CHAPTER-6

6. TO EXPLORE THE ROLE OF ANDROGEN RECEPTOR-MEDIATED EPITHELIAL-STROMAL CROSSTALK IN THE ETIOPATHOLOGY OF BENIGN PROSTATE HYPERPLASIA.

6.1 Introduction

AR plays a critical function in the fetal development of the prostate by influencing AR-positive precursors to fibroblast and smooth muscle cells of urogenital sinus (UGS) and embryonic urogenital mesenchyme (UGM).[1] Further, fetal androgens acting through Stromal-AR maneuver prostate ductal morphogenesis, epithelial differentiation, and proliferation/apoptosis. Hence, the normal epithelial differentiation and function depend on androgen-mediated stromal paracrine signals.[2] During adulthood, epithelial-AR dominates Stromal-AR expression to regulate cellular homeostasis and secretory functions of the prostate gland.[3] Overexpression of epithelial-AR has been reported during BPH and PCa. Though at the cellular level, different expression levels of AR have been observed depending on the stage and grade of the PCa tumors.[4] Nevertheless, Stromal-AR also plays an important role alongside with epithelial-AR for disease progression.

In PCa, not only cancer cells but surrounding stroma, known as carcinoma-associated fibroblasts (CAFs), contributes to the survival and proliferation of tumor cells through its secretory anti-apoptotic factors, growth factors and cytokines.[3, 5, 6] Similarly in BPH also, stromal cells continuously produce cytokines and growth factors to support tumor growth.[7] Thus, growth promotion and inhibition of malignant and non-malignant tumor cells depend on the stromal cell secretory factors. These secretory factors are strongly influenced by activation of AR that regulates the expression and secretion of several cytokines (CCLs, CXCLs, ILs, etc.) and growth factors (IGFs, FGFs, TGFs, etc.) that have paracrine action on epithelial cells during BPH and PCa conditions.[8, 9] Also, it has been observed that gain in Stromal-AR in CAFs rescued rapid growth and progression of the cancerous tumors, whereas loss of Stromal-AR was directly correlated with the advanced pathological stage of PCa.[3] However, the underlying role of Stromal-AR influencing the hyperplastic growth of epithelial cells during BPH condition is still obscure. AR is the central regulatory protein in prostate development and disease pathogenesis. It profoundly functions in epithelial cells during benign and malignant tumors. In addition to epithelia, stromal cell-derived factors are also implicated to regulate prostatic disease progression.

Thus, the present study was designed to understand the role of Stromal-AR mediated epithelial cell growth and disease progression using BPH patient-derived stromal and epithelial cells. As AR targeted therapies are widely used both for BPH and PCa patients, we also aimed to evaluate the implication of such drugs on stromal epithelial crosstalk in disease progression which will enable us to throw more light on the clinical relevance of AR targeted therapies.

6.2 Plan of Work

BPH patient tissue collected from the hospital was digested with collagenase to culture stromal cells. The isolated stromal cells were characterized by specific marker expressions. To study the effect of stromal cells on epithelial cells, the conditioned media (CM) was prepared using culture media without serum-containing AR agonist and antagonist at stated doses. The prepared CM was exposed to epithelial cells to study the effect of Stroma-AR on the growth of epithelial cells using various cellular and molecular approaches. (*Figure 6.1*)

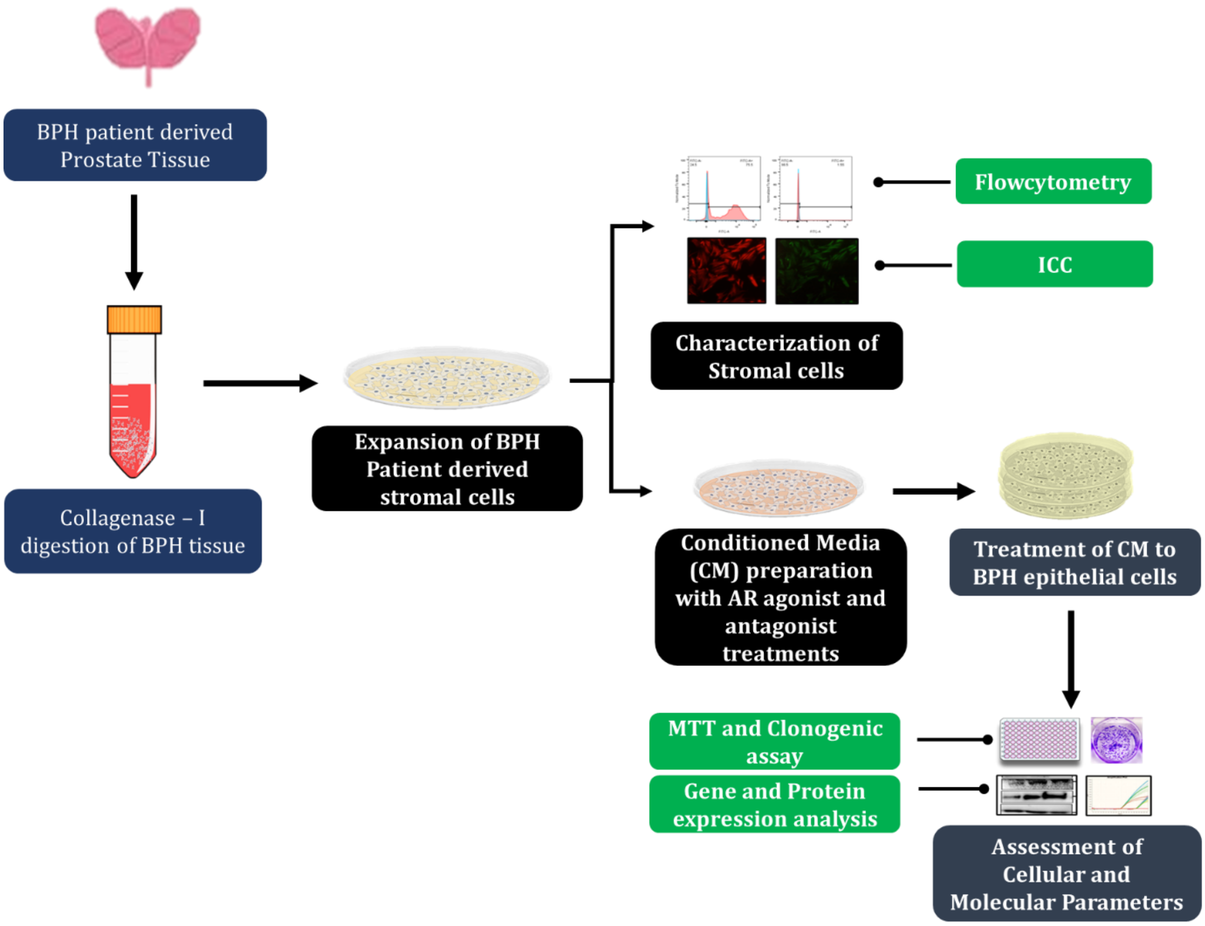
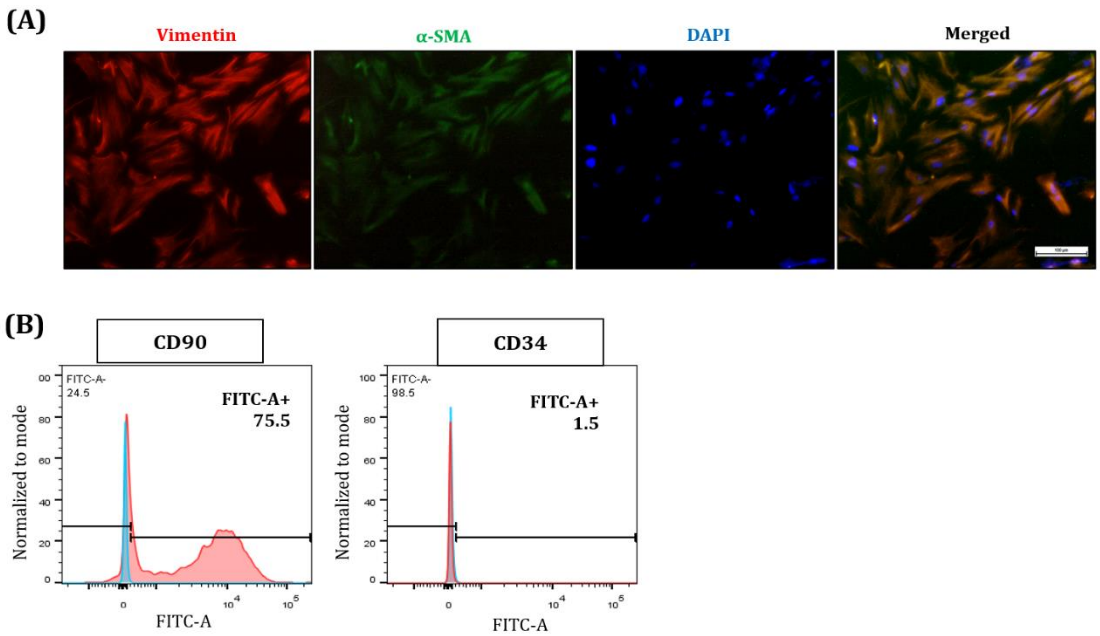


Figure 6. 1: Brief plan of work.

