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Immortalized Cancer-associated Fibroblasts Promote Prostate Cancer Carcinogenesis, Proliferation and Invasion

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Abstract. *Background: Cancer-associated fibroblasts (CAFs) are dominant components of the prostate cancer (PCa) stroma. However, the contrasting effects of CAFs and adjacent normal prostate fibroblasts (NPFs) are still poorly defined. The senescence of non-immortalized CAFs after subculture may limit the cell number and influence experimental results of in vitro studies. In this study, we immortalized CAFs to study their role in PCa carcinogenesis, proliferation, and invasion. Materials and Methods: We cultured and immortalized CAFs and NPFs, then compared their effect on epithelial malignant transformation by using in vitro co-culture, soft agar assay, and a mouse renal capsule xenograft model. We also compared their roles in PCa progression by using in vitro co-culture, cell viability assays, invasion assays, and a mouse xenograft model. For the mechanistic study, we screened a series of growth factors by using real-time polymerase chain reaction. Results: The CAFs and NPFs were successfully cultured, immortalized, and characterized. The CAFs were able to transform prostate epithelial cells into malignant cells, but NPFs were not. The CAFs were more active in promoting proliferation of and invasion by PCa cells, and in secreting higher levels of a series of growth factors. Conclusion: The immortalized CAFs were more supportive of PCa carcinogenesis and progression. Targeting CAFs might be a potential option for PCa therapy. Immortalized CAFs and NPFs will also be valuable resources for future experimental exploration.*

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Key Words: Immortalized cancer-associated fibroblasts, carcinogenesis, proliferation and invasion.

Prostate cancer (PCa) is one of the most common cancers in developed countries, and its incidence is increasing in China (1, 2). Radical prostatectomy and radiotherapy are generally recommended for localized PCa, whilst a comprehensive therapy based on androgen deprivation therapy is first-line treatment for advanced disease (2). Although initially effective at blocking tumor growth, androgen deprivation therapy can eventually fail, leading to a castration-resistant incurable disease stage (3). The current therapeutic options for advanced PCa mostly target cancer cells. However, prostate stromal cells also play key roles in PCa progression (4).

Prostate cancer-associated fibroblasts (CAFs) are dominant components in PCa stroma, and have been reported to promote proliferation of epithelial cells (5, 6). In a previous study, we successfully cultured CAFs from PCa tissues and found that androgen receptors in CAFs promoted PCa epithelial growth and invasion (7). However, the contrasting effects of CAFs and adjacent normal prostate fibroblasts (NPFs) remain poorly defined.

Most researchers use primary CAFs without immortalization. Non-immortalized CAFs can only be subcultured for 6-8 passages, and different sets of primary cells from different PCa tissues may influence the consistency of experimental results. In this study, we primarily cultured and immortalized CAFs and NPFs from the same patient with PCa. Subsequently, we compared their effects on malignant transformation and progression of PCa by using *in vitro* experiments and *in vivo* mouse xenograft models. We also compared expression patterns of growth factors between CAFs and NPFs.

Materials and Methods

Cell culture. PCa specimens and normal prostate tissues were obtained from a patient with PCa (67 years old, primary PCa, Gleason score=3+3) at the Urology Department of Yantai Yuhuangding Hospital. The sample collection and primary cell culture were approved by the Yantai Yuhuangding Hospital Ethics Committee (Approval No. 2013-46). The patient was well informed and agreed to the use of his samples for cell culture and further

research. All procedures abide by the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The detailed procedure of the primary culture of CAFs and NPFs was described elsewhere (7). At passage 3, the plasmid PBabe-SV40-T (neo) was transfected by electroporation (280 V, 960lF) into CAFs and NPFs for cell immortalization (7). Human PCa cell lines LNCaP and non-malignant BPH-1 cells were kind gifts from Dr. Shujie Xia of Shanghai Jiaotong University. All cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37˚C in a humidified incubator containing 5% CO_2.

Immunofluorescence assay. CAFs and NPFs (10^4) were seeded in 4-well chamber slides and cultured overnight. The chamber slides with attached cells were routinely fixed, blocked and then incubated with the following antibodies: anti-vimentin, anti-smooth muscle a-actin (SMA) (both 1:200; Sigma-Aldrich, St. Louis, MO, USA), and IgG control. Subsequently, slides were incubated with fluorescence-labeled secondary antibodies, and mounted in medium containing 4',6-diamidino-2-phenylindole (DAPI).

