Life Sciences 235 (2019) 116791

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Cancer-associated fibroblast regulate proliferation and migration of prostate cancer cells through TGF-β signaling pathway

Dai-Yu Sun[a], Jia-Qi Wu[b], Zhi-Heng He[c], Ming-Fang He[b,*], Hong-Bin Sun[a,**]

[a] Department of Urology, Nanjing Hospital Affiliated to Nanjing Medical University, Nanjing 210006, Jiangsu, China
[b] Institute of Translational Medicine, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, Nanjing 211816, Jiangsu, China
[c] School of Medicine, Yale University, New Haven, CT 06511, USA

ARTICLE INFO

Keywords:
Prostate cancer
CAFs
Tumor microenvironment
TGF-β
Zebrafish

ABSTRACT

Aims: Prostate cancer (PCa) incidence rates are rising in China currently. Cancer-associated fibroblasts (CAFs), as a major component of tumor microenvironment, are crucial for tumor progression. This study was aimed to explore the promotion effect of patient-derived CAFs on the proliferation and migration of prostate cancer cells.
Main methods: CAFs were isolated from tumor tissues of PCa patients. The promotion effect of CAFs on the proliferation and migration of PC-3 and LNCaP cells were evaluated *in vitro* and *in vivo*. The concentration of TGF-β1 was measured by Luminex assay. The blocking activity of LY2109761 on the promotion effect of CAFs was also evaluated.
Key findings: CAFs could significantly promote the proliferation and migration of PC-3 and LNCaP cells both *in vitro* and *in vivo*. TGF-β1 was identified as a highly increased factor in CAFs-CM compared with the normal culture medium of these two cancer cell lines. TGF-β receptor inhibitor LY2109761 could suppress the CAFs-induced cellular proliferation and migration of PC-3 cells but not LNCaP cells.
Significance: Our study suggested a crucial role for CAFs and TGF-β signaling in the progression of PCa. Zebrafish xenograft model was an ideal animal model for the study of CAFs and cancer cell interaction.

1. Introduction

Prostate cancer (PCa) is now becoming an emerging health priority in East Asia. Although much lower than in Western countries, the incidence of PCa has risen rapidly in China over the past few decades [1]. According to the Cancer Statistics in China 2015, PCa ranks as the 6th most prevalent cancer and the 9th leading cause of cancer-related mortality for males [2]. Androgen deprivation therapy (ADT, surgical or medical castration) is the standard treatment for metastatic PCa, but patients invariably relapse and become castration-resistant PCa (CRPC) despite castrate androgen levels [3].

Current therapies focus predominantly on targeting the proliferation of the rapidly growing epithelial cancer cells. Recently, tumor microenvironment in facilitating tumor progression is getting more and more attention [4,5]. As a major component of tumor stroma, cancer-associated fibroblasts (CAFs) actively communicate with cancer cells and contribute to tumor proliferation, invasion and metastasis through paracrine factors in different types of cancers such as lung carcinoma [6], breast cancer [7] and colorectal cancer [8]. Emerging evidence has highlighted the role of CAFs in promoting carcinogenesis and cancer progression in different cancer cell types, including prostate cancer [9]. The mechanisms of how the interactions between CAFs and PCa cells facilitate the growth and metastasis of PCa remain to be a hot topic in PCa research. For example, Cirri group reported that CAFs exerted a mandatory role in PCa progression as they metabolically sustain cancer cell survival and growth, recruit inflammatory and immune cells, and promote cancer cells stemness and epithelial mesenchymal transition, thereby favoring metastatic dissemination of aggressive cancers [10]. Other group summarized that CAFs enhanced the formation of structure by PCa stem cells and involved in the PCa angiogenesis and chemoresistance [11].

Transforming growth factor-β (TGF-β) produced by CAFs was reported to promote the accumulation of fibrotic desmoplastic tissue and the rate of cancer progression [12–14]. Overexpression of TGF-β is reported to be correlated with a poor prognosis for PCa [15], suggesting that TGF-β signaling might have an important role in the progression of

* Correspondence to: M-F. He, Institute of Translational Medicine, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, 30 Puzhu South Road, Nanjing 211816, China.
** Correspondence to: H-B. Sun, Department of Urology, Nanjing Hospital Affiliated to Nanjing Medical University, 68 ChangLe Road, Nanjing 210029, China.
E-mail addresses: mingfanghe@njtech.edu.cn (M.-F. He), docshb@126.com (H.-B. Sun).

https://doi.org/10.1016/j.lfs.2019.116791
Received 3 January 2019; Received in revised form 18 August 2019; Accepted 24 August 2019
Available online 26 August 2019
0024-3205/

PCa cells. There are three TGF-β isoforms, TGF-β1, 2 and 3. Of these, TGF-β1 is the most abundant in humans [16]. In CAFs, TGF-β1 stimulates growth, migration, differentiation and epithelial-mesenchymal transition of cancer cells [7].

In vitro co-culture of cancer cell and the micro-environment, for example CAFs and tumor-associated macrophages (TAMs), were widely used in the studies of the interaction between cancer cell and their stromal cell [17,18]. However, *in vivo* model has the advantage of recapitulating cell-cell interaction more closely to the human body. The teleost zebrafish (*Danio rerio*) is a powerful and genetically tractable model to study human malignancies. Zebrafish xenografts offer fast, single cell resolution, living imaging and the ability to perform large numbers of transplants [19–21]. In recent years, zebrafish model for study cancer progression and cancer microenvironment have become increasingly valuable [19,22–26].

In this study, we showed the evidence that CAFs can promote the proliferation and migration of PCa cells through TGF-β pathway, which could be blocked by the TGF-β receptor inhibitor. Our results suggested that targeting the interaction between PCa cells and CAFs might constitute a novel therapeutic approach for PCa treatment.

2. Material and methods

2.1. Reagents

LY2109761 (L126135, purity > 99%) were purchased from Aladdin (Shanghai, China) and were dissolved in 100% DMSO (Sigma Aldrich, St. Louris, MO, USA) to build a stock concentration of 1 mM. Fetal bovine serum (FBS), phosphate buffer saline (PBS), Roswell Park Memorial Institute basal medium 1640 (RPMI 1640), F12 basal medium, penicillin and streptomycin were purchased from Basal Media Technologies (Shanghai, China).

