CHAPTER-5

5. TO ELUCIDATE THE ROLE OF ANDROGEN RECEPTOR IN THE MAINTENANCE OF STEMNESS IN BENIGN PROSTATE HYPERPLASIA DERIVED EPITHELIAL STEM CELLS.

5.1 Introduction

AR is a transcription factor (TF), that drives the growth and proliferation of prostate cells function as well as the pathologies of the prostate gland.[1] Depending on the severity and stage of PCa disease, AR has been discovered as one of the key transcriptional regulators of stem cells. Cellular expression pattern of AR revealed its abundant expression in luminal cells of prostatic epithelial compartments. However, AR is expressed at lower levels in the basal stem cells (BSCs) of the prostate gland. Interestingly, knocking down AR in BSCs formed malignant lesions in the mouse prostate suggesting an indispensable presence of low AR to maintain tissue homeostasis.[2] A progressive increase of AR expression in BSCs leads to its differentiation into luminal epithelial cells.[3] These BSCs express specific markers like CD133, CD44, CD49f, Sca-1, CD117, Trop2, ΔNP63, LGR4 etc.[4, 5] As discussed in the previous chapter, AR majorly plays a negative regulatory role on the majority of stem/progenitor markers expressed in the prostate cells. Besides BSCs, luminal compartment also exhibits the expression of stem as well as differentiated luminal cells characteristics. The studies have reported the expression of $CK8^+/CK18^+/NKX3.1^+/AR^+$ with low levels of Basal markers $P63^+/CK5^+/SCA1^+/CD49f^{low}/LGR4^+$ in the Luminal Progenitors (LPs) of the mouse and human prostate.[6] Lately, it has been found that overexpression of these stem/progenitor markers acts as the potent inducer of PCa and aggression.[7, 8 9] However, the presence of AR and its transcriptional activity in the LP cells are ambiguous and largely unknown.

Leucine-rich repeats G-Protein Coupled Receptor-4 (LGR4) is a cell surface G-protein coupled receptor, which is one of the most fundamental proteins for the improvement of the liver, kidney, digestive system, bone, regenerative tract, and eye. *LGR4* knockout mice showed 60% embryonic lethality and surviving 40% of mice had multiple developmental deficiencies and male sterility.[10] LGR4 is a Wnt signaling activator that induces β-CATENIN/TCF/LEF mediated transcription in the target cell nucleus dependant canonical Wnt signaling pathway as well as PI3K/AKT signaling in the prostate BSCs.[5, 11, 12] LGR4 mediated activation of β-CATENIN regulates the proliferation and differentiation of

prostate stem cells with its transcriptional activity, suggesting its potential role in the stem cells for the growth and development of the gland.[5] Also, the presence of LGR4 in the LPs became evident by Karthaus *et al*. which has also been recognized as one of the key drivers of PCa progression and metastasis by increasing the CSC phenotype.[9, 13] In the previous chapter, we have demonstrated the increased levels of LGR4 and its correlation with the AR expression in the BPH and PCa patient tissues. However, the role of LGR4 in the development of BPH is largely unknown and unexplored at this point.

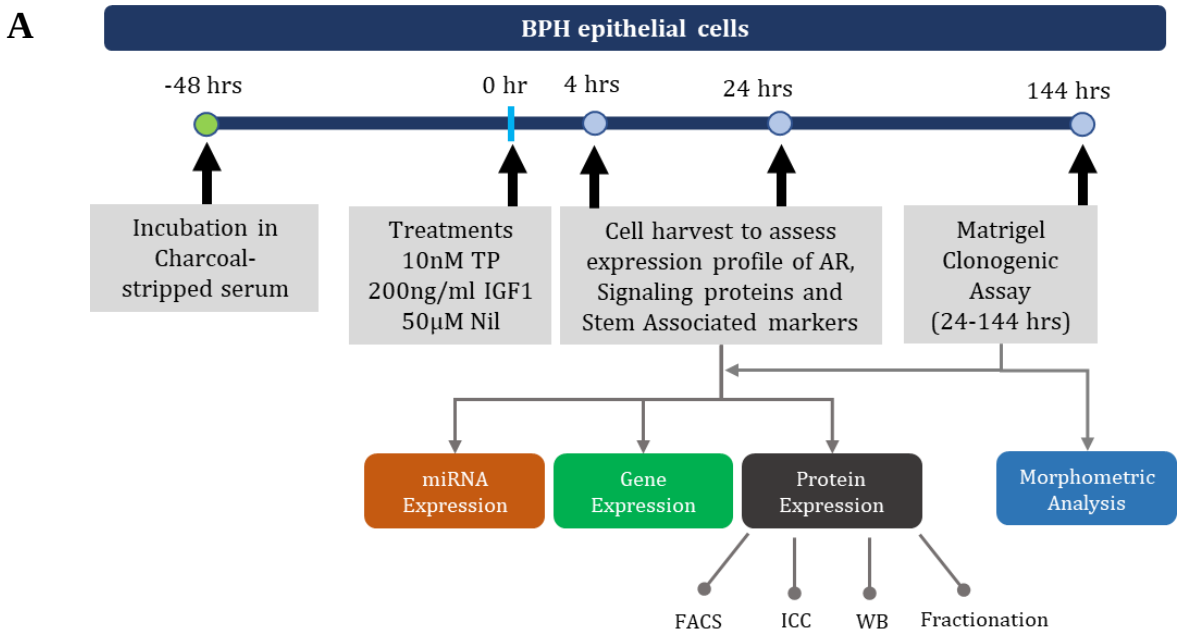
P63 is a prostate BSC specific marker, which belongs to p53 family proteins. P63 has six distinct isoforms; two major isoforms with full-length transactivating (TA) P63 and truncated-NTD (ΔN) P63 proteins each of which have three α, β, γ isoforms. Among these six TFs, TAp63 proteins control the regulation of apoptosis and senescence similar to P53 protein, whereas ΔNp63 TFs regulate fate and proliferation of BSCs in the prostate gland.[14] The presence of P63 isoforms in the stem/progenitor cells has been identified as a vital factor in maintaining homeostasis in the prostate gland. $\Delta NP63^{+}$/Cre knock-in mice study revealed that all epithelial cell types (BSCs, LPS, Luminal cells) of an adult prostate are originated from $\Delta Np63^{+ve}$ stem cells.[15] Additionally, ΔNP63α is the most predominant isotype among ΔNP63α/β/γ variants expressed in prostate BSCs in normal and tumor conditions.[16] Lately, it has been discovered that the expression of P63 is not only restricted to BSCs but also expressed in the LPs An intermediate cell type between BSCs and secretory luminal cells, LPS found to express high levels of luminal markers $CK8^{+}/CK18^{+}/Nkx3.1^{+}/AR^{+}$ with low levels of Basal markers $P63^{+}/CK5^{+}$ along with low androgen dependency.[6] Kwon *et al* also delineated another type of mouse luminal progenitor subpopulation during mouse regeneration experiments which expressed $Sca1^{+}/CD49f^{low}$ that highly expressed AR but devoid of NKX3.1. The organoids produced by these cells were $CK5^{+}/P63^{+}/CK8^{+}/AR^{+}$, suggesting bidirectional differentiation capacity of certain cell populations within the gland. The organoids with the LP phenotype likewise show prostate regeneration potentials *in vivo*.[8] The expression of ΔNP63 TFs has relied on the prevailing cell type within the heterogeneous tumor microenvironment during the PCa. In luminal PCa tumors, ΔNP63α articulation phases out from tumor cells, yet it was increased in the metastasized PCa tumor cells to promote adhesion of CSCs to the bone *in vivo*.[14] As discussed in the previous chapter, the expression of P63 is low in adenocarcinoma patients with higher AR levels, but comparatively increased in the higher

AR expressing BPH patients The importance of AR and P63 co-articulation is a novel finding in the BPH patients and its investigation will be fascinating to understand the novel aspects of AR in LPs.

In this context, our previous lab study showed a high expression of AR, P63, and pluripotent stem cell markers with multi-lineage differentiation capability of BPH epithelial stem cell.[17] Further, a positive correlation of AR with LGR4 and β-CATENIN was determined in BPH and PCa patients in the previous chapter. We have also demonstrated that BPH patients with higher AR protein levels exhibited increased LGR4/β-CATENIN/ΔNP63α. Furthermore, benign epithelial stem/progenitor cells used in the present study have a distinct protein expression contour with co-expression of AR, LGR4, and ΔNP63α. Consequently, the incorporated investigation intends to comprehend the transcriptional role of AR and its implications on LGR4 and ΔNP63α in BPH pathogenesis.

5.2 Plan of work

Epithelial cells isolated previously from BPH patient tissue were used in the present study. To understand the role of AR in prostate stem/progenitor cells, we have characterized the stem-associated CD49f, CD117, CD133, LGR4, and P63 markers in the BPH epithelial cells. After confirming the cells as the stem/progenitor epithelial cells, we have assessed the effect of activation and inhibition of AR to monitor the changes on the stem/progenitor profile by flow cytometry. To understand the effect of ligand-dependent/independent activation and inhibition of AR, nuclear localization of AR and euchromatin accessibility was evaluated in the stem/progenitor cells. Further, the stem-associated miRNA, genes, and protein expression in the BPH stem/progenitor cells have been evaluated. To understand the molecular role, AR-mediated interaction with cell signaling proteins and direct regulation of gene and miRNA expression with its transcriptional ability was assessed with Immunoprecipitation (IP) and Chromatin Immunoprecipitation (ChIP) studies.



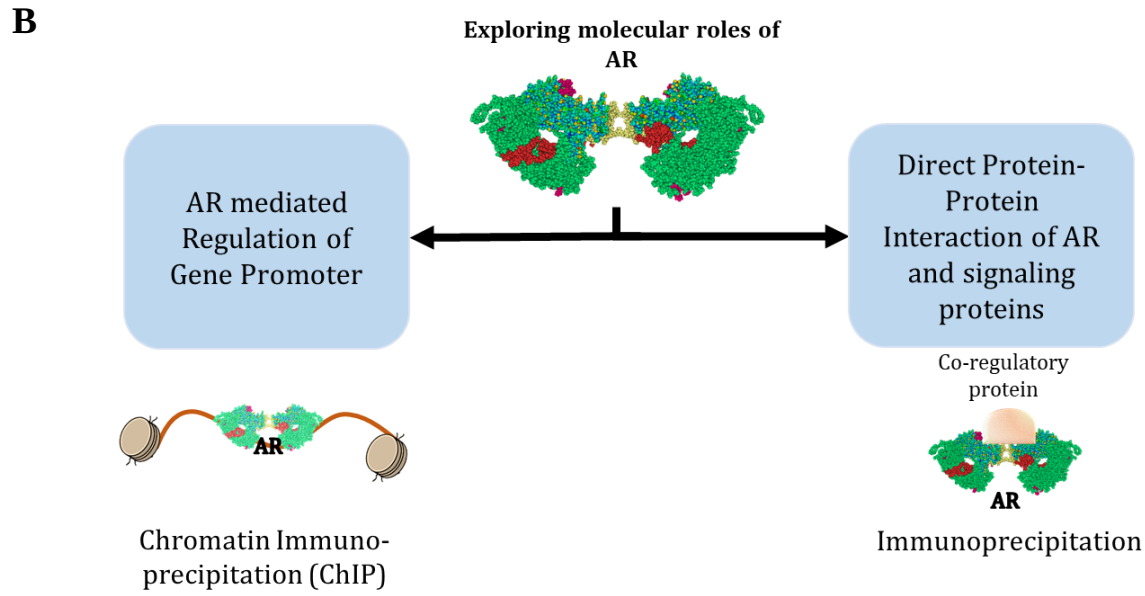


Figure 5. 1: Plan of work. (A) The schematic representation of the treatment exposure with brief experimental design. **(B)** Graphical elucidation of the exploration of AR regulated molecular events in BPH stem/progenitor cells.

5.3 Results

5.3.1 The BPH epithelial cells exhibit the expression of stem cells associated surface markers: CD49f (α6-Integrin), CD117, CD133 (Prominin-1), LGR4, and P63.

The characterization of BPH epithelial cells showed the presence of the stem cell surface markers. The BPH epithelial cells used in the present study are $CD49f^{+ve}$ (99.9%), confirming the presence of BSCs. Furthermore, the cells also presented the differential expression of CD117 (87.8%), CD133 (33.9%), and LGR4 (47.8%) stem/progenitor associated surface receptors. Additionally, the cells also expressed key BSC marker, P63-4A4 (30.5%) in the BPH epithelial cells. *(Figure 5.2)* Thus, the presence of CD49f, CD117, CD133, LGR4, and P63 markers suggested the stem/progenitor state of the BPH epithelial cells.

