



Research paper

Androgen receptor drives hepatocellular carcinogenesis by activating enhancer of zeste homolog 2-mediated Wnt/ β -catenin signaling



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ABSTRACT

Background: Androgen receptor (AR) plays a crucial role as a transcription factor in promoting the development of hepatocellular carcinoma (HCC) which is prone to aberrant chromatin modifications. However, the regulatory effects of AR on epigenetic mediators in HCC remain ill-defined. Enhancer of zeste homolog 2 (*EZH2*), an oncogene responsible for the tri-methylation of histone H3 at lysine 27 (H3K27me3), was identified to be overexpressed in approximate 70–90% of HCC cases, which prompted us to investigate whether or how AR regulates *EZH2* expression.

Methods: Colony formation, soft agar assay, xenograft and orthotopic mouse models were used to determine cell proliferation and tumorigenicity of gene-manipulated HCC cells. Gene regulation was assessed by chromatin immunoprecipitation, luciferase reporter assay, quantitative RT-PCR and immunoblotting. Clinical relevance of candidate proteins in patient specimens was examined in terms of pathological parameters and postsurgical survival rates.

Findings: In this study, we found that AR upregulated *EZH2* expression by binding to *EZH2* promoter and stimulating its transcriptional activity. *EZH2* overexpression increased H3K27me3 levels and thereby silenced the expression of Wnt signal inhibitors, resulting in activation of Wnt/ β -catenin signaling and subsequently induction of cell proliferation and tumorigenesis. In a cohort of human HCC patients, concordant overexpression of AR, *EZH2*, H3K27me3 and active β -catenin was observed in tumor tissues compared with paired non-tumor tissues, which correlated with tumor progression and poor prognosis. These findings demonstrate a novel working model in which *EZH2* mediates AR-induced Wnt/ β -catenin signaling activation through epigenetic modification, and support the application of *EZH2*-targeted reagents for treating HCC patients.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most common and aggressive malignancies worldwide with 800,000 new cases rising annually [1]. A prominent epidemiological feature of HCC is sexual dimorphism with significantly higher incidence in men than in women, with ratios ranging from 5:1 to 7:1. Mounting evidence has unveiled the molecular linkage between androgen signaling and gender disparity in HCC [2,3]. Androgen receptor (AR), rather than androgen, is mainly in charge of androgen signaling, and has been implicated as a major contributor to HCC development with high expression in the tumor tissues. Knockdown of AR in the liver remarkably reduced the incidence of carcinogen- and hepatitis B virus (HBV)-induced HCCs in mouse models, demonstrating the necessity of AR for HCC development

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Research in context

Evidence before this study

Overexpression of androgen receptor (AR) is common in male-predominant hepatocellular carcinoma (HCC). AR serves as an omnipotent transcription factor in promoting the development of HCC through multiple signaling pathways. Aberrant chromatin modification by enhancer of zeste homolog 2 (EZH2) is a hallmark in the development of HCC. Increased expression of EZH2 has been frequently detected in HCC tissues, and it correlates with the aggressiveness and poor prognosis of HCC. However, the regulation of AR on EZH2 is still ill-defined.

Added value of this study

EZH2 is identified as a direct AR-regulated oncogene to mediate hepatocellular proliferation and transformation through epigenetic modification of Wnt/ β -catenin signaling. Targeted inhibition of *EZH2* perturbs the AR/*EZH2*/ β -catenin signaling to suppress cell proliferation *in vitro* and hepatocarcinogenesis *in vivo*. Clinically, hyperactivation of this new epigenetic signaling pathway in HCC tissues correlates with poor prognosis of patients.

Implication of all the available evidence

Both AR and *EZH2* not only play crucial role in the development of HCC, but also act as important biomarkers associated with patient prognosis. The transcriptional activation of *EZH2* by AR represents a novel mechanism underlying their synergistic oncogenic effects in HCC development. Considering the central role of *EZH2* in the AR-controlled signaling, specific inhibition of *EZH2* is a promising regime for the treatment of HCC, or even other male-predominant cancers.

[4]. Indeed, AR acts as a master transcription factor in the nuclear steroid receptor family to regulate target genes that control multiple oncogenic signaling pathways in driving hepatocarcinogenesis [5,6]. *Transformation growth factor β 1* (*TGF β 1*), *cell-cycle related kinase* (*CCRK*), and *vascular endothelial growth factor* (*VEGF*) are identified AR-bound oncogenes that respectively regulate TGF signaling, β -catenin signaling and angiogenesis pathway, and all of which are responsible for cell proliferation and malignant outgrowth [7–9]. Recently, vast genetic and epigenetic aberrations that are involved in the regulations of oncogenic signaling pathways have been associated with AR [10,11]. Beyond the genetic factors affecting tumorigenesis, many new epigenetic regulators are also found to be critical for HCC progression [12]. These findings demonstrate the important correlation of AR and epigenetic regulators in HCC.

Enhancer of zeste homolog 2 (*EZH2*) functions as an epigenetic regulator to modulate chromatin remodeling in many physiological processes, such as deciding cell fate by balancing cell proliferation and differentiation in the body development [13]. Aberrant expression of *EZH2* has been shown to manipulate the process of cancer initiation and progression, and is used as a promising biomarker for the evaluation of staging and prognosis of human cancers including HCC [14–16]. *EZH2* is a core component of polycomb repressive complex 2 (PRC2), which exhibits histone methyltransferase (HMTase) activity to catalyze the trimethylation of histone H3 at lysine 27 (H3K27me3) to alter chromatin configuration [17]. The C-terminal SET domain in *EZH2* is responsible for the trimethylation of H3K27, and this histone mark mediates transcriptional silencing of tumor suppressors to induce carcinogenesis [18]. Interactions of *EZH2* with tumor suppressors on chromatin are associated with increased levels of H3K27me3, whereas

a missense mutation of lysine27 with methionine (K27 M) in the gene encoding H3.3 inhibits *EZH2* activity [19]. Therefore, H3K27me3 acts as an iconic epigenetic mark linked to the oncogenic property of *EZH2* by silencing expression of tumor suppressor genes such as *E-cadherin*, *SLIT2*, and *FOXC1* in a variety of cancers [20–22].

Aberrant expression of *EZH2* in cancer cells usually occurs at the transcriptional level due to the binding of transcriptional activators with *EZH2* promoter. Increasing evidence demonstrates that sex steroid signaling stimulates carcinogenesis through *EZH2*-controlled epigenetic pathways in breast cancer and prostate cancer [23,24]. Intriguingly, it has been documented that AR is responsible for *EZH2* expression in prostate cancer, wherein AR cooperates with *KRAS* to elevate *EZH2* expression and H3K27me3 level through the occupancy on *EZH2* promoter, forming an integrated oncogenic signaling pathway [25]. Recently, aberrant *EZH2*-controlled epigenetic modulation was demonstrated as a major driving force of male-predominant HCC, and HCC cells with high level of AR generally have high level of *EZH2* expression [26]. These findings underscore the positive correlation of AR and *EZH2* in HCC. However, the underlying mechanism of AR regulation on *EZH2* has not yet been elucidated.

Wnt/ β -catenin signaling is another important oncogenic pathway known to be associated with the development of human liver cancer [27]. β -catenin acts as a central player in this signaling pathway, of which dysregulation usually leads to uncontrolled cell proliferation and cell cycle [28]. Apart from infrequent genetic mutations, dysregulation of upstream members such as Wnt signal inhibitors is responsible for the aberrant activation of β -catenin in carcinogenesis [29]. In AR-related HCC samples, our findings underpinned the repressed expression of Wnt signal inhibitors, which correlated with higher AR expression and more active β -catenin, suggesting the existence of certain mediator(s) in the AR regulation of Wnt signal inhibitors. Herein, using chromatin immunoprecipitation combined with gene expression analysis, we revealed that *EZH2* is a direct target of AR to mediate the suppression of Wnt signal inhibitors. *In vitro* and *in vivo* studies showed that AR transcriptionally upregulated *EZH2* expression, which then increased H3K27me3 to silence Wnt signal inhibitors, leading to activated Wnt/ β -catenin signaling and hence increased hepatic neoplastic proliferation and transformation. Furthermore, we evaluated the correlation of the activity of AR/*EZH2*/ β -catenin signaling with tumor stage and poor prognosis in patients.

2. Materials and methods

2.1. Patients and clinical specimens

Liver tissues were collected from patients who underwent hepatectomy for HCC at Shuguang Hospital affiliated to Shanghai University of Traditional Chinese Medicine (Shanghai, China) and Cancer hospital affiliated to Harbin Medical University (Harbin, China) in this study. The excised surgical specimens were immediately snap-frozen in liquid nitrogen for RNA and protein extraction. All patients signed the written consent on the use of clinical specimens for research purposes. Studies using human tissues were reviewed and approved by the Shuguang Hospital and HMU Clinical Research and Trial Ethics Committees, respectively.

2.2. Cell culture and transfection

Human Huh7, PLC5 and SK-Hep1 HCC cell lines and immortal hepatic LO2 cell line were cultured in high-glucose DMEM (Gibco) supplemented with 10% FBS (Hyclone) at 37 °C in a 5% CO₂-containing humidified incubator. Transfections of plasmids and siRNAs into cells were conducted using X-tremeGene Transfection Reagent (Roche) and HiPerfect (Qiagen) following manufacturer's instructions, respectively. For the establishment of stably transfected cell lines, cells were collected after 48 h of transfection and re-seeded into the antibiotic-containing

selective medium until antibiotic-resistant colonies appeared and grew up.

