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Infiltrating macrophages promote prostate tumorigenesis *via* modulating androgen receptor-mediated CCL4-STAT3 signaling

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Abstract

Infiltrating macrophages are a key component of inflammation during tumorigenesis, but the direct evidence of such linkage remains unclear. We report here that persistent co-culturing of immortalized prostate epithelial cells with macrophages, without adding any carcinogens, induces prostate tumorigenesis, and that induction involves the alteration of signaling of macrophage androgen receptor (AR)-inflammatory chemokine CCL4-STAT3 activation as well as epithelial-to-mesenchymal transition (EMT) and down-regulation of p53/PTEN tumor suppressors. *In vivo* studies further showed that $PTEN^{+/-}$ mice lacking macrophage AR developed far fewer prostatic intraepithelial neoplasia (PIN) lesions, supporting an *in vivo* role for macrophage AR during prostate tumorigenesis. CCL4 neutralizing antibody effectively blocked macrophage-induced prostate tumorigenic signaling, and targeting AR *via* an AR degradation enhancer, ASC-J9®, reduced CCL4 expression and xenografted tumor growth *in vivo*. Importantly, CCL4 upregulation was associated with increased Snail expression and down-regulation of p53/PTEN in high-grade PIN and prostate cancer. Together, our results identify the AR-CCL4-STAT3 axis as key regulators during prostate tumor initiation and highlight the important roles of infiltrating macrophages and inflammatory cytokines for the prostate tumorigenesis.

Keywords

androgen receptor; CCL4; macrophages; epithelial-to-mesenchymal transition; prostate cancer

Introduction

Prostate cancer (PCa) is the most frequently diagnosed cancer in men and considered the second leading cause of cancer death in the U.S (1). The Inflammatory microenvironment has been reported to play an important role in tumor initiation, but a direct causal interaction between which specific type of inflammatory infiltrates and epithelial cells results in tumor initiation is still not clear (2). Aged prostate lesions have revealed that focal prostate

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Conflict of Interest: ASC-J9® was patented by the University of Rochester, the University of North Carolina, and AndroScience, and then licensed to AndroScience. Both the University of Rochester and C. Chang own royalties and equity in AndroScience.

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inflammatory atrophy (PIA) is often associated with infiltrating immune cells (3), and transition from PIA to high grade prostatic intraepithelial neoplasia (HGPIN) or PCa, may be also linked to suppression of some selective tumor suppressor genes (4). These results have implicated that inflammatory events in PIA may potentially contribute to protein down-regulation of tumor suppressor genes in prostate and later lead to the development of HGPIN and PCa. Importantly, one previous study demonstrated that pharmacological depletion of macrophages in different mouse tumor models significantly reduced tumor angiogenesis and progression, suggesting that macrophages are critical components in the tumor microenvironment for tumor progression (5). Therefore, research focusing on infiltrating macrophages and their inflammatory activities for promoting early tumor development will lead to discovering new therapeutic agents to block tumor formation.

Interestingly, our previous study identified a novel role of androgen receptor (AR) in a mouse wound healing model via controlling macrophage migration and TNFα production during the wound healing process (6). This study has provided compelling evidence that AR could direct the function of macrophages and established a new regulation of AR from the endocrinal regulation to inflammatory response in the wound healing microenvironment. Given the pro-tumor function of macrophages as described above and the important role of AR in PCa development, further studies are needed to investigate whether AR would modulate macrophage function in the process of PCa initiation through mediating induction of cytokines/chemokines.

Considering that there are no direct experimental models available for addressing the impact of macrophages to directly induce prostate tumorigensis, here we used *in vitro* co-culture/3-D models that recapitulated an interaction between immortalized prostate epithelial cells (RWPE-1 cells) and macrophages, and found for the first time that infiltrating macrophages alone, without adding any carcinogens, could induce prostate tumorigenesis *via* a novel pathway involving AR-inflammatory cytokine CCL4-STAT3 activation, down-regulation of p53/PTEN tumor suppressors, and promotion of epithelial-to-mesenchymal transition (EMT) signaling pathways. These findings may help us to develop new potential therapeutic approaches to battle PCa at the early PIN development stages.

Methods and Materials

Antibodies and reagents

ASC-J9® (5-hydroxy-1,7-bis(3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one) was a gift from AndroScience (7). Antibody information is provided in the Supplementary Methods and Materials.

Cell culture

RWPE-1 and BPH-1 cells (the non-neoplastic, immortalized human prostatic epithelial cell lines) and THP-1 cells (the human acute monocytic leukemia cell line) were obtained from the American Type Culture collection (ATCC, Rockwell, MD). For other cell lines, co-culture and 3-D culture experiments, see Supplementary information.

Human Cytokine Antibody Array and ELISA

The conditioned media (CM) was collected from 48 hr monocultures of RWPE-1, THP-1, or 48 hr co-cultures of RWPE-1/THP-1 cells. The CM collected from monocultures or co-cultures were used to determine relative amounts of cytokine levels using Human Cytokine Array kit (R&D Systems) and for detection of CCL4 using human CCL4 ELISA kits (R&D Systems) according to the manufacturer's instructions.

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Histology and H&E staining/Immunohistochemistry (IHC)

Xenograft tumors and prostate tissues were harvested for histology examination as described in the supplementary Methods and Materials. H&E and IHC staining was performed as described previously (7).

Co-culture and 3-D culture, cell proliferation/migration assay, colony formation assay, Western blot analysis, quantitative real-time PCR, AR silencing in THP-1 cells by lentiviral siRNA, orthotopic implantation, ASC-J9® treatment, generation of MARKO/PTEN$^{+/-}$ (macrophage AR knockout mice), and human prostate tissue microarray analysis were conducted as described in the Supplementary Methods and Materials.

Statistics

The data values were presented as the mean ± SD. *P* values were determined by unpaired Student's *t* test. Differences in CCL4 expression in prostate TMA were analyzed by Fisher's exact test or Chi-square test. *P* 0.05 was considered statistically significant.

Results

Increased macrophage infiltration in HGPIN and PCa lesions

To investigate the potential linkage of infiltrating macrophages in prostate tumorigenesis, we first performed IHC on human prostate tissue microarrays containing benign, HGPIN, and PCa lesions, using an anti-CD68 antibody. Consistent with the findings in an early study (8), the number of CD68-positive macrophages was significantly increased in HGPIN (P=0.004) or PCa (P<0.0001) lesions compared with that in benign prostate (Fig. 1A). There was no significant difference in the number of CD68-positive cells between HGPIN and PCa (P=0.8518). Based on these results, we hypothesized that infiltrating macrophages in the prostate could contribute to the promotion of prostate tumor development.

Co-culture of immortalized human prostate epithelial cells with human macrophage THP-1 cells induces prostate tumorigenesis

To recapitulate the interaction of macrophages with prostate epithelial cells during prostate tumorigenesis, we co-cultured THP-1 cells with RWPE-1 cells in transwell plates. We observed a 50% increase in cell proliferation at 48 hours (Fig. 1B). RWPE-1 cells cultured in the co-culture medium resulted in a 3-fold increase in the migration ability (Fig. 1C). Next, we developed a 3-D co-culture matrigel model to mimic an inflamed microenviroment that would allow interaction between macrophages and prostate epithelial cells, since immortalized prostate epithelial cells, cultured in purified extracellular matrix, can differentiate into well-organized spheroids of glandular prostate epithelial cells, so-called prostaspheres, acinar-like spheroid structures with lumens (9, 10). We found that RWPE-1 cells alone were able to develop prostaspheres after 24 days (Fig. 1D). In contrast, RWPE-1 cells cultured with the co-culture conditioned media (CM) differentiated into a disorganized aggregate structure (Fig. 1D), suggesting that soluble factors derived from the co-culture may disrupt the normal differentiation process of RWPE-1 cells via initiating prostate tumorigenic events (11). Similar results were also obtained when we replaced RWPE-1 cells with another human epithelial cell line, BPH-1 (Fig. S1 A and B). Our results support that soluble factors in the co-culture CM may contribute to transformation of non-tumorigenic RWPE-1 cells and disrupt their differentiation in 3-D Matrigel culture.