2.2. Zebrafish care and handing

All zebrafish experiments were approved by the Nanjing Tech University Experimental Animal Ethical Committee. Zebrafish embryos of the transgenic strain expressing enhanced GFP (EGFP) under the fli1 promoter (*Fli1:EGFP*) were obtained from Model Animal Research Center of Nanjing University and raised at 28.5 °C under standard experimental conditions [27]. The light-dark cycle was 14:10 h. The collection and incubation of embryos were consistent with those previously reported [28]. The age of the embryos is indicated as hours post fertilization (hpf).

2.3. Cell lines culture

PCa cell lines PC-3 and LNCaP were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. PC-3 cell line is androgen insensitive and the LNCaP cell line is androgen sensitive. PC-3 cells were cultured in F12 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. LNCaP cells were cultured in RPMI 1640 supplemented with 20% fetal bovine serum and 1% penicillin-streptomycin.

2.4. CAFs separation and culture

PCa tissues were collected from 3 patients in Sep. 2017, who received no chemotherapy or radiotherapy, at the Nanjing Hospital Affiliated to Nanjing Medical University, Nanjing, China. The study was approved by the Ethics Committee of Nanjing Hospital Affiliated to Nanjing Medical University, and all patients provided written informed consent prior to this study. All the tissue samples were confirmed by two clinical pathologists. CAFs were isolated from freshly resected PCa tissues or biopsy specimen from afore mentioned PCa patients. The PCa samples were washed with sterile phosphate-buffered saline (PBS) two times and transferred directly into the pre-chilled tissue storage solution (Miltenyi, Bergisch Gladbach, Germany) after resected. Within 24 h from resection, tumor tissues were mechanically minced into small pieces (1–2 mm^3) and seeded onto 10 cm petri dishes in RPMI 1640 with 10% FBS, 100 U ml^{-1}, penicillin and 100 μg ml^{-1}streptomycin. The tissue appeared to attach flasks within 8 h. Specimens were inspected daily for the emergence of fibroblasts and the medium was exchanged after 24 h for first time and every third day thereafter. Fibroblasts were allowed to grow out of tumor fragments for 2–3 weeks. Tissue samples were then removed from the cultures and fibroblasts were transferred to larger tissue culture vessels once they had reached 70% confluence. CAF cells used in all our experiments ranged from passage 2–6 to maintain the closest phenotype of the primary tissues.

2.5. Immunocytochemistry

For immunocytochemistry, the fourth passage CAFs were seeded into a 6-well plate and cultured in RPMI 1640 medium with 10% FBS until 80% confluence. Cells were fixed in 4% ice-cold paraformaldehyde plus 0.1% Triton X-100 for 20 min, followed by blocking with 2% bovine serum albumin in PBS for 1 h. Cells were incubated with anti-α-SMA (1:200, Abcam, Cambridge, UK) or anti-vimentin (1:200, Abcam, Cambridge, UK) and then treated with secondary antibodies goat anti-rabbit antibody (1:200, Boster Biological Technology, Wuhan, China), respectively. Images were taken using an OLYMPUS IX71 microscope (OLYMPUS, Tokyo, Japan, magnification ×40 and ×100).

2.6. Fibroblast conditioned medium acquisition

After two passages, cells in confluence were harvested and washed twice with PBS and cultured in RPMI-1640 free from FBS. After 48 h of incubation, the conditioned medium of CAFs (CAFs-CM) from the three plates were collected and mixed in one vial. CAFs-CM was centrifuged for 5 min at 2000 rpm, filtered through 70-μm units. Then the supernatant was filtered with YM3 membrane. Bradford standard method was used to determine the concentration of concentrated CAFs-CM, and 0.75 g l^{-1} of CAF-CM was prepared.

2.7. In vitro cell proliferation

LNCaP (3×10^3 per well) and PC-3 (3×10^3 per well) were seeded in 96-well plate in the appropriate growth medium (GM) for 12 h for attachment. Then cells were treated with GM or growth medium containing CAFs-CM (1:1) with/without 10 μM LY2109761. These culture mediums were replaced on the second, fourth day. Then a group of cells were collected on the second, fourth, sixth day for cell counting. Cells receiving 0.1% DMSO only served as a control. Each treatment was performed in triplicate and each assay was repeated at least three times.

2.8. In vitro wound healing assay

PC-3 and LNCaP cells in GM were seeded into 24-well plates precoated with 0.1% gelatin (Sigma-Aldrich, St. Louis, USA), and grown overnight to confluence. The monolayer cells were wounded by scratching with 10 μl pipette tips and washed with PBS to remove the non-adherent cells. GM or growth medium containing CAFs-CM (1:1) with/without 10 μM LY2109761 was then added into the wells. After 24 h incubation, cells were washed by PBS and fixed with methanol for 10 min and then stained with 0.1% crystal violet solution (Sigma, St. Louris, MO, USA) for 10 min. Then the crystal violet solution was washed by ddH2O. Images were taken at 0 h and 24 h independently through an inverted microscope (IX71, OLYMPUS, Tokyo, Japan). The migrated cells were counted manually. The values were observed from four randomly selected fields.

2.9. In vitro migration assay

AP48 device (designed based on Boyden chamber principle) was used for this assay. It has a total of 48 holes, divided into 12 columns, each column has 4 holes, 27 μl medium (GM/CAF-CM/CAF-CM-LY) was added in each of bottom holes, then covered with microporous membrane coated with 0.1% Gelatin. 50 μl 2×10^5 per ml cell suspension was added in upper holes. After incubation for 6 h, the non-migrated cells from the upper face of the membrane were removed by PBS-soaked cotton swab. The migrated cell on the bottom face of the membrane were fixed with methanol and stained with 0.1% crystal violet staining solution. The migrated cells were counted manually. Four random areas per hole were selected for quantitative analysis.

2.10. TGF-β level determination by Luminex Assay

The Luminex kit (TGFBMAG-64k-01) was obtained from Millipore (Billerica, MA) and assay was performed according to the manufacturer's instructions. Properly diluted medium samples were incubated with the antibody-coupled microspheres and then with biotinylated detection antibody before the addition of streptavidin-phycoerythrin. The captured bead complexes were measured with FLEXMAP 3D system (Luminex Corporation, Austin, TX) using the following instrument settings (events/bead, 18; Sample size, 50 μl; discriminator gate, 8000–15,000). The raw data (mean fluorescence intensity) were collected and further processed for calculating protein concentration by Milliplex Analyst 5.1 (VigeneTech, USA).