2.3. Chromatin immunoprecipitation and quantitative PCR

Chromatin immunoprecipitation was performed as described previously [8]. In brief, 1×10^7 cells were crosslinked with 1% formaldehyde, lysed by lysis buffer, and chromatin in the lysate was fragmented into 100–500 bps by sonication. Protein-DNA complexes were immunoprecipitated (IP) by 2 μ g antibodies coupled with Dynal magnetic beads (Invitrogen). ChIP-grade antibodies used were anti-AR (Millipore; 06-680), anti-EZH2 (Cell signaling; #4905), anti-H3K27me3 (Diagenode; C15200181-50) and anti-IgG (Abcam; ab2410). The IP or input DNA was subjected to elution, reverse crosslink and purification. Purified IP and input DNA were analyzed by PCR quantification using SYBR® Premix Ex Taq™ Kit (TaKaRa). The primer sequences for individual genes were listed in the supplementary information. Promoter enrichment of *EZH2*, *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1* and *SFRP5* conjugated with respective proteins was determined and shown as the percentage of input DNA. IgG antibody was used as a negative control.

2.4. Site-directed mutagenesis and luciferase reporter assay

The *EZH2* promoter region (chr7: 148,807,385–148,808,762) was amplified by PCR and cloned into pGL3-basic vector (Promega) to generate the *EZH2* promoter luciferase reporter. The putative AR binding sites (726-AAGAACA-732; 1084-GGGAACA-1090) were deleted separately or completely to generate *EZH2* promoter mutants using the Quick Change II Site-Directed Mutagenesis Kit (Stratagene). Cells were transiently transfected with the *EZH2*-Luc construct, *EZH2*-Luc mutants, or vectors respectively, accompanied with Renilla luciferase reporters as internal control. After 24 h, siAR (siRNA specifically targeting AR; 5'-GAAAGCACUGCUCACUUCAG-3') or siCtrl (scrambled siRNA; 5'-UUCUCCGAACGUGUCACGU-3') were transfected into PLC5 and Huh7 cells, and AR-expressing plasmids or control vectors were transfected into LO2 and SK-Hep1 cells. Cells were harvested after 2 days and luciferase activity was detected by the Dual Luciferase Reporter Assay System (Promega) using GloMax microplate luminometer instrument (Promega). All experiments were done in triplicate.

2.5. Immunoblotting

Proteins were extracted from cell lines and tissues using protease inhibitor cocktail (Roche) containing lysis buffer (Thermo Scientific) and T-PER Tissue Protein Extraction Reagent (Thermo Scientific), respectively. Protein concentration was determined by Bradford assay (Bio-Rad Laboratories). Equal amounts of proteins were fractionated by 10% SDS-PAGE gel electrophoresis, and transferred into nitrocellulose membrane (Bio-Rad Laboratories). Indicated proteins were detected by primary antibodies, including rabbit anti-AR (Abcam; ab9474; 1:1000), rabbit anti-EZH2 (Cell signaling; #4905; 1:1000), rabbit anti-H3K27me3 (Millipore; 07-449; 1:1000), rabbit anti-AXIN2 (Abcam; ab32197; 1:1000), rabbit anti-NKD1 (Sigma; SAB1401923; 1:1000), mouse anti-PPP2R2B (Sigma; SAB1404234; 1:1000), rabbit anti-PRICKLE1 (Abcam; ab15577; 1:1000), rabbit anti-SFRP5 (Thermo Scientific; PA5-71770; 1:1000), rabbit anti- β -catenin (Cell signaling; #9562; 1:1000), mouse anti-active- β -catenin (Millipore; 05-665; 1:2000), rabbit anti-CCND1 (Thermo Scientific; PA5-12255; 1:1000), mouse anti-EGFR (BD Transduction laboratories; ABIN126775; 1:3000), and mouse anti- β -actin (Sigma; A1978; 1:15000). Signals in protein expression from tissues were quantified by BandScan software (Glyko) and defined as the ratio of target protein relative to β -actin.

2.6. Quantitative RT-PCR

Total RNA was extracted from cells lines and tissues using TRIzol reagent (Invitrogen). cDNA was reverse transcribed from 1 μ g RNA by reverse transcription Master kit (Invitrogen) according to the manufacturer's instruction. For quantitative PCR analysis, aliquots of cDNA were amplified to detect transcript levels of *AR*, *EZH2*, *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1*, *SFRP5*, *CCND1* and *EGFR* using SYBR® Premix Ex Taq™ Kit (TaKaRa) and 7500 Real-Time PCR System (Applied Biosystems). *GAPDH* was used as an internal control. The primer sequences of individual genes used in quantitative PCR were shown in supplementary information. All reactions were performed in triplicate.

2.7. Colony formation

Cells seeded on 12-well plate were cultured overnight for cell attachment on the plate with 80% confluence, followed by transient transfection with gene-expressing plasmids or control vectors. After 2 days, cells were detached, reseeded on 6-well plate and cultured in the antibiotic-containing selective medium for 3 weeks. The antibiotic-resistant colonies formed were stained with 0.2% crystal violet and counted under the microscope. Data were obtained in 3 independent experiments.

2.8. Soft agar assay

Cells seeded on 12-well plate were transiently transfected with gene-expressing plasmids or control vectors. After 2 days, cells were trypsinized, gently mixed with 0.3% antibiotic-containing agar-medium mixture, and reseeded in triplicate onto a 6-well plate covered with a layer of 0.6% agar-medium mixture in priority. Cells in the top agar mixture were then covered with 10%FBS-containing DMEM medium. After 4 weeks, the antibiotic-resistant colonies were stained with 0.2% crystal violet and counted under the microscope. Data were obtained in 3 independent experiments.

2.9. In vivo tumorigenicity assay

Studies using female athymic nude mice (4 to 6 weeks old) were reviewed and approved by the Harbin Medical University Animal Experimentation Ethics Committee. Animal care and experiments were carried out in accordance of the National Institute of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978). In xenograft model, 5×10^5 cells were subcutaneously injected into the rear right dorsal flank of the mice. Tumor size was measured every other day using a caliper, and the tumor volume was calculated as $0.5 \times l \times w^2$, with l indicating length and w indicating width. The mice were euthanized at 5–6 weeks, and the tumors were excised and snap-frozen for protein extraction. In orthotopic model, subcutaneous tumors in xenograft were obtained and minced into 1 mm³ pieces. Each piece was implanted into the left liver lobe of each mouse. The mice were sacrificed after 5 weeks, and the tumor size and weight were measured.

2.10. Statistics

Unless otherwise indicated, data are presented as mean \pm standard deviation of 3 independent experiments. GraphPad Prism 5 (GraphPad Software) was used for data analysis. The independent Student's *t*-test was used to compare colony formation, gene expression, and tumorigenicity between 2 selected groups. The protein levels of AR, EZH2, H3K27me3 and active β -catenin in the tumor tissues and the matched non-tumor tissues were compared using nonparametric Wilcoxon's matched pairs test. The correlation of protein expression was analyzed using Pearson correlation test. Kaplan-Meier survival analysis was used to determine the overall and disease-free survival rates, which

was calculated from the data of curative surgery to death, HCC recurrence or last follow-up. A 2-tailed P value of <0.05 was considered statistically significant.

3. Results

3.1. EZH2 mediates AR-induced hepatocellular proliferation and tumorigenicity

We first investigated the role and function of EZH2 in AR-induced HCC development by performing colony formation and soft agar assay on cells with ectopic gene expression and RNA interference, respectively. Knockdown of AR by specific siRNA (siAR) in PLC5 HCC cells

markedly ablated focus formation (Fig. 1a). Notably, concurrent overexpression of EZH2 by gene transfection significantly restored the proliferation of AR-silenced cells (Fig. 1a). In soft agar assay, AR abrogation impaired the anchorage-independent growth of PLC5 cells, and such impairment could be rescued by parallel ectopic expression of EZH2 (Fig. 1b). In the reciprocal experiments, ectopic expression of AR promoted anchorage-dependent and -independent growths of LO2 human immortal liver cells (Fig. 1c and d). Importantly, shRNA-mediated knockdown of EZH2 (shEZH2) significantly abrogated AR-promoted focus formation and anchorage-independent growth in the same cells (Fig. 1c and d). These results suggested that EZH2 is required to mediate AR-induced cell proliferation and malignant transformation.