To confirm that RWPE-1 cells were indeed undergoing a tumorigenic process after co-culture, RWPE-1 cells (± THP-1 cells) were plated on soft agar to determine their anchorage-independent growth. As expected, non-tumorigenic RWPE-1 cells were able to form colonies on soft agar after co-culture with THP-1 cells (Fig. 1E). Next, RWPE-1 and

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THP-1 cells were either monocultured or co-cultured for 5 days, and then were subcutaneously injected into the flanks of athymic nude mice. The injection of RWPE-1/THP-1 cells resulted in tumor development in 7 out of 7 nude mice. In contrast, none of the mice injected with mono-cultured RWPE-1 or THP-1 cells developed tumors (Fig. 1F and Fig. s2). Taken together, our findings demonstrate, both *in vitro and in vivo*, that induction of prostate tumorigenesis can be achieved in non-tumorigenic prostate RWPE-1 cells by co-culture with THP-1 macrophages.

CCL4 and STAT3 as potential mediators for macrophage-mediated prostate tumorigenesis

Since an earlier study demonstrated that constitutively-active forms of STAT3 promotes epithelial to mesenchymal transition (EMT) and tumorigenesis of RWPE-1 cells (12), we examined the STAT3 signaling pathway and found an increase of pSTAT3 and its downstream genes (COX-2 and c-Myc) in RWPE-1 cells after co-culture with THP-1 cells (Fig. 2A). Consistently, we also observed STAT3 activation in an immortalized mouse prostate epithelial cell line, mPrE, during co-culture with a murine macrophage cell line, RAW264.7 or bone marrow-derived macrophages (BMDMs). (Fig. s3A and B) (13, 14). These data suggest that the crosstalk between macrophages and prostate epithelial cells was able to enhance STAT3 activation in prostate epithelial cells, regardless of whether the macrophages originated from an established cell line or murine bone marrow. In addition, several EMT-associated genes, such as Snail, MMP9, and N-cadherin, were signficantly increased in the co-cultured RWPE-1 cells (Fig. 2B). Simlilar upregulation of EMT-associated genes was found in co-cultured BPH-1 and mPrE cells (Fig. s1C and s3B). These results suggest that STAT3 activation with induction of EMT genes might play important roles in mediating macrophage-induced prostate tumorigeneis (12, 15).

We then applied Western blot-based cytokine array analysis to globally identify inflammatory mediators in the co-culture CM. The most abundant cytokines/chemokines were CCL4, CCL5, IL1β, IL1ra, G-CSF, and IL8 (Fig. 2C and Fig. s4). Interestingly, we found consistent upregulation of CCL4 and CCL5 expression in co-cultured BPH-1 cells (Fig. s1D). We focused on CCL4 since our concurrent study has identified CCL4 as an AR downstream gene linked to prostate tumor initiation (16). The quantitative realtime-PCR analysis confirmed that co-culture led to the greatest increase in mRNA and protein expression in CCL4 (Fig. 2D and E). To validate the role of CCL4 in mediating EMT and prostate tumorigenesis via STAT3 activation, we used a CCL4 neutralizing antibody to determine whether suppression of CCL4 activity might inhibit the crosstalk between THP-1 and RWPE-1 cells. We found significant downregulation of mRNA expression of CCL3, CCL5, and IL8 (Fig. 2F), suggesting that CCL4 induction could possibly be an early and vital event during the crosstalk. Consistently, suppressing CCL4 activity led to a signficant reduction in THP-1-mediated cell migration and EMT gene induction (Fig. 2G–H), with decreases in STAT3 activation and COX-2 induction (Fig. 2I). Taken together (Fig. 2F–I), our results suggest that CCL4 plays an early and crucial role in macrophage-mediated tumorigenic signaling.

CCL4 is a critical mediator for suppression of p53 and PTEN tumor suppressors

To rule out the possiblity that xenografted tumors of RWPE-1/THP-1 cells may be derived from THP-1 cells (Fig. 1F), we used the alternative approach for long-term culture of RWPE-1 cells with the co-culture CM for 60 days, and then implanted these cells into nude mice. Interestingly, we found distinctive morphological changes of RWPE-1 cells, especially in their mesenchymal shape (spindle-like), compared to control cells (Fig. 3A). We also found that long-term culture of RWPE-1 cells with the co-culture CM resulted in increased colony formation (Fig. 3B). More importantly, following orthotopic injection of long-term cultured RWPE-1 cells into the anterior prostate, 8 out of 12 mice developed

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tumors (Fig. 3C), confirming that RWPE-1 cells can become tumorigenic after long-term culture in the co-culture CM. Importantly, immunohistochemistry staining of E6/E7 proteins further revealed that these tumors were originated from injected RWPE-1 cells (Fig. 3C).

To explore the molecular basis for THP-1-mediated tumorigenesis of RWPE-1 cells, we examined two important tumor suppressor pathways, p53 and PTEN, whose suppression plays essential roles in prostate tumorigenesis (17, 18). Although the expression of p53 and PTEN at RNA levels was comparable between parental and long-term cultured RWPE-1 cells after 5 days (**sFig. 5**), their protein levels were significantly decreased after 20 days and almost disappeared after 60 days in the presence of the co-culture CM (Fig. 3D). Next, we further investigated the potential mechanisms underlying increased CCL4 induction for prostate tumorigenesis via downregulation of p53/PTEN proteins. With CCL4-neutralizing antibody, we observed a severe impairment in THP-1-induced downregulation of PTEN and p53 expression (Fig. 3E). Similarly, the anti-CCL4 antibody reduced the induction of EMT markers, N-cadherin and Snail (Fig. 3E). These results suggested that CCL4 plays a consistent and essential role in the entire process of THP-1-induced prostate tumorigenesis in RWPE-1 cells. Together, our study supports a mechanism by which THP-1 cells induced prostate tumorigenensis involves the sequential activation of CCL4- and STAT3-mediated pathways, leading to downregulation of p53/PTEN and induction of EMT for prostate tumorigenesis.

To determine whether CCL4 directly functions as a tumor promoter for prostate epithelial cells, we found that treating RWPE-1 cells with CCL4 failed to induce the expression of EMT markers (MMP9 and Snail) that were upregulated in the co-culture CM (Fig. s6A). We postulated that the reason that naïve RWPE-1 cells were unable to respond to CCL4 could be the chemokine receptor levels. To test this hypothesis, we used quantitative real-time PCR analysis to examine the expression levels of two CCL4 receptors, CCR1 and CCR5, in RWPE-1 cells. CCR1 expression was low in monocultured RWPE-1 cells, but was significantly induced after co-culture with THP-1 cells (Fig. s6B), suggesting the cooperative role of such receptors with CCL4 during the interaction between macrophages and prostate epithelial cells.

