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Pluripotent Stem Cell within the Prostate could be Responsible for Benign Prostate Hyperplasia in Human

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Abstract

Aim: Abnormal prostate growth is the most prevalent pathological sign in aged human males, reflected by high incidence of Benign Prostatic Hyperplasia (BPH) and Prostate Cancer (PCa). The successful isolation and cultivation of prostate cells, is a prerequisite need for establishing a model cell line for understanding the pathogenesis, unique biological properties and also various evidences suggest the role of stem cells in the pathogenesis of these conditions for a therapeutic point of view.

Methods and results: Here we isolated a candidate pluripotent stem cell population from BPH patients underwent TURP which include the isolation of an enriched population of prostate stem cells through cell culture techniques and the cultivation of prostate stem cells *in vitro* and characterization of these cells and their stem potential, including *in-vivo* teratoma generation. Cytogenetic analysis by G-banding assay demonstrated an aneuploid karyotype with a model chromosome number of 60 and normal Y chromosome. Characterization of isolated cells showed the presence of ONS pluripotency stem cell markers. Beside this these cells were also found positive for stem cell surface markers such as CD49b, CD44, CD117, CD34 and prostatic tissue specific markers like p63 and Androgen Receptor. *In-vitro* differentiation of the cells demonstrated formation of a tri-germinal layer into ectodermal, endodermal and mesodermal cell lineages with defined medium conditions and *In-vivo* teratoma formation in excised tumor in Balb/c mouse.

Conclusion: we report here isolation, establishment and characterization of human prostate-derived pluripotent stem cell line. The cell line eventually serves as a potential tool for studies in prostate adult stem cell research, understanding etiopathophysiology and the regulation of BPH and PCa.

Introduction

The prostate is a hormonally regulated organ whose growth accelerates at sexual maturity due to androgen actions on both stromal and epithelial cells. In men over the age of 40-50 years, prostate gland represents a major medical problem in the form of benign prostate hyperplasia (BPH) and prostate cancer (Pca). Epidemiological data from several studies indicated that both diseases are becoming increasingly prevalent worldwide [1,2]. Unavailability of normal/ benign prostate cell lines and suitable animal model made these attributes difficult to study in vitro and in vivo. At histological level, human prostate contains mainly two types of cells, epithelial and stromal cells. The stromal to epithelial ratio in normal prostate of human is 2:1 [3,4]. The epithelial cell layer is composed of four differentiated cell types known as basal, secretory luminal, neuroendocrine (NE), and transit-amplifying (TA) cells that are identified by their morphology, location, and distinct marker expression. The basal cells form a layer of flattened to cuboidal shaped cells above the basement membrane and express p63 (a homolog of the tumor suppressor gene p53), Bc1-2 (an anti-apoptotic factor), Cluster designation (CD) 44, hepatocyte growth factor (HGF), and the high molecular weight cytokeratins (CK) 5 and 14.The expression of androgen receptor (AR) is low or undetectable in the basal cells, which makes the basal cells independent of androgens for their survival [5-7].

Both human and animal studies have shown that stromal cells are essential for functional and morphological differentiation of prostatic epithelium. It has been hypothesized that the basal layer is the proliferative compartment of the prostate, containing a stem cell population, which can differentiate into secretory epithelium and TA cells. Prostatic stem cells are present within the epithelium and are capable of regenerating the adult organ [8]. Several investigations based on stem cell models have elegantly defined role of stem cells in cellular turnover and morphology in normal human prostate [9]. To further support the role of basal stem cells in prostate development, an experiment on p63 null mice was performed and the resultant progeny of these animals were born devoid of prostate gland [10-12]. As the stem cells are key target for mutagenic changes and tumurogenesis in human prostate, an urge arises to understand more about their status in normal and disease prostate tissue and the cellular and molecular mechanism of BPH pathogenesis.

The concept of stem and progenitor cells with the capacity for self-renewal and multilineage differentiation has been important to understand the molecular mechanisms of normal development and functional homeostasis [6,13]. This is also very important to

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understand how tissues are remodeled during inflammatory repair, or in carcinogenesis resulting from oxidative stress, inflammation, genomic and metabolic insults [14,15]. However, at a practical level there have been few human cell lines available that accurately recapitulate prostatic development and that can be used to examine these concepts. To pursue studies relevant to normal human prostate biology with associated disorders as a starting point for studies on human disease, there is an urgent need for human prostate cell lines that show phenotypes that match human tissue samples. There are several non-tumorigenic immortalized human prostate epithelial (HPrE) cell lines have been established using viral SV-40Tag or E6/E7 infection including BPH-1 [16], and RWPE-1 [17], none of these accurately recapitulate normal human prostatic growth and function.

In light of this we made an attempt to isolate and establish a candidate population of Human prostatic stem/progenitor cells from BPH patients (underwent TURP) that expresses pluripotency markers. Characterization of isolated cells showed the presence of pluripotency stem cell markers like Oct 3/4, Nanog and Sox-2 by mRNA expression, western blotting and flowcytometery. We further assessed the expression level of stem cell surface markers to identify normal Prostate stem cells (PSCs) interestingly, these cells were found positive for prostate stem cell markers such as CD49b, CD44, CD117, CD34, p63 and prostatic tissue specific marker like Androgen Receptor. Upon the introduction to specific culture condition isolated prostate cells can differentiate into adipocyte, osteocyte and chondrocyte (mesodermal origin), islet formation (endodermal origin) and neuronal differentiation (ectodermal origin) cell lineages in *In-vitro* and *In-vivo* teratoma formation in *Balb/c* mouse with three germ layers. Isolated prostate cells could provide an ideal source of pluripotent-like stem cells with the potential to have a critical impact on regenerative medicine.

Materials and Methods

Prostate samples

Prostatic tissue was obtained from patients (average age of 70 years; range 55-75 years) who underwent TURP, patient detailed demographic and anthropometric data collected in structured Questionnaire with consent and ethical approval. Benign prostatic hyperplasia (BPH) histology was confirmed by a surgical pathologist and contained no adenocarcinoma of the prostate.

