Published in final edited form as:
Gastroenterology. 2008 September ; 135(3): 947–955.e5. doi:10.1053/j.gastro.2008.05.046.

Androgen receptor is a new potential therapeutic target for the treatment of hepatocellular carcinoma

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Summary

Background—Androgen effects on the hepatocellular carcinoma (HCC) remain controversial and androgen ablation therapy to treat HCC also leads to inconsistent results. Here we examine androgen receptor (AR) roles in hepatocarcinogenesis using mice lacking AR in hepatocytes.

Methods and Designs—Using the Cre-Lox conditional knockout mice model injected with carcinogen, we examined the AR roles in hepatocarcinogenesis. We also tested the possible roles of AR in cellular oxidative stress and DNA damage sensing/repairing systems. Using AR degrading compound, ASC-J9, or AR-siRNA, we also examined the therapeutic potentials of targeting AR in HCC.

Results—We found AR expression was elevated in human HCC compared to normal livers. We also found mice lacking hepatic AR developed later and less HCC than their wild type littermates with comparable serum testosterone in both male and female mice. Addition of functional AR in human HCC cells also resulted in the promotion of cell growth in the absence or presence of 5α-dihydrotestosterone. Mechanistic dissection suggests that AR may promote hepatocarcinogenesis via increased cellular oxidative stress and DNA damage, as well as suppression of p53-mediated DNA damage sensing/repairing system and cell apoptosis. Targeting AR directly via either AR-siRNA or ASC-J9, resulted in suppression of HCC in both ex vivo cell lines and in vivo mice models.

Conclusion—Our data point to AR, but not androgens, as a potential new therapeutic target for the battle of HCC.

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All authors have no interest to disclose in this manuscript.

Introduction

While viral infection and/or environmental carcinogens may lead to the hepatocellular carcinoma (HCC) development, the etiology of this liver cancer remains unclear. Early studies suggested that androgens might contribute to the gender difference of HCC incidence and serum testosterone may have a positive linkage to the development of HCC[1]. However, clinical trials with targeting of androgens via androgen ablation therapy yield inconsistent and disappointing outcomes[2].

Androgen effects are mediated mainly through the androgen receptor (AR)[3]. Androgen/AR signals may modulate many biological events via interaction with various AR coregulators[4]. The biological function of androgen/AR in liver and their detailed consequences, however, remain unclear. We generated the first conditional knockout AR mouse lacking only the hepatic AR (L-AR$^{-/y}$) via mating floxed-AR mice with albumin promoter-driven Cre-recombinase (Alb-Cre) transgenic mice[5]. Results from these mice in which HCC was induced via injection of N'-N'-diethylnitrosamine (DEN) suggest that the AR, rather than androgens, may play a more dominant role in HCC development.

Methods

Human tissue and IHC stain

Ten sets of liver tumors (<3cm) and corresponding normal liver tissues for IHC staining were obtained from ten male patients who received routine liver cancer surgery following informed consent.

Maintenance of animals and generation of T-AR$^{-/y}$, L-AR$^{-/y}$ and T-AR$^{-/-}$, L-AR$^{-/-}$ mice and inducing HCC using DEN

All of the animal experiments followed the Guidance of the Care and Use of Laboratory Animals of the NIH with approval from the University of Rochester, Department of Laboratory Animal Medicine. The strategy to generate flox-AR gene-targeting mice has been described previously[6]. Briefly, we mated male Actb-Cre or Alb-Cre[5] (Cre recombinase under control of Albumin promoter; Jackson Lab., B6.Cg-Tg(Alb-cre)21Mgn/J) mice with flox-AR/AR heterozygous female mice to produce T/L-AR$^{-/y}$ (T: total knockout in whole body; L: liver specific knockout) male and T/L-AR$^{-/+}$ heterozygous female mice. Another mating using T/L-AR$^{-/+}$ female with AR$^{flox/y}$/L-AR$^{-/y}$ also generated T/L-AR$^{-/-}$. We genotyped 21-day-old pups from tail snips by PCR, as described previously[6]. We induce HCC in the liver of 12-day old pups with intraperitoneal (I.P.) injection of a single dose of the HCC initiator, DEN (20 mg/kg/mouse; Sigma-Aldrich)[1]. After genotyping the pups we divided them into 7 different groups. The groups were 1) AR$^{+/y}$, 2) T-AR$^{-/y}$, 3) L-AR$^{-/y}$, 4) AR$^{+/+}$, 5) T-AR$^{-/-}$, 6) L-AR$^{-/-}$, and 7) AR$^{+/y}$ untreated with solvent injection only. Several mice from each group were sacrificed at 20-, 24-, 28-, 32-, 36-, and 40 weeks after DEN-injection. The nude mice used for xenograft experiments were 10-weeks-old male nude mice (Charles River; Crl: CD1-Foxn1^{nu} Origin) and ASC-J9 was provided by AndroScience Corporation (San Diego, CA).

Serum testosterone concentration and tissue preservation

We sacrificed mice at the indicated time points, drew 1 ml of blood by cardiocentesis and immediately assayed for serum testosterone level using the Coat-A-Count Total Testosterone radioimmunoassay (Diagnostic Products). We flash-froze fresh tissues in liquid nitrogen for preservation at −80 °C for gene expression assay. We subjected the hepatic major lobe to 10% neutralized buffered formalin (Sigma) for histological analysis.

Histology and Immunohistochemistry

We fixed the tissues in 10% buffered formalin (Sigma) and embedded them in paraffin. For general histologic inspection, we treated tissue sections with Hematoxylin and Eosin (H&E), and then used an ABC kit (Vector Laboratories) to visualize AR, p53, and 8-oxoG (8-oxodeoxyguanosine) immunostaining by specific antibodies against mouse AR (C-19, Santa Cruz); human AR (441, Dako); p53 (Ab-3, Calbiochem); 8-oxoG (sc-12075, Santa Cruz). We performed the TUNEL staining assay (Calbiochem) as previously described[7]. We injected 5′-Bromo-2′-deoxyuridine (BrdU, Sigma) for 4 consecutive days into 55-weeks-old DEN-induced mice and stained tissue sections with BrdU specific antibody (Zymed) as previously described[7].

Statistical analysis

We analyzed the results using Chi-square tests and Fisher's Exact-tests for cancer incidence using Sigmaplot software, used unpaired T-Test for other experiments, used Standard Deviation (SD) as experimental variation, and considered p-values less than 0.05 to be statistically significant.

Other methods please see supplemental materials.

RESULTS

AR was up-regulated in dysplastic and HCC human livers

We first demonstrated the expression of AR in livers from HCC patients. As shown in Fig. 1A, AR expression was highly expressed in a dysplastic liver nodule. Among ten HCC patients examined, stronger AR expression was found in tumor than surrounding non-tumor in seven patients (Fig. 1B; upper panel). Some of the AR was stained in the tumor border as shown in Fig. 1B (lower panel). Another patient had AR staining in non-tumor part only.

Generation of L-AR$^{-/y}$, L-AR$^{-/-}$ and T-AR$^{-/y}$, T-AR$^{-/-}$ mice with HCC development

We generated AR knockout mice that are either lack hepatic AR (L-AR$^{-/y}$), and their littermates (L-AR$^{-/+}$) or lack AR in the whole body (T-AR$^{-/y}$), and their littermates (T-AR$^{-/+}$) via mating loxP site-AR female transgene (AR$^{flox/flox}$)[6] mice with albumin promoter driven (Alb-Cre)[5] or β-actin promoter driven cre (Actb-Cre)[6] bearing male transgene mice. (Suppl. Fig. 1). We further confirmed AR expression in nuclei of HCC foci in AR$^{+/y}$ mice, but not in L-AR$^{-/y}$ mice by immunohistochemical staining of AR (Fig. 1C–D).