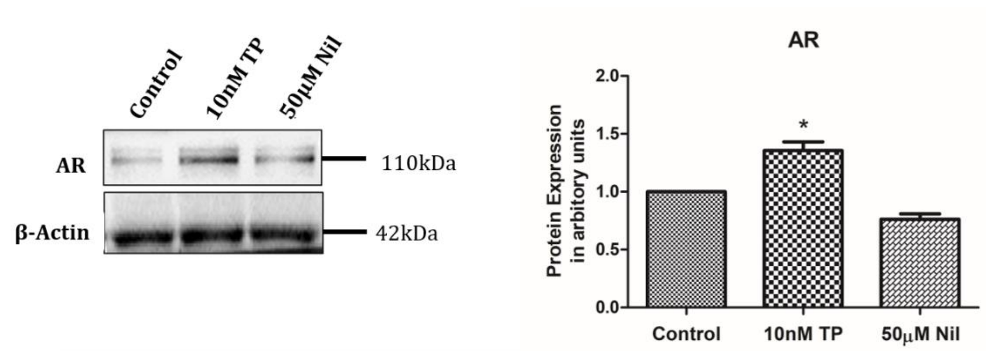
6.3 Results

6.3.1 Effect of AR activation on BPH patient-derived stromal cells.

AR performs diverse functions in the prostate gland via regulating critical genes in epithelial and stromal cells to perform secretory functions. To understand the crosstalk between Stromal and epithelial cells mediated by AR, the stromal cells were isolated and cultured from the surgically excised BPH patient tissue. The isolated cells demonstrated the expression of Vimentin (Red) and α-SMA (Green) confirming the presence of SMCs. (*Figure 6.2A*) Further, the flowcytometric evaluation of the expression of 75.5% CD90 and 1.55% CD34 depicted $CD90^{+ve}/CD34^{low}$ population further confirms the presence of SMCs. (*Figure 6.2B*) The treatment with AR agonist (10nM TP) significantly increased the AR protein levels (1.36±0.08 fold; p≤0.05) in SMCs, whereas AR antagonist (50µM Nil) treatment declined AR protein expression (0.76±0.05 fold; p>0.05). (*Figure 6.2C*) BPH patient-derived stromal cells showed a significant increase in percent cell proliferation with TP treatment at 24 hrs (114.65±3.73 percent; p≤0.01)and 48 hrs (128.85±3.6 percent; p≤0.05) and then decreased at 72 hrs (98.51±6.2; p≤0.05) as compared to control SFM media (100.00±2.2, 109.05±3.3, 120.53±0.3 percent at 24, 48, 72 hrs respectively). However, inhibition of AR with Nil treatment decreased the percent cell proliferation in a time-dependent manner at 24 (82.70±4.13 percent; p≤0.01),48 (92.91±2.4 percent; p≤0.01), 72 (38.63±2.7 percent; p≤0.05). (*Figure 6.2D*) Thus, activation and inhibition of Stromal-AR influences the proliferative potentials of SMCs derived from BPH patients.



(C) **AR protein expression in AR Agonist and Antagonist treated on BPH Patient derived SMCs**



(D)

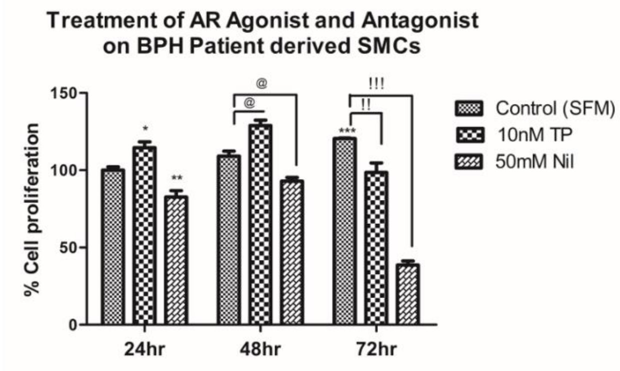
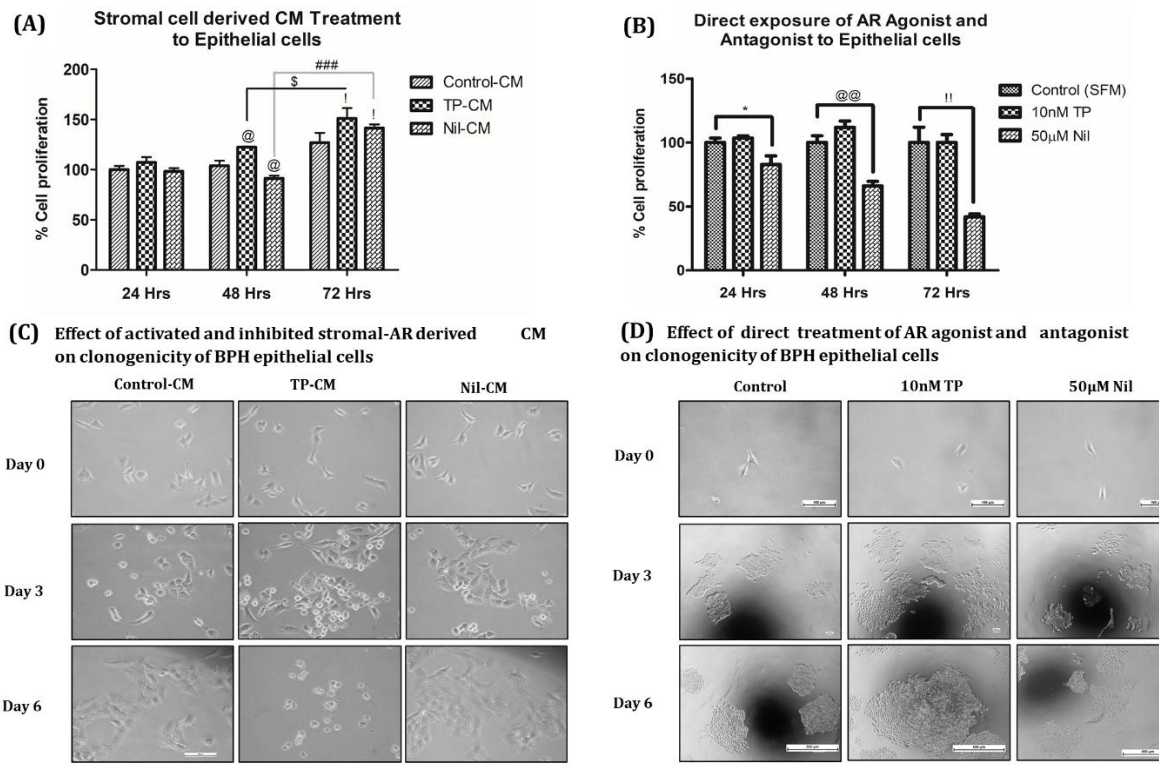


Figure 6. 2*:* Androgens regulates the cell proliferation of BPH patient-derived SMCs. (A) α-SMA and vimentin expression in the isolated cells from BPH patients confirming the presence of stromal cells; image -20X; Scale bar: 100µm. **(B)** Flow cytometric analysis of CD90 and CD34 in BPH patient-derived SMCs. **(C)** AR protein expression in stromal cells upon 10nM-TP and 50µM-Nil treatments: Plotted values in bar graph represent Mean±SEM (n=3; *p≤0.05). **(D)** Assessment of Stromal cell proliferation by MTT upon 10nM-TP and 50µM- Nil treatments: Bars represent percent cell proliferation of each treatment group at different time intervals. Data represented as Mean±SEM. *p≤0.05, **p≤0.01, ***p≤0.001 all groups vs 24-hrs Control (SFM); @P≤0.05, @@P≤0.01 48-hrs Control (SFM) vs 48-hrs treatments; !p≤0.05, !!!!p≤0.0001 72-hrs Control (SFM) vs 72-hrs treatments.