Chemicals

All primary antibodies and cell culture media (Anti-Androgen Receptor cat no A9853, Anti-Vimentin cat no. C9080, Anti Ki-67 cat no. P6834) and FACS antibodies (Anti-Oct3/4, Sox-2 and Nanog) purchased from Sigma-Aldrich, USA and BD Biosciences,USA respectively. RT-PCR reagents from fermentas, Germany. All the reagents were extra pure and of cell culture and molecular biology grade.

Isolation of prostate cells from TURP samples

Fresh TURP tissue obtained from human prostate surgical specimens. Prostate tissue samples were minced into small pieces and digested with Collagenase type I enzyme for 1 hr at 37°C in a shaking incubator at 110 rpm followed by grown in DMEM medium with 10% FBS as previously described [18].

Cell growth kinetics

Fully confluent prostatic cells were trypsinized with 0.1% Trypsin EDTA solution and counted under an inverted phase contrast microscope (Nikon TE2000, Japan). 5×10^4 cells were seeded into each well of 24-well plates for growth curve studies. Cells were eventually trypsinized and counted at different time points (0 h, 24 h, 48 h, 72 h, 96 h, 120 h, 144 h and 168 h). Cell counts were then plotted versus time to demonstrate the growth curve of the cells. Doubling time of the isolated prostate cells were determined using the algorithm Ln (N_t-N_0) Ln (t), where N_t and N_0 were numbered of cells at the final time point and at the initial seeding point respectively, and t was a time period in hours for which cell counts were recorded.

Karyotyping

To study the chromosomal stability isolated human BPH cells (passage7 and 14) were treated with Colcemid (Gibco 15 210-057), trypsinized, resuspended in 75-mM KCl hypotonic solution, fixed in MeOH/acetic Acid (3:1) and stained for metaphase spreads using a standard G-banding protocol technique [19]. For each sample, at least 20 metaphase spreads were examined, in which there was minimal chromosome overlaps, and long chromosome length, little or no cytoplasm, and high banding resolution were selected for detailed analysis.

RNA extraction and semi quantitative Reverse Transcriptase PCR (RT-PCR)

Total RNA was isolated from cells using TRizol Reagent (Sigma Aldrich, USA) extraction following the manufacturer's instructions and immediately treated with DNase l (fermentas). 5 μg of total RNA was reverse transcribed into first strand cDNA using random primers and subjected to PCR amplification of various stem cell genes. One μl of cDNA products was used to amplify genes using a 2X master mix [Sigma Aldrich, USA], containing 1.5 μl Taq Polymerase, 2 mM dNTP, 10X Tris, glycerol reaction Buffer, 25 mM MgCl2, and 20 pM appropriate forward and reverse primers for each gene. GAPDH served as an internal control (Table 1 for primers sequence and annealing temperature). PCR products were separated on a 10% polyacrylamide gels [Sigma Aldrich, USA], visualized and images were captured by Cambridge UV tech, Chemi-doc instrument.

Western blotting

Western blotting of isolated prostate cells performed as previously described [3]. Isolated prostate cells were lysed with urea containing lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas INC.). Total Protein estimation was carried out using

Gene	Primer sequence	Annealing Temp.	Product size
Oct ¾ (NM_002701)	Forward-5'AGCTGGAGAAGGAGAAGCTGG-3' Reverse-5'-TCGGACCACATCCTTCTCGAG-3'	63.5°C	458 bp
Sox-2 (NM003106)	Forward-5'-CACCTACAGCATGTCCTACTC-3' Reverse-5'-CATGCTGTTTCTTACTCTCCTC-3'	60°C	384 bp
Nanog (NM_024865)	Forward-5'GCAAACAACCCACTTCTGC-3' Reverse- 5'AGGCCTTCTGCGTCACAC-3'	55.5°C	287 bp

Table 1: Primers Sequence and annealing temperature.

Bradford reagent according to manufacturer's suggestions (BIO-RAD). Cell lysates (40 µg) were separated on Polyacrylamide gel using the Mini-tetra-cell electrophoresis system (BIO-RAD) and transferred onto a nitrocellulose blotting membrane (Millipore) Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in PBS)s. Primary antibodies were added to blots and incubated overnight at 4°C. Anti-rabbit and Anti-mouse IgG conjugated with HRP were used to develop the blots using Ultra-sensitive enhanced chemiluminiscence reagent (Millipore, USA). And imaged by Chemi-doc instrument Cambridge UV tech, UK.

FACS analysis

Cells were trypsinized, centrifuged, and one million cells were resuspended in 100 µl of wash buffer [PBS containing 10% serum], washed twice with Phosphate buffered saline [PBS] containing 1% bovine serum albumin, and then incubated with primary antibody at 4°C for 1 h. Cells were than labelled with 100 µl of secondary antibody for counter staining, and incubated for an additional 40 min at 4°C [20]. Data were recorded and observations analysed using BD FACS Aria III(BD, USA) and flowjo software respectively.

Immunocytochemistry

Adherent cells were washed with PBS with 2% FBS and then with PBS, the cells were fixed with 2% PFA solution followed by PBS washing and triton-X100 treatment using standard protocol and incubated with primary monoclonal antibodies overnight at 4°C followed by 1 hr incubation with fluorochrome tagged secondary antibodies at room temperature. For negative controls, the primary antibodies were omitted. The expressions of antigens in cells were assessed by immunofluoresense method. Images from Carl Zeiss LSM-710 confocal microscope (Carl Zeiss, Germany) were recorded.

In-vitro Differentiation

Ectodermal lineage

Neuronal differentiation: Isolated prostate cells were plated in six-well plat as described above in the presence of neuro-basal medium (Invitrogen) with N2 supplements and 2 mM glutamate for 10 days. The culture medium was replaced every 4th day. Parallel control cells were cultured without neuronal differentiation medium.

Mesodermal lineage

Osteocyte differentiation: Isolated prostate cells were trypsinized, washed with 10 mM PBS, pH 7.2, and resuspended in DMEM high glucose with 10% FBS medium. Cells were plated into six-well plate at 10^5 cells/well in the presence of osteocyte reagents (20 mM B-glycerol phosphate, 50 ug/ml ascorbic acid and 10 mM dexamethasone) for 10 days. The culture medium was replaced every 3rd day. Parallel control cells were cultured without osteocyte reagents.