To develop HCC in these mice, we used a single injection of the DEN carcinogen as described in Methods and separated them into seven groups: 1) AR$^{+/y}$, 2) L-AR$^{-/y}$, 3) T-AR$^{-/y}$, 4) AR$^{+/+}$, 5) L-AR$^{-/-}$, 6) T-AR$^{-/-}$, and 7) untreated male AR$^{+/y}$ mice.

Reduced HCC incidence in mice lacking hepatic AR with little change of serum testosterone

We found the serum testosterone levels remain comparable between 36-weeks DEN-induced L-AR$^{-/y}$ and AR$^{+/y}$, and between L-AR$^{-/-}$ and AR$^{+/+}$, even though male mice had much higher serum testosterone levels than female mice. Notably, unlike L-AR$^{-/y}$, T-AR$^{-/y}$ mice had much lower serum testosterone levels when compared to littermates AR$^{+/y}$ (Fig. 1E).

We found none of the untreated mice (group 7) developed HCC by 40-weeks of age (data not shown). In contrast, all other six groups developed HCC with different incidence (Fig. 2A). HCC developed in all the DEN-induced male AR$^{+/y}$ mice examined at 28-, 32-, 36- and 40-weeks of age, whereas only 25–60% of DEN-treated female wild-type (AR$^{+/+}$) mice examined at 28~40-weeks of age developed HCC, confirming the gender-difference in HCC

incidence[1, 8]. In contrast, L-AR$^{-/y}$ mice developed less HCC as compared to their wild-type littermates, even though they have comparable serum testosterone. Similar results also occurred in female mice showing L-AR$^{-/-}$ mice developed less HCC with comparable serum testosterone than their wild-type littermates, suggesting that AR, rather than androgens, is crucial for the development of HCC in both male and female mice. Interestingly, HCC incidence in male L-AR$^{-/y}$ and T-AR$^{-/y}$ mice is still higher than female L-AR$^{-/-}$ and T-AR$^{-/-}$ mice, suggesting factors other than AR might also contribute to the gender-differences in HCC incidence.

Due to the multiple origin nature of DEN-induced HCC, we also counted the numbers of tumor foci and found a reduced number of HCC foci in L-AR$^{-/y}$ and T-AR$^{-/y}$ mice compared to AR$^{+/y}$ with a ratio of AR$^{+/y}$: L-AR$^{-/y}$ (or AR$^{+/y}$: T-AR$^{-/y}$)= 20: 6 (Fig. 2B). We also weighed the individual DEN-induced HCC livers and found the ratio of liver weight to whole body weight (LW/BW) was reduced in L-AR$^{-/y}$ and T-AR$^{-/y}$ mice as compared to their littermate AR$^{+/y}$ mice, suggesting that loss of hepatic AR might result in reduction of HCC tumor mass (Fig. 2C). In contrast, the liver weight in non-DEN injected L-AR$^{-/y}$ or T-AR$^{-/y}$ mice was similar to their littermate AR$^{+/y}$ mice (Supplemental Fig. 2), suggesting that loss of hepatic AR has little influence on the steady state of normal liver growth in mice without HCC development.

Loss of hepatic AR results in suppression of HCC growth

Having shown that loss of hepatic AR resulted in reduction of HCC incidence, we determined if loss of hepatic AR might also influence HCC progression that could be correlated with lower proliferation and higher apoptosis rates. We first assessed cell proliferation via intraperitoneal (I.P.) administration of 5′-bromo-2-deoxyuridine (BrdU) in mice for 4 consecutive days. We sacrificed mice and liver tumors were dissected, embedded, sectioned, and stained with anti-BrdU antibody. We counted positive stains for proliferating cells and showed the reduction of BrdU (+) staining in both L-AR$^{-/y}$ and T-AR$^{-/y}$ mice as compared to AR$^{+/y}$ mice (Fig. 2D). We also used the TUNEL apoptosis assay to measure apoptosis, and found more positive TUNEL staining in L-AR$^{-/y}$ and T-AR$^{-/y}$ as compared to AR$^{+/y}$ mice (Fig. 2D), suggesting that loss of hepatic AR might increase cell death in the liver tumor during HCC progression. We then used primary cells isolated from 55-weeks-old DEN-induced AR$^{+/y}$ mice to examine the androgen 5α-dihydrotestosterone (DHT) effects on cell growth. The results from MTT assay showed that the cell numbers increased in a dose-dependent manner upon DHT treatment (Fig. 2E). Together, using various growth and apoptosis assays, our results (Fig. 2D–E) demonstrated that loss of hepatic AR might lead to the suppression of HCC progression.

Human HCC cells transfected with functional AR result in promotion of cell growth

To further strengthen our findings from mice studies showing loss of hepatic AR results in the suppression of HCC growth, we applied human HCC cell lines to examine the AR effects on HCC cell growth (Suppl. Fig. 3). Using cell-counting assay we showed that DHT had little effect on SKpar (parental transfectant) cell growth (Fig. 3A, SKpar-EtOH vs. SKpar-DHT). In contrast, SKAR3 (stable AR transfectant) increased cell growth (Fig. 3A, SKpar-EtOH vs. SKAR3-EtOH) in the absence of DHT and addition of 10 nM DHT further increased cell growth (Fig. 3A, SKAR3-EtOH vs. SKAR3-DHT). These results suggest that both non-androgen-mediated AR and androgen-mediated AR signals might influence HCC cell growth. Addition of functional AR in SKpar cells also resulted in the decreased cell apoptosis in the absence or presence of DHT (Fig. 3B), suggested that AR, rather than androgen may play more important roles in the hepatic cell apoptosis. This conclusion is further supported with the results from the anchorage-independent cell growth assay. Using soft agar colony formation assay, we found that SKAR3, but not SKpar cells, were able to grow in an anchorage-independent environment in the absence of androgen, suggesting increased AR expression via

transfected functional AR resulted in anchorage-independent cell growth (Fig, 3C). Addition of 10 nM DHT showed little influence on the AR-promoted anchorage-independent cell growth. Together, our results in Fig. 3 suggest that the AR, rather than androgen, may play a more important role in the human HCC cells growth.

Loss of hepatic AR reduces cellular oxidative stress and decreases DNA damage in the liver

ROS has been linked to the hepatocarcinogenesis during chronic inflammatory liver injury, such as hepatitis and cirrhosis[9]. Early reports documented the linkage between DEN-induced HCC in mice with innate immune response and the related cellular oxidative stress[10]. We first evaluated the cellular oxidative stress levels via measuring the carbonylated groups[11], the oxidized amino acid side chain of protein (Fig. 4A, left panels). We found that cellular ROS levels in the liver tumor of 36-weeks-old L-$AR^{-/y}$ mice were reduced to 30% as compared to those in DEN-induced $AR^{+/y}$ mice (Fig. 4A, right panel). To further confirm the effect of androgen/AR signals on cellular ROS level, we used AR stably-transfected SKAR3 cells to examine cellular oxidative stress. We measured the cellular ROS level in SKpar and SKAR3 cells treated with 200 μM H_2O_2 in the absence or presence of DHT. The results showed that ROS level in SKAR3 cells is increased upon H_2O_2 treatment and further enhanced in the presence of 1 nM DHT as compared to those in SKpar cells (Fig. 4B).

To further dissect how androgen/AR signals may regulate cellular ROS, we examined several key factors that have been linked to ROS and found mRNA expression of thioreducin-2 and superoxide dismutase 2 (SOD2) were decreased after adding 10 nM DHT in SKAR3 cells treated with H_2O_2 (Suppl. Fig. 4). In contrast, as there is little functional AR available in SKpar cells, addition of 10 nM DHT failed to suppress the H_2O_2-induced thioreducin-2 and SOD2 mRNA expression (Suppl. Fig. 4).