2.11. Cell labeling, xenograft and enumeration procedure

Transgenic zebrafish embryos Tg(*fli1: EGFP*) at 24 hpf were dechorionated with 1 mg/ml of pronase (Sigma-Aldrich, St. Louris, MO, USA). After removing the chorion, embryos were soaked in embryo medium with 0.2 mM 1-phenyl 2-thiourea (PTU) and incubated for further 24 h at 28.5 °C. At 48 hpf, the embryos were anesthetized with 0.16 mg/ml tricaine (Sigma-Aldrich, St. Louris, MO, USA) and positioned with their right side up on a wet 1.6% agarose pad. Cancer cells together with CAFs were co-injected into zebrafish embryos. In order to differentiate these two types of cells, they were fluorescently labeled in different colors. PC-3 and LNCaP cells were fluorescently labeled with CM-DiI (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Primary CAFs was fluorescently labeled with CMAC Blue (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Labeled cells were washed in PBS twice, re-suspended in corresponding culture medium at 2×10^7 cells per ml. Cell viability was assessed by trypan blue staining before the injection and it was higher than 95%. Approximately 200–300 tumor cells or a mixture of equal numbers of 200–300 tumor cells and 200–300 CAFs were injected into yolk sac per zebrafish embryo using a microinjector (IM-31, Narishige, Japan) while under the observation by stereoscope (SMZ 745T, Nikon, Japan). After transplantation, embryos were incubated for 1 h at 28.5 °C, checked for presence of cells at yolk sac and the absence of cells in the circulating system. Then embryos were incubated at 32 °C for the following days [28]. A group of 15–20 embryos was sacrificed and dissociated into a single cell suspension, the number of CM-DiI-labeled cancer cells was enumerated to be the baseline number of PCa cells V.S. coinjection with CAF with/without treatment with inhibitor LY2109761 to ensure cells engraft and proliferate in the zebrafish embryos [29].

2.12. Statistical analyses

All statistical analyses were expressed as mean ± SEM using GraphPad Prism 5.0. Statistical analysis was performed using the standard student's *t*-test for pair comparisons and ANOVA analysis for multiple factors. Significance was considered when P values were lower than 0.05. (***) indicates statistical significance $P < 0.005$, (**) $P < 0.01$, (*) $P < 0.05$. All experiments were done in triplicates and independent experiment was repeated at least three times.

3. Results

3.1. Isolation and characterization of primary CAFs

The fibroblast cell population was first verified by cell morphology. As shown in Supplementary Fig. 1a, the CAFs cells exhibited typical fibroblast characteristics: a spindle-like shape morphology [12], which were further characterized by their high expression of α-SMA and vimentin (Supplementary Fig. 1b, c) [30,31].

3.2. CAFs promoted the proliferation, wound healing and migration of PCa cells

In order to investigate the effect of CAFs on PCa cell proliferation and migration *in vitro*, we cultured PC-3 and LNCaP cells in their normal GM, GM plus CAF-CM at 1:1 (GM + CAF-CM), and GM plus CAF-CM at 1:1 with LY2109761 (GM + CAFs-CM + LY), respectively. Cell counting showed that the cell numbers of PC-3 and LNCaP significantly increased by CAF-CM compared to GM only (Fig. 1a, b). At day 8, the cell numbers in GM + CAF-CM group almost doubled those in GM group for both PC-3 and LNCaP cells. Adding LY2109761 significantly blocked the promotion of proliferation by VAF-CM in PC-3 cells but not

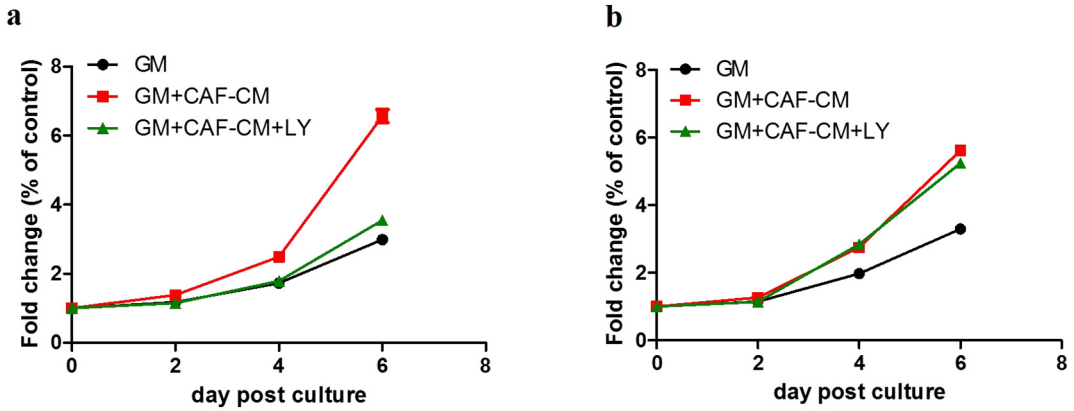

Fig. 1. CAFs promote the proliferation of prostate cancer cells. (a) PC-3 and (b) LNCaP cells were cultured with normal growth medium (GM), or normal GM plus CAF conditioned medium at 1:1 (GM + CAF-CM), or normal growth medium plus CAFs conditioned medium at 1:1 and with LY2109761 (GM + CAFs-CM + LY). Cell numbers were counted at 0, 2, 4, 6 days post culture. Values are represented as mean ± SD of three replicates.