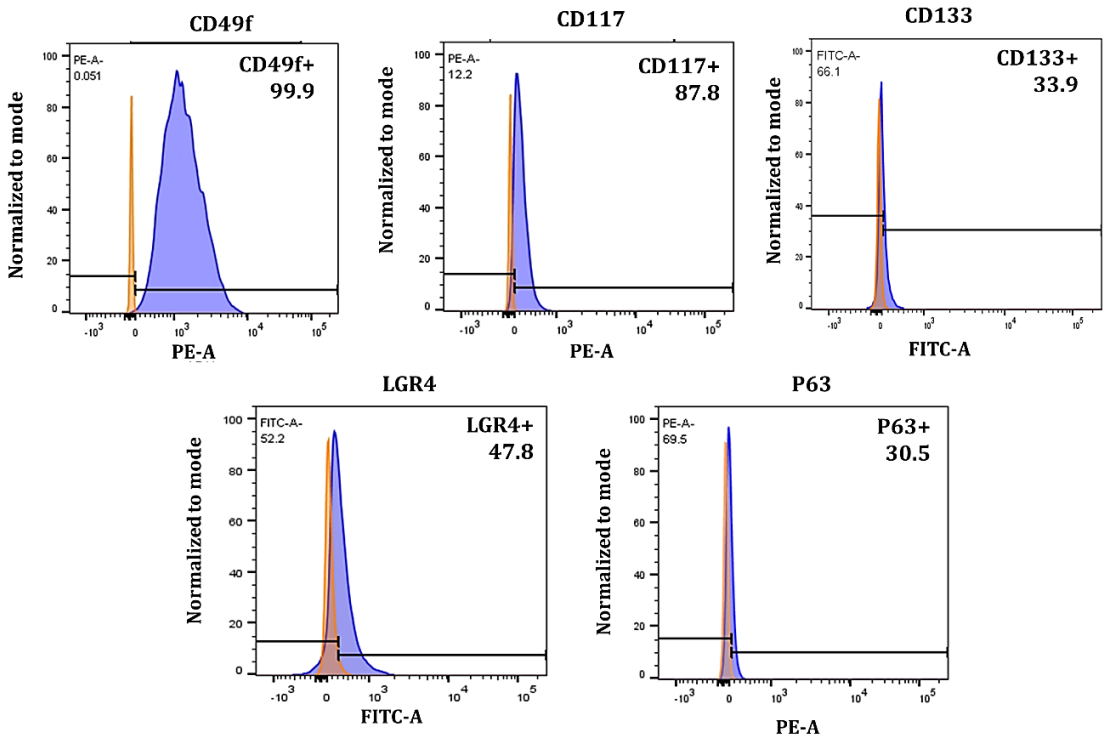


Figure 5. 2: Flowcytometric analysis of the stem cell markers. The graph represents the expression of BSC surface receptors, CD49f (99.9%), CD117 (87.8%), CD133 (33.9%), LGR4 (47.8%), and P63 (30.5%) in the BPH epithelial cells. Minimum 10,000 events were captured on the flow cytometer.

5.3.2 Activation of AR leads to an increase in LGR4$^+$, CD133$^+$ and P63$^+$ cell population in BPH derived epithelial (stem/progenitor) cells.

As the cells are exhibiting the stem/progenitor phenotype, we aimed to explore AR activation and inhibition mediated changes in the stem/progenitor phenotype in BPH stem/progenitor cells. The epithelial (stem/progenitor) cells were subjected to the treatments of 10nM Testosterone Propionate (TP) (AR activator) and 50uM Nilutamide (AR inhibitor) in charcoal-stripped serum (CSS) for 24 hours. The growth medium supplemented with CSS declined the percent population of CD117 (59.9%), CD133 (10.8%), LGR4 (49.4%) and P63 (5.5%) as compared to the media supplemented with FBS (section 5.3.1), suggesting a vital role of hormones and growth factors that were confiscated during the charcoal stripping process.

Treatment with TP in CSS showed an increased percent cell population of basal specific surface markers, CD117 (68.2%) CD133 (22.3%), and LGR4 (49.4%) as compared to control (CSS). Further, treatment with AR inhibitor, Nil, reduced the percent cell populations of CD117 (53.5%), CD133 (6.20%), and LGR4 (18.6%) as compared to

control treatment. The percentage of P63-4A4 positive population slightly increased with TP treatment (7.8%) and decreased with Nil treatment (2.5%). *(Figure 5.3)* However, the expression of CD49f remained unaffected from AR activation and inhibition in BPH stem/progenitor cells. Thus, the alterations in the CD133, CD117, LGR4, and P63 BSC percent populations in the presence of AR activator and inhibitor suggest a plausible regulatory role of AR in stem cells during BPH condition.

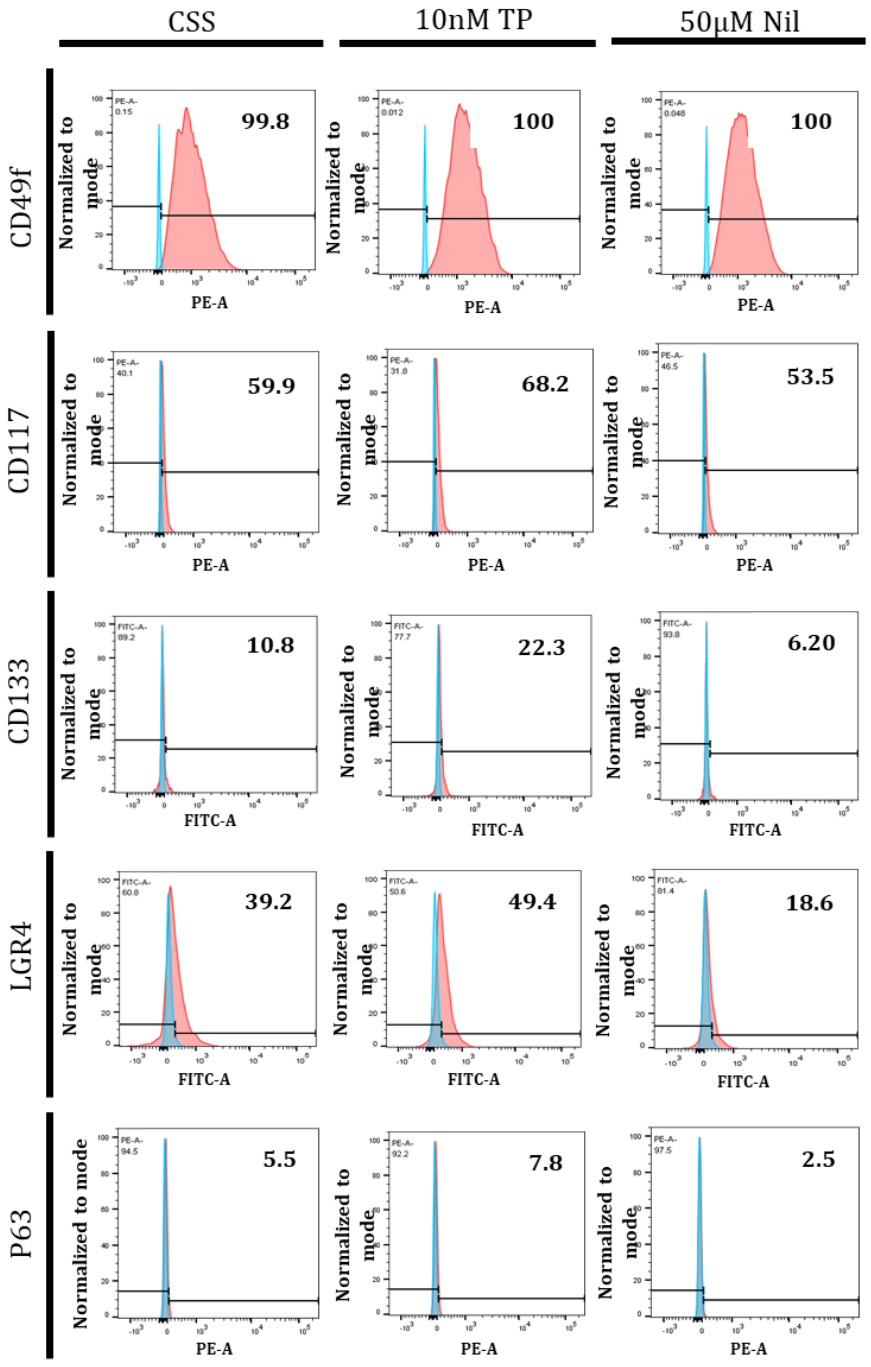
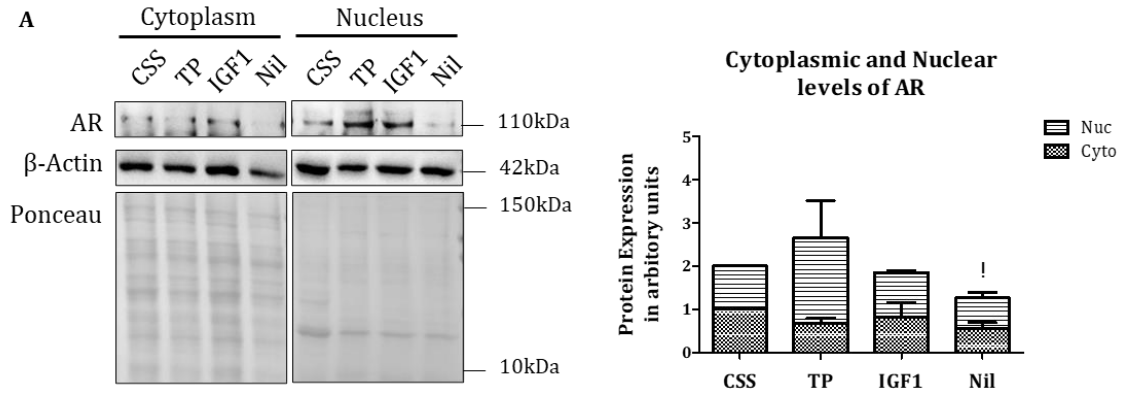


Figure 5. 3: Flow cytometric analysis of stemness specific surface receptors. Expression of CD49f, CD117, CD133, LGR4 (BSC Markers) in BPH stem/progenitor cells treated with TP and Nil; The values represent the percent population of positive cells identified by the flow cytometer.

5.3.3 Testosterone and IGF-1 potentiates nuclear localization of AR and increases its active chromatin accessibility in BPH stem/progenitor cells.

Activation of AR induces Nuclear Localization Signals (NLS) with androgen stimulus and growth factors like IGF-1 through the activation of AKT signaling. Thus, we aimed to investigate the effect of Androgen dependent and Androgen-independent mediated nuclear localization of AR. To assess the AR localization, we performed cytoplasmic and nuclear fractionation in BPH stem/progenitor cells exposed to 10nM TP (direct AR activator), 200µg/ml IGF-1 (indirect AR activator) and 50µM Nil (direct AR inhibitor). Upon TP mediated activation depicted the elevated nuclear AR localization in the cells. (*Figure 5.4A*) Additionally, we have evaluated low, intermediate, high euchromatin accessibility of the AR protein in the cell nucleus under different salt concentrations post-micrococcal nuclease (MNase) digestion. AR was found to be associated with high chromatin accessibility in the BPH stem/progenitor cells. (*Figure 5.4B; right*) The least fraction of AR was observed with the least accessible higher-order euchromatin where the highest AR levels were found with TP treatment. (*Figure 5.4B; left*) The largest fraction of AR was found to be associated with the intermediate accessibility, which is associated with the highest transcriptional activity in the cells due to the presence of active transcriptional machinery. The intermediate accessibility of AR appears to be elevated with TP and IGF-1 stimulation, (*Figure 5.4B; center*) confirming the correlation of nuclear AR with transcriptionally active chromatin upon TP and IGF-1 stimulation.



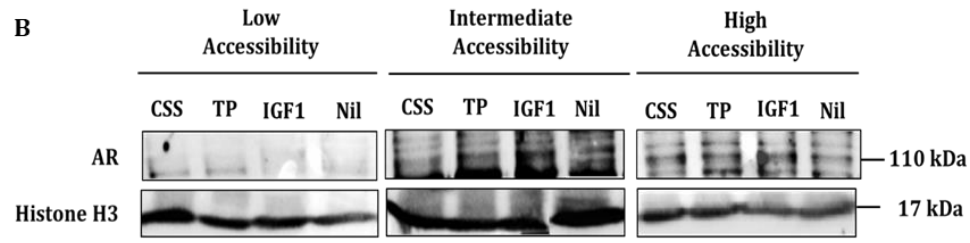


Figure 5. 4: Cellular localization and chromatin accessibility of AR in BPH stem/progenitor cells. (A) Image shows cytoplasmic and nuclear localization (Mean±SEM; n=3; $^{!}p<0.05$ CSS vs treated groups of Cytoplasmic fractions) and **(B)** Euchromatin accessibility (n=1) of AR in BPH stem/progenitor cells exposed to 4-hour treatments of AR agonists and antagonist.

5.3.4 Activation of AR instigated a larger and higher number of tumorsphere formation in BPH stem/progenitor cells.

The sphere formation ability is the inherent characteristics of stem/progenitor cells of the prostate. To evaluate the effect of AR on tumor formation ability of BPH stem/progenitor cells, the cells were cultured in 1:1 mixture of growth media and Matrigel at the periphery of the culture plate. The results showed the formation of tumorspheres in all the treatment groups including control. But, the treatments of TP and IGF-1 formed visibly larger tumorspheres as compared to control and Nil treatment. (*Figure 5.5A Upper Panel*) Further, TP and IGF-1 treatments depicted the highest number of migratory cells from the spheres into the Matrigel as compared to CSS and Nil groups. (*Figure 5.5A-Lower panel*) The average size of the spheres was significantly higher with TP (107.0±1.53µm) and IGF-1 (98.4±1.61µm) treatments as compared to Control (90.1±4.9µm) and Nil (87.2±0.64µm) groups. (*Figure 5.5B*) Further, the number of the larger sphere formation (>150µm and 100-150µm) was found with TP (>150µm: 26±3.2 & 100-150µm: 114.5±8.9) and IGF-1 (>150µm: 20.5±1.85 & 100-150µm: 115.75±8.7) treatments against Control (>150µm: 9.0±2.08 & 100-150µm: 71.5±4.8) and Nil (>150µm: 6.25±1.25 & 100-150µm: 75.0±6.9) groups. Further, increased spheres forming capability of the BPH stem/progenitor cells with Nilutamide treatment (avg size: <90µm) indicate hindered sphere formation due to AR inhibition. (*Figure 5.5C*) Further, evaluation of the expression of AR in the 3D-Matrigel tumors demonstrated a substantial increase in total AR levels with TP treatment (5.8±2.0). Further, the expression of LGR4 is also increased in the spheres treated with TP (1.5±0.08) as compared to the rest of the groups. (*Figure 5.5D*) In summary, the results depicted increased sphere formation ability of the BPH stem/progenitor cells in the

presence of TP and IGF-1. Further, sphere formation is also appeared to be supported by elevated levels of AR and LGR4 in the TP treatment group.