As chronic inflammation induced oxidative stress might result in the breakage or damage of chromosomal DNA, we examined the DNA damage status in the mice liver tumor. By staining for the DNA damage marker, 8-oxoG[12], we found that the positive signal was higher in the liver tumors of $AR^{+/y}$ compared to those in L-$AR^{-/y}$ mice at 36-weeks of DEN induction (Fig. 4C). These results suggest reduced cellular oxidative stress in L-$AR^{-/y}$ mice may suppress the DNA damage, which may then lead to fewer gene mutations and delayed HCC development.

Loss of hepatic AR promotes the p53-mediated DNA damage sensing and repairing system and p53-mediated cell apoptosis

In the normal liver condition, the increased DNA damage via cellular oxidative stress[13] may result in the increase of p53-mediated DNA damage sensing and repairing system. The p53 activation can suppress the function of the anti-apoptotic molecule, Bcl-2; therefore triggering an intrinsic cascade for apoptosis[13]. Interestingly, we found that loss of hepatic AR not only reduced DNA damage, but also enhanced the p53 expression in both normal and liver tumor of L-$AR^{-/y}$ mice (Fig. 5A; 5B). The p53 down-stream target gene, p21, was up-regulated in L-$AR^{-/y}$ as well (Fig. 5B). Comparative results can be consistently observed in human HCC cells (Suppl Fig 5, 6). Furthermore, enhanced p53 expression might also promote the DNA sensing and repairing system. For example, the expressions of the p53 target gene, Gadd45α[14] and β[15], DNA damage repairing executive genes, were increased in liver tumors of L-$AR^{-/y}$ compared to $AR^{-/y}$ mice (Fig. 5C). We also found Gadd45 can be regulated by AR in transcriptional level (Suppl. Fig. 5). The increased DNA damage sensing and repairing system might then result in the reduced DNA damage seen in liver tumors of L-$AR^{-/y}$ mice. Together, results from Fig. 5 suggested that loss of hepatic AR may suppress hepatocarcinogenesis via 2 pathways: 1) suppression of ROS-induced cellular oxidative stress and DNA damage, and 2) increased p53 expression that results in the better DNA sensing and repairing system as well as promoting cell apoptosis.

Therapeutic effects on HCC progression via targeting the AR

Based on the above findings showing AR might play a pivotal role for the HCC progression, we used both ex vivo cells and in vivo mice model to investigate whether AR can be a therapeutic target for the treatment of HCC. We used two therapeutic approaches: 1) transfection with AR-siRNA, and 2) treatment with the anti-AR compound 5-hydroxy-1,7-bis (3,4-dimethoxyphenyl)-1,4,6-heptatrien-3-one (ASC-J9)[16].

1) Targeting AR with AR-siRNA—We first established stable sublines of SKAR3 cells transfected with a retrovirus-based vector that expresses AR-siRNA, which effectively knocked down the AR in MCF-7 cells[7]. We substantially knocked down the AR expression in SKAR3 cells stably-transfected with AR-siRNA (designated SKAR3-si1, 2 or 3) (Fig. 6A). In contrast, AR expressed normally in SKAR3 cells stably transfected with control scramble RNA (designated SKAR3-sc). We then investigated the effect of the AR-siRNA on the AR-mediated transactivation and AR-mediated cell growth in the stable sublines. We treated each stable subline with 1 nM DHT and assessed transactivation by ARE(4)-luciferase promoter assay. We found that addition of 1 nM DHT could induce substantial AR transactivation in SKAR3-sc, but not SKAR3-si1 cells (Fig. 6B). Using the MTT growth assay, we also found that knockdown of AR expression via AR-siRNA resulted in the suppression of DHT-induced cell growth (Fig. 6C).

2) Targeting AR by treatment with the anti-AR compound ASC-J9—The recently developed anti-AR compound ASC-J9 targets AR via dissociating AR from its coregulators, leading to selective degradation of the AR protein[16]. We examined the effects of ASC-J9 on HCC progression in both human HCC cells and in vivo mice model, and found that addition of 5 μM ASC-J9 to the SKAR3 and SKAR7 cells resulted in the suppression of cell growth in the presence of 10 nM DHT (Fig. 6D). Furthermore, addition of 5 μM ASC-J9 also resulted in the increased cell apoptosis in the absence or presence of 10 nM DHT (Fig. 6E). We further confirmed this suppression effect on the HCC cell growth when we replaced human SKAR3 or SKAR7 cells with primary tumor cells isolated from $AR^{+/y}$ mice livers. We found that addition of 5 μM ASC-J9 suppressed the primary tumor cell growth in the absence or presence of 10 nM DHT (Fig. 6F). Furthermore, in the mice inoculated with cells isolated from primary liver tumor of $AR^{+/y}$ mice, we found I.P. injection of ASC-J9 (50 mg/kg/mice twice per week) resulted in the suppression of tumor growth during the course of 17 weeks treatment (Fig. 6G). Together, results from Fig. 6 suggested that directly targeting the AR either via AR-siRNA or ASC-J9 could suppress HCC progression.

Discussion

Up-regulation of AR expression in human HCC compared to normal livers

The AR are expressed in the normal liver tissue from both male and female humans, but their expression and activation was reported to be increased in the tumor tissue and in the surrounding liver tissue of individuals with HCC[17]. Moreover, the expression and activation of AR was reported to be greatly increased in the liver tissue of male and female rodents during chemical-induced liver carcinogenesis[18]. In HBV-related HCC, pathways involving androgen-AR signaling, such as serum testosterone concentration, or length of AR CAG length (<23 repeats) may affect the risk of HBV-related HCC among men[19].

AR, but not androgen could be a better therapeutic target for treatment of HCC

The most important conclusion from these in vivo animal studies with mice lacking hepatic AR and ex vivo studies with human HCC cells transfected with either AR-siRNA or functional AR is a clear demonstration that AR might play pivotal roles for the HCC development and therefore AR, rather than androgens, might represent a new target for treatment of HCC. The

similar findings of AR effect on hepatocarcinogenesis were also observed in HBV transgene mice with subminimum dosage of DEN injection (unpublished results). This conclusion against the conventional concept using androgen ablation therapy that only targets androgens is based on the following evidences. 1) Both male and female mice lacking hepatic AR have less HCC incidence with similar serum testosterone compared to the wild-type littermate mice (Fig. 1 and 2). 2) Stably transfected functional AR increased cell growth in the absence of DHT (Fig. 3). 3) SKAR3, but not SKpar, cells were able to grow in the absence of androgen in an anchorage-independent environment and addition of 10 nM DHT resulted in little influence of the AR-promoted anchorage-independent cell growth (Fig. 3C). 4) Therapeutic targeting of AR via either AR-siRNA or ASC-J9 resulted in the suppression of HCC progression (Fig. 6) and early data suggested that injection of ASC-J9 for 15 weeks resulted in little change in serum testosterone and mice retained normal sexual function and fertility[16].

This conclusion is further supported by early studies showing that in addition to androgen-mediated AR signals, non-androgen-mediated AR signals might also play important roles for the progression of prostate[20] and bladder cancer[21]. For example, protein kinases or growth factors could induce AR activity via signal transduction pathways[4]. Anti-androgen flutamide[22], or Δ5-androstenedione[23] might also be able to induce AR activity in the proper cell environment.

Furthermore, early reports regarding androgen effects on HCC remain controversial and the results of using androgen ablation therapy to treat HCC remains inconsistent[2]. For example, a large cohort investigation of nested case-controls indicated that the serum testosterone in HCC patients were significantly higher than those in non-HCC control patients[24]. The ex vivo studies using human HCC primary cells also demonstrated the positive correlation between androgen/AR signals and HCC progression[25]. Recent studies further suggested that the HBV X protein might function as a coactivator to promote AR-mediated anchorage-independent cell growth via AR-X protein interaction[26]. However, several clinical studies found lower serum testosterone in HCC patients as compared to non-HCC control patients[27]. Furthermore, clinical results using anti-androgens to treat HCC patients remain controversial: using antiandrogen cyproterone acetate (300 mg daily) may result in some positive improvement[28] and an ex vivo study using another antiandrogen, flutamide, may also result in the suppression of androgen-induced HCC cell growth[29]. However, small scale population studies using flutamide in HCC patients has failed in the phase II clinical trial and large scale population studies using leuprorelin and flutamide also failed to show any improvement in patient survival with the use of antiandogens[2].