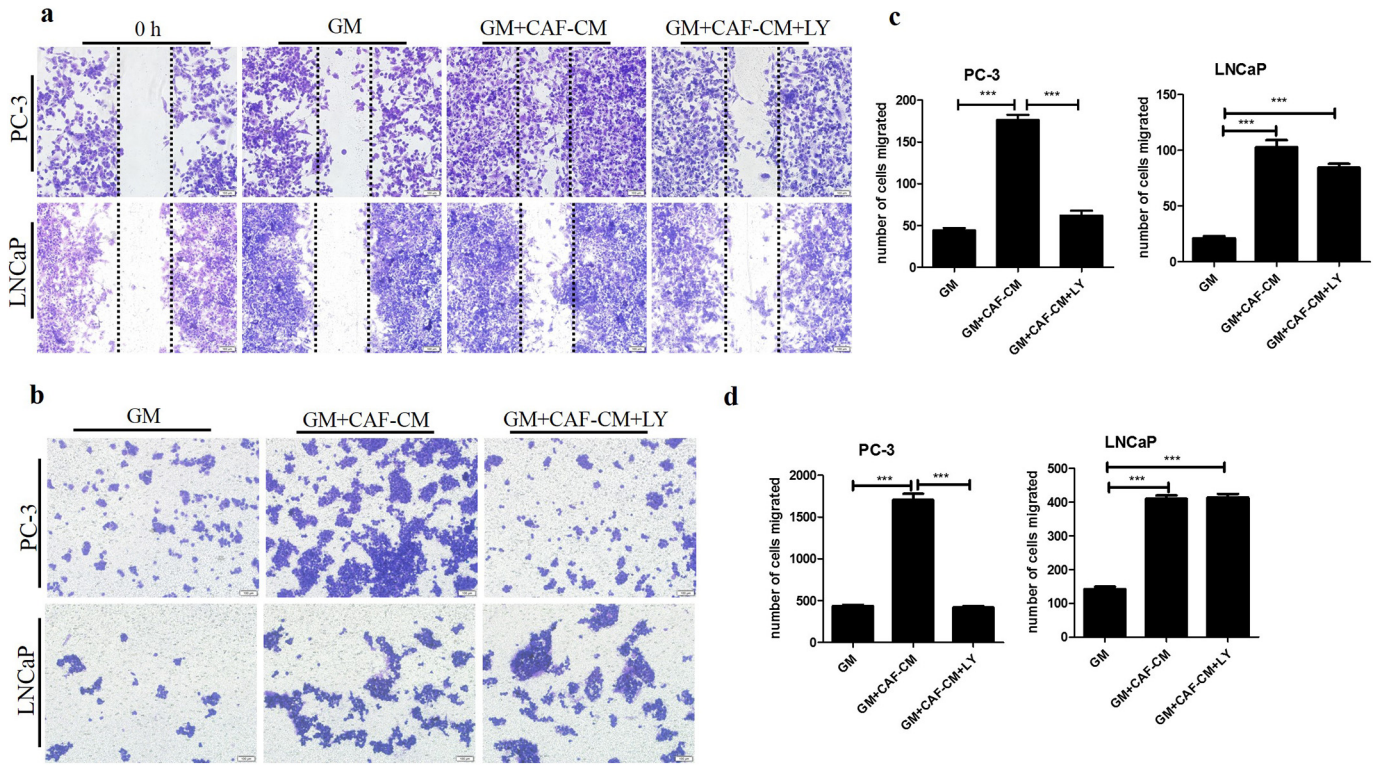

Fig. 2. CAFs enhance the wound healing and migration of prostate cancer cells *via* the secretion of TGF-β. (a) The effect of CAFs on cell wound healing was determined 24 h after in the presence of TGF-βRII inhibitor LY2109761. (b) The effect of CAFs on cell migration was determined 6 h after in the presence of TGF-βRII inhibitor LY2109761. Photographs of migrated cells (magnification, 100×) are shown. Quantitation of (c) wound healing and (d) migrated cells. Migrated cells were counted in four randomly selected microscopic fields. Values are represented as mean ± SD of three replicates. *P < 0.05.

LNCaP cells.

In addition, scratch wound assay was performed to examine whether CAF-CM could affect the wound healing potential of PCa cells. As shown in Fig. 2a and c, CAF-CM had significant promotion effect on the wound-healing potential of PC-3 and LNCaP cells in comparison with GM. LY2109761 could significantly block the promotion effect of CAF-CM in both cells but PC-3 cells showed higher sensitivity to LY2109761 treatment. We also examined the vertical migration of PCa cells induced by CAFs, a key determinant of metastasis in tumor progression. As shown in Fig. 2b and d, CAF-CM treatment greatly accelerated both PCa cells migration rates compared with GM treatment. The migrated cell numbers in GM + CAF-CM treatment group was 4 and 4.8 folds of those in GM treatment group for PC-3 and LNCaP cells, respectively. LY2109761 could significantly block the promotion effect of CAF-CM in PC-3 cells but not LNCaP cells. To summarize, these results elucidated that human primary CAFs could significantly promote the proliferation and migration of PCa cells.

3.3. CAFs promoted tumor growth through the paracrine effect of TGF-β

CAFs secrete a plethora of factors including TGF-β to promote the growth and invasion of the underlying tumor through a paracrine mode [32–35]. Since TGF-β1 was more abundant than TGF-β2 and TGF-β3, we determined the expression level of TGF-β1 in CAF-CM and GM by Luminex technology. As shown in Fig. 3, the expression level of TGF-β1 in GM + CAF-CM group was almost 2 folds higher than that in GM group.

3.4. LY2109761 suppresses TGF-β induced proliferation and migration abilities of PC-3 but not LNCaP cells

LY2109761, a novel inhibitor TGFβR-I and TGFβR-II kinase, has shown antitumor activities in various tumor models such as colorectal cancer, pancreatic cancer, and hepatocellular carcinoma [36–38]. Adding LY2109761 into the CAF-CM co-culture system led to significantly decreased proliferation and migration activities of PC-3 cells (Fig. 1a, 2). LNCaP cells were not as sensitive to LY2109761 treatment as PC-3 cells, its proliferation and migration activities in the co-culture medium were not altered after LY2109761 treatment (Figs. 1b, 2b, and d) while the wound healing activity was slightly decreased by LY2109761 treatment (Fig. 2a, c). Thus, these data suggested that TGF-β has a critical role in CAF driving proliferation and migration of PCa cells.

