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*Original Article*

# Prostate stromal cells express the LMO2 to control prostate cell proliferation and migration

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**Abstract:** We investigated the different gene expression between prostate peripheral zone stromal cells (PZs) and the transitional zone stromal cells (TZs), and found overexpression of LIM domain only 2 (LMO2) gene in PZs. Thus, the biological effects of LMO2 on prostate cell proliferation, migration in the stromal microenvironment were further explored, and the possible molecular mechanisms were explained. To determine if the expression of LMO2 by PZs is able to alter PC3 and BPH-1 cells growth, conditioned media (CM) was collected and was co-incubated with PC3 and BPH-1 cells. The proliferation and migration of both PC3 and BPH-1 were enhanced in the presence of CM derived from stromal cells which stably expressed LMO2, compared with the CM derived from control cells. By contrast, knockdown of the LMO2 in PZs could attenuate the proliferation and migration of cocultured PC3/BPH-1 cells in vitro. Mechanisms of the above phenomenon may be due to that LMO2 overexpressed stromal cells should induce prostate epithelial cell growth and migration via paracrine IL-11. STAT3 signaling pathway may be critical in the mechanisms of these biological effects.

**Keywords:** Prostate cancer, LMO2, stromal cells, proliferation, migration

## Introduction

Peripheral zone of the prostate is the most common site of development of prostatic intraepithelial neoplasia (PIN) and prostatic adenocarcinoma, while transitional zone of the prostate most commonly gives rise to benign prostatic hyperplasia (BPH) [1, 2]. Why do 1ead to distinct localization of prostate cancer between prostate peripheral zone and the transitional zone? Our previous study reported that the stromal cells from different normal prostatic zones have been shown to stimulate cancer cell grown differently in vitro and in vivo. The proliferation of PC3 was found increased when co-culture with prostate peripheral zone stromal cells, while transitional zone stromal cells suppressed PC3 cells growth in the mouse model. We conclude that ultrastructures and gene expression differ between the stromal cells from prostate peripheral zone and the transitional zone of the normal prostate, and stromal-epithelium interactions from different zone might be responsible for the distinct zonal localization of prostate tumor formation [3, 4]. Currently, little is known about the molecular basis for the differences between the prostate zones, but it seems reasonable to assume that the preference for cancer development in peripheral zone is caused by preexisting transcriptome differences between the two zones in normal tissue. Given that the diseases have a strong preference for a specific zone, we assume that this originates from pre-existing molecular differences in the normal zones. Several studies also have shown that differences in gene expression during tumor development in the transition and peripheral zones of the prostate [5-7]. There are differences in gene expression between the two zones, and the precise nature of these differences needs to be investigated further.

LMO2 has been demonstrated to play a crucial role in human acute T-cell leukemia through chromosomal translocation [8]. There is accumulating evidence suggesting the involvement of LMO2 in tumor neo-vasculature and angiogenesis [9]. Furthermore, LMO2 play an important role in prostate cancer progression through