Methyl thiazolyl tetrazolium (MTT) assay. CAFs and NPFs were seeded in 24-well plates (1×10^4 cells per well) and incubated with medium changed every 48 h. The plates were stained with MTT at day 0, 2, 4, 6 for 3 h. The absorbence was read at a wave length of 575 nm.

Malignant transformation of BPH-1 cells in vitro. The BPH-1 cells were co-cultured with CAFs or NPFs in transwell plates. CAFs or NPFs in around 50% confluence were seeded in the lower well, and BPH-1 cells in around 30% confluence were seeded in the upper well. When the BPH-1 cells came to confluence, they were sub-cultured. When the CAFs and NPFs came to confluence, the cells in the lower well were exchanged with new CAFs or NPFs at around 50% confluence. The overall co-culture period was 4 weeks. The resultant BPH-1 cells were used in a soft agar assay.

Soft agar assay. Base agar (0.5 ml of 1%, DNA grade) was put into each well of a 6-well plate. Then 1% agar (DNA grade agarose) was melted in a microwave prior to cooling to 40˚C in a water bath. Agar was subsequently mixed with 3×RPMI-1640 medium and BPH-1 cell suspension in a ratio of 1:1:1, and resulted in a final cell density of 10^5 cells/ml. Add 1ml of the mixture on the top of the base agar. After coagulation, add 2 ml RPMI-1640 supplemented with 10% FBS, and replace medium every 3 days to provide nutrition. After 2 weeks culture in an incubator at 37˚C, the colonies were stained by 1 mg/ml iodonitrotetrazoliumchloride solution.

Malignant transformation of BPH-1 cells in vivo. After anesthesia, five pairs of athymic male nude mice (8 weeks old, from Beijing Wei-tong Li-hua Laboratory Animals and Technology Ltd., Beijing, P.R. China) were put on a heating pad in the prone position. Lifting the back skin with a pair of toothed forceps, and using a coarse scissors, a 2-3 cm dorsal midline incision was made to expose the kidney (8). CAFs or NPFs (5×10^5 cells) and 2×10^5 BPH-1 cells were mixed in 2 mg/ml rat tail collagen (Sigma-Aldrich, St. Louis, MO, USA), then were seeded into the renal capsule of the athymic nude mice. Twelve weeks later, we sacrificed the mice and excised the kidney for further study. The care and use of nude mice in this study were approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University (Approval No. 2013-46).

Hematoxylin and eosin (H&E) staining. Mouse xenograft tissue samples were fixed in 10% formalin and embedded in paraffin by routine. After rehydration, the sections were put into hematoxylin for 8 minutes, then rinsed in running tap water. The slides were then treated with 0.3% acid alcohol, and rinsed in running tap water followed by staining with eosin for 2 min. The sections were dehydrated, cleared and eventually mounted.

LNCaP and CAFs/NPFs in vitro co-culture system. LNCaP cells (2×10^4 cells per well) in serum free RPMI-1640 medium supplied with 1 nM dihydrotestosterone (DHT) were seeded in cell culture inserts of a 24-well transwell plates (2 μm microporous, from BD Biosciences, Franklin Lakes, NJ, USA). CAFs or NPFs (5×10^4) in serum-free RPMI-1640 medium were seeded in the bottom chamber. Serum-free RPMI-1640 medium was changed by 50% every 48 h. The inserts were stained with MTT (Sigma) at day 0, 2, 4, 6 for 3 h. The absorbance was read at a test wavelength of 575 nm.

Invasion assay. LNCaP cells (1×10^5) in serum-free RPMI-1640 medium were added to cell culture inserts with microporous (8 μm) membrane coated with or without (control insert) Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). CAFs or NPFs (1×10^5) were seeded in RPMI-1640 medium containing 10% FBS into the bottom chamber. The cells were incubated for 24 hours at 37˚C, and the upper chamber was then removed. The cells on the bottom of the upper chambers were stained with 1% toluidine blue, and the number of invading cells was counted under a microscope. The invasion ratio was the ratio of the cells invading through the Matrigel-coated insert membrane to those migrating through the control non-coated insert membrane.