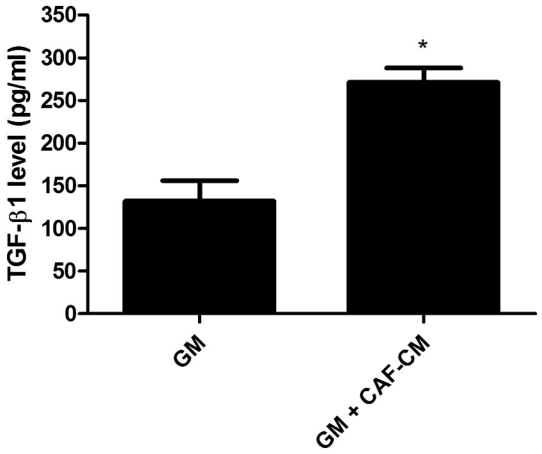

Fig. 3. TGF-β1 level measurement. Growth medium (GM) of PC-3 and conditioned medium of CAFs (CAF-CM) were collected and cryopreserved before cytokine measurement. GM and GM + CAF-CM (1:1) were thawed and processed according to the protocol of Luminex kit. Values are represented as mean ± SD of three replicates. *P < 0.05.

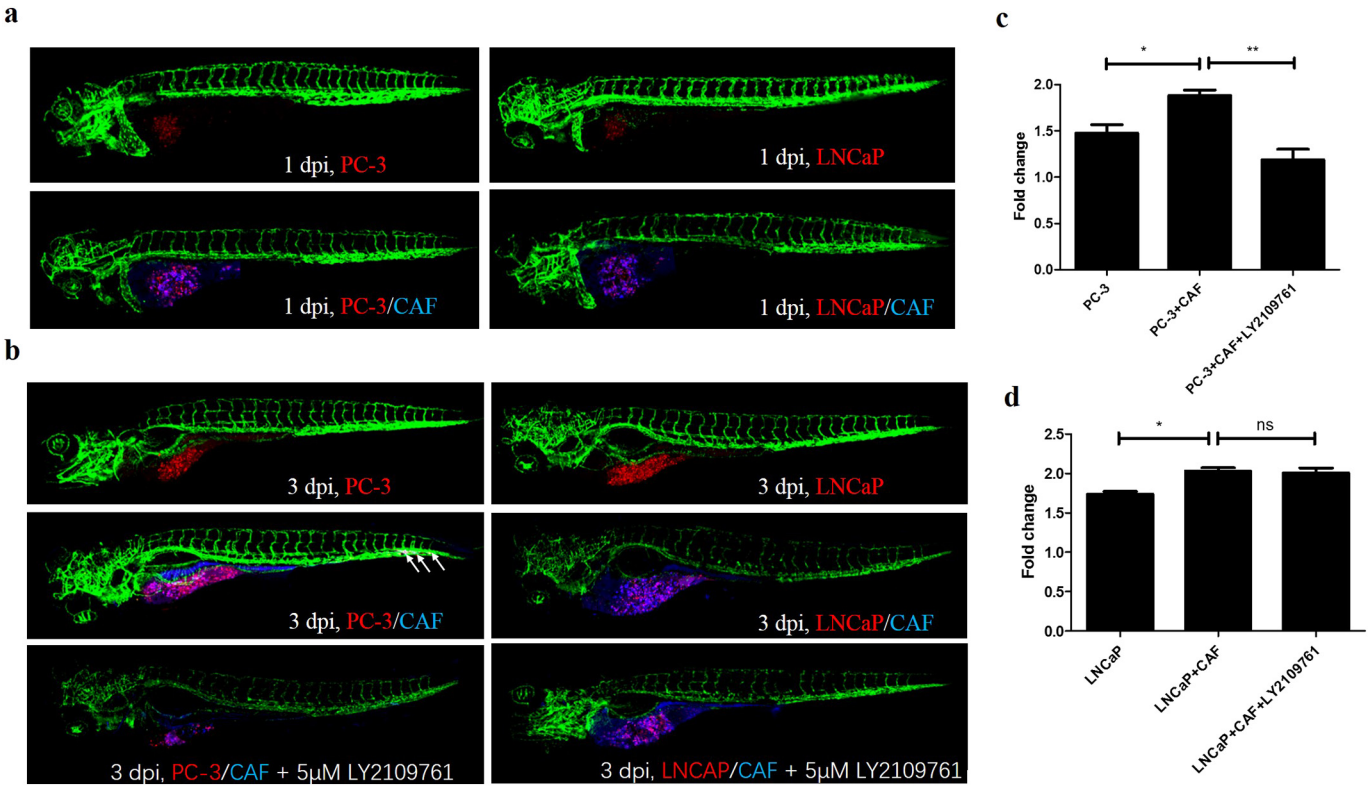

Fig. 4. CAF promoted the proliferation and migration of PCa cells in zebrafish xenograft model. (a) Typical images of CM-DiI-labeled PC-3/LNCaPcells with/without CMAC-Blue-labeled CAFs injected into the zebrafish Tg (*fli-1: EGFP*) yolk sac at 1 dpi (n = 150). (b) Typical images of CM-DiI-labeled PC-3/LNCaP cells with/without CMAC-Blue-labeled CAFs injected into the zebrafish Tg (*fli-1: EGFP*) yolk sac at 1 dpi and were treated with/without LY2109761 for 2 days. (c, d) *In vivo* proliferation of PCa cells. Cell number at 0 dpt was normalized to 1 and set as baseline. Fold change was determined by sacrificing embryos at 0 dpt and 2 dpt. Quantitative values are means ± SEM from 45 to 60 independent individuals. Significance at different treatment group was considered when P values were lower than 0.05. (*) indicates statistical significance $P < 0.05$ and (***) $P < 0.001$. Arrows in (b) point to the metastasized tumor cells in the tail of the zebrafish embryos. dpi: day post injection, dpt: day post treatment.