6.3.2 Activation and inhibition of Stromal-AR affects the growth and clonogenicity of BPH epithelial cells.

To evaluate the paracrine effect of stromal-derived secretome on epithelial cells, CM was prepared via activation of Stomal-AR (TP-CM) and inhibition of Stromal -AR (Nil-CM). Treatment with TP-CM to BPH epithelial cells demonstrated increased cell proliferation at 48 (122.25±0.54 percent; p≤0.05) and 72 (151.01±5.94 percent; p≤0.01) hrs as compared to control-CM treatment (100.00±3.7, 103.91±2.9, 126.81±5.7 percent at 24, 48 and 72 hrs respectively). Whereas, Nil-CM treatment to epithelial cells showed less but significant inhibition of BPH epithelial cell growth at 48 hrs (91.42±1.9 percent; p≤0.05) against control-CM and TP-CM treatments. Strikingly, delayed cell proliferation was observed in Nil-CM treatment at 72 hrs (141.53±2.1 percent; p=0.05). (*Figure 6.3A*) On the contrary, direct exposure of 10nM TP in SFM did not show proliferative effect in BPH epithelial cells. Whereas, direct exposure of 50μM Nil significantly decreased the BPH epithelial cell growth at 24 (89.5±0.7 percent; p≤0.05), 48 (66.03±3.6 percent; p≤0.01), 72 (41.9±2.2 percent; p≤0.01) hrs as compared to control (100±3.45, 96.1±5.03, 89.4±8.03 percent at 24, 48 and 72 hrs respectively). (*Figure 6.3B*)

Further, we investigated the potentials of the secretome to induce clonogenicity in epithelial cells. Results from clonogenic assay showed cell cluster formation with CM treatments of all the groups until day 3. Strikingly, complete epithelial cell death was observed in the case of TP-CM treatment as compared to Control-CM and Nil-CM treatments on the 6th day of clonogenic assay. In contrast, Nil-CM treatment exhibited higher clonogenic expansion of BPH epithelial cells on the 6th day. (*Figure 6.3C*) Moreover, direct treatment of 10nM TP to BPH epithelial cells formed large epithelial cell clusters whereas 50μM Nil exposed cell clusters were less in number and similar in size to untreated control. (*Figure 6.3D*)



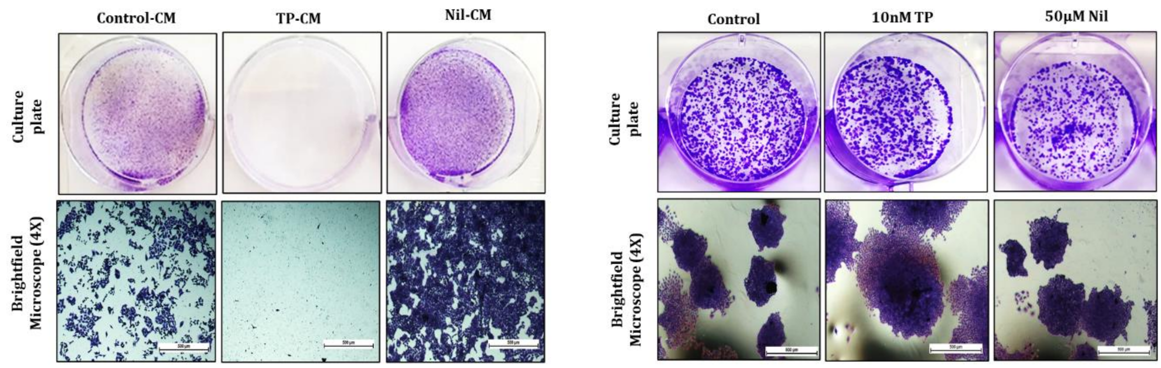


Figure 6. 3: Activation and inhibition of Stromal-AR alter the cell proliferation and clonogenicity of BPH epithelial cells. (A) Cell proliferation of BPH epithelial cells after treatment of Control-CM, TP-CM, and Nil-CM. Data represented as Mean±SEM.; n=3-4; $^{@}p \leq 0.05$ all groups 48-hrs vs 48-hrs Control-CM treatment; $^{!}p \leq 0.05$ 72-hrs of all groups vs 72-hrs Control-CM treatment; $^{\$}p \leq 0.05$ 48 hrs vs 72-hrs TP-CM treatment; $^{\#\#\#}p \leq 0.001$ 48-hrs vs 72-hrs Nil-CM treatment. **(B)** Cell proliferation of BPH epithelial cells after treatment of 10nM TP and 50µM Nil in SFM. Data represented as Mean±SEM.; n=3; $^{*}p \leq 0.05$ vs 24hrs Control; $^{@@}p \leq 0.01$ vs 48hrs Control; $^{!!}p \leq 0.01$ vs 72hrs Control. **(C)** Clonogenic growth of BPH epithelial cells treated with AR agonist and antagonist exposed Stromal cells derived CM (Control-CM, TP-CM, and Nil-CM) and brightfield images captured at Day 0 (left), Day 3 (middle), Day 6 (right) and Right panel show crystal violet staining on 6^{th} Day. **(D)** Clonogenic growth of BPH epithelial cells treated with 10nM TP and 50µM Nil and brightfield images captured at Day 0 (left), Day 3 (middle), Day 6 (right) and Right panel shows crystal violet staining on 6^{th} Day of respective treatments during clonogenic growth of BPH epithelial cells. n=2, image -4x and 20X; Scale bar: 100µm.

Collectively, inhibition of Stromal-AR imparted a higher capacity for clonogenic expansion of epithelial cells. In contrast, activation of Stromal-AR counters the clonogenic growth of epithelial cells. Thus, the results demonstrated a completely antagonistic effect of stromal secretome derived from the modulation of AR and the direct action of AR in BPH epithelial cells.

6.3.3 Stromal-AR does not affect IGF-1 secretion in BPH patient-derived stromal cells.

Expression and secretion of many peptides from stromal cells can regulate epithelial cell growth under the control of AR. One of the key growth factors secreted by prostate stroma is IGF-1 that has a strong mitogenic effect on epithelial cells. The quantitative estimation of IGF-1 levels depicted no significant change between Control-CM (2.58±0.15 pg), TP-CM (3.26±0.38 pg; p>0.05), and Nil-CM (2.58±0.21 pg; p>0.05) groups. (*Figure 6.4A*)

Further, treatment of Control-CM, TP-CM, and Nil-CM supplemented with Anti-IGF-1-mAb, depicted a significant decline in percent cell proliferation of epithelial cells with increasing incubation time. (Control-CM against Control-CM+Anti-IGF-1-mAb (24-hrs: 100.00±2.13 and 89.82±3.33 percent; 48-Hrs: 105.17±5.23 and 75.07±3.7 percent; 72-Hrs: 132.46±1.2 and 77.12±8.8 percent), TP-CM against TP-CM+Anti-IGF-1-mAb (24-hrs: 107.26±3.0 and 104.94±9.3 percent; 48-Hrs: 116.17±6.1 and 85.12±1.5 percent; 72-Hrs: 151.00±5.9 and 86.19±3.2 percent) and Nil-CM against Nil-CM+Anti-IGF-1-mAb (24-hrs: 98.39±1.8 and 175.2±6.0 percent; 48-Hrs: 91.4±1.9and 79.9±0.7 percent; 72-Hrs:141.53±2.1 and 79.54±1.7 percent) (*Figure 6.4B*) Thus, the secretory levels of IGF-1 were not affected by the alterations in Stromal-AR activity in BPH patient-derived stromal cells. However, results suggest a crucial role of stromal secreted IGF-1 on epithelial cell proliferation.