Xenograft model of tumor growth in vivo. Athymic male nude mice were purchased from Beijing Wei-tong Li-hua Laboratory Animals and Technology Ltd. The mixed cells were suspended in 50 μl Matrigel and then implanted subcutaneously into the left flank (1×10^6 LNCaP cells + 1×10^6 CAFs) and right flank (1×10^6 LNCaP cells + 1×10^6 NPFs) of nude mice to form xenograft tumors. All five mice were sacrificed at 8 weeks after inoculation. The tumors were harvested and weighed for comparison between the groups. The care and use of nude mice in this study were approved by the Ethics Committee of the Affiliated Yantai Yuhuangding Hospital of Qingdao University (Approval No. 2013-46).

Real-time quantitative polymerase chain reaction (Q-PCR). Total RNA from CAFs and NPFs was extracted and purified using Trizol (Takara, Carlsbad, CA, USA). Three micrograms of RNA was subjected to reverse transcription of genes encoding growth factors (Table I) using Superscript III (TransGene, Beijing, China). Amplification was performed using SYBR green fluorescence with the following PCR amplification conditions: 1 cycle at 95˚C for 10 min, 45 cycles at 95˚C for 15 s and 60˚C for 60 s (7). The relative expression of mRNAs was calculated using the $2^{-\Delta\Delta CT}$ method to compare the expression levels among different samples. The primer sequences are shown in Table I.

Statistical analysis. Numerical data are presented as the mean±standard deviation. Statistical analysis between groups was performed by using two-sided Student's *t*-test. *p*-Values of less than 0.05 were considered statistically significant.

Table I. *The primer sequences used for real-time quantitative polymerase chain reaction.*

Growth factor (gene)	Sense (5'-3')	Antisense (5'-3')
Insulin-like growth factor 1 (*IGF1*)	CCTCCTCGCATCTCTTCTAC	AATACATCTCCAGCCTCCTTAG
Placental growth factor (*PGF*)	CTCCCCAATCCAGGCATCAA	TGGGACCCATCTTTGCTGAG
Epidermal growth factor (*EGF*)	TACCGAGACCTGAAGTGG	TCTGAGTCCTGTAGTAGTGGG
Fibroblast growth factor 2 (*FGF2*)	GCCTTCTCTTTCAGCATTCAC	CCAACTCGTAACAATCCATCAG
Fibroblast growth factor 7 (*FGF7*)	CCCTGAGCGACACACAAG	CACAATTCCAACTGCCACTG
Fibroblast growth factor 9 (*FGF9*)	ATGGCTCCCTTAGGTGAAGTT	CCCAGGTGGTCACTTAACAAAAC
Fibroblast growth factor 10 (*FGF10*)	CCTCCTTCTCCTCTCCTTCC	GGCAGTTCTCCTTCTTGGTC
Stromal cell-derived factor 1 (*SDF1*)	CTGTGCCCTTCAGATTGTT	GGCGGAGTGTCTTTATGC
Hepatocyte growth factor (*HGF*)	AGGGGCACTGTCAATACCATT	CGTGAGGATACTGAGAATCCCAA
Transforming growth factor β1 (*TGFB1*)	CTAATGGTGGAAACCCACAACG	TATCGCCAGGAATTGTTGCTG
Transforming growth factor β 2 (*TGFB2*)	CCATCCCGCCCACTTTCTAC	AGCTCAATCCGTTGTTCAGGC
Transforming growth factor β 3 (*TGFB3*)	CACCCAGGAAAACACCGAGTC	GCGGAAAACCTTGGAGGTAAT
Vascular endothelial growth factor B (*VEGFB*)	GAGATGTCCCTGGAAGAACACA	GAGTGGGATGGGTGATGTCAG
Vascular endothelial growth factor C (*VEGFC*)	CAGTTACGGTCTGTGTCCAGTGTAG	GGACACACATGGAGGTTTAAAGAAG
β-Actin	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT

Results

Primary culture and characterization of CAFs and NPFs. CAFs and NPFs were isolated from human PCa tissue and adjacent normal prostate tissue, respectively, and were immortalized with SV40-T. The cell morphologies of CAFs and NPFs were similar with elongated or stellated shapes (Figure 1A). The immunofluorescent co-staining of vimentin (fibroblast marker) and SMA (smooth muscle marker) showed the CAFs and NPFs were positive for both these markers. However, the SMA staining in CAFs was much stronger than that in NPFs (Figure 1B), which suggests the CAFs were myofibroblasts and more progenitorial than NPFs. In MTT cell growth assays, the CAFs grew faster than NPFs (Figure 1C).