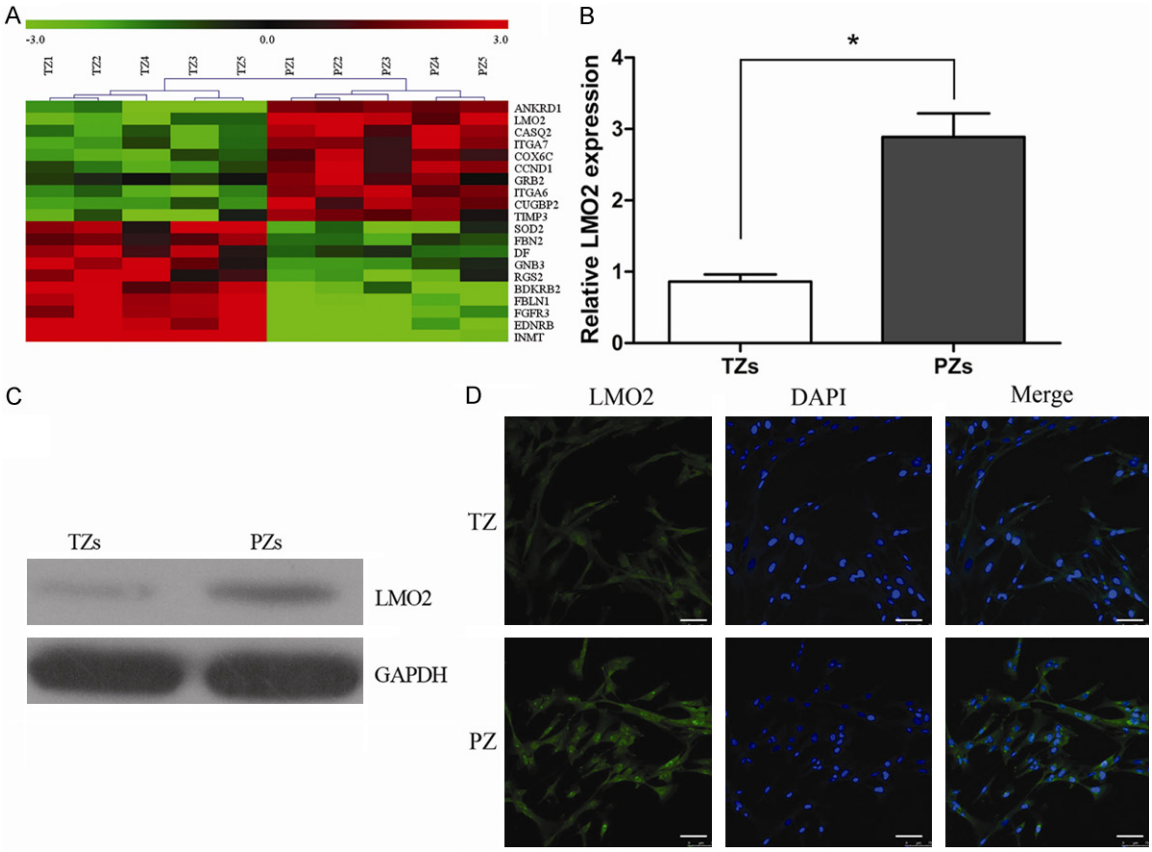


**Figure 1.** Upregulation of LMO2 in prostate peripheral zone stromal cells (PZs). A: Comparison of differentially expressed genes in human prostate peripheral zone stromal cells (PZs) and transitional zone stromal cells (TZs). Red denotes high expression levels, whereas green depicts low expression levels. Each gene listed is significantly differentially expressed between the PZs and TZs. B: Real-time PCR analysis of relative LMO2 expression levels in PZs and TZs. LMO2 is upregulated in PZs compared to TZs. C: Western blotting analysis showed that the LMO2 expression was higher in PZs than inTZs. D: Immunofluorescence staining showed that LMO2 was overexpressed in PZs compared with TZs (original magnification, × 400). *$P$<0.05, when compared with the control group.

the repression of E-cadherin expression [10]. LMO2 has been suggested to function as an important regulator in controlling cell growth and differentiation, while its function in PZs and especially in the prostate stromal microenvironment remains unclear.

In the present study, oligonucleotide microarray analyses was used to compare gene expression between PZs and TZs, and we sought to determine LMO2 expression in PZs and TZs, and extended our studies to investigate the stromal-epithelial crosstalk biological effects of up/down-regulation of LMO2 in prostate stromal cells.

## Materials and methods

### *Cell lines and cell culture*

Fresh prostate tissue was obtained from patients who had bladder cancer and undergoing the operation with a resected prostate. Isolation and culture prostate stromal cells from transitional and peripheral zones were performed as described in our previous report [11, 12]. Primer cells from passages 3-10 were used for all experiments. BPH-1, PC3 and WPMY-1 cells were maintained in RPMI-1640 medium with 10% FBS, and penicillin-streptomycin (1%). Conditioned media (CM) were obtained by 48-hour serum-starved cells, clarified by centrifugation, and used freshly. All cells were cultured in a humidified 5% CO2 atmosphere at 37°C incubator. All experimental procedures were approved by the Institutional Review Board of Shanghai General Hospital and the patient informed consent was obtained before tissue collection.

### *cDNA microarrays analysis*

Total RNA was isolated from PZs and TZs cells samples using TRIzol reagent and purified using

