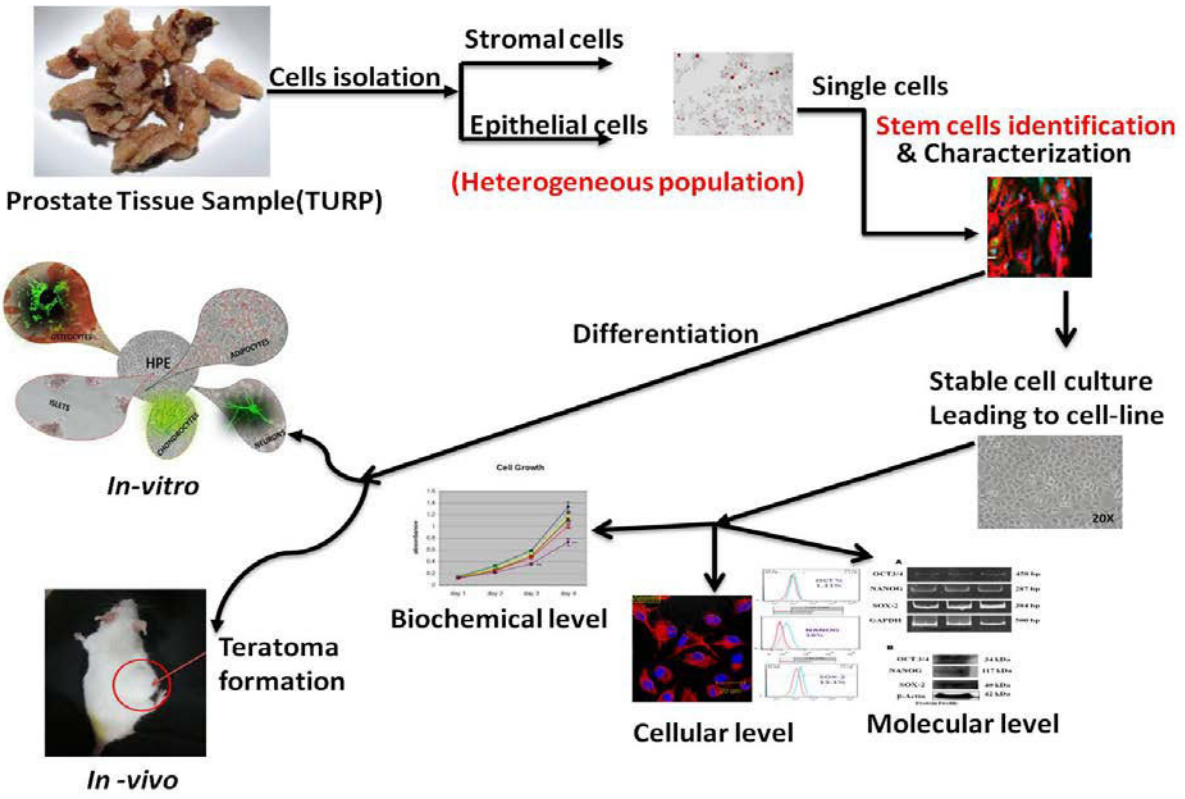
CHAPTER -4(A)

CHAPTER - 4(A)

Isolation of epithelial & stromal cells from Benign Prostate Hyperplasia patient's.

(a): Analysis of cellular and molecular status of both cell types and to understand the role of stem cell in benign prostate hyperplasia tissue.



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

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4(A).1 Introduction

The human prostate is composed of two cellular compartments: (1) an epithelial cell compartment, which includes the exocrine glands with their associated ductal structures, and (2) a surrounding connective tissue stroma. The stroma of the human prostate consists of a number of different cell types. The most abundant cell type in this compartment is the smooth muscle cell, which is derived from the mesenchyme of the embryonic urogenital sinus (UGS). Other cell types located in the stroma of the normal adult prostate include fibroblasts, nerves, endothelial cells and vascular smooth muscle cells. In addition to being the most abundant stromal cell type, the smooth muscle cell appears to be the most important cell type with respect to prostatic development and maintenance of homeostasis. In this regard, changes in the smooth muscle cell may be important in the evolution of prostatic enlargement and carcinogenesis.

Interactions between stromal and epithelium cells appear to be reciprocal in nature. Not only does the developing stroma induce epithelial development, but the developing epithelium also induces primitive mesenchymal cells to differentiate into smooth muscle (Cunha et al. 1992b). Urogenital sinus mesenchyme (UGM) grown in the absence of epithelium will not form smooth muscle. Thus, the development of prostatic smooth muscle *in vivo* requires the presence of both epithelium and appropriate androgenic stimulation. This ability of epithelium to induce the formation of visceral smooth muscle is not restricted to the prostate, but it appears to be a common feature of many organs including the gut, uterus and bladder (Cunha et al. 1989; Baskin et al. 1996).

Many studies have been attempted to identify the signalling molecules acting as paracrine mediators of stromal and epithelial interactions in the developing and adult prostate function. In most studies to date, growth-quiescent adult prostatic tissues have been utilized, and therefore the signalling molecules that have been identified may be involved in homeostasis rather than in carcinogenesis (Cunha et al. 1992a; Cunha 1994) and BPH condition. Currently, there is no detailed information of the spatial and temporal expression patterns of many of the growth factors found in the prostate. Considerations such as the cell of origin of a particular growth factor (epithelium, smooth muscle or fibroblast (stromal)), the precise localization of a growth factor or its receptor in relation to proximal, intermediate or distal ductal architecture, and the specific developmental stages when expression of a particular growth factor occurs have not been adequately examined. Molecular mechanism and pathways involved in hyperplastic

prostate differentiation, especially stem cell differentiation, are poorly understood due to the lack of availability of *in vitro* and *in vivo* models. In order to define better growth factor and cellular expression in the prostate for further study, it is important to isolate both cell types.

In this context, we have standardized isolation protocol for both human and rat prostate cells as a model system using modified method of Chaproniere R. *et al* 1986 with DMEM-F12 medium and collagenase type I enzyme digestion (Chaproniere and McKeehan 1986). Later we were successful to culture human prostate epithelial cells from BPH patients underwent TURP and used them for further investigations towards disease pathogenesis. Characterization of isolated cells were positive for mesenchymal markers vimentin, nestin, cluster designation CD117, CD34 and epithelial markers like p63, E-cadherin, Ki-67, CK19 and Androgen Receptor (AR) by immunocytochemistry, flowcytometry, western blotting and mRNA expression profile. We were also successful to isolate stromal cells. Immunocytochemistry staining of stromal cells showed positive for mesenchymal markers vimentin, nestin and AR.

To pursue studies relevant to normal human prostate biology with associated disorders, there is an urgent need for human prostate cell lines that show phenotypes similar to human tissue samples. To best of knowledge human BPH cell line has been established for the first time in the present study. Many efficient methods have been used to establish cell lines using viral oncogenes, overexpression of human TERT or knockdown of specific proteins, but results alterations to the cell cycle machinery, making the cells susceptible to genomic instability and malignant transformation (Gudjonsson et al. 2004; Bhatia et al. 2008; Wieser et al. 2008). There are several non-tumorigenic immortalized human prostate epithelial (HPrE) cell lines established using viral SV- 40Tag or E6/E7 infection including BPH-1(Hayward et al. 1995), and RWPE-1(Bello et al. 1997), none of these accurately recapitulate normal human prostatic growth and function. In this study, we present new human BPH epithelial cell line, with a self-renewing stem/progenitor population on the basis of expression of stem and basal cell markers in-vitro. Interestingly, the cell line showing both basal as well as secretory epithelial cellular markers expression (Prajapati et al. 2014a), which was previously characterized including p63, AR, and E-cadherin (Uzgare et al. 2004; Tokar et al. 2005; Prajapati et al. 2013)

The concept of stem/progenitor cells with the capacity for self-renewal and multi-lineage differentiation have been important to understand the molecular mechanisms of normal

development and functional homeostasis (Wang et al. 2001; Li et al. 2009). This is also very important to understand how tissues are remodelled during inflammatory repair, or in carcinogenesis resulting from genomic insult, oxidative stress, inflammation or metabolic insult (Isaacs 1987; Tsujimura et al. 2002). In BPH pathogenesis it has been believed that stem cells are playing a crucial role and their reawakening leads to proliferative disorder of prostate. According to stem cell theory, the stem cell population residing in the prostate gland is increased due to abnormal proliferation and apoptosis of stem cells, which may eventually contribute to BPH pathogenesis. Earlier, it was reported by Berry *et al.* that stem cell population is responsible for prostate gland maintenance (Berry et al. 1986). Changes in tissue consistency and cellular hyperplasia are accompanied by down regulation of apoptotic factors and increased level of anti-apoptotic factors that decrease the rate of prostatic cell death and thus, contributing to hype-proliferation of prostatic tissue (Kyprianou et al. 1996). 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 (Tsujimura et al. 2002). Because stromal hyper-proliferative activity is thought to promote the development of BPH, 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 (Lin et al. 2007).

In light of this we isolated and established a candidate population of prostatic stem/progenitor cells from BPH patients underwent TURP. Characterization of isolated cells showed presence of embryonic stem cell markers like Oct 3/4, Sox-2 and Nanog by mRNA expression, western blotting and flow cytometery. Further these cells were also found positive for mesenchymal and other stem cell markers such as CD49b, CD44, CD117, CD34, p63 and prostatic tissue specific marker like Androgen Receptor. *In-vitro* differentiation of the cells demonstrated osteocyte, adipocyte, chondrocyte and neural cell lineage differentiation, and *in-vivo* teratoma formation in balb/c mouse with presence of tri-germinal layer representative in excised teratoma. Our results clearly throw a light that BPH is stem cell associated disorder.

4(A).2 Materials and Methods

4(A).2.1 Prostate samples:

Prostatic tissue was obtained from patients (average age of 70 years; range 55–75 years) undergoing TURP, patient detailed demographic and anthropometric data collected in structured

questionnaire with consent and ethical approval. BPH histology was confirmed by a consultant pathologist and contained no adenocarcinoma of prostate.

4(A).2.2 Chemicals:

All primary (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), cell culture media were purchased from Sigma-Aldrich, USA and BD Biosciences, USA respectively. RT-PCR reagents were obtained from Fermentas, Germany. All the reagents were extra pure and of cell culture and molecular biology grade.

4(A).2.3 Isolation of prostate cells from TURP samples:

Fresh TURP tissue obtained from human prostate surgical specimens. Minced tissue in small pieces and digest with Collagenase type I enzyme for 1hr at 37^0C in a shaking incubator at 110rpm followed by grown in DMEM medium with 10% FBS as previously described (Chapronie R. et al 1986).

4(A).2.4 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 x 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 (0h, 24h, 48h, 72h, 96h, 120h, 144h and 168h). Cell counts were then plotted versus time to establish the growth curve of cells. Doubling time of isolated prostate cells were determined using the algorithm ln (N_t - N_0) ln (t), where 'N_t' and 'N_0' were number of cells at final time point and at initial seeding point respectively, and 't' was time period in hours for which cell counts were recorded.

4(A).2.5 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 (Barch et al. 1997). For each sample, at least 20 metaphase spreads were examined, in which there was minimal chromosome overlap, and long chromosome length, little or no cytoplasm, and high banding resolution were selected for detailed analysis.

4(A).2.6 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). 5µg of total RNA was reverse transcribed into first strand cDNA using random primers and subjected to PCR amplification for various stem cell genes. One µl of cDNA products was used to amplify genes using 2X master mix [Sigma Aldrich, USA], containing 1.5 µl Taq Polymerase, 2mM dNTP, 10X Tris, glycerol reaction Buffer, 25mM $MgCl_2$, and 20pM appropriate forward and reverse primers for each gene. Gapdh served as internal control (for primers sequence and annealing temperature, please refer (*Table 4(A).1*)). PCR products were separated on a 10% polyacrylamide gels [Sigma Aldrich, USA], visualized and images were captured by Cambridge ultra UV-tech chemi-doc instrument.

Gene	Primer Sequence	Annealing Temp.	Product Size
Oct ¾ (NM_002701)	Forward-5'AGCTGGAGAAGGAGAAGCTGG-3' Reverse-5'-TCGGACCACATCCTTCTCGAG-3'	63.5^0C	458bp
Sox-2 (NM003106)	Forward-5'-CACCTACAGCATGTCCTACTC-3' Reverse-5'-CATGCTGTTTCTTACTCTCCTC-3'	60^0C	384bp
Nanog (NM_024865)	Forward-5'GCAAACAACCCACTTCTGC-3' Reverse- 5'AGGCCTTCTGCGTCACAC-3'	55.5^0C	287bp

Table 4(A). 2*: Primer Sequence and annealing temperature*

4(A).2.7 Western blotting:

Western blotting of isolated prostate cells performed as previously described (Carson and Rittmaster, 2003). Isolated prostate cells were lysed with urea containing lysis buffer (1mM 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 Bradford reagent according to manufacturer's suggestions (BIO-RAD). Cell lysates (40µg) were separated on Polyacrylamide gel using Mini-tetracell electrophoresis system (BIO-RAD) and transferred onto nitrocellulose blotting membrane (Millipore) Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in PBS). Dilutions of primary antibodies used, against various stem cell genes. Primary antibodies were added to blots

and incubated overnight at 4^{o}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.

4(A).2.8 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 oC 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^{o}C (Yoon et al. 2010). Data was recorded and observations analysed using BD FACS Aria III and Flowjo™ software respectively.

4(A).2.9 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^{0}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 a Zeiss LSM710 confocal microscope (Zeiss, Germany) were recorded.

4(A).2.10 *In-vitro* Differentiation

4(A).2.10.1 Ectodermal lineage

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

4(A).2.10.2 Mesodermal lineage

Osteocyte differentiation: Isolated prostate cells were trypsinized, washed with 10mM PBS, pH 7.2, and resuspended in DMEM high glucose with % 10 FBS medium. Cells were plated into six- well plated at 10^5 cells/well in the presence of osteocyte reagents (20mM B- glycerol

phosphate, 50ug/ml ascorbic acid and 10mM 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),10mM dexamethasone and 10mg/l insulin for 8 days. The culture medium was replaced every 3rd days. Parallel control cells cultured without adipocyte differentiation medium.

Chondrocyte differentiation: Isolated prostate cells were plated in six- well plate as described above. Chondrocyte differentiation was induced by treatment with 10mM dexamethasone and 10mg/l insulin for 20 days. The culture medium was replaced every 5th day. Parallel control cells cultured without chondrocyte differentiation medium.

4(A).2.10.3 Endodermal lineage

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

4(A).2.11 *In-vivo* Differentiation

Teratoma formation: For each graft, approximately 0.2 million isolated prostate cells, washed and resuspended in 300 µl DMEM complete medium, were 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 (Awad et al. 2004; Chen et al. 2007). Right side of the same animals was used for control or placebo i.e. only agarose plugs were injected in that site. 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, sections were examined for the presence of cells representatives of all three germ layers produced by transplanted cells (Byrne et al. 2009).