Adipocyte differentiation: Isolated prostate cells were plated in six-well plate as described above. Adipogenesis was induced by treatment with IBMax (10 mg/ml),10 mM dexamethasone and 10 mg/l insulin for 8 days. The culture medium was replaced every 3rd day. Parallel control cells cultured without adipocyte differentiation medium.

Chondrocyte differentiation: Isolated prostate cells cells were plated in six-well plate as described above. Chondrocyte differentiation was induced by treatment with 10 mM dexamethasone and 10 mg/l insulin for 20 days. The culture medium was replaced every 5th day.

Parallel control cells were cultured without chondrocyte differentiation medium.

Endodermal lineage

Islet differentiation: Isolated prostate cells were plated in six-well plated as described above in the presence of islet differentiation serum free RPMI1640 medium with 10 ng/l activin-A and 10 mg/l insulin for 10 days. The culture medium was replenished every alternate day. Parallel control cells cultured without islet differentiation medium.

In-vivo Differentiation

Teratoma formation

For each graft, approximately 0.2 million isolated prostate cells, washed and resuspended in 300 µl DMEM complete medium, and transplanted subcutaneously (intraperitoneal body cavity) on left side of six Balb/c mice (maintained in MSU in-house animal house facility) with 1.5% melted agarose using 23G needle [21,22]. Right side of the same animal was used for control or placebo i.e. only agarose plugs were injected in that site. The experiment was approved by the Institute Animal Ethical Committee (CEPSC Reg. No. 938/a/06/CPCSEA). After 3 weeks of transplantation, mice were sacrificed. Visible tumours, were dissected out and fixed overnight with 4% PFA solution. The tissues were then paraffin embedded, sectioned, stained with H&E, and were examined for the presence of cells representatives of all three germ layers produced by transplanted cells [23].

Results

Prostate cells isolation and characterization

Surgically removed TURP prostate tissue samples were digested with collagenase type I enzyme for 1 hr at 37°C. After enzymatic digestion microscopic observation of cultured prostatic cell population showed characteristic fibroblastic and epitheliod shaped cells (Figures 1B and 1C). To isolate epithelial cells from the mix population, epithelial cell patches were picked up using sterile filter paper discs soaked in trypsin and transferred the cells into DMEM medium with 10% FBS for enrichment (Figure 1A). To further confirm the nature of isolated cells, immunocytochemistry was performed with different cell markers (Figure 2A).

Growth curve and kinetics

Cells were plated in 24-well plates and used for determining the population doubling potential, progression and proliferative activity. Cumulative population doublings were calculated by considering initial number of cells seeded at 0 hrs and number of cells harvested at each destined time point respectively without passaging. These observations provide a theoretical growth curve that is directly proportional to the cell number. With the help of the curve generated, doubling time was found to be 26 ± 1.3 hrs.

Cytogenetic analysis

Cytogenetic analysis by G-banding assay demonstrated an aneuploid karyotype with a model chromosome number of 60 (range 58 to 62, n=20) with 4 to 5 marker chromosomes, which were structurally rearranged and the Y chromosome was found to be normal (Figure 1D).
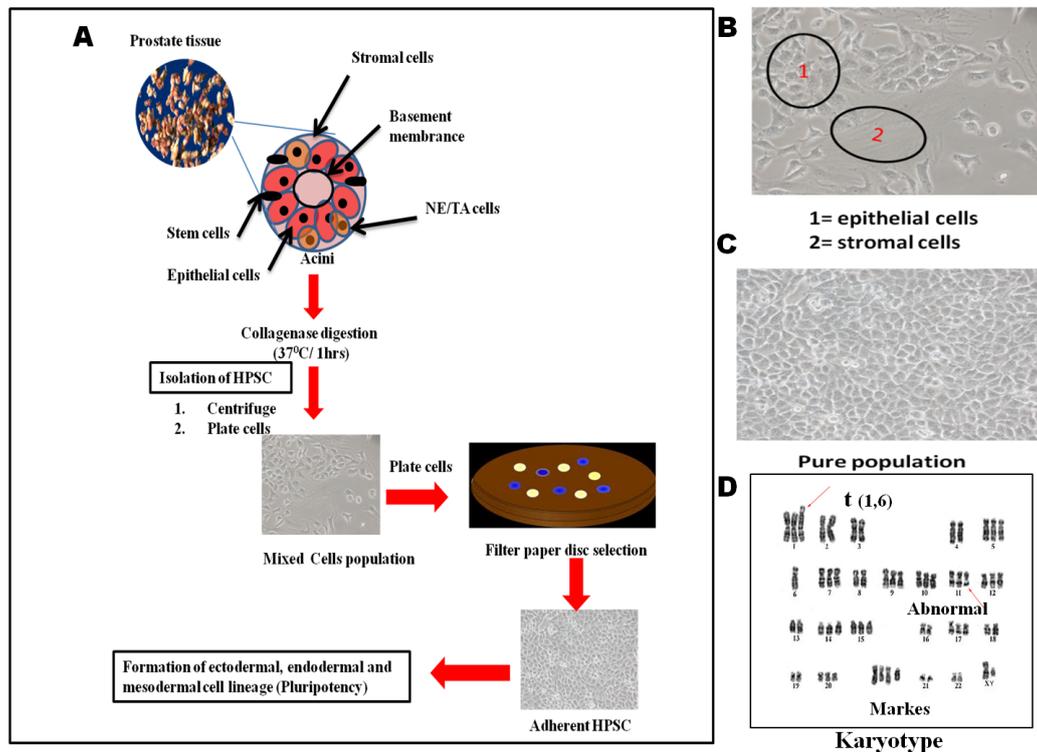
Figure 1: Isolation and cytogenetic characterization of isolated prostate cells from BPH patient. (A) Schematic of prostatic cells isolation from TURP tissue. Cells were obtained after 1 hrs, incubation with collagenase type-I enzyme in DMEM medium without FBS at 37°C. (B and C) Morphological view of isolated cells from mixed population using sterile filter paper discs soaked in trypsin and cultured in DMEM medium with FBS at 37°C under 5% Co_2 (D) karyotype analysis of isolated prostate cells. Cells showed aneuploidy with 4 to 5 marker chromosomes and translocation of 6th chromosome to 1st.