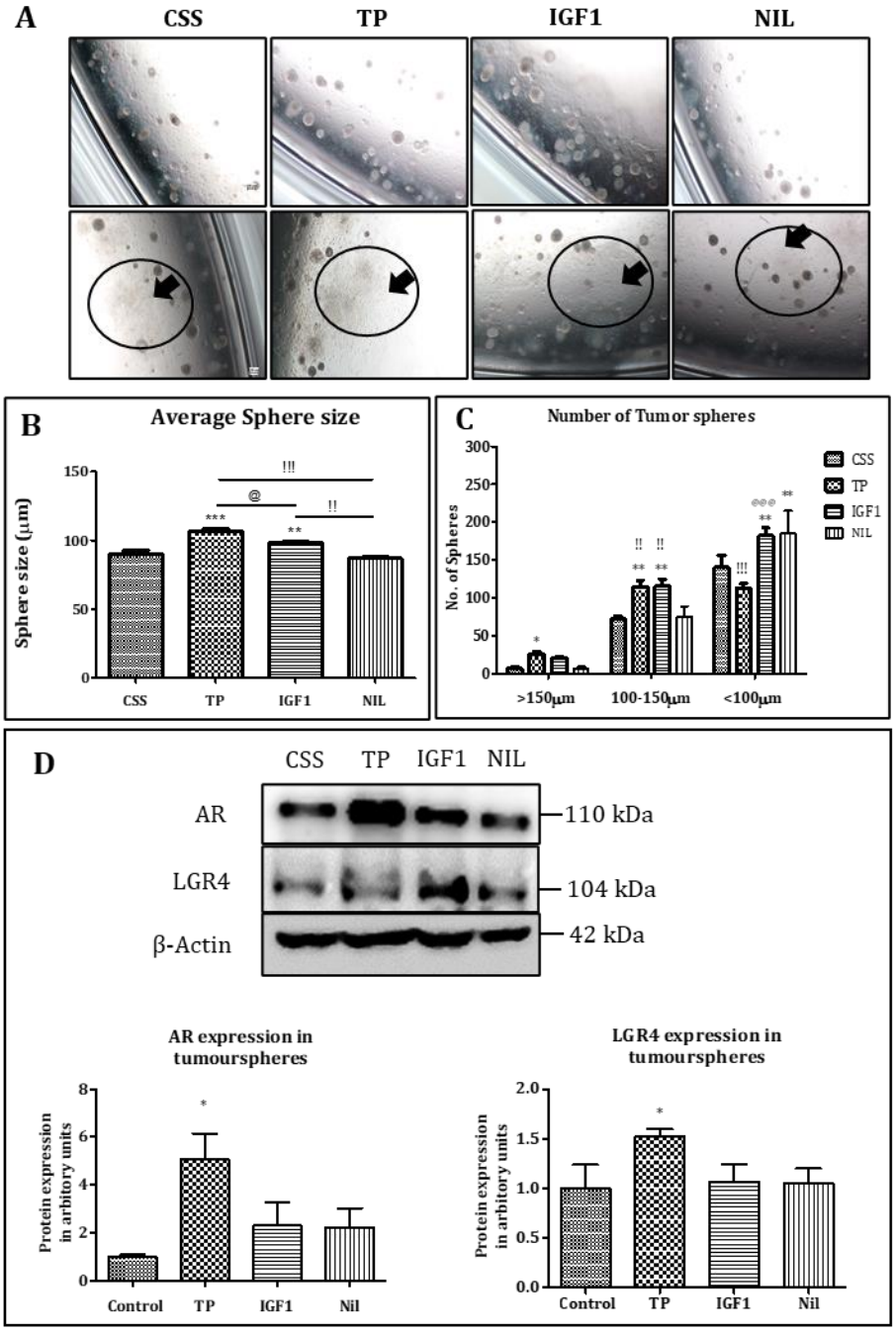


Figure 5. 5: Tumour sphere formation in BPH stem/progenitor cells after treatment with AR agonists and antagonist. (A) Bright-field images of upper panel Matrigel tumorspheres formed by BPH stem/progenitor cells treated with CSS (Control), TP, IGF-1, and Nil and lower panel demonstrate migrated BPH stem/progenitor cells from the spheres (black circle and arrow) after the treatment of CSS (Control), TP, IGF-1, and Nil. Images were captured with 4X objective (Scale- 100µm); **(B)** The graph represents the average size of the tumor formed in >150µm, 150-100µm, and <100µm groups in TP, IGF-

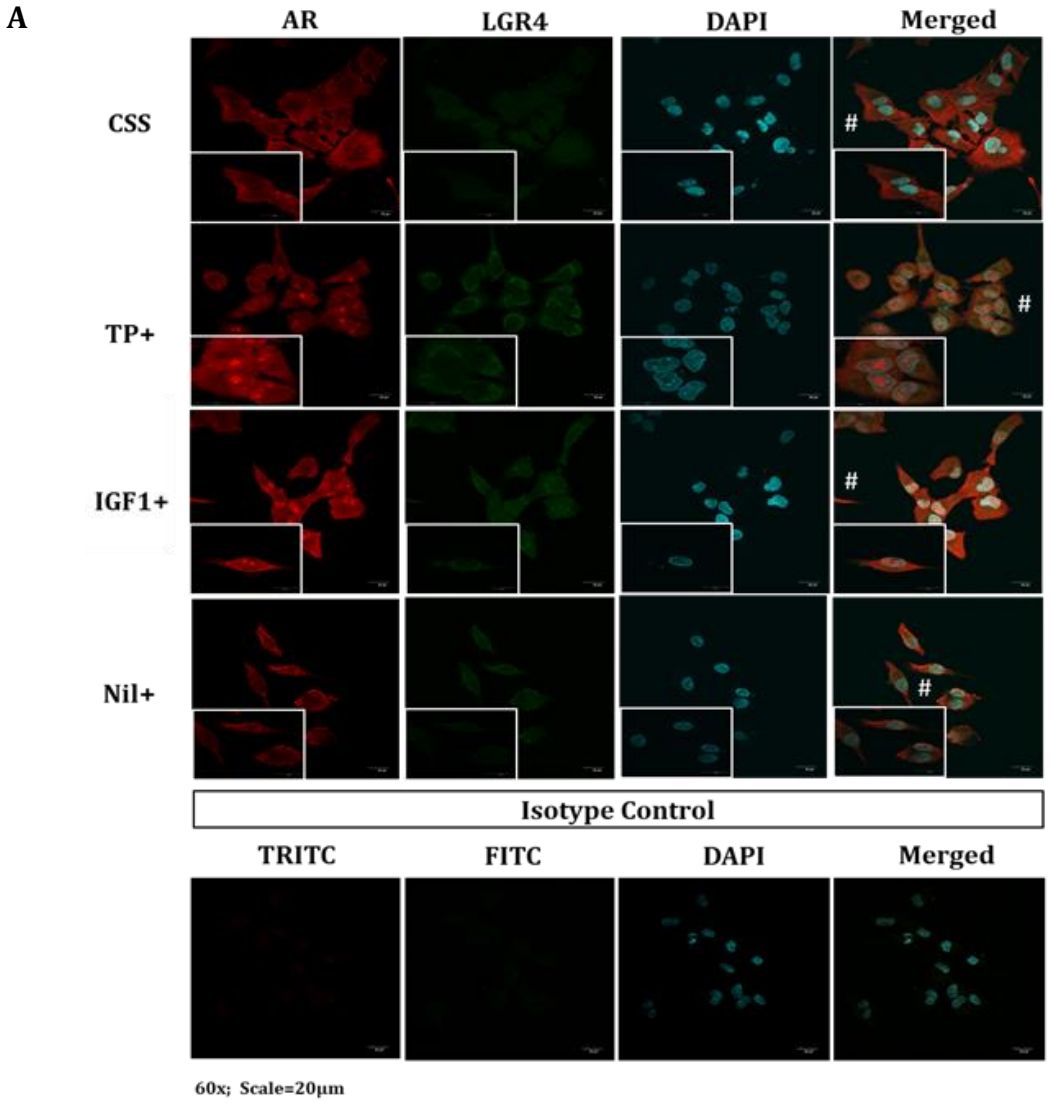
1, and Nil to BPH stem/progenitor cells. Bars represent Mean±SEM (n=4). *p<0.05 Control vs Treatment groups, $^{!}$p<0.05 TP vs Nil groups. **(C)** The graph represents a total number of tumorsphere formed in >150µm, 150-100µm, and <100µm groups with the treatment of TP, IGF-1, and Nil to BPH stem/progenitor cells. Bars represent Mean±SEM (n=4). **p<0.01 Control vs Treatment groups, $^{!!}$p<0.01 TP vs Nil groups. **(D)** Western blot analysis of AR and LGR4 protein expression in the BPH stem/progenitor tumorspheres isolated from Matrigel. The graph represents the densitometric analysis of AR and LGR4 from the western blot image. Bars represent Mean±SEM (n=4). *p<0.05 Control vs Treatment groups.

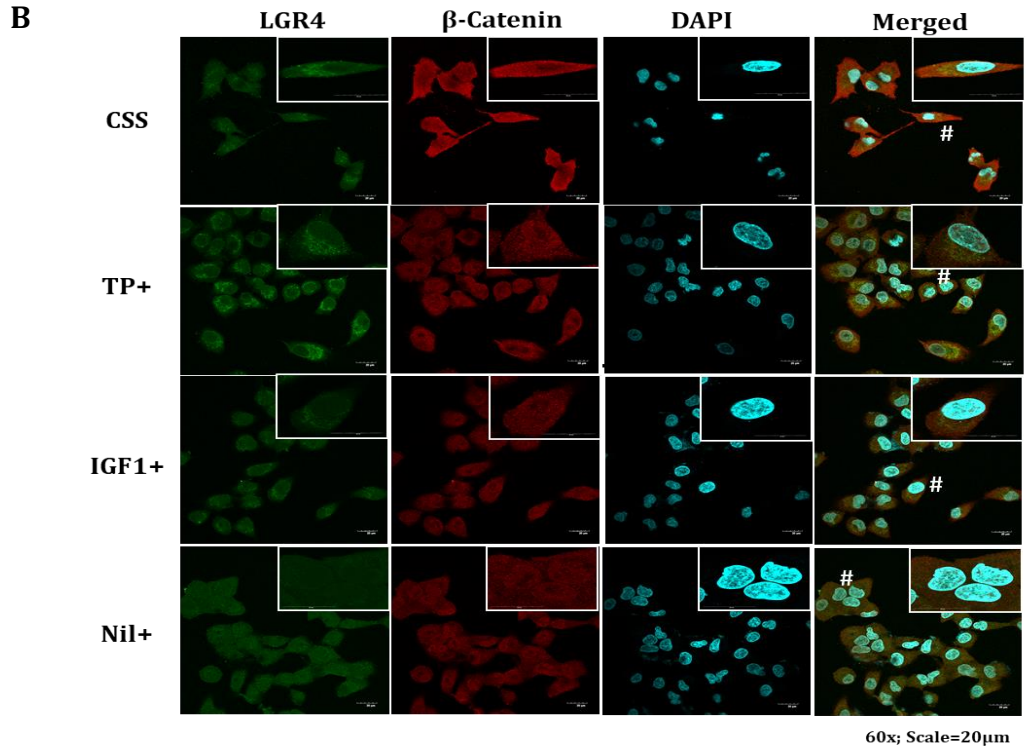
5.3.5 A Ligand-dependent and independent activation of AR increases stem-associated LGR4, β-CATENIN, ΔNP63α expression, and nuclear localization.

To determine the effect of nuclear localization of AR on the expression of LGR4/β-CATENIN/ΔNP63 fluorescent confocal microscopy was performed after exposing the cells with TP, IGF-1, and Nil treatments. The expression of AR was substantially increased in BPH stem/progenitor cells after receiving TP and IGF-1 treatments along with increased nuclear localization of AR (strong nuclear signals) as compared to Control and Nil (hollow nucleus). (*Figure 5.6A-Red*) The strongest LGR4 signals were received with TP treatment, followed by IGF-1 treatment. On the contrary, expression of LGR4 remained low in the Control and Nil treatment groups of BPH stem/progenitor cells. (*Figure 5.6A-Green*) LGR4 potentiates Wnt/β-CATENIN signaling in the cells, leading to enhanced nuclear translocation of β-CATENIN. Total levels of β-CATENIN appeared to be unchanged at the cellular level among the treatment groups. Intriguingly, all the treatments increased nuclear β-CATENIN levels in BPH stem/progenitor cells after 24hr exposure as compared to control. (*Figure 5.6B-Red*). The expression of ΔNP63 variants is the hallmark of the BSC phenotype of cells. BPH stem/progenitor cells exposed to TP and IGF-1 showed increased expression and nuclear localization in the cells. (*Figure 5.6C-Red*) Thus, results indicated that potentiation of AR, through both androgen dependant and independent pathways, leading to increased LGR4 expression and elevated nuclear localization of β-CATENIN and ΔNP63 transcription factors in the BPH stem/progenitor cells.