4(A).3 Results

4(A).3.1 Prostate cells isolation and characterization:

Surgically removed TURP prostate sample were digest with Collagenase type I enzyme for 1hr at 37^0C. After enzymatic digestion microscopic observations of cultured prostatic cell population

showed characteristic fibroblastic and epitheliod shaped cells (*Figure 4(A). 1B &C*). 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(*Figure4(A).2A*). To further confirm the nature of isolated cells, immunocytochemistry were performed with different cell markers. *(Fig 4(A).1)*

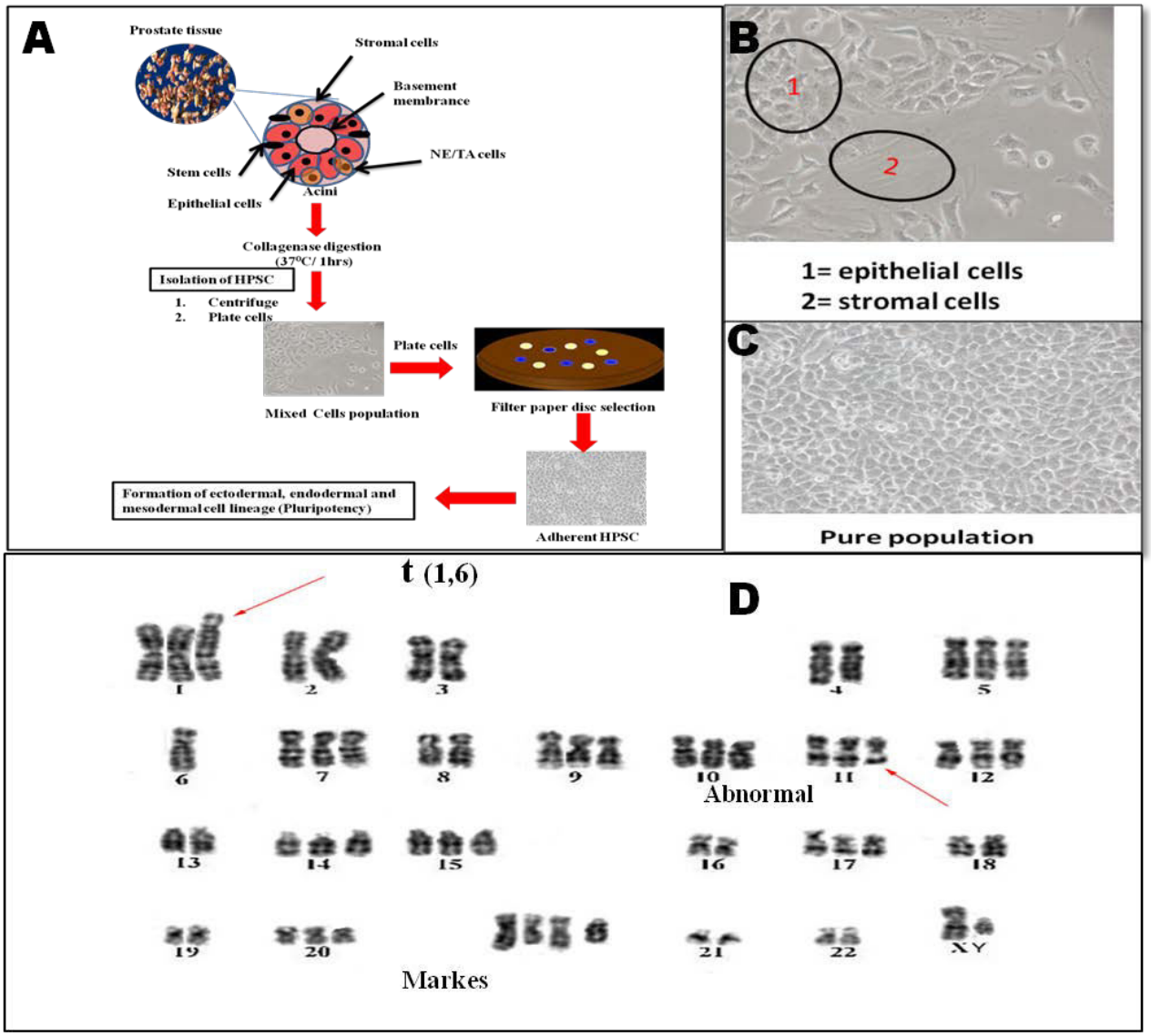

Figure 4(A). 3*: Isolation and cytogenetic characterization of isolated prostate cells from BPH patient. (A) Schematic of prostatic cells isolation from TURP tissue. Cells were obtained after 1hrs, incubation with collagenase type-I enzyme in DMEM medium without FBS at 37^{0}C. (B) and (C) morphological view of isolated cells from mixed population using sterile filter paper discs soaked in trypsin and cultured in DMEM medium with FBS at 370C under 5% Co2 .(D) karyotype analysis of isolated prostate cells. Cells showed aneuploidy with 4 to 5 marker chromosomes and translocation of 6th chromosome to 1st.*

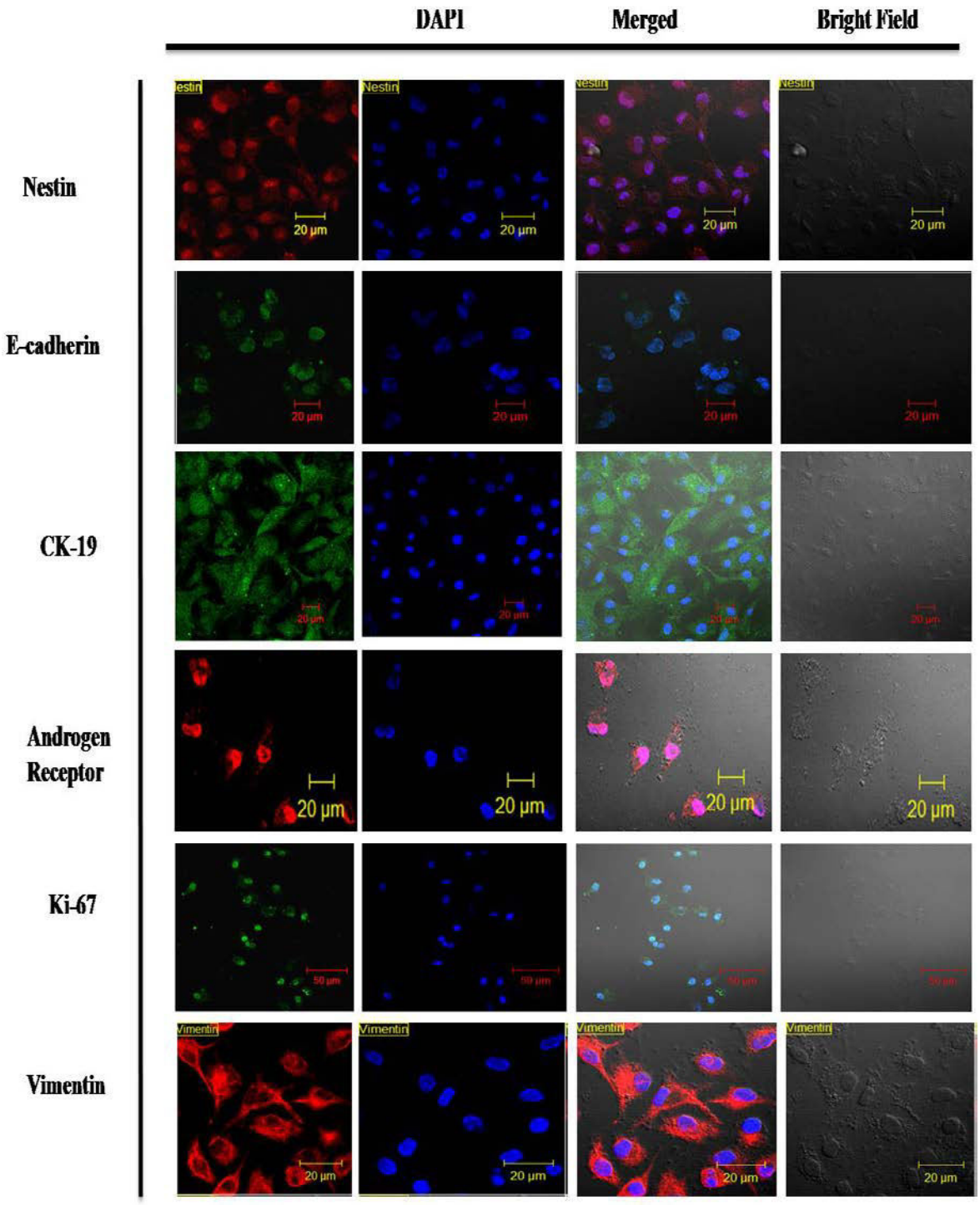

Figure 4(A). 4*: Characterization of isolated prostate cells. Immunofluorescence staining of BPH prostate cells for Nestin, E-cadherin, CK19, Androgen Receptor, Ki-67 and Vimentin (first panel from left side). The second panel from left side shows DAPI staining and the second panel form right side shows the merged images. Bars represent 20µm.*

4(A).3.2 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.

4(A).3.3 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 *(Fig. 4(A).1D).*

4(A).3.4 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 were performed for stem cells specific markers such as CD49b,CD44, CD117, CD34 and p63. Fluorescent microscopic analysis showed cells were positive for stem cell markers *(Fig. 4(A).2A).* FACS analyses 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. *(Fig 4(A).3)*

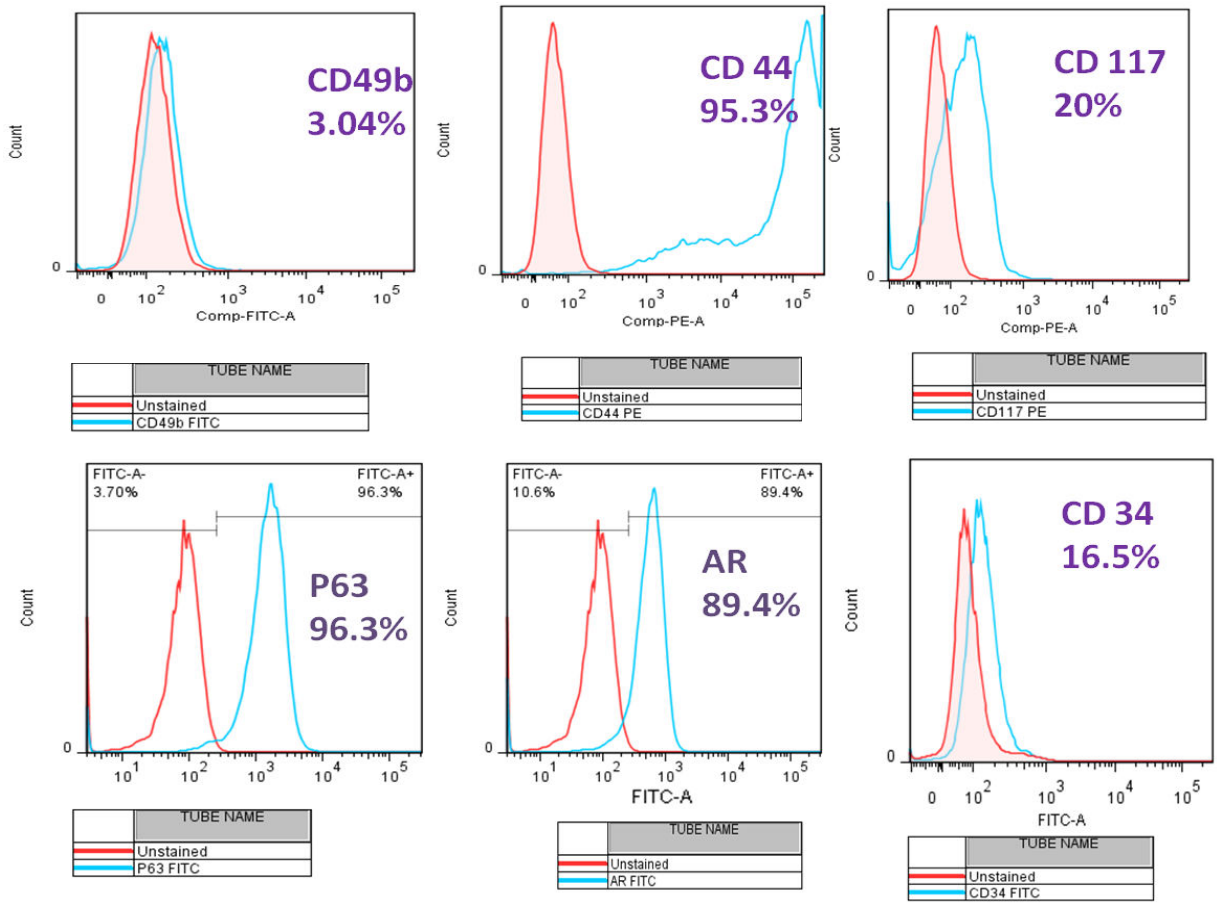

Figure 4(A). 5*: Characterization of isolated prostate cells. FACS analysis of cell surface markers demonstrate 3.04% CD49b, 95.3% CD44, 20%CD117, 96.3% p63, 89.4%AR and 16.5% CD34 positive cells in isolated prostate cell*

4(A).3.5 Pluripotency features:

To elucidate whether the cells possess pluripotent characteristics, cells were further analyzed for 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 *(Fig 4(A).4C)*. RT PCR analysis also showed higher expression of all three ONS genes, Gapdh served as internal control *(Fig 4(A).4A)*. 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 endogenous control *(Fig 4(A).4B)*.

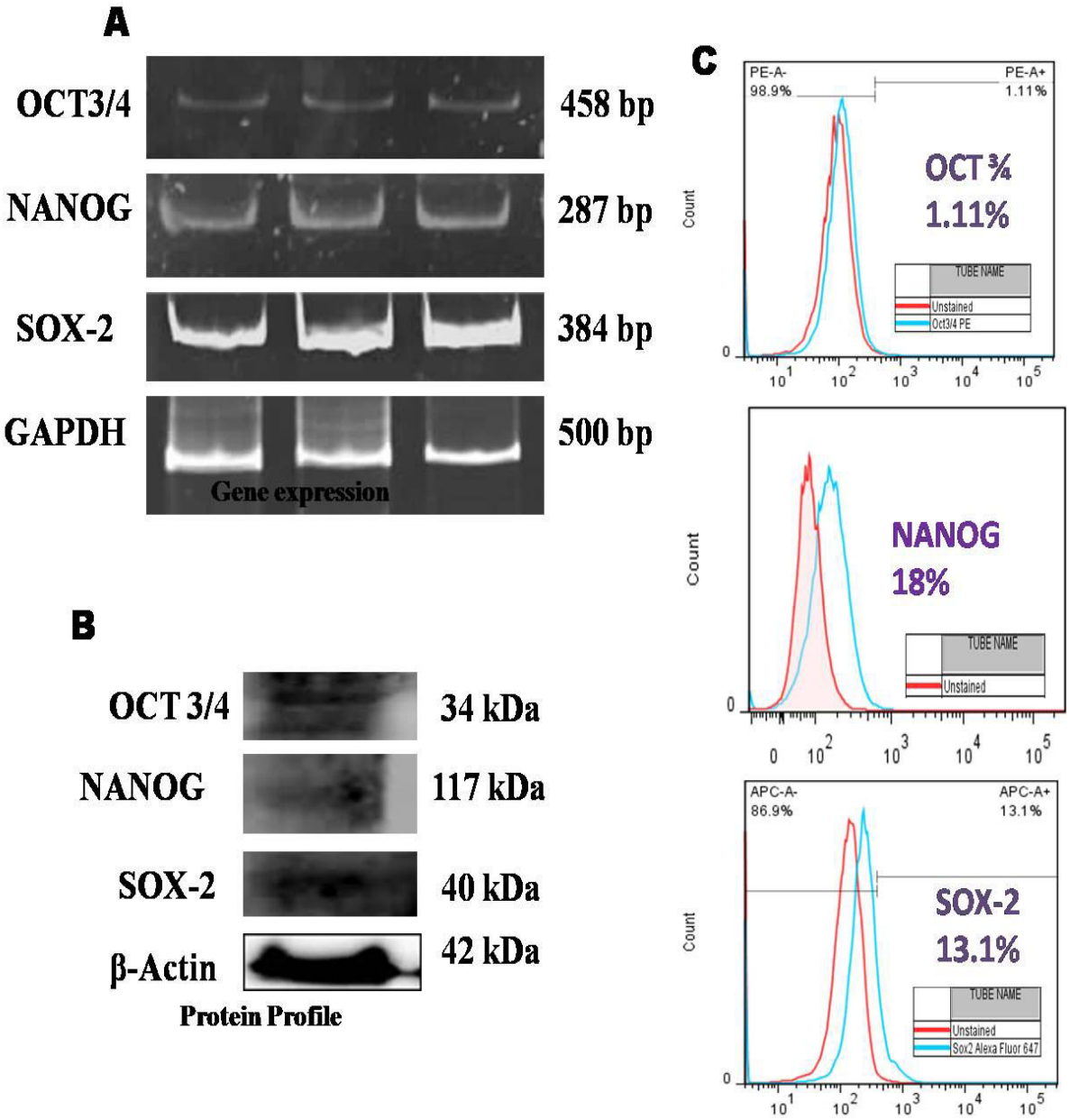

Figure 4(A). 6*: Isolated prostate cells express pluripotent stem cell markers. (A) and (B) At the genomic and protein level. Gel-electrophoresis of RT-PCR products and protein profile of pluripotency specific genes (Oct 3/4., Sox-2 and Nanog) showed expression. (C) Flow cytometry data showed presence of ONS markers in isolated cells.*