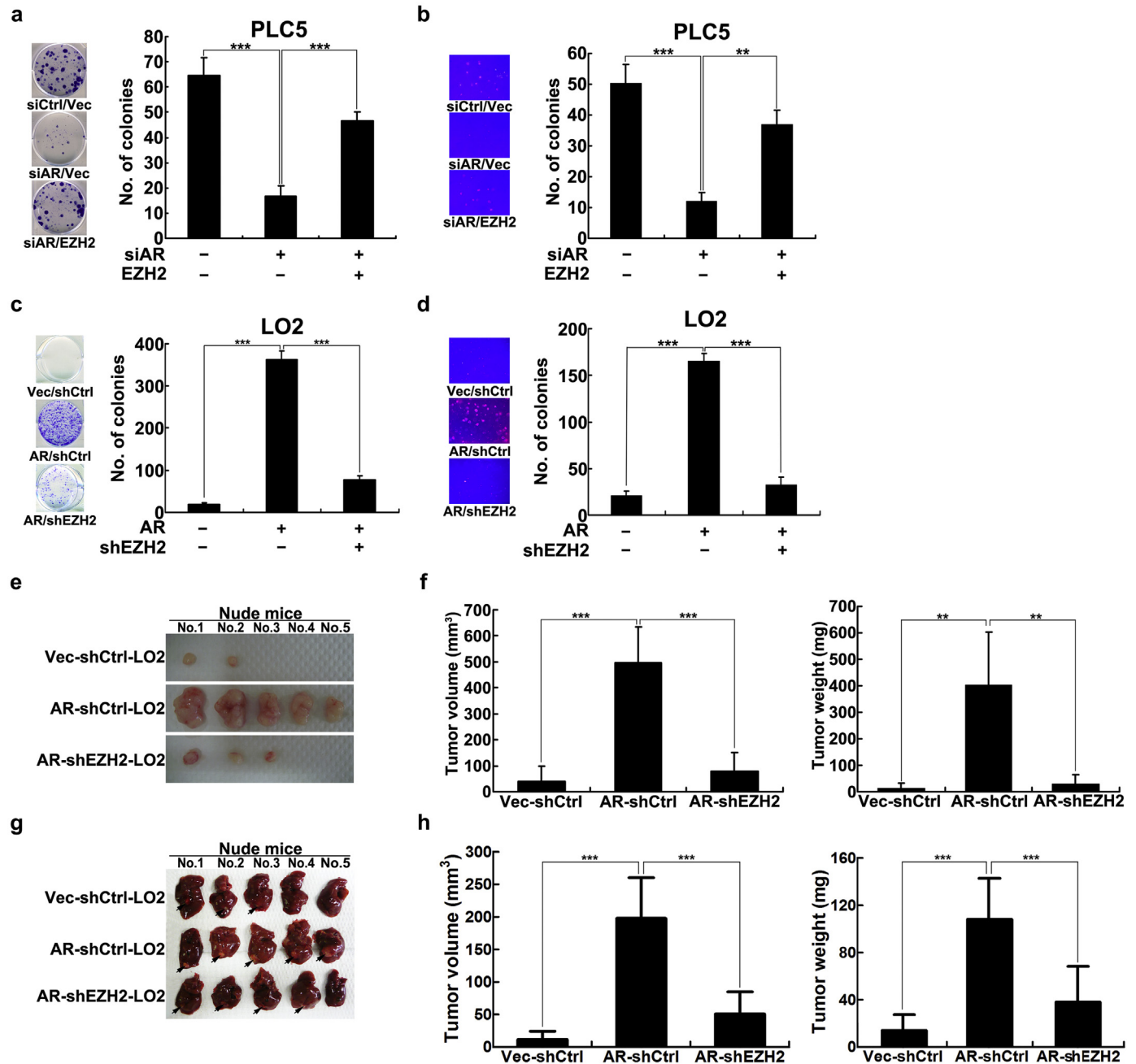


Fig. 1. EZH2 is required for AR-induced hepatocellular proliferation and tumorigenicity. (a) Focus formation and (b) anchorage-independent growth were assessed in control and AR-silenced PLC5 cells with or without EZH2 overexpression. (c) Focus formation and (d) anchorage-independent growth were assessed in control and AR-expressing LO2 cells with or without EZH2 knockdown. (e) Images of xenografts formed from Vec-shCtrl-, AR-shCtrl-, and AR-shEZH2-LO2 stable cells are shown. (f) The volumes and weights of isolated xenograft tumors from the 3 groups were measured and compared. (g) Images of intrahepatic orthografts formed from Vec-shCtrl-, AR-shCtrl-, and AR-shEZH2-LO2 stable cells are shown. (h) The volumes and weights of isolated orthotopic tumors from the 3 groups were measured. $**p < 0.01$; $***p < 0.001$.

In addition to *in vitro* functional analysis, we further verified the mediator role of EZH2 in AR-induced tumorigenicity in mouse models. We constructed LO2 stable cell lines with AR overexpression (AR-shCtrl-LO2), AR overexpression together with EZH2 knockdown (AR-shEZH2-LO2), and empty vector control (Vec-shCtrl-LO2). These different types of cells were subcutaneously injected into the rear dorsal flanks of nude mice for xenograft development, respectively ($n = 5$ per group). In contrast to Vec-shCtrl-LO2 cells, AR-shCtrl-LO2 cells developed into remarkably larger tumors, whereas knockdown of EZH2 significantly attenuated the AR-induced proliferation in AR-shEZH2-LO2 cells (Fig. 1e). Notably, the mean volume and weight of tumors in AR-shCtrl-LO2 cells were nearly 20-fold greater than those of Vec-shCtrl-LO2 and AR-shEZH2-LO2 cells, which exhibited no tumors (3 out of 5; and 2 out of 5) or very small tumors (2 out of 5; and 3 out of 5) after 5 weeks (Fig. 1f). In a complimentary experiment, PLC5 cells with high endogenous expression of EZH2 were used for xenograft development, which could form solid tumors *in vivo*. Consistently, EZH2 knockdown significantly reduced the tumorigenicity of AR-abundant PLC5 cells (Supplementary Fig. 1). In a separate orthotopic mouse model, xenograft pieces developed from Vec-shCtrl-LO2, AR-shCtrl-LO2, and AR-shEZH2-LO2 stable cells were implanted into the livers of nude mice, and the tumor formation were examined after 5 weeks (Fig. 1g). Both the mean volume and weight of AR-induced intrahepatic tumors were dramatically increased compared to those of control group, and such aggressive tumor growth could be effectively attenuated by EZH2 knockdown (Fig. 1h). Altogether, these data confirmed that EZH2 is a major mediator of AR-induced tumorigenesis.

3.2. EZH2 is identified as an AR-directed transcriptional target in HCC cells

To elucidate AR-EZH2 relationship in hepatocellular proliferation and tumorigenicity, we evaluated the regulation of AR on EZH2 expression in HCC cells. In our previous genome-wide promoter profiling, EZH2 was found to be a candidate AR target with moderate but significant score ($p = 0.0082$) [8]. The AR binding sites with high enrichment peaks were mainly detected in EZH2 promoter spanning from -2 kb to -0.5 kb upstream of transcription start site (TSS), in which two consensus motifs of ARE (AAGAACA, -1.2 kb of TSS; GGGAACA, -0.8 kb of TSS; according to JASPAR database <http://jaspar.genereg.net>) are present (Fig. 2a). We then performed ChIP coupled with quantitative PCR targeting both putative AREs to examine the occupancy of AR on EZH2 promoter. A dramatically higher enrichment of EZH2 promoter was obtained using anti-AR antibody compared with anti-IgG antibody in both PLC5 and Huh7 HCC cells, which could be abrogated by knocking down AR using siRNA (siAR) in the same cells (Fig. 2b), demonstrating the specific occupancy of endogenous AR protein at EZH2 promoter. In luciferase reporter assay, EZH2 promoter was cloned into pGL3 reporter, and the promoter construct was then transfected into HCC cells. Endogenous AR protein stimulated the EZH2 promoter activity, which could be effectively suppressed by siAR (Fig. 2c). In addition, removal of individual or both putative 7 bp AR binding sequences in EZH2 promoter by site-directed mutagenesis resulted in the significant reduction of luciferase reporter transcription, confirming the necessity of AREs in the activity of EZH2 promoter (Fig. 2d).

In LO2 and SK-Hep1 cells, the enrichment levels of EZH2 promoter by anti-AR antibody were as low as those by anti-IgG control owing to the absence of endogenous AR protein (Fig. 2e). By contrast, ectopically expressed AR bound to EZH2 promoter, which was shown by the higher enrichment from anti-AR antibody compared with vector controls in ChIP-quantitative PCR assay (Fig. 2e). In luciferase reporter assay, the EZH2 promoter activity remained low in LO2 and SK-Hep1 cells, while ectopic expression of AR greatly induced the EZH2 promoter activity (Fig. 2f). Importantly, deletion of AREs in EZH2 promoter could abolish the induction effect by AR, further confirming the activator role of AR on EZH2 promoter (Fig. 2g). Collectively, these data strongly

demonstrated AR directly regulates EZH2 transcription through promoter binding.

3.3. AR transcriptionally upregulates EZH2 expression to induce histone H3 trimethylation

Since AR directly binds to EZH2 promoter, its transcriptional regulation on EZH2 mRNA and protein level was further characterized. In PLC5 and Huh7 HCC cells with high endogenous levels of EZH2 transcript and AR expression, knockdown of AR by siAR dramatically reduced EZH2 mRNA level as shown in quantitative RT-PCR analysis ($p = 0.00034$; Fig. 3a). Consistently, EZH2 protein expression was also markedly decreased (Fig. 3b), supporting the positive regulatory role of AR on EZH2 gene expression. Moreover, following siAR treatment, we also observed a reduction in EZH2 expression accompanied with H3K27 trimethylation (H3K27me3) (Fig. 3b), indicating the involvement of AR in the EZH2-controlled histone modification. On the other hand, ectopic expression of AR significantly induced EZH2 transcription ($p = 0.00047$; Fig. 3c) and protein expression (Fig. 3d), and also increased H3K27me3 levels in LO2 and SK-Hep1 cells (Fig. 3d). These data demonstrated that AR transcriptionally upregulates EZH2 to increase global H3K27me3 level.