We assessed the localization of the key basal cells TF, ΔNP63α along with β-CATENIN in the BPH stem/progenitor cells with 4 hours of 10nM TP, 20ng/ml IGF-1, and 50µM Nil exposure using the immunoblotting method. The results demonstrated that IGF-1 exposure increased cytoplasmic levels of β-CATENIN localization, whereas nuclear β-CATENIN levels were not affected by any of the treatment groups. ΔNP63α TF is a potent regulator

of the basal (stem) state of the cells which demonstrated significantly enhanced nuclear localization due to TP mediated AR activation. Whereas, IGF-1 and Nil did not considerably affect the localization of ΔNP63α in BPH stem/progenitor cells. (*Figure 5.6D*) Hence, it is clear that AR activation potentiates nuclear localization of ΔNP63α within the first 4 hours of the treatment in BPH stem/progenitor cells, which could be the vital factor to maintain the stem/progenitor state of the stem/progenitor cells. In contrast to our immunocytochemistry and immunoblotting data of 24-hour exposure, β-CATENIN did not show increased nuclear localization with 4-hour treatment exposure and possibly have delayed transcriptional activity in the presence of androgen.

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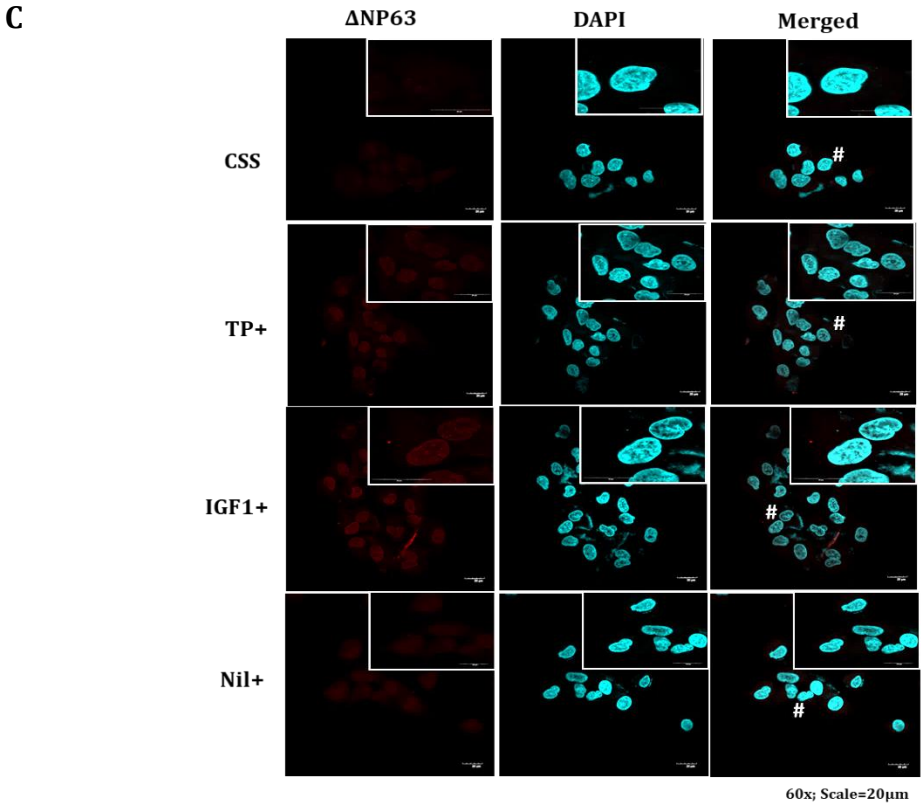
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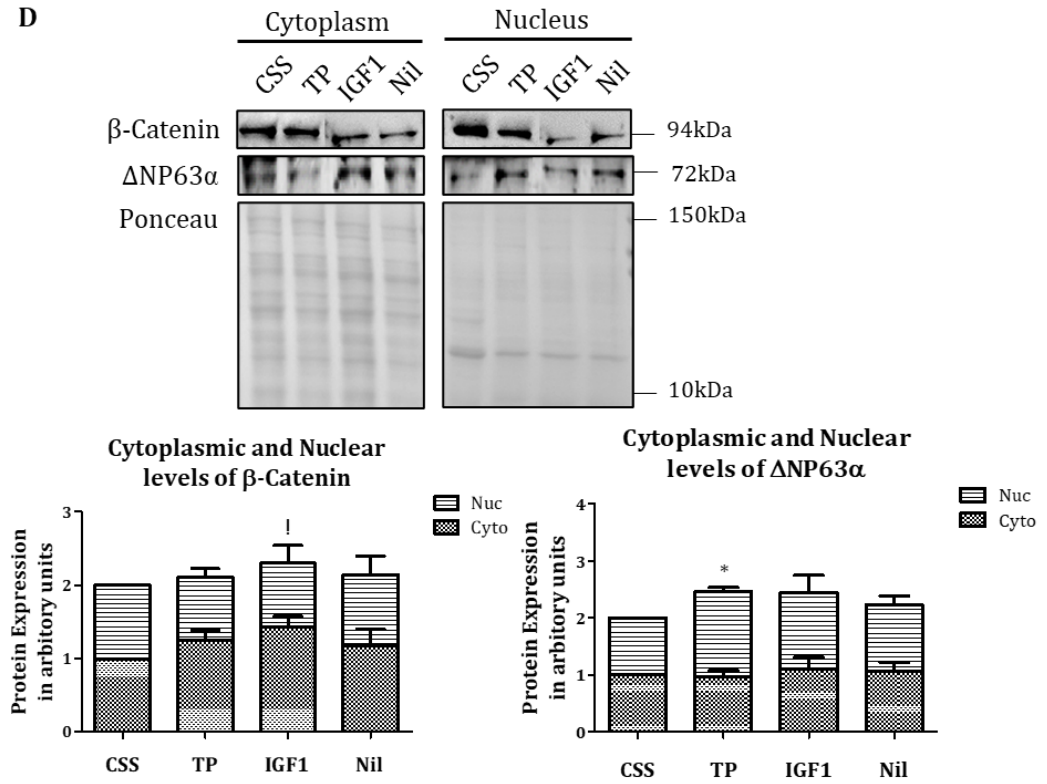


Figure 5. 6: Immunocytochemistry of BPH stem/progenitor cells exposed to TP, IGF-1, and Nil treatments. (A) Representative immunocytochemistry images showing extracellular expression of AR (Red) and LGR4 (Green), isotype control (bottom panel), **(B)** LGR4 (Green) and β-CATENIN (Red), and **(C)** ΔNP63 (Red) in BPH stem/progenitor cells treated with 10nM TP, 200ng/ml IGF-1 and 50μM Nil. Nuclei were stained with DAPI (Blue). Magnified images of specific regions (marked with white '#') were shown in each confocal image shown as a sperate box in the bottom left or top right corner. Images were captured with 60X objective, Scale bar: 20μm. **(D)** Immunoblot images of cytoplasmic and nuclear localization β-CATENIN and ΔNP63α in BPH stem/progenitor cells exposed to 4-hour treatments of TP, IGF-1, and Nil. The graphs indicate the relative protein expression values normalized with ponceau. n=3; Mean±SEM; $^{!}p<0.05$ cytoplasmic levels of control v/s treatment groups, $^{*}p<0.05$ nuclear levels of control v/s treatment groups.

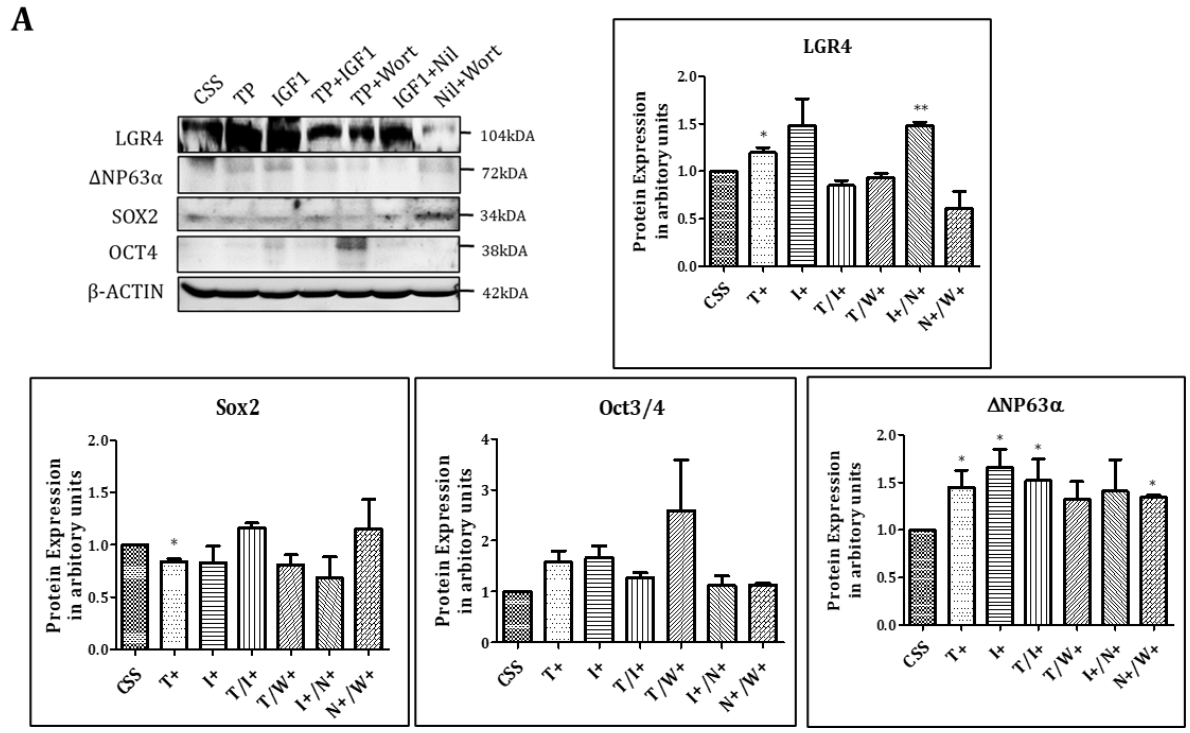
5.3.6 Ligand-dependent and independent regulation of stem/progenitor proteins and miRNA profile

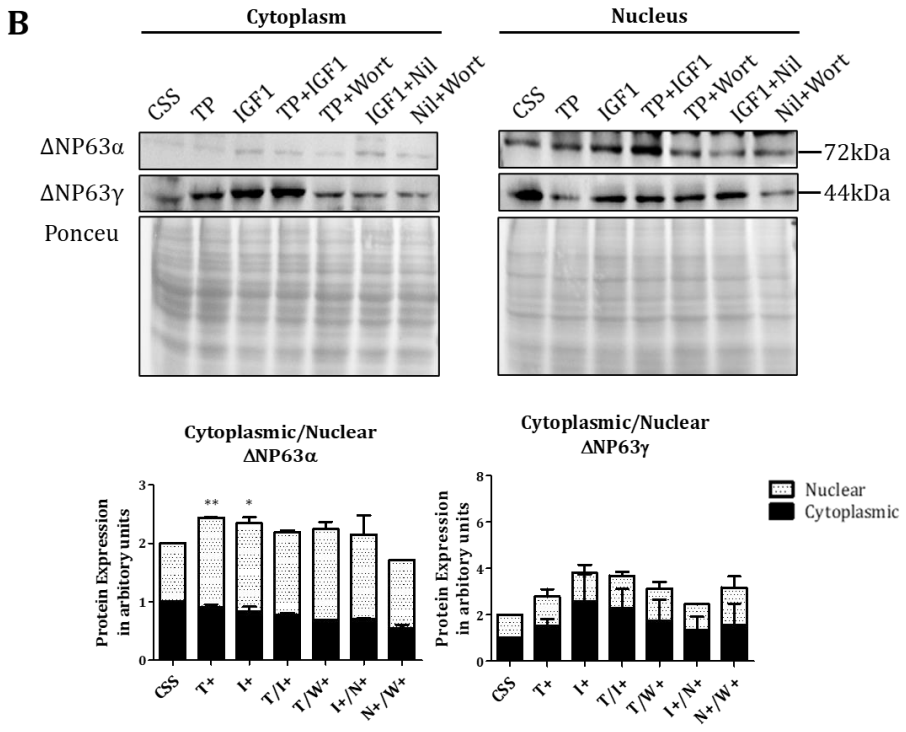
After assessing the immunocytochemistry of the BPH stem/progenitor cells, the protein expression profile of LGR4 and ΔNP63α was also confirmed by immunoblot analysis in presence of AR and AKT activators and inhibitors, TP-IGF-1 and Nilutamide-Wortmannin respectively, along with OCT4 and SOX2 pluripotent stem cell markers. Both LGR4 and ΔNP63α demonstrated increased levels in the BPH stem/progenitor cells exposed to TP

and IGF-1. Moreover, the elevation of LGR4 expression in the presence of IGF-1 and IGF-1/Nil treatments indicates the plausible role of AKT dependent factors as well. (*Figure 5.7A*) In addition to ΔNP63α, SOX2 and OCT4 are important transcriptional regulators of stemness and plasticity of the stem/progenitor state of the prostate cells. Evaluation of the SOX2 expression suggested substantial downregulation with TP treatment and did not exhibit significant changes in other treatment groups. Moreover, OCT4 protein expression did not show significant alterations with the exposure of activators and inhibitors. (*Figure 5.7A*) This pattern is also observed in the cytoplasmic and nuclear localization of ΔNP63-α and -γ TFs, which suggested elevation of nuclear ΔNP63α with TP and IGF-1 treatments but the cytoplasmic restriction of ΔNP63γ proteins with AR activation in all the individual and combinatorial treatment groups. (*Figure 5.7B*) Hence, activation of AR has found to be involved in increasing the cellular levels of LGR4 and ΔNP63 proteins in BPH stem/progenitor cells. Also, AR activation promoted nuclear levels of basal cell-specific ΔNP63α TF and cytoplasmic restriction of EMT specific ΔNP63γ TF.