BPH-1 cells were transformed into malignant cells by CAFs in vitro. For *in vitro* malignant transformation, BPH-1 cells were co-cultured with CAFs or NPFs in transwell plates (Figure 2A). We defined the resultant BPH-1 cells as BPH-1(CAFs) and BPH-1(NPFs). After 4 weeks co-culture, the resultant BPH-1 cells were seeded in soft agar to determine anchorage-independent colony formation ability. After 2 weeks culture, BPH-1(CAFs) were able to form colonies in soft agar, however, BPH-1(NPFs) did not form any colonies (Figure 2B).

BPH-1 cells were transformed into malignant cells by CAFs in vivo. BPH-1 cells combined with CAFs or NPFs in collagen were seeded into the renal capsule of nude mice. Twelve weeks later, tumors had formed in mice seeded with BPH-1 and CAFs, but not in those seeded with BPH-1 and NPFs (Figure 3A). The negative control, *i.e.* seeding with CAFs, NPFs, or BPH-1 cells alone did not lead to tumor formation in renal capsules of nude mice (data not shown). On H&E staining, tumor from mice seeded with BPH-1 and CAFs presented an irregular undifferentiated malignant pattern (Figure 3B). However, the nodules from mice seeded with BPH-1 and NPFs presented a well-arranged gland-like benign pattern (Figure 3C).

CAFs promote the growth of and invasion by PCa LNCaP cells. In order to determine the role of CAFs and NPFs on PCa cell growth, LNCaP cells were co-cultured with CAFs or NPFs in transwell plates with serum-free medium supplemented with 1nM DHT (Figure 4A). In MTT assays, LNCaP cells grew extremely slowly in serum-free medium, and both CAFs and NPFs supported the growth of LNCaP cells. CAFs had a more powerful effect on growth promotion of LNCaP cells (Figure 4B). In transwell invasion assay, there were more invading LNCaP cells when co-cultured with CAFs than when co-cultured with NPFs (Figure 4C), which indicated the CAFs had a promotory effect on invasion by LNCaP cells.

In order to determine the role of CAFs and NPFs *in vivo*, we inoculated LNCaP cells combined with CAFs or NPFs subcutaneously into the flank of nude mice. Eight weeks later, the tumors from mice inoculated with LNCaP and CAFs were much bigger than those arising from inoculated with LNCaP and NPFs (Figure 4D). In summary, CAFs were more effective in promoting the growth of and invasion by PCa LNCaP cells both *in vitro* and *in vivo*.

CAFs secreted more growth factors than NPFs. In order to probe the mechanism of why CAFs were more effective than NPFs in promoting prostate epithelial cell transformation, growth, invasion, and tumorigenesis, we performed Q-PCR