4(A).3.6 *In-vitro* differentiation:

Ectodermal lineage differentiation: To investigate the potential differentiation into ectodermal lineage, isolated prostate cells were cultured for 10days in neuronal differentiating medium. Cells were positive for MAP-2, a marker for mature neurons.(Fig 2.5)

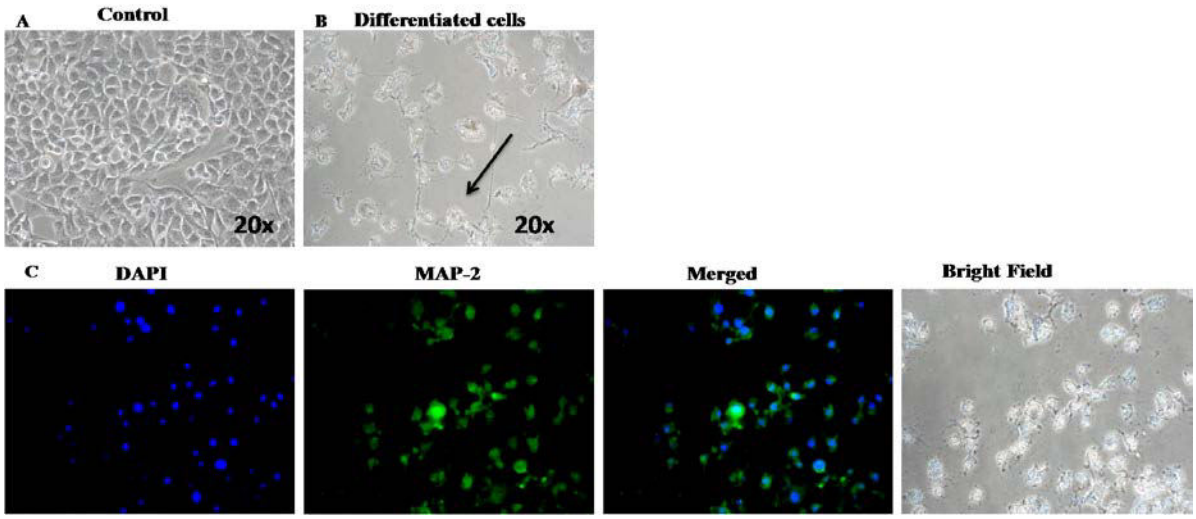

Figure 4(A). 7*: Isolated prostate cells can differentiate to ectodermal cell lineages. (A) Without and (B) with neural differentiation reagents (neuro-basal medium with N2 supplements and 2mM glutamate) for 10 days. (A) Control. (B) neural like cells. (C) neural-like cells were detected by Immunofluorescence staining using MAP-2 antibody. Nuclei were stained with DAPI.*

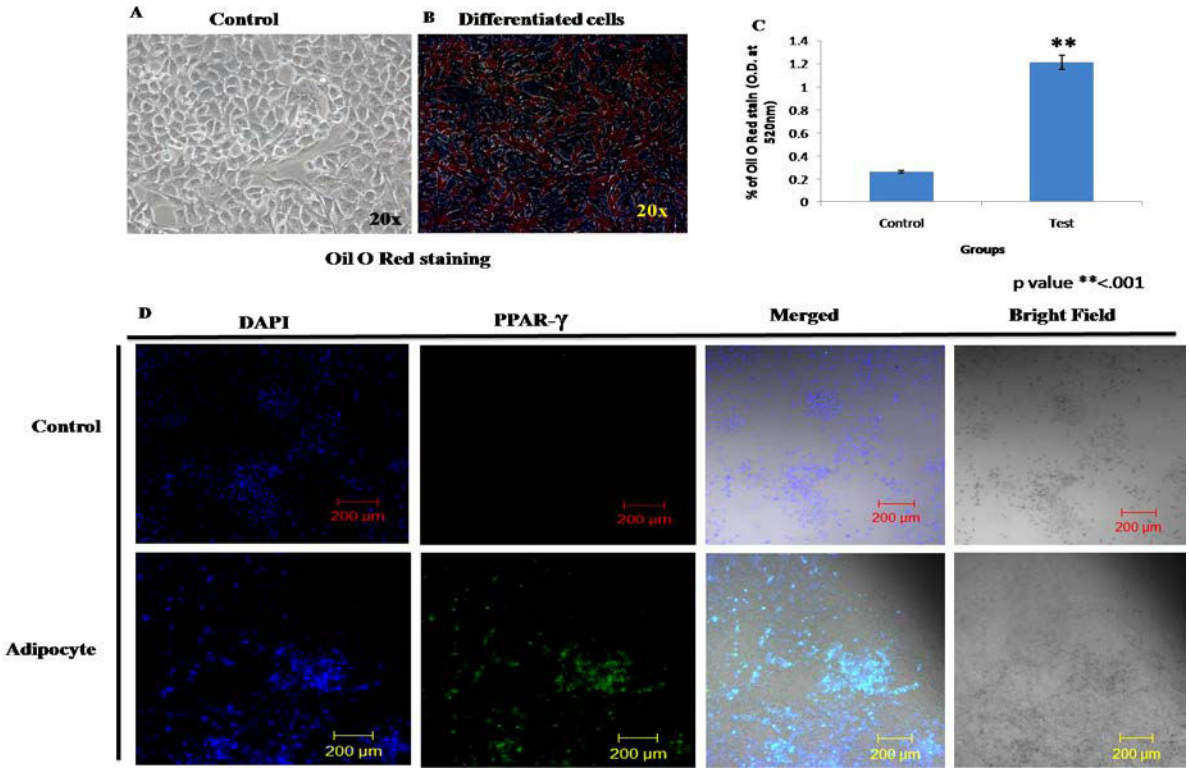

Figure 4(A). 8*: Oil O red staining of isolated prostate cells after culture (A) without and (B) with adipocytic differentiation reagents (IBMax 10 mg/ml, 10mM dexamethasone and 10mg/l insulin) for 8 days. (A) Control cultures showed oil red O negative cells; (B) Positively staining adipocytes. (C) histogram showing the significant oil red O staining in differentiated cells. (D) Immunofluorescence staining of PPAR-γ for differentiated adipocytes. Nuclei were stained with DAPI.*

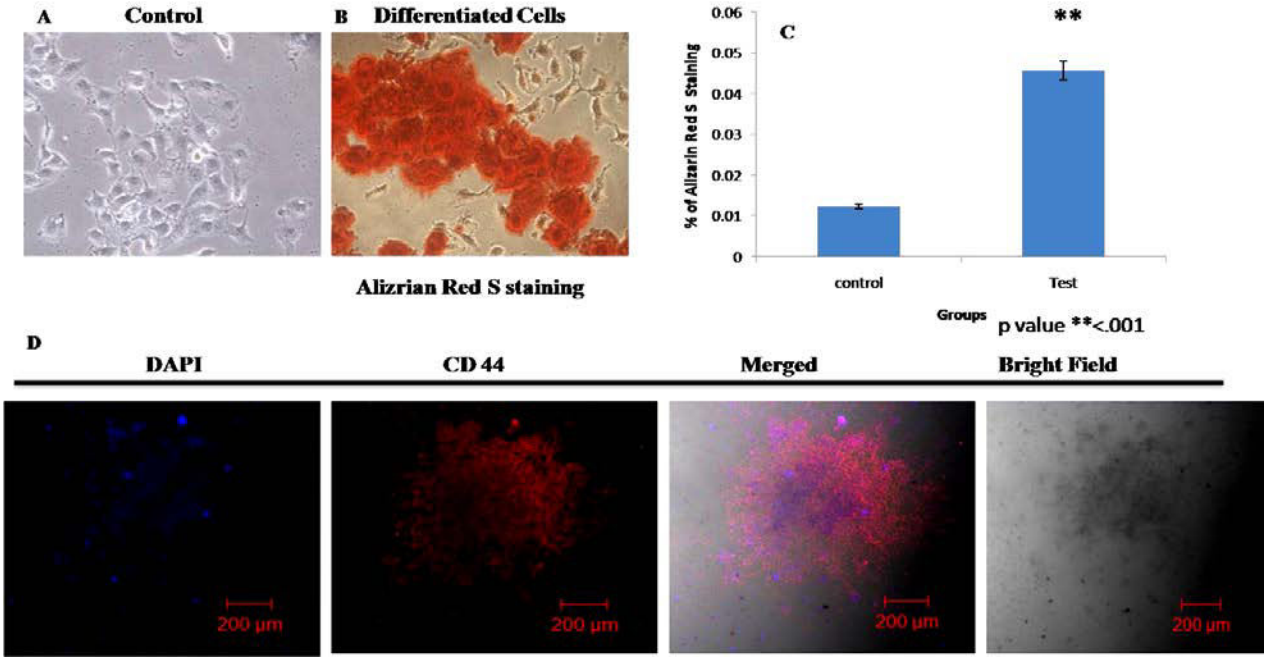

***Figure 4(A). 6(ii)**: Alizarin red S staining of isolated prostate cells after culture (2A) without and (2B) with osteocyte differentiation reagents (20mM glycerol phosphate, 50 µg/ml ascorbic acid, 10mM dexamethasone and 10mg/l insulin) for 10 days. (2A) Control cultures showed Alizarin red S negative cells; (2B) positively stained 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.*

Mesodermal lineage differentiation: To determine the potential of isolated prostate cells to differentiate into cells of mesodermal lineages: adipocytes, chondrocytes and osteocytes. Cells were grown as adherent cells in culture medium of different mesodermal lineages. Differentiation of prostate cells into a mesodermal lineage was determined by immunocytochemistry and specific staining. Mesodermal markers included PPAR-γ, a marker for adipocyte, CD44, marker for osteocytes and CD 90 and CD44 combined marker for chondrocytes. Futher these cells also stained with oil o red, Alizarin red S and Alcian blue stain for adipocytes, osteocytes and chondrocytes respectively. *(Fig.4(A).6(i,ii,iii))*

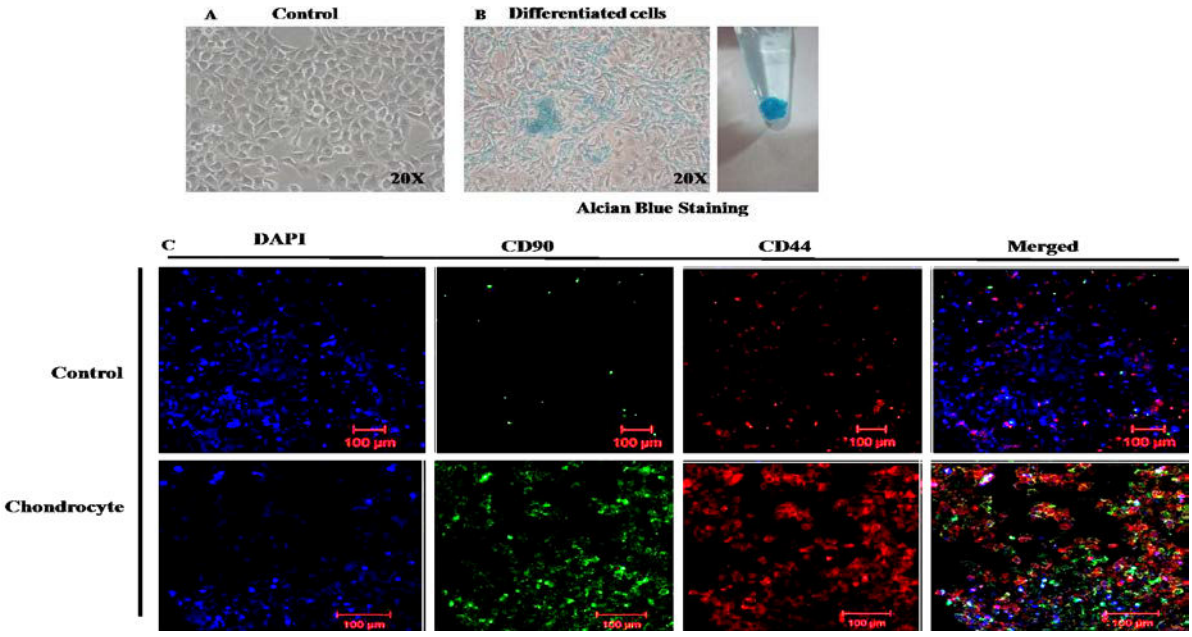

***Figure 4(A). 6(iii)**: Alcian blue staining of isolated prostate cells after culture (A) without and (B) with chondrocyte differentiation reagents (10mM dexamethasone and 10mg/l insulin) for 20 days. (A) Control cultures showed Alcian blue negative cells; (B) positively staining chondrocytes. (C) Immunofluorescence staining of CD90 and CD44 for differentiated chondrocytes. Nuclei were stained with DAPI.*

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 *(Fig. 4(A).7).*

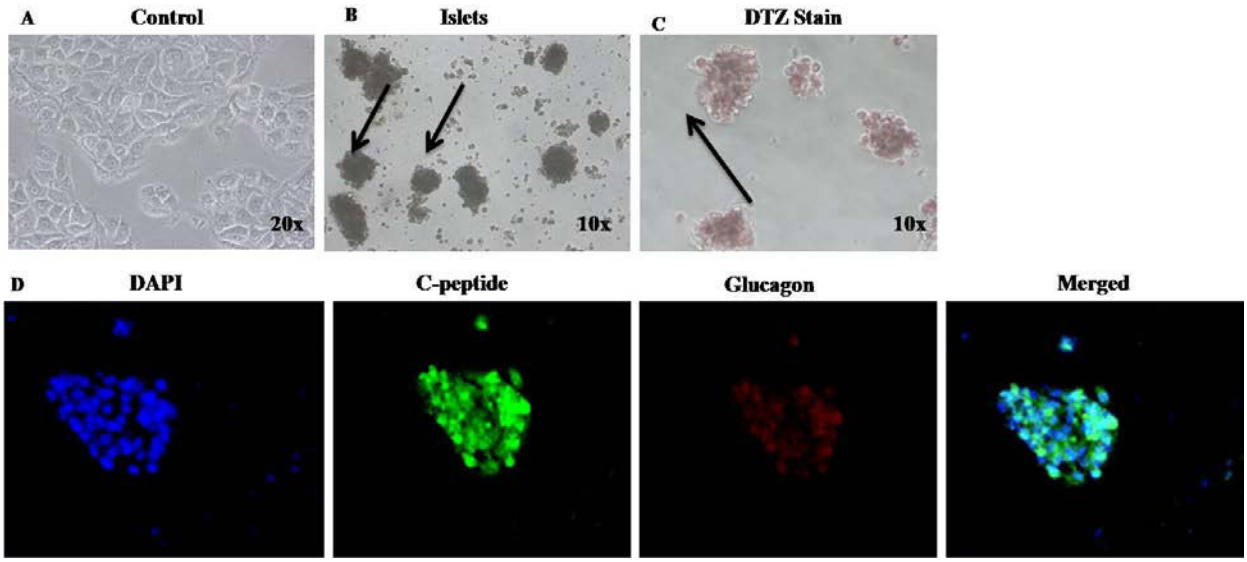

***Figure 4(A). 9**: Isolated prostate cells can differentiate to endodermal cell lineages. (A) without and (B) with islet differentiation reagents (serum free RPMI 1640 medium with 10ng/l activin-A and 10mg/l insulin) for 10 days. (A) Control. (B) Islet like cell cluster. (C) DTZ staining. (D) islet like cell cluster were detected by immunofluorescence staining using Cpeptide and glucagon antibodies. Nuclei were stained with DAPI.*

4(A).3.7 *In-Vivo* differentiation:

Teratomas 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 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 *(Fig. 4(A).8A).*

Histological studies confirmed *in-vivo* functionality and representation of tri-germinal layers in developed teratomas *(Fig. 4(A).8B).* Since the cells were encapsulated in agarose and transplanted subcutaneously beneath the forearms and not directly in vasculature; hence the chances of spreading were minimal to begin with.