Macrophage AR promotes macrophage-induced prostate tumorigenesis via CCL4/STAT3 dependent pathways

Interestingly, the results of our earlier report identified a regulatory role of AR in macrophages during the wound healing process via modulation of chemokine receptors, macrophage migration, and selective pro-inflammatory cytokines (6). However, the precise nature and function of AR in directing macrophages during prostate tumorigenesis is not clear. This is an important yet unanswered question since most AR-PCa research has been focused on AR’s roles within prostate epithelial and stromal cells during PCa development (19, 20). Therefore, we hypothesized that AR in macrophages is also a key player in the crosstalk between macrophages and prostate epithelial cells for macrophage-mediated prostate tumorigenesis shown in Fig. 1. Importantly, we found that AR silencing by lentiviral AR-siRNA in THP-1 cells (THP-1 ARsi), suppressed their ability to promote cell proliferation (Fig. 4A) and migration (Fig. 4B) of RWPE-1 cells during co-culture. Consistently, the *in vitro* transformation capacity of THP-1 ARsi cells on RWPE-1 cells was reduced in both 3D culture and colony formation assays (Fig. 4C and D). Similarly, reduced EMT related gene and inflammatory cytokine expression was oberved in BPH-1 cells that were co-cultured with THP-1 ARsi cells (Fig. s7A–C), supporting a role for AR in macrophage-induced prostate tumorigenesis. We also found that silencing macrophage AR expression in THP-1 cells reduced expression of downstream oncogenic mediators, such as pSTAT3, AR, and c-Myc in RWPE-1 cells (Fig. 4E). THP-1 ARsi cells failed to

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significantly upregulate EMT markers, Snail, Vimentin, and N-cadherin expression in RWPE-1 cells (Fig. 4F). Several cytokines mRNA expression levels were reduced by AR silencing (Fig. 4G) with CCL4 showing the most significant change comparing RWPE-1/THP-1sc (scramble) to RWPE-1/THP-1ARsi cells (Fig. 4H), consistent with our recent study identifying CCL4 as an AR target gene (16). Reduced CCL4 protein levels in the co-culture CM of RWPE-1/THP-1ARsi cells were confirmed by ELISA (Fig. 4I). Interestingly, AR silencing in THP-1 cells also resulted in the reduction of the CCR1 expression levels (Fig. s6B), indicating a similar regulatory mechanism of CCL4/CCR1 upregulation by macrophage AR during co-culture. These results suggest that infiltrated macrophages may simultaneously trigger CCL4 and its cognate receptor expression in RWPE-1 cells and enable RWPE-1 cells to respond to CCL4 stimulation. Collectively, these results suggest that macrophage AR plays a role in mediating CCL4 induction, STAT3, chemokine receptor CCR1 expression, and EMT to promote the macrophage-induced prostate tumorigenesis.

Macrophage AR ablation inhibits PIN formation in PTEN$^{+/-}$ mice

To further investigate the *in vivo* role of macrophage AR in prostate tumorigenesis, we generated a bigenic mouse PTEN mutant line with the genetic background of macrophage AR knockout (MARKO: AR$^{fl/y}$: lyzM-cre^{+}/PTEN$^{+/-}$, see Fig. s8 for details on mating strategy and confirmation of the genotypes), taking advantage of the well-known lysozyme promoter-driven cre enzyme to delete the target gene in mature macrophages at 83–98% efficiency (6, 21, 22). Using this MARKO model we can observe the *in vivo* prostate tumor development while AR is ablated in infiltrated macrophages by the *cre-loxP* system to mimic our *in vitro* co-culture experiments.

Mice were sacrificed at 6 months, when PIN formation can be detected in PTEN$^{+/-}$ mice. We observed increased anterior prostate size in PTEN$^{+/-}$ mice when compared to the controls. Deleting AR in macrophages led to reduced prostate size in MARKO/PTEN$^{+/-}$ mice when compared to PTEN$^{+/-}$ mice (Fig. 5A), along with minimal effects on serum testosterone (Fig. s8C). Strikingly, we found reduced PIN formation in MARKO/PTEN$^{+/-}$ prostate when compared to abundant moderate/low-grade PIN in PTEN$^{+/-}$ prostates (Fig. 5B1), suggesting that defects in AR-deficient macrophages can suppress haplodeficiency of PTEN-induced prostate tumorigenesis.

We then performed F4/80 immunostaining and found that F4/80^{+} macrophages were reduced in MARKO/PTEN$^{+/-}$ prostates when compared to PTEN$^{+/-}$ prostates (Fig. 5B2). These findings suggest a positive correlation of mouse prostate tumorigenicity with the degree of macrophage infiltration modulated by macrophage AR. We also examined the key mediators previously identified in our *in vitro* co-culture models. Our results, consistent with our *in vitro* and *in vivo* findings (Fig. 2–4), demonstrate the consequences of deleting macrophage AR: decreased macrophage infiltration into the prostate, reduced expression of CCL4, pAKT (a marker of PTEN function), pSTAT3, p53, and EMT genes (N-cadherin and Snail), and increased E-cadherin expression in the anterior prostate of MARKO/PTEN$^{+/-}$ mice compared to those found in control PTEN$^{+/-}$ prostate (Fig. 5B3–5B9). Taken together, our bigenic mouse model demonstrates that macrophage AR ablation markedly attenuates PIN formation in PTEN$^{+/-}$ mice. These results provide the first *in vivo* evidence showing essential roles of infiltrating macrophages for the induction of prostate tumorigenesis, and support a role for macrophage AR or its downstream genes (such as CCL4) in PIN formation of PTEN$^{+/-}$ mice.

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Targeting AR with an AR degradation enhancer, ASC-J9®, to suppress macrophage-induced prostate tumorigenesis

We have demonstrated that the AR-CCL4 axis is important for macrophage-induced prostate tumorigenesis. Therefore, we tested a potential therapeutic approach by targeting the upstream AR with an AR degradation enhancer, ASC-J9®, which could selectively degrade AR proteins in certain cells, including prostate cells and macrophages (6, 7, 23).

We first treated the co-cultured RWPE-1 cells with 5 μM ASC-J9® or vehicle control and found that ASC-J9® suppressed THP-1-mediated cell proliferation (Fig. 6A) and migration (Fig. 6B). ASC-J9® also suppressed the THP-1-mediated disruption of the development of acinar-like spheroids of RWPE-1 cells in a 3D culture condition (Fig. 6C). More importantly, as shown in Fig. 6D, ASC-J9® reduced the growth of *in vivo* orthotopic xenografted tumors developed from long-term cultured RWPE-1 cells (left and middle panels) with induced AR degradation in RWPE-1 tumor cells, and prevented p53/PTEN downregulation of xenografted tumors (right panel). When we examined CCL4 expression, we found that ASC-J9® and Casodex (an anti-androgen currently used to treat PCa) effectively suppressed induction of CCL4 expression (Fig. 6E), further confirming a novel role of AR in controlling CCL4 expression since these two drugs target AR in various cell types in the xenografted tumor microenviroment. Consistently, ASC-J9® prevented p53/PTEN downregulation in co-cultured RWPE-1 cells, and inhibited expression of EMT related genes (Snail and N-cadherin) in co-cultured RWPE-1 cells in a 3D culture condition (Fig. 6F and G). In addition to AR protein downregulation, one striking feature of this data is the apparent downregulation of pSTAT3 by ASC-J9® at 5 μM (Fig. 6 F), suggesting that this small molecule can simultaneously target AR and STAT3 function in transformed RWPE-1 cells, and inhibit downstream tumorigenic events. Taken together, results shown in Fig. 6A–G support our working model that therapeutic targeting of prostatic/macrophage AR and STAT3 by ASC-J9® might provide a new approach for treating patients during the early stage of PCa development.