Stem cell characterization

To identify the stem cell properties of isolated cell population, immunocytochemistry for Nestin, E-cadherin, CK19, AR, Vimentin and Ki-67 and FACS analysis for stem cells specific markers such as CD49b, CD44, CD117, CD34 and p63 were performed. Fluorescent microscopic analysis showed cells were positive for stem cell markers (Figure 2A). FACS analysis showed that 3.04% cells were positive for CD49b, 95.3% cells were positive for CD44, 20% cells were positive for CD117, 16.5% cells were positive for CD34 and 96.3% cells were positive for p63 (Figure 2B).

Pluripotency features

To elucidate whether the cells possess pluripotent characteristics, cells were further analyzed for the ONS markers by flow cytometry using BD FACS antibodies. Flowcytometry results showed that 1.11% cells were positive for Oct3/4 a protein involved in the self-renewal of human ES cells; Nanog, another transcription factor involved in self-renewal of human ES were 18% in cells and 13.1 % cells were positive for Sox-2,a transcription factor that control genes involved in embryonic development (Figure 3C). RT PCR analysis also showed higher expression of all the three ONS genes, GAPDH served as an internal control (Figure 3A). Further western blot was performed to confirm the expression of ONS at protein level. Western blot analysis showed clear bands for ONS proteins at 34, 117 and 40 kD, respectively, beta actin served as an endogenous control (Figure 3B).

In vitro differentiation

Ectodermal lineage differentiation: To investigate the potential

differentiation into ectodermal lineage, isolated prostate cells were cultured for 10 days in neuronal differentiating medium. Cells were positive for MAP-2, a marker for mature neurons (Figure 4).

Mesodermal lineage differentiation: To determine the potential of isolated prostate cells to differentiate into cells of mesodermal lineages: adipocytes, chondrocytes and osteocytes where cells were grown as adherent cells in the respective differentiation culture medium. Differentiation of the prostate cells into a mesodermal lineage was determined by immunocytochemistry and specific staining. Mesodermal markers included PPAR-Y, a marker for adipocyte, CD44, marker for osteocytes and CD 90 and CD44 combined marker for chondrocytes. Futher these cells also stained with Oil Red O, Alizarin red S and Alcian blue stain for Adipocytes, osteocytes and chondrocytes respectively (Figure 5(i-iii)).

Endodermal lineage differentiation: Differentiation of isolated prostate cells to an endodermal lineage (islet differentiation) was detected in prostate cells cultured in defined medium for 10 days. Cells were positive for glucagon and C-Peptide by immunocytochemistry (Figure 6).

In vivo differentiation:

Teratoma formation: To further validate the phenotypic properties of isolated prostate cells in terms of pluripotency, in-vivo experiment for teratoma formation in balb/c mice was carried out, since teratomas formation considered as gold standard technique to prove pluripotency. All of the 6 mice developed evident teratomas (left side) wherein skin bulges were bigger in size than that of agarose plugs
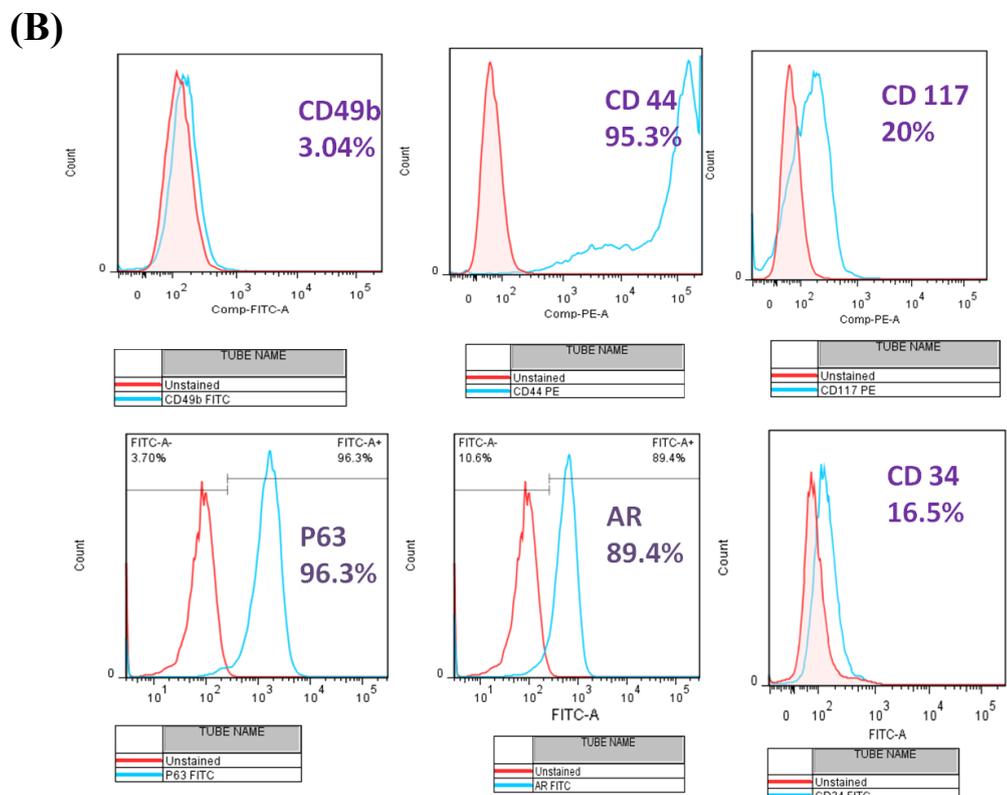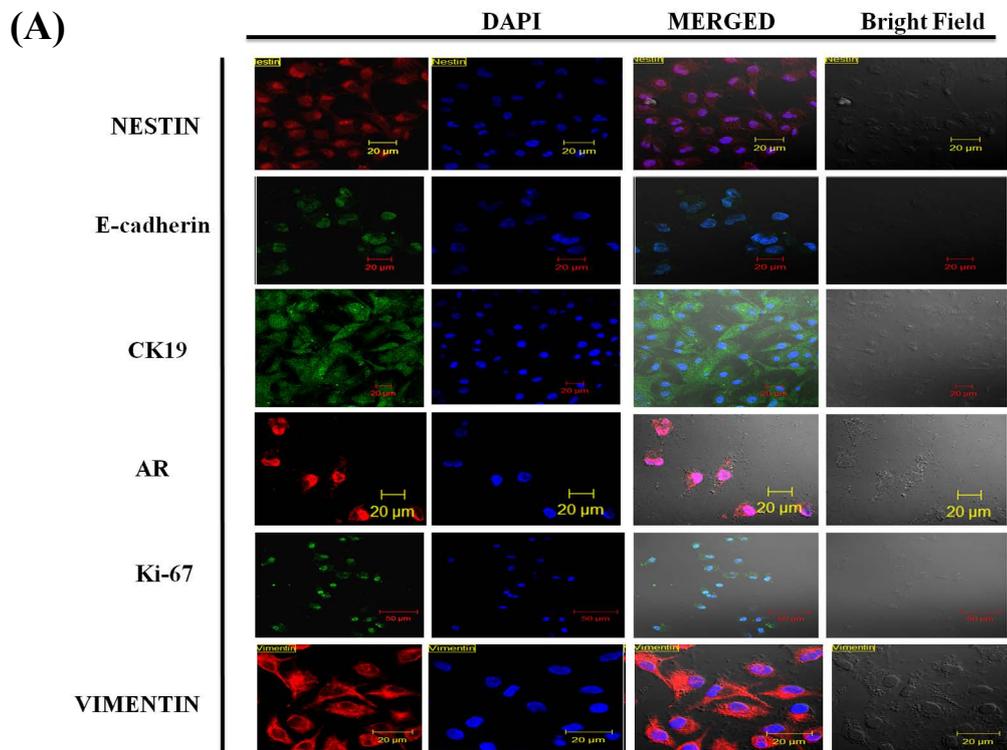
Figure 2: Characterization of isolated prostate cells. (A) Immunofluorescence staining of BPH prostate cells for Nestin, E-cadherin, Ck19, Androgen Receptor, Ki-67 and Vimentin (first panel from left side). The second panel from left side shows DAPI staining and the second panel form right side shows the merged images. Bars represent 20 μm (B) FACS analysis of cell surface markers demonstrate 3.04% CD49b, 95.3% CD44, 20%CD117, 96.3% p63, 89.4%AR and 16.5% CD34 positive cells in isolated prostate cell
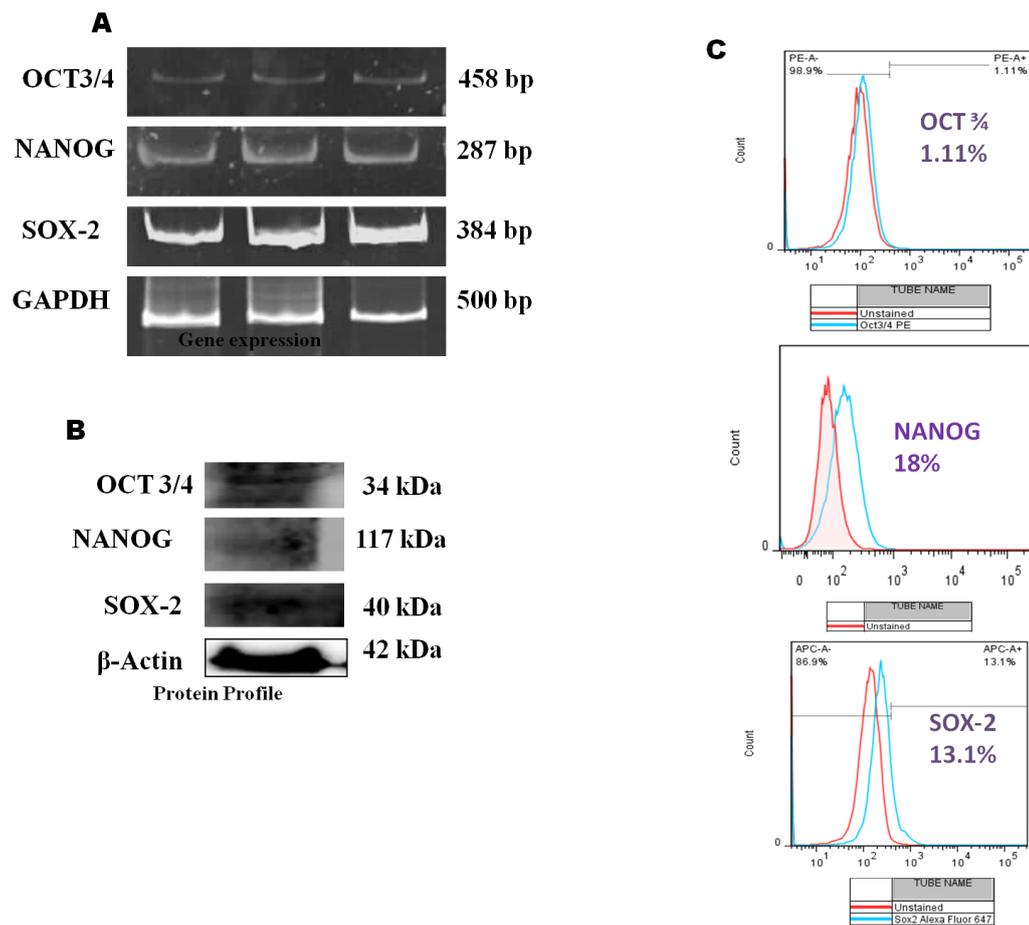
Figure 3: Isolated prostate cells express pluripotent stem cell markers. (A and B) At the genomic and protein level. Gel-electrophoresis of RT-PCR products and protein profile of pluripotency specific genes (Oct 3/4., Sox-2 and Nanog) showed expression. (C) Flow cytometry data showed presence of ONS markers in isolated cells.
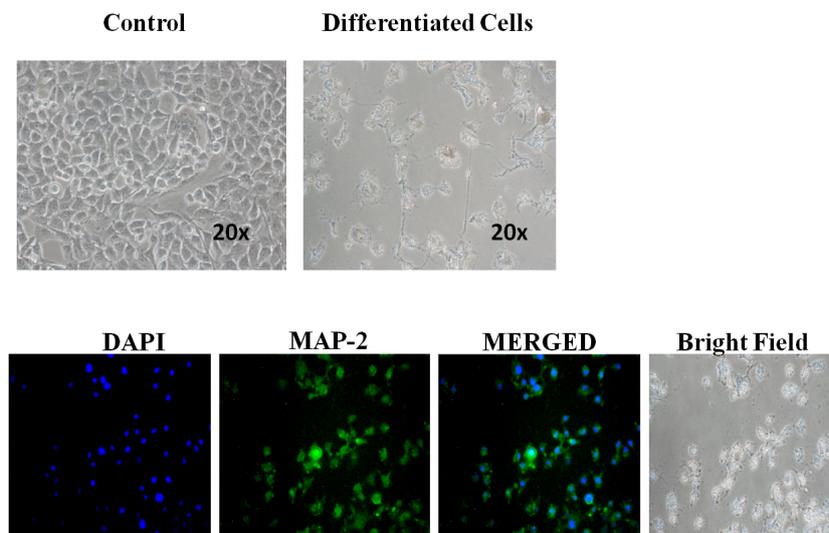