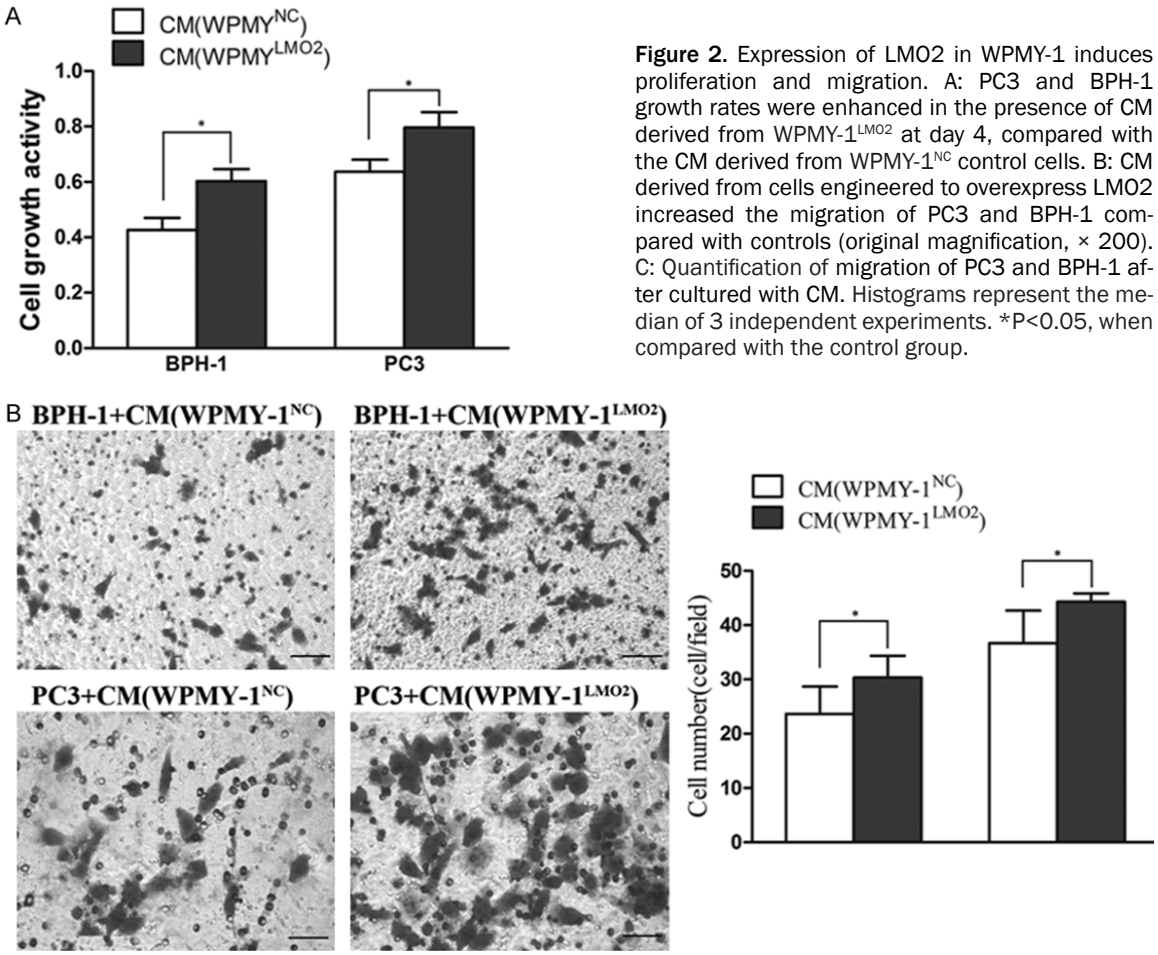


**Figure 2.** Expression of LMO2 in WPMY-1 induces proliferation and migration. A: PC3 and BPH-1 growth rates were enhanced in the presence of CM derived from WPMY-1$^{LMO2}$ at day 4, compared with the CM derived from WPMY-1$^{NC}$ control cells. B: CM derived from cells engineered to overexpress LMO2 increased the migration of PC3 and BPH-1 compared with controls (original magnification, × 200). C: Quantification of migration of PC3 and BPH-1 after cultured with CM. Histograms represent the median of 3 independent experiments. *P<0.05, when compared with the control group.

the RNAeasy Mini Kit (Qiagen GmbH, Hilden, Germany). Affymetrix Human Genome U133 Plus 2.0 Array is used to identify differentially expressed gene between PZs and TZs samples and this GeneChips contains over 47,000 transcripts. After hybridisation, arrays were scanned and data were analyzed using the Gene Chip Operating System (GCOS, Affymetrix, Santa Clara, CA, USA). Only genes under 10% FDR and with more than 2 fold change difference were considered as differentially expressed and chosen for further analysis. All microarray data are available from the Gene Ontology database (http:// www.geneontology.org/).

*Real-time qPCR*

Total RNA was extracted from cell lines using Trizol reagent (Invitrogen, CA, USA). Reverse transcription and quantitative real-time RT-PCR was carried out using the PrimeScript Reverse Transcription System and SYBR Premix Ex Taq™ II kit (Takara, Dalian, China) according to the manufacturer's instructions. The specific primers for LMO2, IL-11, and GAPDH were designed from the respective Genbank sequence, synthesized by Sangon (Shanghai, China). PCR conditions include a denaturation at 94°C for 2 min, followed by 40 cycles of 5 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C. The relative expression fold change of mRNAs was calculated using the $2^{-\Delta Ct}$ method. All reactions were performed in triplicate.

