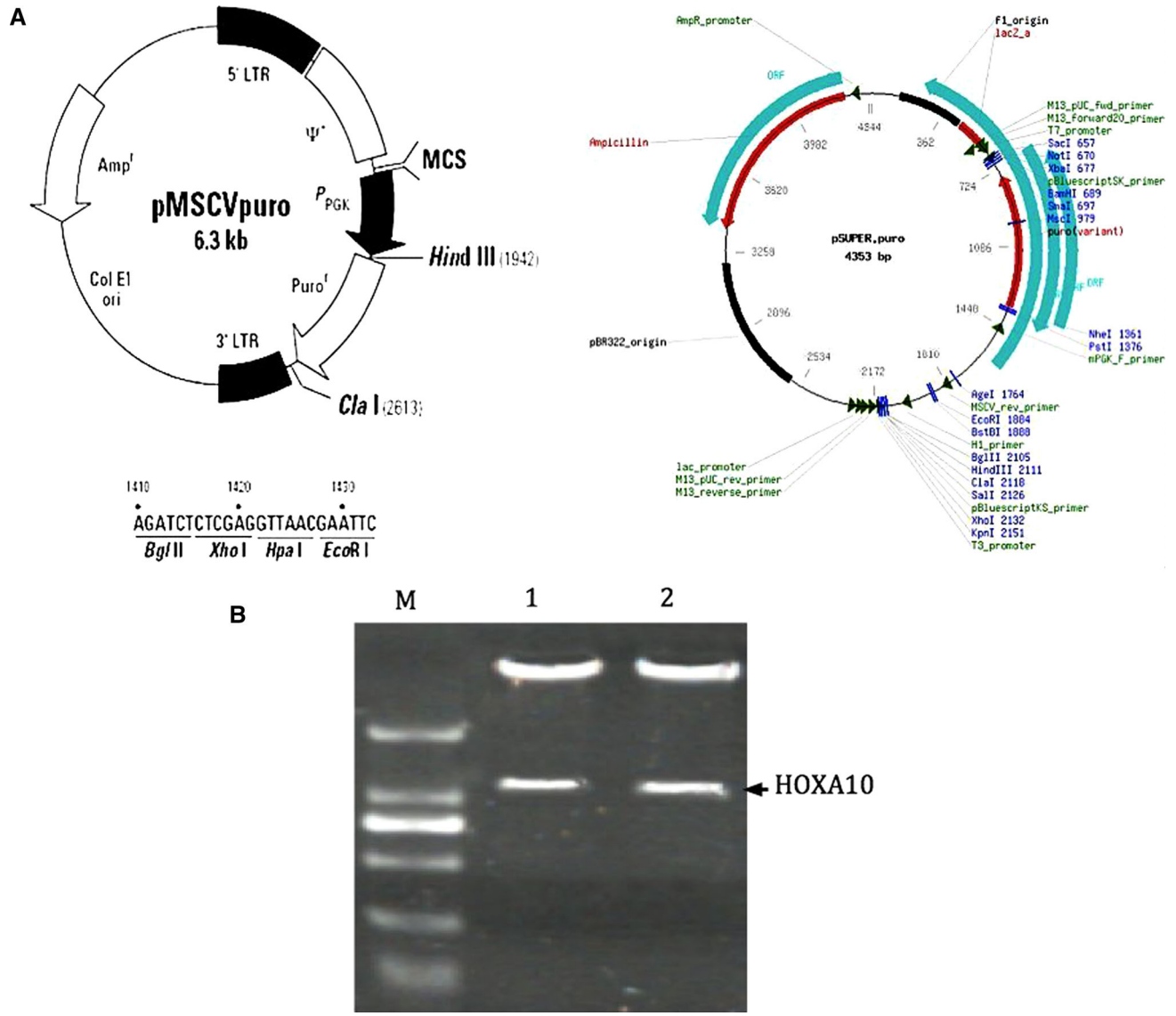

Fig. 1 Cloning of HoxA10 gene. **a** Plasmid maps of pMSCV-puro and pSuper-puro vectors. **b** Restriction analysis of pMSCV-puro-HoxA10 (*lane 1*) and pSuper-puro-HoxA10 (*lane 2*) constructs. Plasmid DNA was digested with XhoI and EcoRI to determine the size of the insert (1233 bp). *Lane M* indicates DNA ladder

described above. Each reaction was repeated in triplicates. Student's *t* test was used for statistical analysis between two groups. $P < 0.05$ was considered as statistically different.

