Supplementary information

Loss of Stromal Androgen Receptor Leads to Suppressed Prostate Tumorigenesis via Modulation of Pro-inflammatory Cytokines/Chemokines

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Supplemental methods

Chromatin Immunoprecipitation

Mouse SV40-T antigen immortalized PrSC has been described previously (Lai et al, 2012) and were cultured in 10% CD-FBS–DMEM/F12 medium for 2 days. Then, cells were treated with vehicle or 10 nM DHT for 4 hr. The cells were subjected to ChIP assay as described in our previous report (Wu et al, 2010). The eluted products were detected using PCR and the AR-binding DNA was pulled down by AR antibody (C-19 for C-terminus from Santa Cruz Biotechnology). The input control served as positive control and IgG control was used as negative control. The primers against MIP-1β and KGF promoter regions were described in the Supplementary Table.

MIP-1β promoter assay

The mouse MIP-1β promoter (-1,989 to +68) was cloned into pGL3-basic vector and confirmed with sequencing. The COS-1 cells were co-transfected with pBabe-mouse AR, pGL3-mMIP-1β or MMTV-Luc, and pRL-TK plasmids by lipofectamine 2000 (Invitrogen) for 6 hr. After transfection, the cells were cultured under 5% CD-FBS media for 2 days. Following, the media were changed to media with 5% CD-FBS containing vehicle or 10 nM DHT for 24 hr incubation. For the IL-1 β stimulation, cells were stimulated with 10 ng/ml human IL-1β recombinant protein (R&D systems) for 4 hr in the 5% CD-FBS media containing EtOH or 10 nM DHT. The NF-kappaB inhibitor 10 μM BAY117082 (Cayman Chemical) was added 1 hr before IL-1β treatment. The standard luciferase assays were conducted and data are presented as mean±SD.

Bone marrow mesenchymal stem cells (BM-MSCs) isolation

For the isolation of mouse BM-MSCs, we followed the protocol described previously (Peister et al, 2004). Briefly, tibias and femurs were dissected from adult mice at 8-10 weeks old. The bone marrow cells were flushed out using syringe, and resuspended in 15% FBS DMEM media. After cell counting, 2 $x10^6$ mononucleated cells were plated in 25 cm^2 plastic flasks with 15% FBS DMEM supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10 mM HEPES. Cells were incubated at 37°C with 5% humidified CO_2 and confirmed with MSCs markers by flow cytometry and QPCR analysis.

Colony-Forming Unit Fibroblast (CFU-F) assay

Primary BM-MSCs were isolated from 8 weeks old Wt and G-ARKO (general ARKO) mice and then plated $2x10^6$ mononucleated cells per well onto 6-well plates. After culturing 21 days, cells were fixed with methanol and washed with ddH_2O. After washing, cells were stained with 1% methylene blue for 15 minutes and cells rinsed with water 2 times. We counted colonies that have over 50 cells as positive CFU-F.

Supplementary references

Lai KP, Yamashita S, Vitkus S, Shyr CR, Yeh S, Chang C (2012) Suppressed prostate epithelial development with impaired branching morphogenesis in mice lacking stromal fibromuscular androgen receptor. Mol Endocrinol 26**:** 52-66

Peister A, Mellad JA, Larson BL, Hall BM, Gibson LF, Prockop DJ (2004) Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood 103**:** 1662-1668

Wu MH, Ma WL, Hsu CL, Chen YL, Ou JH, Ryan CK, Hung YC, Yeh S, Chang C (2010) Androgen receptor promotes hepatitis B virus-induced hepatocarcinogenesis through modulation of hepatitis B virus RNA transcription. Sci Transl Med 2**:** 32ra35

Supplementary Table

The primers sequence used for qRT-PCR and ChIP assays are shown.