Expression of CCL4, p53, and EMT-related proteins in human prostate tissues

To determine the clinical significance of our findings regarding the key mediator, CCL4, in PCa, we examined CCL4 expression in prostatectomy specimens (Fig. 7A). CCL4 was positive in 21/72 (29%) benign, 46/62 (74%) HGPIN, and 50/75 (67%) carcinoma tissues (Table s1). Thus, CCL4 levels were significantly higher in HGPIN and PCa than in non-neoplastic prostate, while there was no significant difference in CCL4 expression between HGPIN and PCa, suggesting its involvement in early PCa development. We also found no strong associations of CCL4 expression with clinicopathologic features of PCa, including Gleason score, pathologic stage (pT), lymph node metastasis, and biochemical recurrence, except higher expression score in GS 6 tumors compared to GS7 (P=0.0088) or GS 7 (P=0.0090) tumors (Table s2 and Fig. s9). Next, we examined the expression of p53, pAKT, E-cadherin, and Snail in these prostate tissues (Fig. 7B). p53 (Table s3) and E-cadherin (Table s5) were significantly reduced in HGPIN, compared to benign prostate. In contrast, pAKT (Table s4) and Snail (Table s6) were significantly increased in HGPIN or PCa, compared to benign prostate. pAKT and E-cadherin were also found to be higher in PCa than in HGPIN. Collectively, results from human prostate tissue array analysis (Fig. 7A–B) confirmed our findings showing that these mediators identified in our *in vitro* co-culture models are important in the early PCa development and could be of prognostic values for PCa development.

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Discussion

One previous study demonstrated that E. coli infections in the prostate induce PIN formation in mice via persistent secretion of free radicals and/or inflammatory cytokines by infiltrating leukocytes during recurrent bacterial infections (24). These results suggest that infiltrating leukocytes are key players in PCa development. Among infiltrating leukocytes, macrophages are generally regarded as the key players in the tumor microenvironment that supports tumor progression (25). Macrophages are often viewed as double agents in the microenvironment since their functional plasticity enables them to switch to a phenotype that is either for or against tumor development and progression (26). Importantly, the THP-1 cells used here were reported to possess characteristics of M2-like TAMs (tumor-associated macrophages) with pro-tumor and immunosuppressive activity in the pre-existing tumor microenvironment (26, 27). However, whether the pre-existing pro-inflammatory environment in prostate glands, before tumorigenesis, would favor M1 or M2 polarization of macrophages remains unclear. It is possible that our *in vitro* co-culture model may provide a pre-neoplastic environment for prostate epithelial cells, and allow THP-1 cells to expand their M2-like roles to promote prostate tumorigenesis (27). Therefore, our findings suggest that macrophages are capable of stimulating prostate tumorigenesis and this is a new discovery distinct from previous studies focusing on macrophage function on tumor angiogenesis/metastasis (28, 29).

Importantly, we identified CCL4 as a novel downstream mediator of macrophage AR, and demonstrated that neutralizing the CCL4 activity can effectively block macrophage-mediated induction of cytokine expression, STAT3 activation, EMT genes, p53/PTEN down-regulation, and cell migration (Fig. 2F–I and 3E–F). These findings suggest a new role for CCL4 and its downstream signaling in early prostate tumorigenesis. Since CCL4 was mainly induced in co-cultured RWPE-1 cells in an AR-dependent fashion, this data raises the intriguing possibility that macrophage AR could be functioning to elicit unknown signals for autocrine action of CCL4 induction in RWPE-1 cells during co-culture. More importantly, additional perplexing data shows that direct CCL4 treatments failed to elicit any downstream signaling pathways in RWPE-1 cells, suggesting that additional signals generated by the macrophages for upregulation of CCL4 receptors may underlie the requirement for RWPE-1 cells to respond to CCL4. It will be fascinating to determine precisely how macrophages induce upregulation of CCL4 receptors in RWPE-1 cells.

An early study demonstrated that STAT3 activation might antagonize the p53/PTEN function in promoting tumor migration and invasion (30), and transformation of non-tumorigenic prostate epithelial cells to PCa cells may require suppression of tumor suppressor genes, such as PTEN and p53 (31, 32). Our demonstration that the long-term co-culture of non-tumorigenic prostate cells in the co-culture medium resulted in CCL4-dependent suppression of p53/PTEN function, suggesting that this key step may be required for prostate tumorigenesis. A delicate regulatory relationship exists between p53 and PTEN in which inactivation of one of the genes results in reduction of the protein levels in the other (33). It will be interesting to dissect how CCL4 downstream signaling can lead to STAT3 activation and down-regulation of both p53 and PTEN in a sequential manner during long-term co-culture-induced transformation.

It is generally believed that EMT is the major focus as the convergence point between inflammation and cancer progression, such as in metastasis (34). Interestingly, our data suggest that the induction of EMT could be an important process involved in early prostate tumorigenesis. It has been demonstrated that expression of constitutively-active STAT3 induces prostate tumorigenesis of immortalized prostate epithelial cells and the induction of EMT has been identified in this study (12), suggesting that the EMT could be one of the key

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components during prostate tumor initiation. In order to obtain the *in vivo* evidence of EMT during prostate tumor initiation, we examined Snail expression in the PIN lesions of prostate of *PTEN$^{f/f}$:probasin-cre^{+}* mice and found that notable Snail expression is associated with PIN lesions (Fig. s10), similar to one recent study using qPCR analysis demonstrating that the EMT inducer, Snail, expression is upregulated in PTEN-null prostates (35). Altogether, our studies support that CCL4-induced EMT in RWPE-1 cells could be a novel and important process involved in prostate tumor initiation.

Using ASC-J9®, an AR protein degradation enhancer, we provide evidence that ASC-J9® can effectively reduce xenografted RWPE-1 tumor size via promoting AR degradation and preventing p53/PTEN down-regulation. Consistent with our recent study (36), our data demonstrated that ASC-J9® (19) suppresses PIN formation and reduces infiltration of inflammatory macrophages in PTEN$^{+/-}$ mice that can spontaneously develop PIN, suggesting that targeting AR activity by ASC-J9® in the tumor microenvironment may not only inhibit prostatic AR function in prostate epithelial cells (36-38), but also prevent macrophages from exerting their pro-tumor function during prostate tumorigenesis. These findings may provide a new therapeutic approach using ASC-J9® for inhibiting PCa at the PIN development stage.