Figure 4: Isolated prostate cells can differentiate to ectodermal cell lineages. (A) Without and (B) with neural differentiation reagents (neuro-basal medium with N2 supplements and 2 mM glutamate) for 10 days. (A) Control. (B) Neural like cells. (C) Neural-like cells were detected by Immunofluorescence staining using MAP-2 antibody. Nuclei were stained with DAPI.
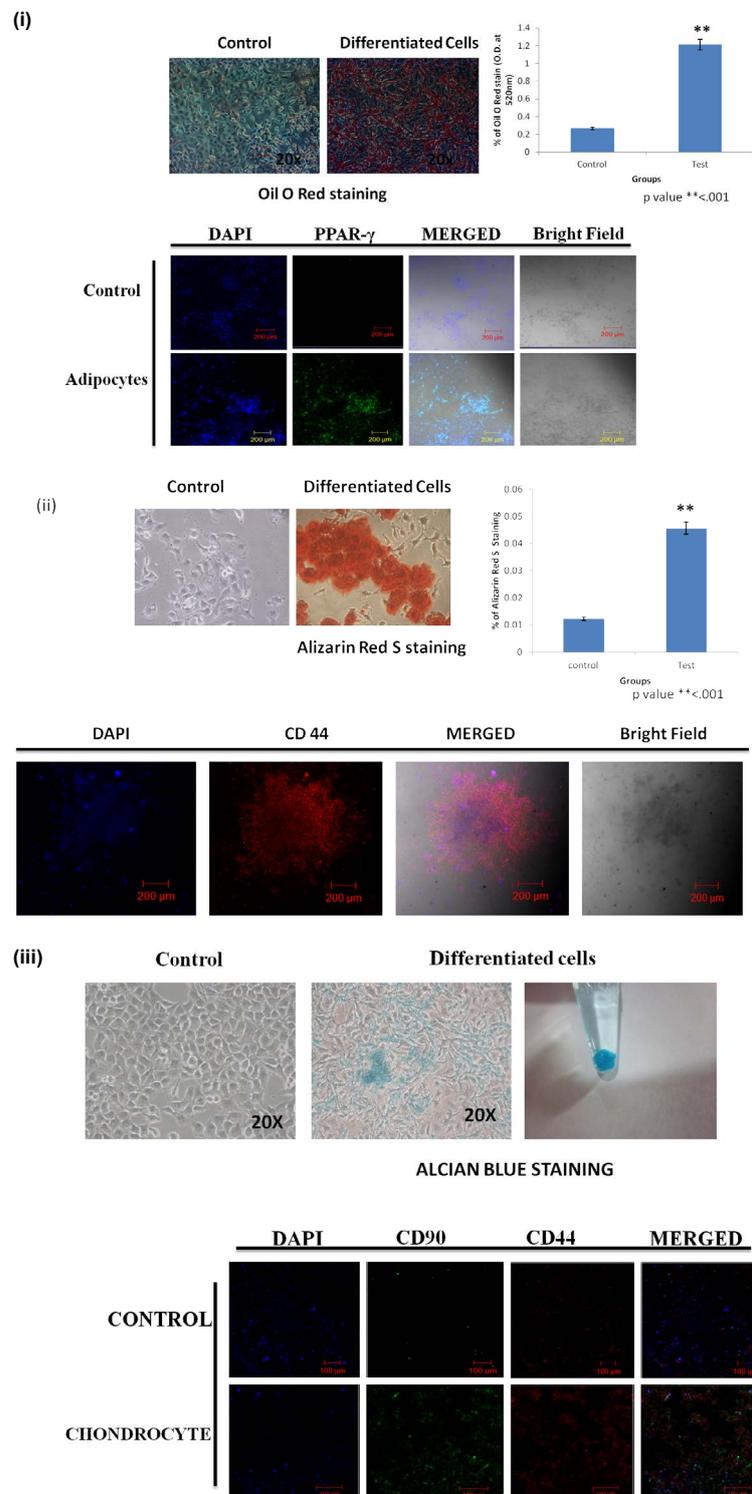
Figure 5: Isolated prostate cells can differentiate to mesodermal cell lineages. (i) Oil red O staining of isolated prostate cells after culture (A) without and (B) with adipocytic differentiation reagents (IBMax 10 mg/ml, 10 mM dexamethasone and 10 mg/l insulin) for 8 days. (A) Control cultures showed Oil Red O negative cells; (B) Positively staining adipocytes. (C) histogram showing the significant oil red O staining in differentiated cells. (D) Immunofluorescence staining of PPAR-γ for differentiated adipocytes. Nuclei were stained with DAPI. (ii) Alizrain red S staining of isolated prostate cells after culture (2A) without and (2B) with osteocyte differentiation reagents (20 mM glycerol phosphate, 50 µg/ml ascorbic acid, 10 mM dexamethasone and 10 mg/l insulin) for 10 days. (2A) Control cultures showed Alizarin red S negative cells; (2B) Positively staining osteocytes. (2C) histogram showing the significant Alizarin red S staining in differentiated cells. (2D) Immunofluorescence staining of CD44 for differentiated osteocytes. Nuclei were stained with DAPI. (iii) Alcian blue staining of isolated prostate cells after culture (A) without and (B) with chondrocyte differentiation reagents (10 mM dexamethasone and 10 mg/l insulin) for 20 days. (A) Control cultures showed Alcian blue negative cells; (B) positively staining chondrocytes. (C) Immunofluorescence staining of CD90 and CD44 for differentiated chondrocytes. Nuclei were stained with DAPI.
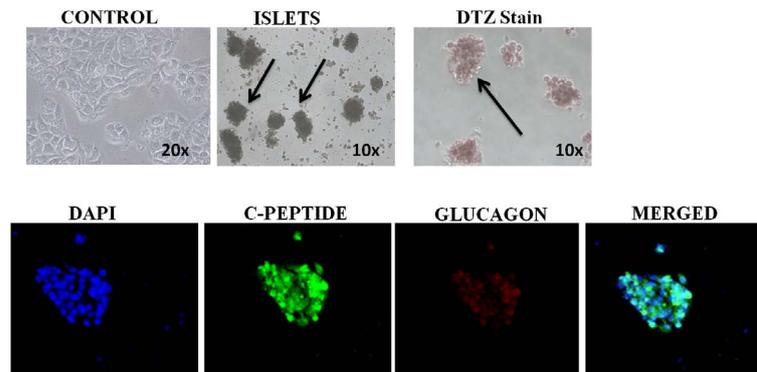
Figure 6: Isolated prostate cells can differentiate to endodermal cell lineages. (A) Without and (B) with islet differentiation reagents (serum free RPMI 1640 medium with 10 ng/l activin-A and 10 mg/l insulin) for 10 days. (A) Control. (B) Islet like cell cluster. (C) DTZ staining. (D) islet-like cell cluster were detected by immunofluorescence staining using C-peptide and glucagon antibodies. Nuclei were stained with DAPI.
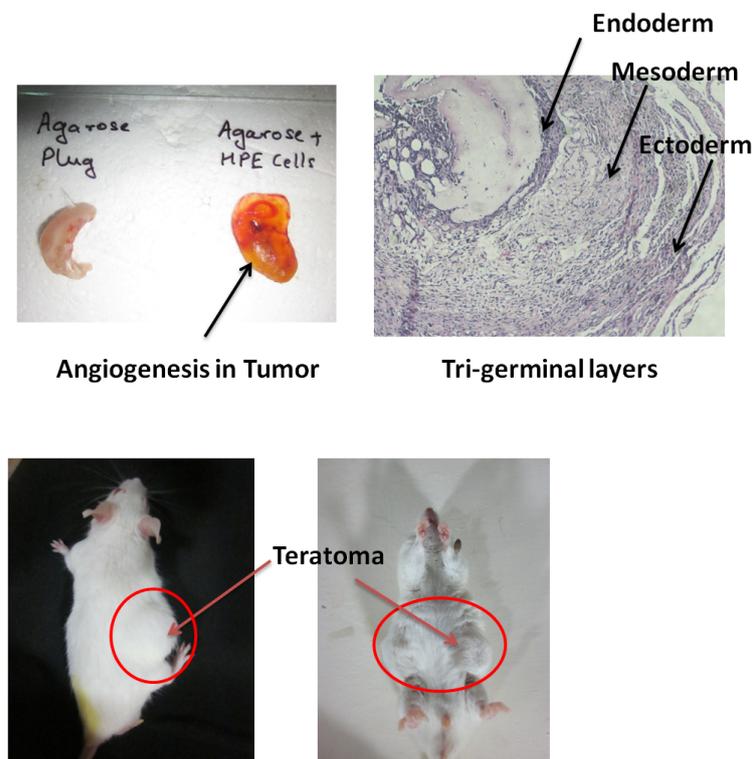