Together, the controversial results of androgens or antiandrogens effects on the HCC progression suggest that targeting androgens for the suppression of HCC progression might have limitations. Therefore, targeting AR may represent a new and better therapeutic approach for treatment of the androgen/AR promoted HCC. Additional dosage studies of ASC-J9 or its derivatives to investigate how HCC may be effectively suppressed, without toxicity, might lead to the better treatment of HCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement

We thank H-Y Lin, M-F Chen, and S-D Yeh for their technical support.

This work was supported by George Whipple Professorship Endowment and NIH grant CA122295.

References

1. Tejura S, Rodgers GR, Dunion MH, Parsons MA, Underwood JC, Ingleton PM. Sex-steroid receptors in the diethylnitrosamine model of hepatocarcinogenesis: modifications by gonadal ablation and steroid replacement therapy. J Mol Endocrinol 1989;3:229–237. [PubMed: 2590384]
2. Randomized trial of leuprorelin and flutamide in male patients with hepatocellular carcinoma treated with tamoxifen. Hepatology 2004;40:1361–1369. [PubMed: 15565568]
3. Chang CS, Kokontis J, Liao ST. Molecular cloning of human and rat complementary DNA encoding androgen receptors. Science 1988;240:324–326. [PubMed: 3353726]
4. Heinlein CA, Chang C. Androgen receptor (AR) coregulators: an overview. Endocr Rev 2002;23:175–200. [PubMed: 11943742]
5. Hung-Yun, LinI-CY.; Ruey-Shen, Wang; Yei-Tsung, Chen; Ning-Chun, Liu; Saleh, Altuwaijri; Cheng-Lung, Hsu; Wen-Lung, Ma; Jenny, Jokinen; Janet D, Sparks; Shuyuan, Yeh; Chawnshang, Chang. Increased hepatic steatosis and insulin resistance in mice lacking hepatic androgen receptor. Hepatology 2008;47(6):1924–1935. [PubMed: 18449947]
6. Yeh S, Tsai MY, Xu Q, Mu XM, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung MC, Zhang S, Gan L, Chang C. Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc Natl Acad Sci U S A 2002;99:13498–13503. [PubMed: 12370412]
7. Yeh S, Hu YC, Wang PH, Xie C, Xu Q, Tsai MY, Dong Z, Wang RS, Lee TH, Chang C. Abnormal mammary gland development and growth retardation in female mice and MCF7 breast cancer cells lacking androgen receptor. J Exp Med 2003;198:1899–1908. [PubMed: 14676301]
8. Ostrowski JL, Ingleton PM, Underwood JC, Parsons MA. Increased hepatic androgen receptor expression in female rats during diethylnitrosamine liver carcinogenesis. A possible correlation with liver tumor development. Gastroenterology 1988;94:1193–1200. [PubMed: 3350289]
9. Pikarsky E, Porat RM, Stein I, Abramovitch R, Amit S, Kasem S, Gutkovich-Pyest E, Urieli-Shoval S, Galun E, Ben-Neriah Y. NF-kappaB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–466. [PubMed: 15329734]
10. Maeda S, Kamata H, Luo JL, Leffert H, Karin M. IKKbeta couples hepatocyte death to cytokine-driven compensatory proliferation that promotes chemical hepatocarcinogenesis. Cell 2005;121:977–990. [PubMed: 15989949]
11. Lim GP, Chu T, Yang F, Beech W, Frautschy SA, Cole GM. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. J Neurosci 2001;21:8370–8377. [PubMed: 11606625]
12. Ichinose T, Nobuyuki S, Takano H, Abe M, Sadakane K, Yanagisawa R, Ochi H, Fujioka K, Lee KG, Shibamoto T. Liver carcinogenesis and formation of 8-hydroxy-deoxyguanosine in C3H/HeN mice by oxidized dietary oils containing carcinogenic dicarbonyl compounds. Food Chem Toxicol 2004;42:1795–1803. [PubMed: 15350677]
13. Hussain SP, Schwank J, Staib F, Wang XW, Harris CC. TP53 mutations and hepatocellular carcinoma: insights into the etiology and pathogenesis of liver cancer. Oncogene 2007;26:2166–2176. [PubMed: 17401425]
14. Gramantieri L, Chieco P, Giovannini C, Lacchini M, Trere D, Grazi GL, Venturi A, Bolondi L. GADD45-alpha expression in cirrhosis and hepatocellular carcinoma: relationship with DNA repair and proliferation. Hum Pathol 2005;36:1154–1162. [PubMed: 16260267]
15. Qiu W, David D, Zhou B, Chu PG, Zhang B, Wu M, Xiao J, Han T, Zhu Z, Wang T, Liu X, Lopez R, Frankel P, Jong A, Yen Y. Down-regulation of growth arrest DNA damage-inducible gene 45beta expression is associated with human hepatocellular carcinoma. Am J Pathol 2003;162:1961–1974. [PubMed: 12759252]
16. Yang Z, Chang YJ, Yu IC, Yeh S, Wu CC, Miyamoto H, Merry DE, Sobue G, Chen LM, Chang SS, Chang C. ASC-J9 ameliorates spinal and bulbar muscular atrophy phenotype via degradation of androgen receptor. Nat Med 2007;13:348–353. [PubMed: 17334372]
17. Nagasue N, Ito A, Yukaya H, Ogawa Y. Androgen receptors in hepatocellular carcinoma and surrounding parenchyma. Gastroenterology 1985;89:643–647. [PubMed: 2991072]

18. Eagon PK, Elm MS, Epley MJ, Shinozuka H, Rao KN. Sex steroid metabolism and receptor status in hepatic hyperplasia and cancer in rats. Gastroenterology 1996;110:1199–1207. [PubMed: 8613010]
19. Yeh SH, Chiu CM, Chen CL, Lu SF, Hsu HC, Chen DS, Chen PJ. Somatic mutations at the trinucleotide repeats of androgen receptor gene in male hepatocellular carcinoma. Int J Cancer 2007;120:1610–1617. [PubMed: 17230529]
20. Yeh S, Lin HK, Kang HY, Thin TH, Lin MF, Chang C. From HER2/Neu signal cascade to androgen receptor and its coactivators: a novel pathway by induction of androgen target genes through MAP kinase in prostate cancer cells. Proc Natl Acad Sci U S A 1999;96:5458–5463. [PubMed: 10318905]
21. Miyamoto H, Yang Z, Chen YT, Ishiguro H, Uemura H, Kubota Y, Nagashima Y, Chang YJ, Hu YC, Tsai MY, Yeh S, Messing EM, Chang C. Promotion of bladder cancer development and progression by androgen receptor signals. J Natl Cancer Inst 2007;99:558–568. [PubMed: 17406000]
22. Yeh S, Miyamoto H, Chang C. Hydroxyflutamide may not always be a pure antiandrogen. Lancet 1997;349:852–853. [PubMed: 9121269]
23. Miyamoto H, Yeh S, Lardy H, Messing E, Chang C. Delta5-androstenediol is a natural hormone with androgenic activity in human prostate cancer cells. Proc Natl Acad Sci U S A 1998;95:11083–11088. [PubMed: 9736693]
24. Yuan JM, Ross RK, Stanczyk FZ, Govindarajan S, Gao YT, Henderson BE, Yu MC. A cohort study of serum testosterone and hepatocellular carcinoma in Shanghai, China. Int J Cancer 1995;63:491–493. [PubMed: 7591255]
25. Yu L, Kubota H, Imai K, Yamaguchi M, Nagasue N. Heterogeneity in androgen receptor levels and growth response to dihydrotestosterone in sublines derived from human hepatocellular carcinoma line (KYN-1). Liver 1997;17:35–40. [PubMed: 9062878]
26. Zheng Y, Chen WL, Ma WL, Chang C, Ou JH. Enhancement of gene transactivation activity of androgen receptor by hepatitis B virus X protein. Virology 2007;363(2):454–461. [PubMed: 17335866]
27. Lampropoulou-Karatzas C, Goritsas P, Makri MG. Low serum testosterone: a special feature of hepatocellular carcinoma. Eur J Med 1993;2:23–27. [PubMed: 8258001]
28. Forbes A, Wilkinson ML, Iqbal MJ, Johnson PJ, Williams R. Response to cyproterone acetate treatment in primary hepatocellular carcinoma is related to fall in free 5 alpha-dihydrotestosterone. Eur J Cancer Clin Oncol 1987;23:1659–1664. [PubMed: 2828073]
29. Jie X, Lang C, Jian Q, Chaoqun L, Dehua Y, Yi S, Yanping J, Luokun X, Qiuping Z, Hui W, Feili G, Boquan J, Youxin J, Jinquan T. Androgen activates PEG10 to promote carcinogenesis in hepatic cancer cells. Oncogene 2007;26(39):5741–5751. [PubMed: 17369855]