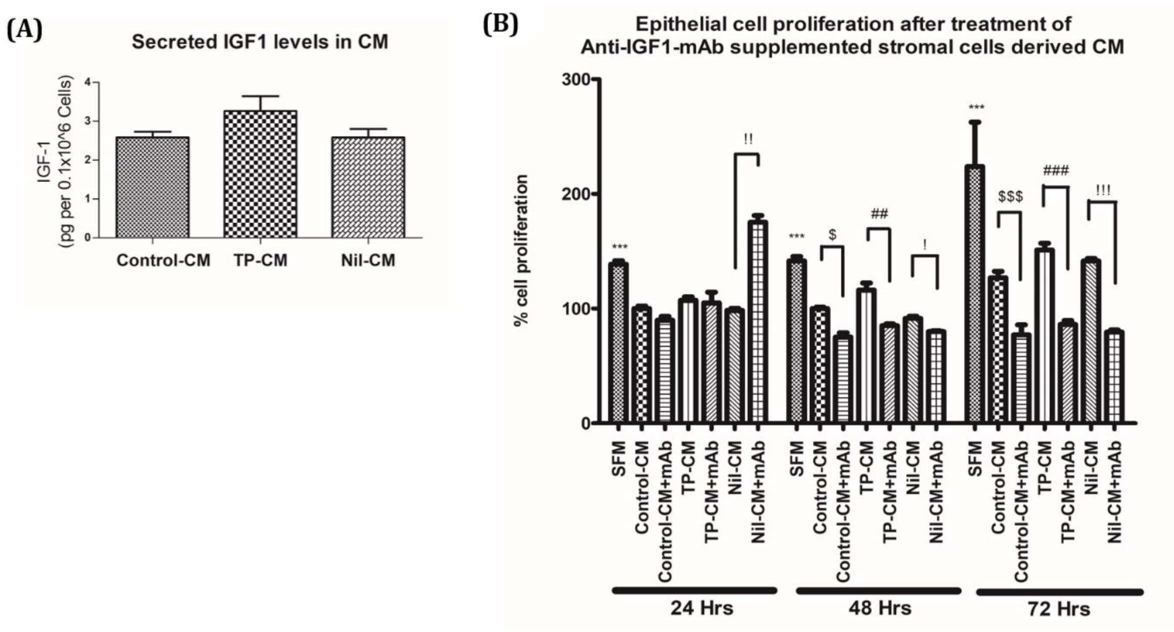


Figure 6. 4: Stromal-AR does not affect the secretion of IGF-1 in BPH patient-derived SMCs. (A) Quantitative estimation of IGF-1 in Control-CM, TP-CM, and Nil-CM groups by ELISA; Mean±SEM; n=3; **(B)** Cell proliferation of BPH epithelial cells after treatment of 10ng/ml anti-IGF-1-mAB in each secretome Control-CM, TP-CM, and Nil-CM. Data represents Mean±SEM; n=3;***p≤0.001 all groups compared with SFM Control; $^{\$}$p≤0.05, $^{\$\$\$}$p≤0.001 Control-CM vs Control-CM+IGF-1-mAb, $^{\#\#}$p≤0.01, $^{\#\#\#}$p≤0.001 TP-CM vs TP-CM+IGF-1-mAb, $^{!}$p≤0.05, $^{!!}$p≤0.01, $^{!!!}$p≤0.001 Nil-CM vs Nil-CM+IGF-1-mAb.

6.3.4 Activation and inhibition of Stromal-AR alters its secretory profile to regulate epithelial cell fate

To comprehend the secretory specific secreted factors, the conditioned media was assessed using HPLC. The results showed the elusion of a distinct peak at 16 min interval, which was common in all the secretome groups including Control-CM, TP-CM, and Nil-CM. Further, we have identified peptide peaks between 36-38 mins in TP-CM, which was not present in Control-CM and Nil-CM groups. Strikingly, a peculiar peptide peak around 41 min was depicted specifically in the Nil-CM group. (*Figure 6.5*) Thus, the data suggests an intriguing and highly specific secretory profile of stromal cells due to activation and inhibition of Stromal-AR. These secreted factors could be the fate-determining elements for the BPH epithelial cells; however, the identification of these factors remains elusive in the present study.

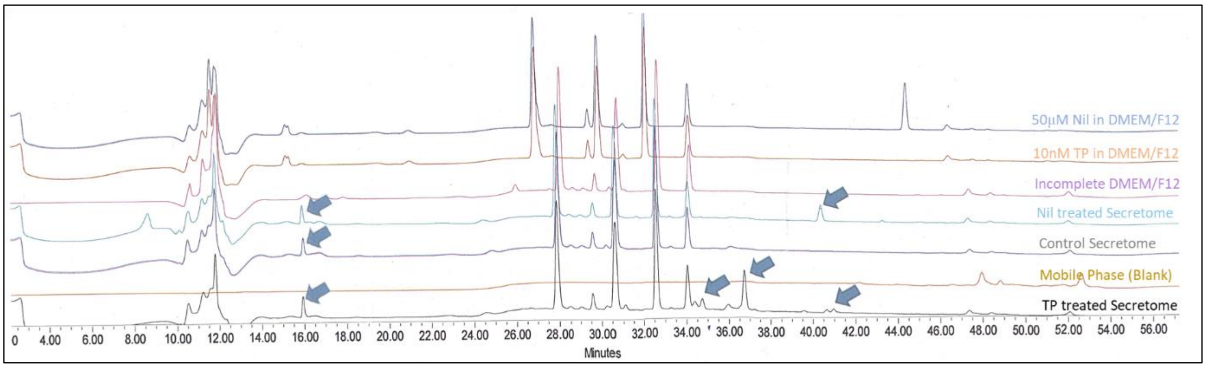


Figure 6. 5: Activation and inhibition of Stromal-AR distinctly affect the secretory factors. The graph represents the specific peaks of stromal cells secreted factors exposed to TP and Nil separated through HPLC. Distinct peaks in comparison to the mobile phase, incomplete media, and drug treatments from stromal cell secretome were highlighted with blue arrows.

6.3.5 Stromal-AR inhibition influences cell survival and clonogenicity via altering AR, AKT, and cMYC expression.

To evaluate the levels of epithelial-AR, AR protein expression was assessed. AR protein levels were significantly upregulated with TP-CM treatment (1.114±0.01 fold; $p \leq 0.01$) and downregulated with Nil-CM treatment (0.80±0.06 fold; $p \leq 0.05$). Apart from AR, IGF-1 plays a substantial role in disease progression via activation of AKT signaling. Evaluation of total AKT and phosphorylated AKT (pAKTS473) depicted a profound increase in BPH epithelial cells upon both TP-CM (Total AKT: 3.53±0.6 fold; $p \leq 0.05$ and pAKT: 1.24±0.08 fold; $p \leq 0.05$ respectively) and Nil-CM (Total AKT: 3.71±0.8; $p \leq 0.05$ and pAKT: 1.46±0.06 fold; $p \leq 0.05$ respectively) treatments as compared to Control-CM group. However, Nil-CM had significantly higher pAKT ($p \leq 0.05$) than the TP-CM group supporting higher cell proliferation and survival capability. (*Figure 6.6A*) The previous report suggested increasing cMYC expression was determined as a vital oncogenic event during the early stage of PCa.[10] As we observed increased clonogenicity in BPH epithelial cells with Nil-CM treatment, we assessed the expression of cMYC in BPH epithelial cells. The significant increase in cMYC transcript levels was detected in BPH epithelial cells treated with Nil-CM (5.63±1.4 folds; p≤0.05) against Control-CM treatment with no change in TP-CM group (0.84±0.06 fold; p>0.05). (*Figure 6.6B*) Collectively, BPH epithelial cells exhibited increased pAKT and cMYC with decreased AR levels due to the paracrine effect of CM derived by Stromal-AR inhibition, supporting cell survival, and enhanced clonogenicity in epithelial cells.