*Western blot analysis*

Cells were washed twice with cold PBS and lysed in ice-cold RIPA buffer (Beyotime, Jiangsu, China) containing phosphatase and protease inhibitors. Total protein was separated by denaturing SDS-PAGE gel electrophoresis and then transferred onto PVDF membranes. Membranes were incubated with the primary antibodies at 4°C overnight and subsequently hybridized with horseradish peroxidase conjugated sec-

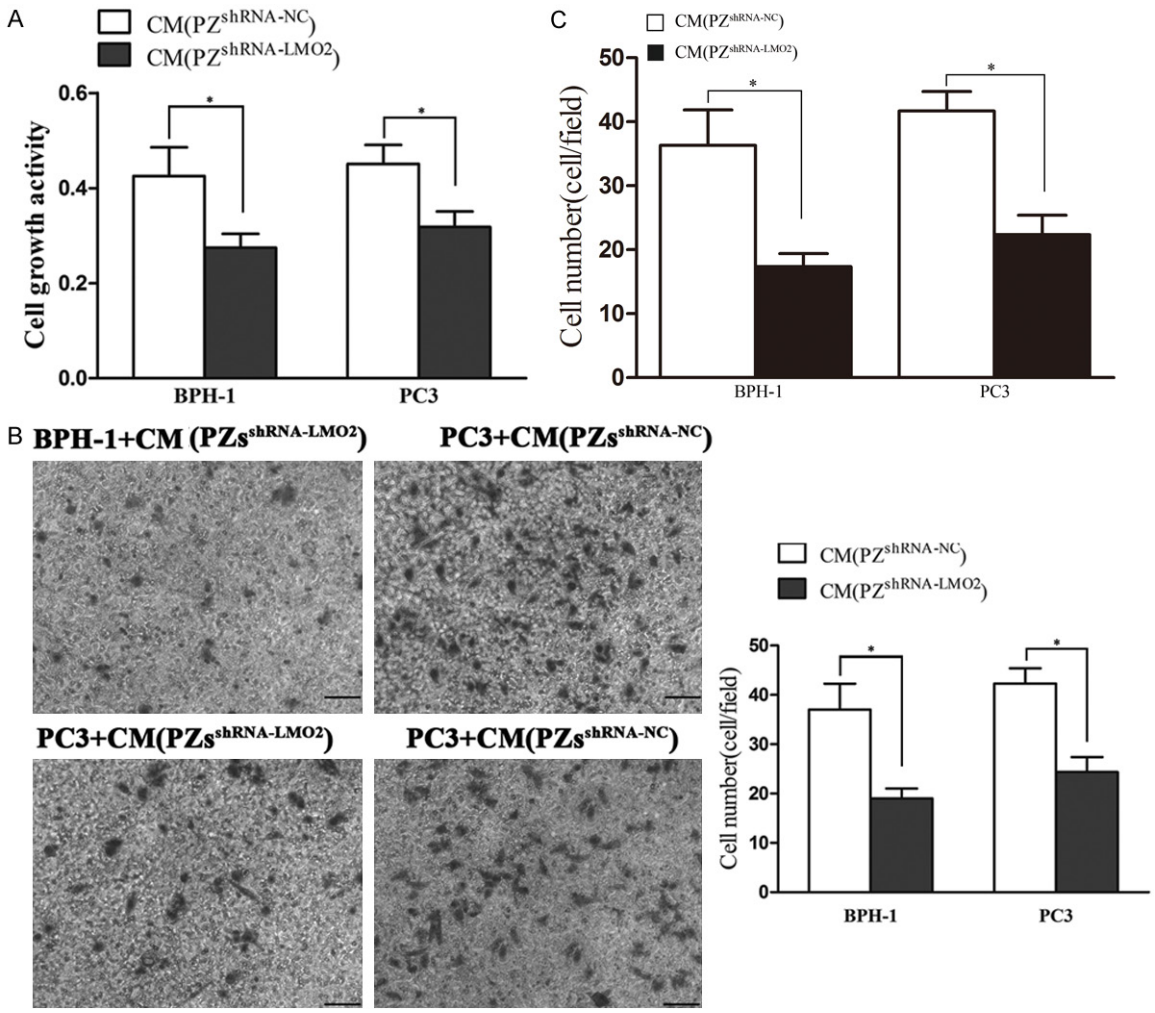


**Figure 3.** Knockdown of LMO2 expression in PZs inhibits proliferation and migration of prostate cells. A: PC3 and BPH-1 growth rates were significantly attenuated in the presence of CM derived from PZs$^{shRNA\text{-}LMO2}$ compared with the CM controls. B: PC3 and BPH-1 showed less migratory potential in the presence of CM derived from PZs$^{shRNA\text{-}LMO2}$ than CM derived from control cells (original magnification, × 200). C: Quantification of migration of PC3 and BPH-1 after cultured with CM. Histograms represent the median of 3 independent experiments. *$P$<0.05, when compared with the control group.

ondary antibody for 2 h at room temperature. The protein bands were visualized using ECL chemiluminescence kit (Boster, Wuhan, China) and exposured to X-ray films. The following antibodies were used for western blotting: goat anti-LMO2 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA); rabbit anti-GAPDH (1:2000; Cell Signaling Technology, Beverly, MA); mouse anti-stat3 and mouse anti-pstat3 (1:1000; Santa Cruz Biotechnology, Dallas, TX, USA).

*Immunofluorescence staining*