Data were further evaluated for the miRNA profile under the influence of TP/IGF and Wort/Nil treatments. The androgen-mediated downregulation of miR-27a and miR-21 also validates the downregulation of both the miRs and upregulation of LGR4 and ΔNP63α in AR^{hi} patients in the previous chapter. Further, we have also attempted to evaluate the influence of AKT on AR-mediated miR regulation via IGF-1 and Wortmannin treatments. The expression of *ΔNP63* targeting miR-203 was not detected in BPH stem/progenitor cells and the expression of other stemness regulating miRs, miR-34b, miR-34c, miR-34b, let-7f, and miR-142, did not substantially express in BPH stem/progenitor cells. Further, previously known AR-mediated positive regulation of miR-21 also depicted decreased levels with TP stimulation in BPH stem/progenitor cells, whereas its expression was substantially increased with IGF-1 and IGF-1/Nil treatments as compared to CSS control. The evidence suggests the AR induced downregulation of miR-21 in BPHstem/progenitor cells. Levels of miR-27a were decreased with TP and TP/Wort (inactive AKT/active AR) treatments suggesting the suppressive role of AR in BPH stem/progenitor cells. However, screening of *LGR4* targeting miR-27a was showed the downregulation with TP stimulation and upregulated in presence of IGF-1 and IGF-1/Nil (active AKT/inactive AR) treatments confirm the suppressive role of AR on miR-27a expression in BPH stem/progenitor cells. The *in vitro* assessment of miR-21 and miR-27a also validates their expression in AR^{hi}

and AR^{lo} BPH patients. Additionally, low levels of miR-34a were expressed which was increased nominally in the presence of AR (Nil) and AKT (Wort) inhibitors. Similarly, the nominal expression of Let-7d was upregulated with all individual and combination treatment groups as compared to the control group, suggesting miR-34a and Let-7d are under mutual regulation of AR and AKT. (*Figure 5.7C*) In summary, BPH and PCa patients suggest that activation of AR is markedly associated with a decrease in miR-27a which could support the unhindered translation of LGR4 transcripts, which is also the case in BPH stem/progenitor cells.





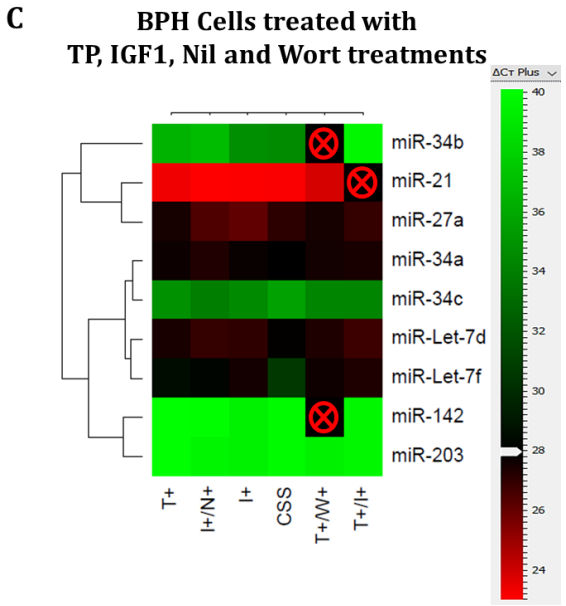
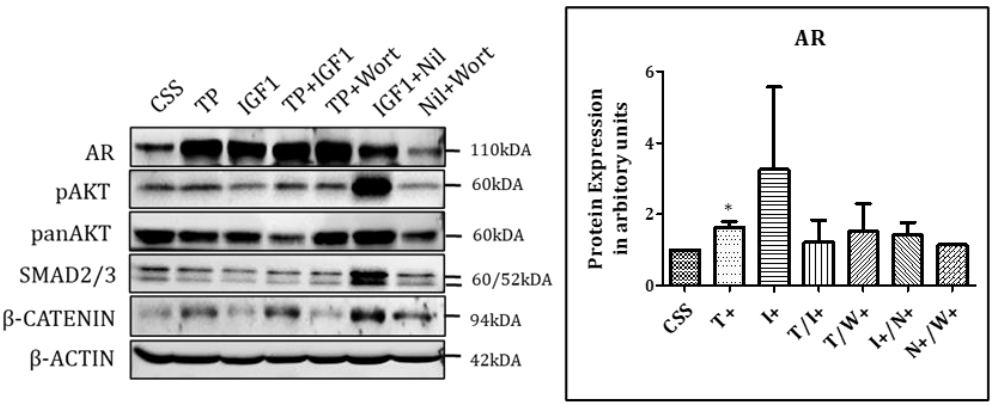


Figure 5. 7: The protein levels of stemness-associated LGR4, SOX2, OCT3/4, and ΔNP63α markers in BPH stem/progenitor cells exposed to TP, IGF-1, Nil, and Wort treatments. (A) Immunoblot and graphs represent the protein expression of LGR4, SOX2, OCT3/4, and ΔNP63α in BPH stem/progenitor cells exposed to the specific treatments; n=3; Mean±SEM; *p<0.05 CSS v/s treated groups; **(B)** Nuclear and cytoplasmic localization of ΔNP63α and γ in BPH stem/progenitor cells exposed to the 24-hour treatment of AR and AKT activators and inhibitors; n=3; Mean±SEM; *p<0.05 CSS v/s treated groups. **(C)** Heatmap represents miRNA expression in BPH stem/progenitor cells exposed to TP, IGF-1, Nil, and Wort treatments. (n=2) ⊗= data not available.

5.3.7 AR protein expression influences β-CATENIN, AKT, and SMAD2/3 proteins in BPH stem/progenitor cells.

Androgen-dependent activation of AR is known to affect AKT that in turn induces s213 phosphorylation. Additionally, it has been observed that AKT and SMAD2/3 regulate the expression and localization of ΔNP63α and γ proteins we further evaluated AKT and SMAD signaling proteins in BPH stem/progenitor cells.[18, 19] Their expression was assessed in the combination treatments of AR and AKT activators (TP and IGF-1 respectively) and AR and AKT inhibitors (Nilutamide and Wortmannin respectively). Results demonstrated increased levels of β-CATENIN and decreased levels of Total AKT and SMAD2/3 in BPH stem/progenitor cells exposed to direct AR/AKT activators TP and IGF-1, where AR, but not pAKT/total AKT, levels were elevated in the cells after 24 hours of treatment. Further, levels of SMAD2/3 were decreased with increased AR expression but increased in cells exposed to AKT activator (IGF-1) and AR inhibitor (Nil), suggesting dominant control of AR in the downregulation of both AKT and SMAD2/3 mediated cell signaling network in BPH stem/progenitor cells. β-CATENIN levels were elevated in BPH stem/progenitor cells with TP and IGF-1 treatments and downregulated with Wortmannin treatment in combination with TP and Nil, suggesting the influence of AR and AKT both over β-CATENIN expression. (*Figure 5.8*) Thus, the expression and activation of AR strongly dominate AKT and SMAD2/3 signaling networks in the BPH stem/progenitor cells.



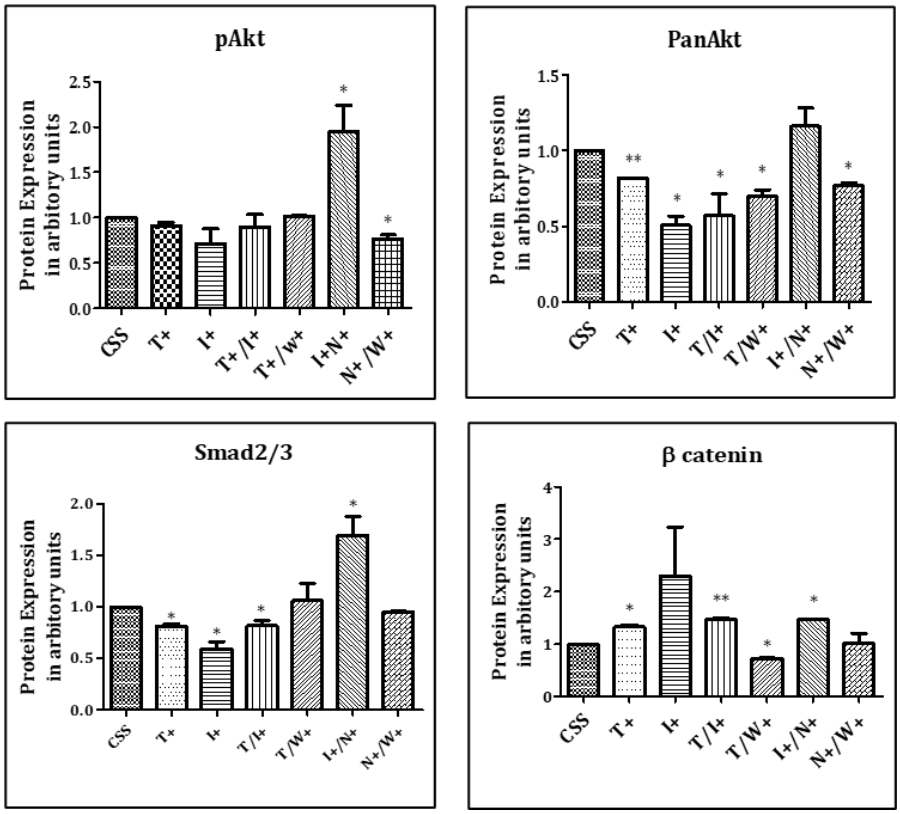
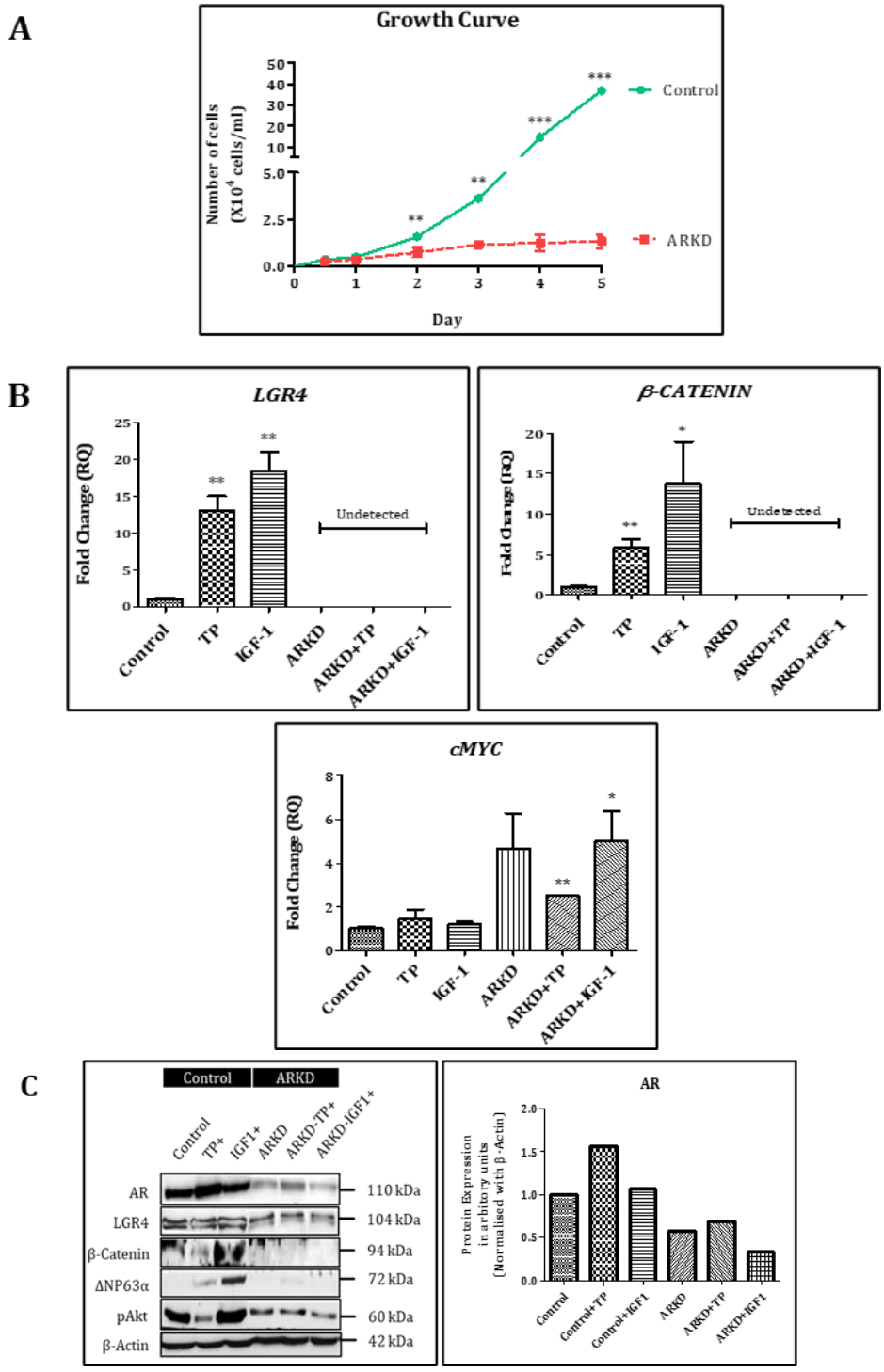


Figure 5. 8: Protein expression analysis of Signaling proteins and stem/progenitor markers. Immunoblot and graphs represent the protein expression of AR, pAKT, Total AKT, and Smad2/3 in BPH stem/progenitor cells exposed to TP, IGF-1, Nil, and Wort treatments for 24 hours; n=3; Mean±SEM; *p<0.05 CSS v/s treated groups.