In addition, we also examined the gene expression profiles in the tumors isolated from our xenograft mouse models. In contrast to the low transcript levels in Vec-shCtrl-LO2 xenografts, AR and EZH2 mRNAs were markedly upregulated in AR-shCtrl-LO2 tumor tissues, whereas EZH2 knockdown abolished the AR-induced EZH2 transcription (Fig. 3e). Consistent with the changes at transcript level, AR overexpression in tumor tissues induced EZH2 protein expression, which could be blocked by EZH2 knockdown (Fig. 3f). Notably, there was concomitant increase of H3K27me3 in AR-shCtrl-LO2 xenografts, while abrogation of EZH2 reduced the level of H3K27me3 in tumor tissues (Fig. 3f). Collectively, these findings further support the upregulation of AR on EZH2 expression at transcription level, thus leading to the initiation of H3K27me3-mediated epigenetic modification.

3.4. AR activates Wnt/ β -catenin signaling through EZH2-mediated epigenetic modulation

We next explored the underlying mechanism whereby AR induces hepatocarcinogenesis through EZH2-mediated epigenetic modification. Gene sets commonly regulated by both AR and EZH2 were identified in our previously reported ChIP-chip database [8,30], and Kyoto Encyclopedia of Genes and Genomes (KEGG) cellular pathway analysis demonstrated the significant enrichment of shared genes on Wnt signaling pathway involved in cell proliferation and oncogenesis (Fig. 4a). Moreover, the majority of the Wnt antagonist genes were occupied by H3K27me3 protein, suggestive of epigenetic repression in HCC cells [30]. Therefore, it was hypothesized that AR activated Wnt/ β -catenin signaling through EZH2-mediated silencing of Wnt signal inhibitors in hepatocellular neoplasm.

To test this speculation, we first examined the AR-induced enrichment of EZH2 and H3K27me3 at the promoters of Wnt signal inhibitors. In PLC5 cells, quantitative ChIP-PCR showed that knockdown of AR strongly reduced the occupancy of EZH2 on the promoters of Wnt signal inhibitors, including AXIN2, NKD1, PPP2R2B, PRICKLE1, and SFRP5, which could be effectively rescued by EZH2 overexpression (Fig. 4b). Moreover, AR silencing also inhibited the occupancy of H3K27me3 on the promoters of Wnt signal inhibitors, which could be recovered by overexpression of EZH2 (Fig. 4c). Quantitative RT-PCR analysis identified marked upregulation of AXIN2, NKD1, PPP2R2B, PRICKLE1 and SFRP5 mRNAs in AR-depleted PLC5 cells compared with control group, while simultaneous overexpression of EZH2 eliminated the siAR effect in the same cells (Fig. 4d). Furthermore, the downstream pro-proliferative effectors of Wnt/ β -catenin signaling, CCND1 and EGFR, were found to be inhibited by AR knockdown but resumed by EZH2 overexpression, such transcriptional outcomes were opposite to that of Wnt signal inhibitors (Fig. 4e). Western blot

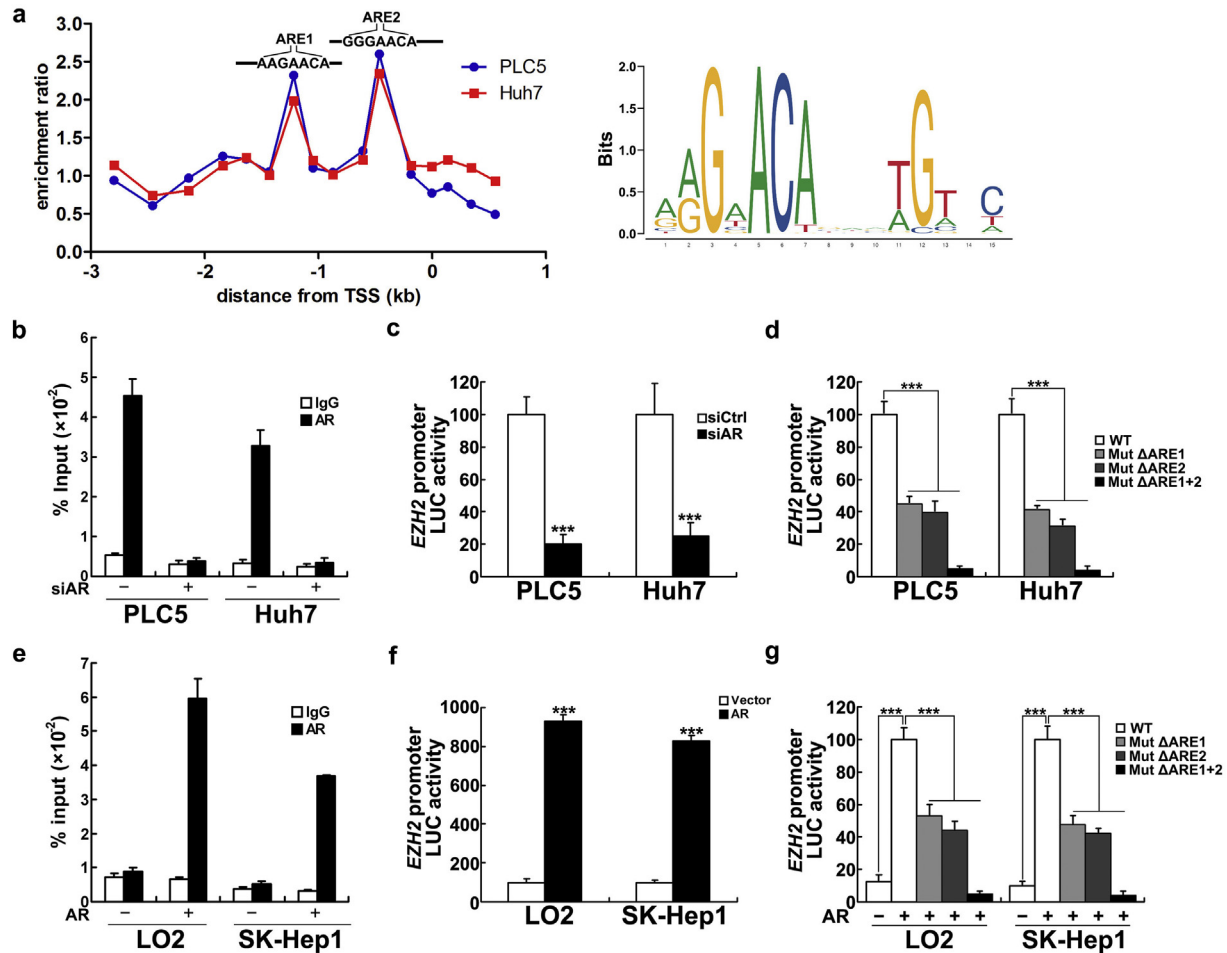


Fig. 2. *EZH2* is a transcriptional target of AR. (a) Enrichment map showing AR binding sites in *EZH2* promoter, wherein the consensus motifs of ARE were identified using JASPAR database. (b) Quantitative ChIP-PCR analysis of AR occupancy on *EZH2* promoter was performed in PLC5 and Huh7 cells transfected with siCtrl- or siAR-transfected PLC5 and Huh7 cells, respectively. (c) *EZH2* promoter activity was assessed by luciferase reporter assay in PLC5 and Huh7 cells transfected with siCtrl or siAR, respectively. Luciferase reporter expression was measured relative to *Renilla* control. (d) The AREs in *EZH2* promoter were essential for transcription activation. The HCC cells were transfected with either the WT or ARE-deleted mutant constructs, respectively. (e) Quantitative ChIP-PCR analysis of AR occupancy on *EZH2* promoter was performed in LO2 and SK-Hep1 cells transfected with vectors or AR-expressing plasmids. (f) The transcriptional activity of *EZH2* promoter regulated by AR was assessed using luciferase reporter assay in LO2 and SK-Hep1 cells transfected with vectors or AR-expressing plasmids. (g) Luciferase reporter transcription controlled by WT or ARE-deleted mutant *EZH2* promoters was measured in LO2 and SK-Hep1 cells transfected with or without AR. *** $p < 0.001$.

analysis further demonstrated that silencing of AR markedly increased the expression of Wnt signal inhibitors, and down-regulated active β -catenin without affecting total β -catenin expression, leading to reduction of downstream targets CCND1 and EGFR (Fig. 4f). Conversely, overexpression of *EZH2* significantly reversed the effects of AR knockdown, resulting in downregulation of Wnt signal inhibitors, activation of β -catenin, and upregulation of CCND1 and EGFR (Fig. 4f).

In the reciprocal experiments, overexpression of ectopic AR in LO2 cells significantly increased the occupancies of *EZH2* and H3K27me3 at Wnt signal inhibitor promoters, which were abolished by knockdown of *EZH2* (Fig. 4g and h). Consistently, mRNA levels of Wnt signal inhibitors were dramatically decreased upon AR overexpression, whereas knockdown of *EZH2* blocked the inhibitory effects of AR on the transcription of Wnt signal inhibitors (Fig. 4i). Moreover, the transcription of CCND1 and EGFR was activated in AR-expressing LO2 cells, which was blocked by *EZH2* knockdown (Fig. 4j). In western blot analysis, overexpression of ectopic AR reduced Wnt signal inhibitors, activated β -catenin, and subsequently increased the expression of CCND1 and EGFR, which were all abrogated by *EZH2* ablation (Fig. 4k). Taken together, these data demonstrated the important role of *EZH2* in mediating AR-induced hepatocarcinogenesis through epigenetic regulation of Wnt/ β -catenin signaling pathways.