PZs and TZs cells were grown on glass coverslips and fixed with 4% paraformaldehyde, permeabilized 0.5% Triton X-100 and then blocked with 5% bovine serum albumin in phosphate buffered saline. The coverslips were then exposed to primary antibodies as mentioned above at 4°C overnight, followed by secondary antibodies. Images were captured using fluorescence microscope (Olympus Axiovert S100, Japan).

*Construction of lentiviral vectors*

The hLMO2 gene fragment was excised from a human cDNA library and subcloned into the retroviral vector pLenti6.3-MCS-IRES-EGFP (Invitrogen, CA, USA) between the BamHI and AscI restriction enzyme sites. pLenti6.3-LMO2 or

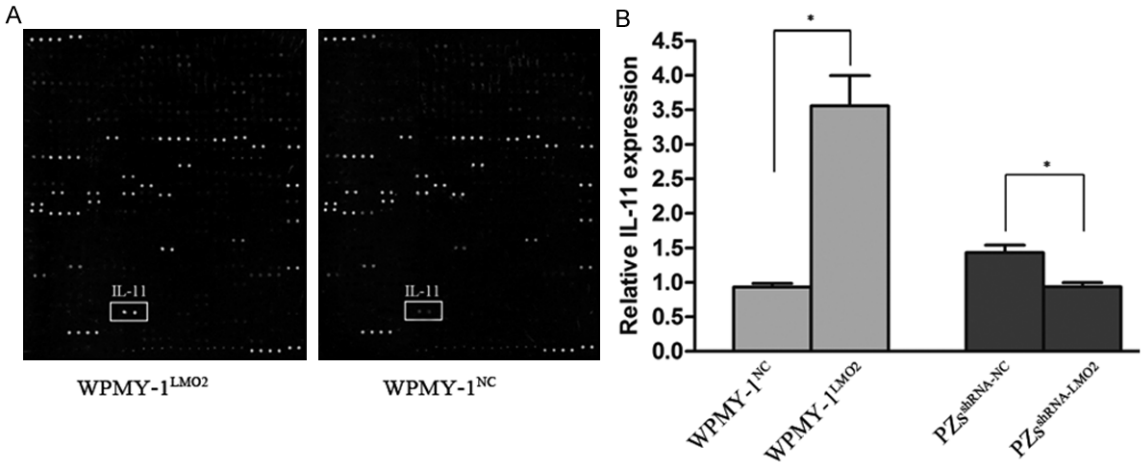


**Figure 4.** IL-11 is a mediator for WPMY-$1^{LMO2}$ cells promotes proliferation and migration in PC3 cells. A: Cytokine array analysis. CM collected for cytokine array analysis from WPMY-$1^{LMO2}$ nd WPMY-$1^{NC}$ cells. B: RT-PCR analysis showing mRNA levels of IL-11 in WPMY-$1^{LMO2}$, WPMY-$1^{NC}$, $PZs^{shRNA\text{-}LMO2}$, and $PZs^{shRNA\text{-}NC}$. *$P$<0.05, when compared with the control group.

pLenti6.3-NC was transfected into HEK293T cells with a mixture of virus packaging plasmid (Invitrogen, CA, USA). Lentivirus was produced according to the previous report [13] and used to transduce WPMY-1, prostate stromal cell. To silence the expression of specific genes, pGLV1/U6/EGFP lentiviral vector expressing shRNA specific for human LMO2 and non-target shRNA were obtained from GenePharma RNAi Company (Shanghai, China). The sequences of the shRNA for hLMO2 gene were identical in previous studies [14].