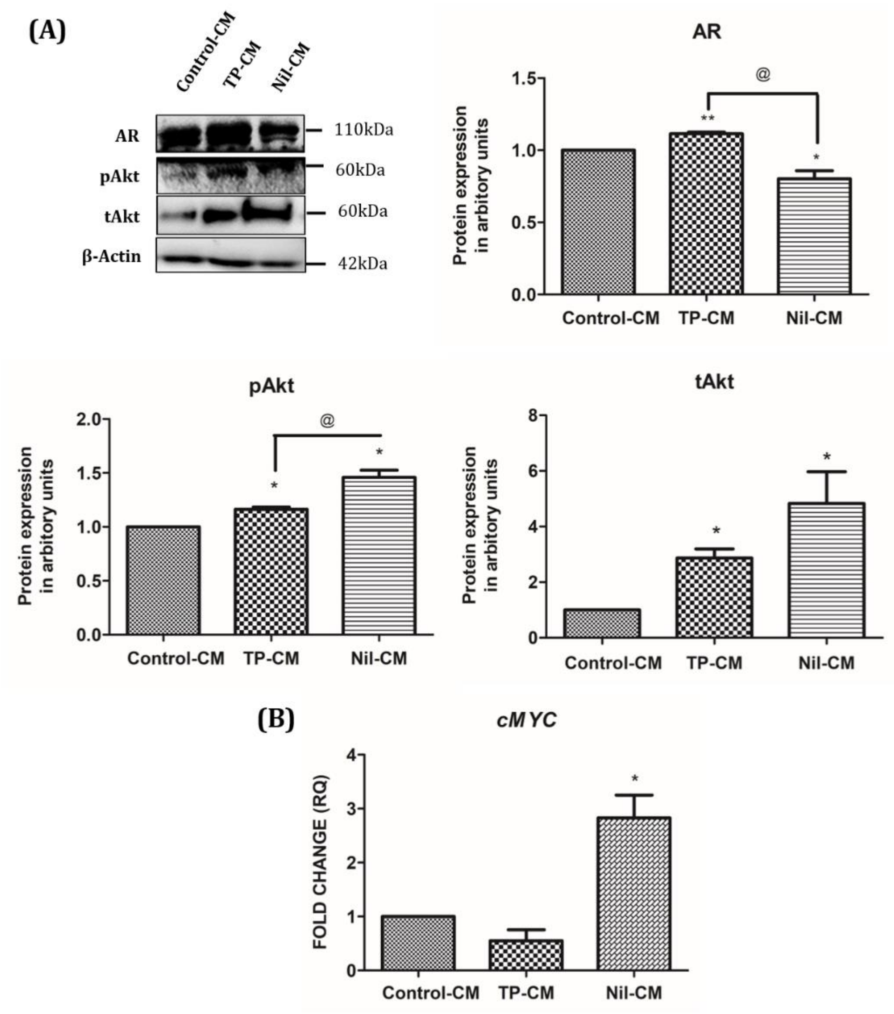


Figure 6. 6: Inhibition of Stromal-AR affects MYC, AR, and AKT levels in BPH epithelial cells through secreted factors. (A) Immunoblot images and graphs representing protein expression of AR, tAKT, and pAKT normalized with β-ACTIN in BPH epithelial cells upon Control-CM, TP-CM and Nil-CM treatments; Data represented as Mean±SEM; n=3; *p≤ 0.05, **p<0.01 as compared to control-CM; $^{@}$P≤0.05, TP-CM

vs Nil-CM. **(B)** Graph representing transcript levels of cMYC in BPH epithelial cells upon Control-CM, TP-CM, and Nil-CM treatments; Data represented as Mean±SEM; n=4; *p≤0.05 as compared to control- CM.

6.3.6 Secretory actions of Stromal-AR activation positively influence the basal/progenitor state of the BPH epithelial cells.

Previously isolated BPH epithelial cells used in this study depicted the expression of pluripotency and basal stem cell markers.[11] As secreted factors from prostate stroma regulate the basal/progenitor state of the epithelial cells,[12] we intended to explore if the basal/progenitor populations are involved in clonogenic growth in BPH. Hence, LGR4 and ΔNP63α basal/progenitor markers were investigated in BPH epithelial cells with the treatment of CM derived from activation and inhibition of Stromal-AR. The transcript levels of LGR4 (12.55±3.1 fold; p≤0.05) and its downstream effector, β-CATENIN (3.59±0.7 fold; p≤0.05) showed upregulation in BPH epithelial cells with TP-CM treatment as compared to Control-CM and Nil-CM treatments. (*Figure 6.7A*) Additionally, the protein expression of LGR4 (2.2±0.2 folds; p≤0.05) and ΔNP63α (1.8±0.16 folds; p≤0.05) were significantly increased with TP-CM treatment no significant alterations in LGR4 (0.74±0.04 fold; p>0.05) and ΔNP63α (0.98±0.25 fold; p>0.05) in Nil-CM treated group in BPH epithelial cells. (*Figure 6.7B*) Thus, the results suggest that activation of Stromal-AR derived CM supports the increased levels of LGR4/β-CATENIN/ΔNP63α suggesting a positive effect on basal/progenitor markers.

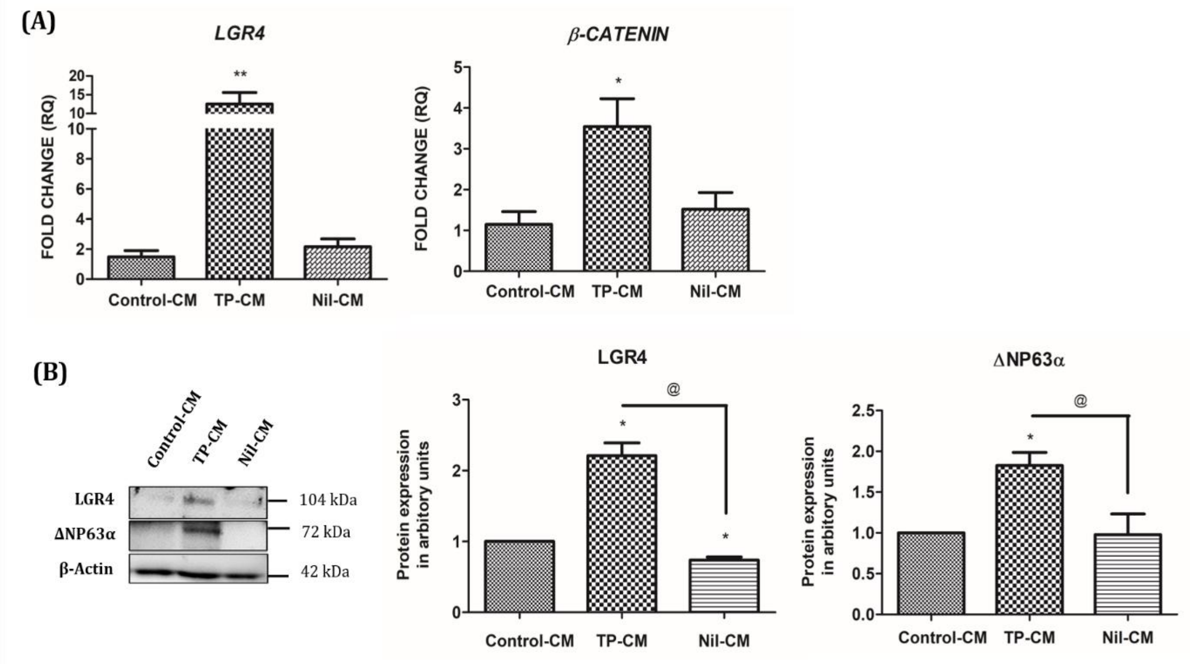


Figure 6. 7: Activation of Stromal-AR influences the basal/progenitor state of BPH epithelial cells through secreted factors. (A) Graphs representing transcript levels of LGR4 and β-CATENIN in BPH epithelial cells upon Control-CM, TP-CM, and Nil-CM treatments; Data represented as Mean±SEM; n=4; $*p \leq 0.05$,$**p \leq 0.01$ as compared to control- CM. **(B)** Immunoblot images and graphs representing protein expression of AR, LGR4 and ΔNP63α normalized with β-ACTIN in BPH epithelial cells upon Control-CM, TP-CM and Nil-CM treatments; Data represented as Mean±SEM; n=3; $*p \leq 0.05$, as compared to control-CM; $^{@}p \leq 0.05$, TP-CM vs Nil-CM.

6.4 Discussion

Crosstalk between stromal and epithelial cells in the prostate tumor microenvironment has immense implications in tumor growth. The involvement of stroma has been identified as a key regulator of PCa tumors.[13] The cells of cancerous stroma, known as Cancer-Associated Fibroblasts (CAFs), can aggressively induce PCa progression, metastasis, and therapy resistance in patients.[14, 15] However, the role of AR in stomal-epithelial crosstalk is not largely explored during BPH condition. Hence, to explore the role of Stromal-AR on epithelial cells, we have isolated fibroblasts from BPH patients expressing Vimentin and α-SMA, ensuring the presence of SMCs, the major cell type of adult prostate stroma.[16, 17] The majority of these isolated SMCs are $CD90^{+ve}/CD34^{-ve}$ and interestingly, $CD90^{+ve}$ stromal cells are in direct contact with the epithelial cells surrounding the acini, that express growth factors and genes related to cell motility, developmental process, and androgen biosynthesis to promote epithelial growth.[18] Hence, the isolated $CD90^{+ve}$ SMCs in the present study must be directly influencing the epithelial cells in a paracrine manner.

AR is the master driver of PCa through epithelial cell proliferation contributing to tumor growth.[19] In agreement with Leimgruber's report, stimulation of TP in the primary cultures of SMCs enhanced their cell proliferation.[20] Additionally, stimulation of androgen in primary fibroblasts of healthy human subjects showed increased AR protein expression.[21] Previously, tissue recombination and mouse knockout depicted that activation of Stromal-AR promotes epithelial growth during gland development.[22, 23] Further, activation and inhibition of Stromal-AR have substantial involvement in the disease progression of the prostate gland.[3] These evidences support that stromal cell secretome derived through Stromal-AR activation is vitally involved in the fetal development of the gland PCa progression also. The co-culture between AR-negative stromal cells and PC3 cells resulted in enhancement of the growth rate of PC-3 cells compared to individually grown PC-3 cells.[24] Similarly, the coculture of AR expressing CAFs with PCa cell-lines showed decreased invasive potentials of the cancer cells.[25-27] Moreover, a greater decrease of Stromal-AR has been reported in the cells of the cancerous tissue as compared to BPH tissue which was also associated with increasing tumor grade.[24] Further, androgen deprivation and loss of Stromal-AR alters paracrine actions by promoting the invasiveness via altering the extracellular matrix in PCa patients.[28, 29] These evidences support the present data where inhibition of Stromal-AR derived from BPH patient imposed clonogenic growth in the BPH epithelial cells through patient-derived stromal cell secretory factors. Moreover, this is the first report to highlight two completely diverse effects of cell-specific AR expression in BPH condition; where epithelia-AR activation increases clonogenicity, but Stromal-AR activation decreases clonogenicity in the BPH patients.