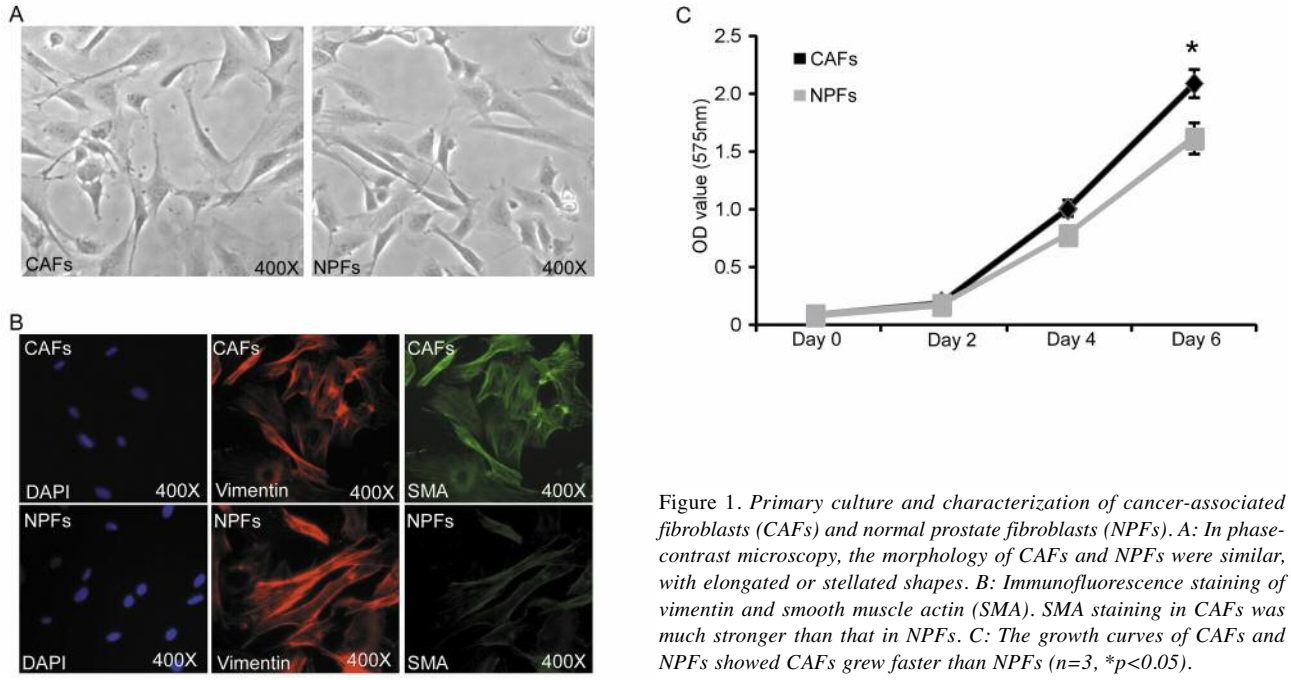

Figure 1. *Primary culture and characterization of cancer-associated fibroblasts (CAFs) and normal prostate fibroblasts (NPFs). A: In phase-contrast microscopy, the morphology of CAFs and NPFs were similar, with elongated or stellated shapes. B: Immunofluorescence staining of vimentin and smooth muscle actin (SMA). SMA staining in CAFs was much stronger than that in NPFs. C: The growth curves of CAFs and NPFs showed CAFs grew faster than NPFs (n=3, *p<0.05).*

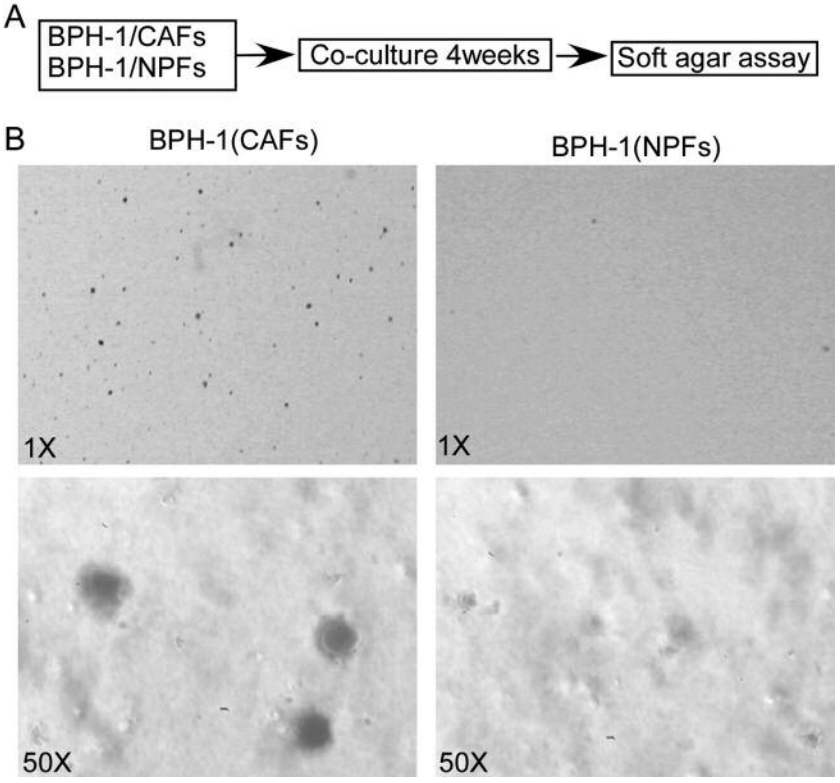

Figure 2. *In vitro malignant transformation of BPH-1 cells. A: Transwell co-culture of BPH-1 cells with cancer-associated fibroblasts (CAFs) or normal prostate fibroblasts (NPFs). B: In soft agar colony formation assay, the BPH-1(CAFs) formed colonies in soft agar (left), however, the BPH-1(NPFs) were almost unable to form any colonies (right).*