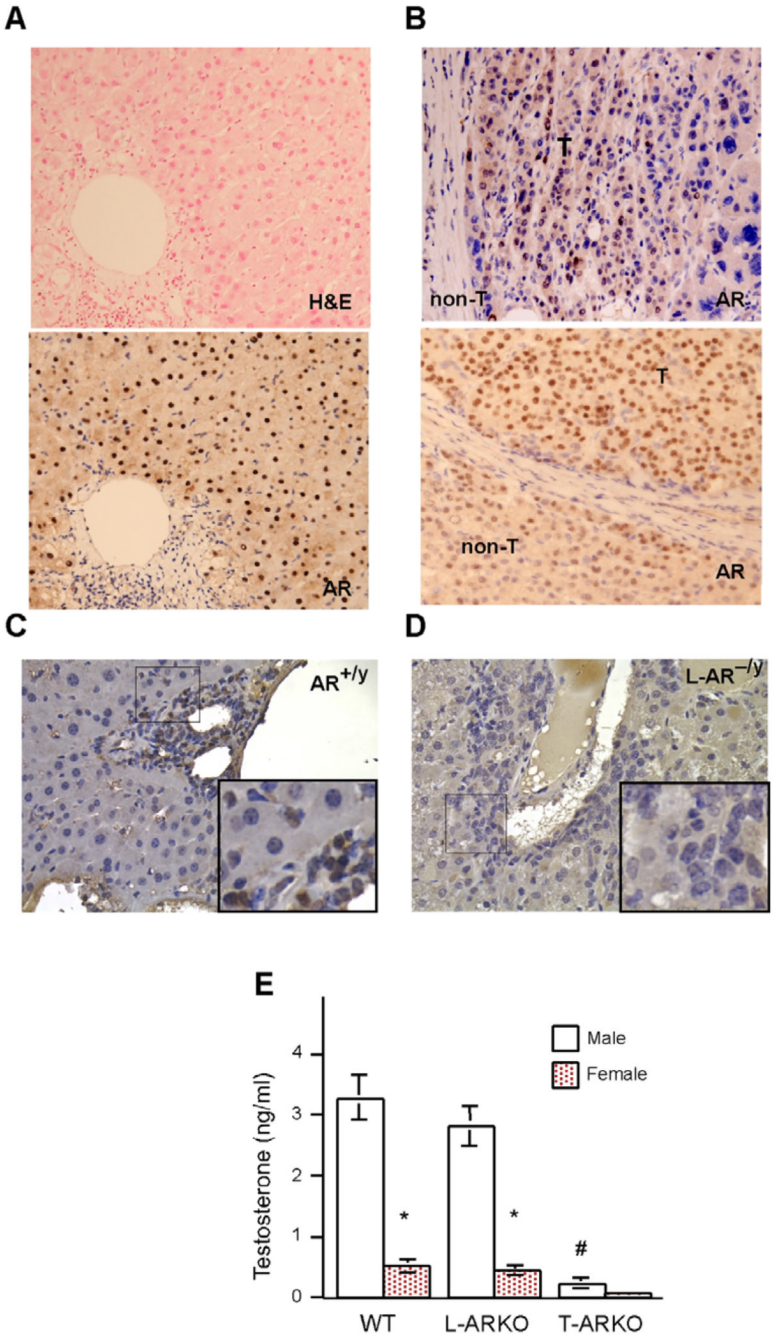

Fig. 1. AR expression in human livers and generation of mice lacking AR in hepatocyte only and serum testosterone level characterization

(A) H&E staining (upper panel) and the nuclear AR staining (lower panel) of a dysplastic liver. (**B**) AR nuclear staining in tumor lesion (T), with less in non-tumor (non-T)(upper panel); AR nuclear staining in tumor margin (lower panel). (**C**, **D**) IHC staining of AR in 28-weeks-old DEN-induced $AR^{+/y}$ and L-$AR^{-/y}$ liver tumor. AR positive staining is brown in $AR^{+/y}$ transformed foci; higher magnification of the indicated area is shown in inset (**C**). In contrast, there is no positive signal in transformed foci of L-$AR^{-/y}$ liver; higher magnification image of the indicated area is shown in inset (**D**). (**E**) Serum Testosterone level measured by ELISA

assay. * represents a significant difference ($p<0.05$) between male and female; # indicates a significant difference ($p<0.05$) between T-AR$^{-/y}$ and AR$^{+/y}$.