Figure 7: Teratomas formation capability of isolated prostate cells with tri-germinal layer. 0.2 million cells with agarose plug were transplanted in left side body cavity of Balb/c mice. Right side only agarose plug transplanted. (A) Angiogenesis in transplanted tumor. (B) H&E staining showing tri-germinal layer formation in excised tumor transplanted with prostate cells. (D) Visible teratomas in animals.

alone (right side). Also we noted that teratomas formed by isolated prostate cells transplanted with agarose plugs were significantly larger than those formed by agarose plugs alone. Imaging of teratoma sections demonstrated that major mass of tumor plugs were mainly derived from isolated prostate cells (Figure7A).

Histological studies confirmed *in-vivo* functionality and representation of tri-germinal layers in developed teratomas (Figure 7B). Since the cells were encapsulated in agarose and transplanted subcutaneously beneath the forearms and not directly in vasculature; the chances of spreading were minimal to begin with.

The isolation and characterization of human prostate stem cells from BPH patient (underwent TURP) have yielded many interesting findings that these prostate cells possess:

1. Pluripotency stem cell markers.

2. Strong proliferative potential with the ability to differentiate into ectodermal, mesodermal and endodermal lineages and teratoma formation with three germ layers. These cell preparations may serve as a potential tool for studies in prostate adult stem cell research and the regulation of Benign Prostatic Hyperplasia.

Discussion

BPH is a slow progressive enlargement of the prostate gland which can lead to lower urinary tract symptoms (LUTS) in elderly men. It is characterized by hyperplasia of epithelial and stromal cells in the transition zone of the prostate gland, which can be observed histopathologically [24]. Stem cells in the human prostate have been identified and isolated using the cell surface markers such as CD44 [25], integrin α2β1 [26], CD133 (Prominin-1) [27] which, are believed to be responsible for the development and progression of proliferative disorders of the prostate such as prostate cancer and benign prostate hyperplasia [9,28-30]. Based on high expression of α2β1 integrin, Collins and colleagues identified PSCs in the basal layer and showed that the α2β1^{high} integrin cells represent ~1% of basal cell population in the human prostate [26].