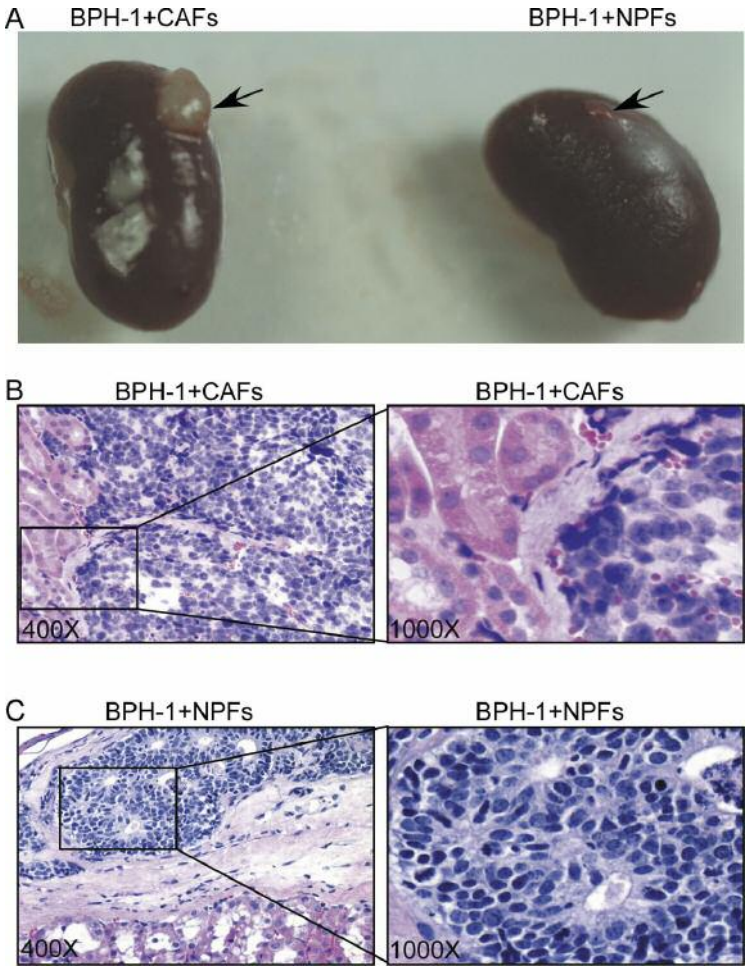

Figure 3. *In vivo malignant transformation of BPH-1 cells. A: In nude mice inoculated with BPH-1 and cancer-associated fibroblasts (CAFs), tumors formed in the renal capsule (left, n=5), however, inoculation with BPH-1 and normal prostate fibroblasts (NPFs) did not lead to tumor (right, n=5). B: Hematoxylin and eosin (H&E) staining of tumor from mice inoculated with BPH-1 and CAFs presented an irregular undifferentiated malignant pattern. C: H&E staining of a tiny nodule from mice inoculated with BPH-1 and NPFs presented a well-arranged gland-like benign pattern.*

assay to screen a series of growth factors. CAFs secreted higher levels of growth factors than NPFs, namely insulin-like growth factor 1 (*IGF1*), placental growth factor (*PGF*), epidermal growth factor (*EGF*), fibroblast growth factor (*FGF*) 2, *FGF7, FGF10*, stromal cell-derived factor 1 (*SDF1*), hepatocyte growth factor (*HGF*), transforming growth factor β1 (*TGFB1*), transforming growth factor β 3 (*TGFB3*), and vascular endothelial growth factor B (*VEGFB*) (Figure 5).