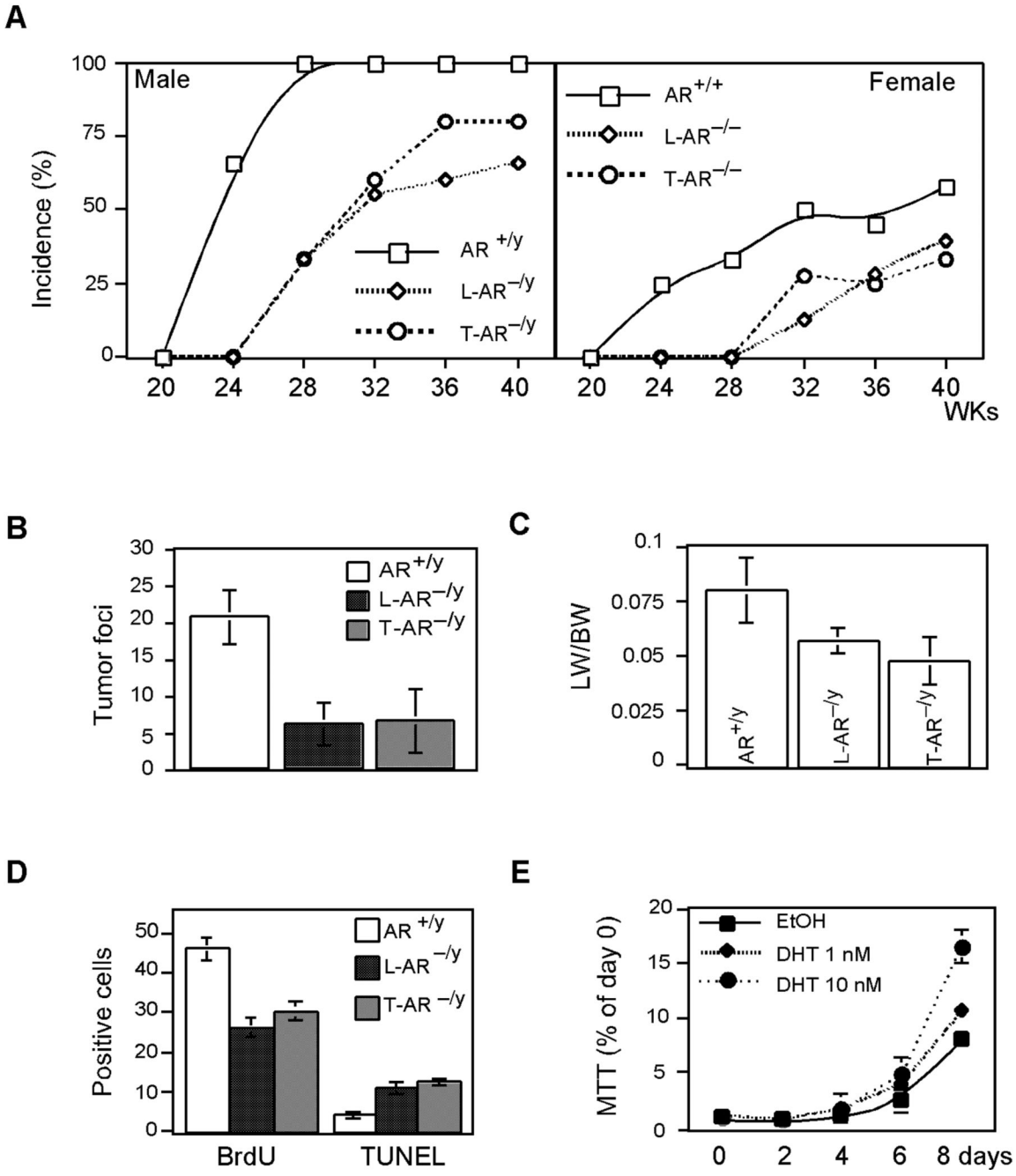

Fig. 2. AR effect on hepatocarcinogenesis

(**A**) HCC incidence of mice. 20 mg/kg/mice of DEN was injected I.P. into 12-days-old mouse pups. After various time periods, 20-, 24-, 28-, 32-, 36-, and 40-weeks, we sacrificed mice and observed hepatocarcinogenesis in all mice. We defined a tumor as positive if it could be observed by the naked eye. Wild-type mice ($AR^{+/y}$ and $AR^{+/+}$) are represented square solid line —□—; T-ARKO ($T\text{-}AR^{-/y}$ and $T\text{-}AR^{-/-}$) are dashed line ┈◇┈; L-ARKO ($L\text{-}AR^{-/y}$ and $L\text{-}AR^{-/-}$) are circle dashed line ┈○┈ (**B**) Tumor foci numbers in 36-weeks DEN-induced male mice decreased in $T\text{-}AR^{-/y}$ and $L\text{-}AR^{-/y}$ compared to $AR^{+/y}$ ($p<0.05$). (**C**) Liver weight//Body weight (LW/BW) ratio in 36-weeks DEN-induced male mice decreased in $T\text{-}AR^{-/y}$ and $L\text{-}AR^{-/y}$ compared to $AR^{+/y}$ ($p<0.05$). (**D**) BrdU (proliferation) and

TUNEL (apoptosis) staining in 36-weeks DEN-induced male mice livers. We found BrdU positive proliferation stains decreased while TUNEL stains increased in T-$AR^{-/y}$ and L-$AR^{-/y}$ compared to $AR^{+/y}$ mice liver. These experiments were from 3 mice and 3 different sections of livers from each genotype. We pooled the numbers of positive stains from each slide from photographed image of sections (3 area/slide; under 10×10 magnification). (**E**) Cell growth analysis using MTT assay on the cells derived from $AR^{+/y}$ primary liver tumor culture in 55-weeks DEN-induced $AR^{+/y}$ mice. We used cells within 3 passages of subculture, treated with ethanol (EtOH) or DHT at different concentrations (1 and 10 nM). We monitored cell growth for a maximum of 8 days and harvested for MTT assay. We subtracted values from background readings at 650 nm and pooled all MTT assay results from 5 independent experiments.

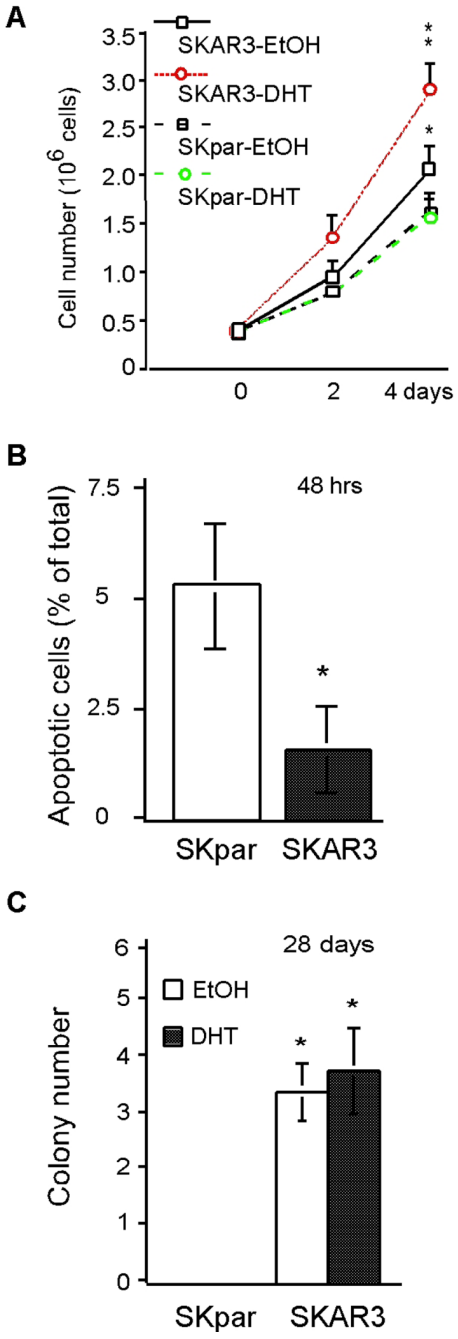

Fig. 3. AR promotes anchorage-dependent and -independent cell growth

(**A**) Androgen and AR effect on anchorage-dependent cell growth. We treated SKpar and SKAR3 cells with EtOH or 10 nM DHT for 4 days and counted the cell numbers to measure cell growth. * indicates the significant difference between SKpar and SKAR3 EtOH treatments. ** indicates the significant difference between SKAR3-DHT to SKAR3-EtOH and SKAR3-DHT to SKpar-DHT ($p<0.05$). (**B**) Apoptotic cells in SKpar and SKAR3 cells. We plated cells and treated with EtOH or 10 nM DHT for 48 hrs then stained with PI for cell apoptosis using flow cytometry. * indicated significant difference between SKpar and SKAR3 cells ($p<0.05$). (**C**) Anchorage-independent cell growth of SKpar and SKAR3 cells. We counted cell clusters

greater than 50 cells as a positive clone. All data were from 3–5 independently repeated experiments that showed similar results with the error bar indicating ±SD of pooled results.