3.5. LY2109761 inhibits PC-3 cell proliferation and metastasis in vivo

Zebrafish is gaining increasing research prominence as a model for measuring cancer cell progression and microenvironment [23,25,26]. Small molecules can be added directly to the water and diffuse into the zebrafish embryo [39]. We therefore set out to investigate the effect of addition of TGF-β receptor type I/II kinase inhibitor on PCa cells proliferation and metastasis in zebrafish xenograft. First, we subjected the zebrafish to different doses of LY210976. The inhibitor was added to the zebrafish embryos at 48 hpf, and monitored for 5 days. At the doses lower than or equal to 5 μM, no or very few deformities were observed. Extensive malformation and death of the embryos occurred at higher doses. Second, CM-DiI-labeled PC-3/LNCaP cells with/without CMAC-Blue-labeled CAFs were injected into the zebrafish yolk sac at 48 hpf and treated with/without 5 μM LY2109761 (Fig. 4a). The incubation water was changed every day. The cancer cell number at 0 dpt (day post treatment) was set as baseline and normalized to 1. PC-3 cells in the control group proliferated to 1.48 folds of baseline. Co-injection of CAFs with PC-3 stimulated cell proliferation to 1.8 folds of baseline, while cell proliferation was decreased to 1.2 folds of baseline at the presence of 5 μM LY2109761 even when CAFs were co-injected (Fig. 4c). Zebrafish transplanted with PC-3 cells did not display micrometastasis at 3 dpi (day post injection). But nearly 54% of zebrafish xenografts co-injected with PC-3 cells and CAFs displayed micrometastasis at 3 dpi, while these micrometastasis could be totally inhibited by LY2109761 (Fig. 4b). LNCaP cells in the control group proliferated to 1.73 folds of baseline. Co-injection of CAFs stimulated cell proliferation to 2 folds of baseline, and this stimulation was not suppressed by LY2109761 (Fig. 4d). Zebrafish transplanted with LNCaP cells only or mixtures of LNCaP cells and CAFs did not display micrometastasis at 3 dpi (day post injection). These data suggested that targeting the TGF-β signaling with small molecular inhibitors proved to be effective to reduce the proliferation and micrometastasis of PC-3 cells, but not LNCaP cells.

4. Discussion

PCa is an androgen-dependent malignant tumor, but the role of a single androgen does not completely explain the progression of PCa disease, especially the conversion of endocrine to castration-resistant (CRPC) after endocrine therapy. We have previously reported that CAFs regulate the proliferation of AR-positive LNCaP cells *via* the androgen receptor (AR) signaling pathway [40]. For AR-negative PC-3 cells, the molecular mechanism of CAFs to promote the growth of PC-3 cells through the secretion of TGF-β1 has become a hot topic [41].

Human bone marrow-derived mesenchymal stem cells (MSC) share striking similarities to CAFs. Previous work had shown that MSC transdifferentiated into CAFs could secrete TGF-β that promotes prostate tumor cell malignancy [42]. It would be necessary to determine whether all activated fibroblasts or only a subset are MSC [43] in our future study. While the role of CAF-secreted TGF-β in PC-3 *vs* LNCaP have been shown in prior works [42] [44], we show it for the first time in the zebrafish xenograft model which provided direct insight of the effects in a living animal.

TGF-β signaling plays a dual regulatory role in the development and progression of cancer. The effects are often context-dependent because TGF-β can induce both growth promotion and growth inhibition in the same cells depending on the cell types and experimental conditions. The molecular mechanisms responsible for inducing proliferation are

less defined than those that lead to inhibition of proliferation [45]. TGF-β controls both the initial development of tumor as well as the cancer progression of a large variety of tumor types. Its role in tumor suppression and its extensive role in cancer progression through direct effects on the cancer cells themselves, as well as the microenvironment were widely discussed [46].

TGF-β1 is mainly produced by prostatic stromal cells [47], whose overexpression is closely related to the poor prognosis of patients with PCa. Yu [7] reported that CAFs-CM derived from human breast cancer stroma contained excessive TGF-β1 to enhance the adhesion, migration and local invasion of cancer cells MCF-7. In the present study, the excessive secretion of TGF-β1 from CAFs was also found by Luminex assay (Fig. 3). It has been reported that, PC-3 cells expressed high levels of TGF-βRII and showed high sensitivity of TGF-β stimulation [48]. This may be one of the molecular mechanisms by which CAFs regulate the proliferation and migration of PC3. LNCaP had a low or negative expression level of TGF-βRII [49]. Although the proliferation and migration of LNCaP could be activated by CAF-CM, the stimulation might not due to TGF-β signaling because LY2109761 could not block this stimulation, other molecular mechanisms might play role instead of TGF-β1 in the modulation of LNCaP. In our present work, we showed LY2109761 could block the stimulation of CAF-CM to the proliferation and migration of PC-3 *in vitro*, but without an additional group of GM + LY, we could hardly rule out the case where the effect of LY was directly on cancer cells and has nothing to do with the CAF-CM.

Zebrafish as several advantages in the tumor cell xenograft assay. First, it has innate immune system and do not to be immunocompromised before xenograft. Second, this model has a unique live imaging ability that single cell movement can be tracked in a living animal. Third, this xenograft model as a medium throughput in the anticancer drug screening. In the present study, we used zebrafish xenografts model and co-injected the PCa cells together with the microenvironment CAFs. Two different types of cells were fluorescently labeled in different colors, this made it possible to track both types of cells during their proliferation, migration and find the interaction between them [22,23]. Recently, patient-derived tumor xenograft model was also established by using zebrafish, this model offers great opportunity for the translational research of cancer as well as a real-time drug sensitivity test for the personalized chemotherapy [19,25]. Zebrafish is a major catch for cancer researchers offering a number of unique advantages for investigating the mechanisms that drive cancer formation and progression [24]. It is no wonder that cancer researchers are taking notice as these advantages provide new way for significant discoveries and a whole animal system for cancer research.

5. Conclusions

Our study showed that CAFs regulated the proliferation and migration of PC3 and LNCaP cells in different molecular mechanisms. CAFs regulated the proliferation of PC-3 through TGF-β1 signaling pathway, which is a potential new therapeutic target for PCa. However, androgen rather than TGF-β signaling pathway might be the main regulative way for the regulation of LNCaP cells by CAFs.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.116791.

Declaration of competing interest

All authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by the Project of Science & Technology of Nanjing (201605023). Key R & D Special Fund of Jiangsu Province (BE2016614, BE2017611). Six Talent Peaks Project in Jiangsu Province (2016-SWYY-024). Innovation Project for Undergraduate and Postgraduate Student of Nanjing Tech University (KYCX17_0961; KYLX16_0624). The Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture (XTE1835).