In summary, our study establishes an *in vitro* model in which the paracrine action was initiated by macrophages induced CCL4 production during co-culture with immortalized prostate epithelial cells. Then, the induction of CCL4 promotes prostate tumorigenesis through STAT3 activation, EMT, and down-regulation of p53/PTEN proteins in RWPE-1 cells (Fig. 2H–I and 3E). Targeting AR with ASC-J9® in xenografted RWPE-1 tumors inhibits tumor growth and reduces CCL4 expression via induction of AR protein degradation and inhibiting STAT3 activation (Fig. 6D–E), suggesting AR and CCL4 in the tumor microenvironment as potential therapeutic targets to effectively block inflammation-associated prostate tumor initiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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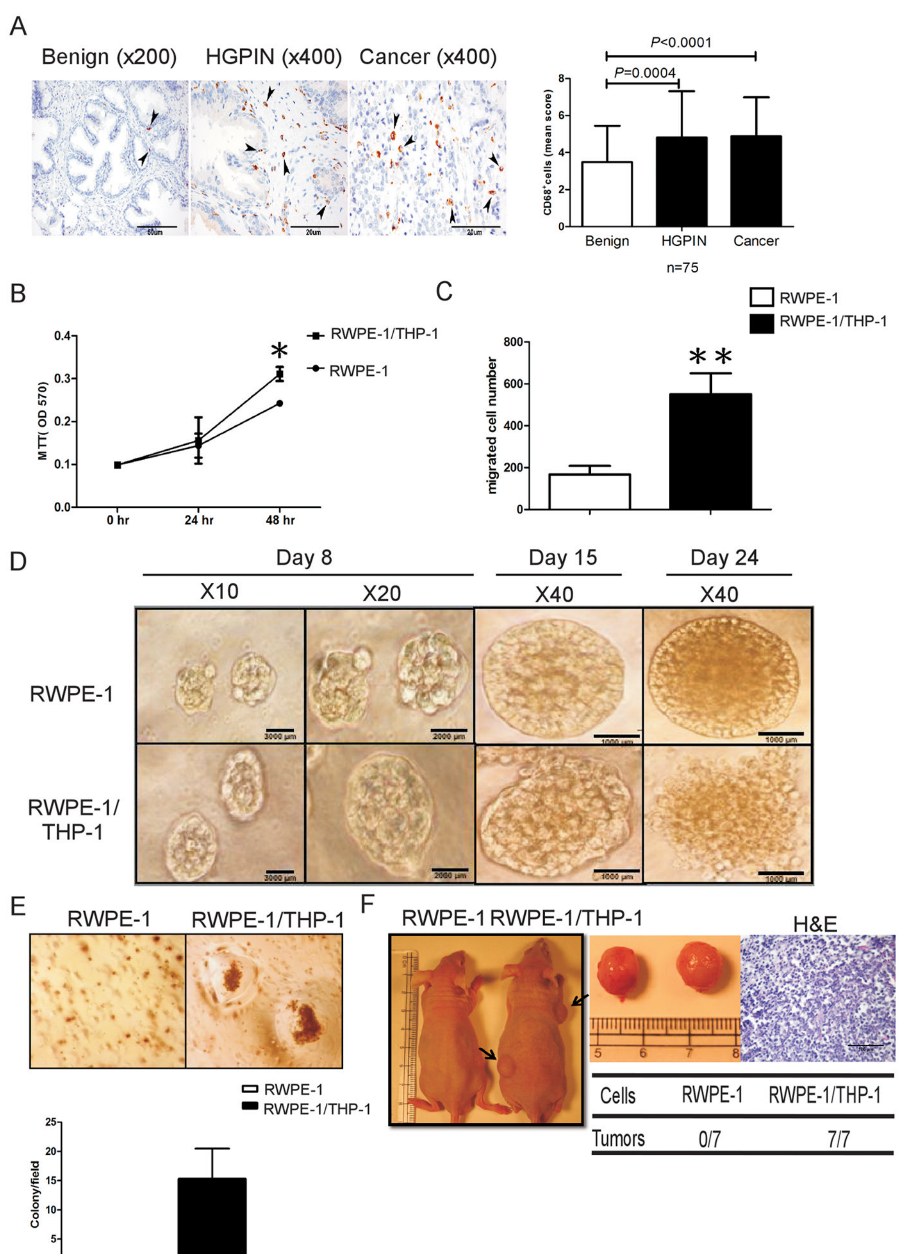

Figure 1.

The co-culture of macrophage-prostate epithelial cells induces prostate tumorigenesis. (**A**) Increased macrophage infiltration was noted in HGPIN and prostate cancer. IHC staining of human prostate tissue array was performed using anti-CD68 antibody. CD68-positive cells were counted and their mean numbers per high-power field are shown at right. Arrowheads indicate CD68-positive macrophages. (**B**) RWPE-1 cells were co-cultured with or without THP-1 macrophages for 24 and 48 hr. Cell proliferation assay was performed using MTT. *, P<0.05. (**C**) RWPE-1 cells were seeded in the upper chamber of 8 μm pore transwells with conditioned media (CM) from co-culture of THP-1/ RWPE1 cells in the lower chamber. Cells were incubated for 20 hours. Cells migrating through pores were stained with toluidine blue and counted in six random fields. Results are expressed as the average number of cells

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per field and are mean ±SD. **, P<0.01. (**D**) Microscopic analysis of acinar morphogenesis and glandular differentiation of RWPE-1 cells in a 3D condition in the presence or absence of the co-culture CM for 8, 15, or 24 days. Magnifications are: X10, X20, X40. (**E**) Colony formation in soft agar assay of RWPE1 cells (alone) and RWPE-1 cells co-cultured with THP-1 cells. (**F**) Left panel: athymic nude mice that received RWPE-1 cells (alone) or RWPE-1/THP-1 cells. Specific tumor growth of xenografts was visible after 10 weeks of subcutaneous injection (indicated by the black arrows). Middle upper panel: tumors from mice. Right upper panel: H.E staining of paraffin sections of xenograft growth after injection of RWPE-1/THP-1 cells. Scale bar: 100 μm. Right lower panel: the incidence of tumorigenesis of RWPE-1 and RWPE-1/THP-1 cells in nude mice.

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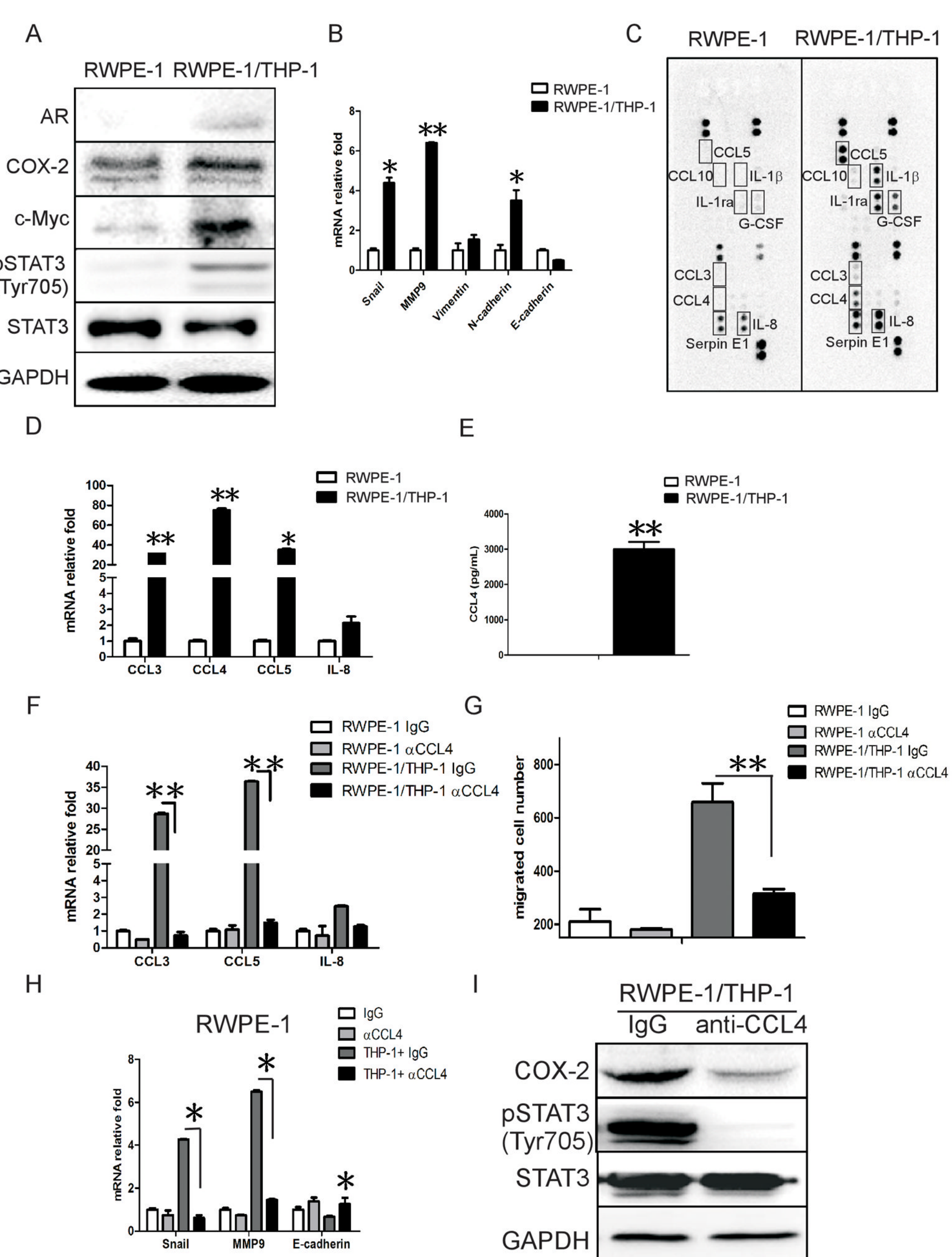

Figure 2.