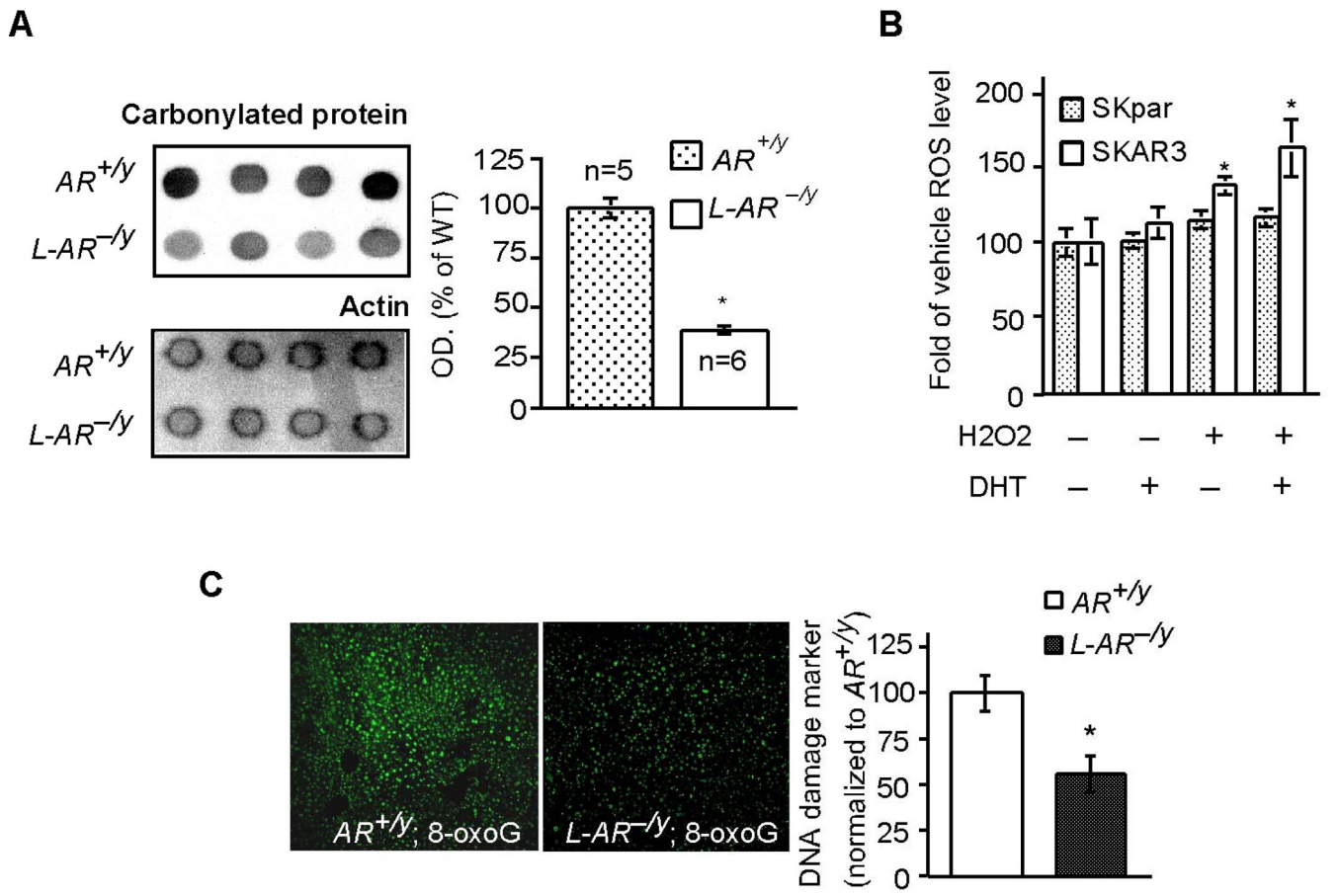

Fig. 4. AR promotes cellular oxidative stress through down-regulating ROS enzymes

(**A**) Oxidative attacked cellular protein decreased in L-$AR^{-/y}$ liver tumors compared to $AR^{+/y}$ liver. We derivitized protein from 36-weeks DEN-induced mice livers to form carbonylated groups that can be recognized by a specific antibody. We dot-blotted the derivitized samples on PVDF membrane and stained for carbonyl group and actin antibody. Representative result from $AR^{+/y}$ mice (n=5) and L-$AR^{-/y}$ (n=6) membrane blots are shown in the left panel, and the quantitative results from three independent blotted membranes of different mice in the right panel that show a similar pattern. * Indicates significant difference ($p< 0.05$). (**B**) We treated SKpar and SKAR3 cells with 200 μM H_2O_2 or 1 nM DHT for 24 hrs and measured ROS level. We performed and pooled three independent experiments. (**C**) IHC staining of DNA damage marker, 8-oxoG, in 36-weeks DEN-induced mice liver tumors. Positive staining (green spot) of 8-oxoG is more abundant in $AR^{+/y}$ (left panel) than L-$AR^{-/y}$ (middle panel) liver tumor. Three liver tumors with 3 different sectioned slides were examined and signals were analyzed, and quantitated using NIH-image software. Quantitated result are shown in right panel. Significant difference in $AR^{+/y}$ and L-$AR^{-/y}$ is indicated using * ($p<0.05$).