Role of the funding source

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

[1] Y. Zhu, H.K. Wang, Y.Y. Qu, D.W. Ye, Prostate cancer in East Asia: evolving trend over the last decade, Asian J. Androl. 17 (2015) 48–57.
[2] W. Chen, R. Zheng, P.D. Baade, S. Zhang, H. Zeng, F. Bray, A. Jemal, X.Q. Yu, J. He, Cancer statistics in China, 2015, CA Cancer J. Clin. 66 (2016) 115–132.
[3] X. Yuan, C. Cai, S. Chen, S. Chen, Z. Yu, S.P. Balk, Androgen receptor functions in castration-resistant prostate cancer and mechanisms of resistance to new agents targeting the androgen axis, Oncogene 33 (2014) 2815–2825.
[4] K.E. Richards, A.E. Zeleniak, M.L. Fishel, J. Wu, L.E. Littlepage, R. Hill, Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells, Oncogene 36 (2017) 1770–1778.
[5] P.T. Xiongyan Wu, Q. Zhou1, J. Li, Y. Zhenjia, X. Wang, J. Li, C. Li, M. Yan, Z. Zhu, B. Liu, S. Liping, IL 6 secreted by cancer associated fibroblasts promotes epithelial mesenchymal transition and metastasis of gastric cancer via JAK2 STAT3 signaling pathway, Oncotarget 8 (2017) 20741.
[6] L. Tao, G. Huang, R. Wang, Y. Pan, Z. He, X. Chu, H. Song, L. Chen, Cancer-associated fibroblasts treated with cisplatin facilitates chemoresistance of lung adenocarcinoma through IL-11/IL-11R/STAT3 signaling pathway, Sci. Rep. 6 (2016) 38408.
[7] Y. Yu, C.H. Xiao, L.D. Tan, Q.S. Wang, X.Q. Li, Y.M. Feng, Cancer-associated fibroblasts induce epithelial-mesenchymal transition of breast cancer cells through paracrine TGF-beta signalling, Br. J. Cancer 110 (2014) 724–732.
[8] C. Pena, M.V. Cespedes, M.B. Lindh, S. Kiflemariam, A. Mezheyeuski, P.H. Edqvist, C. Hagglof, H. Birgisson, L. Bojmar, K. Jirstrom, P. Sandstrom, E. Olsson, S. Veerla, A. Gallardo, T. Sjoblom, A.C. Chang, R.R. Reddel, R. Mangues, M. Augsten, A. Ostman, STC1 expression by cancer-associated fibroblasts drives metastasis of colorectal cancer, Cancer Res. 73 (2013) 1287–1297.
[9] T. Eder, A. Weber, H. Neuwirt, G. Grunbacher, C. Ploner, H. Klocker, N. Sampson, I.E. Eder, Cancer-associated fibroblasts modify the response of prostate cancer cells to androgen and anti-androgens in three-dimensional spheroid culture, Int. J. Mol. Sci. 17 (2016) 1458.
[10] P. Chiarugi, P. Paoli, P. Cirri, Tumor microenvironment and metabolism in prostate cancer, Semin. Oncol. 41 (2014) 267–280.
[11] D.Z. GuocanWang, D.J. Spring, R.A. DePinho, Genetics and biology of prostate cancer, Genes Dev. 32 (2018) 1105–1140.
[12] R. Kalluri, M. Zeisberg, Fibroblasts in cancer, Nat. Rev. Cancer 6 (2006) 392–401.
[13] T. Marsh, K. Pietras, S.S. McAllister, Fibroblasts as architects of cancer pathogenesis, Biochim. Biophys. Acta 1832 (2013) 1070–1078.
[14] N.A. Bhowmick, A. Chytil, D. Plieth, A.E. Gorska, N. Dumont, S. Shappell, M.K. Washington, E.G. Neilson, H.L. Moses, TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia, Science 303 (2004) 848–851.
[15] C.T. Wu, Y.H. Chang, W.Y. Lin, W.C. Chen, M.F. Chen, TGF beta1 expression correlates with survival and tumor aggressiveness of prostate cancer, Ann. Surg. Oncol. 22 (2015) S1587–S1593.
[16] Y.L. Milind Javle, Dongfeng Tan, X. Dong, P.C. Siddhartha Kar, D. Li, Biomarkers of TGF-b signaling pathway and prognosis of pancreatic cancer, PLoS One 9 (January 2014) e85942.
[17] M. Miyake, S. Hori, Y. Morizawa, Y. Tatsumi, Y. Nakai, S. Anai, K. Torimoto, K. Aoki, N. Tanaka, K. Shimada, N. Konishi, M. Toritsuka, T. Kishimoto, C.J. Rosser, K. Fujimoto, CXCL1-mediated interaction of cancer cells with tumor-associated macrophages and cancer-associated fibroblasts promotes tumor progression in human bladder cancer, Neoplasia 18 (2016) 636–646.
[18] H. Yamaguchi, R. Sakai, Direct interaction between carcinoma cells and cancer associated fibroblasts for the regulation of cancer invasion, Cancers (Basel) 7 (2015) 2054–2062.
[19] R. Fior, V. Povoa, R.V. Mendes, T. Carvalho, A. Gomes, N. Figueiredo, M.G. Ferreira, Single-cell functional and chemosensitive profiling of combinatorial colorectal therapy in zebrafish xenografts, Proc. Natl. Acad. Sci. U. S. A. 114 (2017) E8234–E8243.
[20] R. White, K. Rose, L. Zon, Zebrafish cancer: the state of the art and the path forward, Nat. Rev. Cancer 13 (2013) 624–636.
[21] C.J. Veinotte, G. Dellaire, J.N. Berman, Hooking the big one: the potential of zebrafish xenotransplantation to reform cancer drug screening in the genomic era, Dis. Model. Mech. 7 (2014) 745–754.
[22] D.P. Corkery GD, J.N. Berman, Leukaemia xenotransplantation in zebrafish—chemotherapy response assay in vivo, Br. J. Haematol. 153 (2011) 786–799.
[23] S. Liu, S.D. Leach, Zebrafish models for cancer, Annu. Rev. Pathol. 6 (2011) 71–93.
[24] K. Stoletov, R. Klemke, Catch of the day: zebrafish as a human cancer model, Oncogene 27 (2008) 4509–4520.
[25] J. Wang, Z. Cao, X.M. Zhang, M. Nakamura, M. Sun, J. Hartman, R.A. Harris, Y. Sun, Y. Cao, Novel mechanism of macrophage-mediated metastasis revealed in a