One of the key mitotic growth factors synthesized and secreted by stroma is IGF-1, and its receptor (IGF-1R) expressed on prostate epithelial cells that activate AKT signaling.[30] The bioavailability of serum IGF-1 was found to be increased in BPH patients and its overexpression caused the neoplastic transformation of the murine prostate epithelium.[31, 32] In the present study, IGF-1 secretion from BPH stromal cells derived CM was not affected due to alterations in Stromal-AR. Yet, trapping secreted IGF-1 with mAb significantly reduced the growth of BPH epithelial cells suggesting a significant contribution of stromal secreted IGF-1 in cell proliferation. Moreover, liquid chromatography data clearly indicated the presence of differentially secreted factors due to activation and inhibition of Stromal-AR. However, identification of these factors could not be accomplished in the present study. Further, since BPH stroma does not have as extensive chromatin changes as in CAFs, AR may not be able to exert similar regulatory action. Additionally, AR was also found to regulate other secreted factors as well,[33] which may involve in the survival and clonogenicity of BPH epithelial cells.

We showed that the secretome of inhibited Stromal-AR induces cell survival and clonogenicity which is corroborated with increased pAKT and cMYC and decreased AR levels. Previously, higher levels of cMYC expression were detected in PCa patients as compared to BPH patients,[34, 35] which bestow an androgen-independent and invasive PCa tumor growth.[36, 37] Previously, Williams *et al* showed that increased cMYC expression in BPH epithelium is sufficient to induce carcinogenesis.[38] The initiation, advancement, and reoccurrence of PCa are often found to be driven by cMYC protooncogene.[39, 40] Further, MYC overexpression also reduced AR signaling in mouse xenografts and drives resistance against AR inhibitors and profoundly occupied the chromatin sites of decreasing AR levels and promotes AR-independent tumor growth.[41-43] The bigenic (mpAKT/Hi-MYC) mice model discovered that MYC promoted AKT to form drug-resistant PCa and increased pAKT levels inhibits apoptosis and supports the survival and growth of tumor cells in PCa.[44, 45] The majority of the stromal secreted growth factors and cytokines acts via activation of the AKT pathway for cell survival and proliferation.[46, 47] Since AR, cMYC, and AKT expressions are interlinked in PCa survival and tumorigenesis, their role is explicitly relevant in this study also. This study, therefore, corroborates cell survival and clonogenic growth of BPH epithelial cells, which was mediated through paracrine action of Stromal-AR inhibition, which induced the upregulation of oncogene cMYC and AKT. Thus, the progressive loss of Stromal-AR in BPH patients enhances the risk of oncogenic activation and cell proliferation/survival pathways, which can aid malignant changes in the tissue.

The basal/progenitor cells profoundly contribute to the development of BPH and PCa.[11] AR expression is found negative or low in basal/progenitor cells, and increasing AR expression leads to differentiation of Basal/Progenitor cells.[48] Furthermore, stromal secretory factors can regulate resident basal/progenitor cell populations in the prostate gland.[49] Kwon *et al* recently discovered that coculturing stromal cells with basal stem cells increase basal phenotype and organoid forming activity of these cells through its paracrine activity.[18] The stromal cells produce multiple Wnt ligands that induce Wnt/β-CATENIN signaling in prostate basal stem cells,[50] which are regulated by a key stemness marker, LGR4.[51, 52] LGR4 expression was depicted in both human basal and luminal cells and its overexpression drives AR signaling activation with increased cell survival during prostate tumorigenesis in PCa cell-lines.[53, 54] In this study, we have depicted that LGR4 and its downstream effectors AR and β-CATENIN were increased in epithelial cells upon AR activated Stromal-AR secretome treatment. Increased β-CATENIN can bind to the promoter of ΔNP63α and upregulate its expression.[55, 56] And, ΔNP63α is the predominant isotype of P63 that is specifically expressed in prostate basal stem cells to regulates the pool of the basal/progenitor population.[57] Thus, the upregulation of ΔNP63α in BPH epithelial cells supports positive regulation through the activation of the Stromal-AR secretome. The present study exhibited the first key evidence on Stromal-AR mediated positive regulation of ΔNP63α and LGR4/β-CATENIN in basal/progenitor cells of BPH condition, which also rationalizes the pathological condition where increased basal/progenitor cells have been found due to expression of Stromal-AR in the BPH tissue.

The current study reveals the regulatory role of AR that modulates the fate of epithelial cells via adjacently residing stromal cells in BPH condition. The study highlights the first evidence on activation of Stromal-AR that maintains the basal state of the epithelial cells via LGR4, β-CATENIN, and ΔNP63α expression but also limits cell proliferation and inhibits clonogenic expansion in epithelial cells during BPH condition. On the contrary, secretome derived from Stromal-AR inhibition, cause cell survival, and enhanced clonogenicity through cMYC and AKT leading to the hyperproliferative changes in BPH epithelial cells (*Figure 6.7*). Although IGF-1 contributed to epithelial proliferation, the role of other secretory factors remained elusive. It has been reported that loss of AR is substantially more in PCa than in BPH patients, which is associated with poor outcome and relapse in PCa patients.[27, 58] Moreover, instances where BPH often coexists pathologically in a PCa patient,[59, 60] Stromal-AR has found to be lost in the tissue surrounding the PCa tumor.[59] Hence, this study also validates the failure of androgen deprivation therapies in patients with coexisting BPH and PCa where loss or inhibition of Stromal-AR in the BPH stroma can augment the growth or relapse of the PCa tumor. Hence, this *in-vitro* study provides an insight into the complex role of Stromal-AR loss that accomplice the tumorous growth of BPH epithelial cells that could acquire malignant changes with androgen deprivation therapies.

6.5 Summary

This study provides substantial evidence that Stromal-AR activation protects the epithelial cells to acquire proliferative changes during BPH condition. Also, loss of AR and/or use of anti-androgens as BPH therapeutics may bring an undesirable risk of malignant transformation of epithelial cells through Stromal-AR inhibition. Activation of Stromal-AR limits the growth of the epithelial cells and regulates basal/progenitors. In contrast, inhibition of Stromal-AR promotes AR independent growth of the BPH epithelial cells with increases in pAKT and cMYC expression, making them prone to malignant transformation.

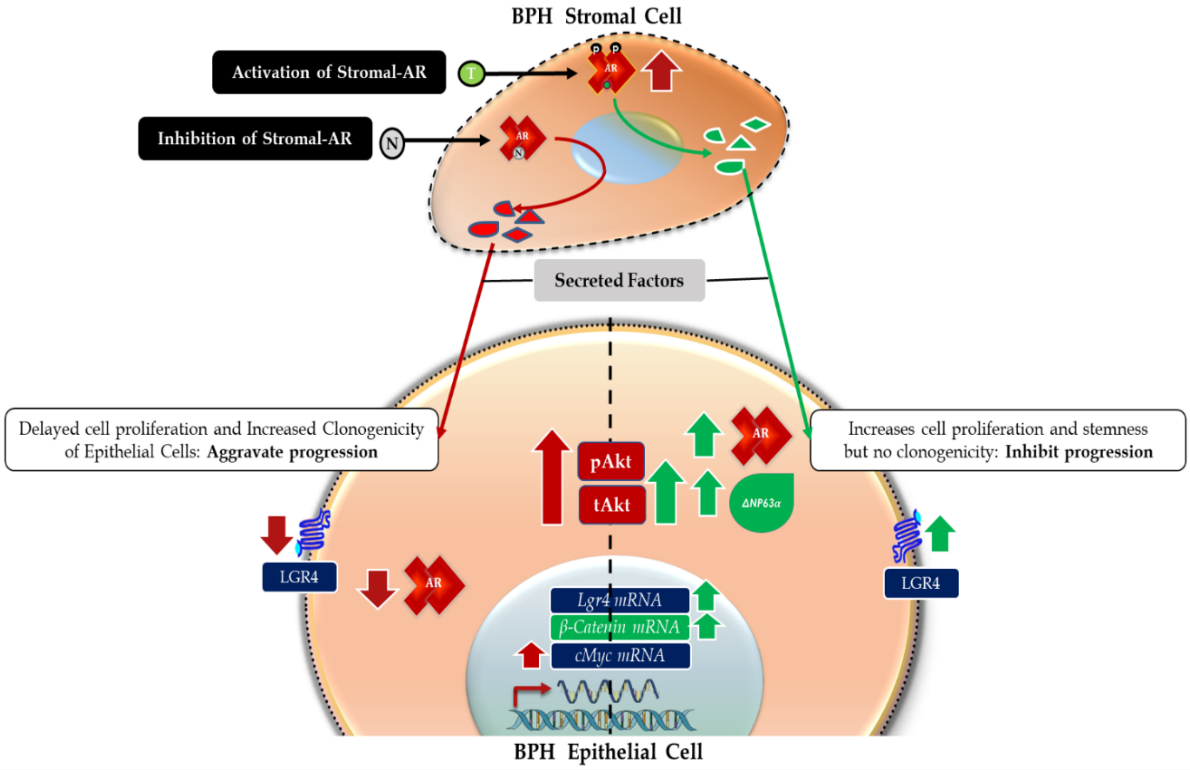


Figure 6. 8: Graphical summary. The image illustrates the role of Stromal-AR activation and inhibition in BPH epithelial cells.