Western Blotting

Cells were lysed in buffer containing 10 mM Tris–HCl pH 7.4, 1 % Triton X-100 and a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific Inc., Rockford, IL). The protein concentration was determined using a BCA kit (Sigma, St. Luois, CA, USA). 20 µg of lysate proteins in Laemmli buffer was fractionated on SDS-PAGE, transferred to polyvinylidene difluoride membranes and blotted. Membranes were treated with 5 % skim milk and incubated with anti-HoxA10 or anti-β-actin primary antibodies overnight at 4 °C. Appropriate IgGs conjugated with HRP were used as secondary antibodies, and the signal was visualized by ECL method.

MTT and Colony Formation Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] colorimetric assay was used to assess cell growth and viability. Cells were grown in a 96-well plate. For a period of 7 days, 20 µl sterile MTT (5 mg/ml, Sigma, St. Louis, CA, USA) was added into each well every 24 h, and cells were incubated with the MTT solution for 4 h at

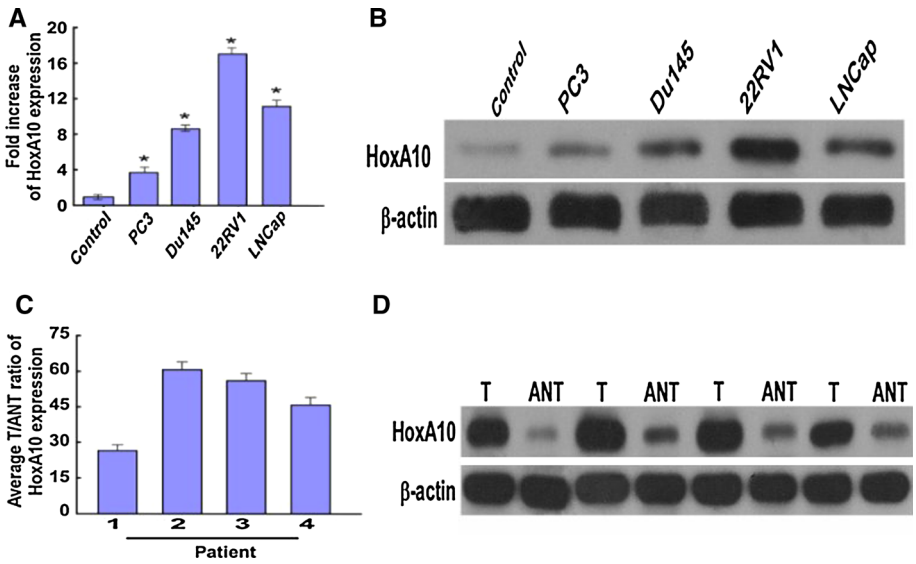

Fig. 2 Expression of HoxA10 in prostate cancer cell lines and tumor tissues. Total RNA and proteins were isolated from PC-3, Du145, 22RV1, and LNcap prostate cell lines, and mRNA expression levels and protein levels of HoxA10 were measured by qPCR **a** and Western blot analysis **b**. RNA and protein extracts from prostate cancer tissues and adjacent normal tissues were analyzed by qPCR **c** and Western blotting **d** to evaluate HoxA10 expression levels. Data expressed as means and SD, $n = 4$, $^*P < 0.05$

37 °C. 150 µl DMSO (Sigma, USA) was then added into each well, and the absorbance was measured at 490 nm using spectrophotometer (EL_x 800; Bio-Tek Instruments-Inc., Winooski, VT, USA). Each sample was measured in a triplicate, and culture medium was used as a control.