5.3.8 ARKD confirms the loss of LGR4 and ΔNP63α gene and protein articulation in BPH stem/progenitor cells.

To further validate the AR-mediated control of LGR4 and ΔNP63α, the cells received the lentiviral transduction of the ARKD construct. The growth of the BPH stem/progenitor cells considerably reduced by ARKD, suggesting a vital role of AR on the growth of the cells. (*Figure 5.9A*) Further, gene expression study showed TP and IGF-1 dependent upregulation of *LGR4* and *β-CATENIN* in control cells, whereas the ARKD cells failed to express their transcript levels. On the contrary, the transcript levels of *cMYC* were substantially elevated in ARKD BPH stem/progenitor cells suggesting the inverse effect of AR inhibition on *cMYC* expression. (*Figure 5.9B*) Moreover, the protein expression of AR, LGR4, β-CATENIN, and pAKT decreased in the ARKD cells as compared to control cells. (*Figure 5.9C*) The control cells showed increased levels of AR and ΔNP63α when exposed to TP stimulation, and increased levels of β-CATENIN, ΔNP63α, and pAKT with

IGF-1 stimulus for 24 hours. Hence, AR plays a substantial role in the regulation of LGR4 and ΔNP63α gene and protein expression in BPH stem/progenitor cells.



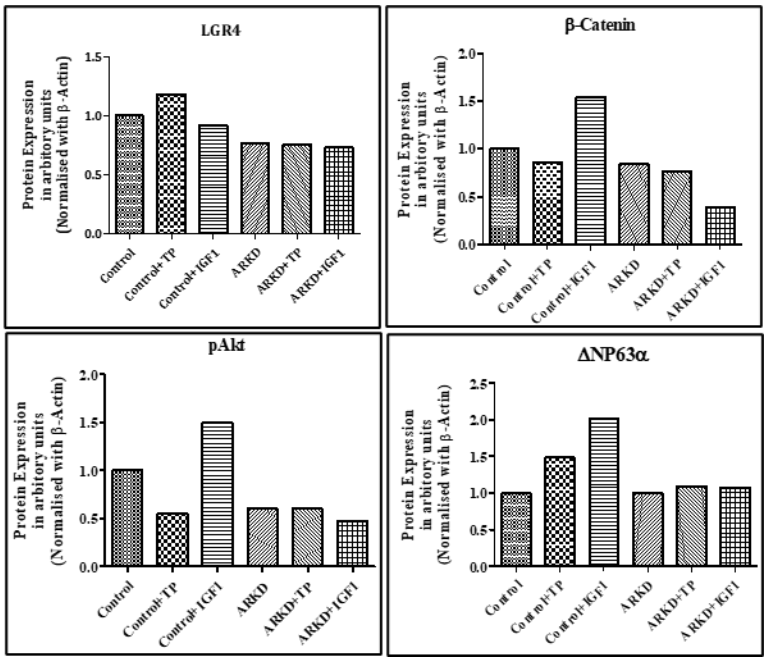


Figure 5. 9: Effect of ARKD in BPH stem/progenitor cells. (A) The growth curve of the BPH stem/progenitor cells monitored for 5 days after developing ARKD condition by the shRNA construct. The graph represents Mean±SEM values; n=5; **p<0.01, ***p<0.001, control v/s ARKD groups; **(B)** The graphs represent the gene expression data of *LGR4*, *β-CATENIN*, and *cMYC* in control and ARKD BPH step/progenitor cells exposed to TP and IGF-1 treatments. n=4; Mean±SEM; *p<0.05, **p<0.01 control v/s all the groups; **(C)** Protein expression analysis of AR, LGR4, β-CATENIN, ΔNP63α, and pAKT normalized with individual β-actin levels in Control and ARKD BPH stem/progenitor cells treated with TP and IGF-1; n=1.

5.3.9 Testosterone stimulation leads to the increased interaction of AR and β-CATENIN in BPH stem/progenitor cells.

To determine the interaction between AR-β-CATENIN, BPH stem/progenitor stem cells were treated with TP, IGF-1, and Nilutamide for activation and inhibition of indigenous AR protein levels. Immunoprecipitation with anti-AR antibody showed increased interaction between AR and β-CATENIN when treated with TP, but not with IGF-1. Further, AR-β-CATENIN interaction was also confirmed with immunoprecipitation with an anti-β-CATENIN antibody. Intriguingly, immunoprecipitation with β-CATENIN showed an enhanced physical interaction with indigenous ΔNP63α when treated with TP. Though, AR did not portray any direct interaction with ΔNP63α. (*Figure 5.10ABC*) The data indicates that β-CATENIN may bridge the complex formation between AR-ΔNP63α. Cumulatively, the protein interaction study depicts that in the presence of AR agonist, the

interaction between AR-β-CATENIN and β-CATENIN-ΔNP63α is increased in BPH stem/progenitor cells.

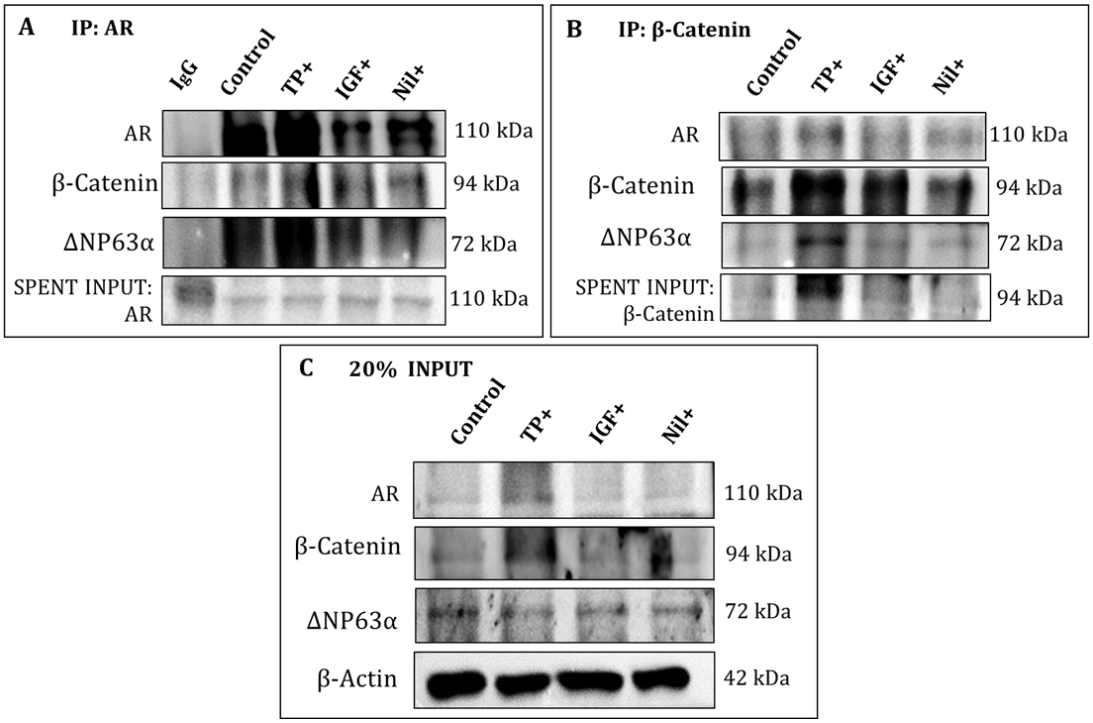


Figure 5. 10: Co-immunoprecipitation (Co-IP) of the endogenous AR and β-CATENIN protein. Complex formation post AR activation (TP^+ and $IGF\text{-}1^+$) and inhibition (Nil^+) treatment for 24 hours from BPH stem/progenitor stem cells. Immuno-precipitations were performed using **(A)** anti-AR antibody, **(B)** anti-β-CATENIN antibody, and immune complexes were probed and developed using the western blot method, and **(C)** Total input of the samples as shown in the image. The anti-rabbit IgG antibody served as a negative control during the Co-IP assay.

5.3.10 Chromatin immunoprecipitation reveals AR and β-CATENIN dependent regulation of LGR4 and ΔNP63 promoters.

The interaction between AR-β-CATENIN is involved in the regulation of gene expressions. AR regulates many genes by binding to ARE sites within gene promoters or enhancer regions. Hence, we aimed to investigate the transcriptional regulation of *LGR4* and *ΔNP63* genes by AR. Firstly, we identified putative AR binding sites (ARE) of *LGR4* and *ΔNP63* genes using EPD (Eukaryotic Promoter Database) and Jasper tools.[20, 21] (*Figure 5.11A and Figure 5.12A*) Concomitant results showed that the promoter region of both genes has full of partial ARE sites within the transcription initiator cluster. (*Figure 5.11A and Figure 5.12A-AR Motif*) To further investigate the physical binding of AR to both of the gene promoters, ChIP assay was performed with 6 and 12 hours of TP treatment

and the time-dependent up-regulation of mRNA levels of both genes was evaluated by real-time PCR. (*Figure 5.11B and Figure 5.12B*) The assessment of AR and β-CATENIN binding on the LGR4 promoter revealed that both AR and β-CATENIN remain to a distal position (-843 to -722) from the transcription start site (TSS) in the Control and Treatment exposures (AR ChIP: CSS: 35.28±0.82, TP: 41.42513±3.72, IGF-1: 74.02±2.68; $p<0.01$, Nil:149.74±28.69; $p<0.001$ and β-CATENIN Chip: CSS: 1.21±0.2, TP: 18.43±1.52; $P<0.01$, IGF-1: 6.22±0.81; $p<0.05$, Nil: 15.35±1.07; $p<0.01$). However, both AR (55.02±0.92; $P<0.01$) and β-CATENIN (18.07±0.61; $P<0.001$) were found to be relocated to the proximal position (-468 to -286) from LGR4-TSS upon androgen stimulus as compared Control (AR: 38.34±1.89; $p<0.01$, β-CATENIN: 18.1±0.61; $p<0.001$) for initiation of transcription of the *LGR4* gene. IGF-1 and Nil treatments did not show significant elevation on AR (IGF-1: 31.93±1.68, Nil: 45.77±6.4) and β-CATENIN (IGF-1: 2.14±0.25, Nil: 7.64±0.5) relocation to the proximal position. (*Figure 5.11C*)

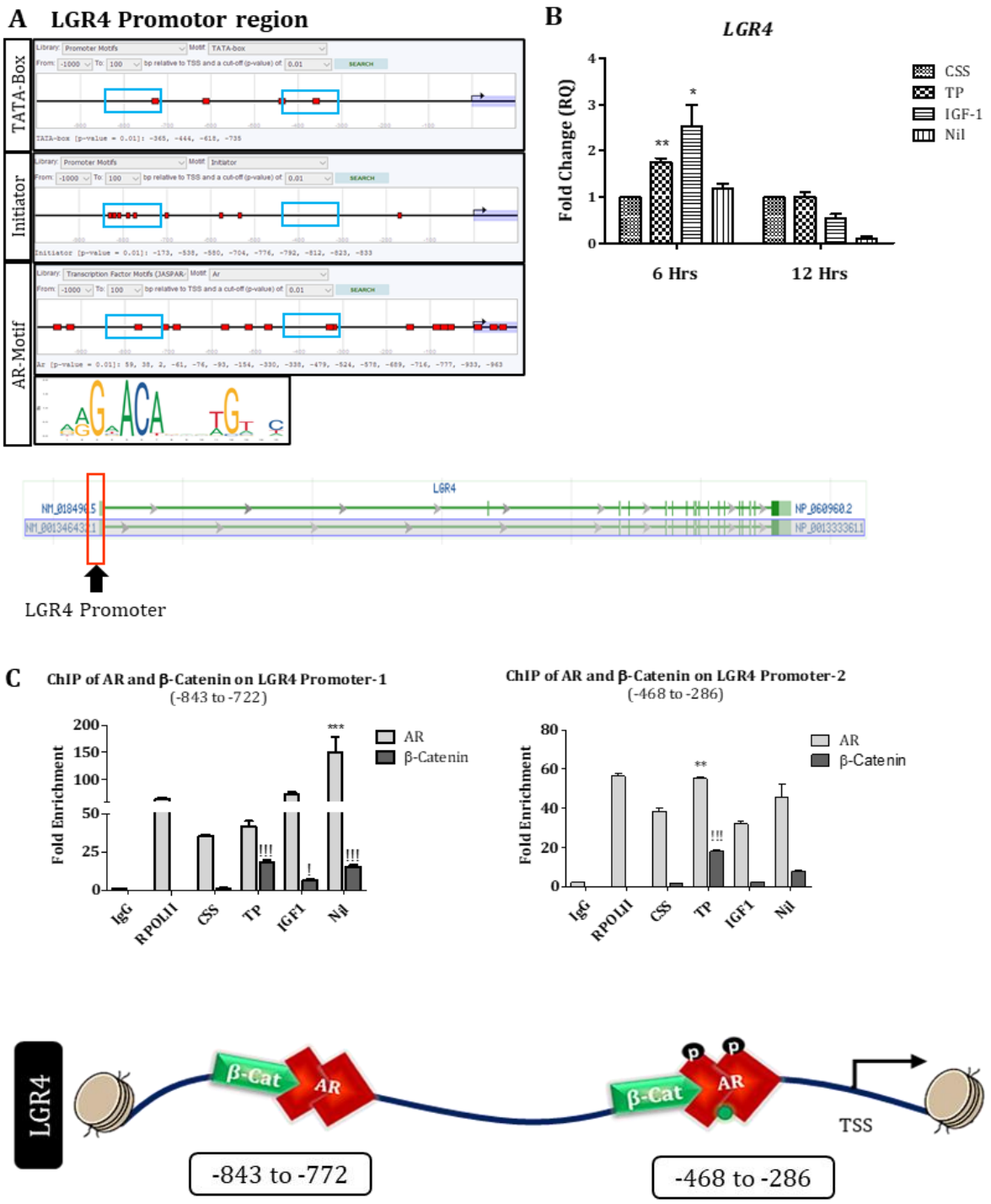


Figure 5. 11: Interaction of AR and β-CATENIN with LGR4 promoters. (A) The image represents the identification of on TATA-box, transcription initiator complex, and within LGR4 promoter and probable AR-motif binding sites on the LGR4 promoter region identified with EPD tools. The specific primer in the promoter region is highlighted with blue boxes. **(B)** The graph represents Mean±SEM values of fold change in gene expression of *LGR4* in BPH stem/progenitor cells exposed to TP, IGF-1, and Nil treatment for 6 and 12 hours. All the treatment groups were compared with control of the respective time point. n=3; *p<0.05. **(C)** The graphs represent Mean±SEM values of ChIP fold enrichment of

AR and β-CATENIN proteins on *LGR4* promoter at -843 to -722 and -468 to -286 regions. n=3; **p<0.01, ***p<0.001 (CSS vs AR ChIP); $^{!}$p<0.05, $^{!!!}$p<0.001 (CSS vs β-CATENIN ChIP).