6.6 References

[1] Cunha GR, Chung LW, Shannon JM, Reese BA: Stromal-epithelial interactions in sex differentiation. Biology of reproduction 1980, 22:19-42.

[2] Simian M, Hirai Y, Navre M, Werb Z, Lochter A, Bissell MJ: The interplay of matrix metalloproteinases, morphogens and growth factors is necessary for branching of mammary epithelial cells. Development 2001, 128:3117-31.

[3] Singh M, Jha R, Melamed J, Shapiro E, Hayward SW, Lee P: Stromal androgen receptor in prostate development and cancer. The American journal of pathology 2014, 184:2598-607.

[4] Li Q, Deng Q, Chao HP, Liu X, Lu Y, Lin K, Liu B, Tang GW, Zhang D, Tracz A, Jeter C, Rycaj K, Calhoun-Davis T, Huang J, Rubin MA, Beltran H, Shen J, Chatta G, Puzanov I, Mohler JL, Wang J, Zhao R, Kirk J, Chen X, Tang DG: Linking prostate cancer cell AR heterogeneity to distinct castration and enzalutamide responses. Nature communications 2018, 9:3600.

[5] Shiao SL, Chu GC, Chung LW: Regulation of prostate cancer progression by the tumor microenvironment. Cancer letters 2016, 380:340-8.

[6] Gascard P, Tlsty TD: Carcinoma-associated fibroblasts: orchestrating the composition of malignancy. Genes & development 2016, 30:1002-19.

[7] Schauer IG, Rowley DR: The functional role of reactive stroma in benign prostatic hyperplasia. Differentiation; research in biological diversity 2011, 82:200-10.

[8] Zhu M-L, Kyprianou N: Androgen receptor and growth factor signaling cross-talk in prostate cancer cells. Endocrine-related cancer 2008, 15:841-9.

[9] Izumi K, Mizokami A, Lin WJ, Lai KP, Chang C: Androgen receptor roles in the development of benign prostate hyperplasia. The American journal of pathology 2013, 182:1942-9.

[10] Imura M, Kojima Y, Kubota Y, Hamakawa T, Yasui T, Sasaki S, Hayashi Y, Kohri K: Regulation of cell proliferation through a KIT-mediated mechanism in benign prostatic hyperplasia. The Prostate 2012, 72:1506-13.

[11] Prajapati A, Gupta S, Mistry B, Gupta S: Prostate stem cells in the development of benign prostate hyperplasia and prostate cancer: emerging role and concepts. BioMed research international 2013, 2013:107954.

[12] Li Q, Alsaidan OA, Rai S, Wu M, Shen H, Beharry Z, Almada LL, Fernandez-Zapico ME, Wang L, Cai H: Stromal Gli signaling regulates the activity and differentiation

of prostate stem and progenitor cells. The Journal of biological chemistry 2018, 293:10547-60.

[13] Schroeder FH, Westerhof M, Bosch RJ, Kurth KH: Benign prostatic hyperplasia treated by castration or the LH-RH analogue buserelin: a report on 6 cases. European urology 1986, 12:318-21.

[14] Kyprianou N, Davies P: Association states of androgen receptors in nuclei of human benign hypertrophic prostate. The Prostate 1986, 8:363-80.

[15] Feneley MR, Puddefoot JR, Xia S, Sowter C, Slavin G, Kirby RS, Vinson GP: Zonal biochemical and morphological characteristics in BPH. British journal of urology 1995, 75:608-13.

[16] Gabbiani G, Schmid E, Winter S, Chaponnier C, De Ckhastonay C, Vandekerckhove J, Weber K, Franke WW: Vascular smooth muscle cells differ from other smooth muscle cells: predominance of vimentin filaments and a specific alpha-type actin. Proceedings of the National Academy of Sciences 1981, 78:298-302.

[17] Gravina GL, Mancini A, Ranieri G, Di Pasquale B, Marampon F, Di Clemente L, Ricevuto E, Festuccia C: Phenotypic characterization of human prostatic stromal cells in primary cultures derived from human tissue samples. International journal of oncology 2013, 42:2116-22.

[18] Kwon OJ, Zhang Y, Li Y, Wei X, Zhang L, Chen R, Creighton CJ, Xin L: Functional Heterogeneity of Mouse Prostate Stromal Cells Revealed by Single-Cell RNA-Seq. iScience 2019, 13:328-38.

[19] Zhou Y, Bolton EC, Jones JO: Androgens and androgen receptor signaling in prostate tumorigenesis. J Mol Endocrinol 2015, 54:R15-29.

[20] Leimgruber C, Quintar AA, Garcia LN, Petiti JP, De Paul AL, Maldonado CA: Testosterone abrogates TLR4 activation in prostate smooth muscle cells contributing to the preservation of a differentiated phenotype. Journal of cellular physiology 2013, 228:1551-60.

[21] Cano P, Godoy A, Escamilla R, Dhir R, Onate SA: Stromal-epithelial cell interactions and androgen receptor-coregulator recruitment is altered in the tissue microenvironment of prostate cancer. Cancer research 2007, 67:511-9.

[22] Yu S, Zhang C, Lin CC, Niu Y, Lai KP, Chang HC, Yeh SD, Chang C, Yeh S: Altered prostate epithelial development and IGF-1 signal in mice lacking the androgen receptor in stromal smooth muscle cells. The Prostate 2011, 71:517-24.

[23] Yu S, Yeh CR, Niu Y, Chang HC, Tsai YC, Moses HL, Shyr CR, Chang C, Yeh S: Altered prostate epithelial development in mice lacking the androgen receptor in stromal fibroblasts. The Prostate 2012, 72:437-49.

[24] Li Y, Li CX, Ye H, Chen F, Melamed J, Peng Y, Liu J, Wang Z, Tsou HC, Wei J, Walden P, Garabedian MJ, Lee P: Decrease in stromal androgen receptor associates with androgen-independent disease and promotes prostate cancer cell proliferation and invasion. Journal of cellular and molecular medicine 2008, 12:2790-8.

[25] Cioni B, Nevedomskaya E, Melis MHM, van Burgsteden J, Stelloo S, Hodel E, Spinozzi D, de Jong J, van der Poel H, de Boer JP, Wessels LFA, Zwart W, Bergman AM: Loss of androgen receptor signaling in prostate cancer-associated fibroblasts (CAFs) promotes CCL2- and CXCL8-mediated cancer cell migration. Molecular oncology 2018, 12:1308-23.

[26] Leach DA, Buchanan G: Stromal Androgen Receptor in Prostate Cancer Development and Progression. Cancers 2017, 9.

[27] Leach DA, Need EF, Toivanen R, Trotta AP, Palethorpe HM, Tamblyn DJ, Kopsaftis T, England GM, Smith E, Drew PA, Pinnock CB, Lee P, Holst J, Risbridger GP, Chopra S, DeFranco DB, Taylor RA, Buchanan G: Stromal androgen receptor regulates the composition of the microenvironment to influence prostate cancer outcome. Oncotarget 2015, 6:16135-50.

[28] Wikstrom P, Marusic J, Stattin P, Bergh A: Low stroma androgen receptor level in normal and tumor prostate tissue is related to poor outcome in prostate cancer patients. The Prostate 2009, 69:799-809.

[29] Kuniyasu H, Chihara Y, Kondo H, Ohmori H, Ukai R: Amphoterin induction in prostatic stromal cells by androgen deprivation is associated with metastatic prostate cancer. Oncol Rep 2003, 10:1863-8.

[30] Denduluri SK, Idowu O, Wang Z, Liao Z, Yan Z, Mohammed MK, Ye J, Wei Q, Wang J, Zhao L: Insulin-like growth factor (IGF) signaling in tumorigenesis and the development of cancer drug resistance. Genes & diseases 2015, 2:13-25.