Supplementary Figures

Figure S1. PIN lesions are not restricted to proximal or distal ducts of Pten+/- mice. (**A**) Histological examination of 7 months old Wt-AR/Pten+/+, Wt-AR/Pten+/-, and dARKO/Pten+/- mouse DLPs subjected to H&E staining. The prostate ducts were separated into proximal and distal regions. The PIN lesions are presented as multi-layered epithelium and typical cribriform structures pointed-out by arrows. (**B**) IHC staining against p-Akt at Ser473 to confirm PIN lesion in proximal and distal ducts of three genotypes of mice. Arrows indicate p-Akt positive epithelium. N=4-5 for each group for mouse data collection. Scale Bars=200 μm (100X).

Figure S2. Stromal AR regulates the similar proinflammatory cytokines/chemokines expression in PrSC isolated from Pten+/- mouse APs. (**A**) Real-time PCR analysis was used to detect cytokine changes in Pten+/- PrSC ARscr and Pten+/- PrSC ARsi isolated from APs. Each bar represents the mean±SD of at least 2 independent determinations performed in triplicate.

Figure S3. AR does not directly bind to MIP-1β promoter region in mouse prostate stromal cells. (A) The partial promoter sequences of mouse MIP-1β are shown. The primers used for ChIP assay are labeled with black bold colors with