However, mutual transcriptional regulation by AR and β-CATENIN could not be detected in the *ΔNP63* promoter region. The majority of the promoter distal promoter sites (-630 to -442) from TSS were dominantly occupied by β-CATENIN against AR despite the stimulatory or inhibitory treatments of TP (22.91±0.87; p<0.01), IGF-1 (23.88±2.36; p<0.001), and Nil (32.59±0.69; p<0.001) as compared to Control group (5.29±0.53). On the contrary, AR is dominantly appended on the proximal site (-514 to -275) from TSS of the *ΔNP63* promoter region. Intriguingly, TP and IGF-1 stimulation significantly increased β-CATENIN binding (TP:14.19±1.42; p<0.05; IGF-1: 2.67±0.37; p<0.05) as compare to Control (0.31±0.12) and Nil (5.49±4.01) treatments. Conversely, TP stimulation significantly decreased AR binding (TP: 34.55±1.65; p<0.05) to the proximal site (-514 to -275) from *ΔNP63*-TSS as compared to Control (42.60±0.89) group. However, IGF-1 (71.22±4.35; p<0.01) and Nil (77.71±2.68; p<0.05) treatment groups showed significant elevation in AR binding to the proximal site (-514 to -275) from *ΔNP63*-TSS as compared to Control group. suggesting the regulation of *ΔNP63* promoter by β-CATENIN and not by AR- β-CATENIN interaction complex. (*Figure 5.12C*) Hence, ChIP data notably confirms the direct upregulation of LGR4 gene expression by AR-β-CATENIN interaction complex in response to androgen stimulus. However, *ΔNP63* promoter is possibly inhibited by AR protein and majorly regulated by β-CATENIN in response to androgen stimulus, but remained independent of AR protein.

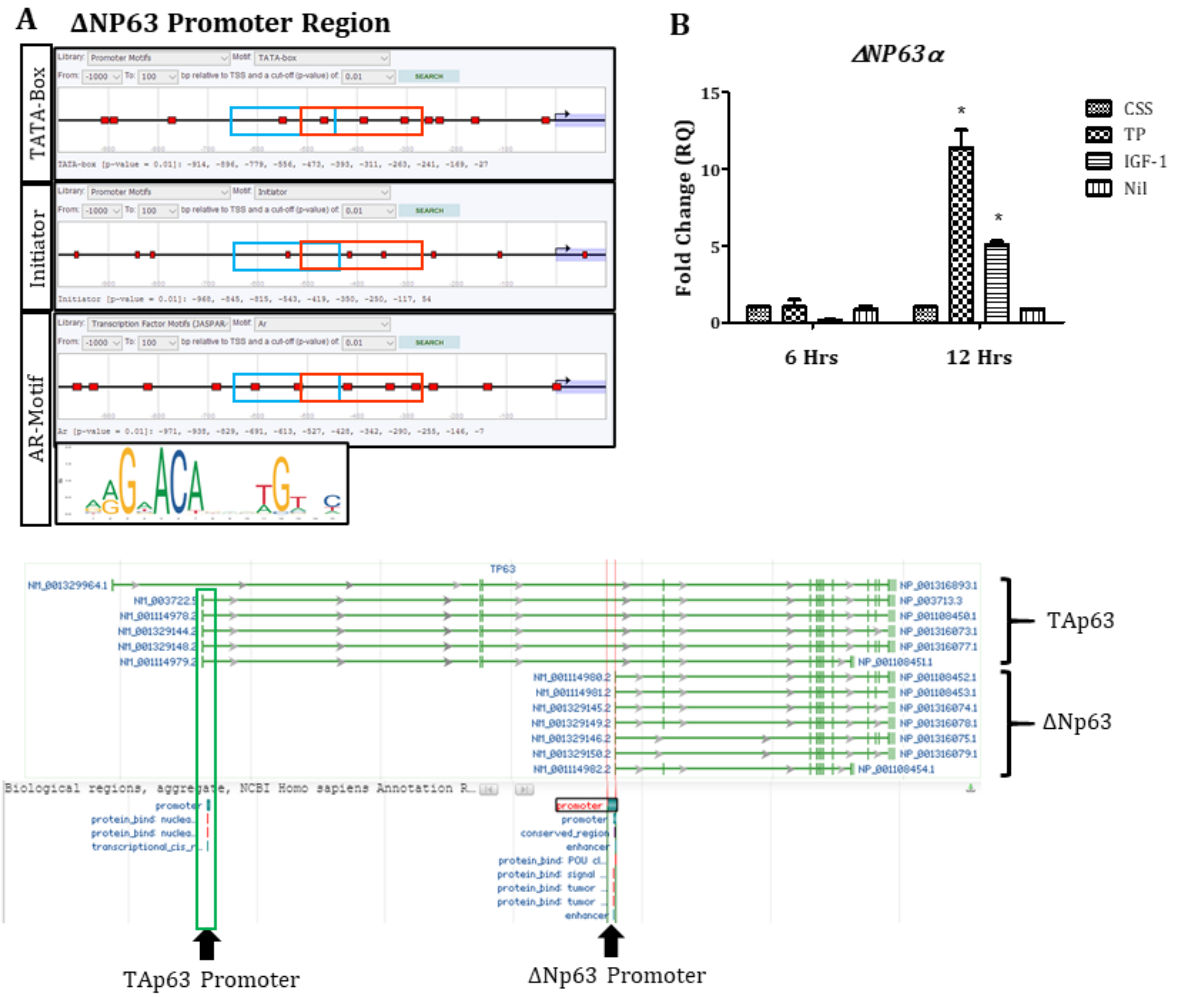

A
ΔNP63 Promoter Region
TATA-Box
Initiator
AR-Motif
B
ΔNP63α
Fold Change (RQ)
CSS
TP
IGF-1
Nil
6 Hrs
12 Hrs
TAp63
ΔNp63
TAp63 Promoter
ΔNp63 Promoter
C
ChIP of AR and β-Catenin on ΔNP63promoter
(-630 to -442)
ChIP of AR and β-Catenin on ΔNP63 Promoter
(-514 to -275)
Fold Enrichment
AR
β-Catenin
IgG
RPOLII
CSS
TP
IGF1
Nil
ΔNP63
β-Cat
AR
TCF
TSS
-630 to -442
-514 to -275

Figure 5. 12: Interaction of AR and β-CATENIN with ΔNP63 promoters. (A) The image represents the identification of on TATA-box, transcription initiator complex, and within Δ*NP63* promoter and probable AR-motif binding sites on the *LGR4* promoter region identified with EPD tools. The specific primer in the promoter region is highlighted with blue and red boxes. **(B)** The graph represents Mean±SEM values of fold change in gene expression of Δ*NP63* in BPH stem/progenitor cells exposed to TP, IGF-1, and Nil treatment for 6 and 12 hours. All the treatment groups were compared with control of the respective time point. n=3; *p<0.05. **(C)** The graphs represent Mean±SEM values of ChIP fold enrichment of AR and β-CATENIN proteins on Δ*NP63* promoter at -630 to -442 and -514 to -275 regions. n=3; *p<0.05, **p<0.01 (CSS vs AR ChIP); $^{!!}$p<0.01, $^{!!!}$p<0.001 (CSS vs β-CATENIN ChIP). IgG and RNA-Polymerase II (RPOLII) served as negative and positive control respectively during ChIP.

5.4 Discussion

The role of AR in prostate stem cells has been explored widely, however, due to vast crosstalk with the signaling network and transcription activity, its precise mechanism over stemness regulation has remained unclear. Moreover, androgen/AR signaling axis in concordance with stem/progenitor cells also contributes to the development of the BPH. In the present study, we demonstrated alteration in the stem/progenitor populations of BPH stem/progenitor cells exposed to AR agonist and antagonist stimuli.

To understand the role of AR in BPH stem cells, the BPH patient-derived stem/progenitor cells were characterized for the assessment of the presence of stem-associated markers. Prostate BSCs explicitly express several surface receptors like CD49f, CD133, CD117, and LGR4 which are also the key regulator of the basal state of the cells.[22-25] The flowcytometric analysis showed CD49^{hi} phenotype along with the presence of CD133, CD117, and LGR4 expression in the varying percentage of certain subpopulations, confirming stem/progenitor cells in the BPH stem/progenitor cell population. All the BPH stem/progenitor cells were CD49f^{+ve} (α6 Integrin) which has been proved as the sphere-forming cells during BPH and PCa conditions in human patients.[26] Moreover, it has been observed that CD49f^{+} stem cells substantially co-express TROP2 and SCA-1 BSC markers, which can serve as a cell of origin of the PCa in patient tissues.[27] Similarly, CD133 (Prominin-1) positive cells can also serve as a tumor-initiating cell and therapy resistance during PCa.[28, 29] CD117 (c-Kit) retains the capacity of regenerating the entire prostate gland.[24] Assessment of CD117 expression in BPH and PCa tissue samples suggested its elevated expression in BPH condition as compare to PCa tissues and circulatory tumor stem cells.[30, 31] LGR4, expressed by the BPH stem/progenitor cells, is

also a prostate BSC marker that controls the differentiation of the BSCs.[25] Apart from the stemness associated surface receptors, one of the most known BSC markers, P63 was also found to be present in the BPH stem/progenitor cells.[32] Further, these cells are also evidently AR-positive, suggesting the presence of stem-associated markers along with the AR expression. Hence, the characterization of the BPH stem/progenitor cells in the present study indicates the luminal progenitor (LP) nature of the BPH stem/progenitor cells which exhibit Basal and luminal markers and have bipotent differentiation ability.

To further substantiate the role of AR in the BPH stem/progenitor stem/progenitor cells, the cells were exposed to AR agonist and antagonist treatments. Increasing expression of CD133, CD117, and LGR4 upon TP exposure suggested that they may be under the influence of AR activation in the BPH stem/progenitor cells. However, a study based on the molecular exploration of $CD49f^{+ve}$ cells of BPH patient tissue suggested low or no expression of AR in these cells,[26] supporting the unaffected $CD49f^{+ve}$ population with AR modulation in the present study. The positive changes in the $CD133^{+ve}$ population upon androgen stimulus is upheld by a previous study demonstrating the positive regulation of CD133 by AR in endometrial cancer cells.[33] However, co-expression of CD133 and active AR were detected at lower frequencies in normal human prostate.[34] Further, BPH and PCa patient-derived $CD49b^{hi}/CD133^{+ve}$ cells were detected with AR protein expression by flow cytometry, however, the AR expression was significantly less as compared to luminal cells. And the AR expression was found to be essential in $CD49b^{hi}/CD133^{+ve}$ for the disease progression.[34] Interestingly, the changes in CD117 and LGR4 population by AR activation in BPH stem/progenitor cells are the novel findings of this study. Further, co-expression of AR and P63 was also discovered in the benign tissue surrounding the cancerous prostate tissue of the patients.[35] However, this is the first study to report the changes in P63 levels in BPH stem/progenitor cells due to modulation in AR activity. Thus, AR implies a positive regulatory effect on the expression of CD133, CD117, and LGR4 expression.

Androgen dependent or independent nuclear translocation of AR is the primary requirement to activate target gene regulatory actions as a TF. To achieve nuclear localization, NTD/LBD mutually enhances cytoplasmic retention, and the Nuclear Localization Signal (NLS) in NTD, DBD, and LBD regions promotes nuclear translocation of AR.[50] We have found increased nuclear translocation of AR with ligand-dependent and

independent activation via TP and IGF-1 respectively. Further, AR had substantially higher euchromatin accessibility upon activation with TP and IGF-1 suggesting the activation of its transcriptional function. A similar observation of chromatin accessibility of AR with full and half ARE sites has been demonstrated by Tewari *et al* in LNCaP cells.[51] Thus, both androgen-dependent and independent signals are potent to enhance the transcriptional regulatory actions of AR.