For colony formation assay, cells were seeded on 6-well plates at a density of 1,000 cells/well, cultured at 37°C, 5 % CO_2 until the appearance of cell colonies, fixed with methanol, stained with hematoxylin, and colonies were counted.

Statistical Analysis

Statistical analysis was performed using SPSS18.0 software for Windows (IBM, standard version 18.0). Each set of experimental data was presented as mean ± standard deviations. While comparing multiple samples, one-way ANOVA was used. $P < 0.05$ (two-tailed) was considered statistically different.

Results

The Expression of HoxA10 in Human Prostate Cancer Cells

We first evaluated the expression of HoxA10 in prostate cancer cell lines and tissues by qPCR and western blot analysis. PC-3, DU145, 22RV1, and LNCap prostate cancer cells exhibited significantly elevated levels of HoxA10 mRNA expression (over 3-, 7-, 16- and 12-fold, respectively) and increased protein expression levels, compared to control (Fig. 2a, b). Similarly, we detected high levels of HoxA10 mRNA and protein expression in human prostate cancer tissues compared to adjacent normal tissue (Fig. 2c, d).

The Effect of HoxA10 Overexpression and Silencing on Proliferation of PC-3 Prostate Cancer Cells

We altered the expression of HoxA10 in PC-3 prostate cancer cells by stably overexpressing HoxA10 using pMSCV-HoxA10 (PC-3/HoxA10) construct or, alternatively, by silencing the gene using pSuper-puro system for expression of short interfering RNA (PC-3/HoxA10-RNAi). The expression of HoxA10 was over fourfold increase in cells transfected with PC-3/HoxA10, compared to control PC-3/Vector–transfected cells. In contrast, lentiviral transduction with HoxA10-RNAi resulted in a markedly decreased HoxA10 mRNA and protein levels, comparing to cells transfected with vector alone (PC-3/scramble), as indicated by qPCR and Western blotting (Fig. 3a, b). We next evaluated the effect of different HoxA10 levels on the proliferation of PC-3 cells. Cell proliferation was measured by MTT and colony formation assays. We found that HoxA10 overexpression induced cell proliferation and increased the number of cell clones over threefold, comparing to control (Fig. 3c, d). On the other hand, silencing HoxA10 significantly inhibited cell proliferation and colony formation. These results suggest that HoxA10 may play a role in regulating cell proliferation in prostate cancer cells.

Discussion

Prostate cancer is the second leading cause of cancer mortality in men, with an estimated 238,000 new cases annually in the United States alone [14]. Currently, the main treatment options for early stages of prostate cancer include surgery, radiotherapy, or endocrine therapy. However, these methods are all associated with various degrees