underline and potential AREs motifs are labeled with red colors predicated by TESS (U of Pennsylvania). **(B)** Stromal AR fails to bind to MIP-1β promoter by ChIP assay. The immunoprecipitated MIP-1β and KGF promoters (as positive control) are shown. The input and IgG controls are also provided.

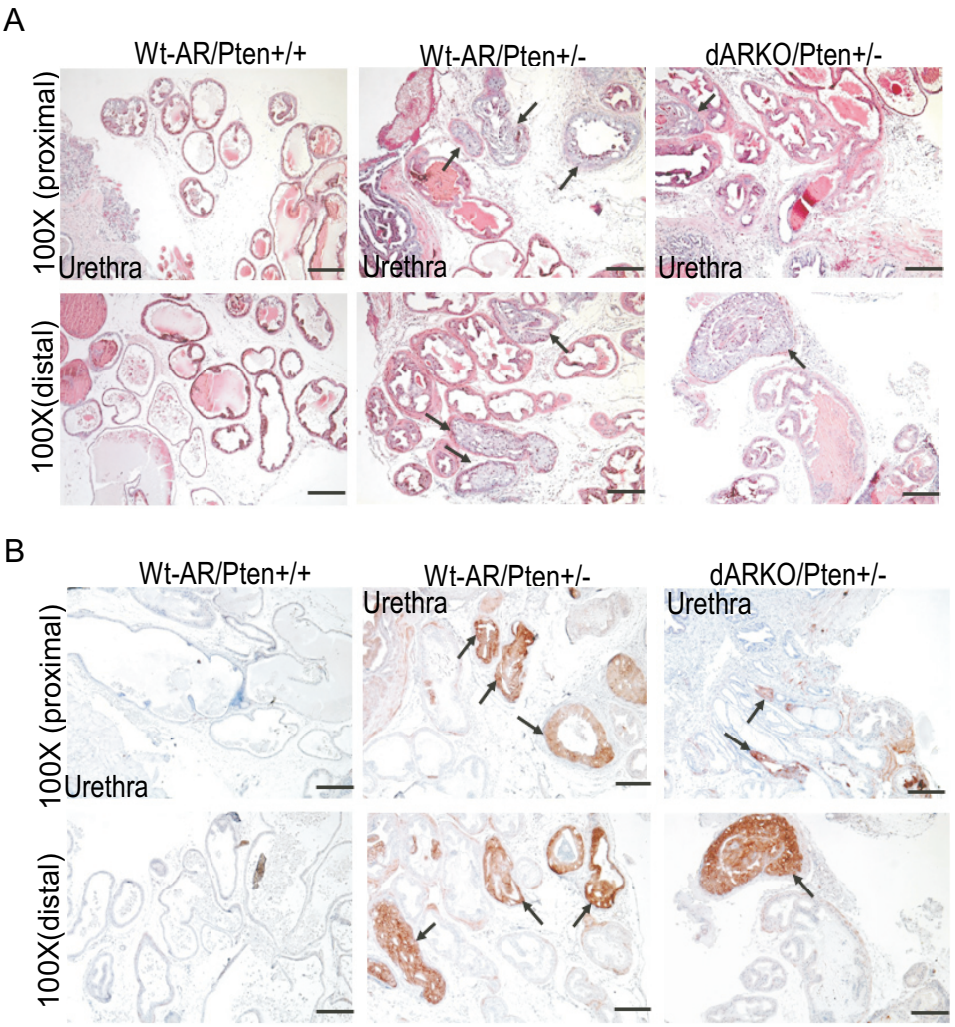
Figure S4. AR can potentiate NF-kappaB activity to activate MIP-1β promoter. (A) The COS-1 cells were transfected with pGL3-mMIP-1β (-1,989 to +68) to examine the possible AR regulation. The COS-1 cells were transfected with pBabe-mouse AR, MMTV-Luc or mouse MIP-1β–Luc, and pRL-TK by lipofectamine 2000. The cells were cultured with 5% CD-FBS for 2 days then stimulated with EtOH or 10 nM DHT for 24 hr. For the stimulation of MIP-1 β promoter and to analyze the potential mediator NF-kappaB, we applied the recombinant human IL-1β protein (10 ng/ml) for 4 hr before harvest. We also treated cells with 10 μM NF-KappaB inhibitor BAY117082 for 1 hr prior to the IL-1β stimulation. The cells were lysed and subjected to standard reporter assay. The Renilla luciferase was monitored and served as control for normalization. Each bar represents the mean±SD of at least 2 independent determinations performed in triplicate.