AR belongs to the NR superfamily TF which works in an androgen dependant and independent manner.[36] It has been observed previously that stimulation of androgen (TP) and growth factor (IGF-1) lead to the activation of NLS motifs of AR that increased its nuclear localization, leading to increased cell proliferation in BPH and PCa conditions.[38-41] Androgen-independent activation of AR was regulated by growth factors like IGF-1 via AKT and MAPK pathways.[36, 37] Further, *in vitro* sphere-formation ability is one of the explicit characteristics of the human stem/progenitor cells in normal as well as PCa conditions.[42, 43] In presence of DHT, AR-positive LNCaP cells exhibited higher sphere-forming capacity associating them to higher cell proliferation as compared to AR-KO LNCaP clones.[44] Similar observations were also made in the present study, where TP and IGF-1 increased sphere formation of BPH stem/progenitor cells. Previously, it has been demonstrated that the prostaspheres derived from primary stem/progenitor cell cultures expressing stem-associated markers, like CD117, Trop2, CD49f, and ABCG2, of normal human prostate exhibited undetectable levels of AR mRNA and no expression of AR protein.[45] In contrast to prostate, the sphere formation ability of patient-derived primary breast cancer stem cells was stimulated by AR.[46] Previously, the expression of AR was found to be patchy in $CK5^+/P63^+$ spheres formed from human stem/progenitor, whereas AR was strongly expressed in CK8 (luminal) spheres derived from luminal cells which also produced few $CK5^+/P63^+$ (basal) spheres,[9] suggesting its bipotent characteristics. Karthaus *et al* also demonstrated the expression of LGR4 in both basal and luminal cell type derived tumors.[9] Another study by Huang *et al* showed prominent expression of LGR4 in P63 expressing BSCs derived spheres of the normal mouse prostate.[47] In stem/progenitor and non-stem/progenitor PCa cells, AR exhibited differential function in terms of sphere formation, where AR decreased sphere formation ability in stem/progenitor cells, but increased in non-stem/progenitor cells.[48] However, we have observed increased sphere formation ability with AR activation and LGR4 expression in

BPH stem/progenitor cells, suggesting the disease-specific role of AR in the stem/progenitor cells. Further, Bisson and Prowse found that increasing WNT/β-CATENIN levels increased the sphere formation in prostate cells,[49] which supports the increased sphere formation ability due to increased LGR4 levels and its downstream β-CATENIN activation as observed in the present study. Thus, androgen-dependent and independent activation of AR can increase the sphere-forming ability of BPH stem/progenitor cells in AR and LGR4 dependent manner. But, the link between the AR, LGR4, and P63 is yet to be addressed in the stem/progenitor cells.

In BPH patients, we have observed the positive association of AR with LGR4 and ΔNP63α markers. The investigation of their articulation with AR activation and inhibition suggested increased LGR4 and ΔNP63α expression. Previously, Luo *et al* also showed the co-localization of LGR4 and P63 proteins in the prostate BSCs.[5] Additionally, the evidence of LGR4 mediated elevation of AR expression to promote PCa tumorigenesis has also been reported previously.[54] In the present study, we have found TP and IGF-1 treatment mediated increase in LGR4 and β-CATENIN expression in BPH stem/progenitor cells. Supported by the previous reports suggesting the elevated expression of the downstream effector of LGR4, β-CATENIN in BPH and PCa conditions.[49, 55] Moreover, β- CATENIN and AR both can directly control *cMYC* gene transcription in PCa cells,[56, 57] but in the present study, we showed knockdown of AR exhibited stronger upregulation in cMYC expression. ΔNP63 variants (ΔNP63α/β/γ) of TP63 are vital to stem cell transcription factors in the prostate gland, that inhibits cell cycle regulation and apoptosis regulators TP63 and P53.[58] TP and IGF-1 treatment showed significant upregulation of ΔNP63α protein. And, nuclear fractionation data showed that TP and IGF-1 treatments potentiates nuclear localization of ΔNP63α in BPH stem/progenitor cells. Studies have shown that ΔNP63 variants act as a key transcriptional regulator of the prostate BSCs.[32] ΔNp63-specific knockout has demonstrated that this isoform is the most critical for stratified stem/progenitor development, consistent with the higher levels of this isoform in the prostate and other epithelial tissues.[59]

Activation of AR intervenes in several cell signaling pathways like AKT and TGFβ/SMAD pathways. Previously, Carver *et al* discovered that blockade of AR increases the activation of AKT in the prostate of PTEN-null mice,[40] which supports the findings of the present study depicting increased total AKT and pAKT in the Nil/IGF-1 treated cells. We also

found that the expression of LGR4 is dependent on AR and AKT levels. The inhibition of either of them impairs the expression pattern of LGR4. Further, DHT mediated AR-LBD activation directly inhibits the association of SMAD3 to the SMAD-binding element and inhibits SMAD3 mediated transcriptional activities. Thus, ligand-mediated AR activation inhibits EMT promoting the TGFβ signaling pathway in the prostate stem/progenitor cells.[52, 53] The SMAD2/3 complex is found to be a transcriptional potentiator of ΔNP63γ proteins, which regulate the EMT in the stem/progenitor cells. Here, we have demonstrated the downregulation of SMAD2/3, which caused cytoplasmic restriction of ΔNP63γ and enhanced nuclear localization of ΔNP63α proteins due to AR activation in the BPH stem/progenitor stem/progenitor cells. Thus, AKT and SMAD2/3 play a vital role in the expression and function of LGR4 and ΔNP63proteins in BPH stem/progenitor cells.

AR has over 300 direct/indirect interacting partners, making cellular functions of AR even more complex and unprecedented in the cells during prostate diseases.[60] Increased LGR4 expression also inhibits β-CATENIN degradation to strengthen its transcriptional activation in the prostate stem cells.[8] Among many interacting signaling proteins, β-CATENIN was found to directly interact with the AF-2 activation region of AR and modulates the transcriptional activity of AR.[61] Further, Androgen stimulation to AR leads to the formation of AR-β-CATENIN-TCF4 complex that affects transcription of normal prostate stem/progenitor cells.[62] However, β-CATENIN has found to work in AR independent manner also to regulate the self-renewal of stem/progenitor cells in PCa cells.[49] In the present study, complex formation between AR-β-CATENIN was found, which became more pronounced upon TP stimulation in BPH stem/progenitor cells.

Transcription factors bind to DNA in a sequence-specific manner, and positively or negatively affect gene expression through the control of transcription. The activation of AR induces its transcriptional activity through full and half ARE sites in gene promoters and enhancers and enormous numbers of chromatin binding sites.[63, 64] Moreover, direct interaction between AR and β-CATENIN forms a transcriptionally active complex, which was also reported previously in LNCaP cells.[65, 66] Here, we demonstrated that ligand-dependent and independent activation of AR increased the AR-β-CATENIN interaction which regulates the gene transcription of LGR4 and ΔNP63. Thus, we confirm the regulation of the *LGR4* gene is via AR/β-CATENIN interaction complex. Intriguingly, we also report a unique feature of AR in the regulation of ΔNP63 promoter in the present

study, where unstimulated AR remained bound to the ΔNP63 promoter and androgen stimulation caused retraction of AR and promotion of β-CATENIN binding to the ΔNP63 promoter region. Similar to our findings, Ruptier *et al* previously demonstrated TCF/β-CATENIN mediated positive regulation of ΔNP63 promoter.[67] The protein interacting domain of β-CATENIN was found to be overlapping between AR and TCF-4,[68, 69] hence either one of them can interact with β-CATENIN at a time. Further, the interactions between AR/β-CATENIN and β-CATENIN/TCF was regulated by Peptidyl-prolyl isomerase (Pin-1) protein in the PCa cells in dose-dependent response of DHT, which is also a regulator of AR phosphorylations [70, 71] Since the ΔNP63 promoter regulation was delayed with hormone stimulation as compared to LGR4 promoter, the transcriptional activity of AR could be influenced by Pin-1, that strongly promotes β-CATENIN/TCF interaction. Thus, we assumed that the regulation of the Δ*NP63* promoter is possibly through the TCF/β-CATENIN complex instead of the AR/β-CATENIN complex in presence of Testosterone. However, the vitality of Pin-1 and activation of AR in the formation and potentiation of the TCF/β-CATENIN interaction complex remains elusive in BPH stem/progenitor cells.

Here, we report a synchronized activation of AR and β-CATENIN ultimately increased the expression of LGR4 and ΔNP63α in the BPH stem/progenitor cells. The elevation in these expressions is the primary causal factor of the clonal expansion of BPH stem/progenitor cells. Moreover, ligand-dependent activation of AR elevated the interaction between AR/β-CATENIN and a novel complex formation between β-CATENIN/ΔNP63α proteins. We also confirm the direct regulatory role of the AR/β-CATENIN complex on LGR4 promoter regulation. Further, the present findings also suggest that the regulation of ΔNP63 promoter surely depends on β-CATENIN in response to hormone and AR might have a negative regulatory impact over ΔNP63 promoter in BPH stem/progenitor cells.

5.5 Summary

In summary, the substantial involvement of AR in the regulation of two stem-associated proteins, LGR4 and ΔNP63α has been discovered in the stem/progenitor cells of BPH condition. In the present study, we have unrevealed the tumorigenic potential of BPH stem/progenitor cells with augmented LGR4 levels in the tumorspheres. Also, the positive effect of ligand-dependent and independent activation, nuclear localization, and euchromatin accessibility of AR on LGR4 and ΔNP63α expression has been demonstrated. Further, we also discovered the interaction between AR-β-CATENIN and a novel interaction between β-CATENIN-ΔNP63α proteins upon Testosterone stimulation for the first time in BPH stem/progenitor cells. However, the functional implications of β-CATENIN-ΔNP63α proteins are yet not discovered in the prostate stem/progenitor cells. The AR-β-CATENIN interaction complex has found to regulate the LGR4 promoter and its expression in the present study. We have also demonstrated a possible inhibitory role of AR over ΔNP63 promoter which is under the positive regulation of β-CATENIN in the BPH stem/progenitor cells. (*Figure 5.13*)

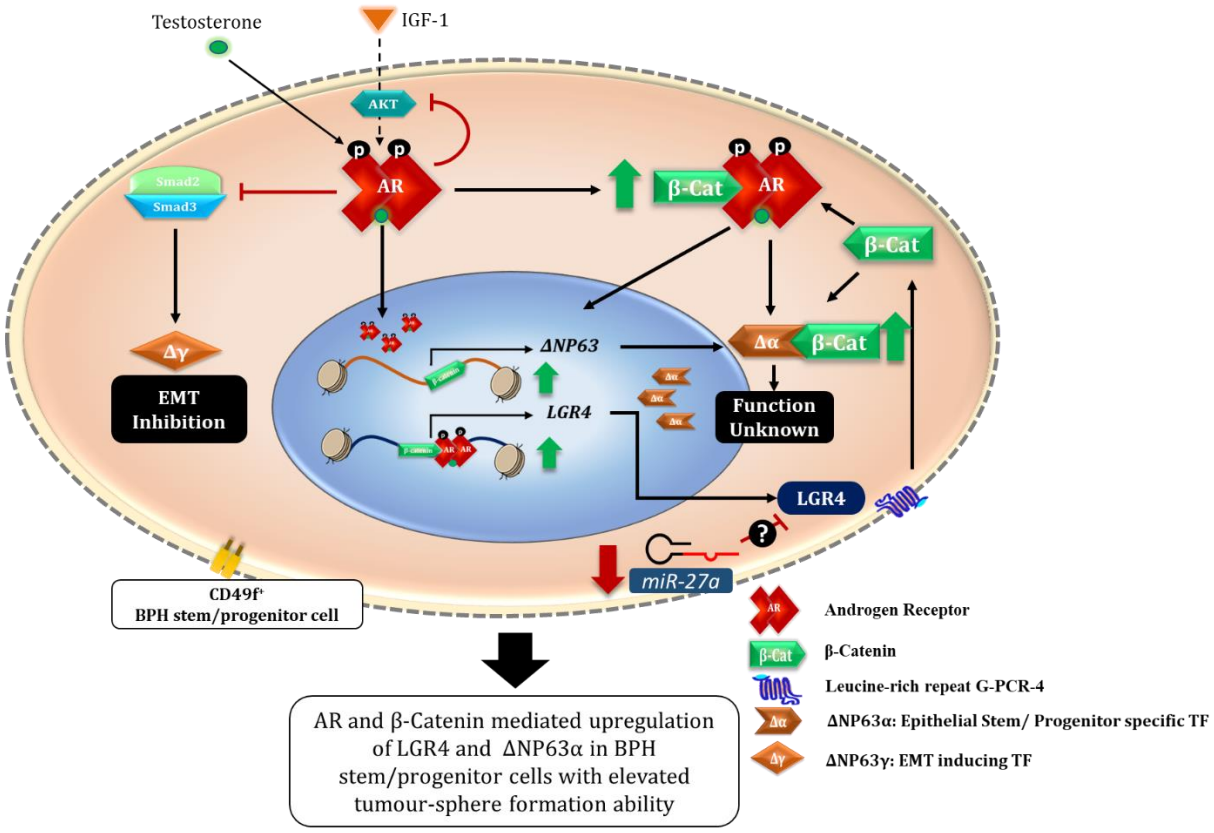


Figure 5. 13: Graphical summary. The molecular role of AR in the regulation of LGR4 and ΔNP63α articulation in BPH stem/progenitor cells.

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