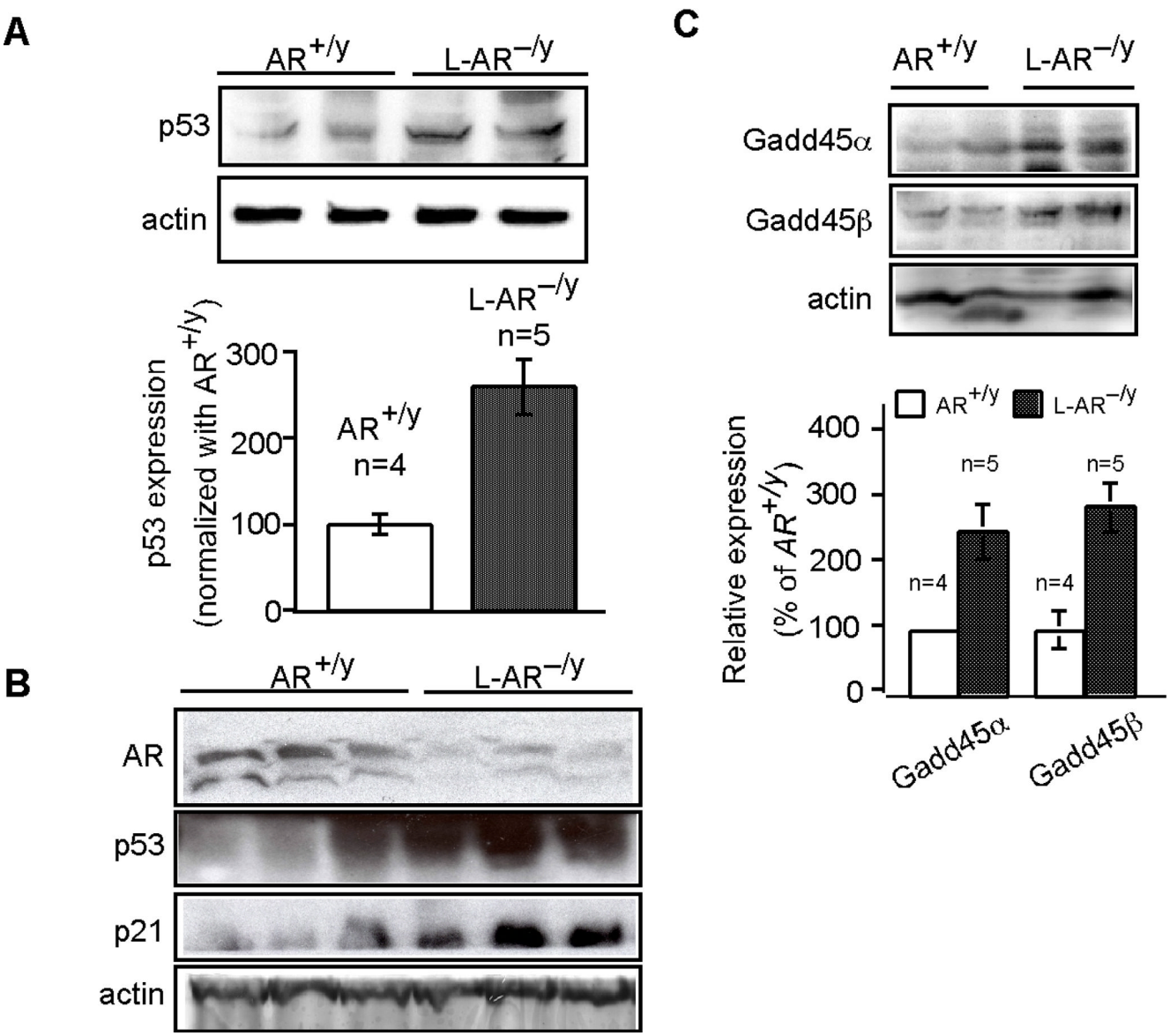

Fig. 5. AR suppresses p53 and down-stream target genes

(**A**) p53 protein expression in 36-weeks DEN-induced mouse HCC livers. We measured p53 expression with immunoblotting and show in our quantitative results that p53 expression in the $AR^{+/y}$ mice is lower than L-$AR^{-/y}$. GAPDH served as loading control. (**B**) AR, p53 and p21 protein expression in 36-weeks DEN-injected mouse normal livers. Immunoblotting demonstrates thatp53 expression in $AR^{+/y}$ mice is lower than that in L-$AR^{-/y}$. β-Actin served as loading control. Higher expression of p53 and p21 proteins were detected in L-$AR^{-/y}$ livers compared to $AR^{+/y}$ (n=4 of each group). (**C**) We examined Gadd45α and β protein expression using specific antibodies, and compared $AR^{+/y}$ and L-$AR^{-/y}$ liver tumors. Quantitation of Gadd45α and β protein expression in lower panel. * indicates significant difference in $AR^{+/y}$ and L-$AR^{-/y}$ ($p<0.05$).

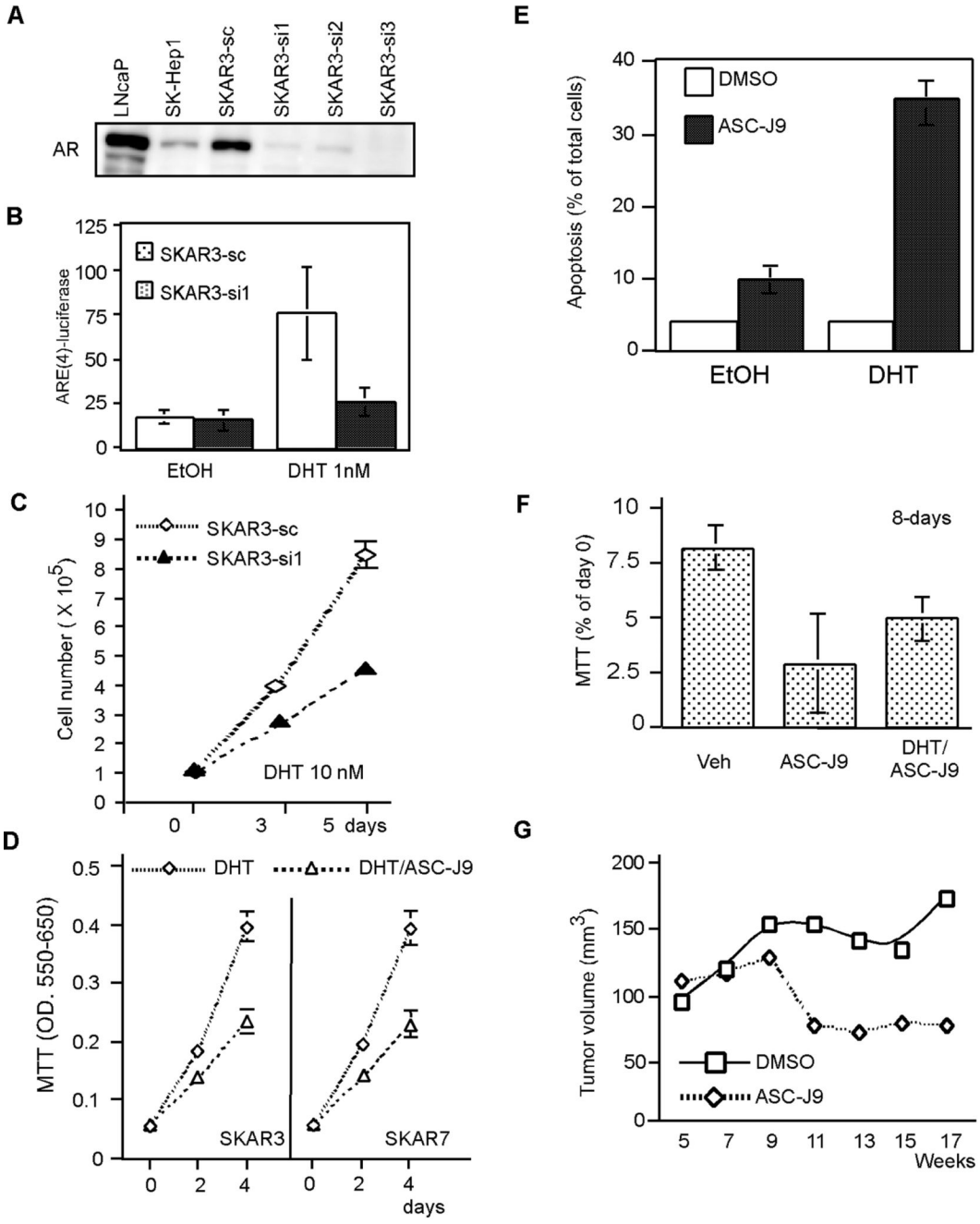

Fig. 6. Targeting AR as therapeutic strategy

(**A**) Establishment of AR siRNAstable transfectants of human SKAR3 cells. Scrambled siRNA (SKAR3-sc, lane3) and different siRNA targeting AR (SKAR3-si1, SKAR3-si2, and SKAR3-si3;lane 4, 5, 6 respectively) stable transfectants derived from SKAR3 cells. LNCaPand SK-Hep1 cells served as positive and negative controls of AR expression, respectively. (**B**) We used AR transactivation activity to examine the knockdown efficiency of AR siRNA in the SKAR3 cells. We used the SKAR3-si1 cells to compare with SKAR3-sc cells and treated with EtOH and 1 nM DHT for 24 hrsafter ARE(4)-luciferase transfection. We normalized the readings with the readout of pRL-TK cotransfection and pooled three individual experiments. (**C**) AR siRNA effect on SKAR3 cell growth. We treated SKAR3-sc and SKAR3-si1 cells with

EtOH or 1 nM DHT, then observed cell growth by counting cells on different days. (**D**) ASC-J9 effect on SKAR3 and SKAR7 cells. We plated and cultured cells with EtOH, 10 nM DHT and 5 μM ASC-J9 for different days and examined cell growth using MTT assay. (**E**) ASC-J9 effect on SKAR3 cell apoptosis and proliferation. We cultured cells with 10 nM DHT, or 5 μM ASC-J9 for 24 hrs, then detached, stained with PI, assayed immediately by flow cytometry to observe cell apoptosis. (**F**) We derived primary cells from 55-weeks DEN-induced $AR^{+/y}$ liver tumors and cultured ex vivo. We treated cells with ASC-J9 or cotreated with 10nM DHT for 8 days, then harvested for MTT assay. The result represents three independent experiments. (**G**) ASC-J9 suppressed liver cancer growth in vivo. We derived primary cells from 55-weeks DEN-induced AR+/y liver tumors and subcutaneously inoculated into nude mice (2×10^6 cells/site) flank. After 3 weeks, we IP injected mice with ASC-J9 50 mg/kg/mice) twice per-week for 17wks. We measured and pooled results from six injection sites from 3 mice. Solvent (DMSO) group is shown as solid line, and ASC-J9 group is shown as dashed line.