3.5. AR induces cell proliferation and tumorigenesis by activating Wnt/ β -catenin signaling

Next we performed functional experiments to assess whether AR promotes cell proliferation and tumorigenesis through the activation of Wnt/ β -catenin signaling. Ectopic AR expression in LO2 cells markedly induced anchorage-dependent growth in culture plate (Fig. 5a) and anchorage-independent growth in soft agar (Fig. 5b). Notably, silencing of β -catenin by specific shRNA interference (sh β -catenin) significantly downregulated cell proliferation and malignant transformation of AR-expressing cells (Fig. 5a and b). In addition, Vec-shCtrl-, AR-shCtrl- and AR-sh β -catenin-expressing LO2 stable cell lines were established, and subcutaneously injected into nude mice for xenograft development. Stable expression of sh β -catenin in AR-expressing LO2 cells impaired the induction of active β -catenin and its targets CCND1 and EGFR (Fig. 5c). Compared with Vec-shCtrl-LO2 controls, AR-shCtrl-LO2 cells exhibited much faster tumor growth, whereas knockdown of β -catenin in AR-sh β -catenin-LO2 cells significantly inhibited tumorigenicity (Fig. 5d). Following quantifications of dissected tumors showed that the mean volume and weight of AR-sh β -catenin-LO2 tumors decreased >3 fold relative to AR-shCtrl-LO2 xenografts, to levels comparable to those of Vec-shCtrl-LO2 xenografts (Fig. 5e). These results corroborated

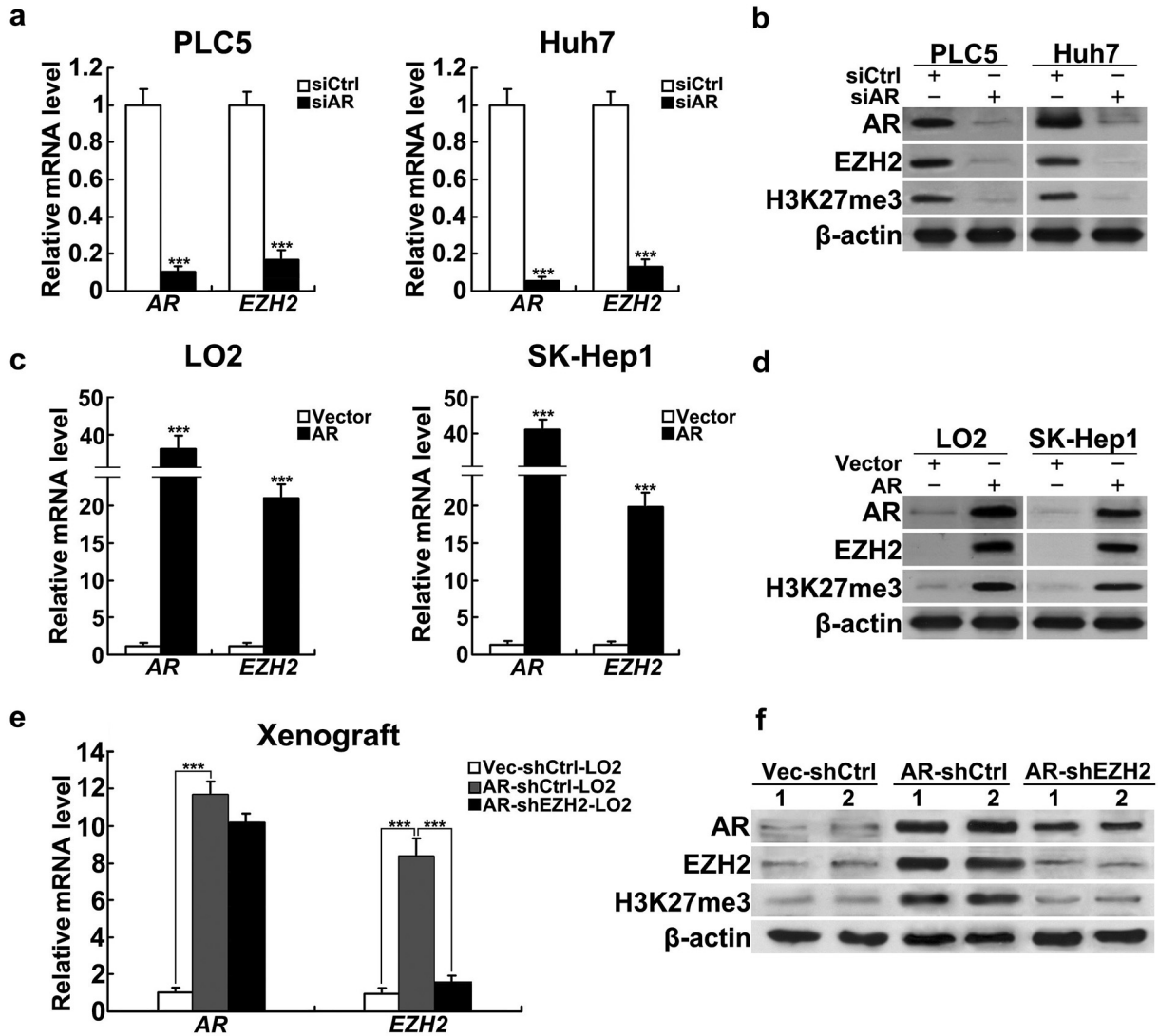


Fig. 3. AR upregulates EZH2 and H3K27me3 levels. (a) Transcript levels of AR and EZH2 and (b) protein expression of AR, EZH2 and H3K27me3 were detected in control or AR-silenced PLC5 and Huh7 cells. (c) Transcript levels of AR and EZH2 and (d) protein expression of AR, EZH2 and H3K27me3 were detected in LO2 and SK-Hep1 cells transiently transfected with empty vector or AR-expressing plasmids, respectively. (e) Transcription of *Ar* and *Ezh2* and (f) protein expression of AR, EZH2 and H3K27me3 were determined in tumor tissues isolated from xenograft mouse models. *** $p < 0.001$.

that AR promotes cell proliferation and tumorigenicity by activating Wnt/ β -catenin signaling pathway.

3.6. AR expression positively correlates with EZH2 and active β -catenin in human HCCs

To investigate the clinical significance of AR/EZH2/ β -catenin signaling cascade in HCC progression, we examined the protein expression of the constitutive components of this signaling cascade in 32 pairs of human HCC tissues and matched normal liver tissues. In contrast to the low basal levels in normal liver samples, AR, EZH2, H3K27me3 and active β -catenin were concordantly upregulated in HCC tissues (Fig. 6a). Following quantification of Western blot results showed that the expression of these proteins was progressively induced from normal tissues to non-tumor tissues to tumor tissues (Fig. 6b). The average upregulation of these proteins in tumor tissues was >1.5 fold relative to those in the adjacent precancerous tissues, among which overexpression of AR, EZH2, H3K27me3 and active β -catenin were detected in 62.5% (20/32), 71.9% (23/32), 65.6% (21/32) and 77.5% (25/32) of HCCs, respectively. The majority of AR-expressing HCCs (83%) showed higher expression of EZH2 and active β -catenin. Moreover, we

performed association analysis on the expression levels of these signaling components. Notably, the expression of AR, EZH2, H3K27me3 and active β -catenin significantly and positively correlated with each other in the same cohort of HCC samples (Fig. 6c). Taken together, these results demonstrated the consistent overexpression of AR, EZH2, H3K27me3 and active β -catenin in HCCs.

3.7. Hyperactivity of AR/EZH2/ β -catenin signaling cascade associates with poor prognosis in HCC patients

Based on the average fold changes of AR, EZH2, H3K27me3 and active β -catenin expression in tumors versus the paired precancerous tissues, we defined the activities of this signaling cascade in individual HCC samples. Next we evaluated whether the activity of AR/EZH2/ β -catenin signaling cascade correlated with HCC progression. The cohort of HCC samples were classified based on tumor staging, and the protein levels of all components in the signaling cascade were found to increase from early to advanced stages of HCCs (Fig. 7a). Furthermore, AR, EZH2, H3K27me3 and active β -catenin were all increased in poorly differentiated HCC samples compared to those in moderately- or well-differentiated ones, suggesting a correlation between the activity of