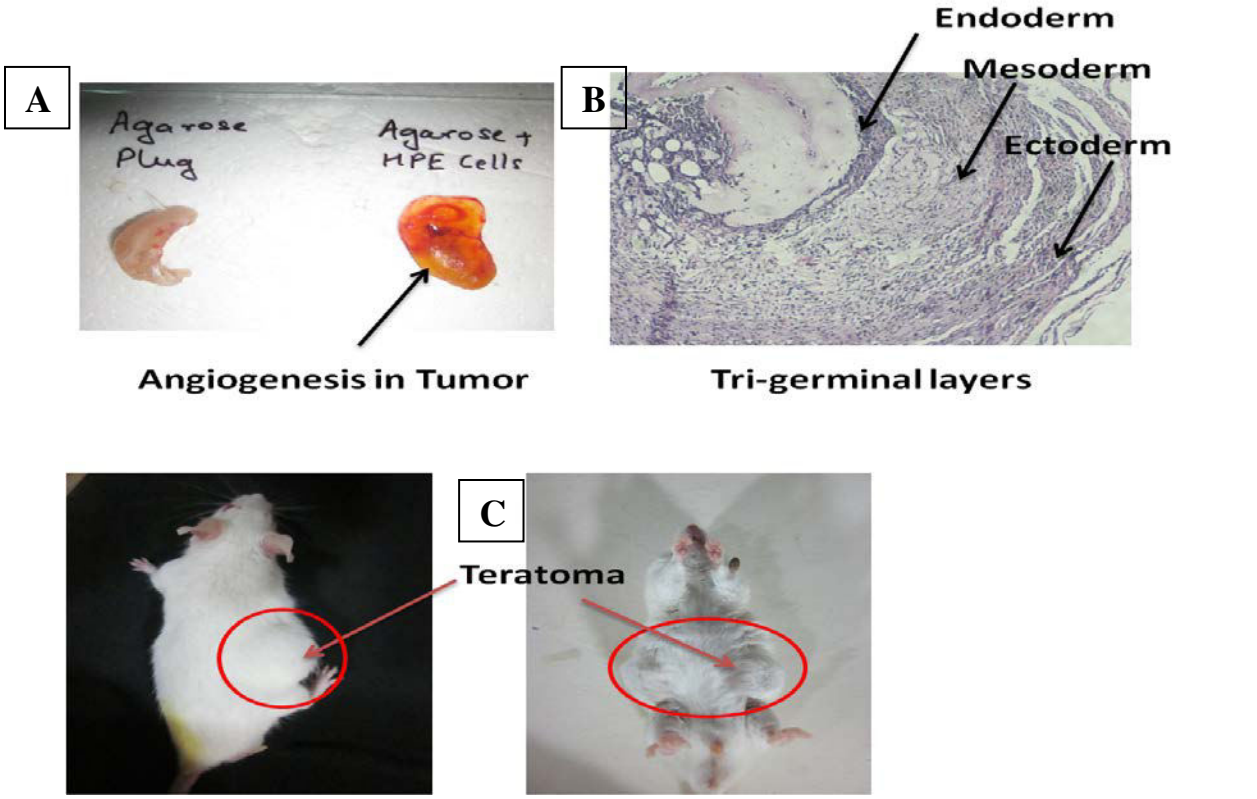

Figure 4(A). 10*: Teratoma formation capability of isolated prostate cells with tri-germinal layer.0.2 million cells with agarose plug were transplanted in left side body cavity of Balb/c mice. Right side only agarose plug transplanted. (A) Angiogenesis in transplanted tumor. (B) H&E staining showing tri-germinal layer formation in excised tumor transplanted with prostate cells. (C) Visible teratoma in animals.*

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 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 BPH.

4(A).6 Discussion

BPH is characterized by hyper proliferation of epithelial and stromal cells in the transition zone of the prostate gland, which can be observed histopathologically (Schuster and Schuster 1999). Stem cells in the human prostate have been identified and isolated using the cell surface markers such as CD44 (Liu et al. 1997), integrin $\alpha_2\beta_1$ (Collins et al. 2001) , CD133 (Prominin-1) (Richardson et al. 2004) which, are believed to be responsible for the development and progression of proliferative disorders of the prostate such as prostate cancer and BPH. (Reya et al. 2001; Lawson and Witte 2007; Isaacs 2008; Prajapati et al. 2013). Based on high expression of $\alpha_2\beta_1$ integrin, Collins and colleagues identified PSCs in the basal layer and showed that the $\alpha_2\beta_1^{high}$ integrin cells represent ~1% of basal cell population in the human prostate (Collins et al. 2001).

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 (Le Magnen et al.2013). However, role of ONS and other stem cell markers in prostate hyperplasia 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 level of CD49b in the present study is high when compared to previous report that showed presence of only 1% of this marker (Bhatt et al. 2003). 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 (Signoretti et al. 2000; Davis et al. 2002). Previous investigation revealed a role of p63 in stem cell functions (McKeon 2004). 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,* 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 up-regulated after castration-induced prostate involution, consistent with prostate stem cell identity and function (Leong et al. 2008). CD44 was used as the marker to identify basal stem cells with tissue-regeneration abilities (Signoretti et al. 2000). Interestingly, isolated cells showed both CD117and 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 (Tsujimura et al. 2002), 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 (Lin et al. 2007). 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 (Isaacs and Coffey 1989; Wang et al. 2001; Tsujimura et al. 2002).

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 isolated prostate cells has demonstrated an aneuploid DNA content and translocation of chromosome 6 to 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 (Balachandar et al. 2008). Chromosome 1 has a breakage-prone site, which has been reported to be sensitive to environmental clastogens and responsible for tumor development and progression (Paraskeva et al. 1988; Grosovsky et al. 1996; Conforti-Froes et al. 1997).

Most interesting finding of our study is that isolated pluripotent like 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 (Evans and Chandler 1987). 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. 2014a; Prajapati et al. 2014b).

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 (Gudjonsson et al. 2004; Bhatia et al. 2008; Wieser et al. 2008) 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 tumorigenesis.

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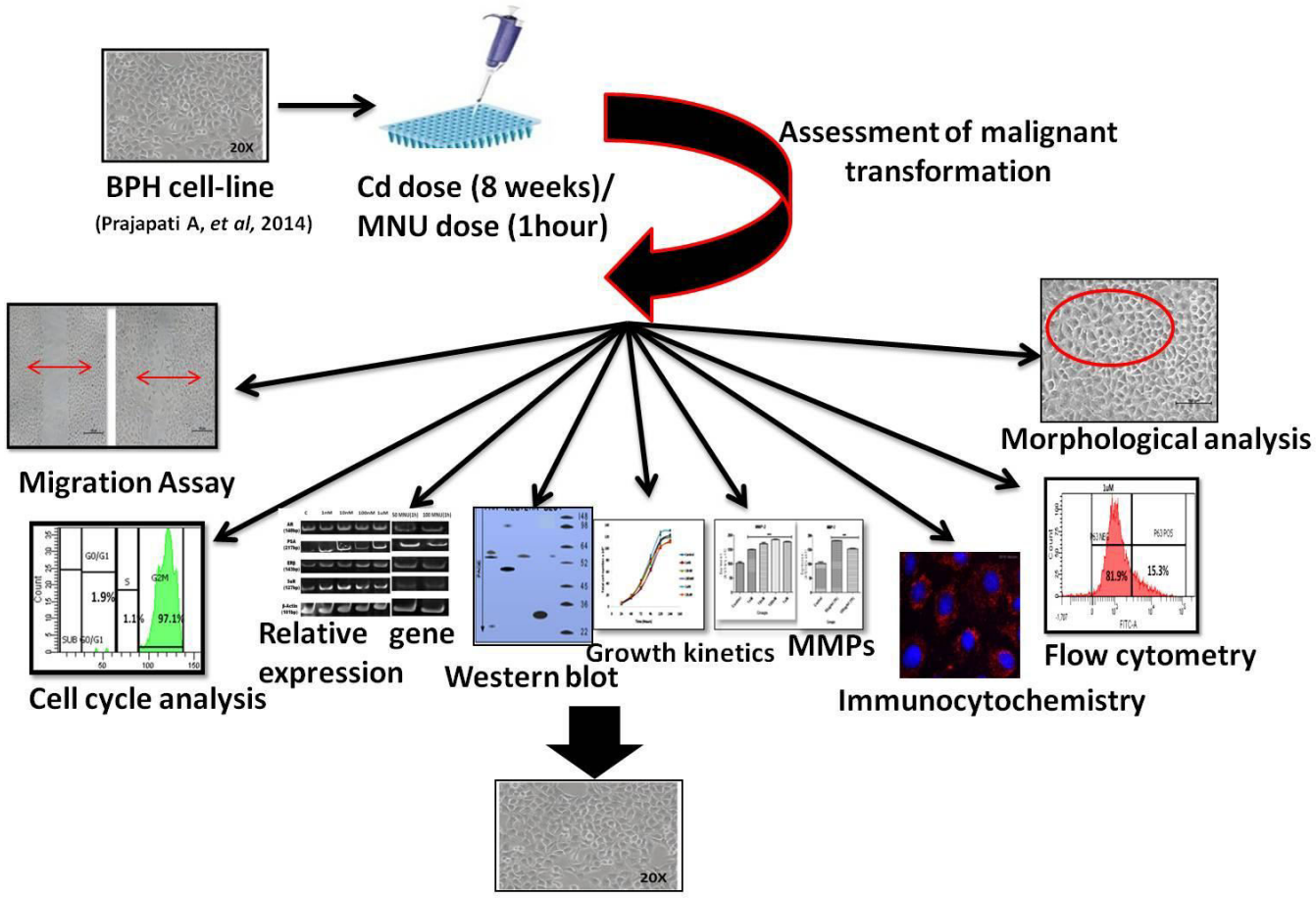
CHAPTER -4(B)

CHAPTER - 4 (B)

To study the link between Benign Prostate Hyperplasia and Prostate Cancer by using Human BPH epithelial cell-line using Cadmium as model.



4(B).1 Introduction

Benign prostatic hyperplasia (BPH) and prostate cancer (PCa) are among the most common pathologies of the prostate gland and represent a significant health risk in men across the world. The two diseases share some important features, including hormone-dependent growth and response to anti-androgen therapy. Moreover, risk factors such as inflammation and metabolic disruption are also playing very important roles in the development of both diseases. Despite these commonalities, BPH and PCa exhibit important differences in terms of histology and localization. Although large-scale epidemiological studies have shown that men with BPH have an increased risk of PCa and its related mortality, it remains unclear whether BPH is the first step in the pathway to PCa.

Cadmium (Cd) is a well known environmental pollutant and potent carcinogen. The major source of Cd exposure is food and smoking. Approximately 3–10% of the Cd absorbed by food whereas, 50% is absorbed through smoking into the bloodstream (Jarup et al. 1998). Smokers absorb an additional 1–3 mg Cd/d from the respiratory tract (Fay et al. 1999), which is quite higher than non-smokers and increase Cd content has been observed with severity of BPH (Pandya et al. 2013).

Most of the Cd in the body is bound to metallothioneins, low molecular weight proteins that function in the homeostasis of essential metals (e.g., zinc) (Hamer 1986; De Lisle et al. 1996). The Cd-metallothionein complex is distributed to various organs and is reabsorbed in kidney tubuli (Ohta and Cherian 1991). Half-life of Cd in kidney cortex is approximately 10–30 years as human body has no mechanism to excrete Cd leading to its accumulation in tissues (Vuori et al. 1979).

The growth and development of the prostate gland is dependent on androgens and its signaling. Studies suggest that Cd has potent androgenic and estrogen-like activity, where it binds with high affinity to androgen receptor and the hormone-binding domain of Estrogen Receptor (ER) and activates the receptors (Stoica et al. 2000; Prajapati et al. 2014b).The interaction of Cd with ERα involves several amino acids, some of which are also conserved in the hormone-binding domain of the Androgen Receptor (AR) (Wurtz et al. 1996; Tanenbaum et al. 1998). Several studies have been demonstrated proliferative and carcinogenic effect of Cd on the prostate *in vitro* and *in vivo* (Sahmoun et al. 2005) (Waalkes 2000). Many reports accuse Cd for its potency

of malignant transformation of non-tumorigenic human prostate epithelial (HPrE) cells into tumorigenic cells *in-vitro* (Achanzar et al. 2001; Nakamura et al. 2002). But reports on BPH to prostate cancer conversions are very scanty. To increase our understanding of the intricate pathways and mechanisms connecting between BPH and PCa, further study is needed.

In this context, we have established a BPH epithelial cell line from BPH patient as mentioned in the initial part of the chapter. The cell line has opened new avenues for defining mechanisms in prostate carcinogenesis. In present study we exposed human BPH cells to Cd with different concentrations (i.e 1 nM, 10 nM, 100 nM, 1 µM till eight weeks) for cancer induction. Methyl Nitrosuria (MNU) served as a positive control in our study for PCa induction. Characterization of Cd exposed BPH cells showed subtle morphological changes. The study has been evaluated for conversion of hyperplasia cells into cancer by using flowcytometry, zymography, gene expression and protein profiling.

This study provides compelling evidence that Cd has the potency to convert BPH epithelial cells into cancerous in dose dependant manner. Further, comparison between the transformed and BPH cells should lead to a better understanding of the mechanism involved in Cd induced carcinogenesis. Establishing BPH as a causal factor for PCa development could improve the accuracy of prognosis which will help in potentially reducing the number of men who die from PCa.

4(B).2 Materials and Methods

4(B).2.1 Chemicals:

All primary and secondary antibodies, cell culture reagents media (Anti-Androgen Receptor cat no A9853, Anti Ki-67 cat no. P6834) were purchased from Sigma-Aldrich, USA. RT-PCR and molecular biology reagents from Fermentas, Germany. All other required reagents were purchased locally.

4(B).2.2- Determination of Cytotoxic dose of Cd:

BPH epithelial cells were cultured as previously described in the initial part of the chapter (Prajapati et al. 2014a). 0.5 Million cells from passage no. 11 of BPH cell line were seeded in 96 well plates in triplicates. To determine the cytotoxic dose, Cd treatment with concentration ranging from 10^{-10} M to 10^{-3}M was done for 48 hours. Control cells did not receive any

treatment. After 48 hours incubation with Cd, cells were incubated with 50ug/ml MTT containing cell culture medium for 4 hours in CO_2 incubator. Medium (with MTT) was replaced after 4 hours and added 100µl/well of DMSO subjected to OD at 570nm using ELISA reader (Multiskan^{TM} ThermoScientific). Graph was plotted on Cd concentration versus % Viability value and cytotoxic Concentration (CC_{50}) was calculated using Prism Software v-5.0.