Figure S5. Pten+/- PrSC contains the tumor-promoting potentials of CAFs but AR itself does not affect the MSCs marker expression. (**A**) Real-time PCR analysis was used to detect AR transcript in WT MSCs, ARKO MSCs, Pten+/- PrSC ARscr, and Pten+/- PrSC ARsi in the presence of 10 nM DHT. (**B**) CD29 real-time PCR analysis. (**C**) CD44 real-time PCR analysis. (**D**) CD90 real-time PCR analysis. (**E**) Colony-forming Unit Fibroblast assay was used to determine the self-renewal ability of WT MSCs and ARKO MSCs after 21 days culture. (**A-D**) Each bar represents the mean±SD of at least 2 independent determinations performed in triplicate. (**E**) N=5-6 for each group for mouse data.

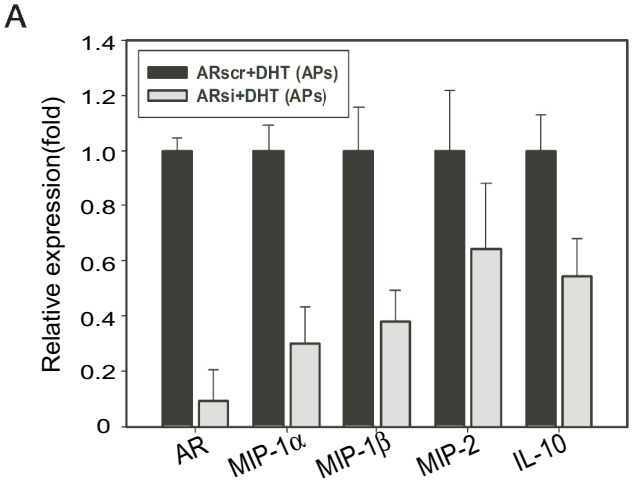
Supplementary Table

	Sense	Anti-sense
Mouse GAPDH	5'-GCTCCTGGAAGATGGTGATG-3'	5'–GGTGAAGGTCGGTGTGAAAC-3'
Mouse AR	5'-GGACAGTACCAGGGACCATG-3'	5'-TCCGTAGTGACAGCCAGAAG-3'
Mouse CD29	5'-CTACTTCTGCACGATGTGATGAT-3'	5'-TTGGCTGGCAACCCTTCTTT-3'
Mouse CD44	5'-ACTTTGCCTCTTGCAGTTGAG-3'	5'-CGTAGCGGCAGGTTACATTCA-3'
Mouse CD90	5'-GCTCTCAGTCTTGCAGGTGTC-3'	5'-CAGGCGAAGGTTTTGGTTCA-3'
Mouse MIP-1α	5'–TTCTCTGTACCATGACACTCTGC-3'	5'-CGTGGAATCTTCCGGCTGTAG-3'
Mouse MIP-1β	5'–TTCCTGCTGTTTCTCTTACACCT-3'	5'–CTGTCTGCCTCTTTTGGTCAG-3'
Mouse MIP-2	5'-CCAACCACCAGGCTACAGG-3'	5'-GCGTCACACTCAAGCTCTG-3'
Mouse IL-10	5'–GCTCTTACTGACTGGCATGAG-3'	5'-CGCAGCTCTAGGAGCATGTG-3'
Mouse MIP-1β promoter AR ChIP-1	5'-TGCTTAGGTTGGCTACACATTTTG-3'	5'-ATATTGACCACTCCAGACTCTTCC-3'
Mouse MIP-1β promoter AR ChIP-2	5'-TCCTCTCCTGTCCTTGTTCTAG-3'	5'-CAGTGGAGCCTTCAAGTCAATC-3'
Mouse KGF promoter AR ChIP	5'-TCACTAAGAACATCATCATACC-3'	5'-AGACCAAATAGAGACAGAGAC-3'

Supplementary Figure 1



Supplementary Figure 2



A

Mouse MIP-1β AR ChIP primer 1 (-689 to -446)

GGGAAACACCCATATGCTTAGGTTGGCTACACATTTTGTCTGCAGCAAACACTATCA
CTAGGTATCAATCCTTATTTATACTAGAACATTCATTGGGTCTTTCAAACTTTCAAACT
GTTTCTCTTATTCCAATAAAATGTCCTGAAATCTCTATTTTTTCCTCTCCTGTCCTTGTT
CTAGATAGGCTAAACCAACTCTTCTGAATGATGCTGATTGAATGGTATTTCTGTGTGT
GGAAGAGTCTGGAGTGGTCAATATCTTCTCTCTTGTGGTCACACCCTATGTTTGTTCT
GGAAATCACAGCCACCTGGAAAGATTGACTTGAAGGCTCCACTGCCTGGAGGTGC
CGACCCCCAAGTGAAGTG

Mouse MIP-1β AR ChIP primer 2 (-545 to -368)