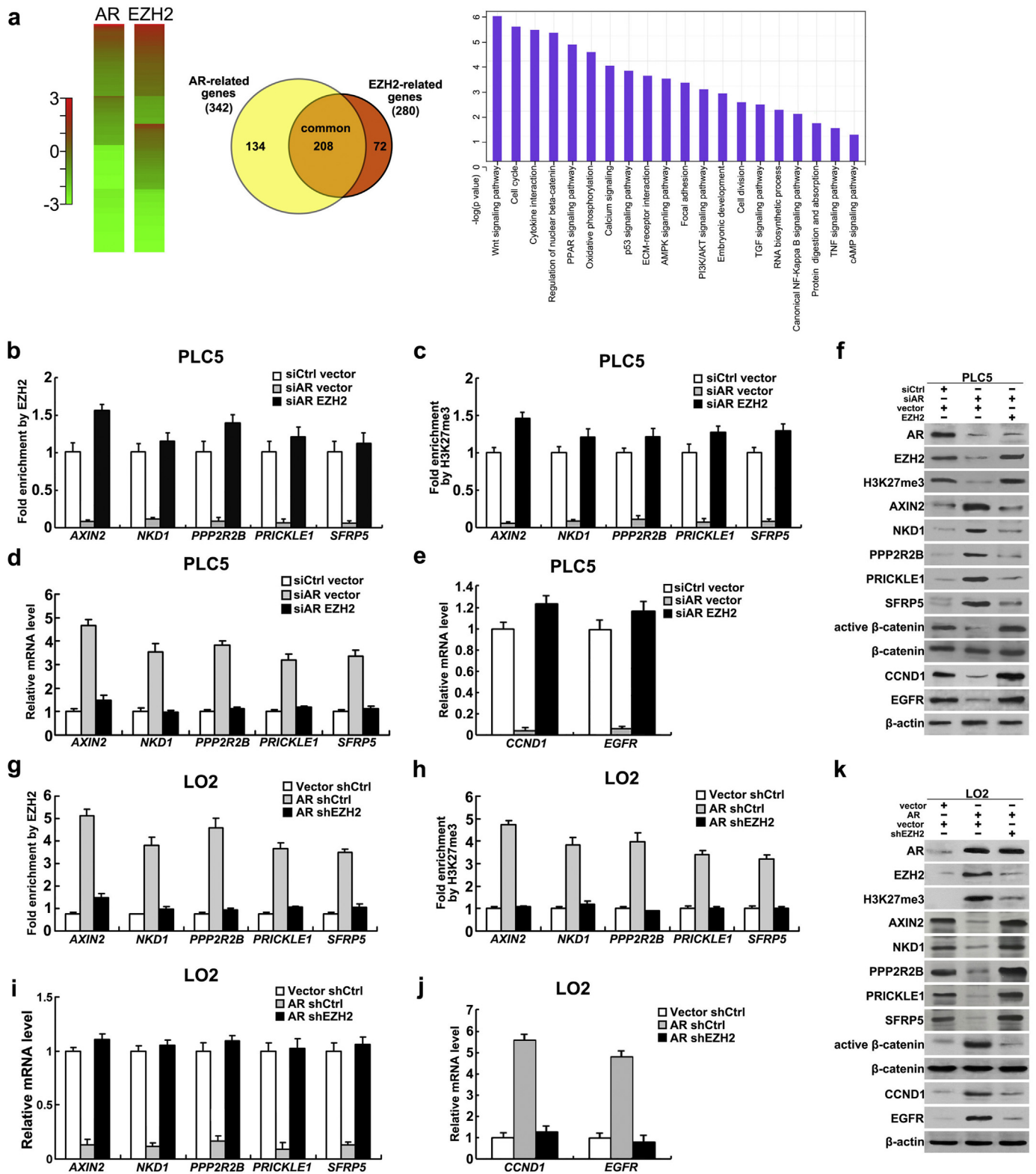


Fig. 4. AR activates Wnt/ β -catenin signaling through EZH2-mediated silencing of Wnt signal inhibitors. (a) Heat map (left) showing expression levels of AR- and EZH2-regulated genes, respectively; Venn diagram (middle) showing common gene set of AR- and EZH2-regulated genes, and KEGG cellular pathway analysis (right) showing the significant enrichment of Wnt signaling pathway in the common gene set. (b–c) Occupancies of (b) EZH2 and (c) H3K27me3 at the promoters of *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1* and *SFRP5* were detected by quantitative ChIP-PCR analysis in siCtrl- and siAR-PLC5 cells with or without EZH2 expression. (d–e) Transcript levels of (d) *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1*, *SFRP5* and (e) *CCND1* and *EGFR* were assessed by quantitative RT-PCR in siCtrl- and siAR-PLC5 cells with or without EZH2 expression. (f) Protein expression of AR, EZH2, H3K27me3, Wnt signal inhibitors, active and total β -catenin, *CCND1* and *EGFR* were detected by western blot in siCtrl-vector, siAR-vector, siAR-EZH2 co-transfected PLC5 cells. β -actin was used as a loading control. (g–h) Enrichments of (g) EZH2 and (h) H3K27me3 at the promoters of Wnt signal inhibitors were determined in vector-shCtrl, AR-shCtrl, and AR-shEZH2 co-transfected LO2 cells. (i–j) mRNA levels of (i) Wnt signal inhibitors and (j) *CCND1* and *EGFR* were detected in vector-shCtrl, AR-shCtrl, and AR-shEZH2 co-transfected LO2 cells. (k) Protein expression of AR, EZH2, H3K27me3, Wnt signal inhibitors, active and total β -catenin, *CCND1* and *EGFR* were detected in vector-shCtrl, AR-shCtrl and AR-shEZH2 co-transfected LO2 cells. β -actin was used as a loading control.

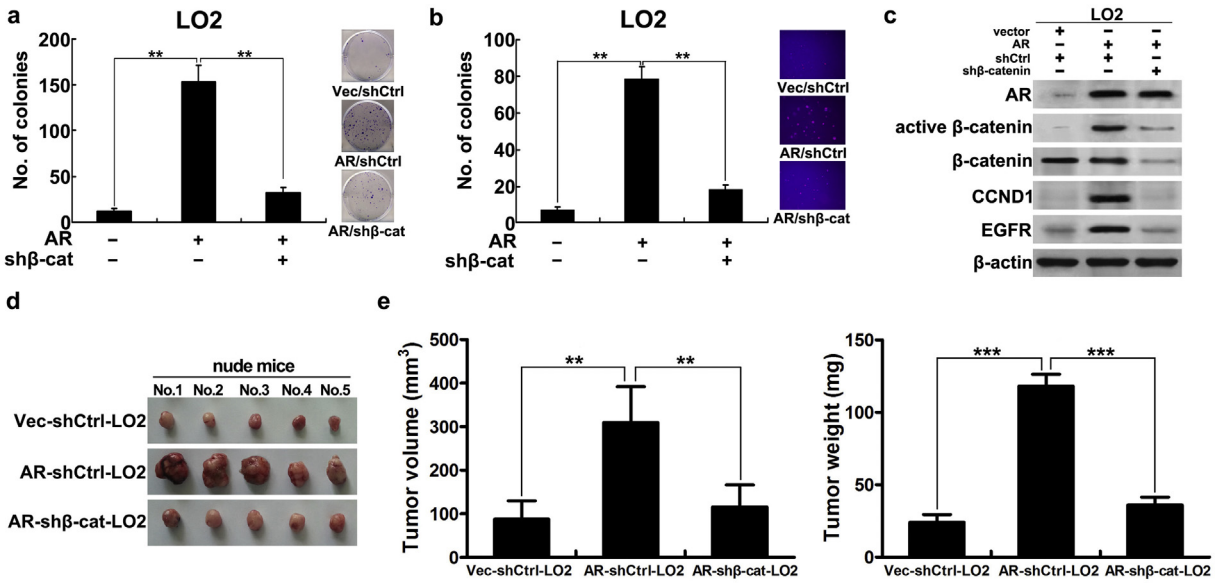


Fig. 5. AR promotes hepatocellular proliferation and tumorigenesis by activating Wnt/β-catenin signaling. (a) Focus formation assay and (b) anchorage-independent growth by soft agar assay were assessed in control and AR-expressing LO2 cells with or without β-catenin knockdown. (c) Protein expression of AR, active and total β-catenin, CCND1 and EGFR was determined by western blot. LO2 cells were co-transfected with the combination of vector and shCtrl, AR and shCtrl, and AR and shβ-catenin respectively. β-actin was used as a loading control. (d) Images of xenografts formed from Vec-shCtrl, AR-shCtrl-, and AR-shβ-catenin-LO2 stable cells are shown. (e) The volumes and weights of isolated xenograft tumors from the 3 groups were measured. ***P* < 0.01; ****P* < 0.001.

this signaling cascade and tumor differentiation (Fig. 7b). More importantly, Kaplan-Meier analysis showed that HCC patients with higher activities of AR/EZH2/β-catenin signaling cascade exhibited significantly shorter overall survivals (Fig. 7c) and disease-free survivals (Fig. 7d).

Taken together, our findings in clinical samples unravel the positive correlation between the activity of AR/EZH2/β-catenin signaling and HCC progression, suggesting that these indicators may be useful for evaluation of HCC prognosis. Overall we identified a novel working model