*Cell proliferation assays*

PC3 and BPH-1 cells were seeded in a 96-well plate at the density of 3000 cells per well for 24 h and then 50 ng $ml^{-1}$ IL-11 or CM derived from $PZs^{shRNA\text{-}LMO2}$, $PZs^{shRNA\text{-}NC}$ WPMY-$1^{LMO2}$, WPMY-$1^{NC}$ were added to each well. Cell proliferation was evaluated using the CCK-8 assay kit at indicated time point (Dojindo, Kumamoto, Japan), according to the Manufacturer's instructions. Each assay was performed in six replicates and each experiment was repeated three times.

*Migration assay*

The migration capacity of prostate cancer cells were assessed using Corning transwell assay as described. Cells were plated at density of 5 × $10^4$ cells per well with serum-free culture medium in the upper chamber of transwell migration chambers (8.0 μm), and CM derived from $PZs^{shRNA\text{-}LMO2}$, $PZs^{shRNA\text{-}NC}$, WPMY-$1^{LMO2}$, WPMY-$1^{NC}$ were added to the bottom chambers. After 24 h of incubation, cells remaining on the upper surface of the membrane were removed with a cotton swab, and the transmigrated cells were fixed in methanol and stained with crystal violet and stained cells were counted by photographing five fields per membrane at least 3 independent experiments.

*L-based human antibody array*

L-based human antibody array was performed by Full Moon BioSystems, Inc. (Sunnyvale, CA). WPMY-$1^{LMO2}$ and WPMY-$1^{NC}$ cells were seeded in 6 cm culture dishes serum-starved for 24 h and cell culture supernatant samples were biotinylated using Biotin reagent. Biotin-labeled supernatant samples were performed by L-based human antibody array following the manufacturer's instructions (RayBiotech, Inc.). The chip was scanned by Axon Genepix 4000B scanner and the raw data was read using software GenePix Pro 6.0.

*Statistical analysis*

Statistical analyses were performed using the SPSS 17.0 statistical software (Chicago, IL, USA). Data were presented as mean ± standard deviation (SD) from at least three independent experiments. The Student's $t$ test was employed to assess statistically significant differences. A $P$ value <0.05 was considered as statistically significant difference.

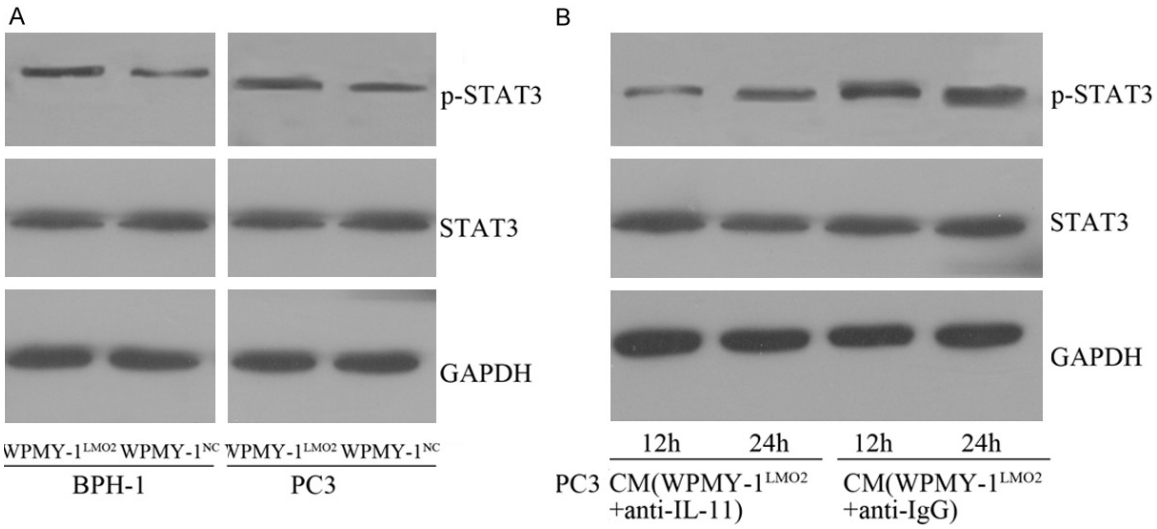


**Figure 5.** IL-11 promotes prostate cells proliferation and migration via activating STAT3. A: PC3 and BPH-1 cells were incubated with CM from WPMY-$1^{LMO2}$ and WPMY-$1^{NC}$. Levels of pSTAT3 were assessed using Western blot. B: PC3 were incubated with CM from WPMY-$1^{LMO2}$ with or without IL-11-blocking antibodies.