Co-culture with THP-1 cells induces various cytokines and downstream signaling in RWPE-1 cells. (**A**) Whole protein extracts isolated from RWPE-1 cells and RWPE-1/THP-1 cells were analyzed for the protein levels of COX-2, pSTAT3, AR, and c-myc. STAT3 and GAPDH proteins were used as loading controls. (**B**) qPCR analysis of EMT-related genes in RWPE-1 cells and RWPE-1/THP-1 cells. (**C**) Cytokine array analysis of CM isolated from RWPE-1 or RWPE-1/THP-1 cells for 48 hours, and the expression of soluble mediators was determined by Human cytokine array (R&D Systems). (**D**) qPCR analysis of cytokine expression levels in RWPE-1 cells 48 hr after co-culture with THP-1 cells. CCL3 and CCL4 were more highly expressed after co-culture. (**E**) The amount of CCL4 was determined by

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ELISA in the media of RWPE-1/THP-1 or RWPE-1 cells alone. (**F–I**) Neutralization of CCL4 by a specific antibody attenuates THP-1-induced cytokine expression, cell migration, EMT-related genes, and downstream signaling mediators. (**F**) qPCR analysis of the expression of various cytokines in RWPE-1 ± THP-1 cells cultured in the presence of anti-CCL4 neutralizing antibody (αCCL4) or isotype control antibody (IgG). (**G**) RWPE-1 ± THP-1 cells were plated in a transwell plate as described in **F** for cell migration assay. (**H**) qPCR analysis of Snail, MMP-9, and E-Cadherin in RWPE-1 cells ± THP-1 cells as described in **F**. (**I**) Western blot analysis of COX-2 and pSTAT3 in RWPE-1 cells that were cultured as described in **F**. STAT3 and GAPDH served as protein loading controls. All data shown are mean ± SD. *, $P < 0.05$, **, $P < 0.01$.

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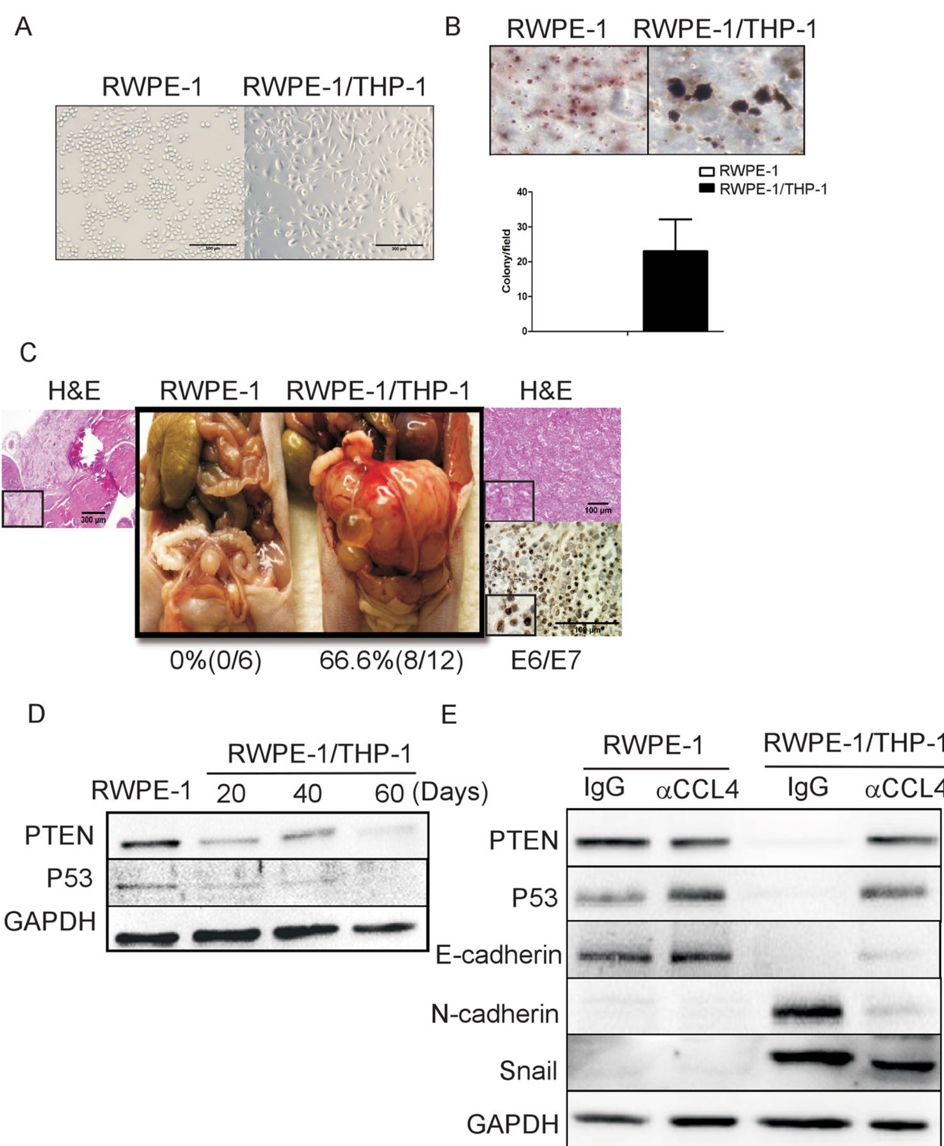

Figure 3.

Characterization of macrophage-mediated RWPE-1 cell transformation after long-term culture with the co-culture CM. (**A**) Image of representative fields of parental and transformed RWPE-1 cells. (**B**) Increased colony formation of RWPE-1 cells with co-culture CM was observed after 2 weeks. (**C**) H&E staining and IHC (let and right upper panels) analysis of cross-sections through the anterior prostate of athymic nude mice after 10 weeks of orthotopic injection of parental RWPE-1 or long-term cultured RWPE-1 cells. Gross observation and histological analysis of the anterior prostate of athymic nude mice after orthotopic injection of long-term cultured RWPE-1 cells (middle panel). An antibody against E6/E7 was used as a marker for detecting HPV18 E6 and E7 oncoproteins in RWPE-1 cells (lower right panel). (**D**) p53 and PTEN protein expression in RWPE-1 cells with the co-culture CM for 20, 40, and 60 days. (**E**) Neutralizing CCL4 activity by an anti-CCL4 antibody inhibits THP-1-mediated PTEN/ P53 protein down-regulation and induction of EMT-related genes.