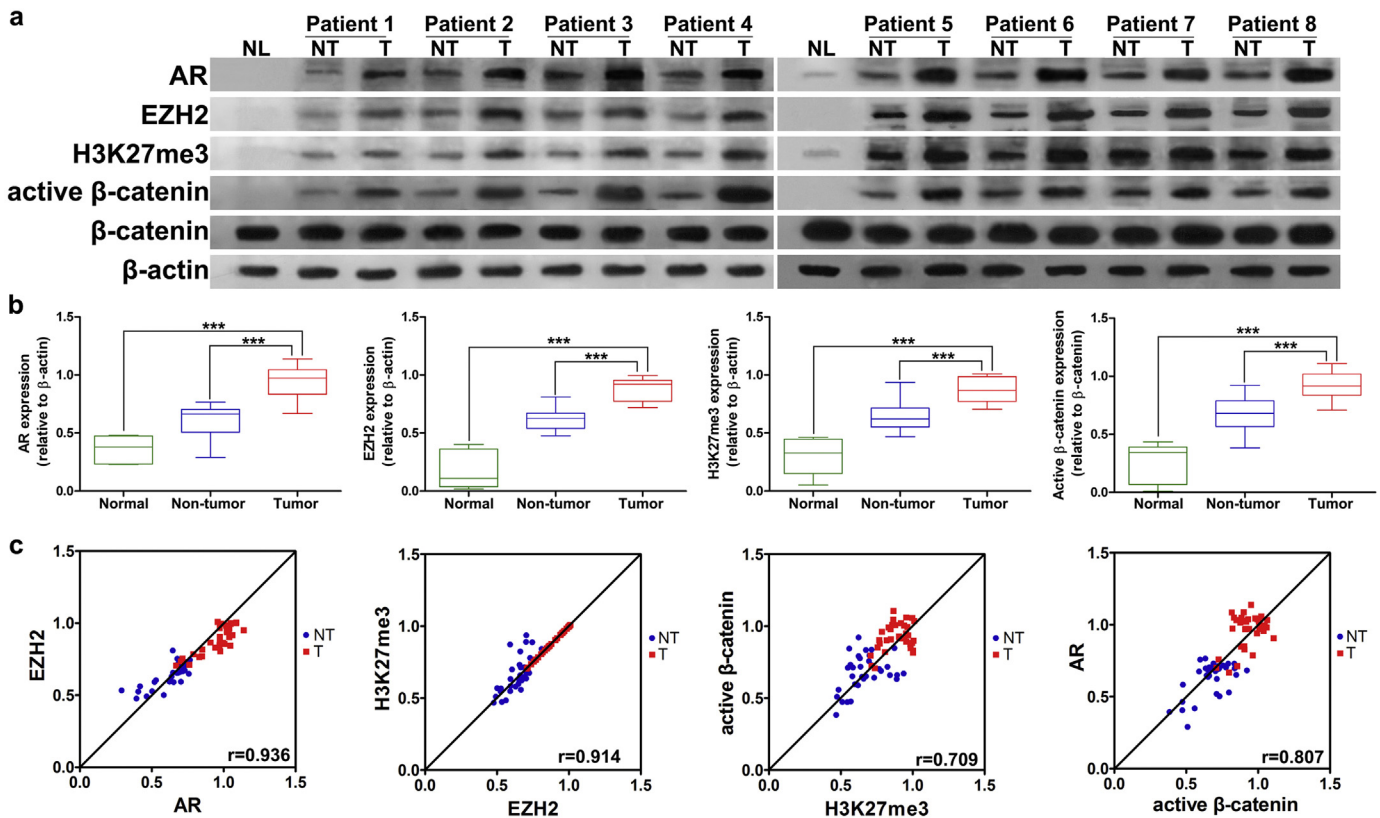


Fig. 6. AR overexpression was associated with EZH2-mediated epigenetic activation of β-catenin in human tumor tissues. (a) Representative western blot of AR, EZH2, H3K27me3, active and total β-catenin in human normal liver (NL), and 32 pairs of non-tumor (NT) and tumor (T) tissues. (b) Relative protein levels of AR, EZH2, H3K27me3 and active β-catenin in normal, adjacent non-tumor and tumor tissues. The lines within the box denote median, and whiskers represent 10–90 percentiles. (c) Correlation of the expression levels among AR, EZH2, H3K27me3 and active β-catenin in 32 pairs of HCC samples denoted by Pearson's correlation coefficients. ****p* < 0.001.

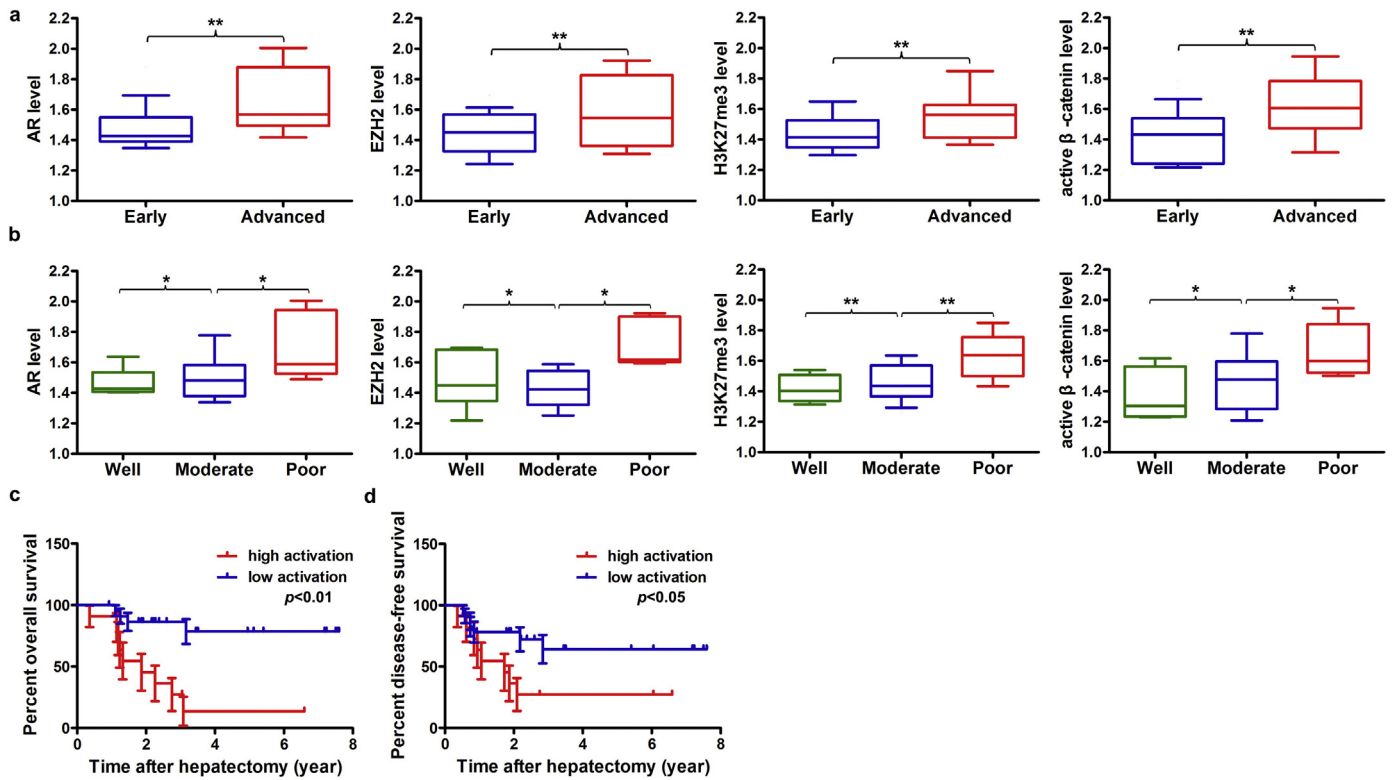


Fig. 7. Elevated activity of AR/EZH2/ β -catenin signaling in human tumor tissues correlated with poor survival of HCC patients. (a) Relative protein levels of the components of AR/EZH2/ β -catenin signaling were classified by tumor progression and (b) differentiation. The lines within the box denote median, and whiskers represent 10–90 percentiles. (c–d) Kaplan-Meier analysis of (c) overall survival and (d) disease-free survival curves of HCC patients with high ($n = 10$) or low ($n = 22$) activity of AR/EZH2/ β -catenin signaling. * $p < 0.05$; ** $p < 0.01$.

that AR binds to the *EZH2* promoter to transcriptionally upregulate *EZH2* expression, subsequently promotes H3K27me3-mediated silencing of Wnt signal inhibitors leading to activated Wnt/ β -catenin signaling, then induces the expression of downstream pro-proliferative target genes (Fig. 8).

4. Discussion

It has been widely recognized that AR plays a crucial role in connecting sexual hormone signaling with gender disparity in the development of HCC. *EZH2* overabundance has been linked to aggressive

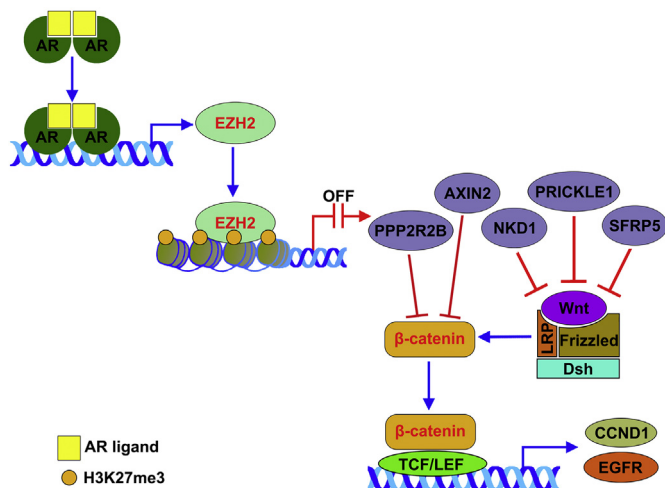


Fig. 8. A schematic model of AR/EZH2/ β -catenin signaling in hepatocarcinogenesis. AR binds to *EZH2* promoter for upregulation of *EZH2*, which in turn increases H3K27me3 for transcriptional silencing of Wnt signal inhibitors, thereby activates Wnt/ β -catenin signaling and further stimulates downstream pro-proliferative target genes.

and advanced stage of male-predominant HCCs, and is strongly associated with poor clinical outcome and prognosis [11,31]. Although previous studies have demonstrated the synergistic crosstalk between AR and *EZH2* in HCC cells, the molecular linkage between them has not yet been fully elucidated. In this study, our data revealed the transcriptional regulation of AR on *EZH2* expression. Importantly, *EZH2*, being a core polycomb group protein, acts as an oncoprotein by catalyzing H3K27me3 in HCCs [32], which could be abolished by AR knockdown. This finding suggests an alternative mechanism underlying AR-induced hepatocellular proliferation through epigenetic regulation. Furthermore, upregulation of *EZH2* activated Wnt/ β -catenin signaling through H3K27me3-mediated silencing of Wnt signal inhibitors, supporting that *EZH2* acts as a mediator in the AR/*EZH2*/ β -catenin signaling cascade in promoting HCC progression. Clinically, elevated activity of AR/*EZH2*/ β -catenin signaling was associated with tumor progression and poor prognosis of HCC patients, highlighting the biological significance of AR-managed epigenetic programming in liver cancer.