4(B).2.3 Cell Culture:

Based on 50% viability by cytotoxic dose determination study, BPH epithelial cells were treated with different concentrations of Cd i.e. 1 nM, 10 nM, 100 nM and 1µM till eight weeks as reported in the literatures. Medium was replenished at every alternative day. The cells were also treated with 50µg/ml and 100µg/ml Methyl Nitrosuria (MNU) (Boileau et al. 2003) for 1 hours. After 8 weeks of Cd exposure, the cells were assessed for malignant transformation study.

4(B).2.4 Cell Growth kinetics:

0.5 million cells of each Cd (1nM, 10nM, 100nM and 1µM) and MNU (50µM and 100µM for 1 hour) treated cells were seeded into 12-well plates in triplicates for different time points (i.e 0h, 24h, 48h, 72h, 96h, 120h, 144h, 168h). Fully confluent prostatic cells were trypsinized and counted under inverted phase contrast microscope (Nikon TE2000, Japan). Doubling time of treated prostate cells were determined using the algorithm ln (N_t - N_0) ln (t), where N_t and N_0 were number of cells at final time point and at initial seeding point respectively, and t was time period in hours for which cell counts were recorded.

4(B).2.5- RNA extraction and semi quantitative Reverse Transcriptase PCR (RT-PCR):

Total RNA was isolated from the treated cells after eight weeks Cd exposure using TRizol Reagent (Sigma Aldrich, USA) extraction following the manufacturer's instructions and immediately treated with DNase-l (Fermentas). 5µg of total RNA was reverse transcribed into first strand cDNA using random primers and subjected to PCR amplification for various prostatic genes. One µl of cDNA product was used to amplify genes using 2X master mix (Sigma Aldrich, USA), containing 1.5 µlTaq Polymerase, 2mM dNTP, 10X Tris, glycerol reaction Buffer, 25mM MgCl2, and 20pM appropriate forward and reverse primers for each gene. β-Actin served as an internal control (*Table: 4(B).1).* PCR products were separated on a 10% polyacrylamide gels (Sigma Aldrich, USA), visualized and images were captured by Cambridge ultra uv tech chemi-doc instrument.

GENE	PRIMER SEQUENCE	ANNEALING TEMP. (°C)	PRODUCT SIZE
AR	F - 5' CCTGGCTTCCGCAACTTACAC 3'	63.1	148bp
	R - 5' GGACTTGTGCATGCGGCCGTACTCA 3'		
5α-Reductase	F - 5' TACACAGACATACGGTTTAGC 3'	55.5	127bp
	R - 5' CTTGTGGAATCCTGTAGCTGA 3'		
PSA	F - 5' CCTCCTGAAGAATCGATTCCT 3'	58.1	217bp
	R - 5' GAGGTCCACACACTGAAGTT 3'		
ERβ	F - 5' AGAGTCCCTGGTGTGAAGCAAG 3'	63.8	143bp
	R - 5' GACAGCGCAGAAGTGAGCATC 3'		
β - ACTIN	F - 5' ATGGATGACGATATCGCTGC 3'	58	101bp
	R - 5' CTTCTGACCCATACCCACCA 3'		

Table 4(B). 1: *Primer Sequence and annealing temperature*

4(B).2.6- Western blotting:

Western blotting of the treated cells performed as previously described (Carson and Rittmaster 2003). The cells were lysed with urea containing lysis buffer (1mM 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 Bradford reagent according to manufacturer's suggestions (BIO-RAD). Cell lysates (40µg) were separated on Polyacrylamide gel using Mini-tetracell electrophoresis system (BIO-RAD) and transferred onto nitrocellulose blotting membrane (Millipore). Blots were then incubated with blocking milk buffer (5% fat free skimmed milk with 0.1% Tween-20 in Phosphate Buffer Saline (PBS)). Dilutions of primary antibodies against various prostatic proteins 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 blot images were documented using chemi-doc instrument Cambridge UV tech, Uk.

4(B).2.7- FACS analysis:

Cd and MNU treated 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. Data were recorded and observations analysed using BD FACS Aria III and Flowjo™ software respectively.

4(B).2.8- Immunocytochemistry:

Cells of all groups 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 4^0C 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 were captured using Zeiss LSM710 confocal microscope (Zeiss, Germany).

4(B).2.9- Cell cycle analysis:

Cd and MNU treated cells with passage matched control human benign prostate epithelial cells were harvested and single cell suspension was obtained in PBS. After centrifugation, pellet was washed twice with PBS containing 1% bovine serum albumin and added with chilled absolute ethanol drop wise with vortexing to minimize clumping and cell loss. Cells were kept for fixation for overnight at -20^{o}C. After overnight incubation, cells were pellet down followed by PBS wash containing 1% bovine serum albumin. 500 µl Propidium Iodide (PI) (20 µg/ml in PBS) staining solution was added to cell pellet and mixed well and incubated for 1 hour at room temperature in dark. Cell cycle distribution (DNA histograms) data were recorded using a BD FACS Aria-III flow cytometer and data were analyzed by BD FACS Diva software (v6.3.1). Cell doublets and aggregates were avoided during gating.

4(B).2.10- Migration assay:

Cells of all treated groups along with control cells were seeded in 12 well plates in triplicates and after achieving 80% confluence, cells were starved in serum free medium for overnight. A wound was created in monolayer of cells using sterile tip. The scratched cell monolayer images were captured. Then, complete medium with 10% FBS containing 2µg/ml Mitomycin C was added and after 24 hours, images of the same were recorded using Nikon Inverted Phase-contrast Microscope using 20X objective magnification.

4(B).2.11 Zymographic analysis of MMP activity:

Cells at 70% confluency were washed twice with PBS, and the medium was changed to Tumorsphere SFM^{TM} media (generous gift from Irvine scientific, USA) without supplements. After 48h, the conditioned medium was collected and centrifuged for 5 min at 400 x g. A 500ul

aliquot was concentrated to<100 µl in a lyophilizer at 4^0 C. Protein concentration was determined using Bradford method and 5ug of the total protein from each sample was electrophoresed on a 10% zymographic gel containing 0.1% gelatin. MMP activity was detected by incubating the gel in1x zymogram renaturing buffer for 30 min at room temperature and then in 1x zymogram developing buffer overnight at room temperature, followed by staining with coomassie blue dye. After staining, the bands were quantified using Image J software.

4(B).3 Results

4(B).3.1 Evaluation of Cytotoxic Concentration of Cd (CC_{50}):

BPH cells exposed with different concentration of Cd ranging from 10^{-10}M to 10^{-3}M for 48 Hours. CC_{50} of the Cd was assessed by MTT assay. 26.6µM Cd concentration showed 50% cell viability, which was considered as 50% cytotoxic concentration. Data were analyzed using Prism™ software v5.0 *Figure 4(B). 2.*

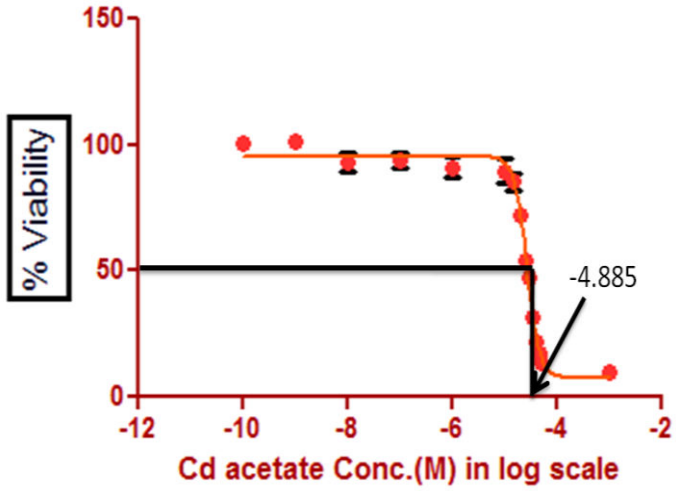


***Figure 4(B). 1**: Determination of Cd cytotoxic concentrations: Anti-log (-4.885=26 x 10^{-6}) (n=3)*

4(B).3.2 Growth curve and kinetics:

Cd and MNU treated 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 points (i.e 24h, 48h, 72h, 96h, 120h, 144h, 168h) respectively without passaging. These observations provide a theoretical growth curve that is directly proportional to the cell number. 1µM Cd treated and, both 50 and 100µg/ml MNU treated cells for 1 hour. exposure showed significant decrease in doubling time which was around 24 hours as compared to control cells *(Table 4(B).2).*

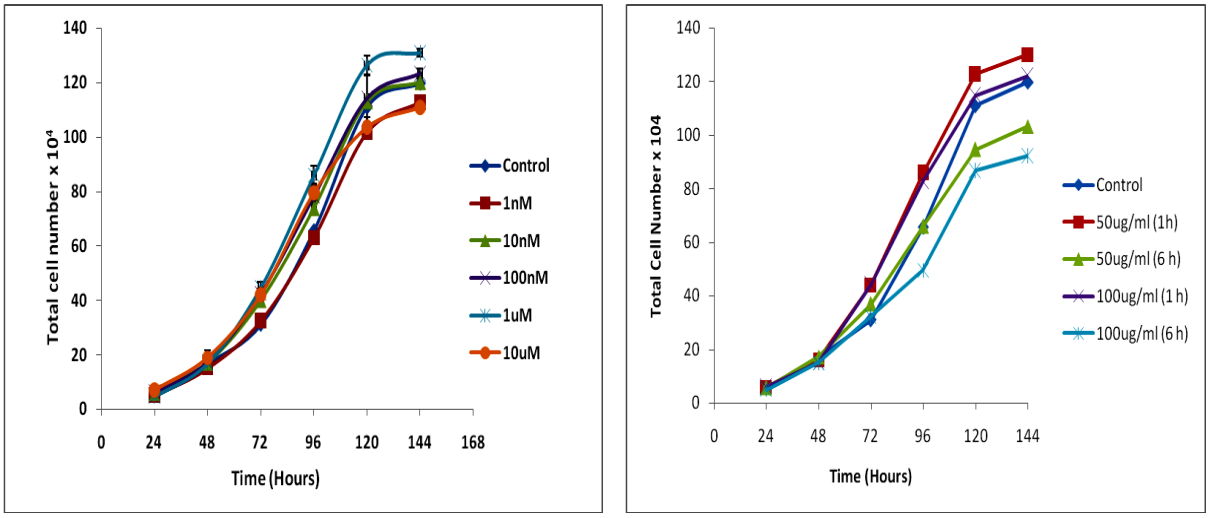


***Figure 4(B). 3**: Growth curve of Cd and MNU treated cells.*

CADMIUM TREATED GROUPS	
Group	**Doubling time (hours)**
Control	**26.5±0.374**
1nM	26±0.48
10nM	26±0.50
100nM	27±1.1
1µM	**24.7±0.25***
MNU TREATED GROUPS	
50µg/ml (1 hour)	**24±0.3 ***
100µg/ml (1 hours)	**24.6±0.3***

***Table 4(B). 2**: Doubling time of Cadmium treated and MNU treated cells. Results represent the mean of three observations ± SEM. *p< 0.05(Control vs Cd /MNU treated)*

4(B).3.3 Morphology:

Cancerous cells are charcterized by irregular size and shape with large nucleus (Baba and Catoi 2007). All Cd and MNU treated cells with passage matched control were seeded in 12 well plate in equal cell number and images were captured at 80% confluence. 1µM concentration of Cd treated cells were showed distorted epitheliod structure, irregular shape and increase in cell size as compared to control and other Cd groups. Similar observations were also noted in MNU treated cells.

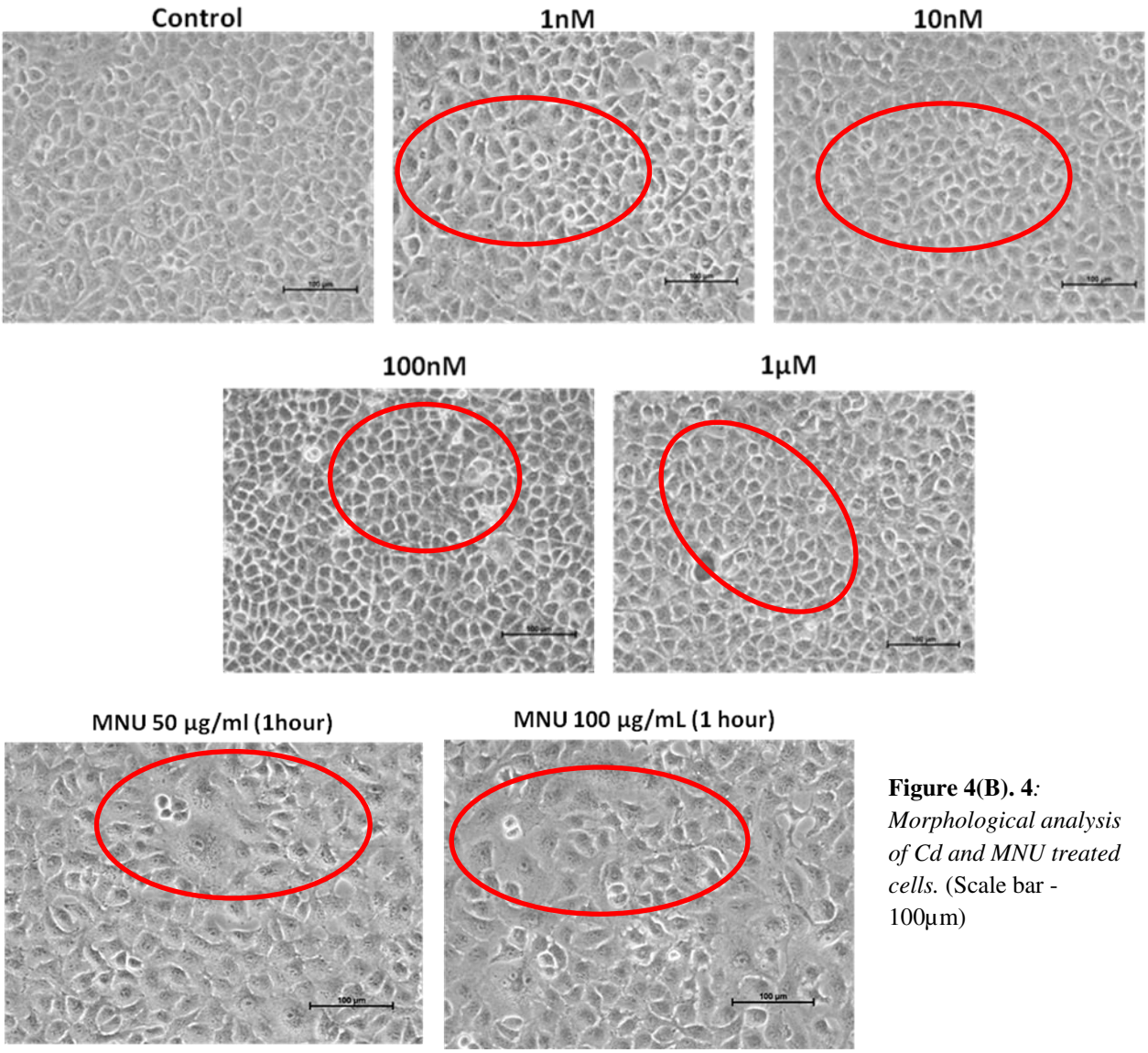


Figure 4(B). 4*: Morphological analysis of Cd and MNU treated cells.* (Scale bar - 100µm)

4(B).3.4 Cell cycle analysis:

Cell cycle analysis was done by flow cytometry. 10nM, 100nM and 1µM Cd treated cell population were showed significant peak shift as compare to control cells. 10nM and 100nM cells showed 66.9% and 74.2% increase in S phase respectively, where as 1µM Cd treated cells showed 97.1 % increase in G_2/M phase which was concurred with other available reports (Martin et al. 2002). Both 50 and 100µg/ml MNU treated cells for 1 hour treatment showed significant increase in G_2M phase i.e. 98.2% and 96.4% respectively.