Discussion

The cross-talk between epithelium and stroma plays critical roles in normal prostate development and cancer (9, 10). In tissue recombination experiments, in nude mice with a tissue recombinant of epithelia and urogenital sinus mesenchyme, prostate-like tissue formed, but did not in those with epithelia without stroma (11). During the progression of cancer, tumor cells alter the properties of the surrounding stroma to create a supportive micro-environment (12). In turn, the modified stromal cells become more active and promote the progression of cancer (10). Different types of growth factors and cytokines secreted by reactive stromal cells and the direct stroma–epithelium interactions are thought to play key roles in cancer progression (13).

PCa stroma is composed of fibroblasts, myofibroblasts, endothelial cells and immune cells (14). PCa cells produced growth factors and cytokines to activate peripheral stromal cells and resulted in the accumulation of CAFs (15). CAFs share a similar morphology with myofibroblasts observed in wound healing (13), and might originate from transformation

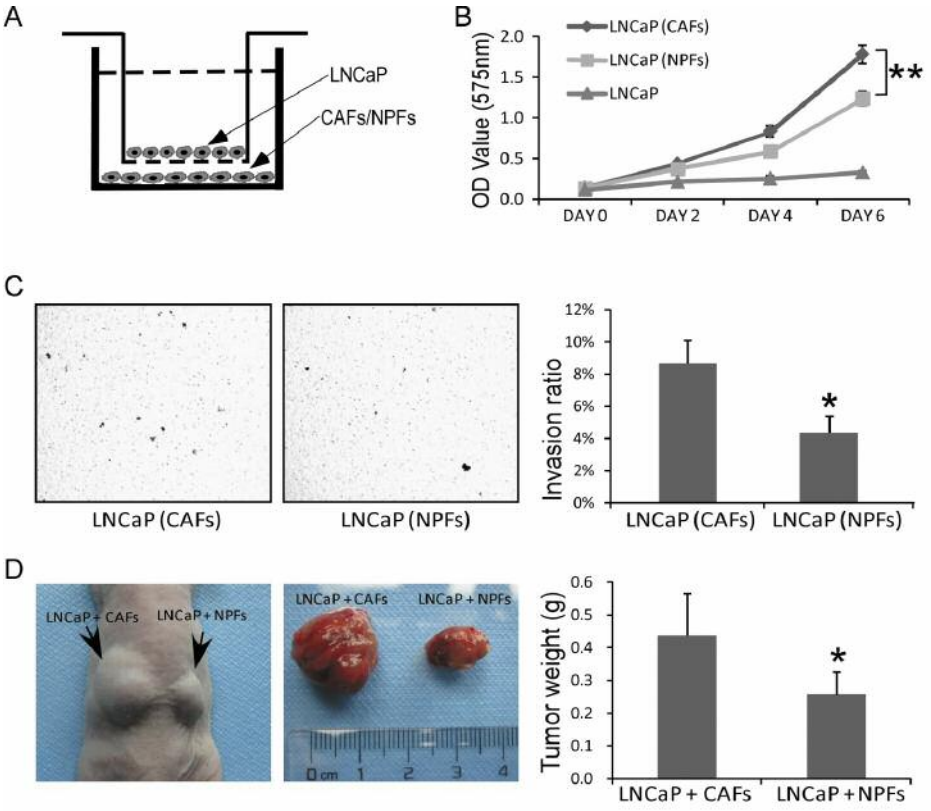

Figure 4. *The effect of cancer-associated fibroblasts (CAFs) and normal prostate fibroblasts (NPFs) on the growth and invasion of LNCaP cells. A: In the co-culture transwell system, LNCaP cells were seeded in the insert of the transwell, and CAFs or NPFs were seeded on the bottom. The culture medium was serum-free RPMI-1640 supplied with 1 nM dihydrotestosterone. B: In Methyl thiazolyl tetrazolium (MTT) assay, LNCaP cells co-cultured with CAFs grew faster than those co-cultured with NPFs (n=3, **p<0.01). C: In invasion assay, the invasion ratio was significantly higher for LNCaP (CAFs) cells than for LNCaP (NPFs) cells (n=3, *p<0.05). D: LNCaP+CAFs formed larger tumors in nude mice than LNCaP+NPFs (n=5, *p<0.05).*

of normal fibroblasts (16), bone marrow-derived mesenchymal stem cells (17), or epithelial to mesenchymal transition (EMT) (18). CAFs are dominant components in PCa stroma (5, 6), and were reported to promote epithelial proliferation and invasion (19), regulate the deposition of extracellular matrix (20, 21), mediate inflammation and angiogenesis (22), and induce EMT and stemness of cancer cells (23).