[31] Niu Y-N, Xia S-J: Stroma–epithelium crosstalk in prostate cancer. Asian journal of andrology 2009, 11:28.

[32] Saikali Z, Setya H, Singh G, Persad S: Role of IGF-1/IGF-1R in regulation of invasion in DU145 prostate cancer cells. Cancer cell international 2008, 8:10.

[33] Yamamoto H, Masters JR, Dasgupta P, Chandra A, Popert R, Freeman A, Ahmed A: CD49f is an efficient marker of monolayer- and spheroid colony-forming cells of the benign and malignant human prostate. PloS one 2012, 7:e46979.

[34] Le Magnen C, Bubendorf L, Ruiz C, Zlobec I, Bachmann A, Heberer M, Spagnoli GC, Wyler S, Mengus C: Klf4 transcription factor is expressed in the cytoplasm of prostate cancer cells. European journal of cancer 2013, 49:955-63.

[35] Bhowal A, Majumder S, Ghosh S, Basu S, Sen D, Roychowdhury S, Sengupta S, Chatterji U: Pathway-based expression profiling of benign prostatic hyperplasia and prostate cancer delineates an immunophilin molecule associated with cancer progression. Scientific reports 2017, 7:9763.

[36] Bernard D, Pourtier-Manzanedo A, Gil J, Beach DH: Myc confers androgen-independent prostate cancer cell growth. J Clin Invest 2003, 112:1724-31.

[37] Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, Sawyers CL: Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer cell 2003, 4:223-38.

[38] Williams K, Fernandez S, Stien X, Ishii K, Love HD, Lau YF, Roberts RL, Hayward SW: Unopposed c-MYC expression in benign prostatic epithelium causes a cancer phenotype. The Prostate 2005, 63:369-84.

[39] Hawksworth D, Ravindranath L, Chen Y, Furusato B, Sesterhenn IA, McLeod DG, Srivastava S, Petrovics G: Overexpression of C-MYC oncogene in prostate cancer predicts biochemical recurrence. Prostate cancer and prostatic diseases 2010, 13:311-5.

[40] Koh CM, Bieberich CJ, Dang CV, Nelson WG, Yegnasubramanian S, De Marzo AM: MYC and Prostate Cancer. Genes & cancer 2010, 1:617-28.

[41] Dardenne E, Beltran H, Benelli M, Gayvert K, Berger A, Puca L, Cyrta J, Sboner A, Noorzad Z, MacDonald T, Cheung C, Yuen KS, Gao D, Chen Y, Eilers M, Mosquera JM, Robinson BD, Elemento O, Rubin MA, Demichelis F, Rickman DS: N-Myc Induces an EZH2-Mediated Transcriptional Program Driving Neuroendocrine Prostate Cancer. Cancer cell 2016, 30:563-77.

[42] Barfeld SJ, Urbanucci A, Itkonen HM, Fazli L, Hicks JL, Thiede B, Rennie PS, Yegnasubramanian S, DeMarzo AM, Mills IG: c-Myc Antagonises the Transcriptional Activity of the Androgen Receptor in Prostate Cancer Affecting Key Gene Networks. EBioMedicine 2017, 18:83-93.

[43] Hoang DT, Iczkowski KA, Kilari D, See W, Nevalainen MT: Androgen receptor-dependent and -independent mechanisms driving prostate cancer progression: Opportunities for therapeutic targeting from multiple angles. Oncotarget 2017, 8:3724-45.

[44] Clegg NJ, Couto SS, Wongvipat J, Hieronymus H, Carver BS, Taylor BS, Ellwood-Yen K, Gerald WL, Sander C, Sawyers CL: MYC cooperates with AKT in prostate tumorigenesis and alters sensitivity to mTOR inhibitors. PloS one 2011, 6:e17449.

[45] Das TP, Suman S, Alatassi H, Ankem MK, Damodaran C: Inhibition of AKT promotes FOXO3a-dependent apoptosis in prostate cancer. Cell death & disease 2016, 7:e2111.

[46] Siu MK, Suau F, Chen WY, Tsai YC, Tsai HY, Yeh HL, Liu YN: KLF4 functions as an activator of the androgen receptor through reciprocal feedback. Oncogenesis 2016, 5:e282.

[47] Lin VK, Wang SY, Vazquez DV, C CX, Zhang S, Tang L: Prostatic stromal cells derived from benign prostatic hyperplasia specimens possess stem cell like property. The Prostate 2007, 67:1265-76.

[48] Vander Griend DJ, Litvinov IV, Isaacs JT: Conversion of androgen receptor signaling from a growth suppressor in normal prostate epithelial cells to an oncogene in prostate cancer cells involves a gain of function in c-Myc regulation. International journal of biological sciences 2014, 10:627-42.

[49] Giannoni E, Bianchini F, Masieri L, Serni S, Torre E, Calorini L, Chiarugi P: Reciprocal activation of prostate cancer cells and cancer-associated fibroblasts stimulates epithelial-mesenchymal transition and cancer stemness. Cancer research 2010, 70:6945-56.

[50] Wei X, Zhang L, Zhou Z, Kwon OJ, Zhang Y, Nguyen H, Dumpit R, True L, Nelson P, Dong B, Xue W, Birchmeier W, Taketo MM, Xu F, Creighton CJ, Ittmann MM, Xin L: Spatially Restricted Stromal Wnt Signaling Restrains Prostate Epithelial Progenitor Growth through Direct and Indirect Mechanisms. Cell Stem Cell 2019, 24:753-68 e6.

[51] Luo W, Rodriguez M, Valdez JM, Zhu X, Tan K, Li D, Siwko S, Xin L, Liu M: Lgr4 is a key regulator of prostate development and prostate stem cell differentiation. Stem Cells 2013, 31:2492-505.

[52] Qian Y, Liu S, Guan Y, Pan H, Guan X, Qiu Z, Li L, Gao N, Zhao Y, Li X, Lu Y, Liu M, Li D: Lgr4-mediated Wnt/beta-CATENIN signaling in peritubular myoid cells is essential for spermatogenesis. Development 2013, 140:1751-61.

[53] Karthaus WR, Iaquinta PJ, Drost J, Gracanin A, van Boxtel R, Wongvipat J, Dowling CM, Gao D, Begthel H, Sachs N, Vries RGJ, Cuppen E, Chen Y, Sawyers CL, Clevers HC: Identification of multipotent luminal progenitor cells in human prostate organoid cultures. Cell 2014, 159:163-75.

[54] Zhang J, Li Q, Zhang S, Xu Q, Wang T: Lgr4 promotes prostate tumorigenesis through the Jmjd2a/AR signaling pathway. Experimental cell research 2016, 349:77-84.

[55] Chu WK, Dai PM, Li HL, Chen JK: Glycogen synthase kinase-3beta regulates DeltaNP63 gene transcription through the beta-CATENIN signaling pathway. J Cell Biochem 2008, 105:447-53.

[56] Ruptier C, De Gasperis A, Ansieau S, Granjon A, Taniere P, Lafosse I, Shi H, Petitjean A, Taranchon-Clermont E, Tribollet V, Voeltzel T, Scoazec JY, Maguer-Satta V, Puisieux A, Hainaut P, Cavard C, Caron de Fromentel C: TP63 P2 promoter functional analysis identifies beta-CATENIN as a key regulator of DeltaNP63 expression. Oncogene 2011, 30:4656-65.

[57] Di Como CJ, Urist MJ, Babayan I, Drobnjak M, Hedvat CV, Teruya-Feldstein J, Pohar K, Hoos A, Cordon-Cardo C: P63 expression profiles in human normal and tumor tissues. Clinical cancer research : an official journal of the American Association for Cancer Research 2002, 8:494-501.

[58] Henshall SM, Quinn DI, Lee CS, Head DR, Golovsky D, Brenner PC, Delprado W, Stricker PD, Grygiel JJ, Sutherland RL: Altered expression of androgen receptor in the malignant epithelium and adjacent stroma is associated with early relapse in prostate cancer. Cancer research 2001, 61:423-7.

[59] Olapade-Olaopa EO, MacKay EH, Taub NA, Sandhu DP, Terry TR, Habib FK: Malignant transformation of human prostatic epithelium is associated with the loss of androgen receptor immunoreactivity in the surrounding stroma. Clinical Cancer Research 1999, 5:569-76.

[60] Orsted DD, Bojesen SE, Nielsen SF, Nordestgaard BG: Association of clinical benign prostate hyperplasia with prostate cancer incidence and mortality revisited: a nationwide cohort study of 3,009,258 men. European urology 2011, 60:691-8.