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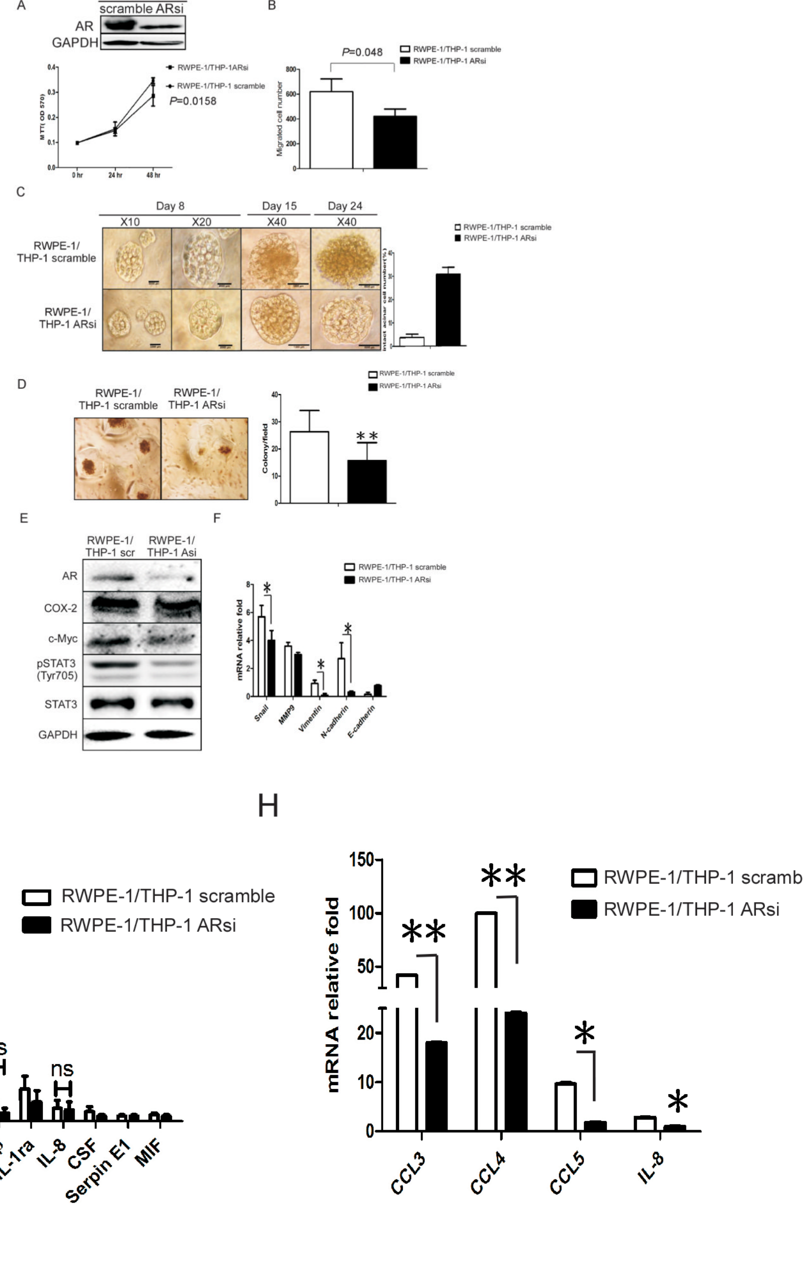

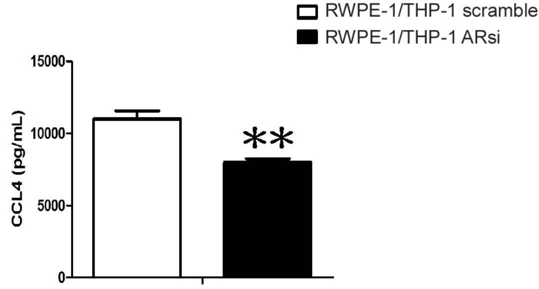

Figure 4.

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Effects of macrophage AR silencing on cell proliferation, migration, acinar morphogenesis, and colony formation. (**A**) RWPE-1 cells were co-cultured with THP-1 scramble or ARsi macrophages and assayed with MTT at 24 and 48 hours. (**B**) RWPE-1 cells (1×10^5/well) were incubated with co-culture CM of THP-1 scramble or ARsi macrophages for 20 hours in transwell plates (8 μm). Migrated cells were stained with toluidine blue and counted in six random fields. Results are expressed as the average number of cells per field and are mean ± SD. (**C**) Reduced AR expression in THP-1 cells attenuated the inhibitory effects of THP-1 cells on the acinar morphogenesis and glandular differentiation of RWPE-1 cells in culture at day 24. Magnifications are: X10, X20, X40. Results are expressed as the mean number of the intact acinar cells (right panel). (**D**) Colony formation in soft agar of RWPE-1 cells that were plated with either THP-1 scramble or THP-1 ARsi macrophages and the numbers of colonies shown (right panel). **, $P<0.01$. (**E**) Silencing AR expression in THP-1 cells inhibits induction of COX-2, pSTAT3, AR, and c-myc proteins in RWPE-1 cells during co-culture. (**F**) Reduced EMT-related gene expression in RWPE-1/THP-1 ARsi cells. (**G**) Quantification of cytokine array analysis of the co-culture CM from RWPE-1/THP-1 scramble or RWPE-1/THP-1 ARsi cells. (**H**) qPCR analysis of CCL3, CCL4, CCL5, and IL-8 mRNA levels in RWPE-1/THP-1 scramble and RWPE-1/THP-1 ARsi cells. (**I**) The amount of CCL4 was determined by ELISA in the media of RWPE-1/THP-1 scramble and RWPE-1/THP-1 ARsi cells co-cultured for 48 hr. All data shown are mean ± SD, *, $P<0.05$ **, $P<0.01$.

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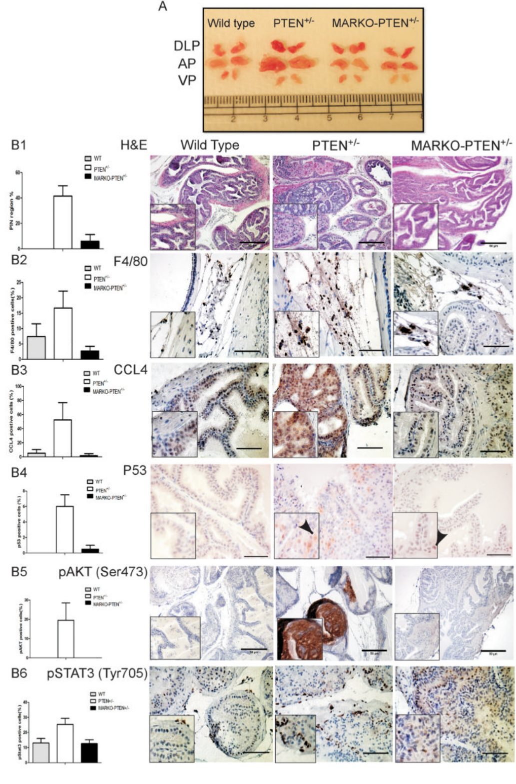