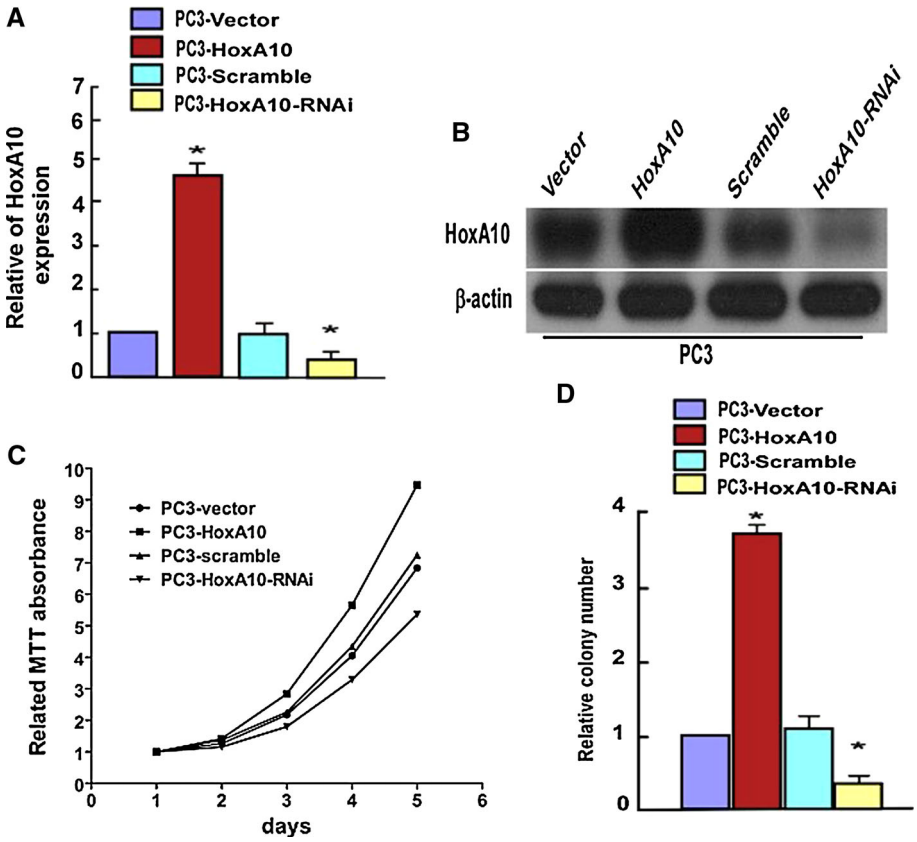

Fig. 3 The effect of HoxA10 overexpression and silencing on PC-3 proliferation. PC-3 cells were transfected with pMSCV-puro-HoxA10 (PC-3-HoxA10), pSuper-puro-HoxA10 (PC-3-HoxA10-RNAi) constructs, or with pMSCV (PC-3-Vector) and pSuper-puro scramble (PC-3-Scramble) as negative controls. Levels of HoxA10 expression were confirmed by qPCR **a** and Western blot analysis **b**. The effect of HoxA10 levels on PC-3 proliferation was analyzed by MTT **c** and colony formation assays **d**. Data expressed as means and SD, $n = 4$, $^{*}P < 0.05$

of complications and side effects [15, 16]. For advanced hormone-refractory prostate treatment, effective methods are still lacking. The option of gene therapy has the advantages of high selectivity and low rate of adverse side effects. Current studies have been focusing on specific target genes to induce apoptosis and the efficient clinical routes to deliver genes inside tumor cells [17], and there is a great need in identifying novel target genes for gene therapy.

The development of tumor is a complex, multi-stage process, with excessive cell proliferation considered as one of the most important basic biological characteristics of malignant transformation [18]. In the present study, we report that the expression of HoxA10, a multifunctional regulatory gene that is involved in regulating cell development and maturation, is increased in prostate cancer tissues and cell lines. In order to investigate the regulatory effect of this gene on tumor proliferation, we used lentiviral transduction system that was successfully and efficiently utilized in the previous reports [19–21] to express HoxA10 in prostate cancer cell line PC-3. Overexpressing HoxA10 led to significant increase in cancer cell proliferation, while silencing HoxA10 expression with RNAi resulted in reduced proliferation rates. Overall, our work provided an important insight into the molecular mechanisms of the development and proliferation of prostate tumor cells and the role of HoxA10 in regulating these processes.

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References

1. Shah, N., & Sukumar, S. (2010). The HOX genes and their roles in oncogenesis. *Nature Reviews Cancer, 10*, 361–371.
2. Cillo, C., Cantile, M., Faiella, A., et al. (2001). Homeobox genes in normal and malignant cell. *Journal of Cellular Physiology, 188*(2), 161–169.
3. Daftary, G. S., & Taylor, H. S. (2004). Pleiotropic effects of HOXA10 on the functional development of peri-implantation endometrium. *Molecular Reproduction and Development, 67*(1), 8–14.
4. Kim, J. J., & Fazleabas, A. T. (2004). Uterine receptivity and implantation: The regulation and action of insulin-like growth factor binding protein 1 (IGFBP1), HOXA10 and forkhea transcription factor 1 (FOXO1) in the baboon endometrium. *Reproductive Biology and Endocrinology, 16*(2), 34–40.
5. Abem, J., Hamada, J., Takahashi, O., et al. (2006). Disordered expression of HOX gebes in human non small cell cancer. *Oncology Reports, 15*(4), 797–802.