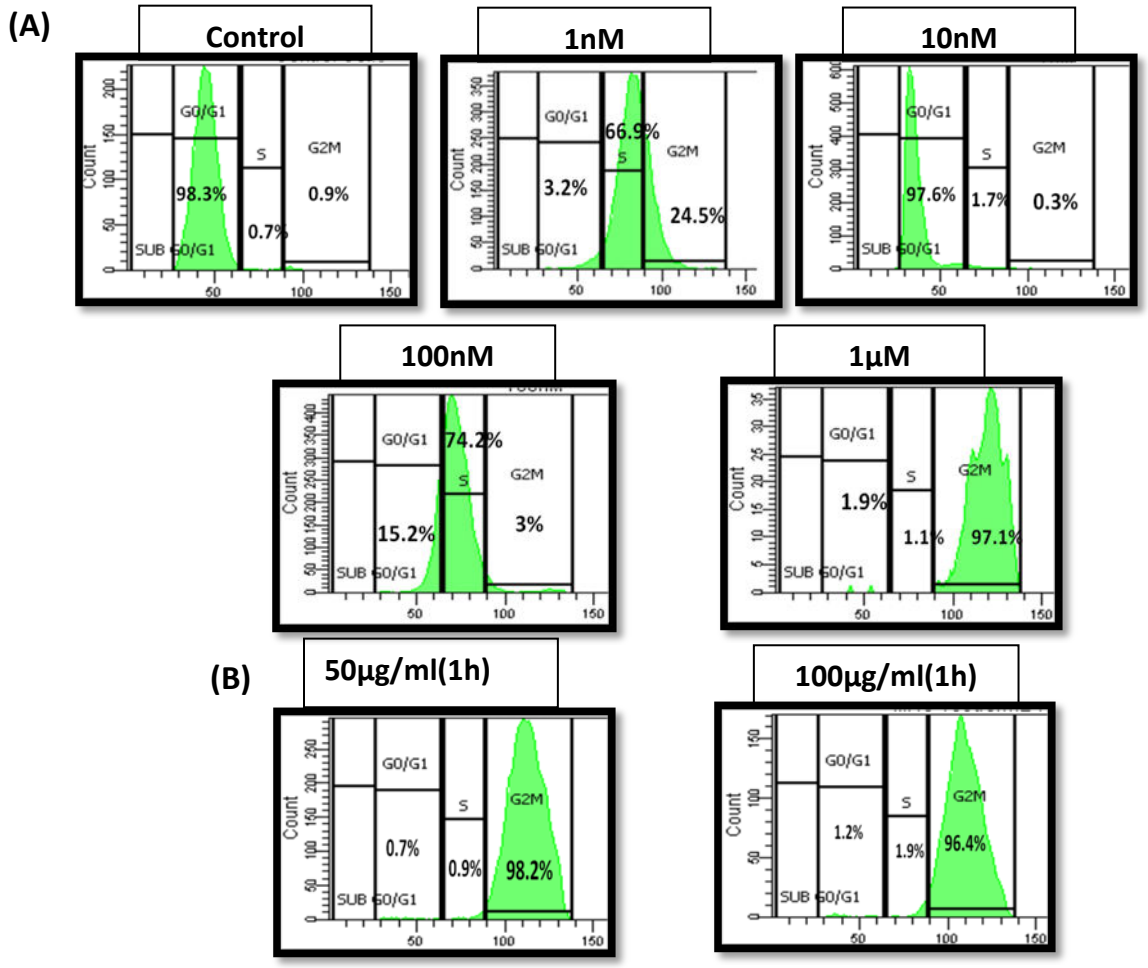


Figure 4(B). 5*: Cell cycle analysis by flowcytometry. (A) after 8 weeks of Cd treatment with different concentrations (1nM, 10nM, 100nM, 1µM) (B) MNU treatment (50µg/ml and 100µg/ml for 1 hour).*

4(B).3.5 Gene expression study:

The Cd has an ability to bind with steroid hormone receptors leading to stimulate proliferation and transactivation of AR . To shed light into the gene expressions underlying the response of Cd and MNU treated cells were examined for gene expression profile (AR, PSA, ERβ , and 5α-Reductase II) by Reverse Transcriptase -PCR method *(Fig 4(B).5)*. Significant increased expression of AR, PSA, ERβ and 5α-Reductase II were observed in 10nM, 100nM and 1µM of Cd treated cells. In MNU treated cells significant increase in PSA (50µg- and 100µg/ml for 1 hour), AR (in all four groups) and ERβ (50and 100µg/ml for 1 hour) were observed where as 5α-Reductase II showed significant decrease in all MNU groups. β – Actin served as an internal control.

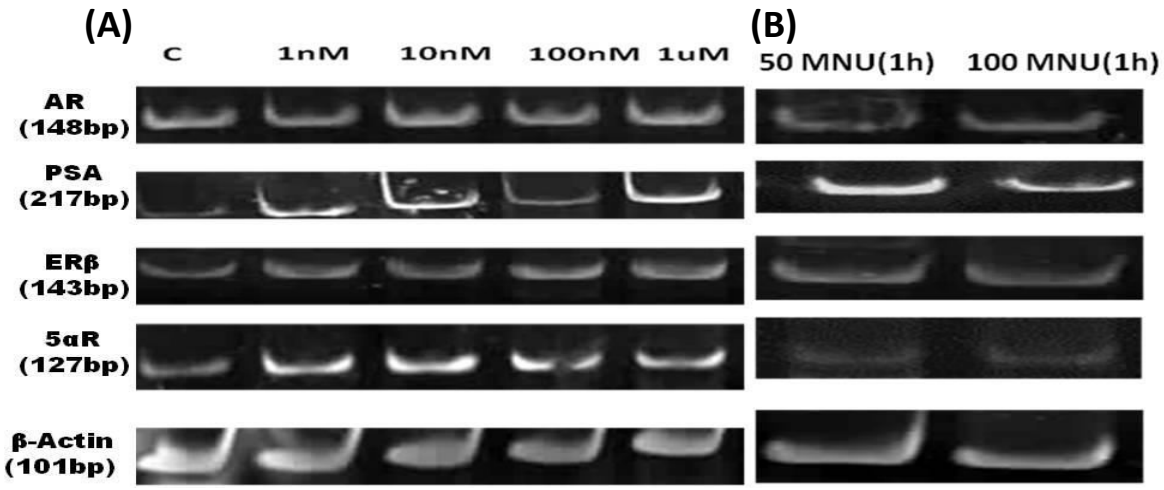
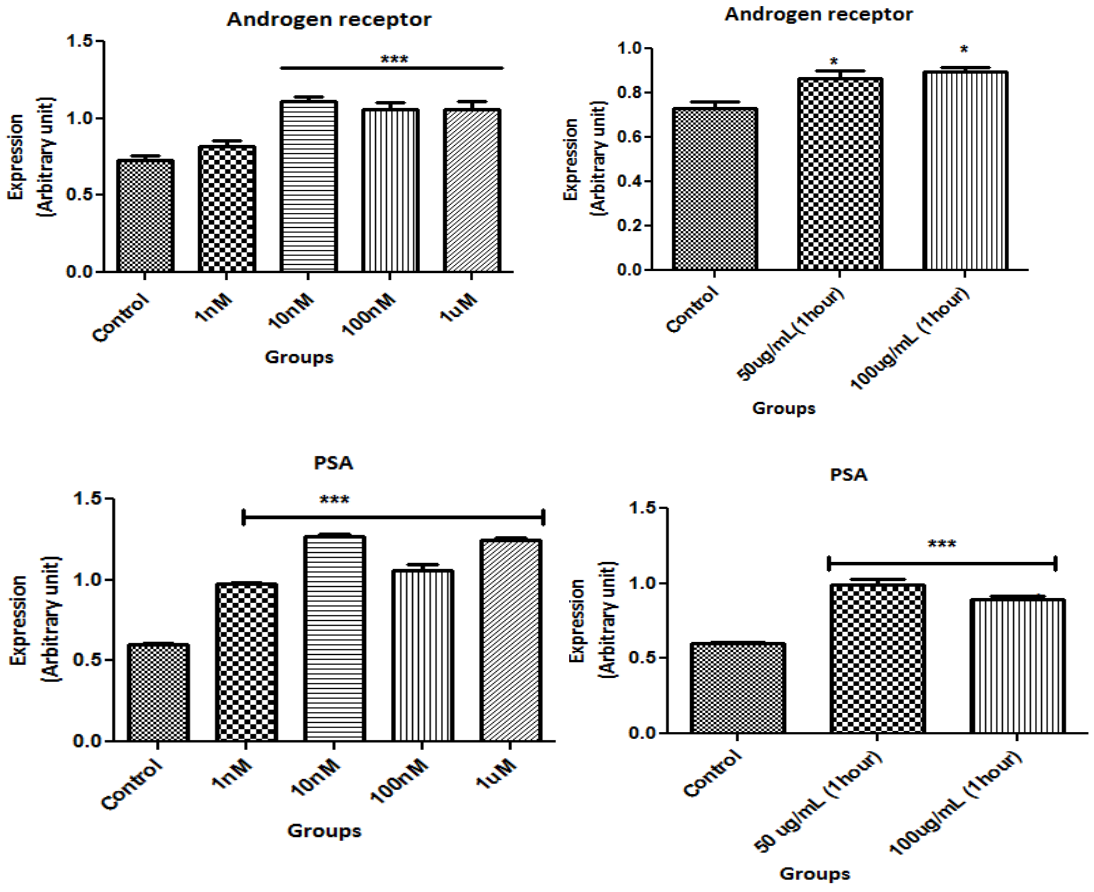


***Figure 4(B). 6**: Gene expression profile: DNA-PAGE (15%) gel picture showing PCR amplification of AR, PSA, ER- β, 5 α reductase II , β – actin gene in (A) after 8 weeks of Cd treatment with different concentrations (1nM, 10nM, 100n and, 1μM)and (B) MNU treated cells (50and 100μg/ml for 1 hour).*



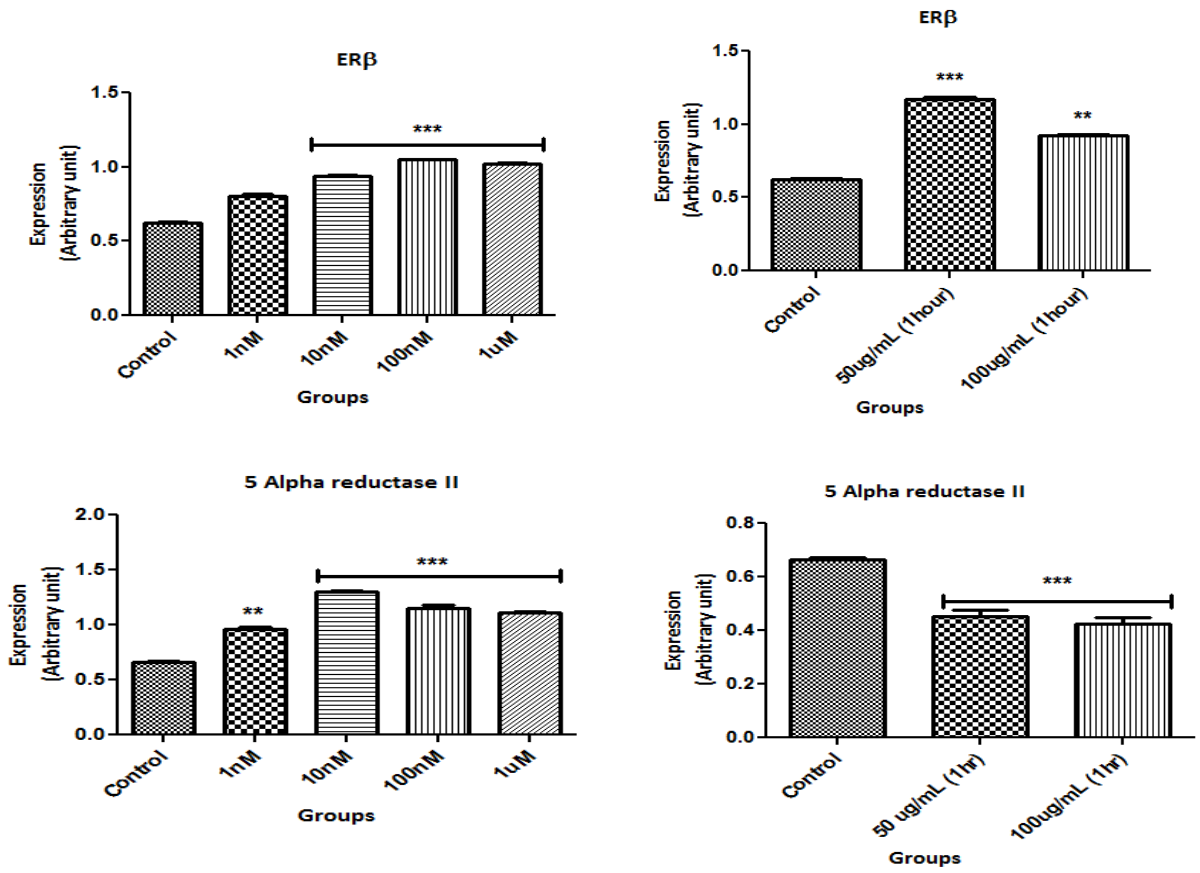


Figure 4(B). 7*: Desitometric analysis of Gene expression of AR, PSA, ERβ and 5-α reductase –II. The results represent the mean of three observation ± SEM. ***p<0.001, **p<0.01,*p<0.05, Control vs Cd /MNU treated cells*

4(B).3.6 Protein profiling:

Western blotting data of protein expression of p63 showed significant increase in 1nM, 10nM and 100nM Cd treated cells and decreased expression of 1µM and MNU treated cells. Similar observations were also found in both time group of 50µg/ml MNU groups. Indicating, the transformation of hyperplasia conditions into carcinoma. No significant difference was observed in expression of Androgen receptor in the Cd treated cells. At higher dose of Cd and MNU treated cells (50µg/ml for 1 hour) showed decrease in AR expression. Significant increase in AR expression was found in MNU treated cells with 100µg/ml for 1 hour.