GGGAAACACCCATATGCTTAGGTTGGCTACACATTTTGTCTGCAGCAAACACTATCAC
TAGGTATCAATCCTTATTTATACTAGAACATTCATTGGGTCTTTCAAACTTTCAAACTGT
TTCTCTTATTCCAATAAAATGTCCTGAAATCTCTATTTTTTCCTCTCCTGTCCTTGTTCTA
GATAGGCTAAACCAACTCTTCTGAATGATGCTGATTGAATGGTATTTCTGTGTGTGGA
AGAGTCTGGAGTGGTCAATATCTTCTCTCTTGTGGTCACACCCTATGTTTGTTCTGGAA
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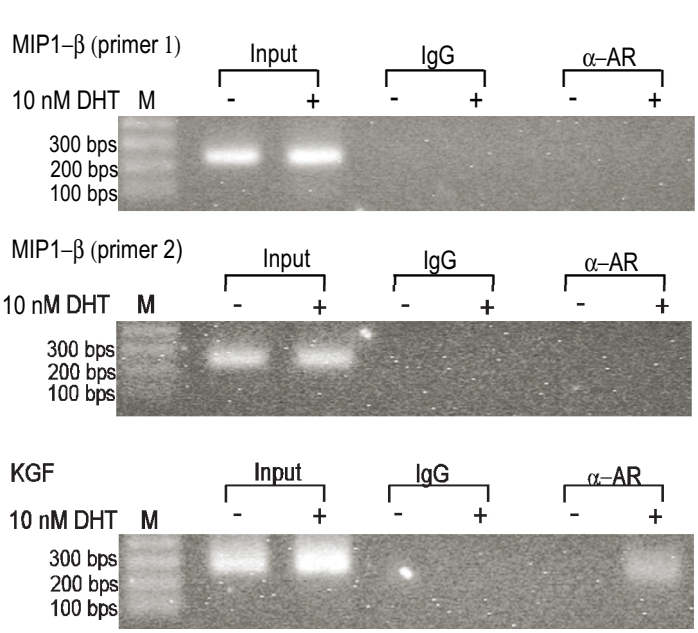
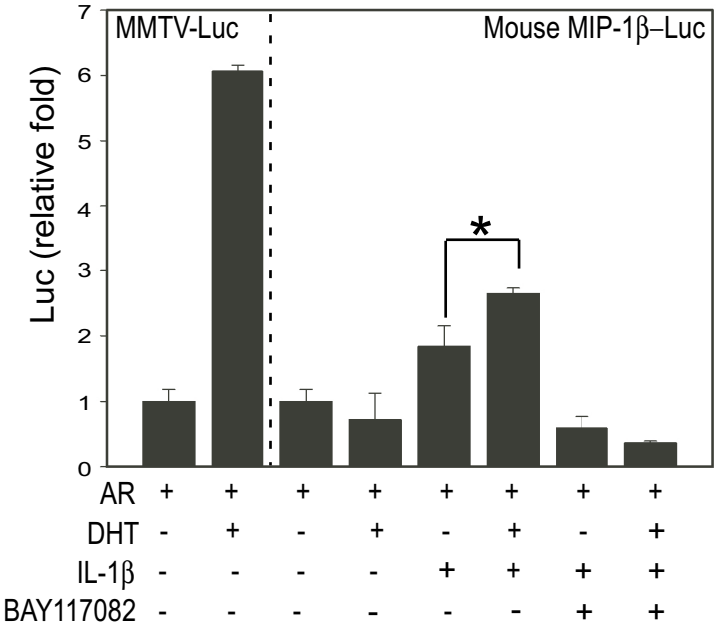
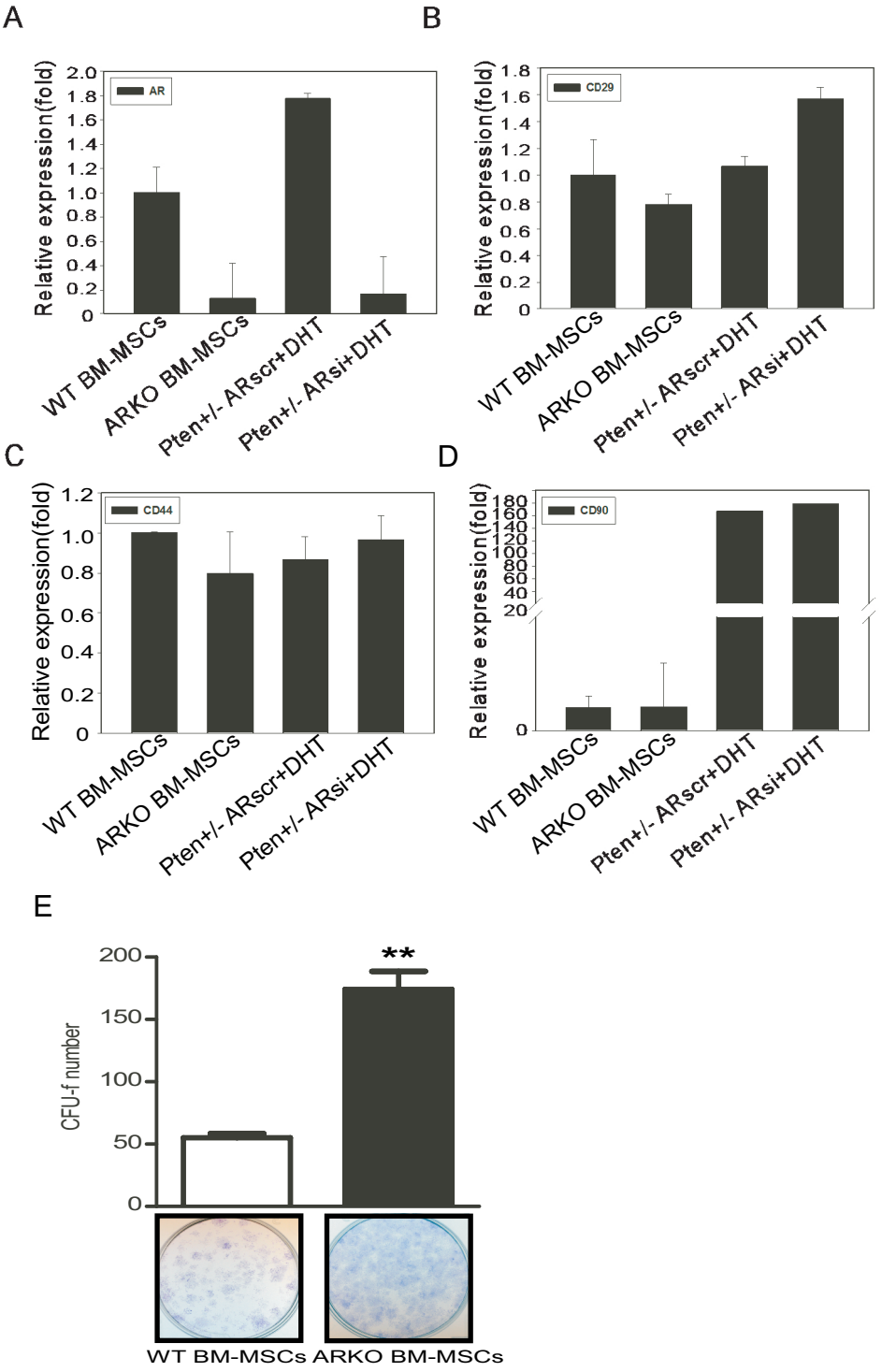
CCCAAGTGAAGTG

B



A


MMTV-Luc
Mouse MIP-1β–Luc
Luc (relative fold)
0
1
2
3
4
5
6
7
*
AR + + + + + + + +
DHT - + - + - + - +
IL-1β - - - - + + + +
BAY117082 - - - - - - + +


A
AR
Relative expression(fold)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
WT BM-MSCs
ARKO BM-MSCs
Pten+/- ARscr+DHT
Pten+/- ARsi+DHT
B
CD29
Relative expression(fold)
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
WT BM-MSCs
ARKO BM-MSCs
Pten+/- ARscr+DHT
Pten+/- ARsi+DHT
C
CD44
Relative expression(fold)
1.2
1.0
0.8
0.6
0.4
0.2
0
WT BM-MSCs
ARKO BM-MSCs
Pten+/- ARscr+DHT
Pten+/- ARsi+DHT
D
CD90
Relative expression(fold)
180
160
140
120
100
80
60
40
20
0
WT BM-MSCs
ARKO BM-MSCs
Pten+/- ARscr+DHT
Pten+/- ARsi+DHT
E
CFU-f number
200
150
100
50
0
**
WT BM-MSCs
ARKO BM-MSCs