6. Plowright, L., Harrington, K. J., Pandha, H. S., et al. (2009). HOX transcription factor scare potential therapeutic targets in non small cell lung cancer. *British Journal of Cancer, 100*(3), 470–475.
7. Calvo, R., west, J., et al. (2000). Altered HOX and WNT7A expression in human lung cancer. *Proceedings of the National Academy of Sciences of the United States of America, 97*(23), 12776–12781.
8. Bjornsson, J. M., Andersson, E., Lundström, P., et al. (2001). Proliferation of primitive myeloid progenitors can be reversibly induced by HOXA10. *Blood, 98*(12), 3301–3308.
9. Lim, J. Y., Yoon, S. O., Seol, S. Y., et al. (2013). Overexpression of miR-196b and HOXA10 characterize a poor-prognosis gastric cancer subtype. *World Journal of Gastroenterology, 19*(41), 7078–7088.
10. Jiang, Y., Chu, Y., Tang, W., et al. (2013). Transcription factor WT1 and promoter CpG hypomethylation coactivate HOXA10 expression in ovarian cancer. *Current Pharmaceutical Design, 20*(11), 1647–1654.
11. Qu, Y., Dang, S., & Hou, P. (2013). Gene methylation in gastric cancer. *Clinica Chimica Acta, 23*(424), 53–65.
12. Ferlay, J., Shin, H. R., Bray, F., et al. (2010). Estimates of worldwide burden of cancer in 2008 GLOBOCAN 2008. *International Journal of Cancer, 127*, 2893–2917.
13. Waltregny, D., Alami, Y., Clausse, N., et al. (2002). Overexpression of the homeobox gene HOXC8 in human prostate cancer correlates with loss of tumor differentiation. *Prostate, 50*, 162–169.
14. Siegel, R., Naishadham, D., & Jemal, A. (2013). Cancer statistics, 2013. *CA: A Cancer Journal for Clinicians, 63*, 11–30.
15. Hanahan, D., & Weinberg, R. A. (2000). The hallmarks of cancer. *Cell, 100*(1), 57–70.
16. Huggins, C., & Hodges, C. V. (1972). Studies on prostatic cancer. I. The effect of castration, of estrogen and androgen injection on serum phosphatases in metastatic carcinoma of the prostate. *CA: A Cancer Journal for Clinicians, 22*, 232–240.
17. Dash, R., Azab, B., Shen, X. N., et al. (2011). Developing an effective gene therapy for prostate cancer: New technologies with potential to translate from the laboratory into the clinic. *Discovery medicine, 11*(56), 46–56.
18. Prenzel, N., Fischer, O. M., Streit, S., et al. (2001). The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocrine-Related Cancer, 8*(1), 11–31.
19. Liu, J., Jones, K. L., Sumer, H., et al. (2009). Stable transgene expression in human embryonic stem cells after simple chemical transfection. *Molecular Reproduction and Development, 76*(6), 580–586.
20. Wang, H., Shayakhmetov, D. M., Leege, T., et al. (2005). A capsid-modified helper-dependent adenovirus vector containing the beta-globin locus control region displays a nonrandom integration pattern and allows stable, erythroid-specific gene expression. *Journal of Virology, 79*(17), 10999–11013.
21. Soifer, H. S., & Kasahara, N. (2004). Retrotransposon-adenovirus hybrid vectors:efficient delivery and stable integration of transgenes via a two-stage mechanism. *Current Gene Therapy, 4*(4), 373–384.