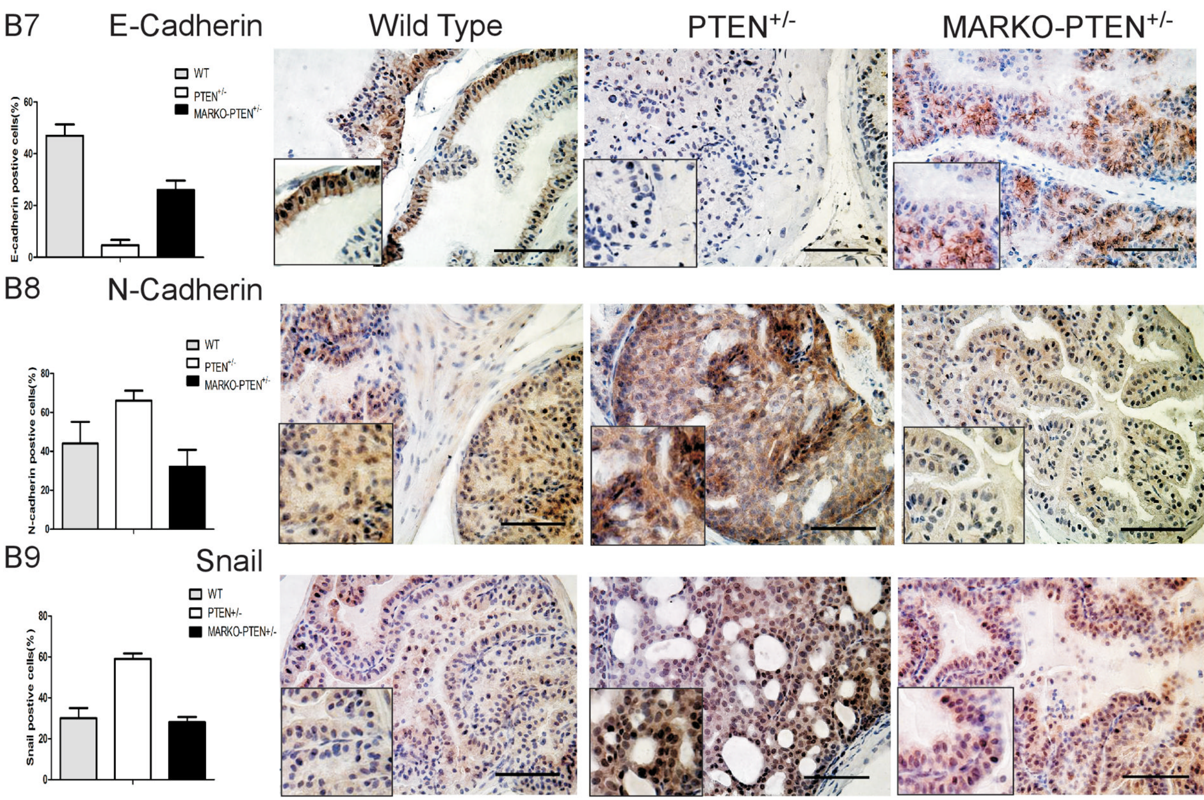

Figure 5.

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Characterization of MARKO-PTEN$^{+/-}$ prostate. (**A**) Gross observation of the dorsolateral prostate (DLP), anterior prostate (AP), and ventral prostate (VP) from Wild Type, PTEN$^{+/-}$, and MARKO/PTEN$^{+/-}$ mice. Reduced size of AP in MARKO/PTEN$^{+/-}$ mice. (**B1–B9**) IHC analysis of F4/80, CCL4, p53, pAKT, pSTAT3, E-Cadherin, and N-Cadherin expression in AP of Wild Type, PTEN$^{+/-}$, and MARKO/PTEN$^{+/-}$ mice. The AR ablation in macrophage reduced expression levels of F4/80, CCL4, pAKT, pSTAT3, p53, and N-Cadherin in PTEN$^{+/-}$ prostate. Magnifications are x10. Inset magnifications are x40. Arrowheads indicate p53-positive cells.

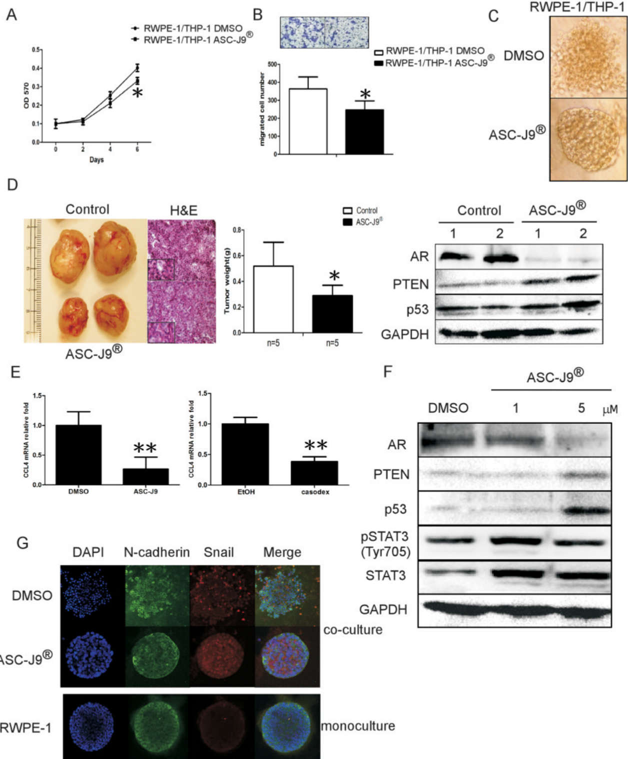

Figure 6.

Targeting macrophage AR and its downstream consequences. (**A, B**) ASC-J9® effects on cell growth (**A**) and migration (**B**). (**C**) ASC-J9® effect on acinar morphogenesis by 3D culture. ASC-J9® degraded AR in THP-1 can reverse the macrophages caused glandular structure change. (**D**) Gross observation, H&E, and weight analysis of xenografted tumors (left and middle panels), and Western blot analysis of *in vivo* orthotopic xenografted tumors (right panel). (**E**) Effects of ASC-J9® or casodex on CCL4 mRNA level using Q-PCR. (**F**) Effects of ASC-J9® on PTEN and p53 protein downregulation in co-cultured RWPE-1 cells. (**G**) ASC-J9® suppressed macrophages induced EMT marker expression (N-Cadherin and Snail) in RWPE-1 cells. Differentiated monoculture or co-culture RWPE-1 spheroids were photographed by confocal microscopy in a 3D culture condition. Data shown are mean ± SD, *, $P < 0.05$ **, $P < 0.01$.

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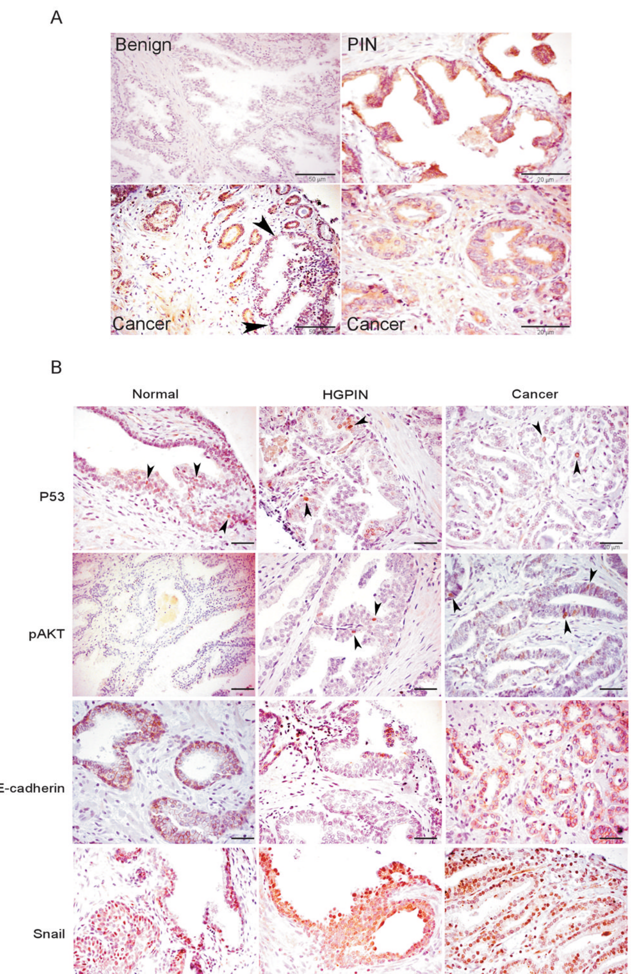

Figure 7.

IHC analysis of CCL4 (**A**) and p53/pAKT/E-cadherin/Snail (**B**) in prostate TMAs. Arrowheads indicate benign glands adjacent to cancer glands (**A**) or p53/pAKT cells (**B**).