## Results

*LMO2 expression is upregulated in PZs versus TZs*

To investigate the expression profiles of genes in PZs and TZs cell lines, global gene expression levels were measured using Affymetrix Human Genome U133 Plus 2.0 Array containing over 47,000 transcripts. The expression levels of select genes differed significantly between PZs and TZs cell lines and the top ten downregulated and upregulated genes are presented in **Figure 1A**. Among those genes whose expression was most highly upregulated in PZs versus TZs was LMO2. We chose to focus our investigation on LMO2 because it was reported to be involved with the regulation of cell proliferation, apoptosis, and migration [10, 15, 16]. To verify the accuracy of these microarray results, expression level of LMO2 was measured by a real-time quantitative PCR and western blot method. **Figure 1B** showed LMO2 differentially expressed between PZs and TZs cells by qRT-PCR measurement. We examined the expression levels of LMO2 among the PZs and TZs cells by western blot. Relative to PZs, TZs showed lower LMO2 protein levels (**Figure 1C**). Furthermore, immunofluorescence staining was performed on PZs and TZs cells. This analysis showed that LMO2 expression is upregulated in PZs compared with TZs (**Figure 1D**). Consistent with the microarray data, our results suggest that expression of LMO2 was upregulated in PZs compared with TZs.

*LMO2 expression in WPMY-1 stromal cells stimulates prostate cells proliferation and migration*

To test the function of LMO2 in stromal cells, we first asked if overexpression of LMO2 in WPMY-1 stromal cell can affect their ability to stimulate tumor cell growth and migration. CM was collected and co-incubated with PC3 and BPH-1 cells. PC3 and BPH-1 cells growth rates were enhanced in the presence of CM derived from WPMY-$1^{LMO2}$, at day 4, compared with the CM derived from WPMY-$1^{NC}$ (**Figure 2A**). Transwell assay was introduced to investigate whether the stromal cells over expression of LMO2 regulated prostate epithelial cells migration. CM derived from cells engineered to overexpress LMO2 increased the migration of PC3 and BPH-1 (**Figure 2B**, **2C**). These assays demonstrated that overexpression of LMO2 in stromal cells can promote the proliferation, migration of epithelial cells and may thus induce their malignant transformation.

*Knockdown of LMO2 expression in PZs attenuate proliferation and migration of prostate cells*

In order to investigate the effects of LMO2-shRNA reconstituted PZs on tumor proliferation

and migration, we prepared a 24 h CM from PZs with shRNA-LMO2 or empty vectors and evaluated their ability to stimulate prostate cell proliferation and migration. $PZs^{shRNA\text{-}LMO2}$ CM reduced the proliferative capacity of PC3 and BPH-1 cells as compared with empty vectors CM (**Figure 3A**). Given that alterations in the expression of LMO2 in PZs were able to alter the proliferation of prostate cells, it was next determined if similar effects could be observed on the motility activity of prostate cells. CM derived from cells engineered to decreasing LMO2 expression reduced the migrative abilities of PC3 and BPH-1 compared with controls (**Figure 3B**).

*IL-11 is a mediator for WPMY-$1^{LMO2}$ cells promote proliferation and migration in prostate cells*

LMO2 overexpression in stromal cells can promote PCa cells proliferation and migration, we speculated that WPMY-$1^{LMO2}$ cells could secrete some chemokines/cytokines/growth factors to exert their effects. We conducted cytokine array to investigate whether the secreted chemokines/cytokines were changed in WPMY-1 cell after transfected with LMO2 gene, and the level of IL-11 was increased (**Figure 4A**). We then assayed the mRNA level of IL-11 in stromal cells. Overexpression of LMO2 in WPMY-1 cells up-regulated IL-11 mRNA, while knockdown of LMO2 in PZs down-regulated IL-11 mRNA compared with their control (**Figure 4B**).