Most previous studies used primary CAFs without immortalization. Non-immortalized CAFs can only be subcultured for 6-8 passages and may not produce enough cells for further study or for repeat experiments. Furthermore, different sets of primary cells from different PCa tissues may influence the consistency of experimental results. In the present study, we immortalized CAFs and paired NPFs from the same patient by using SV40-T, then investigated the contrasting effects of CAFs and NPFs on epithelial cells.

The morphology of immortalized CAFs and NPFs were similar with elongated or stellated shapes. The CAFs were double positive for vimentin and SMA, which was consistent with previous reports (13). However, the SMA expression level was very weak in NPFs, which means the CAFs were myofibroblasts, but the NPFs were not. The CAFs also grew faster than NPFs.

For the *in vitro* malignant transformation of benign prostate epithelial BPH-1 cells, we co-cultured the BPH-1 with CAFs or NPFs, producing BPH-1(CAFs) and BPH-1(NPFs) cells. The BPH-1(CAFs) cells formed colonies in soft agar, but the BPH-1 (NPFs) cells did not. Since anchor-independent growth is a property of malignant cells, the BPH-1 were transformed *in vitro* by CAFs, but not by NPFs. We then recombined the BPH-1 cells with CAFs and NPFs and inoculated them in the renal capsules of nude mice for *in vivo* transformation. Similarly, the BPH-1 with CAFs formed tumors, but the BPH-1 with NPFs did not. This confirms that CAFs promote the carcinogenesis of prostate epithelial cells.

To study the effect of CAFs and NPFs on the proliferation and invasion of PCa tumors cells, we co-cultured LNCaP cells with CAFs or NPFs in transwell

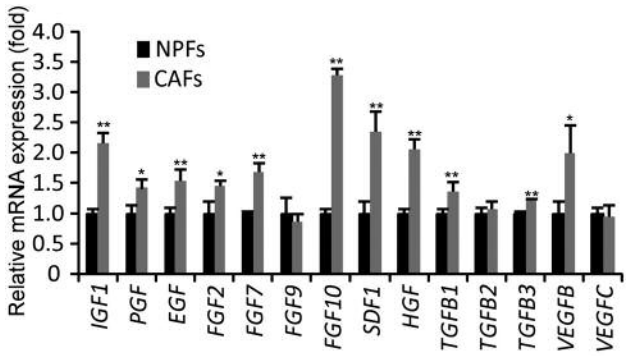

*Figure 5. The mRNA expression level of multiple growth factors secreted by cancer-associated fibroblasts (CAFs) and normal prostate fibroblasts (NPFs). The expression levels of insulin-like growth factor 1 (IGF1), placental growth factor (PGF), epidermal growth factor (EGF), fibroblast growth factor (FGF) 2, FGF7, FGF10, stromal cell-derived factor 1 (SDF1), hepatocyte growth factor (HGF), transforming growth factor β1 (TGFB1), transforming growth factor β3 (TGFB3), and vascular endothelial growth factor B (VEGFB) were higher in CAFs than those in NPFs (n=3, *p<0.05, **p<0.01). The expression levels of FGF9, TGFB2, and VEGFC did not significantly differ between cell types.*

plates with serum free medium. The LNCaP cells co-cultured with CAFs grew faster, and invaded more than the cells co-cultured with NPFs. In the subcutaneous tumor model, the tumors from LNCaP with CAFs were much bigger than those from LNCaP with NPFs. In summary, the CAFs were more powerful in promoting the proliferation of and invasion by PCa cells. To identify potential mechanism(s) we found that CAFs secreted higher levels of growth factors than NPFs. Growth factors are important for regulating a variety of cellular processes, including tumor growth, differentiation, invasion, and blood vessel differentiation.

Overall, we successfully cultured and immortalized CAFs and NPFs from the same patient, and found the CAFs secreted more growth factors, and were more active in promoting PCa carcinogenesis, proliferation and invasion. Targeting CAFs might be a potential option for PCa treatment. The immortalized CAFs and NPFs also provide good experimental tools for further PCa studies.

Conflicts of Interest

The Authors declare there are no conflicts of interest in regard to this study.

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