A major finding presented here is that AR functions as a master transcription factor to directly activate the transcription of *EZH2* which in turn remodels the epigenetic landscape. Compared with activating and inactivating mutations frequently identified in hematologic malignancies, *EZH2* is consistently overexpressed in solid tumors [24,33]. A number of transcription factors have been identified to directly bind to the *EZH2* promoter and activate its mRNA expression in different cancer models [34,35]. In human HCCs, *EZH2* is significantly upregulated at both mRNA and protein levels [36]. However, the regulatory mechanism of *EZH2* expression remains elusive. In this study, the functional link between AR and *EZH2* is confirmed by the facts that knockdown of *EZH2* in AR-expressing cells significantly abrogates anchorage-dependent and -independent growth *in vitro*, and tumorigenesis *in vivo*, demonstrating the mediator role of *EZH2* in AR-induced HCC formation. Using promoter enrichment and luciferase report assay, we have demonstrated that AR directly bound to *EZH2* promoter and

activated its transcriptional activity. In accord, knockdown of *AR* decreased *EZH2* mRNA level in HCC cells in gene expression analysis, manifesting the existence of specific *AR* binding site in *EZH2* promoter. Two AREs were identified in *EZH2* promoter, and ARE deletion could abolish the *AR*-driven transcription of *EZH2*. Notably, deletion of either ARE only partly impaired the promoter activity, which is totally eliminated by both AREs deletion, indicating the two AREs have complementary and synergistic function on promoter activity regulation. Considering the recently reported interaction of *AR* and *EZH2* in HCC cells [37], our data suggest that *EZH2* may act as a co-activator of *AR*. Intriguingly, it has been reported that the oncogenic function of *EZH2* in prostate cancer cells is dependent on the co-activator role of *EZH2* in *AR* signaling [38]. Thus, further experiments regarding *AR* and *EZH2* interactome would be warranted in HCC cells.

Furthermore, global H3K27me3 level was found to be increased following *EZH2* induction, suggesting *AR* epigenetically promotes carcinogenesis through *EZH2*-regulated histone modification. Indeed, *EZH2* is the catalytic unit of PRC2, which has HMTase activity to methylate H3 at lysine 27. It has been reported that *EZH2* can catalyze di- and trimethylation of histone H3 at lysine27 (H3K27me2 and H3K27me3), both of which execute PRC2-dependent epigenetic silencing of tumor repressors in prostate cancer, though H3K27me3 is the major product catalyzed by the SET domain of *EZH2* [39]. In addition, Xu et al. have shown that *EZH2* exhibits PRC2-independent oncogenic activity by promoting the recruitment of H3K4me3 to oncogenes [40]. In our previous study, we found that *EZH2* was activated through phosphorylation at Serine21, which could increase H3K4me3 level in HCC cells [26]. In this study, we also found that *AR*-induced *EZH2* could change H3K4me3 levels in LO2 and SK-Hep1 cells (data not shown). The change of H3K4me3 implies that *EZH2* acts as an epigenetic activator to stimulate oncogenes, indicating an alternative role of *AR*-induced *EZH2* in gene activation *via* modulation of active chromatin state in HCC development. Further investigation is necessary to corroborate the speculation.

Mounting evidence has shown that β -catenin acts as an intracellular signal transducer of the Wnt signaling pathway, and is involved in many physiological processes such as cell adhesion and cellular metabolism [41,42]. In addition, aberrant activation of β -catenin by Wnt signals has been reported to contribute to the pathogenesis of liver cancer [43]. We have previously shown that overexpression of *EZH2* stimulates β -catenin activity in HCC cells through the epigenetic inhibition of Wnt signal inhibitors [30]. Our present findings demonstrated that *AR* increased global H3K27me3 level after inducing *EZH2* expression, suggesting the *AR* is an upstream regulator of *EZH2*-controlled epigenetic modification. Using promoter enrichment assay, we showed that the occupancy of *EZH2* on the promoters of Wnt signal inhibitors, including *AXIN2*, *NKD1*, *PPP2R2B*, *PRICKLE1* and *SFRP5*, were inhibited by knockdown of *AR* in PLC5 cells, whilst ectopic *AR* expression promoted the recruitment of *EZH2* on these promoters in LO2 cells. Since the *EZH2*-mediated H3K27me3 is responsible for transcriptional suppression, we also examined the change of H3K27me3 enrichment at the promoters of Wnt signal inhibitors. As expected, *AR* increased H3K27me3 enrichment at these promoters in HCC cells, which could be abolished by *EZH2* knockdown. In accord, gene expression analysis showed that *AR* negatively regulated Wnt signal inhibitors mRNA levels through induction of *EZH2* and H3K27me3 expression. This compelling evidence showed that *AR* activates β -catenin through *EZH2*-mediated epigenetic silencing of Wnt signal inhibitors. Gao et al. showed that *EZH2* suppresses tumor suppressor *TP53* in H3K27me3-independent manner in HCC, suggesting a putative non-epigenetic function of *EZH2* on the regulation of target genes [44]. However, we did not have any data supporting such H3K27me3-independent non-epigenetic role of *EZH2* in HCCs. Glycogen synthase kinase-3 β (GSK3 β) binds with Wnt signal inhibitors to form a complex that degrades β -catenin through phosphorylation. When phosphorylated by oncogenic factors like CCRK, GSK3 β dissociates from Wnt signal inhibitor complex, and hence β -

catenin activity is restored [8]. These findings showed an alternative non-epigenetic mechanism for regulation of Wnt/ β -catenin signaling, and further experiments are warranted to investigate the role of *EZH2* in this process. *CCND1* and *EGFR* are the two pro-proliferative downstream effectors of β -catenin signaling [45,46]. In our study, their transcription was also found to be increased by *AR* overexpression, which was blocked by *EZH2* knockdown. Furthermore, protein expression analysis consolidated that *AR* induced β -catenin activation and the expression of target genes *CCND1* and *EGFR*, in which *EZH2* is a critical mediator. Thus our findings suggest that *AR*/*EZH2* epigenetic programming may play a key role in the activation of Wnt/ β -catenin signaling in HCCs.

The clinical relevance of *AR*/*EZH2*/ β -catenin signaling activity was further addressed in this study. In a cohort of HCC samples, we observed increased activity of *AR*/*EZH2*/ β -catenin signaling indicated by overexpression of all these members in tumor tissues compared with matched adjacent non-malignant liver tissues. Our data also demonstrated elevated activity of *AR*/*EZH2*/ β -catenin signaling in >60% of HCCs, which significantly correlated with HCC staging. More importantly, hyperactivity of *AR*/*EZH2*/ β -catenin signaling in tumor tissues could distinguish a subset of patients with increased risk of poor overall survival and disease-free survival, demonstrating the clinical significance of this *AR*/*EZH2*/ β -catenin signaling in HCC prognosis. In addition to our study about Wnt/ β -catenin signaling, the major signaling pathways involved in HCC development include *RAF*/*MEK*/*ERK* pathway, *PI3K*/*AKT* pathway, insulin-like growth factor pathway, etc. In other related study, we found that the *AKT* activity and *E2F1* expression are different between tumor tissues and their respective surroundings [26]. Based on these results, it is conceivable that *AKT* signaling and *pRB*-*E2F1* signaling are different between tumors and surrounding tissues. Examination of their expression patterns in HCC samples for clinical significance is warranted in further investigation. In conclusion, our current study identifies a novel molecular pathway driving HCC progression through epigenetic programming, and delineates the mechanistic details that *EZH2* functions as a mediator bridging *AR* and Wnt/ β -catenin signaling during tumorigenesis. Thus, exploiting the epigenetic vulnerabilities of *EZH2* in *AR*/*EZH2*/ β -catenin cascade is highly relevant to the design of therapeutic interventions in HCC and potentially the other male-predominant cancers.

Declaration of interests

The authors declare that they have no competing interests.

Author's contributions

Study concept and design: H.F., Y.Q.G., A.S.L.C.; acquisition of data: H.B.S., Z.Y., X.H.S., J.F., Q.Y., H.K.; analysis and interpretation of data: H.B.S., Z.Y., X.H.S.; drafting of the manuscript: Z.Y.; H.F.; critical revision of the manuscript for important intellectual content: M.T.S.M., A.S.L.C., Y.Q.G.; statistical analysis: X.J.Z., L.Y.H., M.L.; obtained funding: Z.Y., H.F., Y.Q.G., A.S.L.C.; administrative, technical, or material support: X.J.Z., L.Y.H., M.L.; study supervision: H.F., Y.Q.G.; Final approval of the version to be submitted: H.F., A.S.L.C.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ebiom.2018.08.043>.

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