A very recent finding has demonstrated a relatively high expression of stemness-associated genes, including Oct4A, Sox2, c-Myc, Nanog, and Klf4, in BPH as compared to normal prostate tissue [31]. However, role of ONS and other stem cell markers in hyperplastic prostatic epithelium remain to be established. In the present study, cells were isolated from human TURP (Trans Urethral Resection of Prostate) tissue excised from BPH patients. Stemness nature of isolated cells can be proved by 1. expression of stem cell marker genes, 2. *In vivo* teratoma formation, 3. and *In vitro* multiple-cell lineage differentiation. The expression levels of ONS markers of isolated prostate cells clearly prove pluripotent nature. These cells do possess high level of prostate stem cell markers like CD44 (95%),CD117(c-kit) (20%), p63 (96%), CD49b (3%) and Nestin. The expression levels of CD49b in the present study is high when compared to previous report that showed presence of just 1% of this marker [32]. The p63 a homolog of p53, is present in the basal epithelium of the prostate and in primary cell cultures from normal tissues and its expression is absent in prostate cancer [12,33]. Previous investigation revealed a role of p63 in stem cell functions [34]. In our study isolated cells showed approximately 96.3% p63 positive cells by flow cytometry, further supporting stemness characteristics of isolated prostate cells.

Leong et al. [35] identified CD117 (c-Kit, stem cell factor receptor) as a new marker of a rare adult mouse PSC population which showed self-renewal and full differentiation potential characteristics of stem cells. The CD117(+) with CD44(+) phenotype regenerated functional prostate after transplantation *in vivo*. Moreover, CD117(+) PSCs showed long-term self renewal capacity after serial isolation and transplantation *in vivo*. CD117 expression was predominantly localized to the proximal region of the mouse prostate and was upregulated after castration-induced prostate involution, consistent with prostate stem cell identity and function [35]. CD44 was used as the marker to identify basal stem cells with tissue-regeneration abilities [36]. Interestingly, isolated cells showed both CD117 and CD44 i.e 20% and 95% of these marker respectively, which eventually supports the above fact.

The presence of all the three germ layers (Ecto, Meso and Endoderm) in *in vivo* study clearly demarcates the ability of these cells to form teratomas similar to that of embryonic stem cells. Moreover, results from *in vitro* study suggest that the isolated cells are capable of differentiating into multiple-cell lineages ie. Osteocyte, adipocyte and chondrocyte (Meso-dermal origin), Neural differentiation (Ectodermal origin) and Islet like cell formation (Endo-dermal origin) further providing evidence of pluripotent nature.

It has been reported that stromal to epithelial ratio is altered in BPH, where the ratio increases from 2:1 in normal glands to 5:1 in BPH [15], because the existence of adult stem cells in the prostate stromal compartment is speculated to expand the stroma in response to stimuli during the pathogenesis of BPH [37]. However, involvement of epithelial cells in the prostate development has been less well-understood. It has long been hypothesized and further supported by experiments that a stem/progenitor cell hierarchy exists within the prostate epithelium as well [6,15,38].

The presence of these high proliferative and plastic stem cells isolated from BPH patient in our investigation suggests that BPH could occur as a result of amelioration of stem cell properties that could ultimately give rise to a clonal expansion of specific cell population.

Further cytogenetic study of the isolated prostate cells has demonstrated an aneuploid DNA content and translocation of chromosome 6 to chromosome 1 in prostate. Earlier reports also showed deletion, translocation, inversion and mosaics on chromosomes 1, 7, 16 and Y in south Indian BPH patients and Chromosome 1 showed deletion and translocation in both PC and BPH Patients [39]. Chromosome 1 has a breakage-prone site, which has been reported to be sensitive to environmental clastogens and responsible for tumor development and progression [40-42].

Most interesting finding of our study is that the isolated pluripotent stem cells from BPH patients are expressing both basal(CD44, CD49b, p63 etc.) and secretory (AR,CD117,CK19 etc.) epithelial cell markers and capable to form teratomas when transplanted into balb/c mice along with three germ layers formation. Evidence has been shown that basal and secretory cells have the ability to self-renew [43]. Molecular mechanism and pathways involved in hyperplastic prostate differentiation, especially stem cell differentiation, are poorly understood due to the lack of suitable models. Hence, we made an attempt to develop cell line as referred in present study and cost effective animal model in our earlier study which can be used for understanding the prostate pathology. (Prajapati et al. [30] communicated).

Many methods have been used to establish cell lines using viral oncogenes, overexpression of human TERT or knockdown of specific proteins to inactivate regulatory key pathways, making the cells susceptible to genomic instability and malignant transformation [44-46] which were used to understand pathogenesis and effective therapy for the disease. In light of this, aim of the present investigation was to develop *in vitro* model system to study the pathogenesis of BPH and its potential for assessing therapeutics. In this study, we used method of serial passaging to establish immortalize cell line. The advantages of this approach include limiting genetic damage to key cell cycle checkpoints and allowing for the derivation of cells that are able to recapitulate key aspects of physiology.

This BPH stem/progenitor cell line with pluripotent stem cell characteristics provide the first *in vitro* model which can be used to enhance our understanding of human benign tumor development and provide a tool for testing diagnostic, treatment, and prevention strategies for BPH and cancer patients. Furthermore, this established cell line provides in-depth knowledge to study the role of stem cells, cancer stem cells and epithelial cell differentiation mechanism in disease progression. Because many epithelial cancers and benign tumors seem to arise from cancer stem cells and often exhibit similar characteristics, knowledge generated by the BPH epithelial stem/progenitor cell line will likely be applicable to other epithelial tumurogenesis.

Conclusion

One of the major strength of our study is that the pluripotent cells we obtained from human BPH form a connecting link between embryonic pluripotent stem cells and mesenchymal stem cells. This is evidenced by the expression of beautiful admixture of pluripotent (ONS expression, trilineage *in vitro* differentiation, teratoma formation) and mesenchymal markers (adipo, chondro, osteogenic differentiation and CD marker expression) exhibited by this pluripotent stem cell line we established. Multi-lineage differentiation characteristics of prostate cells can be exploited for stem cell treatment in patients suffering with other disease. While there are chromosomal alterations associated with with crisis and subsequent immortalization, these cells are behaviorally benign as assessed with histopathological and immunocytochemistry criteria. As such, this cell line represent potentially useful model to investigate mechanisms associated with both benign and malignant prostatic disorders and may open avenues for developing intervention therapies for prevention of BPH and cancer progression.

Acknowledgements

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