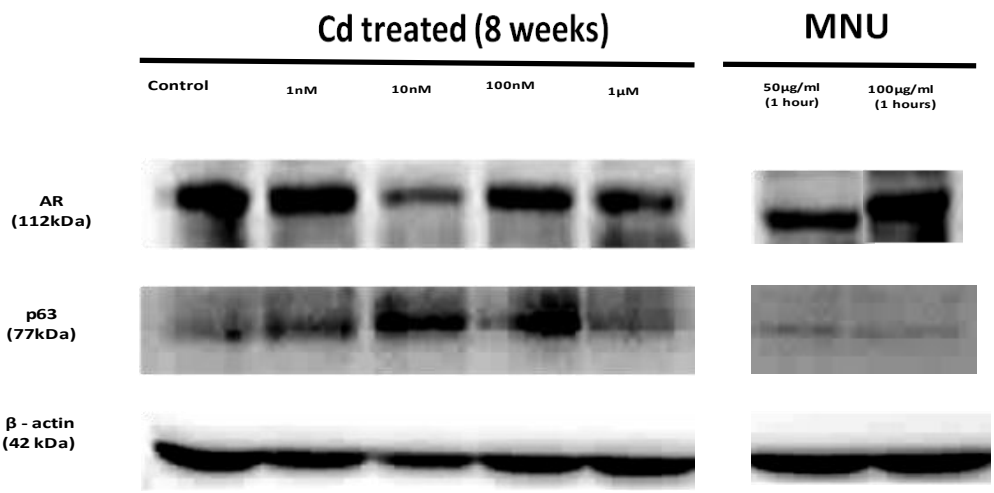


Figure 4(B). 8: *Protein expression of AR, p63 and β–actin in all Cd and MNU treated cells by Western blot*

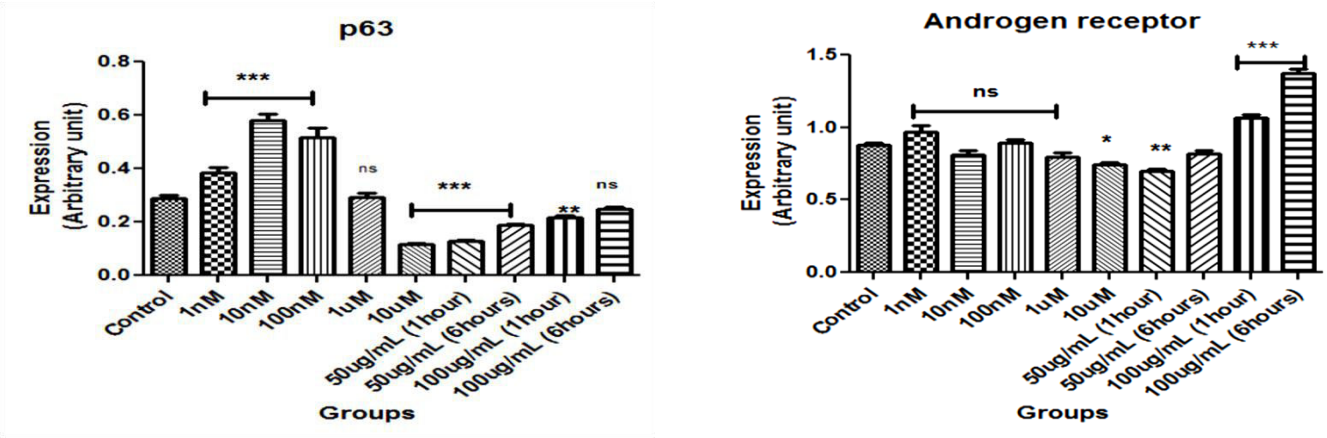


Figure 4(B). 9*: Densitometric analysis of p63 and AR protein expression in all Cd and MNU treated cells. Results represent mean of three observation ± SEM ***p<0.001, **p<0.01, *p<0.05 Control vs Cd /MNU treated. Ns: Non significant*

4(B).3.7 p63 expression by Flow cytometry:

To further validate p63 expression. An experiment was done by flow cytometry. These data clearly indicated that number of cell populations of Cd treated groups showed decrease expression of p63 with increasing Cd dose compared to control and indicated malignant transformation of BPH cells into PCa.

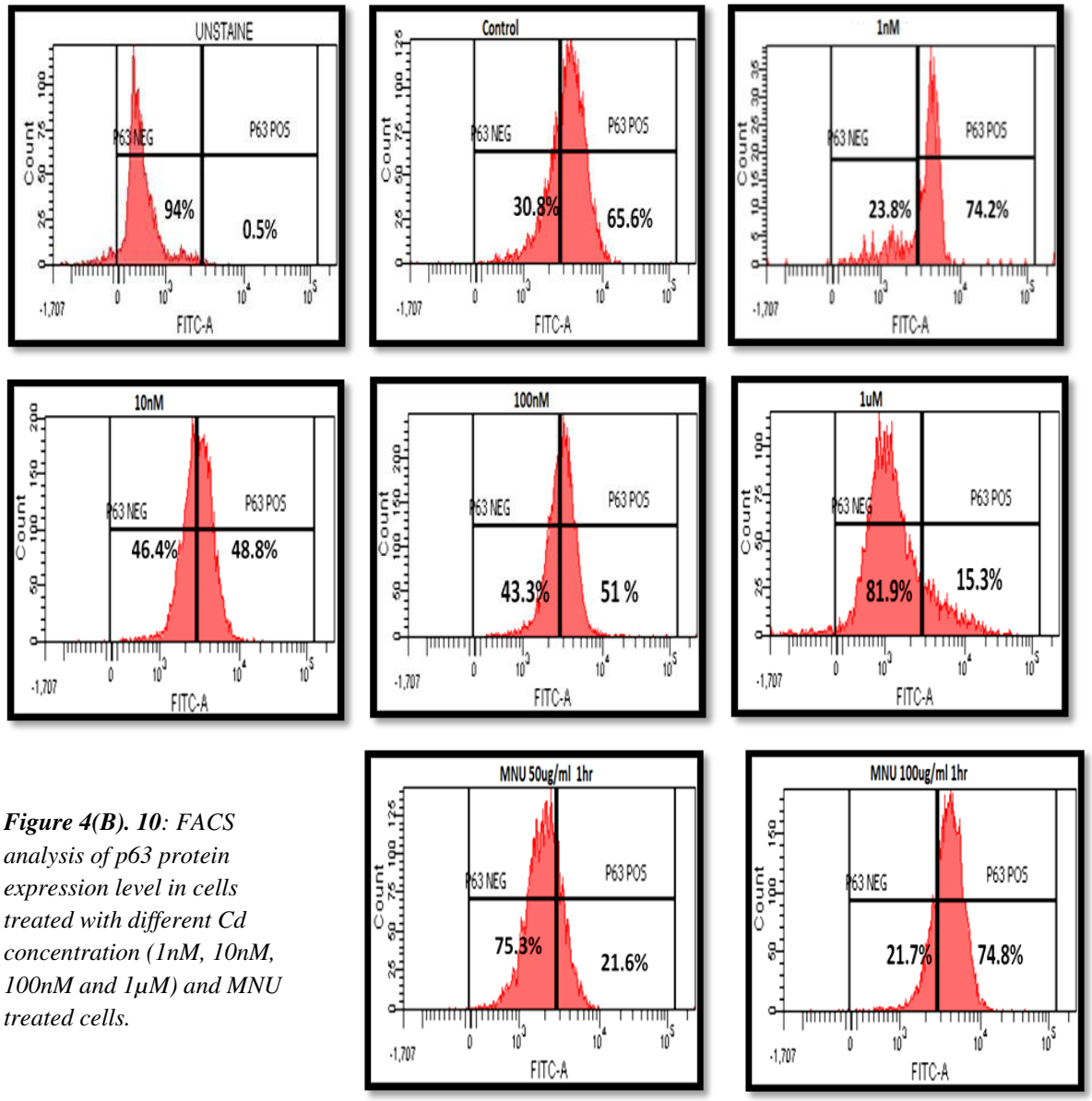


***Figure 4(B). 10**: FACS analysis of p63 protein expression level in cells treated with different Cd concentration (1nM, 10nM, 100nM and 1µM) and MNU treated cells.*

4(B).3.8 Immunocytochemistry:

To further authenticate the action of Cd on BPH cells, immunocytochemistry was performed. Immunocytochemistry data suggested increase in nuclear localization of AR in all Cd treated cells in dose dependant manner. Basal cytoplasmic level of ER-α was also observed in all Cd treated cells. Both nuclear and cytoplasmic expressions of AR were observed in MNU treated cells. Nuclear localization of Ki-67 increased in all Cd treated cells in dose dependant manner and in 50µg/ml MNU group, which indicated increase in cell proliferation.

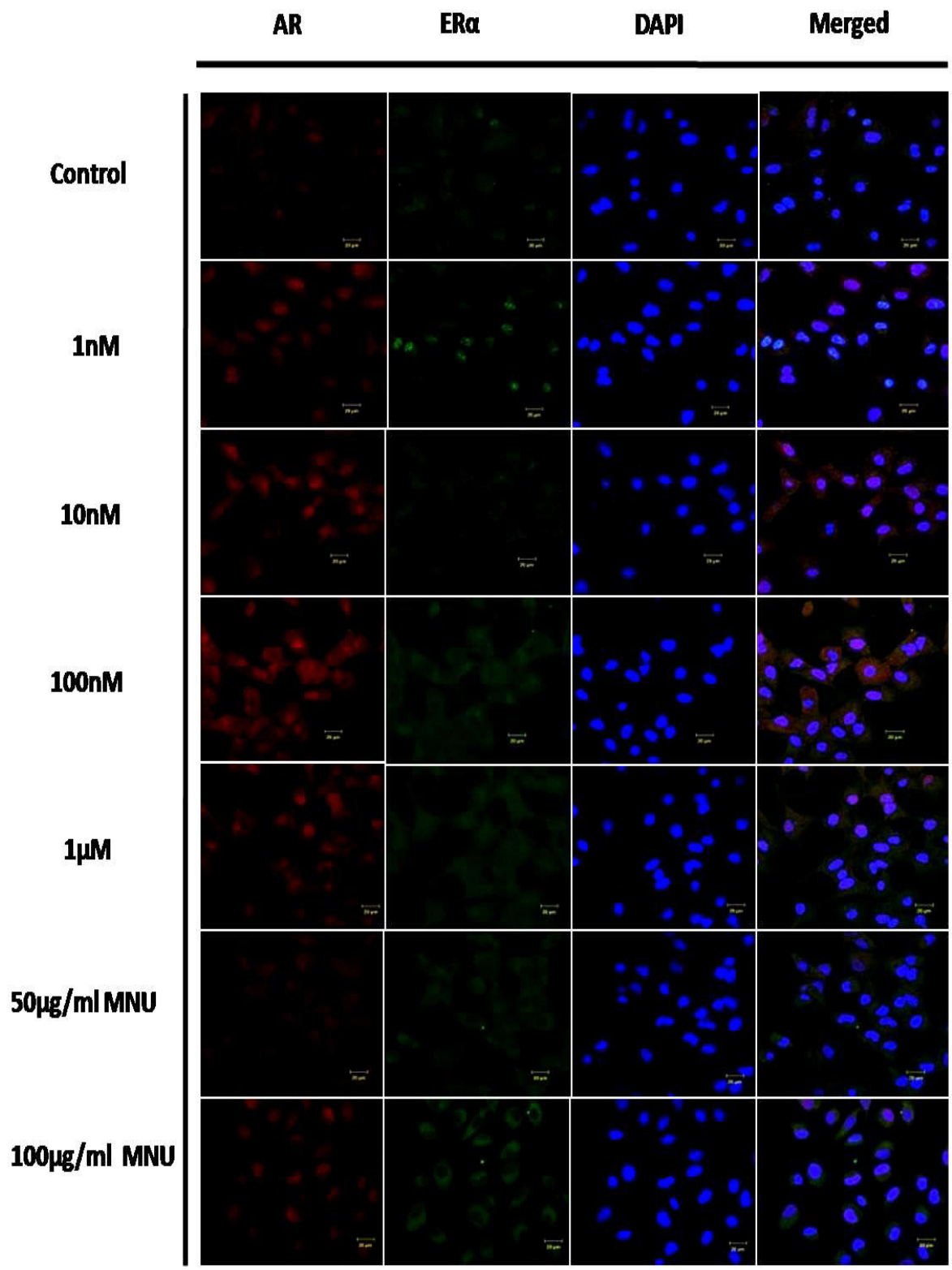


Figure 4(B). 11: *Immunocytochemistry of AR and ER-α receptor of Cd and MNU treated cells. (*Scale bar: 20µm)

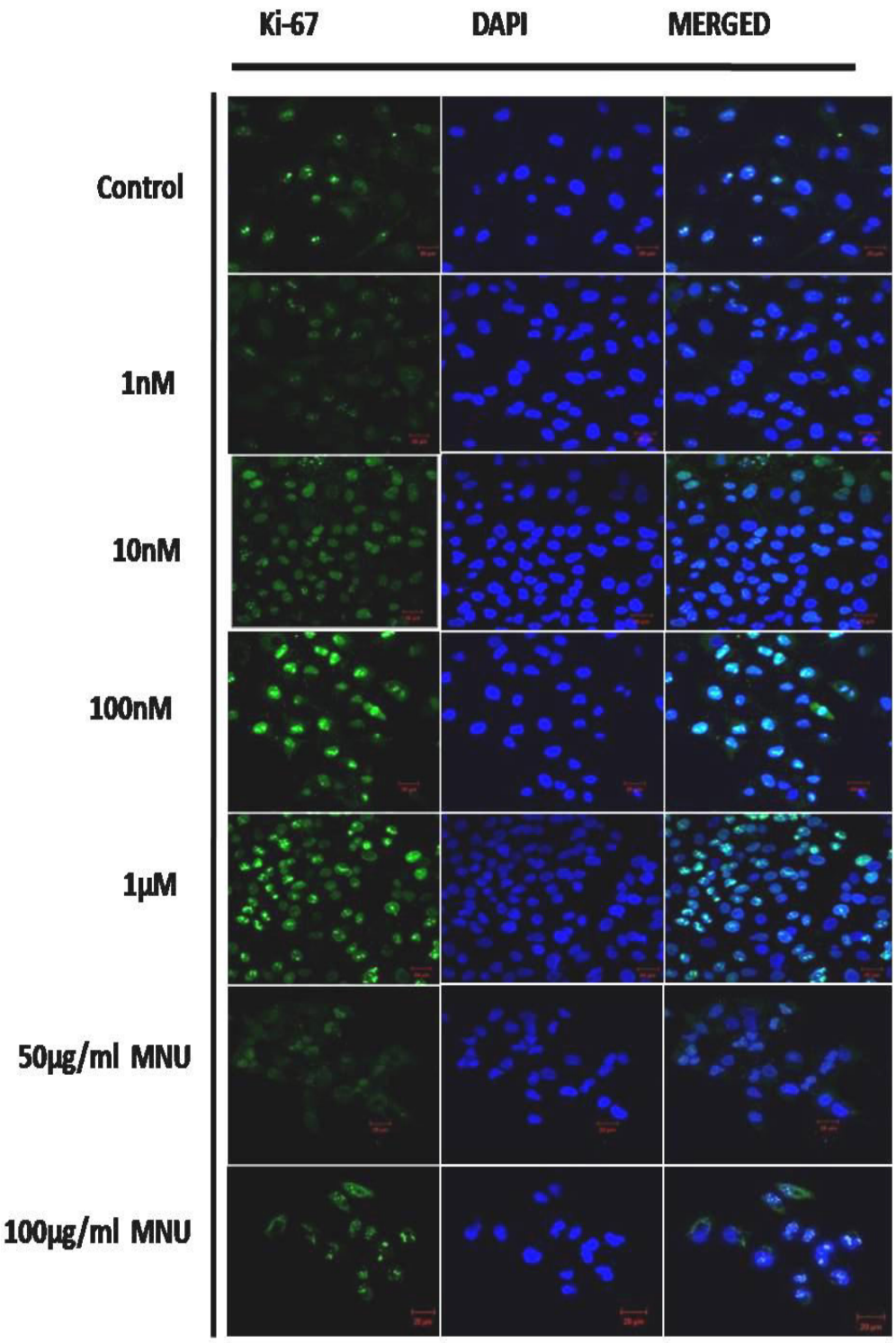


Figure 4(II). 12*: Immunocytochemistry of ki-67 in Cd and MNU treated cells.(*Scale bar: 20µm)

4(B).3.9- Migration Assay:

To assess the migratory potential of the Cd and MNU treated cells, migration assay was performed. A Wound was created on monolayer of cells before Mitomycin C treatment. 1µM of Cd treated cells depicted migratory potential as compared to control. Moreover, the other groups of Cd and MNU treated cells showed less migratory potential as compared to 1µM Cd treated cells. Images were captured and analyzed using Nikon NIS- Elements software™.