*Paracrine of IL-11 by prostate stromal cells promote prostate cells proliferation and migration via activating STAT3*

Phospho-STAT3 protein levels increased after incubating PC3 and BPH-1 with WPMY-$1^{LMO2}$ condition medium (**Figure 5A**). The key role of IL-11 mediated signaling is confirmed by treatment of PC3 with IL-11 blocking antibodies during their exposure to WPMY-$1^{LMO2}$ CM. IL-11 blocking antibodies are able to attenuate STAT3 protein phosphoralytion levels in PC-3 cells (**Figure 5B**). These data indicate that IL-11 are the best possible candidate molecules secreted by WPMY-$1^{LMO2}$ cells to affect the proliferation and migration ability of prostate cells. Recently, IL-11 has been reported to be implicated with ability to growth of many human cancer [17] and has been associated with increased metastatic potential in cancer cells [18, 19]. IL-11 signals through a specific IL-11Rα and a common signal-transducing, gp130, to activate the JAK-STAT pathway [20].

## Discussion

To investigate differences in gene expression in different zones of the prostate by microarray analyses, to better understand why aggressive tumors predominantly occur in the peripheral zone (PZ), whereas benign prostatic hyperplasia (BPH) occurs almost exclusively in the transition zone (TZ) The present study aimed to investigate differences in gene expression in different zones of the prostate stromal cells, to better understand the difference in propensity of tumors in the zones of the prostate. Heul-Nieuwenhuijsen et al have elucidated the differences in mRNA expression between PZ and TZ in normal prostate tissue [5]. Noel et al [6] have reported that 43 genes were differentially expressed between the zones in normal tissues. The miRNA expression was explored in different zones of the prostate, and to investigate the relationship between miRNA expression and incidence of cancer in the PZ and TZ. The results revealed that the major differences in miRNA expression are found between the two zones of normal prostate tissues [21]. The two normal prostate zones consist of both stromal and epithelial cells. The regulation of stromal cells on prostatic epithelial cells is the key factor for the development of prostate cancer [22]. Therefore, it is important to consider stromal cells characteristics when studying the prostate zones. In the present study, we used microarray analyses to show that there is differential expression between the PZs and TZs, and LMO2 gene was found to be expressed at a higher level in the PZs. The mechanism by which LMO2 expression levels are increased in PZs cells is unknown.

LMO2 proteins are important regulators in determining cell fate and controlling cell growth and differentiation [23, 24]. Our studies demonstrate that LMO2 gene and protein levels are increased in prostate peripheral zone stromal cells and that LMO2 exerts promotion impacts to prostate cell proliferation and migration through modulating the expression of an important cytokines, IL-11. These observations suggest that LMO2 expression in prostate peripheral zone stromal cells alters the balanced prostatic microenvironment, which may con-

tribute to prostate epithelial cells proliferation and migration. The result of cytokines microarray confirmed that multiple cytokines have changed when increased LMO2 expression in WPMY-1 cells, and the changes of IL-11 was the most obvious. Moreover STAT3 pathways are activated in PC3 and BPH-1 cells when cultured with CM from WPMY-1 over-expressing LMO2. Activation of STAT3 pathways in PC3 and BPH-1 cells by WPMY-1 overexpressing LMO2 indicates that stromal cells support tumor growth and migration. We identify that IL-11 is an important genes, which mediate actions of stromal LMO2 to prostate epithelial cells. Consistent to the roles of IL-11 in enhancing tumor cell proliferation and mobility, we observed that CM from WPMY-1$^{LMO2}$ cells promoted not only prostate cell proliferation, but also migration. In addition, LMO2 may play an important role in prostate cancer progression, via repression of E-cadherin expression in vitro [10]. Signal transducer and activator of transcription 3 (STAT3) is known to be involved in the progression of prostate cancer (PCa) and is a key factor in drug resistance and tumor immunoescape [25]. Inhibition of STAT3 activation suppresses IL-6-mediated malignant conversion and the associated invasive phenotype [26]. IL-11 is a member of the IL6 family of cytokines. It can induce the activation of three independent downstream signaling pathways: the signal transducer and activator of transcription (STAT) pathway, the Ras-mitogen activated protein kinase (MAPK) pathway; and the phosphotidylinositol-3 kinase (PI-3K) pathway [27, 28]. IL-11 can be produced by tumor associated fibroblasts, and can be upregulated in cancer cells as part of an autocrine signaling loop, and promotes tumor progression [29, 30]. Studies have indicated that IL-11 plays an important role in the process of prostate cancer [31].

The overexpression of LMO2 in stromal cells enhanced proliferation and modulated prostate epithelial cells migration in vitro. Mechanisms of the above phenomenon may be due to that LMO2 overexpressed stromal cells should supports prostate epithelial cell growth and invasion via paracrine IL-11. Prostate epithelial-stromal interaction is the importance of the progress in prostate cancer, especially the key role of the stromal cells.

## Acknowledgements

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## Disclosure of conflict of interest

None.

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