zebrafish model of tumor development, Cancer Res. 75 (2015) 306–315.
[26] C. Liu, Y. Zhang, S. Lim, K. Hosaka, Y. Yang, T. Pavlova, T. Alkasalias, J. Hartman, L. Jensen, X. Xing, X. Wang, Y. Lu, G. Nie, Y. Cao, A zebrafish model discovers a novel mechanism of stromal fibroblast-mediated cancer metastasis, Clin. Cancer Res. 23 (2017) 4769–4779.
[27] N.D. Lawson, B.M. Weinstein, In vivo imaging of embryonic vascular development using transgenic zebrafish, Dev. Biol. 248 (2002) 307–318.
[28] J.Q. Wu, J. Zhai, C.Y. Li, A.M. Tan, P. Wei, L.Z. Shen, M.F. He, Patient-derived xenograft in zebrafish embryos: a new platform for translational research in gastric cancer, J. Exp. Clin. Cancer Res. 36 (2017) 160.
[29] D.P. Corkery, G. Dellaire, J.N. German, Leukaemia xenotransplantation in zebrafish-chemotherapy response assay in vivo, Br. J. Haemetol. 153 (2011) 786–789.
[30] J.J. Tomasek, G. Gabbiani, B. Hinz, C. Chaponnier, R.A. Brown, Myofibroblasts and mechano-regulation of connective tissue remodelling, Nat. Rev. Mol. Cell. Biol. 3 (2002) 349–363.
[31] G. Gabbiani, The myofibroblast in wound healing and fibrocontractive diseases, J. Pathol. 200 (2003) 500–503.
[32] K. Shiga, M. Hara, T. Nagasaki, T. Sato, H. Takahashi, H. Takeyama, Cancer-associated fibroblasts: their characteristics and their roles in tumor growth, Cancers (Basel) 7 (2015) 2443–2458.
[33] W.D. Bai, X.M. Ye, M.Y. Zhang, H.Y. Zhu, W.J. Xi, X. Huang, J. Zhao, B. Gu, G.X. Zheng, A.G. Yang, L.T. Jia, MiR-200c suppresses TGF-beta signaling and counteracts trastuzumab resistance and metastasis by targeting ZNF217 and ZEB1 in breast cancer, Int. J. Cancer 135 (2014) 1356–1368.
[34] R.Y. Liu, Y. Zeng, Z. Lei, L. Wang, H. Yang, Z. Liu, J. Zhao, H.T. Zhang, JAK/STAT3 signaling is required for TGF-beta-induced epithelial-mesenchymal transition in lung cancer cells, Int. J. Oncol. 44 (2014) 1643–1651.
[35] H. Zhang, L. Liu, Y. Wang, G. Zhao, R. Xie, C. Liu, X. Xiao, K. Wu, Y. Nie, H. Zhang, D. Fan, KLF8 involves in TGF-beta-induced EMT and promotes invasion and migration in gastric cancer cells, J. Cancer Res. Clin. Oncol. 139 (2013) 1033–1042.
[36] B. Zhang, S.K. Halder, S. Zhang, P.K. Datta, Targeting transforming growth factor-beta signaling in liver metastasis of colon cancer, Cancer Lett. 277 (2009) 114–120.
[37] D. Melisi, S. Ishiyama, G.M. Sclabas, J.B. Fleming, Q. Xia, G. Tortora, J.L. Abbruzzese, P.J. Chiao, LY2109761, a novel transforming growth factor beta receptor type I and type II dual inhibitor, as a therapeutic approach to suppressing pancreatic cancer metastasis, Mol. Cancer Ther. 7 (2008) 829–840.
[38] E. Fransvea, U. Angelotti, S. Antonaci, G. Giannelli, Blocking transforming growth factor-beta up-regulates E-cadherin and reduces migration and invasion of hepatocellular carcinoma cells, Hepatology 47 (2008) 1557–1566.
[39] H.T.C. Hendrian Sukardi, E.C.Y. Chan, Z. Gong, S.H. Lam, Zebrafish for drug toxicity screening bridging the in vitro cell-based models and in vivo mammalian models, Drug Metab. Toxicol. 7 (2011) 579–589.
[40] Z.-Y.W. Yi-Feng Li, X.-G. Chen, H.-B. Sun, Effects of anti-androgen-induced cancer-associated fibroblasts on proliferation of prostate cancer cell line LNCaP, J. Contemp. Urol. Reprod. Oncol. 5 (2013) 96–100.
[41] D.A. Barron, D.R. Rowley, The reactive stroma microenvironment and prostate cancer progression, Endocr. Relat. Cancer 19 (2012) R187–R204.
[42] P. Barcellos-de-Souza, G. Comito, C. Pons-Segura, M.L. Taddei, V. Gori, V. Becherucci, F. Bambi, F. Margheri, A. Laurenzana, M. Del Rosso, P. Chiarugi, Mesenchymal stem cells are recruited and activated into carcinoma-associated fibroblasts by prostate cancer microenvironment-derived TGF-beta1, Stem Cells 34 (2016) 2536–2547.
[43] R. Kalluri, The biology and function of fibroblasts in cancer, Nat. Rev. Cancer 16 (2016) 582–598.
[44] H. Ye, J. Cheng, Y. Tang, Z. Liu, C. Xu, Y. Liu, et al., Human bone marrow-derived mesenchymal stem cells produced tgfbeta contributes to progression and metastasis of prostate cancer, Cancer Investig. 30 (2012) 513–518.
[45] Y. Zhang, P.B. Alexander, X.F. Wang, TGF-beta family signaling in the control of cell proliferation and survival, Cold Spring Harb. Perspect. Biol. 9 (2017).
[46] M. Morikawa, R. Derynck, K. Miyazono, TGF-beta and the TGF-beta family: context-dependent roles in cell and tissue physiology, Cold Spring Harb. Perspect. Biol. 8 (2016).
[47] B. Costanza, I.A. Umelo, J. Bellier, V. Castronovo, A. Turtoi, Stromal modulators of TGF-beta in cancer, J. Clin. Med. 6 (2017) 7.
[48] G.-F.Z. Kai-Jie Wu, D. Zhang, J. Zeng, X.-Y. Wang, Y. Xue, L.-L. Zhang, D.-L. He, Identification and significance of TGF_Smads pathway key proteins in prostate cancer cell lines, Natl. J. Androl. 15 (2009) 920–924.
[49] Kyprianou Emban, Dihydrotestosterone enhances transforming growth factor-beta-induced apoptosis in hormone-sensitive prostate cancer cells, Endocrinology 142 (2000) 2419–2426.