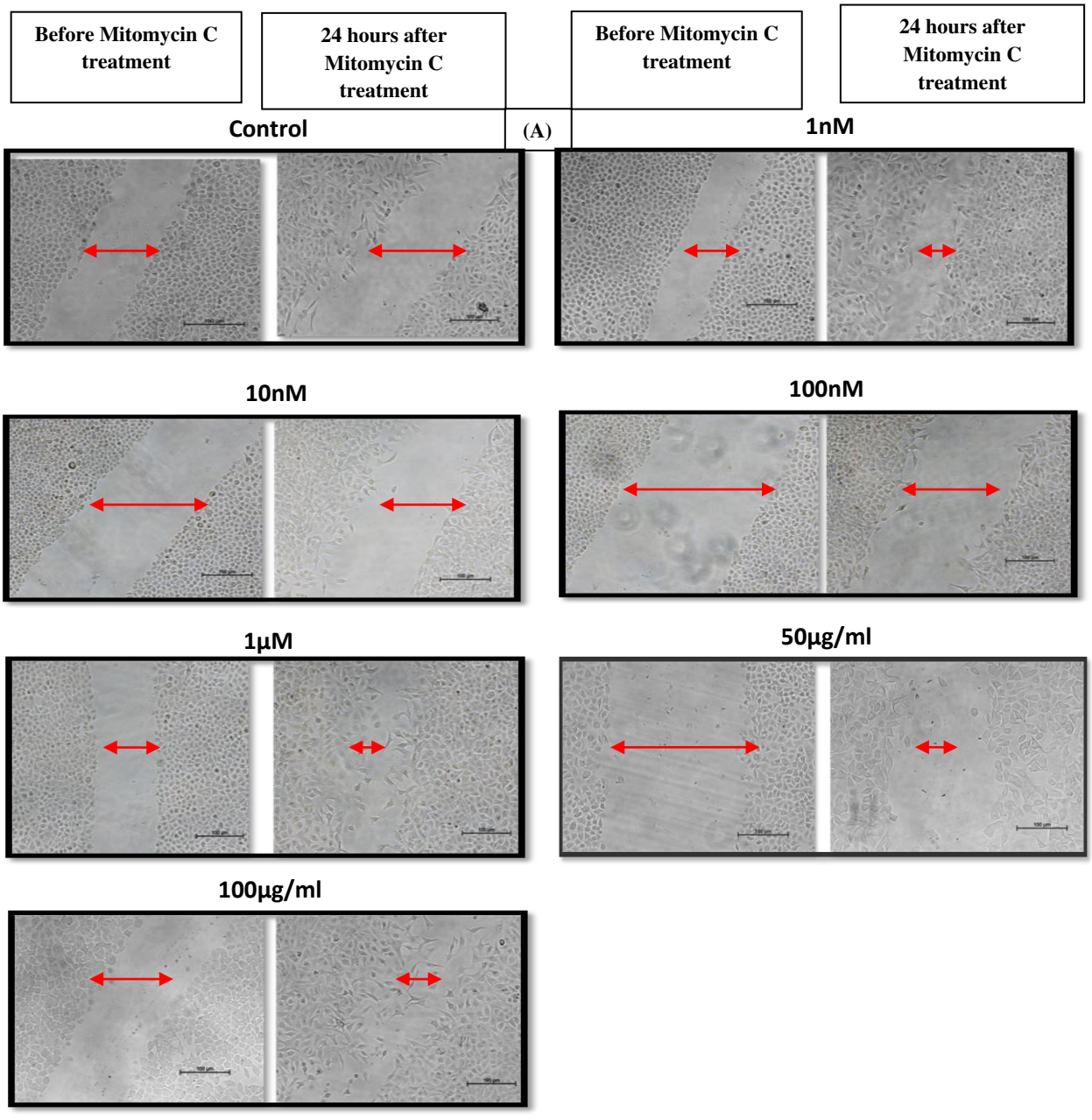


***Figure 4(B). 13**: Wound healing assay in (A) Cd treated cells and (B) MNU treated cells. Wound scratch is done by 10ul tip.(Scale Bar - 100µm)*

4(B).3.10 Zymogyraphy:

MMPs are secreted enzymes that selectively degrade the extracellular matrix and have been implicated in tumor cell invasion. Zymographic analysis revealed significant increased in secretion of active MMP-2 and MMP-9 in all treatment groups. Our results are similar with other previous available reports. The hyper secretion of the MMPs is consistent with the highly aggressive nature of the tumors derived from malignant transformed cells

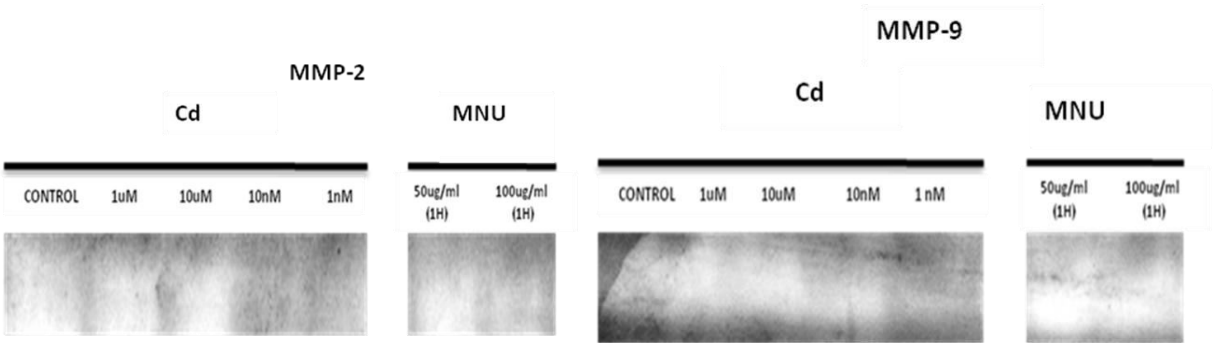


Figure 4(B). 14: *Zone of clearance in Gelatin Zymography gel for MMPs*

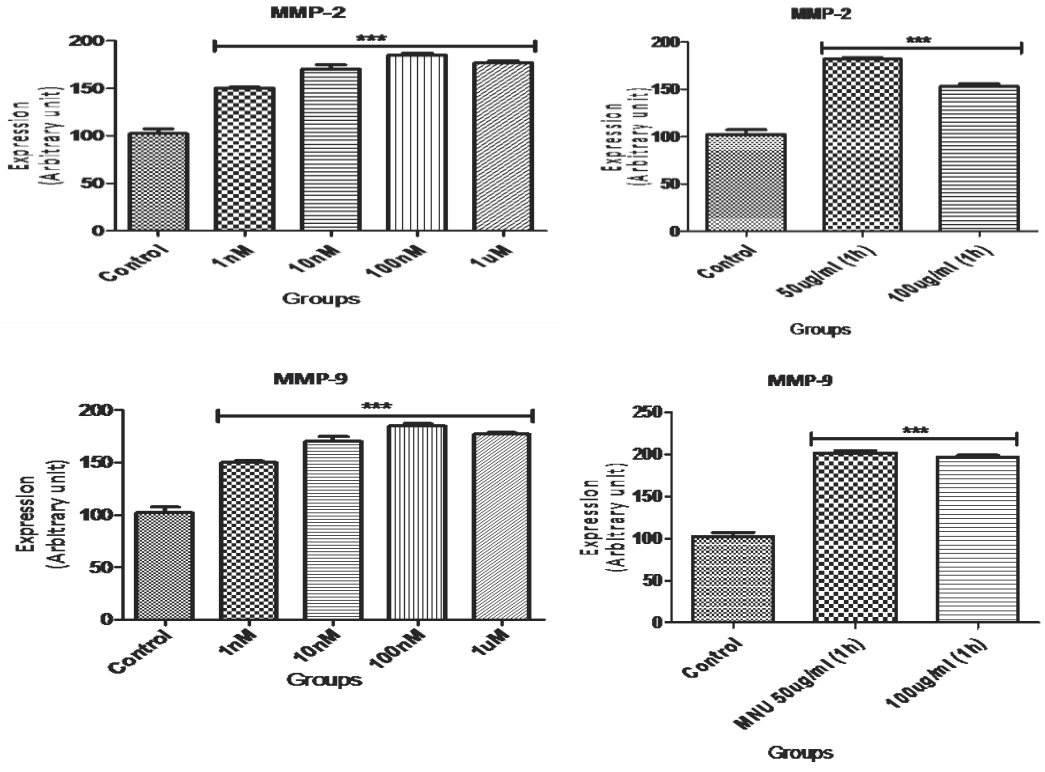


Figure 4(B). 15*: Zymography analysis for MMPs .Results represent mean of three observation ± SEM ***p<0.001 Control vs Cd /MNU treated.*

4 (B).4 Discussion

Cd is depicted as major factor for carcinogenesis in many tissues and playing pivotal role in the prostate cancer as well (Waalkes 2003). Population based cohort studies demonstrated increased risk of PCa in BPH patients (Orsted et al. 2011) Several reports also showed *in vitro* transformation of human normal prostate epithelial cells into cancerous prostate cells (Achanzar et al. 2001; Nakamura et al. 2002). However, the underlying mechanisms involved in Cd carcinogenesis remain unclear. Our previous lab data showed positive association of Cd concentration with increased severity of BPH pathogenesis(Pandya et al. 2013). In the present study we successfully demonstrated malignant transformation of human BPH epithelial cell line by chronic exposure to Cd to understand the link between BPH to PCa.

The dose dependent study of Cd on BPH epithelial cell line determined cytotoxic dose concentration. Further we used lower concentration to understanding pathogenesis. Growth kinetics experiment showed increase in growth rate of 1µM Cd treated and 1 hour MNU (50 and 100ug/ml) treated cells. Similar results were obtained by Benbrahim.T et al 2007 in CTPE cells (Cd derived malignant transformed human prostate epithelial cells) compared to RWPE-1 cell line (non-tumorigenic human prostate epithelial cell line) (Benbrahim-Tallaa et al. 2007).

Cd is potent oxidative stress inducer by enhancing the production of H_2O_2 which leads to DNA damage, chromosomal aberration, gene mutation and morphological transformation (Zhong et al. 1990; Koizumi and Li 1992; Meplan et al. 1999; Liu and Jan 2000) with irregular large size nucleus (Baba and Catoi 2007). In present study, BPH cells with transformation showed increased cell size with distorted epitheliod shape in 1µM Cd and MNU treated cells. Beside these Cd is also known as spindle poison, which depolymerizes microtubule and actin filament followed by microtubules disassembly and cause cell cycle arrest (Perrino and Chou 1986; Wang and Templeton 1996; Li et al. 2005). G_2/M arrest was observed in Chinese hamster ovary K1 (CHOK1) cells with Cd treatment (Yang et al. 2004). In another report, where, normal prostate epithelial cells were exposed to $CdCl_2$ (2.5uM) for 32 hours, transformation into cancerous cells also showed G_2 phase arrest and decreased G1 phase (Bakshi et al. 2008). Similarly, our results of cell cycle analysis confirms G_2/M arrest with 1µM Cd and both 50 and 100 µg/ml concentrations of MNU treated cells, suggesting progression of BPH transformation into cancer.

To further substantiate the role of Cd in cancerous conversion, gene expression and immuno-cytochemistry were performed. The expression profile of AR, PSA, ERβ and 5α Reductase genes were found significantly increased in Cd treated cells. MNU treated cells also showed similar trend of AR, PSA, ERβ genes expression. Cd interacts with the hormone-binding domain of AR and ER, (Stoica et al. 2000) and activates nuclear translocation of AR at higher concentration (Martin et al. 2002).Increased expression of AR in MNU exposed tumor derived cell lines has been reported earlier(Bentel et al. 1999). In concordance with the above literature, our Immunocytochemistry data depicted increased nuclear localization of AR and cytoplasmic level of ER-α with the increased Cd concentration, providing the fact that Cd would probably mediate its effect by binding to the AR with more affinity, causing nuclear localization and induced malignancy in BPH cells. It has been reported that PSA gene expression increases with different stages of PCa progression (Gurova et al. 2002). ERβ expression is reported in early phases of low grade PCa, perhaps, to counteract proliferative stimuli (Signoretti and Loda 2001). Bennbrahim et al. 2006 showed higher expression of ERβ gene in CTPE cells as compared to normal prostate epithelial cells (Benbrahim-Tallaa et al. 2007). In our study, ERβ expression was significantly increased in the Cd and MNU treated cells compared to control, strengthening our hypothesis that Cd induces conversion of BPH into PCa. Report from Meplan et al 1999, indicated that Cd replaces Zn in p53 and impairs its DNA binding activity and subsequently cause cell cycle arrest after DNA damage (Meplan et al. 1999), thus, affecting key regulatory factors in prostatic epithelium, which potentially result in aberrant gene expression and cellular transformation. Expression of 5α reductase in Cd treated cells exhibited increased expression suggesting more conversion of testosterone to DHT which has more androgenic activity. However, MNU treated cells demonstrated significantly decreased expression of 5α Reductase suggesting different mechanism. Similar Luo and colleagues found no significant difference in the 5α Reductase mRNA level between PCa, BPH and normal samples (Luo et al. 2003). Further Wako *et al.*, found no significant change in both 5α Reductase-I and II level between localized prostate cancer samples and normal prostate tissue (Wako et al. 2008). Thus, 5α Reductase level with PCa development and progression is controversial.

Ki-67 is proliferation marker of epithelial cells and depicts mitotic activity. Report from PCa tissue showed higher ki-67 expression compared to BPH tissue (Davidsson et al. 2011). Immunocytochemistry of the treated cells demonstrated increased nuclear expression of ki-67 in

the Cd treated cells in dose dependant manner as compared to MNU treated cells. p63 is basal stem cell marker and homologue of a tumor suppressor gene p53, its expression has been observed to be decreased in PCa which can be used as a differential diagnostic marker (Signoretti et al. 2000). Our results also demonstrated decreased protein expression of p63 with higher concentration of the Cd and MNU treated BPH epithelial cells, confirming loss of basal cells, a hall mark of PCa. Further, confirming cancerous conversion of BPH cells.

Cancerous cells have migratory potential. To elucidate invasiveness of the Cd treated BPH cells, we performed cell migration assay. 1µM Cd treated cells showed migratory potential similar to the MNU treated cells. It is known that cell migration is central to homeostatic processes such as the repair of injured tissues. It also contributes to various pathologies including tumor formation and metastasis (Horwitz and Webb 2003) further suggesting transformation.

Matrix Metallo proteinases (MMPs) are known to secrete in its active form and responsible factor of cancer invasion by degrading the extracellular matrix and basement membrane of surrounding cells (Curran and Murray 2000). Increased level of MMP-2 (62kDa) and MMP-9 (82kDa), also known as Gelatinase-A and Gelatinase-B respectively (Piedagnel et al. 1999), is linked with prostate cancer progression (Webber et al. 1995; Stearns and Stearns 1996). In this context, increased secretions of MMP-2 and MMP-9 are demonstrated in present study which is consistent with other reports from human prostate tumors (Festuccia et al. 1996; Achanzar et al. 2001). Additionally, an increased ratio of MMP-2 and 9 activity has been observed in cells from PCa when compared with cells from benign tumors (Festuccia et al. 1996), providing persuasive evidences that Cd can enhance tumor progression.

The biokinetics of the Cd, is known to exhibit for its accumulation and an extremely long residence time in human body. It is earlier reported that chronic exposure of Cd can induce malignant transformation of normal human prostatic epithelial cells *in vitro*, producing highly aggressive tumors upon inoculation into Nude mice (Achanzar et al. 2001). The present study fortifies the human epidemiological data by providing clear and compelling evidences that human prostate epithelial cells are susceptible to Cd- induced malignant transformation due to environmental exposure even, if an individual receives small but repeated exposure.

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